ISSN 1881-7815 Online ISSN 1881-7823

BST
BioScience Trends

Volume 11, Number 1
February, 2017

IRCA
BSSA

IACMHR

www.biosciencetrends.com

BST
BioScience Trends

ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

BioScience Trends is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA and Shandong University China-Japan Cooperation Center for Drug Discovery & Screening (SDU-DDSC).

BioScience Trends devotes to publishing the latest and most exciting advances in scientific research. Articles cover fields of life science such as biochemistry, molecular biology, clinical research, public health, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

BioScience Trends publishes Original Articles, Brief Reports, Reviews, Policy Forum articles, Case Reports, News, and Letters on all aspects of the field of life science. All contributions should seek to promote international collaboration.

Editorial Board

Editorial Office

Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku, Tokyo 112-0003, Japan
Tel: +81-3-5840-8764 Fax: +81-3-5840-8765
E-mail: office@biosciencetrends.com

BioScience Trends

Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

Tel: +81-3-5840-8764, Fax: +81-3-5840-8765
E-mail: office@biosciencetrends.com
URL: www.biosciencetrends.com

Editorial Board Members

CONTENTS

Volume 11, Number 1, 2017

Reviews

Original Articles

CONTENTS (*Continued*)

Review

DOI: 10.5582/bst.2016.01250

Steering of carcinoma progression by the YIN/YANG interaction of STAT1/STAT3

Karlheinz Friedrich[1], Helmut Dolznig[2], Xiaonan Han[3,4], Richard Moriggl[5,6,7,*]

[1] *Institute of Biochemistry II, University Hospital Jena, Jena, Germany;*
[2] *Institute of Medical Genetics, Medical University of Vienna, Vienna, Austria;*
[3] *Key Laboratory of Human Disease Comparative Medicine, Ministry of Health; the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS) & Peking Union Medical College (PUMC), Beijing, China;*
[4] *Division of Gastroenterology, Hepatology and Nutrition, Cincinnati Children's Hospital Medical Center (CCHMC), Cincinnati, Ohio, USA;*
[5] *Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria;*
[6] *Medical University of Vienna, Vienna, Austria;*
[7] *Institute of Animal Breeding and Genetics, University of Veterinary Medicine, Vienna, Austria.*

Summary **STAT1/STAT3 transcription factors are important regulators for development of normal, infected or inflammed cells. They are also critically involved in the progression of various malignant tumours, including epithelial-derived carcinomas. Here, we focus on colorectal cancer (CRC) insights for STAT1/3, where controversial functions for STAT3 were reported. For a long time STAT3 has been regarded as a driver of tumour malignancy and its activation was associated with negative clinical outcome. In contrast, STAT1 was generally viewed as an independent tumour suppressor and positive prognostic marker. Here we discuss the experimental evidence for the tight association and regulation of oncogenic STAT3 transcription kept at bay by nuclear STAT1. We summarise current research and describe cellular models of different STAT1/STAT3 expression ratios. STAT1/3 expression levels are influenced by the mutational status of carcinoma cells associated with nuclear unphosphorylated STAT1. Animal tumour models and results from *in vitro* experiments allow for the conclusion that both proteins interact as antagonistic transcription factors in CRC cells. These STATs steer also important processes during infection and inflammation that influence development and progression of CRC. The STAT1/3 interplay is important to understand gene regulation and we describe it here similar like the YIN/YANG dualism. Thus, we propose to evaluate both STAT1 and STAT3 expression patterns in cancers in a dual manner instead of regarding them as independent transcription factors. This conceptual dualistic view could advance diagnostic predictions in the future.**

Keywords: Colorectal carcinoma, JAK-STAT pathway, transcription factors, STAT1/STAT3 interplay, prognostic marker

1. Introduction

The JAK-STAT pathway was recognized as one of the twelve core cancer pathways based on 'omics' technologies and from insights into cancer genome sequencing (*1*). JAK-STAT signalling contributes to many hallmarks of cancer, where survival, growth promotion, angiogenesis or activation of key other signalling pathways like PI3K-AKT-mTOR or RAS-RAF signalling are very well documented (*2-4*).

Cytokines bind to their corresponding receptor chains upon which significant structural changes are induced that also lead to a close proximity interaction of their transmembrane domains. The key event upon cytokine activation is separation of the intracellular Box1 sequences in the cytoplasmic tails of cytokine

Released online in J-STAGE as advance publication February 2, 2017.

*Address correspondence to:
Dr. Richard Moriggl, Ludwig Boltzmann Institute for Cancer Research (LBI-CR), Währingerstr. 13a, A-1090 Vienna, Austria.
E-mail: richard.moriggl@lbicr.lbg.ac.at

receptors that bind the JAK tyrosine kinases that are bound *via* their FERM domains. This separation results in a sliding motion and removal of the pseudokinase inhibitory domain of one JAK molecule to the other JAK molecule (*5*). Both JAK kinases are pre-assembled at the receptor level in antiparallel dimer fashion. Understanding the structural requirements and mode of JAK kinase activation is important in cancer since they are most frequently mutated among all tyrosine kinases in mammals in cancer. The majority of cancer types are associated with JAK hyperactivation, which transactivate each other and trans-phosphorylate the cytoplasmic tails of receptor chains c*a*using subsequently the activation of other important key signalling molecules (*5*). STAT molecules and other important signalling pathways such as activation of the RAS-RAF-MAPK axis or the PI3K-AKT-mTOR signalling route are downstream of JAK kinase action. Activation of the pathway acts in a cell type-specific and mutational, context-dependent way. It can involve controversial outcomes, such as tumour suppressive as well as oncogenic properties. Gene dosage and expression levels are also relevant, since amplification of the *stat3/5* locus on human chromosome #17q was associated with epithelial or mesenchymal tumour progression [*a PubMed search for #17q amplification revealing 412 publications as of Dec. 2016*]. Next Generation Sequencing data together with histopathology studies revealed that JAK/STAT signalling is in most cancers hyperactivated and it is among the most intensely mutated signal transduction pathways in cancer tightly linked with RAS transformation or MYC upregulation in cytokine responses *e.g*. It is therefore not surprising the pathway belongs to the prime targets of pharmaceutical research and developments. Today, more than ten JAK kinase inhibitors are evaluated in clinical trials. Ruxolitinib potently targets JAK1 and JAK2, while Tofacitinib inhibits JAK1, JAK2 and JAK3 (*2*) [currently, one finds multiple phase 1-III clinical trials *via clinicaltrials.gov* that lists 51 active trials which used Ruxolitinib as of Dec. 2016].

Deficiencies or aberrations in activity caused by JAK-STAT mutations have severe consequences, leading *e.g*. to developmental and immune defects, dysregulated inflammation, metabolic disease and malignant cell transformation (*6-12*). The JAK-STAT pathway is highly conserved and diseases caused by germ-line or somatic mutations in JAKs or STATs are almost equivalent when studied in transgenic mice or found in patients and explored molecularly in human/patient-derived cell systems (*13-15*).

2. STAT1/3 signalling in tumour biology

STAT1/3 are pleiotropic transcription factors that make complex contributions to cancer (Figure 1). Knockout studies on STAT1 unravelled its central role in innate and acquired immunity upon infection, but also in the control of anti-tumourigenic functions, *e.g*. due to tumour surveillance control mechanisms. Nowadays, STAT1 is predominantly viewed as a tumour suppressor, with the exception of MHC class I regulation, which controls NK cell-mediated killing of tumour cells (*16*). STAT1 was shown to have a function in the control of pro-apoptotic and anti-proliferative signalling pathways (*17*). STAT1 was also described as a favourable prognostic marker in carcinomas of the breast or colon (*18,19*). The gene encoding for the SOCS1 Ubiquitine E3 Ligase is, for example a direct target gene of STAT1, and Socs1-deficient mice show spontaneous CRC tumour development, associated with hyper-activation of STAT1 (*20*) due to higher cytokine receptor and JAK kinase activity. Hypermethylation of SOCS family members or their genetic loss was frequently reported in cancer (*21,22*).

STAT3 is a key immunomodulatory and anti-infectious transcription factor downstream of interferon signalling and its effects are often opposite to those of STAT1(*23*). STAT3 is also a master transcription factor controlling differentiation under physiological conditions, *e.g*. it blocks differentiation in association with leukaemia inhibitory factor (LIF) signalling in embryonic stem cells and, hence, a key process for mammalian development (*24*). Moreover, STAT3 has an essential steering function for Th17 cell generation that control autoimmunity and infection through the IL-23-IL-17 cytokine signalling axis, particular important for chronic inflammatory processes of the GI tract (*25*). STAT3 action in cancer is generally viewed in a simplistic manner, and most studies use pYSTAT3 or nuclear STAT3 predominantly as a surrogate marker of its oncogene activity. Patients with gain-of-function (GOF) mutations in STAT3 shape the cancer gene expression landscape of inflammatory liver adenomas or of mature T cell neoplasia (*26,27*). Importantly, STAT3 regulates also stem cell to tissue fate and invasive processes through epithelial to mesenchymal transitions or mesenchymal to epithelial transitions during embryo or cancer progression (*28*). Thus, STAT3 can control key differentiation processes under physiologic or malignant conditions. These functions of STAT3 are consistent with early embryonic death when fully depleted in mice (*29*). In conclusion, STAT1 is a tumour suppressor in CRC, but STAT3 function is promiscuous. It can promote growth, survival, migration or attachment of cancer cells and it may play an important role in cancer immune escape or tumour neo-vascularisation (*4*).

3. Influence of STAT1/3 on CRC

3.1. *STAT1/3 as YIN/YANG antagonists*

The activities of STAT1/3 in cancer are reminiscent of the YIN/YANG dualism, describing two opposite forces in nature. Here, YIN characterises negative

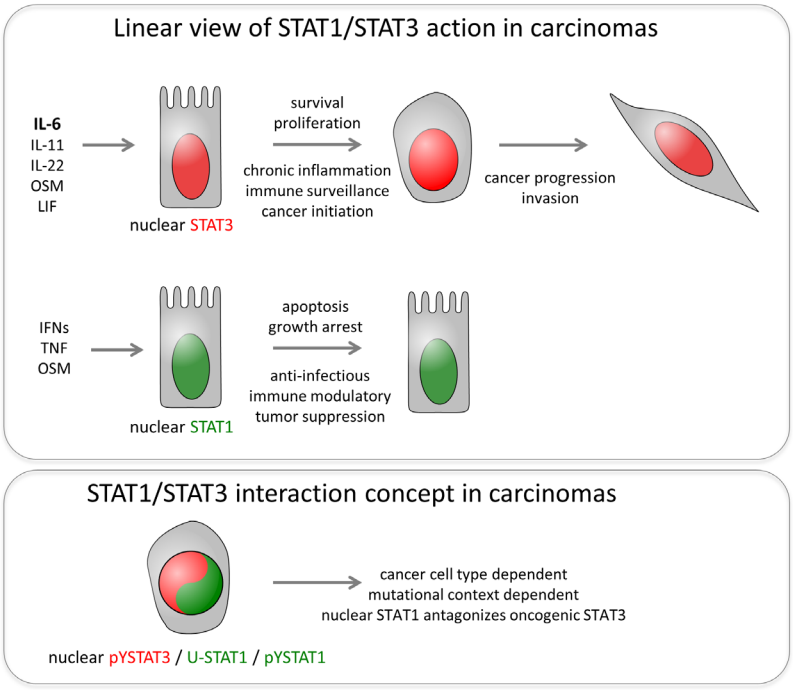

Figure 1. Roles of STAT1/3 expression and activity status in colorectal carcinoma. The pleiotropic transcription factors STAT1 and STAT3 often make antagonistic contributions to cancer initiation or progression associated with chronic infection and inflammation. STAT3 is involved in proliferation, survival and invasion of colorectal carcinoma (CRC) cells and cytokines such as interleukin 6 (IL-6), IL-11, IL-22, oncostatin M (OSM) or leukemia inhibitory factor (LIF) among others were shown to induce potently pYSTAT3 activity levels. STAT1 activation is mostly triggered by interferon (IFN) signalling, however, nuclear STAT1 is found in the unphosphorylated (= non-tyrosine phosphorylated state, U-STAT1) which is *e.g.* also triggered by inflammatory stimuli such as tumour necrosis factor α (TNF) signalling. Surprisingly, nuclear U-STAT1 is constitutively found in CRC cells, however, at varying expression levels antagonising STAT3 signalling.

action of processes, whereas YANG stands for positive action. YIN/YANG, though of contrasting character, are not thought of as static or separated entities. They rather interfere with each other, as exemplified by the mutual interplay of the female and the male principle. Thus, the nature of YIN/YANG lies in the interchange between and the unification of two complementary beings. Recent investigations suggest that STAT1/3 are operative in CRC in a combined analogous manner and they should be viewed and studied as fraternal twins to understand their combined mode of action for CRC biology. Thus, we view STAT1/3 transcription factors as YIN/YANG molecules in the malignant disease process of CRC, reminiscent of ideas laid down in the earliest known Chinese medical book called Huangdi Neijing (Yellow Emperor's Classic of Medicine) two millenniums ago. Here, we will discuss the implications of the STAT1/STAT3 interplay for cancer progression.

3.2. *Predictive potential of STAT1/3 in CRC*

As for other cancers, both STAT1/3 have been evaluated for their suitability as prognostic markers in CRC. In accordance with its generally assumed tumour suppressive functions, STAT1 was shown to be associated with favourable prognosis (*18*). However, it was also claimed that increased tumour expression of STAT1 was associated with impaired patient survival (*30*).

Contradictory reports were published with regard to the predictive validity of STAT3 expression and activation levels in CRC. Some investigations supported the notion of STAT3 as an oncogene, while others concluded that STAT3 is a tumour suppressor. Several studies reported oncogenic effects of elevated STAT3 signalling (*30-36*). In contrast, others reported on STAT3 as a determinant of positive outcome in intestinal tumour progression (*37,38*). Moreover, we could show recently that absence of STAT1 and/or STAT3 proteins is correlated with shorter survival of CRC patients (*37,39*). We performed a systematic study to shed light on the functional interplay and the tumour growth-associated roles of STAT1/3 in CRC progression. Importantly, we could demonstrate that oncogenic function of STAT3 in CRC depends to a large extent on the ratio of STAT1/STAT3 expression and the isolated view on STAT3 activity alone is not sufficient (*39*). A low STAT1/high STAT3 ratio proved to be a valid predictor of poor disease outcome, whereas high STAT1 levels in combination with low STAT3 levels were a favourable determinant (Figure 2). From these results we conclude that STAT3 activation or nuclear function as a transcription factor can be antagonised by STAT1. Hence, we postulate that STAT3 function should be viewed in context with STAT1 expression levels in CRC (*39*).

These results can explain earlier conflicting views on

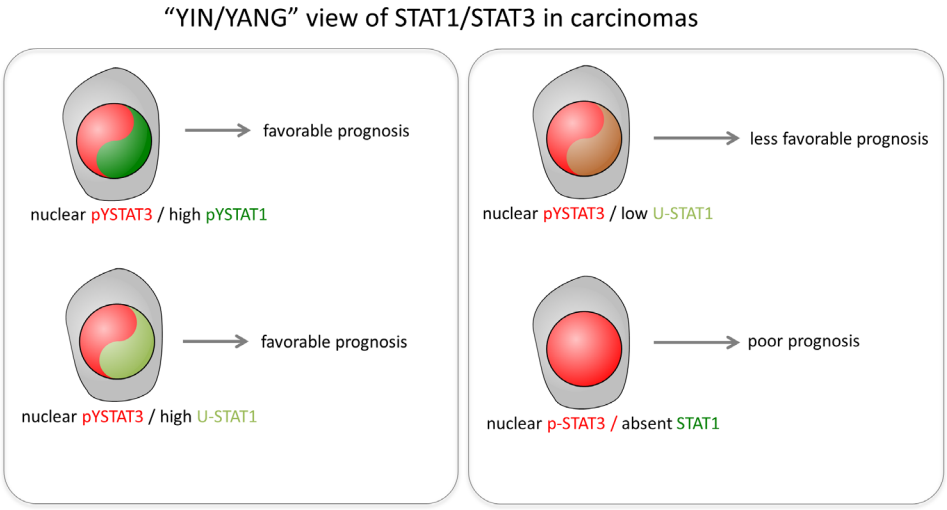

Figure 2. The YIN/YANG interplay of STAT1/3 irrespective of tyrosine phosphorylation gains predictive power on colorectal cancer progression. STAT1/3 proteins interact both without and with DNA. Furthermore, STAT1 and STAT3 physically interact irrespectively of their tyrosine phosphorylation status. Mechanistically, assessment of STAT1/3 cellular distribution and activation suggested that U-STAT1 is mainly nuclear and antagonises STAT3 activity in a YIN/YANG mode. Thus, sole STAT3 activity in patient samples is not a clear predictor of disease progression and one should always control for STAT1 expression since that largely counteracts oncogenic STAT3 and combined analysis of STAT1 and STAT3 nuclear and cytoplasmic expression levels gains prognostic power in the analysis of patient-derived carcinomas.

the predictive tendency of elevated STAT3 expression/activity in CRC that is antagonised by nuclear STAT1 expression levels even without the prerequisite of STAT1 tyrosine phosphorylation (Figure 2). We further could show that different results from various studies on the impact of STAT3 on CRC must be further viewed in context with distinct driver mutations since STAT1 expression upon genetic knockdown or loss of STAT3 can either go up or down. Thus, genetically distinct patient cohorts will influence together with an individual gut microbiota STAT3 signalling. Furthermore, STAT3 signalling is influenced in different cell types through chronic inflammation/infection or by complex tumour-stromal cell interactions (*35*).

Our recent study based on CRC tissue microarrays, cellular and tumour models correlated the ratio of STAT1 to STAT3 expression as well as the IL-6 receptor α chain expression status with disease outcome. While concomitant absence of STAT1/3 was clearly associated with longer patient survival, higher ratio of STAT1/3 expression influenced significantly CRC prognosis. Using four well characterized CRC cell lines with experimentally ablated STAT3 expression and SCID mouse xenografting confirmed that it is the ratio of STAT1/STAT3 expression which gains predictive power for disease progression. Thus, these findings support the view of STAT1/3 as a pair of YIN/YANG counteraction.

4. Mechanisms of STAT1/3 interaction and consequence to cancer biology

The importance of the STAT1/3 protein ratio could be recapitulated in xenograft models using paired cell lines engineered for knockdown of STAT3 expression. Surprisingly and depended on the employed CRC cell line, we found either a repressive or inductive effect on endogenous STAT1 expression upon STAT3 knockdown (*39*). Low STAT1/high STAT3 ratio showed faster tumour growth in xenografts (Figure 2). In contrast, xenografts of cell lines showing high STAT1 and low STAT3 levels grew significantly slower. The molecular basis of the combined activities of STAT1/3 in CRC is a multifaceted issue. The YIN/YANG interplay of STAT1/3 manifests in several ways: *i*) STAT1/3 proteins interact both without and with DNA engagement and their tyrosine phosphorylation status is despite common pathway drawings dispensable for that. STATs can form antiparallel dimers as also revealed through crystal structure insights of unphosphorylated STAT molecules (*40*) and both dock to cytokine receptor cytoplasmic chains *via* their N-terminal sequences. *ii*) STAT1/3 transcription factors have largely the same DNA binding consensus sites. Thus, they bind to similar DNA sequences at regulatory gene transcription regions, but they have very distinct target gene spectra due to cell type specific cofactors, corepressors or transcription factor interaction. *iii*) STAT1/3 expression can be modulated by each other's expression level through STAT regulatory elements in their respective promoters. *iv*) STAT1/3 are activated or inhibited in unique ways. They can be induced by either distinct or by the same set of cytokines or growth factors. Furthermore, their ability to induce SOCS proteins, which then downregulate the JAK kinase and cytokine receptor chains by degradation is unique, all allowing for transcriptional fine tuning giving selectivity to signalling.

With regard to cancer, the situation is more complex

since changes in the mutational landscape of the JAK-STAT-SOCS pathway or epigenetic differences influence gene regulation drastically. In general, overlapping tyrosine kinase action can lead to both of their STAT1/3 activation. Pathways that contribute to their inactivation or inhibition are frequently lost or mutated in cancer, such as inactivation of tyrosine phosphatases (*e.g.* by reactive oxygen species inactivating the catalytic cysteine of tyrosine phosphatases) or diminished proteolytic degradation (*e.g.* methylation of SOCS proteins, change in sumoylation or ubiquitinylation pathways is frequently deregulated escaping then proteasome degradation) (*41,42*). We have currently an incomplete mechanistic understanding how mutations or epigenetic changes together with changes in cytokine or growth factor signaling influence the ratio of STAT1/3 protein expression affecting CRC progression.

4.1. *Formation of STAT1/STAT3 heterodimers and gene regulation*

The heterodimeric ratio of STAT1/3 complexes and its pre-association or nuclear complex formation is both relevant for docking and gene regulation *via* STAT1/3. We postulate that gene regulation control is simply dictated by its YIN/YANG expression ratio and interaction control. Upon cytokine receptor engagement, STAT activation *via* the JAK/STAT pathway is a rapid process, but not essential for nuclear translocation in cancer cells. Under physiologic conditions in normal cells signals are transferred into the nucleus of cells to reprogram gene transcription through the STAT transcription factors in minutes. It was early on recognized that the activity and cellular abundance of individual STAT proteins can alter the expression of other STAT protein family members (*21,43*), but so far there were limited functional studies regarding the influence of STAT1 expression levels on STAT3 signalling during CRC tumour growth. To make the picture even more complex, STAT oligomerization reported for STAT1, STAT3, STAT4, STAT5A and STAT5B allows for loop formation on DNA among each other. This will also influence STAT1/3 action including autoregulatory expression change. Furthermore, other transcription factors that bind to similar or the identical target gene sequences then STAT1, STAT3 or STAT5 such as BCL6 (an important oncogene in B cell lymphomas), will change the fait and outcome of gene regulation through the STAT1/STAT3 YIN/YANG interplay. It is likely that U-STAT1 participates in gene transcription in CRC, possibly of interferon regulated genes involved in anti-tumour activities. It was postulated that the mutual interdependence of STAT1/3 through heterodimer formation within DNA binding complexes in tumour cells has a crucial influence on cancer cell fate (*44*). We have shown that STAT1/STAT3 heterodimers are detectable in the majority of CRC biopsies and form independent upon stimulation with IL-6 in various CRC cell lines due to the predominant nuclear expression of STAT1 that we will next illuminate further (*37*).

4.2. *Potential role of non-phosphorylated STATs and non-canonical STAT signalling in CRC*

The formation of STAT1/STAT3 heterodimers was experimentally mostly demonstrated in a DNA-bound state. However, it is noteworthy that U-STAT1 was also found persistently in the nucleus of epithelial cells as early described by the George Stark laboratory (*45*). Non-canonical STAT pathways employ STATs independently of tyrosine phosphorylation (*21,46*) .

In general, pYSTAT1 signalling was found to be mostly associated with interferon signalling, whereas pYSTAT3 signalling is connected with responses emanating from the activated distal four tyrosine residues within the gp130 receptor cytoplasmic chain (*43*). Importantly, U-STAT1/3 proteins have functions distinct from those of the pY proteins (*21*): *i*) as specific transcription factors and modifiers of transcription in case of U-STAT1 and U-STAT3 (*43*), *ii*) as effectors of mitochondrial function (serine phosphorylated STAT3) (*47*). U-STAT1 was shown to extend expression of IFN-induced genes (*45*). The fact that U-STAT1, if present in the nucleus can bind to activated pYSTAT3 controlling its proliferative and pro-survival function was so far neglected. Based on co-immunoprecipitation studies and structural insights derived from crystallography of the unphosphorylated STAT5A dimer (*40*) and integrative modelling of the U-STAT3 dimer (*48*) one can assume that STAT1/STAT3 heterodimers can bind in anti-parallel fashion in a non-tyrosine phosphorylated manner also to receptor chains. This antiparallel STAT dimerization at the cytokine receptor chains is reminiscent of JAK kinase dimerisation at the Box1 motif and the escape mechanism of inhibitory kinase action through the sliding model described above also suggesting an antiparallel juxtaposition of the kinase and the pseudokinase domains (*5*). Important here is the concept that STAT1/3 protein-protein interaction can be observed without the necessity to be fully activated by tyrosine phosphorylation. We could confirm early reports for STAT1/STAT3 interaction without the prerequisite of tyrosine phosphorylation (*39,49*). Furthermore, we also demonstrated significant nuclear presence of STAT1 independent of cytokine stimulus in CRC cells, where it can heterodimerize with pYSTAT3, for example activated by IL-6 (*39*). We were able to show that also upon tyrosine phosphorylation of STAT3 the interaction of U-STAT1 and pYSTAT3 is strongly interdependent. We conclude that it is the level of U-STAT1 expression that counter-regulates the activity of tyrosine phosphorylated, pro-oncogenic STAT3.

So far the specific activities of unphosphorylated

STATs in cancer have attracted limited attention, but there are indications that the non-canonical functions of U-STAT1 in particular in CRC have a key role in many physiological and pathophysiological conditions. In regard to gastrointestinal diseases this may very well include chronic Inflammatory Bowel Disease, Chron's disease, and Ulcerative Colitis (*36*), Hepatitis B and C driven liver inflammation and hepatocellular carcinoma progression. Processes such as chronic infection and inflammation can trigger adenoma initiation, they contribute to carcinoma formation and they are critically involved for metastatic spread, all processes dominated by cytokine and JAK-STAT action (Figure 1). Expression and nuclear import of U-STAT1 as an important break counteracting inflammation and infection as well as cancer progression should obtain more attention, particular in light of pYSTAT3 action. Here, more mechanistic work both from animal studies and comparative pathology examinations of patient pools will be required. U-STAT1 as well as non-canonical signalling *via* STAT1 are of great importance to understand the YIN/YANG nature of the STAT1/STAT3 interplay. The medical relevance of the STAT1/STAT3 dualism is strong because one third of the global population suffers from GI tract disease. Many aspects causing GI tract disease originate from genetics, behaviour or infectious disease. Fatty liver disease and metabolic syndrome are associated with obesity and type II diabetes, which has a chronic inflammatory component. Both, hepatitis infection as well as chronic inflammatory colonic diseases have a connex to pathogens or infectious disease, but also to individual genetic polymorphisms. Ultimately these epithelial layer tissue damaging conditions can culminate into four times higher risk to develop CRC closely associated with chronic inflammatory colonic disease (*36,50*). Therefore, future drug targeting approaches should consider changes in the ratio of STAT1 to STAT3 expression, since it strongly impacts on carcinoma progression.

5. Conclusions

Abundance of nuclear STAT1 dictates oncogenic or proliferative/survival of STAT3 activity for disease outcome in CRC. The mechanisms by which combinatorial effects of STAT1/3 modulate transcription deserve more detailed studies. Our current view provides an explanation for the controversial influence of STAT3 on CRC development and progression. U-STAT1 can be regarded as a guardian to keep oncogenic STAT3-induced gene regulation at bay. Therefore, STAT1 has important regulatory activity on STAT3-mediated transcription and it is a key tumour suppressor, which can control too much STAT3 activity in a dose dependent manner. The antagonism of STAT1/3 in CRC balances also chronic inflammation and infection. We suggest that the ratio of STAT1/3 expression has prognostic power for progression of CRC or GI tract cancers. Despite a high degree of CRC heterogeneity, meaningful clinical predictions may be possible if both STAT3 and counterbalancing STAT1 are analysed in biopsies instead of the centric view on only one STAT protein, as the field did in the past. We hope this concept will be tested in other carcinoma settings to define its implications for the biology of major cancer killers better. We predict that the YIN/YANG STAT1/3 nature will be relevant for many different cancer cell types and inflammatory or infectious disease might follow a similar trait.

Acknowledgements

This work was supported by grant SFB-061 and SFB-F47 from the Austrian Science Fund (FWF) to RM; R21 AI103388/ NIAID /United States to XH and RM, and scholarships to the group of KHF by the Interdisziplinäres Zentrum für Klinische Forschung (IZKF) Jena and Deutsche Krebshilfe e.V. We are grateful for generous support by a private foundation from Liechtenstein to RM.

References

1. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. Science. 2013; 339:1546-1558.
2. Buchert M, Burns CJ, Ernst M. Targeting JAK kinase in solid tumors: Emerging opportunities and challenges. Oncogene. 2016; 35:939-951.
3. Hanahan D, Weinberg Robert A. Hallmarks of cancer: The next generation. Cell. 2011; 144:646-674.
4. Yu H, Lee H, Herrmann A, Buettner R, Jove R. Revisiting STAT3 signalling in cancer: New and unexpected biological functions. Nat Rev Cancer. 2014; 14:736-746.
5. Brooks AJ, Dai W, O'Mara ML, *et al*. Mechanism of activation of protein kinase JAK2 by the growth hormone receptor. Science. 2014; 344:1249783.
6. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: Impact on human disease and therapeutic intervention. Annu Rev Med. 2015; 66:311-328.
7. Rauch I, Muller M, Decker T. The regulation of inflammation by interferons and their STATs. JAKSTAT. 2013; 2:e23820.
8. O'Shea JJ, Plenge R. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. Immunity. 2012; 36:542-550.
9. Chen E, Staudt LM, Green AR. Janus kinase deregulation in leukemia and lymphoma. Immunity. 2012; 36:529-541.
10. Leitner NR, Witalisz-Siepracka A, Strobl B, Müller M. Tyrosine kinase 2 – surveillant of tumours and bona fide oncogene. Cytokine. 2015.
11. Meissl K, Macho-Maschler S, Müller M, Strobl B. The good, the bad and the unknown faces of STAT1 in carcinogenesis. Cytokine. 2015.
12. Yang S, Luo C, Gu Q, *et al*. Activating JAK1 mutation

may predict the sensitivity of JAK-STAT inhibition in hepatocellular carcinoma. Oncotarget. 2016; 7:5461-5469
13. Van Roosbroeck K, Cox L, Tousseyn T, Lahortiga I, Gielen O, Cauwelier B, De Paepe P, Verhoef G, Marynen P, Vandenberghe P, De Wolf-Peeters C, Cools J, Wlodarska I. JAK2 rearrangements, including the novel SEC31A-JAK2 fusion, are recurrent in classical Hodgkin lymphoma. Blood. 2011; 117:4056-4064.
14. Boisson B, Quartier P, Casanova JL. Immunological loss-of-function due to genetic gain-of-function in humans: Autosomal dominance of the third kind. Curr Opin Immunol. 2015; 0:90-105.
15. Casanova JL. Human genetic basis of interindividual variability in the course of infection. Proc Natl Acad Sci U S A. 2015; 112:E7118-E7127.
16. Kovacic B, Stoiber D, Moriggl R, Weisz E, Ott RG, Kreibich R, Levy DE, Beug H, Freissmuth M, Sexl V. STAT1 acts as a tumor promoter for leukemia development. Cancer Cell. 2006; 10:77-87.
17. Kim HS, Lee MS. STAT1 as a key modulator of cell death. Cell Signal. 2007; 19:454-465.
18. Simpson JAD, Al-Attar A, Watson NFS, Scholefield JH, Ilyas M, Durrant LG. Intratumoral T cell infiltration, MHC class I and STAT1 as biomarkers of good prognosis in colorectal cancer. Gut. 2010; 59:926-933.
19. Widschwendter A, Tonko-Geymayer S, Welte T, Daxenbichler G, Marth C, Doppler W. Prognostic significance of signal transducer and activator of transcription 1 activation in breast cancer. Clin Cancer Res. 2002; 8:3065-3074.
20. Hanada T, Kobayashi T, Chinen T, Saeki K, Takaki H, Koga K, Minoda Y, Sanada T, Yoshioka T, Mimata H, Kato S, Yoshimura A. IFNγ-dependent, spontaneous development of colorectal carcinomas in SOCS1-deficient mice. J Exp Med. 2006; 203:1391-1397.
21. Stark GR1, Darnell JE Jr. The JAK-STAT pathway at twenty. Immunity. 2012; 36:503-514.
22. Chen E, Staudt Louis M, Green Anthony R. Janus kinase deregulation in leukemia and lymphoma. Immunity. 2012; 36:529-541.
23. Kortylewski M, Yu H. Role of Stat3 in suppressing anti-tumor immunity. Curr Opin Immunol. 2008; 20:228-233.
24. Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated *via* activation of STAT3. Genes Dev. 1998; 12:2048-2060.
25. Gaffen SL, Jain R, Garg AV, Cua DJ. The IL-23-IL-17 immune axis: From mechanisms to therapeutic testing. Nat Rev Immunol. 2014; 14:585-600.
26. Pilati C, Letouzé E, Nault J-C, *et al*. Genomic profiling of hepatocellular adenomas reveals recurrent FRK-activating mutations and the mechanisms of malignant transformation. Cancer Cell. 2014; 25:428-441.
27. Crescenzo R, Abate F, Lasorsa E, *et al*. Convergent mutations and kinase fusions lead to oncogenic STAT3 activation in anaplastic large cell lymphoma. Cancer Cell. 2015; 27:516-532.
28. Yao C, Su L, Shan J, Zhu C, Liu L, Liu C, Xu Y, Yang Z, Bian X, Shao J, Li J, Lai M, Shen J, Qian C. IGF/STAT3/NANOG/Slug signaling axis simultaneously controls epithelial-mesenchymal transition and stemness maintenance in colorectal cancer. Stem Cells. 2016; 34:820-831.
29. Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc Natl Acad Sci U S A. 1997; 94:3801-3804.
30. Klupp F, Diers J, Kahlert C, Neumann L, Halama N, Franz C, Schmidt T, Lasitschka F, Warth A, Weitz J, Koch M, Schneider M, Ulrich A. Expressional STAT3/STAT5 ratio is an independent prognostic marker in colon carcinoma. Ann Surg Oncol. 2015; 22:1548-1555.
31. Corvinus FM, Orth C, Moriggl R, Tsareva SA, Wagner S, Pfitzner EB, Baus D, Kaufmann R, Huber LA, Zatloukal K, Beug H, Ohlschläger P, Schütz A, Halbhuber KJ, Friedrich K. Persistent STAT3 activation in colon cancer is associated with enhanced cell proliferation and tumor growth. Neoplasia. 2005; 7:545-555.
32. Tsareva SA, Moriggl R, Corvinus FM, Wiederanders B, Schütz A, Kovacic B, Friedrich K. Signal transducer and activator of transcription 3 activation promotes invasive growth of colon carcinomas through matrix metalloproteinase induction. Neoplasia. 2007; 9:279-291.
33. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. IL-6 and STAT3 are required for survival of intestinal epithelial cells and development of colitis associated cancer. Cancer cell. 2009; 15:103-113.
34. Bollrath J, Phesse TJ, von Burstin VA, *et al*. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell. 2009; 15:91-102.
35. Quante M, Varga J, Wang TC, Greten FR. The gastrointestinal tumor microenvironment. Gastroenterology. 2013; 145:63-78.
36. Grivennikov SI. Inflammation and colorectal cancer: Colitis-associated neoplasia. Semin Immunopathol. 2013; 35:229-244.
37. Gordziel C, Bratsch J, Moriggl R, Knösel T, Friedrich K. Both STAT1 and STAT3 are favourable prognostic determinants in colorectal carcinoma. Br J Cancer. 2013; 109:138-146.
38. Musteanu M, Blaas L, Mair M, Schlederer M, Bilban M, Tauber S, Esterbauer H, Mueller M, Casanova E, Kenner L, Poli V, Eferl R. Stat3 is a negative regulator of intestinal tumor progression in Apc^{Min} mice. Gastroenterology. 2010; 138:1003-1011.e1005.
39. Nivarthi H, Gordziel C, Themanns M, *et al*. The ratio of STAT1 to STAT3 expression is a determinant of colorectal cancer growth. Oncotarget. 2016; 7:51096-51106
40. Neculai D, Neculai AM, Verrier S, Straub K, Klumpp K, Pfitzner E, Becker S. Structure of the unphosphorylated STAT5a dimer. J Biol Chem. 2005; 280:40782-40787.
41. Jayavelu AK, Muller JP, Bauer R, *et al*. NOX4-driven ROS formation mediates PTP inactivation and cell transformation in FLT3ITD-positive AML cells. Leukemia. 2016; 30:473-483.
42. Themanns M, Mueller KM, Kessler SM, *et al*. Hepatic deletion of janus kinase 2 counteracts oxidative stress in mice. Sci Rep. 2016; 6:34719.
43. Cheon H, Yang J, Stark GR. The functions of signal transducers and activators of transcriptions 1 and 3 as cytokine-inducible proteins. J Interferon Cytokine Res. 2011; 31:33-40.
44. Regis G, Pensa S, Boselli D, Novelli F, Poli V. Ups and downs: The STAT1:STAT3 seesaw of Interferon and gp130 receptor signalling. Semin Cell Dev Biol. 2008; 19:351-359.
45. Cheon H, Stark GR. Unphosphorylated STAT1 prolongs the expression of interferon-induced immune regulatory

genes. Proc Natl Acad Sci U S A. 2009; 106:9373-9378.
46. Brown S, Zeidler MP. Unphosphorylated STATs go nuclear. Curr Opin Genet Dev. 2008; 18:455-460.
47. Gough DJ, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, Levy DE. Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. Science. 2009; 324:1713-1716.
48. Sgrignani J, Olsson S, Ekonomiuk D, Genini D, Krause R, Catapano CV, Cavalli A. Molecular determinants for unphosphorylated STAT3 dimerization determined by integrative modeling. Biochemistry. 2015; 54:5489-5501.
49. Stancato LF, David M, Carter-Su C, Larner AC, Pratt WB. Preassociation of STAT1 with STAT2 and STAT3 in separate signalling complexes prior to cytokine stimulation. J Biol Chem. 1996; 271:4134-4137.
50. Triantafillidis JK, Nasioulas G, Kosmidis PA. Colorectal cancer and inflammatory bowel disease: Epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies. Anticancer Res. 2009; 29:2727-2737.

(Received December 29, 2016; Revised January 26, 2017; Accepted January 27, 2017)

Review

DOI: 10.5582/bst.2016.01226

Role of TRPV channels in regulating various pancreatic β-cell functions: Lessons from *in vitro* studies

Marek Skrzypski[1], Maria Billert[1], Stefan Mergler[2], Noushafarin Khajavi[3], Krzysztof W. Nowak[1], Mathias Z. Strowski[4,5,*]

[1] *Department of Animal Physiology and Biochemistry, Poznań University of Life Sciences, Poznań, Poland;*
[2] *Department of Ophthalmology, Charité University Medicine Berlin, Germany;*
[3] *Institute for Experimental Pediatric Endocrinology, Charite, University-Medicine Berlin, Berlin, Germany;*
[4] *Department of Hepatology and Gastroenterology and Interdisciplinary Centre of Metabolism: Endocrinology, Diabetes and Metabolism; Charité-University Medicine Berlin, Berlin, Germany;*
[5] *Department of Internal Medicine-Gastroenterology, Park-Klinik Weissensee, Berlin, Germany.*

Summary **Pancreatic β-cell functions are regulated by a variety of endogenous and exogenous factors. Calcium is one of the most potent triggers of β-cell growth, insulin production and exocytosis. Recently, others and we showed that TRPV channels are expressed in insulin producing cell lines and/or primary β-cells. These channels modulate calcium ions, insulin secretion and cell proliferation. Besides the classical roles of TRPV channels in the sensory system, there are also novel functions described in non-excitable cells such as in insulin-producing β-cells. This review summarises the current knowledge about the expression and the role of TRPV channels in controlling β-cell functions based upon studies performed in isolated primary β-cells as well as permanent β-cell models.**

Keywords: Apoptosis, beta cell, calcium, insulin, TRPV, proliferation

1. Introduction

Pancreatic islets are composed of several types of endocrine cells including α, β, δ, ε and PP cells, which produce glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide, respectively (*1*). Approximately 80% of endocrine cells composing pancreatic islet are represented by β-cells (*2*). Insulin is the major hormone responsible for maintenance of normoglycemia by promoting glucose transport to insulin-depended tissues, suppression of gluconeogenesis and enhancing glycogenesis (*3*). Furthermore, insulin has pleiotropic effects such as modulation of learning, memory and reproduction processes (*4*). The loss of pancreatic β-cells and impaired insulin production are hallmarks of type 1 and type 2 diabetes (*5*). Late complications of persistent chronic hyperglycemia and other metabolic derangements are nephropathy, neuropathy, retinopathy or other cardiovascular diseases (*6,7*). Notably, the prevalence of both types of diabetes is currently increasing (*8*). There is growing evidence that changes in intracellular calcium levels play a prominent physiological role in β-cells. Ca^{2+} ions modulate insulin secretion, expression and proliferation as well as β-cell growth and apoptosis (*9,10*). Calcium changes related to β-cell death or insulin exocytosis were reported in beta cells derived from diabetic subjects as well as in insulinomas (a neuroendocrine tumor derived from β-cells). It is well-known that in β-cells Ca^{2+} influx depends on the activity of a large number of ion channels. However, voltage-operated Ca^{2+} channels (VOCCs) play a predominant role (*11*). In addition, recent studies in pancreatic primary β-cell and insulin producing permanent β-cell lines indicate that Ca^{2+} ions entrance is regulated by transient receptor potential vanilloid (TRPV) channels. In the present work, we review the current knowledge about the role of these channels in controlling primary β-cell functions including calcium homeostasis, insulin secretion as well as cell growth and death.

Released online in J-STAGE as advance publication February 2, 2017.

**Address correspondence to:*
Dr. Mathias Z. Strowski, Department of Hepatology and Gastroenterology & the Interdisciplinary Centre of Metabolism: Endocrinology, Diabetes and Metabolism, Charité-University Medicine Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany.
E-mail: mathias.strowski@charite.de

2. TRPV as subfamily of TRP channels

Transient potential receptor (TRP) channels are members of a superfamily of cation channels. They are subdivided into six groups with differences in their amino acid sequences, activation factors and modulatory mechanisms. TRP channels (TRPs) were firstly described in *Drosophila,* where *trp* gene mutation carried a characteristic defect in phototransduction (*12*). All TRPs consists of six putative transmembrane protein subunits with the pore loop between the fifth and sixth segment (S5-S6). They differ in cytoplasmic amino and carboxy termini (*13,14*). The 28 mammalian TRP channels are divided into six different subfamilies based on sequence homology: canonical (TRPC), melastatin (TRPM), vanilloid (TRPV), polycystin (TRPP), ankyrin (TRPA), and mucolipin (TRPML1-3) receptors. TRPs are activated by wide range of stimuli including mechanical and osmotic changes, temperature or intracellular ligands. The TRPV channel family consists of six members (TRPV1-6). All of them contain 3-6 intracellular N-terminal ankyrin repeats domain, common protein-protein interaction motif. They can form homomeric and/or heteromeric, tetrameric configurations. TRPVs can be subdivided into two groups: TRPV1-4 and TRPV5-6. TRPV1-4 are thermosensitive, non-selective calcium channels having permeability ratio P_{Ca}/P_{Na} between 5 and 10, whereas TRPV5-6 are highly Ca^{2+}-selective ion channels, with P_{Ca}/P_{Na} over 100 (*15-17*). TRPV1 was the first TRPV subtype to be identified and is therefore the most extensively characterized channel. TRPV1 is activated by vanilloid compounds such as capsaicin (an active ingredient in hot chili peppers), noxious heat (≥ 43°C) (*18*), low pH (≤ 5.9) or voltage (*19,20*). TRPV1 is not only involved in classical pain sensations *via* nociceptor activation (*21*), but also contribute to novel functions in non-neuronal or tumor cells (*22*). TRPV1 activity can be modulated by protein kinase A (*23,24*), protein kinase C (*25*) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation (*26*). Another regulatory molecule of TPRV1 is PIP2, which binds to the C- and N-terminal region of TRPV1 and thus regulates this channel activation (*27,28*). TRPV2 is approx. 50% identical to TRPV1, but is insensitive to capsaicin and low pH. Very recent results indicated a link between TRPV1 and VEGF in benign tumor cells (*29*). The high-heat sensor TRPV2 is activated by noxious heat with an activation threshold greater than 52°C (*30*). Moreover, TRPV2 is activated by physical stimuli including mechanical stretch and osmotic swelling (*31*). This channel is highly expressed in various tissue types such as nervous system, immune-related tissues and cells, vascular smooth muscle, and endothelial cells. TRPV3 is a moderate heat-activated TRP channel with 40% identity to TRPV1 (*32*). It's temperature activation threshold in the physiological temperatures is in the range of 32 to 39°C. TRPV3 can be also activated by camphor, thymol and 2- aminoethoxydiphenyl borate (2-APB). This channel plays a major role in regulating physiological skin homeostasis by regulating proliferation and apoptosis in epidermal keratinocytes (*33*). In addition, it has been reported that TRPV3 is involved in hairlessness combined with dermatitis (*34*). TRPV4 can be activated by physical stimuli such as cell swelling, moderate heat (> 24-27°C), shear stress and chemical ligands (*e.g*. phorbol esters, endocannabinoids, arachidonic acids or synthetic compound GSK1016790) (*35-37*). This channel is widely expressed in various tissues such as brain, vascular endothelium, bladder, kidney and multiple excitable, and non-excitable peripheral cell types including eye surface cells (*38*). TRPV5 and TRPV6 are the only calcium selective TRP channels. Although they share 74% amino acids identity, they are only 22-24% identical to other TRPVs. TRPV5 and TRPV6 maintain calcium homeostasis in epithelial tissues (*39*). Both channels can be activated by fluid shear force (*40*). TRPV5 is highly expressed in the kidney, where it regulates Ca^{2+} excretion into the urine (*41,42*). It is regulated by Klotho, a β-glucuronidase that hydrolyzes extracellular sugar residues on TRPV5 (*43*). TRPV6 is involved in the active Ca^{2+} absorption and is predominantly expressed in the small intestine (*44*). Increased expression of TRPV6 has been observed in various human tumors, including prostate cancer (*45*).

3. Expression of TRPVs in pancreatic β-cells and insulin producing cell lines and their role in controlling insulin secretion

3.1. *TRPV1*

Initially, TRPV1 mRNA expression was detected in rat INS-1 and RINm5F insulin producing (insulinoma) β-cell lines (*46*). Immunofluorescence staining revealed that TRPV1 is expressed in endocrine cells of rat islets. Furthermore, it was reported that TRPV1 activation by the agonist capsaicin stimulated insulin secretion from INS-1 cells (*46*). Capsaicin-induced insulin secretion was attenuated by EGTA or the TRPV1 blocker capsazepine. In the same study, insulinotropic action of capsaicin was confirmed in animal experiments. It was found that in rats s.c. injection of capsaicin resulted higher plasma insulin levels as compared with vehicle-treated animals. The presence of TRPV1 protein was also identified in rat insulinoma INS-1E cells (cell line derived from INS-1 cells) (*47,48*).

Consistent with previous data, it was reported that the TRPV1 agonists capsaicin and AM404 are able to rise intracellular calcium levels in INS-1E cells, which depends upon TRPV1 channel activation (*47*). However, the same research group failed to detect TRPV1 immunoreactivity in human primary β- as well as human insulinoma cells (*47*). In agreement with these

observations, capsaicin did not increase Ca^{2+} levels in human primary β-cells. Furthermore, capsaicin failed to affect calcium influx in rat primary β-cells (*47*). Consistent with the incapability of capsaicin to affect calcium levels in rat primary β-cells, others reported that rat pancreatic islets lack TRPV1 (*49*). Similar data were obtained by Diaz-Garcia *et al*. who studied whether TRPV1 is expressed in rat pancreatic β-cells (*50*). This study showed that primary β-cells isolated from adult rats do not express TRPV1 mRNA. To further identity factors that can induce TRPV1 expression in β-cells, the same group tested whether TRPV1 can be induced by the nerve growth factor (NGF). It was found that NGF stimulates TRPV1 mRNA expression. However, NGF failed to induce TRPV1 protein production. The lack of TRPV1 expression was also reported in β-cells isolated from neonate rats (*50*). Furthermore, this research laboratory was not able to detect TRPV1 protein production in RINm5F cells, which was inconsistent with previous work (*46*). Overall, these data indicate that TRPV1 protein and mRNA are present in rat insulinoma cells (INS-1 and INS-1E), whereas TRPV1 is absent in rat and human primary β-cells as well as human insulinoma. Moreover, it appears that TRPV1 directly stimulates insulin secretion from rat insulinoma cells but not from human or rodent primary β-cells or human insulinoma. However, contribution of TRPV1 in modulating insulin secretion *in vivo* cannot be completely excluded. For instance, animal studies indicated that pancreatic islets do not express TRPV1 but they are innervated by TRPV1-expressing sensory nerve fibers and systemic capsaicin treatment potentiated glucose induced insulin secretion (*49*). Thus, it is likely TRPV1 may modulate insulin levels despite its absence in β-cells.

3.2. *TRPV2*

Both TRPV2 mRNA and protein were detected in murine insulin producing min6 cell line as well as in primary β-cells (*51*). The vast part of data describing the role of TRPV2 in controlling intracellular calcium levels and insulin secretion was obtained using cells with TRPV2 downregulation. Hisanaga *et al*. found that insulin stimulates intracellular calcium levels in min6 cells, which is mediated through insulin-induced TRPV2 translocation to the plasma membrane (*51*). Suppression of TRPV2 protein production by shRNA or blockade by tranilast (an inhibitor of TRPV2) inhibited insulin-induced Ca^{2+} influx in min6 cells (*51*). Furthermore, this study reported that transilast supressed high glucose- and potassium-induced insulin secretion from min6 cells. Importantly, the same work found that insulin-induced TRPV2 translocation from cytoplasm to the plasma membrane accurse also in mouse primary β-cells. In addition, downregulation of TRPV2 protein production resulted in lower rate of glucose- and potassium-induced insulin secretion in mouse primary β-cells. The contribution of insulin to the translocation of TRPV2 to the plasma membrane was confirmed by another study, which showed that this action is mediated *via* PI3K. Furthermore, this study showed also that TRPV4 is involved in first but not second phase of insulin release (*52*). Another work investigated the role of Klotho gene in controlling insulin secretion from min6 cells. It was found that overexpression of Klotho leads to increased plasma membrane retention of TRPV2 in min6 cells (*53*). By contrast, Klotho knockout reduced plasma membrane retention of TRPV2. Furthermore, pharmacological inhibition of TRPV2 by tranilast supressed both, Klotho-induced calcium influx and glucose-induced insulin secretion in min6 cells. In summary, these data collectively showed that TRPV2 stimulates insulin secretion from min6 and mouse primary β-cells. Furthermore, there is convincing evidence that its translocation to the plasma membrane is controlled by insulin as well as Klotho-dependent manner.

3.3. *TRPV3*

According to our knowledge data indicating functional expression of TRPV3 in primary β-cells or insulin producing cells are not available.

3.4. *TRPV4*

Expression of TRPV4 mRNA was found in min6, INS-E cells and rat pancreatic islets (*54,55*). There are two studies addressing the question whether TRPV4 modulates intracellular calcium levels in insulin producing cells. Casas *et al*. using min6 cells reported that human islet amyloid polypeptide (hIAPP) increases that intracellular calcium level in min6 cells (*54*). This effects of hIAPP was supressed by addition of gadolinium a non-specific TRP channel inhibitor. Another experiments showed that the hIAPP-induced intracellular Ca^{2+} rise in min6 cells can be also suppressed by ruthenium red, which is a non-competitive pan inhibitor of TRPs, in particularly TRPV channels including TRPV4 (and also TRPV1) (*56,57*). To assess whether TRPV4 mediates hIAPP-induced intracellular Ca^{2+} rise the authors of this study utilised siRNA technique. These experiments demonstrated that elevation of Ca^{2+} by hIAPP was supressed in cells with TRPV4 protein downregulation. Overall, this study showed for the first time that TRPV4 activation leads to increase Ca^{2+} levels in min6 cells. Recently, we studied the effects of TRPV4 activation on intracellular Ca^{2+} levels in INS-1E cells. We found that TRPV4 activation by moderate heating, hypotonic challenge as well as its agonist 4αPDD increased Ca^{2+} levels (*55*). Stimulation of Ca^{2+} elevation was attenuated in INS-1E cells with TRPV4 protein downregulation suggesting these effects are mediated *via* TRPV4. Moreover, we found elevation

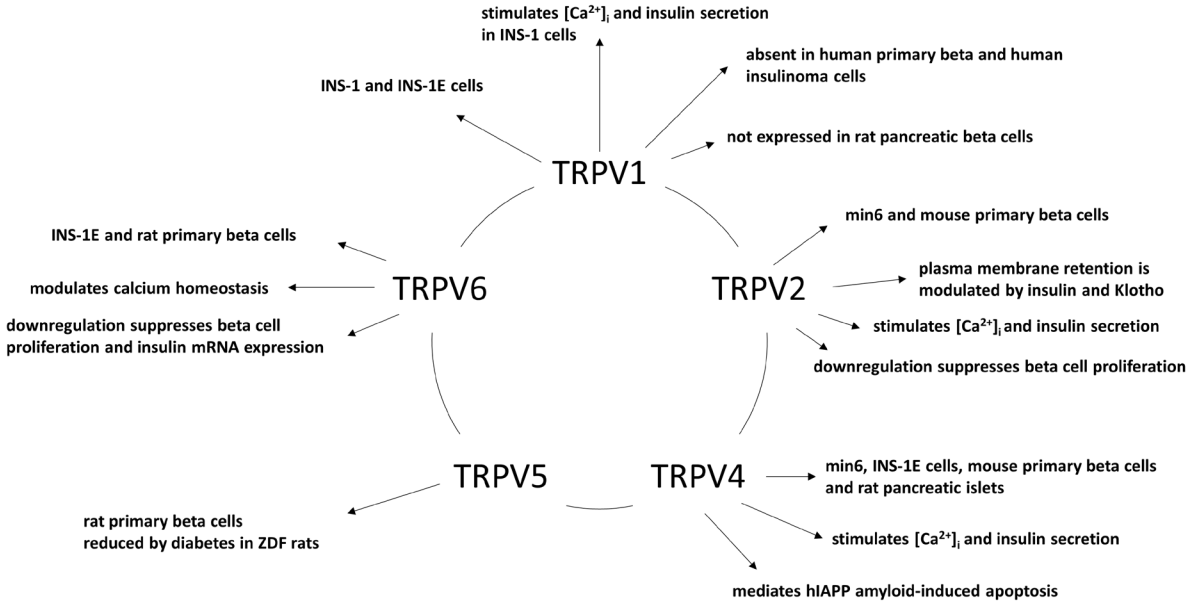

Figure 1. Expression and potential function of TRPV channels in the endocrine pancreas.

of Ca^{2+} influx induced by 4αPDD was accompanied by increased insulin secretion. Stimulation of insulin secretion by 4αPDD was additionally confirmed in isolated rat pancreatic islets. In summary, these data revealed that TRPV4 modulates calcium homeostasis and insulin secretion in insulin producing INS-1E cells, suggesting its influence on insulin secretion in primary β-cells. Nevertheless, whether TRPV4 contribute to insulin secretion *in vivo* remains an open question.

3.5. *TRPV5*

Janssen *et al*. reported that TRPV5 mRNA is present in RNA isolated from whole rat pancreas (*58*). The authors of this work also studied immunofluorescence localization of this channel in pancreatic islets. Double immunofluorescence studies revealed that TRPV5 colocalize with β- but not α-cells. It was also observed that TRPV5 expression decreases during the progression of diabetes in ZDF Rats (*58*). In contrast to rat islets, others reported no expression of TRPV5 in mouse islets (*59*). To our best knowledge, there are no data demonstrating contribution of TRPV5 to calcium homeostasis and insulin secretion in β-cells.

3.6. *TRPV6*

Human studies appeared that TRPV6 expression is restricted only to exocrine pancreas (*60*). Recently, however, we reported that TRPV6 mRNA is expressed in rat INS-1E cells and rat pancreatic islets (*61*). Using double immunofluorescence staining we detected that TRPV6 is expressed in rat β-cells. Furthermore, we found that TRPV6 downregulation is associated with impaired calcium influx in INS-1E cells. On the other hand, we did not find any difference in basal and glucose-induced insulin secretion between cells with normal and supressed TRPV6 protein production. However, we showed that suppression of TRPV6 expression leads to suppression of insulin mRNA expression in INS-1E cells. These data suggest that TRPV6 modulates insulin mRNA expression but not secretion. However, since these data were obtained using INS-E cells further studies are needed to elucidate the role of TRPV6 in primary β-cells.

4. TRPVs in controlling β-cell growth and death

Keeping mind the pivotal role of calcium ions in modulating cell proliferation and apoptosis, several studies investigated whether selected TRPVs are involved in pancreatic β-cell proliferation or death. Hisanaga *et al*. found that suppression of TRPV2 protein production in min6 is associated with impaired glucose- and serum-induced cell proliferation but not enhanced cell death (*51*). Their results showed that TRPV2 is required to maintain min6 cell proliferation. A promitogenic role of TRPVs in insulin producing cells is also supported by our recent data. We found that suppression of TRPV6 protein production by siRNA technique resulted in reduced cell proliferation and viability in INS-1E cells (*61*). Likewise previous studies, these effects were detected in the presence of glucose and serum. Studying potential mechanism by which TRPV6 may control cell growth, we identified that decreased cell proliferation in TRPV6 siRNA transfected cells was accompanied by reduced activity of calcineurin/NFAT

signalling and ERK1/2 phosphorylation. Since these pathways were involved in β-cell proliferation (*62,63*), these data strongly suggested that TRPV6 may trigger INS-E cell growth *via* calcineurin/NFAT- and ERK1/2-dependent mechanisms. Notably, similar data indicating the link between TRPV6/NFAT signalling and cell proliferation were described in human neuroendocrine pancreatic BON cells (*64*). By contrast, to promitogenic effects of TRPV2 and TRPV6 in insulin producing cells, it was demonstrated that TRPV4 activation by hIAPP stimulate β-cell death. Casas *et al*. found that activation of TRPV4 by hIAPP induces the endoplasmic reticulum (ER) stress response and apoptosis in min6 and murine primary β-cells (*54*). Since increased accumulation of hIAPP aggregates associates with β-cell death in type 2 diabetic patients (*65*), these re*sul*ts suggest that TRPV4 can contribute to β-cell loss in type 2 diabetes. The role of other TRPVs in controlling β-cell growth remains unknown.

The TRPV channel expression and function in the endocrine pancreas are shown in Figure 1.

5. Concluding remarks

In summary, there is evidence that TRPV1, TRPV2, TRPV4 and TRPV6 are expressed in rodent insulinoma cell lines which are involved in calcium regulation. TRPV1, TRPV2 and TRPV4 were implicated in insulin secretion in rodent insulin producing cells. Furthermore, there is evidence that TRPV2 controls Ca^{2+} influx and insulin secretion in mouse primary β-cells. By contrast, TRPV1 is rather not expressed in rat primary β-cells or human insulinoma. The loss of TRPV2 protein production in murine min6 cells and TRPV6 in rat INS-1E cells leads to supressed cell proliferation. On the other hand, TRPV4 activation by hIAPP results in min6 and mouse primary β-cell apoptosis.

Acknowledgements

M. Skrzypski was supported by Iuventus plus grant (IP 2014 042273) from the Polish Ministry of Science and Higher Education, and Mathias Z. Strowski was supported by the Deutsche Forschungsgemeinschaft (STR 558/9-1).

References

1. Steiner DJ, Kim A, Miller K, Hara M. Pancreatic islet plasticity: Interspecies comparison of islet architecture and composition. Islets. 2010; 2:135-145.
2. Peiris H, Bonder CS, Coates PT, Keating DJ, Jessup CF. The beta-cell/EC axis: How do islet cells talk to each other? Diabetes. 2014; 63:3-11.
3. Wilcox G. Insulin and insulin resistance. Clin Biochem Rev. 2005; 26:19-39.
4. Duarte AI, Moreira PI, Oliveira CR. Insulin in central nervous system: more than just a peripheral hormone. J Aging Res. 2012; 2012:384017.
5. Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes. 2005; 54 Suppl 2:S97-107.
6. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev. 2013; 93:137-188.
7. Devi TS, Lee I, Huttemann M, Kumar A, Nantwi KD, Singh LP. TXNIP links innate host defense mechanisms to oxidative stress and inflammation in retinal Muller glia under chronic hyperglycemia: implications for diabetic retinopathy. Exp Diabetes Res. 2012; 2012:438238.
8. Balakumar P, Maung UK, Jagadeesh G. Prevalence and prevention of cardiovascular disease and diabetes mellitus. Pharmacol Res. 2016; 113:600-609.
9. Chang-Chen KJ, Mullur R, Bernal-Mizrachi E. β-cell failure as a complication of diabetes. Rev Endocr Metab Disord. 2008; 9:329-343.
10. Ramadan JW, Steiner SR, O'Neill CM, Nunemaker CS. The central role of calcium in the effects of cytokines on beta-cell function: implications for type 1 and type 2 diabetes. Cell Calcium. 2011; 50:481-490.
11. Rorsman P, Braun M, Zhang Q. Regulation of calcium in pancreatic alpha- and beta-cells in health and disease. Cell calcium. 2012; 51:300-308.
12. Cosens DJ, Manning A. Abnormal electroretinogram from a Drosophila mutant. Nature. 1969; 224:285-287.
13. Venkatachalam K, Montell C. TRP channels. Annu Rev Biochem. 2007; 76:387-417.
14. Harteneck C, Plant TD, Schultz G. From worm to man: three subfamilies of TRP channels. Trends Neurosci. 2000; 23:159-166.
15. Ramsey IS, Delling M, Clapham DE. An introduction to TRP channels. Annu Rev Physiol. 2006; 68:619-647.
16. Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential cation channels in disease. Physiol Rev. 2007; 87:165-217.
17. den Dekker E, Hoenderop JG, Nilius B, Bindels RJ. The epithelial calcium channels, TRPV5 & TRPV6: from identification towards regulation. Cell calcium. 2003; 33:497-507.
18. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature. 1997; 389:816-824.
19. Gunthorpe MJ, Harries MH, Prinjha RK, Davis JB, Randall A. Voltage- and time-dependent properties of the recombinant rat vanilloid receptor (rVR1). J Physiol. 2000; 525 Pt 3:747-759.
20. Voets T, Droogmans G, Wissenbach U, Janssens A, Flockerzi V, Nilius B. The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. Nature. 2004; 430:748-754.
21. Price DD. Psychological and neural mechanisms of the affective dimension of pain. Science. 2000; 288:1769-1772.
22. Fernandes ES, Fernandes MA, Keeble JE. The functions of TRPA1 and TRPV1: moving away from sensory nerves. Br J Pharmacol. 2012; 166:510-521.
23. Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt. cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron. 2002; 35:721-731.
24. Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C, Kress M. PKA/AKAP/VR-1 module:

A common link of Gs-mediated signaling to thermal hyperalgesia. J Neurosci. 2002; 22:4740-4745.
25. Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW 4th. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). Proc Natl Acad Sci U S A. 2003; 100:12480-12485.
26. Jung J, Shin JS, Lee SY, Hwang SW, Koo J, Cho H, Oh U. Phosphorylation of vanilloid receptor 1 by Ca^{2+}/calmodulin-dependent kinase II regulates its vanilloid binding. J Biol Chem. 2004; 279:7048-7054.
27. Ufret-Vincenty CA, Klein RM, Hua L, Angueyra J, Gordon SE. Localization of the PIP2 sensor of TRPV1 ion channels. J Biol Chem. 2011; 286:9688-9698.
28. Grycova L, Holendova B, Bumba L, Bily J, Jirku M, Lansky Z, Teisinger J. Integrative binding sites within intracellular termini of TRPV1 receptor. PloS One. 2012; 7:e48437.
29. Garreis F, Schroder A, Reinach PS, Zoll S, Khajavi N, Dhandapani P, Lucius A, Pleyer U, Paulsen F, Mergler S. Upregulation of Transient Receptor Potential Vanilloid Type-1 Channel Activity and Ca^{2+} Influx Dysfunction in Human Pterygial Cells. Invest Ophthalmol Vis Sci. 2016; 57:2564-2577.
30. Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D. A capsaicin-receptor homologue with a high threshold for noxious heat. Nature. 1999; 398:436-441.
31. Peralvarez-Marin A, Donate-Macian P, Gaudet R. What do we know about the transient receptor potential vanilloid 2 (TRPV2) ion channel? The FEBS J. 2013; 280:5471-5487.
32. Xu H, Ramsey IS, Kotecha SA, Moran MM, Chong JA, Lawson D, Ge P, Lilly J, Silos-Santiago I, Xie Y, DiStefano PS, Curtis R, Clapham DE. TRPV3 is a calcium-permeable temperature-sensitive cation channel. Nature. 2002; 418:181-186.
33. Nilius B, Biro T, Owsianik G. TRPV3: time to decipher a poorly understood family member! J Physiol. 2014; 592:295-304.
34. Xiao R, Tian J, Tang J, Zhu MX. The TRPV3 mutation associated with the hairless phenotype in rodents is constitutively active. Cell calcium. 2008; 43:334-343.
35. Thorneloe KS, Sulpizio AC, Lin Z, *et al. N*-((1*S*)-1-{[4-((2*S*)-2-{[(2,4-dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1-piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part I. J Pharmacol Exp Ther. 2008; 326:432-442.
36. Vriens J, Watanabe H, Janssens A, Droogmans G, Voets T, Nilius B. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. Proc Natl Acad Sci U S A. 2004; 101:396-401.
37. Watanabe H, Davis JB, Smart D, Jerman JC, Smith GD, Hayes P, Vriens J, Cairns W, Wissenbach U, Prenen J, Flockerzi V, Droogmans G, Benham CD, Nilius B. Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. J Biol Chem. 2002; 277:13569-13577.
38. Mergler S, Valtink M, Taetz K, Sahlmuller M, Fels G, Reinach PS, Engelmann K, Pleyer U. Characterization of transient receptor potential vanilloid channel 4 (TRPV4) in human corneal endothelial cells. Exp Eye Res. 2011; 93:710-719.
39. van Abel M, Hoenderop JG, Bindels RJ. The epithelial calcium channels TRPV5 and TRPV6: regulation and implications for disease. Naunyn Schmiedebergs Arch Pharmacol. 2005; 371:295-306.
40. Cha SK, Kim JH, Huang CL. Flow-induced activation of TRPV5 and TRPV6 channels stimulates Ca^{2+}-activated K^+ channel causing membrane hyperpolarization. Biochim Biophys Acta. 2013; 1833:3046-3053.
41. Dimke H, Hoenderop JG, Bindels RJ. Molecular basis of epithelial Ca^{2+} and Mg^{2+} transport: insights from the TRP channel family. J Physiol. 2011; 589:1535-1542.
42. Hoenderop JG, van Leeuwen JP, van der Eerden BC, Kersten FF, van der Kemp AW, Merillat AM, Waarsing JH, Rossier BC, Vallon V, Hummler E, Bindels RJ. Renal Ca^{2+} wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. J Clin Inves. 2003; 112:1906-1914.
43. Chang Q, Hoefs S, van der Kemp AW, Topala CN, Bindels RJ, Hoenderop JG. The β-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. Science. 2005; 310:490-493.
44. van de Graaf SF, Boullart I, Hoenderop JG, Bindels RJ. Regulation of the epithelial Ca^{2+} channels TRPV5 and TRPV6 by 1α,25-dihydroxy Vitamin D_3 and dietary Ca^{2+}. J Steroid Biochem Mol Biol. 2004; 89-90:303-308.
45. Lehen'kyi V, Raphael M, Prevarskaya N. The role of the TRPV6 channel in cancer. J Physiol. 2012; 590:1369-1376.
46. Akiba Y, Kato S, Katsube K, Nakamura M, Takeuchi K, Ishii H, Hibi T. Transient receptor potential vanilloid subfamily 1 expressed in pancreatic islet beta cells modulates insulin secretion in rats. Biochem Biophys Res Commun. 2004; 321:219-225.
47. Fagelskiold AJ, Kannisto K, Bostrom A, Hadrovic B, Farre C, Eweida M, Wester K, Islam MS. Insulin-secreting INS-1E cells express functional TRPV1 channels. Islets. 2012; 4:56-63.
48. Malenczyk K, Jazurek M, Keimpema E, Silvestri C, Janikiewicz J, Mackie K, Di Marzo V, Redowicz MJ, Harkany T, Dobrzyn A. CB1 cannabinoid receptors couple to focal adhesion kinase to control insulin release. J Biol Chem. 2013; 288:32685-32699.
49. Gram DX, Ahren B, Nagy I, Olsen UB, Brand CL, Sundler F, Tabanera R, Svendsen O, Carr RD, Santha P, Wierup N, Hansen AJ. Capsaicin-sensitive sensory fibers in the islets of Langerhans contribute to defective insulin secretion in Zucker diabetic rat, an animal model for some aspects of human type 2 diabetes. Eur J Neurosci. 2007; 25:213-223.
50. Diaz-Garcia CM, Morales-Lazaro SL, Sanchez-Soto C, Velasco M, Rosenbaum T, Hiriart M. Role for the TRPV1 channel in insulin secretion from pancreatic beta cells. J Membr Biol. 2014; 247:479-491.
51. Hisanaga E, Nagasawa M, Ueki K, Kulkarni RN, Mori M, Kojima I. Regulation of calcium-permeable TRPV2 channel by insulin in pancreatic beta-cells. Diabetes. 2009; 58:174-184.
52. Aoyagi K, Ohara-Imaizumi M, Nishiwaki C, Nakamichi Y, Nagamatsu S. Insulin/phosphoinositide 3-kinase pathway accelerates the glucose-induced first-phase insulin secretion through TrpV2 recruitment in pancreatic β-cells. Biochem J. 2010; 432:375-386.
53. Lin Y, Sun Z. Antiaging gene Klotho enhances glucose-induced insulin secretion by up-regulating

plasma membrane levels of TRPV2 in MIN6 β-cells. Endocrinology. 2012; 153:3029-3039.
54. Casas S, Novials A, Reimann F, Gomis R, Gribble FM. Calcium elevation in mouse pancreatic beta cells evoked by extracellular human islet amyloid polypeptide involves activation of the mechanosensitive ion channel TRPV4. Diabetologia. 2008; 51:2252-2262.
55. Skrzypski M, Kakkassery M, Mergler S, Grotzinger C, Khajavi N, Sassek M, Szczepankiewicz D, Wiedenmann B, Nowak KW, Strowski MZ. Activation of TRPV4 channel in pancreatic INS-1E beta cells enhances glucose-stimulated insulin secretion *via* calcium-dependent mechanisms. FEBS lett. 2013; 587:3281-3287.
56. Sullivan MN, Francis M, Pitts NL, Taylor MS, Earley S. Optical recording reveals novel properties of GSK1016790A-induced vanilloid transient receptor potential channel TRPV4 activity in primary human endothelial cells. Mol Pharmacol. 2012; 82:464-472.
57. Sanchez JC, Lopez-Zapata DF, Wilkins RJ. TRPV4 channels activity in bovine articular chondrocytes: regulation by obesity-associated mediators. Cell calcium. 2014; 56:493-503.
58. Janssen SW, Hoenderop JG, Hermus AR, Sweep FC, Martens GJ, Bindels RJ. Expression of the novel epithelial Ca^{2+} channel ECaC1 in rat pancreatic islets. J Histochem Cytochem. 2002; 50:789-798.
59. Szallasi A. TRP Channels as Therapeutic Targets: From Basic Science to Clinical Use. Academic Press, 2015. *http://www.sciencedirect.com/science/book/9780124200241* (accessed January 23, 2017).
60. Wissenbach U, Niemeyer BA, Fixemer T, Schneidewind A, Trost C, Cavalie A, Reus K, Meese E, Bonkhoff H, Flockerzi V. Expression of CaT-like, a novel calcium-selective channel, correlates with the malignancy of prostate cancer. J Biol Chem. 2001; 276:19461-19468.
61. Skrzypski M, Khajavi N, Mergler S, Szczepankiewicz D, Kolodziejski PA, Metzke D, Wojciechowicz T, Billert M, Nowak KW, Strowski MZ. TRPV6 channel modulates proliferation of insulin secreting INS-1E beta cell line. Biochim Biophys Acta. 2015; 1853:3202-3210.
62. Heit JJ, Apelqvist AA, Gu X, Winslow MM, Neilson JR, Crabtree GR, Kim SK. Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. Nature. 2006; 443:345-349.
63. Lawrence M, Shao C, Duan L, McGlynn K, Cobb MH. The protein kinases ERK1/2 and their roles in pancreatic beta cells. Acta Physiol (Oxf). 2008; 192:11-17.
64. Skrzypski M, Kolodziejski PA, Mergler S, Khajavi N, Nowak KW, Strowski MZ. TRPV6 modulates proliferation of human pancreatic neuroendocrine BON-1 tumour cells. Biosci Rep. 2016; 36:e00372.
65. Khemtemourian L, Killian JA, Hoppener JW, Engel MF. Recent insights in islet amyloid polypeptide-induced membrane disruption and its role in β-cell death in type 2 diabetes mellitus. Exp Diabetes Res. 2008; 2008:421287.

(Received December 1, 2016; Revised January 26, 2017; Accepted January 27, 2017)

Review

DOI: 10.5582/bst.2016.01169

Neuron-specific splicing

Nor Hakimah Ab Hakim[1,*], Burhanuddin Yeop Majlis[1], Hitoshi Suzuki[2], Toshifumi Tsukahara[2]

[1] *Institute of Microengineering and Nanoelectronics (IMEN), Universiti Kebangsaan Malaysia, Selangor Darul Ehsan, Malaysia;*
[2] *School of Materials Science, Japan Advanced Institute of Science and Technology, Nomi, Ishikawa, Japan.*

Summary **During pre-mRNA splicing events, introns are removed from the pre-mRNA, and the remaining exons are connected together to form a single continuous molecule. Alternative splicing is a common mechanism for the regulation of gene expression in eukaryotes. More than 90% of human genes are known to undergo alternative splicing. The most common type of alternative splicing is exon skipping, which is also known as cassette exon. Other known alternative splicing events include alternative 5′ splice sites, alternative 3′ splice sites, intron retention, and mutually exclusive exons. Alternative splicing events are controlled by regulatory proteins responsible for both positive and negative regulation. In this review, we focus on neuronal splicing regulators and discuss several notable regulators in depth. In addition, we have also included an example of splicing regulation mediated by the RBFox protein family. Lastly, as previous studies have shown that a number of splicing factors are associated with neuronal diseases such as Alzheime's disease (AD) and Autism spectrum disorder (ASD), here we consider their importance in neuronal diseases wherein the underlying mechanisms have yet to be elucidated.**

Keywords: Alternative splicing, gene regulation, RNA splicing, splicing regulator

1. Introduction

Posttranscriptional regulation is vital for gene expression. Furthermore, posttranscriptional regulation provides a mechanism for rapid changes in the protein composition of cells. In eukaryotic cells, the conversion of the initial primary transcript synthesized by RNA polymerase II into a functional mRNA involves three major events: 5′-capping, 3′-cleavage/polyadenylation, and RNA splicing. The addition of these specific modifications to both the 5′ and 3′ ends of the pre-mRNA is crucial, as they protect mRNAs from enzymes that will quickly digest uncapped RNAs generated by RNA processing, such as RNA transcribed downstream from a polyadenylation site and spliced-out introns. Moreover, in the nucleus, the 5′-cap and 3′-poly(A) tail distinguish pre-mRNA molecules from various other kinds of RNA (*1,2*).

As the nascent mRNA precursor is being transcribed, the pre-mRNA processing events (*i.e.*, capping, polyadenylation, and splicing) take place in the nucleus. Therefore, pre-mRNA processing occurs co-transcriptionally. The 5′-end is instantly modified as the RNA emerges from the surface of RNA polymerase II by the addition of the 5′-cap structure found on all mRNA molecules, and is immediately bound by members of a complex group of RNA-binding proteins as the nascent pre-mRNA continues to develop. These RNA-binding proteins subsequently assist in both RNA splicing and the exportation of fully processed mRNA molecules into the cytoplasm through nuclear pore complexes. In the cytoplasm, some of these proteins remain associated with the mRNA. However, most of them either remain in the nucleus or shuttle back into the nucleus shortly after the mRNA is exported to the cytoplasm (*1,2*).

In addition, it is important to note that mRNAs never occur as free RNA molecules in the cell, as they are always associated with proteins as ribonucleoprotein complexes (RNP). First, they exist as nascent pre-mRNPs that are capped and spliced as they are transcribed, and

Released online in J-STAGE as advance publication December 31, 2016.

**Address correspondence to:*
Dr. Nor Hakimah Ab Hakim, Institute of Microengineering and Nanoelectronics (IMEN), Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia.
E-mail: hakimah_nor@ukm.edu.my

then become nuclear mRNPs following cleavage and polyadenylation. Finally, following the exchange of proteins that accompanies the export to the cytoplasm, they are referred to as cytoplasmic mRNPs (*1,2*).

2. Splicing

Pre-mRNA splicing is a process where introns are removed from the pre-mRNA and the remaining exons are connected to form a single continuous molecule. Introns are regions on RNA transcripts that do not code for protein. Transcripts with many exons can be spliced co-transcriptionally, although most pre-mRNA splicing events take place after the completion of mRNA synthesis. Other transcript modifications by RNA processing, such as end-capping of the pre-mRNA, assist pre-mRNA splicing. A large protein complex, known as the spliceosome, catalyses the splicing reaction. Proteins and small nuclear RNA molecules form the spliceosome that recognizes splice sites in pre-mRNA (*1,2*).

2.1. *Alternative splicing*

Alternative splicing is a common mechanism used to regulate gene expression in eukaryotes (*3-7*). It is recognized as a major contributor of proteomic diversity because it allows for the generation of multiple proteins from a single gene (*8,9*). It is known that 90% of human genes undergo alternative splicing (*10*). One notable example is the *Drosophila Dscam*, which can encode more than 38,000 proteins from a single gene (reviewed in *11*).

2.2. *Types of alternative splicing*

There are several types of alternative splicing, the most common type being "exon skipping" (also known as "cassette exon"). Other known alternative splicing events are: alternative 5′ splice sites, alternative 3′ splice sites, intron retention, and mutually exclusive exons (reviewed in *11,12*). Owing to alterations in exon combinations during alternative splicing events, the resulting protein isoforms may have different functions or activities.

3. Regulation of gene expression through alternative splicing

Regulation of splicing involves both *trans*- and *cis*-components, including sequences in pre-mRNA as well as cellular factors (RNA or protein, respectively) (*12*). In most cases, the *trans*-factors and cis-elements involved in a particular alternative splicing event are difficult to predict. Similarly, because many pseudo-exons that are flanked by predicted splice sites are not spliced, it is challenging to predict exons from the genome sequence information alone in the absence of transcript sequence information. It has been previously demonstrated that the presence of a pair of strong splice sites was not sufficient to define an exon (*12*).

To gain a better understanding of the underlying mechanisms, global analyses have been performed and the results indicate that relative enrichment in exon splicing enhancers (ESEs) or exon splicing silencers (ESSs) can help distinguish authentic exons from pseudo-exons. Despite extensive variation in the sequence of auxiliary splicing elements, they are crucial to the determination of alternative exons (*12*). Many ESEs were reported to contain binding sites for members of the serine-arginine rich (SR) protein family. SR proteins play important roles in the assembly of the spliceosome and function as both essential splicing and regulatory factors. Members of this protein family have a modular structure with one or two N-terminal RNA recognition motif (RRM)-type domains that bind RNA, in addition to C-terminal domains that are enriched in serine and arginine residues (RS domains). Additionally, RS domains are found in other core splicing factors, such as U2AF65 and U2AF35. They function to facilitate both protein-protein and protein-RNA interactions (*12*).

4. Neuronal splicing regulators

Certain pre-mRNA-binding regulatory proteins direct changes in splicing patterns and consequently enhance or silence particular splicing options. Previous studies have shown that these factors can be classified as positive or negative factors. SR proteins, as well as their related proteins, are among the most well-known and best characterized positive factors. These proteins bind to elements on ESEs and promote exon inclusion. Negative factors include members of the heterogeneous nuclear ribonucleoprotein (hnRNP) group of RNA-binding proteins, such as hnRNPA1 and the polypyrimidine tract-binding protein (PTB/PTBP1) (reviewed in *5,13*).

In addition, several proteins have the ability to either enhance or repress exon inclusion, depending on the position of their binding sites relative to the target exon. These RNA-binding proteins maintain developmentally regulated splicing patterns, albeit partially; however, the proteins that mediate changes in splicing in response to neuronal activity are largely unknown. Certain splicing factors are exclusively expressed in neurons for instance PTBP1, RBFox proteins and Nova 1, while others are enriched in neurons in the brain such as PTBP2 (nPTB) and RBFox proteins. However, splicing factors that are enriched in neurons in the brain can also be found in other tissues. Additionally, other factors can determine neuronal splicing patterns by their absence in neurons. Several proteins have been shown to control various groups of target transcripts in a coordinated

Table 1. Proteins known to regulate splicing in the nervous system

Neuronal splicing regulators	Homologues	Binding motifs	Tissue specificity	Known targets	References
PTBP1	Not applicable	UCUCU UUCU	Widespread / ubiquitous	Many, including GABA, α-actinin, α-tropomyosin, FGFR1 and 2	*5,20*
PTBP2 (nPTB)	Not applicable	CUCUCU	Brain	Many, including Bin 1, PMCA, Mef2, Nasp, and SRC	*5,20*
Nova1	Nova2	UCAUY	Neurons	Many, including GABAAγ2 and GlyRα2	*5,22,23*
RBFox1	RBFox3	UGCAUG	Brain, heart, neurons and skeletal muscle	α-actinin, EWS, FGFR2, fibronectin, and SRC	*5,27,28,32*
RBFox2	RBFox3	UGCAUG	Brain, heart, neurons, skeletal muscle and embryo	FGFR2, fibronectin, non-muscle myosin LC, and SRC	*5,27,28,29, 32*
KHDRBS1	Not applicable	UAAA UUUA	Nucleus	SMN2	*31*

Y indicates C or U; R indicates A or G; V indicates A, C, or G; H indicates A, C, or U. This table has been modified from Li Q, *et al*. (*5*).

manner, despite exon regulation by multiple factors (*5*).

5. Splicing regulators

As mentioned above, changes in splicing pattern are directed by positive and negative regulatory factors that bind to pre-mRNA and enhance or silence particular splicing choices (reviewed in *5,12,14*). Notable regulatory proteins that participate in neuronal alternative splicing are reviewed below and summarized in Table 1.

5.1. *PTBP1 and PTBP2*

Polypyrimidine tract-binding proteins are RNA-binding proteins that contain four RNA-binding domains of the RRM type (*15*). They are abundantly and widely expressed nuclear RNA-binding proteins. In addition, PTBP1 (Figure 1) has been shown to be involved in various cellular processes that affect gene expression, specifically alternative splicing (*14-17*). Furthermore, PTBP1 was reported to bind short RNA elements containing mixtures of U and C, such as UCUCU and UUCU (*15,18-20*). PTBP1 and hnRNP A1 are classified as hnRNP proteins. They have been implicated in repressing certain splicing events, thereby creating a layer of negative regulation. Moreover, PTBP1 has been recognized as a key splicing repressor in mammalian cells (*13*).

In contrast, the paralogue protein PTBP2, or nPTB, is present in the nervous system and testis (*21*). The function of PTBP2 in the brain remains unclear, although PTBP1 has been suggested to act as a negative regulator of neuron-specific exons in non-neuronal tissues (*21*). PTBP2 has been shown to be expressed in neuronal progenitors and it was detected in differentiated neurons and some astrocytes. Moreover, PTBP2 has been shown to be critical for postnatal survival and proper splicing of hundreds of mRNAs in the brain (*21*).

5.2. *Nova1*

The neuro-oncological ventral antigen (Nova1) is a neuron-specific RNA-binding protein that binds to the RNA sequence UCAUY. Nova1 has been reported to regulate the alternative splicing of several genes, including the glycine receptor a2 (*22,23*). In humans, the *NOVA1* gene is highly expressed in motor neurons. In contrast, *NOVA2* is abundantly expressed in neurons of the cortex, dorsal spinal cord, and hippocampus. Both factors were first identified in patients with paraneoplastic opsoclonus-myoclonus-ataxia (POMA) as autoantigens (reviewed in *5*).

5.3. *RBFox proteins*

Originally identified in *Caenorhabditis elegans*, the feminizing locus on X (*RBFox1*) gene functions as the numerator element to determine the number of X chromosomes relative to the ploidy. Additionally, *RBFox1* plays a role in the determination of male or hermaphrodite development (*24-26*). RBFox1 is an RNA-binding protein that contains an RRM. RBFox1 was reported to be expressed in the brain, heart, and skeletal muscle in mouse (*27*). Furthermore, Jin Y., *et al*. (*28*) showed that the zebrafish RBFox1 protein binds specifically to the pentanucleotide sequence GCAUG in their SELEX experiments.

In mammals, three members of the Fox family

proteins – RBFox1, RBFox2, and RBFox3 – have been identified. RBFox1 and RBFox2 are closely related because they share an identical RRM domain (illustrated in Figure 2) (*29*). RBFox1 and RBFox2 are also known as the ataxin-2 binding protein 1 (A2BP1) and RNA-binding motif protein 9 (RBM9), respectively. The latest addition to the family, RBFox3, has not been extensively characterized and its role is still not very well understood.

5.4. *KHDRBS1*

KH domain-containing, RNA binding, signal transduction-associated 1 (KHDRBS1), previously known as Src-associated in mitosis 68 kDa protein (SAM68), is a nuclear RNA-binding protein. KHDRBS1 has been reported to be involved in numerous mRNA metabolism events, including nuclear export, translation, and somatodendritic transport, as well as splicing (*30,31*). KHDRBS1 belongs to the signal transduction and activation of RNA (STAR) domain protein family. KHDRBS1 possesses a GSG domain that binds to A- or U-rich RNA sequences, such as UAAA or UUUA, and forms homodimers. Consistent with its role in alternative splicing, KHDRBS1 is primarily localized in the nucleus (*30,31*).

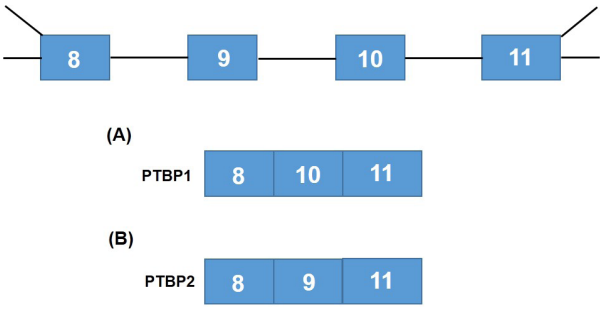

Figure 1. Schematic diagrams of *PTBP1* and *PTBP2*. Figure shows comparison between **(A)** *PTBP1* gene and **(B)** *PTBP2* gene structure.

6. An example of splicing regulation mediated by the RBFox protein family

In this section, we describe a mechanism for neuronal alternative splicing based on our previously published report (*32*), consistent with several others (summarized in Figure 3). The RBFox1 protein family functions as either a splicing enhancer or a repressor in the regulation of alternative splicing (*27,33*). The RBFox1 protein family specifically binds to the (U)GCAUG target sequence. It functions as an exon repressor when the target pentanucleotide is located in the upstream intronic flanking (UIF) region of the regulated exon. In contrast, the RBFox1 protein family promotes the inclusion of the skipping exon (cassette exon) if the (U)GCAUG element is located in the downstream intronic flanking (DIF) region.

As an example, in many heterologous cells, RBFox1 expression promotes skipping of exon 9 in the F1γ mini-gene by specifically binding to the GCAUG stretches located within the UIF region (*28,33*). Another example of RBFox-mediated regulation was demonstrated in the CaV1.2 L-type calcium channel gene, where the expression of RBFox1 and RBFox2 repressed exon 9* through (U)GCAUG motifs in the intronic and exonic regions (*34*).

As previously mentioned, the RBFox1 family can also mediate the inclusion of skipping exon (cassette exon) *via* (U)GCAUG sequences located within the DIF region. In the rat fibronectin gene, RBFox1 expression promoted the inclusion of an EIIIB exon *via* highly repeated and evolutionarily conserved (U)GCAUG elements in the DIF region. Additionally, several studies have shown that RBFox2 can positively regulate the

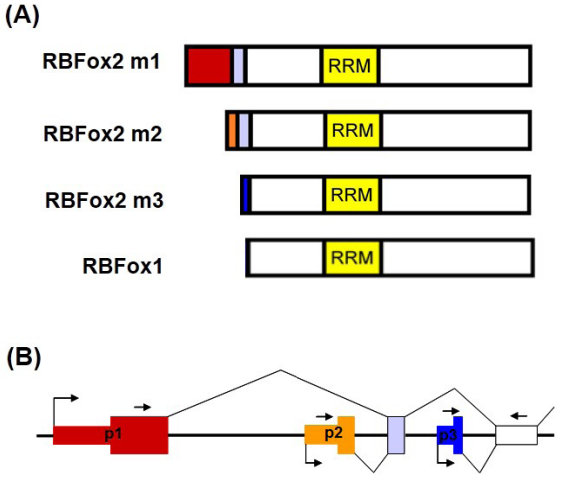

Figure 2. Alternative promoters in RB*Fox2*. Schematic diagrams of **(A)** RBFox2 isoforms and **(B)** *RBFox2* gene structure. The *RBFox2* gene has at least three alternative splicing promoters expressed in P19 cells. This figure has been modified from Hakim NHA, *et al*. (*29*).

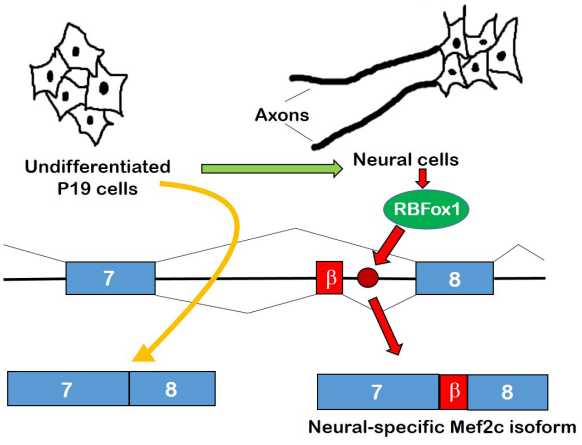

Figure 3. A model for the regulation of *Mef2c* exon β alternative splicing by RBFox1. Findings from our previous study suggested that RBFox1, which is specifically expressed in the neuronal cell stage, promoted Mef2c exon β inclusion *via* the GCAUG motif (*32*).

inclusion of exon 16 in the protein 4.1 R pre-mRNA during late erythroleukemia cell differentiation and in heterologous HeLa cells *via* conserved triple (U)GCAUG repeats within the DIF region (*33,35,36*).

7. A comprehensive analysis of alternative splicing and functionality in the neuronal differentiation of P19 cells

In order to thoroughly investigate the neural splicing event in the P19 mouse embryonic carcinoma cell line, we previously performed a GeneChip exon array study using total RNA purified from undifferentiated P19 cells (Day 0), neuronal-differentiated cells (Day 7), and cells from the glial cell stage (Day 10) (*37*). We employed nine filtering conditions for probe sets based on annotations, estimated gene expression levels, splicing index (SI), and detection above background (DABG) values, as well as alternative splicing predictions. We extracted 262 differentially alternatively spliced (DAS) candidate exons for neural splicing. Among them, 30 exons were validated by RT-PCR. Results from RT-PCR suggested that 87% of these exons were altered between undifferentiated and neuronal-differentiated cells. Furthermore, bioinformatics approaches, such as gene ontology (GO) analysis, text-mining, and pathway analysis, also suggested that many candidate exons were associated with neural events. In addition to the gene-level informatics analyses, we also performed text-mining for the alternative isoforms. This type of analysis is a new approach to detecting exon-level annotations (*37*).

In order to test whether the 262 DAS exons of 236 DAS genes were involved in neural events, GO analysis was performed using the Biological Process category. These terms were categorized into 10 groups: neural-related process, differentiation and development, signalling, post-translational regulation, transcription, cytoskeleton and cell adhesion, cellular transport, cell cycle and proliferation, apoptosis, and others. The most enriched category was neural-related process (20.8%), followed by differentiation and development (12.5%), cytoskeleton and cell adhesion (12.5%), and signalling (9.7%) (*37*).

The functions (cell processes) of 151 of the 236 DAS genes have been previously described; however, the remaining 85 genes were classified as unknown genes. Because their functions in neural cells or organs have been previously reported, 66 of the 151 DAS genes with known functions were categorized as well-known genes in neural processes. The remaining 85 genes were categorized as functional DAS genes owing to limited evidence for their neural regulatory roles. Our results suggested that many DAS genes are associated with neural events, which was also demonstrated by the GO analyses. Because the 66 well-known genes have been studied in neural cells or organs, further studies on functional differences between the alternative isoforms may reveal their functions in non-neural cells or organs (*37*).

We subsequently identified biological relationships between the 189 cell processes and 85 functional DAS genes. The analysis showed that 47 DAS genes were linked to these cell processes. These relationships are potentially important because isoforms created by neural splicing may have novel functions that have not been previously reported in neural cells. Furthermore, 11 of the 47 genes were involved in cell cycle-related events, as well as in G1 and/or S phase processes (*37*).

In addition, we also performed GO analysis on the 66 genes whose functions are known in neural cells and tissues. Surprisingly, RNA splicing was the most frequent GO term for these genes. Remarkably, the *RBFox1* homologue was among the 66 genes. The exon array analysis results also suggested that *RBFox2* has one alternative exon, which was altered in neuronal differentiation (*37*).

8. Alternative splicing and disease

In certain regulators, such as RBFox2, hnRNP, and SR proteins, changes in the nuclear level occur frequently in cancer (*39*). Furthermore, previous studies have shown that a number of splicing factors, including SR, RBFox, and hnRNPA1 proteins, are associated with Alzheimer's disease (AD) (*39*). Technically, the expression is not misregulated in the disease, but in the patient that has the disease. On the other hand, splicing components (*e.g.*, U1 snRNP) seem to be depleted from the nucleus, thus forming cytoplasmic aggregates (*39*).

RBFox proteins have been known to play an important role in brain development and function. Thus, the haploinsufficiency of this protein has been implicated in various neuropsychiatric disorders, including Autism spectrum disorder (ASD). Since RBFox proteins affect other pathways in transcription in addition to RNA processing, it is challenging to identify clinically relevant sets of splicing events. In addition, there are three highly related RBFox proteins expressed in the brain, with partially overlapping functions (*39*).

9. Conclusions

Alternative splicing is a major contributor to proteomic diversity. This mechanism allows multiple proteins to be generated from a single gene. In addition, the resulting isoforms may have different functions or activities, as exon combinations are altered in alternative splicing events. Changes in splicing patterns are influenced by regulatory proteins that bind to pre-mRNA and enhance or silence particular splicing choices. In addition, it has been noted that some splicing factors have been linked to neuronal diseases for instance AD and ASD as well as cancer (*39*).

Acknowledgements

Nor Hakimah Ab Hakim and Burhanuddin Yeop Majlis are supported in part by a HI-CoE Grant from the Higher Education Ministry of Malaysia. Toshifumi Tsukahara and Hitoshi Suzuki are supported in part by a Grant-in-aid for Scientific Research (B) from the Japan Society for the Promotion of Science (JSPS).

References

1. Lodish H, Berk A, Kaiser CA, Krieger M, Scott, MP, Bretscher A, Ploegh H, Matsudaira P. Molecular cell biology. W. H. Freeman, New York, USA, 2008; pp. 325-335.
2. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cells. Garland Science, New York, USA, 2007; pp. 479-480.
3. Suzuki H, Tsukahara T. A view of pre-mRNA splicing from RNase R resistant RNAs. Int J Mol Sci.. 2014; 15:9331-9342.
4. Sharma S, Kohlstaedt LA, Damianov A, Rio D, Black DL. Polypyrimidine tract binding protein controls the transition from exon definition to an intron defined spliceosome. Nat Struct Mol Biol. 2008; 15:183-191.
5. Li Q, Lee JA, Black DL. Neuronal regulation of alternative pre-mRNA splicing. Nat Rev Neurosci. 2007; 8:819-831.
6. Shin C, Manley JL. Cell signalling and the control of pre-mRNA splicing. Nat Rev Mol Cell Biol. 2004; 5:727-738.
7. Smith CW, Valcarcel J. Alternative pre-mRNA splicing: The logic of combinatorial control. Trends Biochem Sci. 2000; 25:381-388.
8. Black DL. Protein diversity from alternative splicing: A challenge for bioinformatics and post – genome biology. Cell. 2000; 103:367-370.
9. Graveley BR. Alternative splicing: Increasing diversity in the proteomic world. Trends Genet. 2001; 17:100-107.
10. Johnson JM, Castle J, Garrett-Engele P, Kan Z, Loerch PM, Armour CD, Santos R, Schadt EE, Stoughton R, Shoemaker DD. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science. 2003; 302:2141-2144.
11. Blencowe BJ. Alternative splicing: New insights from global analyses. Cell. 2006; 126:37-47.
12. Matlin AJ, Clark F, Smith CW. Understanding alternative splicing: Towards a cellular code. Nat Rev Mol Cell Biol. 2005; 6:386-398.
13. Wagner EJ, Garcia-Blanco MA. Polypyrimidine tract binding protein antagonizes exon definition. Mol Cell Biol. 2001; 21:3281-3288.
14. Black DL. Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem. 2003; 72:291-336.
15. Amir-Ahmady B, Boutz P, Markovtsov V, Phillips ML, Black DL. Exon repression by polypyrimidine tract binding protein. RNA. 2005; 11:699-716.
16. Valcárcel J, Gebauer F. Post-transcriptional regulation: The dawn of PTB. Curr Biol. 1997; 7:R705-708.
17. Smith CW, Valcárcel J. Alternative pre-mRNA splicing: The logic of combinatorial control. Trends Biochem Sci. 2000; 25:381-388.
18. Singh R, Valcárcel J, Green MR. Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract binding proteins. Science. 1995; 268:1173-1176.
19. Pérez I, McAfee JG, Patton JG. Multiple RRMs contribute to RNA binding specificity and affinity for polypyrimidine tract-binding proteins. Biochemistry. 1997; 36:11881-11890.
20. Simpson PJ, Monie TP, Szendroi A, Davydova N, Tyzack JK, Conte MR, Read CM, Cary PD, Svergun DI, Konarev PV, Curry S, Matthews S. Structure and RNA interactions of the N-terminal RRM domains of PTB. Structure. 2004; 12:1631-1643.
21. Licatalosi DD, Yano M, Fak JJ, Mele A, Grabinski SE, Zhang C, Darnell RB. Ptbp2 represses adult-specific splicing to regulate the generation of neuronal precursors in the embryonic brain. Genes Dev. 2012; 26:1626-1642.
22. Jensen KB, Musunuru K, Lewis HA, Burley SK, Darnell RB. The tetranucleotide UCAY directs the specific recognition of RNA by the Nova K-homology 3 domain. Proc Natl Acad Sci U S A. 2000; 97:5740-5745.
23. Dredge BK, Darnell RB. Nova regulates GABAA receptor γ2 alternative splicing *via* a distal downstream UCAU-rich intronic splicing enhancer. Mol Cell Biol. 2003; 23:4687-4700.
24. Auweter SD, Fasan R, Reymond L, Underwood JG, Black DL, Pitsch S, Allain H. Molecular basis of RNA recognition by the human alternative splicing factor Fox -1. The EMBO J. 2006; 25:163-173.
25. Hodgkin J, Zellan J, Albertson D. Identification of a candidate primary sex determination locus, fox-1, on the X -chromosome of Caenorhabditis elegans. Development. 1994; 120:3681-3689.
26. Skipper M, Milne CA, Hodgkin J. Genetic and molecular analysis of fox-1, a numerator element involved in *Caenorhabditis elegans* primary sex determination. Genetics. 1999; 151:617-631.
27. Fukumura K, Kato A, Jin Y, Ideue T, Hirose T, Kataoka N, Fujiwara T, Sakamoto H, Inoue K. Tissue-specific splicing regulator Fox-1 induces exon skipping by interfering E complex formation on the downstream intron of human *F1γ* gene. Nucleic Acids Res. 2007; 35:5303-5311.
28. Jin Y, Suzuki H, Maegawa S, Endo H, Sugano S, Hashimoto K, Yasuda K, Inoue K. A vertebrate RNA-binding protein Fox-1 regulates tissue-specific splicing *via* the pentanucleotide GCAUG. EMBO J. 2003; 22:905-912.
29. Hakim NHA, Majlis BY, Suzuki H, Tsukahara T. Expression Analysis of Fox-2 Alternative Isoforms during Neuronal Differentiation. Int'l Journal of Advances in Chemical Engineering & Biological Sciences (IJACEBS). 2016; 3:102104
30. Chawla G, Lin CH, Han A, Shiue L, Ares M, Black DL. Sam68 regulates a set of alternatively spliced exons during neurogenesis. Mol Cell Biol. 2009; 29:201-213.
31. Pedrotti S, Bielli P, Paronetto MP, Ciccosanti F, Fimia GM, Stamm S, Manley JL, Sette C. The splicing regulator Sam68 binds to a novel exonic splicing silencer and functions in SMN2 alternative splicing in spinal muscular atrophy. EMBO J. 2010; 29:1235-1247.
32. Hakim NH, Kounishi T, Alam AH, Tsukahara T, Suzuki H. Alternative splicing of Mef2c promoted by Fox-1 during neuronal differentiation in P19 cells. Genes Cells. 2010; 15:255-267.
33. Kuroyanagi H. Fox-1 family of RNA-binding proteins. Cell Mol Life Sci. 2009; 66:3895-3907.

34. Yang L, Embree LJ, Hickstein DD. TLS-ERG leukemia fusion protein inhibits RNA splicing mediated by serine-arginine proteins. Mol Cell Biol. 2000; 20:3345-3354.
35. Ponthier JL, Schluepen C, Chen W, Lersch RA, Gee SL, Hou VC, Lo AJ, Short SA, Chasis JA, Winkelmann JC, Conboy JG. Fox-2 splicing factor binds to a conserved intron motif to promote inclusion of protein 4.1 R alternative exon 16. J Biol Chem. 2006; 281:12468-12474.
36. Newman A. Molecular biology. RNA enzymes for RNA splicing. Nature. 2001; 413:695-696.
37. Suzuki H, Osaki K, Sano K, Alam AH, Nakamura Y, Ishigaki Y, Kawahara K, Tsukahara T. Comprehensive analysis of alternative splicing and functionality in neuronal differentiation of P19 cells. PLoS One. 2011; 6:e16880.
38. Yang G, Huang SC, Wu JY, Benz EJ Jr. Regulated Fox-2 isoform expression mediates protein 4.1 R splicing during erythroid differentiation. Blood. 2008; 111:392-401.
39. Chabot B, Shkreta L. Defective control of pre-messenger RNA splicing in human disease. J Cell Biol. 2016; 212:13-27.

(Received September 6, 2016; Revised November 23, 2016; Accepted December 6, 2016)

Review

DOI: 10.5582/bst.2016.01220

Regulation of Ras signaling and function by plasma membrane microdomains

Lawrence E. Goldfinger[1,*], James V. Michael[2]

[1] *Department of Anatomy & Cell Biology and The Sol Sherry Thrombosis Research Center, Lewis Katz School of Medicine at Temple University, and Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA, USA;*
[2] *Department of Medicine, Center for Translational Medicine, Thomas Jefferson University, Philadelphia, PA, USA.*

Summary **Together H-, N- and KRAS mutations are major contributors to ~30% of all human cancers. Thus, Ras inhibition remains an important anti-cancer strategy. The molecular mechanisms of isotypic Ras oncogenesis are still not completely understood. Monopharmacological therapeutics have not been successful in the clinic. These disappointing outcomes have led to attempts to target elements downstream of Ras, mainly targeting either the Phosphatidylinositol 3-Kinase (PI3K) or Mitogen-Activated Protein Kinase (MAPK) pathways. While several such approaches are moderately effective, recent efforts have focused on preclinical evaluation of combination therapies to improve efficacies. This review will detail current understanding of the contributions of plasma membrane microdomain targeting of Ras to mitogenic and tumorigenic signaling and tumor progression. Moreover, this review will outline novel approaches to target Ras in cancers, including targeting schemes for new drug development, as well as putative re-purposing of drugs in current use to take advantage of blunting Ras signaling by interfering with Ras plasma membrane microdomain targeting and retention.**

Keywords: Ras, lipid rafts, membrane microdomains, tumor progression

1. Introduction

The Ras homologues most prominently associated with cancers, H-, N-, and KRAS, are ubiquitously expressed with overlapping yet non-redundant functions (*1-4*). Ras propagates growth factor signaling, most prominently the MAPK mitogenic pathway (Raf/Mitogen-Activated Protein Kinase/ERK Kinase (MEK)/Extracellular signal-Regulated Kinase (ERK)) and PI3K/molecular target of rapamycin (mTOR) survival pathways (*1,3,4*). Constitutively active (CA) Ras mutations are highly transforming and tumorigenic (*5*). Combined, CA Ras mutations are associated with as high as ~30% of human malignancies (*6*). H- and NRAS mutations account for a substantial proportion, most prominently for HRAS in cervix (9%), salivary gland (15%), and urinary tract (11%), and for NRAS, skin (18%) and hematopoietic cancers (> 10%), as well as many other cancer types; the remainder harbor KRAS mutations (*6*). The molecular mechanisms of isotypic Ras oncogenesis are still not completely understood, and Ras inhibition remains an important anti-cancer strategy (*1,3,4*).

Ras function is modulated by its localization within microdomains at the plasma membrane and putatively at internal membranes. Lipid bilayers are comprised of subdomains enriched in cholesterol and glycosphingolipids, which are referred to as lipid ordered domains, or lipid rafts. The fatty-acid side chains present in lipid ordered membranes tend to be more saturated than those in the surrounding membrane. Due to the presence of cholesterol and fatty acid saturation, a lipid ordered domain exhibits less fluidity than the surrounding plasma membrane. Lipid ordered domains are classically characterized by the resistance to extraction with nonionic detergents. Many proteins involved in cell signaling have been shown to be enriched within lipid ordered microdomains, creating signaling nodes in these domains. By condensing pathway components, lipid

Released online in J-STAGE as advance publication February 7, 2017.

**Address correspondence to:*
Dr. Lawrence E. Goldfinger, Lewis Katz School of Medicine at Temple University, 3420 N Broad Street, MRB 200, Philadelphia, PA 19140, USA.
E-mail: goldfinger@temple.edu

rafts may promote signal transduction by responding to agonist stimulation causing cluster formation, therefore leading to downstream signal activation. Alternatively, lipid rafts may negatively regulate signal transduction by spatially segregating pathway proteins, leading to reduced downstream signaling (7).

Distinctions in plasma membrane microdomain targeting and regulation between H-/N-/KRAS and related Ras paralogues are proving highly instructive in elucidating the mechanistic basis for Ras signaling in homeostasis and pathophysiology. This in turn provides a basis for design of new anti-Ras drugs and re-appropriation of existing drugs to inhibit Ras, *via* targeting the membrane microdomain localization of Ras. This review will outline the contributions of plasma membrane microdomains and Ras membrane targeting to Ras signaling and functional outcomes, and implications for anti-Ras therapies. The review will focus principally on HRAS, NRAS and KRAS, the isotypes most prominently associated with human cancers, and will include a discussion of the RRAS subfamily, as distinct membrane targeting of RRAS and the other isotypes has revealed important contributions of membrane targeting to Ras signaling, cellular function, and roles in tumor progression.

2. Ras GTPases

2.1. *Ras subfamily*

The Ras superfamily is comprised of over 150 small GTPases, which can bind and hydrolyze guanine triphosphate (GTP). The Ras isotypes most prominently associated with human cancers, H-, N-, and KRAS (that consists of KRAS4A and KRAS4B splice variants), have overlapping yet nonredundant cellular functions, and constitutive activation of each isotype is associated with distinct cancer subtypes. In contrast to the H-/N-/KRAS subfamily, RRAS1 shares similar overall structure but displays different cellular functions. RRAS1 has limited transforming ability in cell lines, and activating RRAS1 mutations are at best weakly associated with human cancers (*8*). In response to growth factor receptor activation, Ras-specific guanine exchange factors (GEFs) are recruited and activated, facilitating GTP binding to Ras by displacing GDP. GTP-loading of Ras enacts a conformational change in the Ras protein, exposing the effector-binding domain to support effector interactions, leading to propagation of downstream signaling pathways promoting cell proliferation, differentiation, and survival. GTP-loading of wildtype (WT) Ras is reversed by the action of GTPase-Activating Proteins (GAPs), which increase the low intrinsic GTPase activity of Ras, causing GTP hydrolysis to guanine diphosphate (GDP), and Ras inactivation. Ras GTPases are generally characterized as "molecular switches" based on the ability to turn signaling on or off through GTP binding and hydrolysis (*9*). Multiple GAPs have been identified as tumor suppressors, including p120-RasGAP (RASA1) and neurofibromin (*10,11*). CA Ras mutations are characterized by 'locking' Ras in the GTP-bound state, either by inhibiting GAP sensitivity, increasing GTP binding affinity or other mechanisms. These Ras mutants are transforming when over-expressed in cells in culture, and drive tumor formation in animal models. Together activating mutations in H-, N-, and KRAS (hereafter referred to collectively as proto-oncogenic (WT) or oncogenic (CA) Ras) are associated with ~30% of all human tumors, and reports have shown Ras mutations in up to 90% of pancreatic cancers (*1,3,4*). Ras signal propagation, and hence cellular and tumorigenic effects, are also tightly regulated by Ras plasma membrane targeting and microdomain association. Although CA Ras is insensitive to activity modulation, spatial regulation by Ras microdomain targeting at the plasma membrane has emerged as a mechanism for modulating Ras signaling.

2.2. *Ras structure*

Ras proteins each comprise an effector-binding domain, guanine nucleotide binding domains, and C-terminal hypervariable region, and include regions of high and low sequence homology across isotypes (Figure 1). Crystal structures of Ras indicate that the exchange of guanine nucleotides GDP-to-GTP results in an allosteric conformational change in adjacent regions, referred to as "switch I" and "switch II" (*12*). The core effector binding domain is located within the switch I region, and is highly conserved among isotypes. Switch I is a loop structure which is exposed upon GTP binding, and is sometimes referred to as the effector loop (*13*). The area which displays the least sequence homology is located in the C-terminal region, named the hypervariable region (HVR). Within the HVR lie residues subject to distinct and isotype-specific post-translational modifications. These post-translational modifications are responsible for the differential membrane targeting among Ras isotypes (*14-16*) (Figure 1).

2.3. *Ras membrane association driven by post-translational lipid modifications*

The C-terminal 23/24 amino acids of Ras constitute the HVR, the sequence of which dictates Ras isotype-specific post-translational modifications (*17*). The extreme C-termini contain a cysteine-aliphatic-aliphatic-X motif (CaaX), in which the X is usually serine, methionine, or glutamine. Following translation in cytosolic ribosomes, the Ras polypeptide is enzymatically isoprenylated with a 15-carbon farnesyl group by farnesyl transferase (Figure 2). The farnesyl transferase binds Ras within the cytosol and attaches the lipid moiety to the cysteine residue of the CaaX motif (*18*). RRAS contains a cysteine-aliphatic-aliphatic-Leucine (CaaL) motif (Table 1). The Leucine in

the CaaL motif drives covalent addition of a 20-carbon geranylgeranyl lipid group to the cysteine residue *via* geranylgeranyl transferase (*19*). Both farnesyl transferase and geranylgeranyl transferase attach the respective lipid group *via* an irreversible thioether bond.

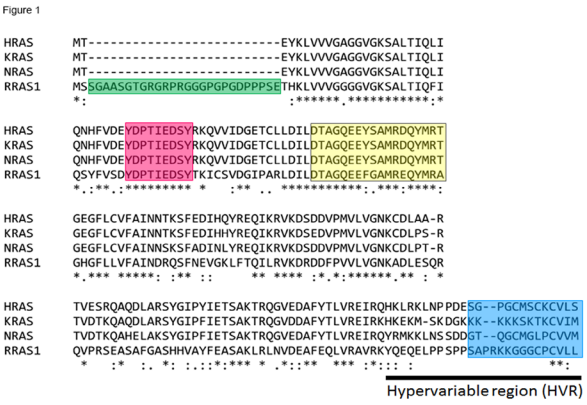

Figure 1. Primary structure alignment of proto-oncogenic RAS subfamily and RRAS1. Green: Unique RRAS N-terminal extension. Pink: Core effector binding domain. Yellow: "switch I" and "switch II" guanine nucleotide-binding region. Cyan: membrane targeting domain. The underlined amino acids indicate the hypervariable region (HVR). Asterisk (*): conserved amino acids.

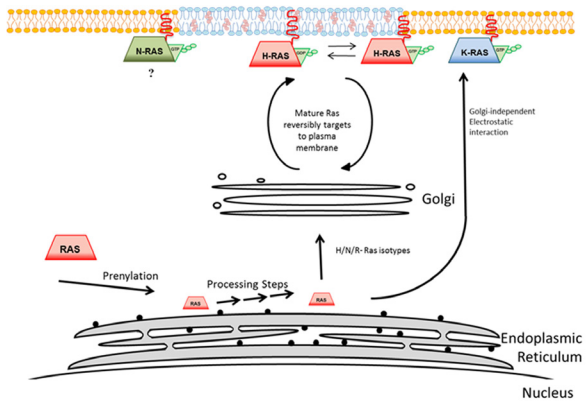

Figure 2. Post-Translational Modifications of Ras and plasma membrane targeting. Ras proteins are translated by cytosolic ribosomes, which are subsequently isoprenylated at the Cysteine of the CaaX consensus sequence. Isoprenylation increases affinity for the endoplasmic reticulum, where the protein undergoes further modification. RceI proteolytically cleaves the –aaX tripeptide, then Icmt carboxy-methylates the newly prenylated Cysteine. Most Ras proteins also require palmitoylation, catalyzed by the DHHC family of isoprenyl transferases, to leave the Golgi and traffic properly to the plasma membrane. Different Ras isoforms laterally segregate at the plasma membrane, to propagate downstream signaling. Upon depalmitoylation, Ras cycles back to the Golgi, where it is capable of being palmitoylated many times during the half-life of the protein.

Once Ras proteins are isoprenylated by either a farnesyl (H-,K-,N-, TC21) or geranylgeranyl (R-, M-) group the protein hydrophobicity is increased, thus causing higher affinity to the endoplasmic reticulum membrane (Figure 2). Thereafter, the CaaX or CaaL sequence targets Ras to the cytosolic surface of the endoplasmic reticulum where Ras and a-factor converting enzyme (Rce1), proteolytically removes the –aaX tripeptide (*20*). Next, the newly C-terminal prenylcysteine is targeted by isoprenylcysteine carboxyl methyltransferase (Icmt), which methyl-esterifies the α carboxyl group (*21*). Covalently linked lipid moieties have been shown to account for specific GEF activation. For example, Ras-GRF1 and Ras-GRF2 can activate HRAS, while Ras-GRF2 is unable to activate RRAS specifically because of the geranylgeranyl attachment (*22,23*).

The isoprenylated Ras proteins weakly bind endomembranes, yet a second motif within the hypervariable region strengthens membrane interaction and supports subsequent Ras trafficking. One common secondary motif is the reversible addition of a 16 carbon palmitate group (*24*). Palmitoyl groups are added to H-, N-, R-, and splice variant K(A)RAS. The more physiologically predominant splice variant K(B)RAS (hereafter referred to as KRAS) contains a polylysine sequence which allows an electrostatic interaction with the acidic headgroups of lipid bilayers (Table 1). KRAS traffics to the plasma membrane through a poorly understood route which is Golgi-independent (*25,26*). N- and K-(A)RAS are monopalmitoylated and thus require a third HVR motif for plasma membrane association, which consists of a stretch of hydrophobic residues (*27*). Proto-oncogenic Ras palmitoylation (H-, N-, K(A)-RAS) is carried out by a heterodimeric complex consisting of Palmitoyltransferase ZDHHC9 (DHHC9) and Golgin subfamily A member 7 (GCP16) (*28*). DHHC9 is one member of a family of DHHC-motif-containing protein S-acyltransferases (PATs) (*28*). Alternatively, the precise function of GCP16 is unclear. GCP16 is required for the heterodimeric complex localization, and plays a role in DHHC9 protein stability (*29*). Another DHHC family member, DHHC19, is responsible for palmitate transfer to RRAS, but not H-, N-, or K-(4A)RAS (*30*). Palmitoylated Ras proteins are mainly targeted to recycling endosomes, which function as a shuttle along the post-Golgi exocytic pathway to the plasma membrane. HRAS is palmitoylated on two

Table 1. Ras Lipid Modifications

Ras isoform	C-Terminal prenylation motif	Isoprenylation	Secondary Lipid Binding Motif	Palmitoylation
HRAS	CaaX	Farnesyl	Palmitoylation	2 sites
NRAS	CaaX	Farnesyl	Palmitoylation, Hydrophobic Residues	1 site
KRAS4A	CaaX	Farnesyl	Palmitoylation, Hydrophobic Residues	1 site
KRAS4B	CaaX	Farnesyl	Electrostatic interaction	None
RRAS1	CaaL	Geranylgeranyl	Palmitoylation	1 site

Cysteine residues, while NRAS is palmitoylated at one site (Table 1). Palmitoylation is required for shuttling of Ras to recycling endosomes, and the lack of proper palmitoylation leads to Ras mis-localization (*16*). In addition to H-, N-, and KRAS4A, RRAS also requires palmitoylation to exit from the Golgi, and to traffic anterograde *via* vesicles to the plasma membrane (*31*).

Palmitate groups are attached *via* a reversible thioester bond. This reversible addition of a lipid moiety allows for spatio-temporal regulation of Ras proteins. Depalmitoylating enzymes, such as acyl protein thioesterase I (AptI) or FKBP12, cleave the thioester bond between the Cysteine residue and the palmitate. Palmitate removal reduces the Ras protein's affinity for the plasma membrane, which triggers recycling back to the Golgi where Ras can be palmitoylated. This cycle can be repeated multiple times during the half-life of the protein (*32-34*).

3. Plasma membrane targeting of Ras

Localization of Ras proteins within the plane of the plasma membrane appears to be more dynamic than originally believed (*35-37*). Biochemical and electron microscopy studies suggest that approximately 50% of the inactive form of HRAS (GDP bound) is localized to lipid ordered domains, while activated HRAS (GTP bound) exits lipid ordered domains and preferentially resides in the lipid disordered membrane(*38,39*). Recently, semi-atomic in silico simulations provided evidence indicating that GTP-bound HRAS is localized to the border between the lipid ordered/lipid disordered domains (*40*). Such evidence provides a handy explanation for GTP-HRAS being observed enriched in both fractions, as gradient sedimentation techniques reveal bulk distributions and a microdomain "split" in GTP-HRAS likely supports its targeting to the microdomain borders. Similarly, inactive (GDP bound) NRAS is found in both lipid ordered and lipid disordered membranes. Atomic force microscopy has shown GTP-bound activated NRAS is also likely shuttled to the lipid ordered/lipid disordered boundary, where it may help reduce line tension at the phase boundary (*41,42*). However, NRAS PM microdomain localization is more controversial, as fluorescence recovery after photobleaching (FRAP) studies indicated that GTP-bound NRAS reside within lipid rafts (*36,43*). KRAS is targeted to the lipid disordered subdomain, regardless of activity state, directly from the endoplasmic reticulum without modification from the Golgi. Despite the electrostatic force generating the association with the plasma membrane, approximately 85% of KRAS is found to localize within the lipid disordered domain. Importantly, KRAS propagates signals from the lipid disordered membrane that is spatially distinct from HRAS (*37,44*). Collectively, the farnesyl lipid moiety appears to preferentially localize Ras proteins to the lipid disordered domain, while the palmitate group prefers the lipid ordered domain. This trend accounts for similar targeting of H- and NRAS to the lipid raft border, and for the nonpalmitoylated KRAS to preferentially propagate signals from the lipid disordered domain. RRAS is geranylgeranylated and palmitoylated (not farnesylated), and in line with the apparent role of Ras lipidation in microdomain targeting, RRAS localizes to lipid ordered microdomains in both active and inactive states, *i.e.*, RRAS does not shuttle to the lipid ordered/disordered domain border upon GTP binding (*40,45*) (Figure 2).

The processes which regulate Ras localization to distinct microdomains at the plasma membrane and lateral movement are incompletely understood. However, recent reports indicate that Galectin proteins play a critical role. Galectin over-expression has been observed in several tumor types, and has been associated with tumor progression (*46*). Galectins are a family of carbohydrate-binding proteins, with high affinity for β-galactosides. GTP-bound HRAS has been demonstrated to selectively bind Galectin-1 (Gal-1), and GTP-bound KRAS selectively binds Galectin-3 (Gal-3) (*47,48*). While Gal-3 does not directly bind to NRAS, increased Gal-3 expression simultaneously increases KRAS signaling while decreasing NRAS activation (*49*). This is due to an interaction of the N-terminus of Gal-3 with Ras exchange factor, RasGRP4, which diminishes NRAS GTP loading (*50*). At the time of this publication, there are no reports of a Galectin protein that selectively interacts with NRAS.

In the case of HRAS, Gal-1 contains a prenyl-binding pocket, which interacts with the farnesyl group in GTP-HRAS, independent of lectin function. This interaction is thought to alter the orientation of the HRAS globular domain with respect to the plasma membrane, and thereby regulate lateral segregation of HRAS and promote MAPK signaling (*47,51*). Indeed, ectopic Gal-1 over-expression or suppression increases or abrogates GTP-bound HRAS nanoclustering, respectively (*52*). Thus, upon GTP-loading, the conformational shift of Ras promotes affinity for Galectin binding, which subsequently allows lateral movement at the plasma membrane, allowing for distinct signaling platforms (*51*).

4. Ras signaling (H, K, N)

Ras can be activated in response to a diverse array of upstream extracellular signals such as growth factors, cytokines, hormones and neurotransmitters which stimulate cell surface receptors that include receptor tyrosine kinases (RTKs), non-receptor tyrosine kinase-associated receptors, and G protein- coupled receptors (GPCRs) (*53,54*) (Figure 3).

Perhaps the most studied pathway of Ras activation involves the mitogen-stimulated RTK epidermal growth factor (EGF) receptor (EGFR) (*54,55*). Upon stimulation, EGFR dimerizes and undergoes autophosphorylation of tyrosine residues in its cytoplasmic domain. These

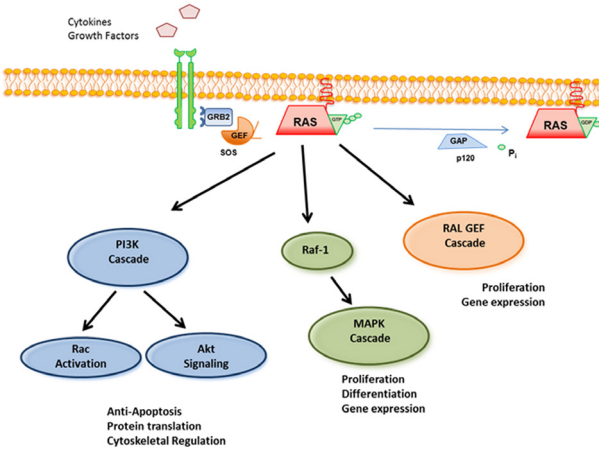

Figure 3. Overview of Ras Signaling Pathways. Ras is activated in response to a variety of extracellular stimuli, which facilitate localization of guanine nucleotide exchange factors (GEFs) to catalyze the replacement of GDP for GTP. Upon GTP-loading (activation) Ras functions like a molecular switch, propagating a variety of downstream signal cascades leading to proliferation, differentiation, altered gene expression, and other biological processes. The intrinsic ability of RAS to hydrolyze GTP to GDP + P*i* is catalyzed by a large family of GTPase activating proteins (GAPs) including p120, which effectively turns off Ras signaling

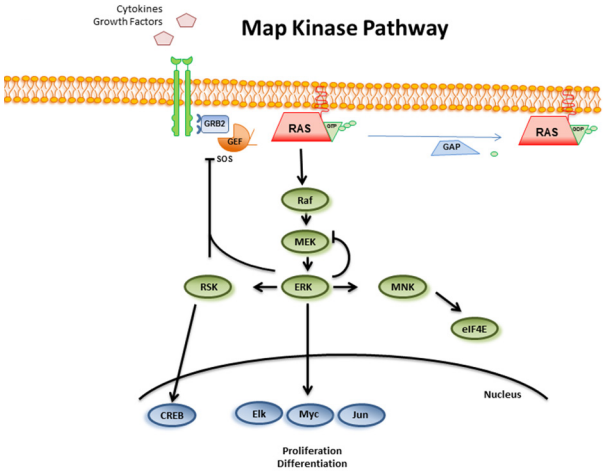

Figure 4. Overview of mitogen activated protein kinase (MAPK) signaling pathway. Upon activation, Ras binds and translocates Raf to the plasma membrane. Activation of Raf subsequently starts a signal cascade which leads to MEK and ERK activation. Activated ERK can translocate to the nucleus and alter transcription, or phosphorylate other cytosolic proteins which push the cell towards proliferation and differentiation. Downstream ERK has been shown to turn off signaling by negatively regulating MEK. In addition, both RSK and ERK can phosphorylate Ras-GEF SOS, which destabilizes its interaction with the RTK and terminates signaling.

phosphorylation events enable binding for adaptor proteins such as Shc and/or Grb2 *via* Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (*56*). The tri-complex Shc/Grb2/SOS has been implicated as the major pathway for EGF stimulation of Ras (*57*). Upon interaction with the activated RTK, Shc becomes autophosphorylated which creates recognition sites for the SH2 domain of Grb2. Grb2 is stably associated with son of sevenless (SOS), a Ras GEF. Thus, the Shc/Grb2/SOS or Grb2/SOS complex translocation to the plasma membrane mediates an increase in GTP-bound, active Ras (*58,59*). Shc, Grb2 and SOS provide the link between many types of activated cell surface receptors and Ras (*60*). Another receptor which activates Ras proteins is the Tyrosine Kinase Receptor-A (TrkA), which is stimulated by the neurotrophic factor nerve growth factor (NGF) (*61*). Once activated, Ras activates downstream signal cascades (Figure 3).

4.1. *Raf/MAPKinase pathway*

The mitogen-activated protein kinase (MAPK) pathway plays a key role in many physiological responses such as cell proliferation, apoptosis, and differentiation. GTP binding and effector loop exposure in Ras creates a docking site for a high affinity interaction with Raf kinases, which is reversed upon Ras inactivation (*62,63*). The Ras-Raf complex translocates the cytoplasmic Raf to the plasma membrane, which supports activation of Raf through a complex and incompletely understood mechanism (Figure 4) (*64*). Thus, Raf activation by Ras is a function of the ability of Ras to recruit Raf to the plasma membrane where it can be phosphorylated by cognate kinases and not by direct action of Ras itself (Ras lacks a kinase domain and kinase activity); this is supported by constitutive Raf activation by direct fusion of a CaaX sequence to the Raf C-terminus (*65,66*). Once activated, Raf phosphorylates MEK1 and MEK2 proteins at residues Ser218 and Ser222, thus propagating the mitogenic signal (*67*). Raf directly associates and activates MEK through its C-terminal catalytic domain (*68*). Activated MEK 1 and 2 are dual-specificity kinases which phosphorylate tandem residues in the TEY motif in their substrates, the Extracellular Signal Regulated Kinases 1 and 2 (ERK1 and ERK 2, also denoted as p44MAPK (ERK1) and p42MAPK (ERK2)) at Thr202/Tyr204 and Thr185/Tyr187, respectively, allowing for a conformational shift and full enzymatic activation (Figure 4) (*69*). Scaffold proteins, such as protein Kinase suppressor of Ras (KSR), help aid in the MAPK signaling by coordinating the assembly of a Raf-MEK-ERK complex (*70*).

Upon activation, ERK 1/2 can translocate to the nucleus where it phosphorylates and activates multiple transcription factors such as Elk-1, c-Ets-1, and c-Ets-2, c-Myc, c-Jun, and c-Fos (Figure 4) (*71,72*). ERK also has the ability to activate cytoplasmic kinases, such as p90 RSK1, MAP kinase-interacting kinase 1 (MNK1), and 2 (MNK2) (*73-75*). P90RSK can translocate to the nucleus and potentiate transcription *via* the activation of the transcription factor cyclic AMP-responsive element-binding protein (CREB) (*76,77*). MNK proteins may modulate translation by binding and phosphorylating eukaryotic translation initiation factor 4E (eIF4E), which

is a component of the translation initiation machinery (*78*). The various transcription factors regulated by ERK or RSK regulate gene expression to induce cellular phenotypes including cell proliferation, apoptosis, and differentiation (Figure 4).

4.2. *PI3Kinase pathway*

Another well-characterized pathway of Ras signaling involves phosphatidylinositol 3-kinases (PI3Ks), which play important roles in cell growth, adhesion, survival, motility, and transformation (*79,80*). The PI3Ks are heterodimeric lipid kinases with a catalytic and an adaptor/regulatory subunit, encoded by separate genes and subject to alternative splicing to generate multiple subtypes. The PI3K family of enzymes are organized into three main classes (class I, II, and III), and various subgroups have been categorized based on their primary structure, substrate specificity, and regulation (*81*). Class I PI3K is perhaps the best characterized family, and most clearly implicated in human cancer. The catalytic subunits for the class I PI3Ks are p110α, p110β, p110γ, and p110δ (*82,83*). The p110 subunits are divided into two classes; one which binds a p85 regulatory subunit (class IA: p110α, p110β, and p110δ) and the other which does not (class IB-p110γ). The regulatory p85 subunit serves a dual role by protecting p110 from degradation while inhibiting enzymatic activity. However, once RTKs become stimulated, p85 PI3K mediates p110 translocation to the plasma membrane, relocating the heterodimer to propagate downstream signaling (*84,85*).

PI3K activation can occur in multiple ways, which begin with extracellular activation of RTKs. One method of PI3K activation is by the regulatory p85 subunit directly binding to phosphorylated YXXM motifs (consisting of a Tyrosine-X-X-Methionine, where X denotes any residue) within the RTK, which triggers the P110 subunit to be catalytically active. A second form of activation has been characterized by the scaffolding protein Gab1, which binds regulatory p85 PI3K and associates with the EGF receptor both directly and indirectly through adaptor protein Grb2. Gab1 is recruited to the plasma membrane in response to EGFR activation, that is eventually turned off by conversion of Phosphatidylinositol (3,4,5)-triphosphate (PIP_3) to Phosphatidylinositol 4,5-bisphosphate (PIP_2) by Phosphatase and tensin homolog (PTEN), which triggers Gab1 dissociation from the plasma membrane (*86*). Finally, a third method of PI3K activation is through Ras (Figure 5). Adaptor protein Grb2 creates a cascade by binding and activating the Ras GEF SOS, thus activating downstream Ras and p110. Ras binds the catalytic p110 subunit of PI3K in a GTP-dependent manner which can stimulate PI3-kinase activity independently of p85 (*87,88*). Moreover, Grb2 can exist in complex with SOS, Gab1, and Ras which would bring these various PI3K activators in close proximity (*89*). It is unclear which of these pathways predominates in physiological situations; however there is evidence that RTK activation of the p85 subunit and Ras activation of the p110 subunit are complementary, and synergistically activates the pathway (*90*).

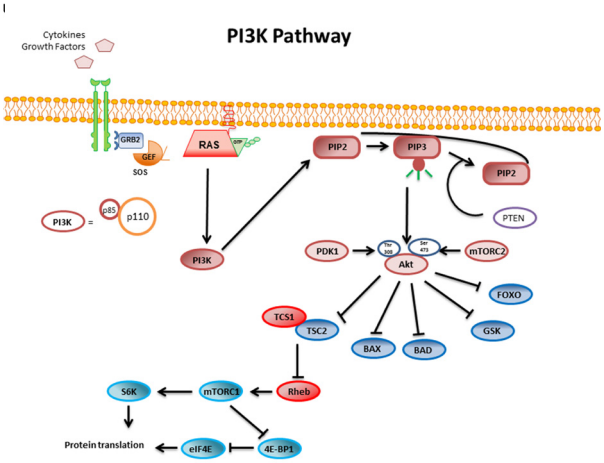

Figure 5. Overview of Ras/phosphatidylinositol-3 kinase (PI3K) signaling pathways. Upon activation, Ras can activate the p110 catalytic subunit of PI3K, which is a lipid kinase that phosphorylates PIP2 to generate PIP3. PIP3 recruits proteins that harbor a PH domain, such as Akt. Akt pushes the cell toward survival by negatively regulating a multitude of pro-apoptotic proteins such as BAX or BAD. Akt also inhibits the Rheb GAP protein TSC2. An increase in active Rheb leads to mTORC1 activation, which regulates multiple proteins that results in increased protein translation. PTEN dephosphorylates PIP3 back to PIP2, and effectively shuts off PI3K signaling.

Upon activation, PI3K generates PIP_3 from PIP_2 by phosphorylation. PIP_3 then acts as a second messenger facilitating downstream signaling, by recruiting proteins that contain a pleckstrin homology (PH) domain to the plasma membrane. PI3K activation and subsequent production of PIP_3 propagate various downstream pathways that regulate a number of cellular functions including those involved in tumor development and progression (Figure 5) (*91*). PTEN dephosphorylates PIP_3 to PIP_2, resulting in release of PH proteins and curtailing PI3K signaling (*92*).

One particularly significant downstream signaling cascade is the recruitment and activation of Akt/Protein Kinase B (PKB) (Figure 5). Akt activation is a multistep process in which residues Thr308 and Ser473 become phosphorylated. Initially, 3-Phosphoinositide-dependent protein kinase-1 (PDK1) and Akt are recruited to the plasma membrane in a PIP_3-dependent manner. Experimentally, PDK1 has been shown to phosphorylate Akt at Thr308, while the kinase for Ser473 has remained subject to controversy. Mammalian target of rapamycin (mTOR) in complex with GβL and Rictor (which is collectively referred to as mTORC2) is the kinase currently thought to be responsible for phosphorylating Ser473 (*93-95*). Akt promotes cell survival *via* negative regulation of numerous pro-apoptotic family members.

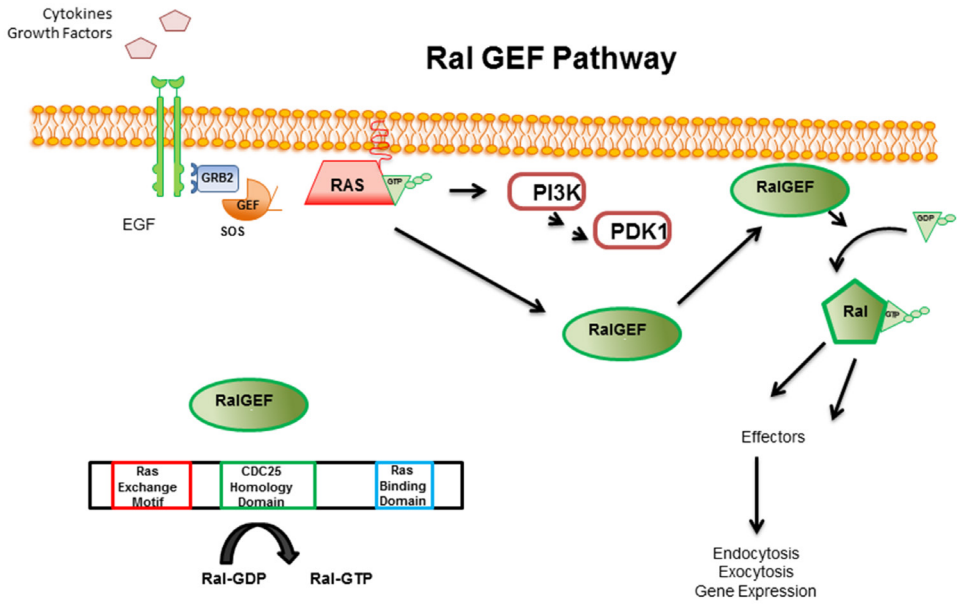

Figure 6. Overview of Ral-guanine exchange factor (Ral GEF) signaling pathway. Upon activation, Ras can bind RalGEFs via a RBD, which triggers translocation of the protein complex to the plasma membrane. At the membrane, the RalGEF encounters Ral, leading towards nucleotide exchange and downstream signaling. *In vitro* the RalGEF protein only requires a CDC25 homology domain for catalytic activity, while *in vivo* studies demonstrate that the upstream REM domain is needed. Activation of Ral alters gene expression, and regulates endocytosis/exocytosis.

For example, Akt can phosphorylate Bcl-2 family members BAD and BAX, rendering them inactive (*96,97*) (Figure 5).

Another important downstream signaling protein of Akt is mTOR in a complex with GβL and Raptor (named mTORC1). This is a multi-step signaling pathway which begins with Akt phosphorylating tuberous sclerosis 2 (TSC2). TSC2 is GAP protein for GTPase Ras homolog enriched in brain (Rheb), which predominately forms a heterodimeric pair with tuberous sclerosis 1 (TSC1). Upon phosphorylation the TSC1/TSC2 complex becomes destabilized, thereafter inhibiting GAP activity for Rheb (*98*). This event relieves a negative regulatory affect, allowing Rheb to activate the mTORC1 complex (*99,100*). The mTORC1 complex phosphorylates S6 Kinase 1 (S6K1) and 4E-BP1, which play a role in the regulation of cell growth and proliferation (*101*). Downstream S6K1 activation ultimately forms a negative feedback loop by inhibiting insulin receptor-substrate function, thus decreasing PI3K activation (*102,103*).

4.3. *RalGEF pathway*

Another well-studied Ras effector is the RalGEF family of proteins, which comprises 4 members; RalGDS, RGL, RGL2/Rlf and RGL3, each of which can interact with members of the Ras family (*104-107*). All the RalGEF family members share a common structure, and display the highest sequence homology in three critical domains: a CDC25 homology domain, Ras Exchange Motif (REM) domain, and a C-terminal RBD (Figure 6) (*108*). RalGEFs act as a guanine exchange factor for Ral, by stimulating the GDP/GTP exchange. The GEF properties come from the CDC25 homology domain. *In vitro* studies show that the CDC25 homology domain is sufficient for RalGDS catalytic activity, while *in vivo* studies show the requirement for the upstream REM domain (*108,109*). The N-terminal region of RalGDS (potentially a portion of the REM domain) has been reported to associate with the catalytic domain and thus blocks GEF activity which may be regulated by phosphorylation by Protein Kinase C (PKC) (*110,111*).

Upon the interaction of activated Ras and RalGEF, the complex is translocated to the plasma membrane where further downstream signaling occurs (Figure 6). Importantly, RalGEFs do not undergo a conformational shift, rather the complex functions by allowing access of the RalGEF with Ral at the membrane (*112,113*). However, the contributions of Ras plasma membrane microdomains to Ras/Ral signaling remain poorly understood. Once Ral is activated in a RalGEF-dependent manner, it subsequently interacts with numerous effectors such as; Sec5, Filamin, RalBP1, and ZONAB, and likely other proteins yet to be identified. Through these interactions, Ral proteins regulate endocytosis, exocytosis, actin organization, and control of gene expression (Figure 6) (*114-116*). Within the literature there is currently debate on the role of RalGEFs in Ras-mediated transformation. Although initial studies in mice had indicated a relatively minor role for RalGEFs in Ras-mediated transformation, following studies indicated a crucial role of RalGEFs in promoting Ras-mediated transformation and tumorigenic growth of human cells. This difference may be in part because of potential variation in mice and human Ras-transformation, which could have some distinctive underlying mechanisms. In

addition, studies which reduce RalGEF function show a diminished effect on cell transformation (*117-119*). RalGDS null mice are viable; however, histological analysis has indicated that proper RalGDS signaling is required for normal apoptosis of papillomas in response to mitogenic signals (*120*) (Figure 6).

Ras-mediated PI3K activation has been shown to increase the GEF activity of RalGDS. EGF-mediated activation of PI3K promotes the association of the N-terminus of the PDK1 with the N-terminus of RalGDS. Currently it is not known whether the N-terminal domains directly interact or if a scaffold protein links them. The PDK1-RalGDS interaction relieves the auto-inhibition of RalGDS, which does not require the catalytic domain of PDK1 (*111*). Collectively, after Ras-dependent movement of RalGDS to the membrane, PDK1 (activated *via* Ras/PI3K/PIP3/PDK1 pathway) is within proximity to associate by some unknown mechanism, which enhances RalGEF catalytic activity.

4.4. *PI3Kinase and MAPK pathway cross-talk*

The PI3K and MAPK pathways interact in numerous ways, which may both negatively and positively regulate downstream cellular responses (*121*). For example, Akt activated by the PI3K pathway has an ability to directly bind and phosphorylate Raf (at Ser259). The phosphorylation of Ser259 negatively regulates the MAPK pathway. The Akt-mediated phosphorylation of Raf allows for the binding partner 14-3-3 to inhibit Raf, and this interaction may shift the cellular response of breast tumor tissue from cell cycle arrest to proliferation through an incompletely understood feedback mechanism (*122*). Alternatively, in cultured mouse fibroblasts, constitutive Raf-MEK1 signaling leads to a negative feedback loop of Ras and PI3K signaling, causing Akt inhibition through Ephrin receptor Eph2A. The Ras-PI3K inhibition from Raf-MEK1 activation is necessary for Ras-induced cell cycle arrest (*123*). The PI3K pathway also activates PAK, which is involved in cytoskeletal dynamics. PAK is regulated by the Rho family of GTPases, and capable of phosphorylating Raf (*124*). This PAK1 dependent phosphorylation of Raf1 may regulate Raf localization to the mitochondria (*125*).

Signals from the MAPK and PI3K pathways have also been shown to converge on common downstream proteins or act synergistically. The mTORC1 complex functions as a point of convergence from multiple signaling networks, and aberrant signaling has been implicated in pathologies including cancer. mTOR is activated downstream of PI3K/Akt; PI3K is a major effector of activated Ras oncogenes. However, mTORC1 is also subject to Akt-independent activation of mTORC1 signaling; *e.g.*, mTOR is activated by mitogenic signaling through activation of the Ras/MEK/ERK pathway. The PI3K/Akt or Ras/MEK/ERK

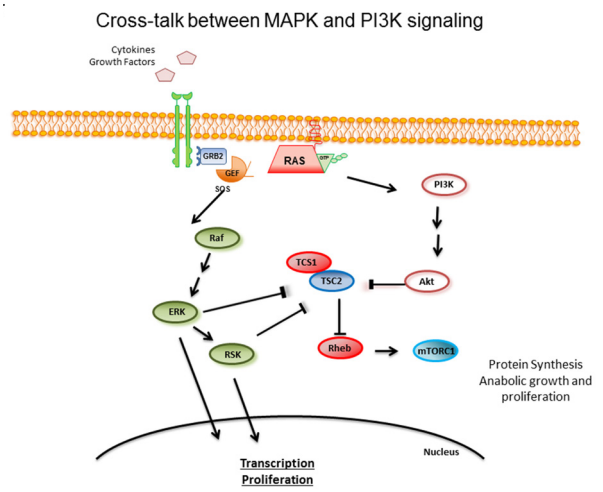

Figure 7. MAPK/PI3K cross-talk in Ras signaling. The Raf/ERK pathway is highlighted in green, and PI3K/mTOR pathways in red and blue.

pathways induce phosphorylation of distinct residues in the mTORC1 negative regulator, TSC2, each resulting in activation of the mTORC1 complex (*126*). Both PI3K/Akt and MAPK/ERK are able to inactivate the proapoptotic protein BAD (*127*). In addition, Raf-MAPK signaling in epithelial cells strongly induces transcription of autocrine expression of EGF-like growth factors, such as HB-EGF, TGFα, and amphiregulin. This autocrine signaling can be a potent inducer of Ras and PI3K, and is implicated in protection of the cells from apoptosis in response to extracellular matrix (ECM) detachment (*128*) (Figure 7).

5. Plasma membrane microdomains and Ras function

Lateral separation at the plasma membrane dictates Ras-isotype specific signaling. Activated HRAS signals from the lipid disordered membrane, which has been shown to be necessary for efficient activation of downstream effector Raf (*38,129,130*). Alternatively, activated RRAS resides within lipid ordered membranes. Initial reports using yeast two-hybrid screening showed RRAS could directly bind Raf-1 (*131*). However, subsequent studies show RRAS does not active the MAPK pathway in cells, suggesting a spatial regulation affecting these differences in HRAS and RRAS signaling (Figure 8) (*132*). Our group recently exploited distinct membrane microdomain targeting of RRAS and HRAS, by swapping the C-terminal targeting domains and stably expressing these Ras variants in non-transformed cells, to explore the contributions of this feature to HRAS signaling, stimulation of proliferation, and tumorigenic potential. Earlier studies had shown that HRAS harboring the RRAS targeting domain (which we refer to as tR), displayed RRAS-like regulation of integrin activation (another Ras function not discussed in detail here), indicating adoption of an RRAS function

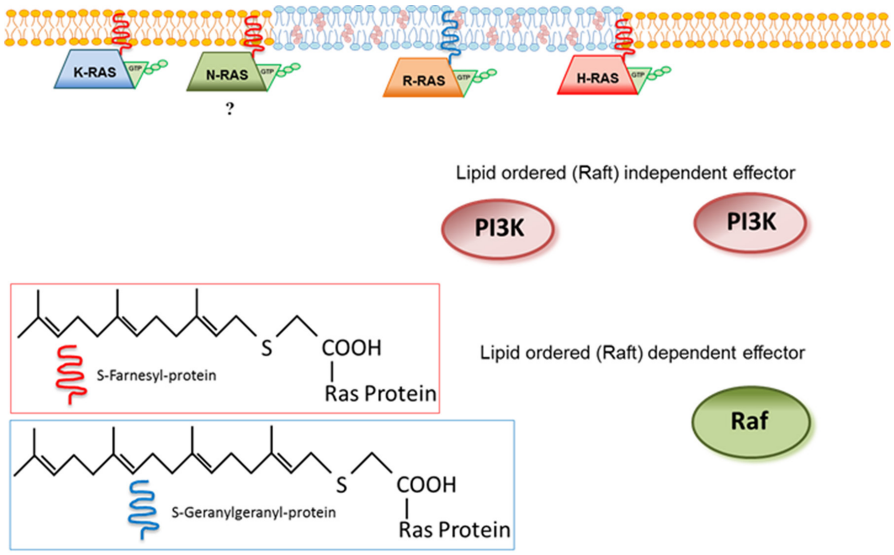

Figure 8. The C-termini modulate Ras signaling by targeting HRAS and RRAS to different membrane microdomains. The lipid ordered membrane (rafts) are distinct microdomains which have increased concentrations of cholesterol and glycosphingolipids. Ras isoforms are laterally segregated at the plasma membrane depending upon the post-translational modifications dictated by the HVR. Shown are the locations in which each isoform signals from upon GTP-loading. The location specific signaling of HRAS has been shown to be necessary for efficient activation of downstream effector Raf, while PI3K activation can occur along the entire membrane.

by HRAS mediated solely by exchange of the plasma membrane targeting domain (*22,45*). We found that the targeting domain (tD) swaps resulted in isotypic switches in Ras mitogenic signaling through the Raf pathway: HRAS harboring the RRAS tD (HRAS-tR) lost the ability to bind and activate Raf, stimulate MEK and ERK activation, and to promote cell proliferation as well as transformation, whereas the converse swap in RRAS, RRAS-tH, showed a gain of all these functions (*39*). However, HRAS-tR retained the ability to activate PI3K, whereas RRAS showed only weak PI3K activation regardless of the associated tD. We proposed a model for regulation of Ras isotypic signaling by lateral segregation in membrane microdomains, outlined in Figure 8. In this model, Ras localized to the lipid ordered/disordered border (*e.g.*, with tH), gains access to Raf interaction, supporting propagation of the MAPK pathway, and mitogenesis, and transforming Ras outcomes in the case of CA Ras (*39*). Thus, targeting to the lipid ordered/disordered membrane border is sufficient for a Ras protein to recruit Raf and propagate the associated signaling and functional effects.

6. Targeting Ras in cancer

There have been a variety of approaches taken to combat Ras oncogenic signaling. The most straightforward approach would be to directly target Ras, though this has proven to be challenging (*133*), in part because Ras binds GTP with an affinity in the picomolar range (*134*). Identification of small molecules which bind with high affinity to the surface of Ras proteins has been difficult due to the globular domain of Ras, which does not have an easily accessible active site or pocket, and Ras was once thought of as 'undruggable' (*135,136*). Few screened inhibitors have shown promising efficacy past pilot *in vitro* experiments (*136-138*). Targeting plasma membrane microdomain localization of H-/N-RAS as well as KRAS holds promise as an alternative approach to interfere with CA Ras signaling and effects.

6.1. *Development of Ras inhibitors and current anti-Ras modalities*

Given the difficulties in targeting Ras directly, some initial efforts focused on intervention of Ras signaling through blocking post-translational modification. Therapeutics designed to interfere with Ras prenylation can be divided into at least two groups. One strategy is development of peptidomimetics that compete with unmodified Ras for farnesyltransferase. Another would be nonpeptidomimetics, such as farnesylpyrophosphate (FPP) analogs, which compete for binding to the farnesyltransferase protein (*139-141*). Collectively, small molecule inhibitors which inactivate the enzymatic function of farnesyltransferase are labeled FTIs (farnesyltransferase inhibitors). Treatment with FTIs (such as SCH 66336) inhibit cell growth in a variety of cancer cell lines when treated *in vitro* and *in vivo* tumor xenografts (*142*). Subsequently, many studies focused on the effect of FTIs on HRAS, showing great efficacy in disrupting membrane association, and blunting colony formation in soft agar (*143,144*). Despite a wealth of data showing blunted cancer growth using various *in vitro* systems, clinical trials using FTIs alone have had disappointingly poor outcomes (*142,145,146*). Further investigation revealed that cells treated with FTIs can

yield alternatively prenylated mutant KRAS or NRAS, by attachment of a geranylgeranyl group (*147,148*). This led to a new approach for developing a class of inhibitors for Geranylgeranyltransferase (GGTIs), though monotherapy or in conjunction with FTIs are not effective due to toxicity issues (*149*). A class of inhibitors targeting both farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase) (such as L-778, 123) were developed and failed to make it through phase I clinical trials. Despite dual inhibition, similar to treatment with FTIs, Ras activity was not inhibited (*150*).

Another strategy for targeting Ras in cancer has been through inhibition of effector pathways directly downstream of Ras. Efforts have largely focused on either the MAPK or PI3K pathways. Sorafenib was the first Raf inhibitor to be approved by the US Food and Drug Administration (FDA) for treatment of carcinomas (*151,152*), though its effects appear largely due to inhibition of tumor angiogenesis (*153*). However, tumors bearing B-Raf activating mutations (such as the most common V600E) do not benefit from treatment with sorafenib, due to the reduced affinity from change in tertiary structure (*154*). This finding facilitated the development for inhibitors targeting CA B-Raf, such as Vemurafenib (PLX4032), which is an ATP-competitive inhibitor of B-Raf V600E (*155*). While successfully inhibiting the mutant Raf, the therapeutic enhances dimer formation and transactivation of the nonmutant Raf dimer, subsequently causing a paradoxical increase in signaling (*156*). Only recently has a new generation of Raf inhibitor has become available, so called 'paradox-breaking' Raf inhibitors. This presents an exciting new avenue, which is expected to make its way to clinical trials within the next few years (*157*).

Increased activity in PI3K signaling is commonly seen in cancer, and p110α is critical for Ras-driven tumorigenesis (*121,158*). Interestingly, Ras and p110α activating mutations are seen together in some cancers, such as colorectal cancer (*159-161*). Quite a few therapeutic agents have been developed to target proteins along this axis which are currently under clinical evaluation. The mTORC1 complex is targeted by Rapamycin analogs, everolimus and temsirolimus, which were the first therapeutics of their kind to be FDA approved to treat cancer (*162,163*). Rapamycin potently inhibits mTOR, and is FDA approved for its immunosuppressant properties, and more recently to treat lymphangioleiomyomatosis (LAM) (*164,165*). Other developed inhibitors currently in clinical trials include targeting of either specific class I PI3-kinase isoforms, or so-called 'pan-PI3K' inhibitors (*166-168*). Despite some preclinical promise, using PI3K-pathway drugs as a monotherapy often leads to acquired resistance (*168-170*).

Often single-pathway inhibitors will induce feedback to the opposite pathway (see PI3K and MAPK pathway cross-talk). As such, the lack of *in vivo* efficacy in targeting either the MAPK or PI3K pathway independently has driven the idea of combinatorial therapies. Studies utilizing *in vitro* systems or animal models which use treatments blocking both pathways have shown some preclinical success, which increases enthusiasm for potential clinical success (*170-172*).

6.2. *Targeting Ras and Ras tumor growth with membrane microdomain-targeting drugs*

6.2.1. *HRAS-induced tumor growth regulated by plasma membrane microdomains*

Based on our model, we predicted that cells stably expressing RRAS-tH would drive tumor progression in mice similar to HRAS cells, due to adoption of HRAS-like signaling by RRAS as a result of tH-directed targeting. We also predicted that HRAS-tR cells would show converse effects, with loss of tumorigenicity on a CA HRAS background by tR-mediated lipid raft sequestration. Surprisingly, both HRAS and HRAS-tR cells yielded robust tumor growth in orthotopic models, yielding tumors up to 2500 mm^3, whereas RRAS-tH cells formed small tumors but they did not progress to volumes > ~50-100mm^3. This was an unexpected result, as we anticipated that tumor promotion by HRAS-tR would be attenuated compared with HRAS. Inhibition of MEK resulted in ~70% attenuation of tumor growth by HRAS cells, but had no effect on HRAS-tR tumor growth. However, blockade of PI3K using LY294002 substantially inhibited growth of both HRAS and HRAS-tR tumors (*39*). Thus, MAPK and PI3K signaling contribute to HRAS-driven proliferation, transformation and tumor progression, and PI3K activation is a major pathway driving tumor progression by lipid raft-sequestered HRAS, which is deficient in MAPK signaling.

We investigated whether mTORC1 pathways represented a point of convergence for HRAS signaling, by monitoring phosphorylation of S6 by S6 kinase, a downstream mTORC1 effector. HRAS induced robust S6 phosphorylation (pS6) at Serine 240/244 in low serum, and PI3K inhibition ablated HRAS-induced pS6, as expected. However, MEK inhibition also diminished pS6. Interestingly, HRAS-tR stable cells, in which HRAS-induced phospho-ERK (pERK) is inhibited, also showed attenuated pS6 in low serum, comparable to MEK-inhibited HRAS expressing cells. Thus, inhibition of MEK partially blocks HRAS-induced mTORC1 signaling. In allograft tumor models, treatment with the mTOR inhibitor rapamycin blunted HRAS cell tumor growth; however, HRAS-tR tumors were much more sensitive to rapamycin. IHC analysis of resected tumors indicated a marked reduction in pS6 in rapamycin-treated HRAS tumors, but also in untreated HRAS-tR tumors. Interestingly, pERK was also reduced in HRAS

tumors with rapamycin (*173*). Together, our recent results indicate that genetically enforced lipid ordered domain sequestration inhibits HRAS-induced mTORC1 signaling, and sensitizes HRAS tumors to rapamycin treatment.

6.2.2. *Gal-1 and combinatorial inhibition*

As described above, we found that dual pathway blockade by a combination of genetically enforced microdomain sequestration (inhibiting Raf pathway signaling) and PI3K (downstream mTOR) inhibition, yields additive effects in blocking progression of HRAS-driven tumor growth. To investigate a more translational approach taking advantage of Ras plasma membrane microdomain targeting, we considered whether inhibition of Gal-1 would alter HRAS PM membrane microdomain localization, and thereby disrupt CA HRAS in a manner similar to genetically-enforced HRAS sequestration (*i.e.*, HRAS-tR). We utilized OTX008, a small molecule allosteric inhibitor of Gal-1, which binds on the opposite side from the β-galactoside-binding site, and has been demonstrated as an anticancer agent (*46,174,175*). We found that OTX008 treatment shifts HRAS to the lipid ordered domain and yields a marked inhibition of tumor growth. However, we found that OTX008 combined with rapamycin provides an additive effect resulting in nearly undetectable tumor growth. These profound results indicate that combined mTOR/Gal-1 inhibition yields stasis of HRAS-driven tumor growth. Thus, inhibition of Gal-1 results in a blockade of GTP-HRAS shuttling from the lipid ordered domain and inhibition of MAPK signaling, and sensitizes tumors driven by CA HRAS to mTOR inhibition (Figure 9A) (*173*). Inhibition of Gal-1 using OTX008 is currently under clinical evaluation, and our findings support the development of Gal-1 targeting schemes to limit progression of mutant HRAS cancers. Moreover, preclinical and clinical trials support using mTOR inhibitors to combat Ras-propagated cancer (*46*); hence, a dual pathway inhibition through Gal-1/mTOR targeting may present increased efficacy over current anti-Ras modalities.

6.2.3. *Gal-3 as a putative approach to combat KRAS cancers*

A majority of Ras-driven cancer cases in the United States (*e.g.*, in lung, pancreas, and colorectal cancers) are caused by mutant KRAS *(6)*. As highlighted above, direct targeting of KRAS has proven clinically intractable due to a lack of drug-binding pockets, and drugs against the membrane anchors have not been successful (*176*). However, alternative strategies to modulate KRAS anchorage to the plasma membrane may prove viable for combating KRAS-driven cancer progression. MEK inhibition alone seems to have

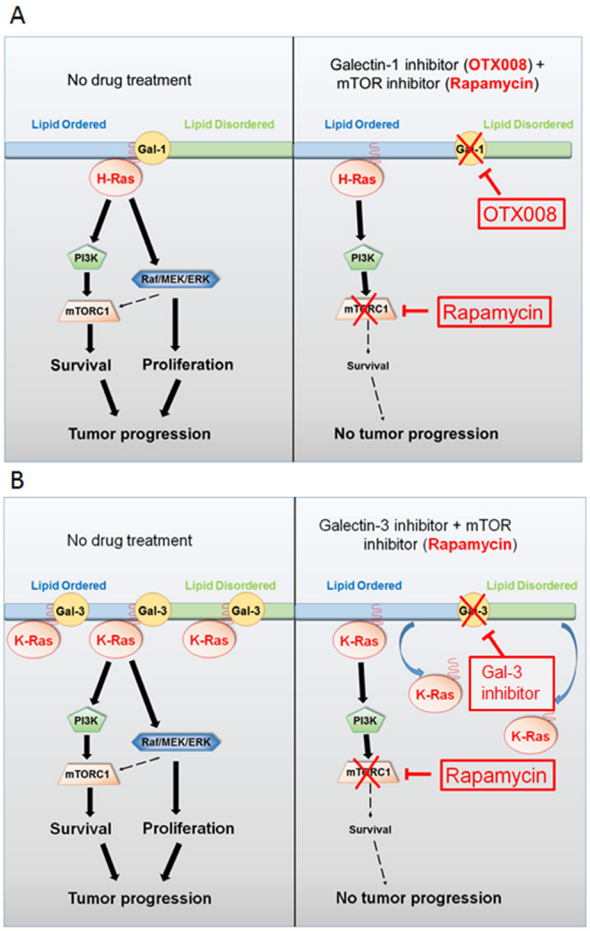

Figure 9. Ras signaling and tumorigenesis in response to inhibition of Galectins and mTOR. (A) Schematic of predicted effects of dual targeting in HRAS cancers. Galectin-1 inhibition using OTX008 results in HRAS mistargeting to the PM and disruption of MAPK mitogenic signaling. Rapamycin treatment potently reduces mTOR survival signaling; analogue mTOR inhibitors should have similar effects. Combinatorial use of these inhibitors results in an additive effect over either monotherapy. **(B)** Predicted effects of dual inhibition of Galectin-3, and mTOR (rapamycin or other mTOR inhibitors), in KRAS mutant tumors. Gal-3 inhibition reduces KRAS membrane anchorage, greatly reducing Raf activation which is lipid ordered domain-restricted, but allowing PI3K activation which is distributed across membrane microdomains. Combined Gal-3 and mTOR inhibition blocks both pathways and inhibits tumor progression.

variable outcomes in KRAS tumors (*177*). Although KRAS does not segregate in PM microdomains like H- and NRAS, targeting KRAS membrane anchorage may be an effective anti-tumor approach in KRAS mutant cancers. Whereas HRAS is localized to the PM with Gal-1, KRAS PM anchorage is supported by the Galectin-3 (Gal-3) scaffold (*178,179*). Gal-3 over-expression leads to chronic KRAS activation, potentiation of Ras signaling, tumor cell activation including increased proliferation and migration, and tumor progression (*180-182*). Gal-3-mediated KRAS activation is associated with ERK, but not PI3K, signaling (*48*). There is substantial evidence for Gal-3 inhibition as a potential tumor blocker (*183-190*).

Thus, a parallel strategy to inhibit KRAS cancers to the Gal-1/mTOR strategy for HRAS, using Gal-3/mTOR inhibition, may provide a new direction (Figure 9B). Our group is currently investigating dual inhibition of Gal-3 and mTOR in blocking progression of KRAS-driven cancers.

7. Conclusions and future prospects

Taken together, how can furthering our understanding of the roles of plasma membrane microdomain Ras targeting help in developing effective therapies to treat patients with Ras-driven cancer? Currently, Ras-positive cancers are treated with a cocktail of therapeutics, generally targeting EGFR or VEGF, or downstream pathway components as described above. However effective the treatment may initially be, it is common to develop an acquired resistance or secondary mutations which requires alternative strategies. Preclinical data largely support positive results using a cocktail of PI3K and MAPK inhibitors, and a large number of inhibitors are currently in clinical trials. We propose a novel rationale for an alternative strategy in HRAS-driven cancers using clinically viable and commercially available therapeutics to disrupt Ras signaling by interfering with localization-dependent effector binding. This approach warrants further analysis as to whether it would be advantageous to individuals who have developed resistance to the common targeted therapies. Future studies are needed to provide further mechanistic insight into the molecular, cellular, and physiological outcomes of combinatorial mTOR/Galectin-1 inhibition.

HRAS mutations are considerably rarer in cancer than mutations in NRAS or KRAS. However, KRAS-positive anti-cancer approaches may still benefit from this basic dual treatment paradigm. Whereas HRAS is localized to the PM with Gal-1, KRAS anchorage is supported by the Gal-3 scaffold. This suggests that inhibition of Gal-3 and mTOR may mimic HRAS results in the context of KRAS mutant cancers. Scaffold proteins that specifically bind NRAS, and PM localization remain controversial, so implications in targeted therapies will require further exploration. However, it is likely that an analogous scaffolding protein which localizes NRAS to the PM exists. This suggests that both N- and KRAS-driven tumorigenesis may benefit through a modified form of the proposed targeted therapy. Overall, new approaches to combat Ras-driven cancers are still needed, and disrupting plasma membrane microdomain-based Ras function holds promise as an effective strategy.

Acknowledgements

This work was supported by AHA grant 16GRNT27260319 to L.E.G.

References

1. Downward J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer. 2003; 3:11-22.
2. Johnson L, Doron Greenbaum, Karen Cichowski, Kim Mercer, Elizabeth Murphy, Eric Schmitt, Roderick T. Bronson, Heywood Umanoff, Windfried Edelmann, Raju Kucherlapati, and Tyler Jacks. K-ras is an essential gene in the mouse with partial functional overlap with N-ras. Genes Dev. 1997; 11:2468-2481.
3. Fernández-Medarde A, Santos E. Ras in Cancer and Developmental Diseases. Genes and Cancer. 2011; 2:344-359.
4. Adjei A. Blocking oncogenic Ras signaling for cancer therapy. J Natl Cancer Inst. 2001; 93:1062-1074.
5. Cheng CM, Li H, Gasman S, Huang J, Schiff R, Chang EC. Compartmentalized Ras proteins transform NIH 3T3 cells with different efficiencies. Mol Cell Biol. 2010; 31:983-997.
6. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: Weaving a tumorigenic web. Nat Rev Cancer. 2011; 11:761-774.
7. Pike L. Lipid rafts: Bringing order to chaos. Lipid Res. 2003; 44:655-657.
8. Cox AD, Brtva TR, Lowe DG, Der CJ. R-Ras induces malignant, but not morphologic, transformation of NIH3T3 cells. Oncogene. 1994; 9:3281-3288.
9. Goldfinger L. Choose your own path: Specificity in Ras GTPase signaling. Mol Biosyst. 2008; 4:293-299.
10. Ballester R, Marchuk D, Boguski M, Saulino A, Letcher R, Wigler M, Collins F. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. Cell. 1990; 63:851-859.
11. Gibbs J, Schaber MD, Allard WJ, Sigal IS, Scolnick EM. Purification of ras GTPase activating protein from bovine brain. Proc Natl Acad Sci U S A. 1988; 85:5026-5030.
12. Vetter I, Wittinghofer A. The guanine nucleotide-binding switch in three dimensions. Science. 2001; 294:1299-1304.
13. Spoerner M, Herrmann C, Vetter IR, Kalbitzer HR, Wittinghofer A. Dynamic properties of the Ras switch I region and its importance for binding to effectors. Proc Natl Acad Sci U S A. 2001; 98:4944-4949.
14. Eisenberg S, Laude AJ, Beckett AJ, Mageean CJ, Aran V, Hernandez-Valladares M, Henis YI, Prior IA. The role of palmitoylation in regulating Ras localization and function. Biochem Soc Trans. 2013; 41:79-83.
15. Rocks O, Peyker A, Kahms M, Verveer PJ, Koerner C, Lumbierres M, Kuhlmann J, Waldmann H, Wittinghofer A, Bastiaens PI. An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. Science. 2005; 307:1746-1752.
16. Misaki R, Morimatsu M, Uemura T, Waguri S, Miyoshi E, Taniguchi N, Matsuda M, Taguchi T. Palmitoylated Ras proteins traffic through recycling endosomes to the plasma membrane during exocytosis. J Cell Biol. 2010; 191:23-29.
17. Henis YI, Hancock JF, Prior IA. Ras acylation, compartmentalization and signaling nanoclusters (Review). Mol Membr Biol. 2009; 26:80-92.
18. Reiss Y, Goldstein JL, Seabra MC, Casey PJ, Brown MS. Inhibition of Purified p2lras Farnesyl: Protein Transferase by Cys-AAX Tetrapeptides. Cell. 1990; 62:81-88.
19. Wright LPaMRP. CAAX modification and membrane

targeting of Ras. J Lipid Res. 2006; 47:883-891.
20. Boyartchuk VL, Matthew N. Ashby, Jasper Rine. Modulation of Ras and a-Factor Function by Carboxyl-Terminal Proteolysis. Science. 1997; 275:1796-1800.
21. Hrycyna CA, S K Sapperstein, S Clarke, and S Michaelis. The Saccharomyces cerevisiae STE14 gene encodes a methyltransferase that mediates C-terminal methylation of a-factor and RAS proteins. EMBO J. 1991; 10:1699–1709.
22. Oertli B, Han J, Marte BM, Sethi T, Downward J, Ginsberg M, Hughes PE. The effector loop and prenylation site of R-Ras are involved in the regulation of integrin function [In Process Citation]. Oncogene. 2000; 19:4961-4969.
23. Hancock JF, Magee AI, Childs JE, Marshall CJ. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell. 1989; 57:1167-1177.
24. Buss J, Sefton BM. Direct identification of palmitic acid as the lipid attached to p21ras. Mol Cell Biol. 1986; 6:116-122.
25. Apolloni A, Prior IA, Lindsay M, Parton RG, Hancock JF. H-ras but not K-ras traffics to the plasma membrane through the exocytic pathway. Mol Cell Biol. 2000; 20:2475-2487.
26. Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, Ivanov IE, Philips MR. Endomembrane trafficking of ras: The CAAX motif targets proteins to the ER and Golgi. Cell. 1999; 98:69-80.
27. Laude A, Prior IA. Palmitoylation and localisation of RAS isoforms are modulated by the hypervariable linker domain. J Cell Sci. 2008; 121:421-427.
28. Swarthout JT, Lobo S, Farh L, Croke MR, Greentree WK, Deschenes RJ, Linder ME. DHHC9 and GCP16 constitute a human protein fatty acyltransferase with specificity for H- and N-Ras. J Biol Chem. 2005; 280:31141-31148.
29. Ohta E, Misumi Y, Sohda M, Fujiwara T, Yano A, Ikehara Y. Identification and characterization of GCP16, a novel acylated Golgi protein that interacts with GCP170. J Biol Chem. 2003; 278:51957-51967.
30. Baumgart F, Corral-Escariz M, Perez-Gil J, Rodriguez-Crespo I. Palmitoylation of R-Ras by human DHHC19, a palmitoyl transferase with a CaaX box. Biochim Biophys Acta. 2010; 1798:592-604.
31. Wurtzel JG, Kumar P, Goldfinger LE. Palmitoylation regulates vesicular trafficking of R-Ras to membrane ruffles and effects on ruffling and cell spreading. Small GTPases. 2012; 3:139-153.
32. Dekker FJ, Rocks O, Vartak N, *et al*. Small-molecule inhibition of APT1 affects Ras localization and signaling. Nat Chem Biol. 2010; 6:449-456.
33. James G, Olson EN. Identification of a novel fatty acylated protein that partitions between the plasma membrane and cytosol and is deacylated in response to serum and growth factor stimulation. J Biol Chem. 1989; 264:20998-21006.
34. Ahearn IM, Tsai FD, Court H, Zhou M, Jennings BC, Ahmed M, Fehrenbacher N, Linder ME, Philips MR. FKBP12 binds to acylated H-ras and promotes depalmitoylation. Mol Cell. 2011; 41:173-185.
35. Niv H, Gutman O, Kloog Y, Henis YI. Activated K-Ras and H-Ras display different interactions with saturable nonraft sites at the surface of live cells. J Cell Biol. 2002; 157:865–872.
36. Roy S, Plowman S, Rotblat B, Prior IA, Muncke C, Grainger S, Parton RG, Henis YI, Kloog Y, Hancock JF. Individual palmitoyl residues serve distinct roles in H-ras trafficking, microlocalization, and signaling. Mol Cell Biol. 2005; 25:6722–6733.
37. Hancock JF, Parton RG. Ras plasma membrane signalling platforms. Biochem J. 2005; 389:1-11.
38. Prior IA, Harding A, Yan J, Sluimer J, Parton RG, Hancock JF. GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. Nat Cell Biol. 2001; 3:368-375.
39. Michael JV, Wurtzel JGT, Goldfinger LE. Regulation of H-Ras-driven MAPK signaling, transformation and tumorigenesis, but not PI3K signaling and tumor progression, by plasma membrane microdomains. Oncogenesis. 2016; 5:e228.
40. Janosi L, Li Z, Hancock JF, Gorfe AA. Organization, dynamics, and segregation of Ras nanoclusters in membrane domains. Proc Natl Acad Sci U S A. 2012; 109:8097-8102.
41. Nicolini C, Baranski J, Schlummer S, Palomo J, Lumbierres-Burgues M, Kahms M, Kuhlmann J, Sanchez S, Gratton E, Waldmann H, Winter R. Visualizing association of N-ras in lipid microdomains: Influence of domain structure and interfacial adsorption. J Am Chem Soc. 2006; 128:192-201.
42. Matallanas D, Imanol Arozarena, María T. Berciano, David S. Aaronson, Angel Pellicer, Miguel Lafarga, Piero Crespo. Differences on the Inhibitory Specificities of H-Ras, K-Ras, and N-Ras (N17) Dominant Negative Mutants Are Related to Their Membrane Microlocalization. J Biol Chem. 2002; 278:4572-4581.
43. Eisenberg S, Beckett AJ, Prior IA, Dekker FJ, Hedberg C, Waldmann H, Ehrlich M, Henis YI. Raft protein clustering alters N-Ras membrane interactions and activation pattern. Mol Cell Biol. 2011; 31:3938-3952.
44. Hancock JF. Ras proteins: Different signals from different locations. Nat Rev Mol Cell Biol. 2003; 4:373-384.
45. Hansen M, Prior IA, Hughes PE, Oertli B, Chou FL, Willumsen BM, Hancock JF, Ginsberg MH. C-terminal sequences in R-Ras are involved in integrin regulation and in plasma membrane microdomain distribution. Biochem Biophys Res Commun. 2003; 311:829-838.
46. Astorgues-Xerri L, Riveiro ME, Tijeras-Raballand A, Serova M, Neuzillet C, Albert S, Raymond E, Faivre S. Unraveling galectin-1 as a novel therapeutic target for cancer. Cancer Treat Rev. 2014; 40:307-319.
47. Paz A, Haklai R, Elad-Sfadia G, Ballan E, Kloog Y. Galectin-1 binds oncogenic H-Ras to mediate Ras membrane anchorage and cell transformation. Oncogene. 2001; 20:7486-7493.
48. Elad-Sfadia G, Haklai R, Balan E, Kloog Y. Galectin-3 augments K-Ras activation and triggers a Ras signal that attenuates ERK but not phosphoinositide 3-kinase activity. J Biol Chem. 2004; 279:34922-34930.
49. Shalom-Feuerstein R, Cooks T, Raz A, Kloog Y. Galectin-3 regulates a molecular switch from N-Ras to K-Ras usage in human breast carcinoma cells. Cancer Res. 2005; 65:7292-7300.
50. Shalom-Feuerstein R, Levy R, Makovski V, Raz A, Kloog Y. Galectin-3 regulates RasGRP4-mediated activation of N-Ras and H-Ras. Biochim Biophys Acta. 2008; 1783:985-993.
51. Belanis L, Plowman SJ, Rotblat B, Hancock JF, Kloog Y. Galectin-1 is a novel structural component and a major regulator of h-ras nanoclusters. Mol Biol Cell. 2008; 19:1404-1414.
52. Prior I, Muncke C, Parton RG, Hancock JF. Direct

visualization of Ras proteins in spatially distinct cell surface microdomains. J Cell Biol. 2003; 160:165-170.
53. Satoh T, Kaziro Y. Ras in signal transduction. Semin Cancer Biol. 1992; 3:169-177.
54. Khosravi-Far R, Der CJ. The Ras signal transduction pathway. Cancer Metastasis Rev. 1994; 13:67-89.
55. Egan SE, Weinberg RA. The pathway to signal achievement. Nature. 1993; 365:781-783.
56. Mayer BJ, Ren R, Clark KL, Baltimore D. A putative modular domain present in diverse signaling proteins. Cell. 1993; 73:629-630.
57. Sasaoka T, Langlois WJ, Leitner JW, Draznin B, Olefsky JM. The signaling pathway coupling epidermal growth factor receptors to activation of p21ras. J Biol Chem. 1994; 269:32621-33625.
58. Feig L. Guanine-nucleotide exchange factors: A family of positive regulators of Ras and related GTPases. Curr Opin Cell Biol. 1994; 6:204-211.
59. Bowtell D, Fu P, Simon M, Senior P. Identification of murine homologues of the Drosophila son of sevenless gene: Potential activators of ras. Proc Natl Acad Sci U S A. 1992; 89:6511-6515.
60. Chardin P, Camonis JH, Gale NW, van Aelst L, Schlessinger J, Wigler MH, Bar-Sagi D. Human Sos1: Aguanine nucleotide exchange factor for Ras that binds to GRB2. Science. 1993; 260:1338-1343.
61. Thomas SM, DeMarco M, D'Arcangelo G ,Halegoua S, Brugge J.S., . Ras Is Essential for Nerve Growth Factor and Phorbol Ester-Induced Tyrosine Phosphorylation of MAP Kinases. Cell. 1992; 68:1032-1040.
62. Van Aelst L, Barr M, Marcus S, Polverino A, Wigler M. Complex formation between RAS and RAF and other protein kinases. Proc Natl Acad Sci U S A. 1993; 90:6213-6217.
63. Moodie S, Willumsen BM, Weber MJ, Wolfman A,. Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. Science. 1993; 260:1658-1661.
64. Morrison D, Cutler RE. The complexity of Raf-1 regulation. Curr Opin Cell Biol. 1997; 9:174-179.
65. Leevers S, Paterson HF, Marshall CJ. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature. 1994; 369:411-414.
66. Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF. Activation of Raf as a result of recruitment to the plasma membrane. Science. 1994; 264:1469-1467.
67. Kyriakis J, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR, Avruch J. Raf-1 activates MAP kinase-kinase. Nature. 1992; 358:417-421.
68. Gardner A, Vaillancourt RR, Lange-Carter CA, Johnson GL. MEK-1 phosphorylation by MEK kinase, Raf, and mitogen-activated protein kinase: Analysis of phosphopeptides and regulation of activity. Mol Biol Cell. 1994; 5:193-201.
69. Crews C, Erikson RL. Purification of a murine protein-tyrosine/threonine kinase that phosphorylates and activates the Erk-1 gene product: Relationship to the fission yeast byr1 gene product. Proc Natl Acad Sci U S A. 1992; 89:8205-8209.
70. Clapéron A, Therrien M. KSR and CNK: Two scaffolds regulating RAS-mediated RAF activation. Oncogene. 2007; 26:3143-3158.
71. Suzuki T, K-Tsuzuku J, Ajima R, Nakamura T, Yoshida Y, Yamamoto T. Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. Genes Dev. 2002; 16:1356-1370.
72. Davis R. Transcriptional regulation by MAP kinases. Mol Reprod Dev. 1995; 42:459-467.
73. Waskiewicz AJ FA, Proud CG, Cooper JA. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO J. 1997; 16:1909-1920.
74. Dalby K, Morrice N, Caudwell FB, Avruch J, Cohen P. Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. J Biol Chem. 1998; 273:1496-1505.
75. Fukunaga R, Hunter T. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J. 1997; 16:1921-1933.
76. Xu S, Robbins D, Frost J, Dang A, Lange-Carter C, Cobb MH. MEKK1 phosphorylates MEK1 and MEK2 but does not cause activation of mitogen-activated protein kinase. Proc Natl Acad Sci U S A. 1995; 92:6808-6812.
77. Doehn U, Hauge C, Frank SR, Jensen CJ, Duda K, Nielsen JV, Cohen MS, Johansen JV, Winther BR, Lund LR, Winther O, Taunton J, Hansen SH, Frödin M. RSK is a principal effector of the RAS-ERK pathway for eliciting a coordinate promotile/invasive gene program and phenotype in epithelial cells. Mol Cell. 2009; 35:511-522.
78. Pyronnet S, Imataka H, Gingras AC, Fukunaga R, Hunter T, Sonenberg N. Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. EMBO J. 1999; 18:270-279.
79. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer. 2002; 2:489-501.
80. Cantley L. The phosphoinositide 3-kinase pathway. Science. 2002; 296:1655-1657.
81. Courtney K, Corcoran RB, Engelman JA. . The PI3K pathway as drug target in human cancer. . J Clin Oncol. 2010; 28:1075-1083.
82. Engelman J, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet. 2006; 7:606-619.
83. Yuan T, Cantley LC. PI3K pathway alterations in cancer: Variations on a theme. Oncogene. 2008; 27:5497-5510.
84. Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer JM. Regulation of the p85/p110 phosphatidylinositol 3'-kinase: Stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. Mol Cell Biol. 1998; 18:1379-1387.
85. Skolnik E, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, Ullrich A, Schlessinger J. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell. 1991; 65:83-90.
86. Rodrigues G, Falasca M, Zhang Z, Ong SH, Schlessinger J. A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. Mol Cell Biol. 2000; 20:1448-1459.
87. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Downward J. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature. 1994; 370:527-532.
88. Kodaki T, Woscholski R, Hallberg B, Rodriguez-Viciana P, Downward J, Parker PJ. The activation of phosphatidylinositol 3-kinase by Ras. Curr Biol. 1994; 4:798-806.

89. Ong S, Hadari YR, Gotoh N, Guy GR, Schlessinger J, Lax I. Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. Proc Natl Acad Sci U S A. 2001; 98:6074-6079.
90. Chan T, Rodeck U, Chan AM, Kimmelman AC, Rittenhouse SE, Panayotou G, Tsichlis PN. Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit. Cancer Cell. 2002; 1:181-191.
91. Shirai T, Tanaka K, Terada Y, Sawada T, Shirai R, Hashimoto Y, Nagata S, Iwamatsu A, Okawa K, Li S, Hattori S, Mano H, Fukui Y. Specific detection of phosphatidylinositol 3,4,5-trisphosphate binding proteins by the PIP_3 analogue beads: An application for rapid purification of the PIP_3 binding proteins. Biochim Biophys Acta. 1998; 1402:292-302.
92. Maehama T, Dixon JE. PTEN: A tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol. 1999; 9:125-128.
93. Alessi D, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 1996; 15:6541-6551.
94. Huang B, Akbar M, Kevala K, Kim HY. Phosphatidylserine is a critical modulator for Akt activation. J Cell Biol. 2011; 192:979-992.
95. Sarbassov D, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005; 307:1098-1101.
96. Gardai S, Hildeman DA, Frankel SK, Whitlock BB, Frasch SC, Borregaard N, Marrack P, Bratton DL, Henson PM. Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. J Biol Chem. 2004; 279:21085-21095.
97. Datta S, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997; 91:231-241.
98. Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 2003; 17:1829-1834.
99. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 2002; 4:648-657.
100. Sato T, Umetsu A, Tamanoi F. Characterization of the Rheb-mTOR signaling pathway in mammalian cells: Constitutive active mutants of Rheb and mTOR. Methods Enzymol. 2008;307-320.
101. Kim D, Sarbassov DD, Ali SM, Latek RR, Guntur KV, Erdjument-Bromage H, Tempst P, Sabatini DM. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell. 2003; 11:895-904.
102. Manning B. Balancing Akt with S6K: Implications for both metabolic diseases and tumorigenesis. J Cell Biol. 2004; 167:399-403.
103. Um S, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J, Thomas G. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature. 2004; 431:200-205.
104. Peterson S, Trabalzini L, Brtva TR, Fischer T, Altschuler DL, Martelli P, Lapetina EG, Der CJ, White GC 2nd. Identification of a novel RalGDS-related protein as a candidate effector for Ras and Rap1. J Biol Chem. 1996; 271:29903-29908.
105. Hofer F, Fields S, Schneider C, Martin GS. Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. Proc Natl Acad Sci U S A. 1994; 91:11089-11093.
106. Spaargaren M, Bischoff JR. Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras, and Rap. Proc Natl Acad Sci U S A. 1994; 91:12609-12613.
107. Shao H, Andres DA. A novel RalGEF-like protein, RGL3, as a candidate effector for rit and Ras. J Biol Chem. 2000; 275:26914-26924.
108. Quilliam L, Rebhun JF, Castro AF. A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. Prog Nucleic Acid Res Mol Biol. 2002;391-444.
109. Boriack-Sjodin P, Margarit SM, Bar-Sagi D, Kuriyan J. The structural basis of the activation of Ras by Sos. Nature. 1998; 394:337-343.
110. Rusanescu G, Gotoh T, Tian X, Feig LA. Regulation of Ras signaling specificity by protein kinase C. Mol Cell Biol. 2001; 21:2650-2658.
111. Tian X, Rusanescu G, Hou W, Schaffhausen B, Feig LA. PDK1 mediates growth factor-induced Ral-GEF activation by a kinase-independent mechanism. EMBO J. 2002; 21:1327-1338.
112. Murai H, Ikeda M, Kishida S, Ishida O, Okazaki-Kishida M, Matsuura Y, Kikuchi A. Characterization of Ral GDP dissociation stimulator-like (RGL) activities to regulate c-fos promoter and the GDP/GTP exchange of Ral. J Biol Chem. 1997; 272:10483-10490.
113. Matsubara K, Kishida S, Matsuura Y, Kitayama H, Noda M, Kikuchi A. Plasma membrane recruitment of RalGDS is critical for Ras-dependent Ral activation. Oncogene. 1999; 18:1303-1312.
114. Rodriguez-Viciana P, McCormick F. RalGDS comes of age. Cancer Cell. 2005; 7:205-206.
115. Feig L. Ral-GTPases: Approaching their 15 minutes of fame. Trends Cell Biol. 2003; 13:419-425.
116. Mitin N, Rossman KL, Der CJ. Signaling interplay in Ras superfamily function. Curr Biol. 2005; 15:563-574.
117. Repasky G, Chenette EJ, Der CJ. Renewing the conspiracy theory debate: Does Raf function alone to mediate Ras oncogenesis? Trends Cell Biol. 2004; 14:639-647.
118. Hamad N, Elconin JH, Karnoub AE, Bai W, Rich JN, Abraham RT, Der CJ, Counter CM. Distinct requirements for Ras oncogenesis in human versus mouse cells. Genes Dev. 2002; 16:2045-2057.
119. Rangarajan A, Hong SJ, Gifford A, Weinberg RA. Species- and cell type-specific requirements for cellular transformation. Cancer Cell. 2004; 6:171-183.
120. Gonzalez-Garcia A, Pritchard CA, Paterson HF, Mavria G, Stamp G, Marshall CJ. RalGDS is required for tumor formation in a model of skin carcinogenesis. Cancer Cell. 2005; 7:219-226.
121. Rodriguez-Viciana P, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, Waterfield MD, Ridley A, Downward J. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell. 1997; 89:457-467.
122. Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science. 1999; 286:1741-1744.
123. Menges C, McCance DJ. Constitutive activation of the

Raf-MAPK pathway causes negative feedback inhibition of Ras-PI3K-AKT and cellular arrest through the EphA2 receptor. Oncogene. 2008; 27:2934-2940.
124. King A, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S, Marshall MS. The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature. 1998; 396:180-183.
125. Jin S, Zhuo Y, Guo W, Field J. p21-activated Kinase 1 (Pak1)-dependent phosphorylation of Raf-1 regulates its mitochondrial localization, phosphorylation of BAD, and Bcl-2 association. J Biol Chem. 2005; 280:24698-24705.
126. Memmott R, Dennis PA. Akt-dependent and -independent mechanisms of mTOR regulation in cancer. Cell Signal. 2009; 21:656-664.
127. Danial N. BAD: Undertaker by night, candyman by day. Oncogene. 2009; 27:S53-S70.
128. Schulze A, Lehmann K, Jefferies HB, McMahon M, Downward J. Analysis of the transcriptional program induced by Raf in epithelial cells. Genes Dev. 2001; 15:981-984.
129. Hansen M, Rusyn EV, Hughes PE, Ginsberg MH, Cox AD, Willumsen BM. R-Ras C-terminal sequences are sufficient to confer R-Ras specificity to H-Ras. Oncogene. 2002; 21:4448-4461.
130. Jaumot M, Yan J, Clyde-Smith J, Sluimer J, Hancock JF. The linker domain of the Ha-Ras hypervariable region regulates interactions with exchange factors, Raf-1 and phosphoinositide 3-kinase. J Biol Chem. 2002; 277:272-278.
131. Spaargaren M, Martin GA, McCormick F, Fernandez-Sarabia MJ, Bischoff JR. The Ras-related protein R-ras interacts directly with Raf-1 in a GTP-dependent manner. Biochem J. 1994; 300:303-307.
132. Marte BM, Rodriguez-Viciana P, Wennstrom S, Warne PH, Downward J. R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways. Curr Biol. 1997; 7:63-70.
133. Spiegel J, Cromm PM1, Zimmermann G1, Grossmann TN2, Waldmann H1. Small-molecule modulation of Ras signaling. Nat Chem Biol. 2014; 10:613-622.
134. John J, Schlichting I, Schiltz E, Rösch P, Wittinghofer A. C-terminal truncation of p21H preserves crucial kinetic and structural properties. J Biol Chem. 1989; 264:13086-13092.
135. Brunger AT, Milburn MV, Tong L, deVos AM, Jancarik J, Yamaizumi Z, Nishimura S, Ohtsuka E, Kim SH. Crystal structure of an active form of RAS protein, a complex of a GTP analog and the HRAS p21 catalytic domain. Proc Natl Acad Sci U S A. 1990; 87:4849-4853.
136. Cox A, Fesik SW2, Kimmelman AC3, Luo J4, Der CJ1. Drugging the undruggable RAS: Mission possible? Nat Rev Drug Discov 2014; 13:828-851.
137. Peri F, Airoldi C, Colombo S, Martegani E, van Neuren AS, Stein M, Marinzi C, Nicotra F. Design, synthesis and biological evaluation of sugar-derived Ras inhibitors. Chembiochem. 2005; 6:1839-1848.
138. Wang W, Fang G, Rudolph J. Ras inhibition *via* direct Ras binding--is there a path forward? Bioorg Med Chem Lett. 2012; 22:5766-5776.
139. End D, Smets G, Todd AV, Applegate TL, Fuery CJ, Angibaud P, Venet M, Sanz G, Poignet H, Skrzat S, Devine A, Wouters W, Bowden C. Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 *in vivo* and *in vitro*. Cancer Res 2001; 61:131-137.
140. Yonemoto M, Satoh T, Arakawa H, Suzuki-Takahashi I, Monden Y, Kodera T, Tanaka K, Aoyama T, Iwasawa Y, Kamei T, Nishimura S, Tomimoto K. J-104,871, a novel farnesyltransferase inhibitor, blocks Ras farnesylation *in vivo* in a farnesyl pyrophosphate-competitive manner. Mol Pharmacol. 1998; 54:1-7.
141. Basso A, Kirschmeier P, Bishop WR. Lipid posttranslational modifications. Farnesyl transferase inhibitors. J Lipid Res. 2006; 47:15-31.
142. Liu M, Bryant MS, Chen J, *et al*. Antitumor activity of SCH 66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and wap-ras transgenic mice. Cancer Res. 1998; 58:4947-4956.
143. Ashar H, James L, Gray K, Carr D, McGuirk M, Maxwell E, Black S, Armstrong L, Doll RJ, Taveras AG, Bishop WR, Kirschmeier P. The farnesyl transferase inhibitor SCH 66336 induces a $G_2 \rightarrow M$ or G_1 pause in sensitive human tumor cell lines. Exp Cell Res. 2001; 262:17-27.
144. Chen X, Makarewicz JM1, Knauf JA2, Johnson LK3, Fagin JA4. Transformation by $Hras^{G12V}$ is consistently associated with mutant allele copy gains and is reversed by farnesyl transferase inhibition. Oncogene. 2014; 33:5442-5449.
145. Winquist E, Moore MJ, Chi KN, Ernst DS, Hirte H, North S, Powers J, Walsh W, Boucher T, Patton R, Seymour L. A multinomial Phase II study of lonafarnib (SCH 66336) in patients with refractory urothelial cancer. Urol Oncol. 2005; 23:143-149.
146. Nakajima A, Tauchi T, Sumi M, Bishop WR, Ohyashiki K. Efficacy of SCH66336, a farnesyl transferase inhibitor, in conjunction with imatinib against BCR-ABL-positive cells. Mol Cancer Ther. 2003; 2:219-224.
147. Whyte D, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, Bishop WR, Pai JK. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem. 1997; 272:14459-14464.
148. Fiordalisi J, Johnson RL 2nd, Weinbaum CA, Sakabe K, Chen Z, Casey PJ, Cox AD. High affinity for farnesyltransferase and alternative prenylation contribute individually to K-Ras4B resistance to farnesyltransferase inhibitors. J Biol Chem. 2003; 278:41718-41727.
149. Lobell R, Omer CA, Abrams MT, Bhimnathwala HG, Brucker MJ, Buser CA, Davide JP, deSolms SJ, Dinsmore CJ, Ellis-Hutchings MS, Kral AM, Liu D, Lumma WC, Machotka SV, Rands E, Williams TM, Graham SL, Hartman GD, Oliff AI, Heimbrook DC, Kohl NE. Evaluation of farnesyl:protein transferase and geranylgeranyl:protein transferase inhibitor combinations in preclinical models. Cancer Res. 2001; 61:8758-8768.
150. Lobell R, Liu D, Buser CA, Davide JP, DePuy E, Hamilton K, Koblan KS, Lee Y, Mosser S, Motzel SL, Abbruzzese JL, Fuchs CS, Rowinsky EK, Rubin EH, Sharma S, Deutsch PJ, Mazina KE, Morrison BW, Wildonger L, Yao SL, Kohl NE. Preclinical and clinical pharmacodynamic assessment of L-778,123, a dual inhibitor of farnesyl:protein transferase and geranylgeranyl:protein transferase type-I. Mol Cancer Ther. 2002; 1:747-758.
151. Llovet J, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP

Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med. 2008; 359:378-390.
152. Abou-Alfa G, Schwartz L, Ricci S, Amadori D, Santoro A, Figer A, De Greve J, Douillard JY, Lathia C, Schwartz B, Taylor I, Moscovici M, Saltz LB. Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. J Clin Oncol. 2006; 24:4293-4300.
153. Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, Schwartz B, Simantov R, Kelley S. Discovery and development of sorafenib: A multikinase inhibitor for treating cancer. Nat Rev Drug Discov. 2006; 5:835-844.
154. Ott P, Hamilton A, Min C, Safarzadeh-Amiri S, Goldberg L, Yoon J, Yee H, Buckley M, Christos PJ, Wright JJ, Polsky D, Osman I, Liebes L, Pavlick AC. A phase II trial of sorafenib in metastatic melanoma with tissue correlates. PLoS One. 2010; 5.
155. Yang H, Higgins B, Kolinsky K, Packman K, Go Z, Iyer R, Kolis S, Zhao S, Lee R, Grippo JF, Schostack K, Simcox ME, Heimbrook D, Bollag G, Su F. RG7204 (PLX4032), a selective BRAFV600E inhibitor, displays potent antitumor activity in preclinical melanoma models. Cancer Res. 2010; 70:5518-5527.
156. Poulikakos P, Zhang C, Bollag G, Shokat KM, Rosen N. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature. 2010; 464:427-430.
157. Girotti M, Lopes F, Preece N, Niculescu-Duvaz D, Zambon A, Davies L, Whittaker S, Saturno G, Viros A, Pedersen M, Suijkerbuijk BM, Menard D, McLeary R, Johnson L, Fish L, Ejiama S, Sanchez-Laorden B, Hohloch J, Carragher N, Macleod K, Ashton G, Marusiak AA, Fusi A, Brognard J, Frame M, Lorigan P, Marais R, Springer C. Paradox-breaking RAF inhibitors that also target SRC are effective in drug-resistant BRAF mutant melanoma. Cancer Cell. 2015; 27:85-96.
158. Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, Nye E, Stamp G, Alitalo K, Downward J. Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. Cell. 2007; 129:957-968.
159. Velho S, Oliveira C, Ferreira A, Ferreira AC, Suriano G, Schwartz S Jr, Duval A, Carneiro F, Machado JC, Hamelin R, Seruca R. The prevalence of PIK3CA mutations in gastric and colon cancer. Eur J Cancer. 2005; 41:1649-1654.
160. Ollikainen M, Gylling A, Puputti M, Nupponen NN, Abdel-Rahman WM, Butzow R, Peltomäki P. Patterns of PIK3CA alterations in familial colorectal and endometrial carcinoma. Int J Cancer. 2007; 121:915-920.
161. Barault L, Veyrie N, Jooste V, Lecorre D, Chapusot C, Ferraz JM, Lièvre A, Cortet M, Bouvier AM, Rat P, Roignot P, Faivre J, Laurent-Puig P, Piard F. Mutations in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in a population-based series of colon cancers. Int J Cancer. 2008; 122:2255-2259.
162. Motzer R, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grünwald V, Thompson JA, Figlin RA, Hollaender N, Kay A, Ravaud A; RECORD-1 Study Group. Phase 3 trial of everolimus for metastatic renal cell carcinoma : Final results and analysis of prognostic factors. Cancer. 2010; 116:4256-4265.
163. Hess G, Herbrecht R, Romaguera J, Verhoef G, Crump M, Gisselbrecht C, Laurell A, Offner F, Strahs A, Berkenblit A, Hanushevsky O, Clancy J, Hewes B, Moore L, Coiffier B. Phase III study to evaluate temsirolimus compared with investigator's choice therapy for the treatment of relapsed or refractory mantle cell lymphoma. J Clin Oncol. 2009; 27:3822-3829.
164. McCormack F, Inoue Y, Moss J, Singer LG, Strange C, Nakata K, Barker AF, Chapman JT, Brantly ML, Stocks JM, Brown KK, Lynch JP 3rd, Goldberg HJ, Young LR, Kinder BW, Downey GP, Sullivan EJ, Colby TV, McKay RT, Cohen MM, Korbee L, Taveira-DaSilva AM, Lee HS, Krischer JP, Trapnell BC; National Institutes of Health Rare Lung Diseases Consortium; MILES Trial Group. Efficacy and safety of sirolimus in lymphangioleiomyomatosis. N Engl J Med. 2011; 364:1595-1606.
165. Groth C, Bäckman L, Morales JM, Calne R, Kreis H, Lang P, Touraine JL, Claesson K, Campistol JM, Durand D, Wramner L, Brattström C, Charpentier B. Sirolimus (rapamycin)-based therapy in human renal transplantation: Similar efficacy and different toxicity compared with cyclosporine. Sirolimus European Renal Transplant Study Group. Transplantation. 1999; 67:1036-1042.
166. De Buck S, Jakab A, Boehm M, Bootle D, Juric D, Quadt C, Goggin TK. Population pharmacokinetics and pharmacodynamics of BYL719, a phosphoinositide 3-kinase antagonist, in adult patients with advanced solid malignancies. Br J Clin Pharmacol. 2014; 78:543-555.
167. O'Brien S, Lamanna N, Kipps TJ, Flinn I, Zelenetz AD, Burger JA, Keating M, Mitra S, Holes L, Yu AS, Johnson DM, Miller LL, Kim Y, Dansey RD, Dubowy RL, Coutre SE. A phase 2 study of idelalisib plus rituximab in treatment-naïve older patients with chronic lymphocytic leukemia. Blood. 2015; 126:2686-1694.
168. Dienstmann R, Rodon J, Serra V, Tabernero J. Picking the point of inhibition: A comparative review of PI3K/AKT/mTOR pathway inhibitors. Mol Cancer Ther. 2014; 13:1021-1031.
169. Engelman J. Targeting PI3K signalling in cancer: Opportunities, challenges and limitations. Nat Rev Cancer. 2009; 9:550-562.
170. Gysin S, Salt M, Young A, McCormick F. Therapeutic strategies for targeting ras proteins. Genes Cancer. 2011; 2:359-372.
171. Engelman J, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, Maira M, McNamara K, Perera SA, Song Y, Chirieac LR, Kaur R, Lightbown A, Simendinger J, Li T, Padera RF, García-Echeverría C, Weissleder R, Mahmood U, Cantley LC, Wong KK. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nat Med. 2008; 14:1351-1356.
172. Halilovic E, She QB, Ye Q, Pagliarini R, Sellers WR, Solit DB, Rosen N. PIK3CA mutation uncouples tumor growth and cyclin D1 regulation from MEK/ERK and mutant KRAS signaling. Cancer Res. 2010; 70:6804-6814.
173. Michael JV, Wurtzel JGT; Goldfinger, LE. Inhibition of Galectin-1 Sensitizes HRAS-driven Tumor Growth to Rapamycin Treatment. Anticancer Res. 2016; 36:5053-5061.
174. Astorgues-Xerri L, Riveiro ME, Tijeras-Raballand A, Serova M, Rabinovich GA, Bieche I, Vidaud M, de Gramont A, Martinet M, Cvitkovic E, Faivre S, Raymond E. OTX008, a selective small-molecule inhibitor of galectin-1, downregulates cancer cell proliferation, invasion and tumour angiogenesis. Eur J Cancer. 2014;

50:2463-2477.
175. Dings RP, Miller MC, Nesmelova I, Astorgues-Xerri L, Kumar N, Serova M, Chen X, Raymond E, Hoye TR, Mayo KH. Antitumor agent calixarene 0118 targets human galectin-1 as an allosteric inhibitor of carbohydrate binding. J Med Chem. 2012; 55:5121-5129.
176. Pan J, Yeung SC. Recent advances in understanding the antineoplastic mechanisms of farnesyltransferase inhibitors. Cancer Res. 2005; 65:9109-9112.
177. Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A, Ye Q, Lobo JM, She Y, Osman I, Golub TR, Sebolt-Leopold J, Sellers WR, Rosen N. BRAF mutation predicts sensitivity to MEK inhibition. Nature. 2006; 439:358-362.
178. Shalom-Feuerstein R, Plowman SJ, Rotblat B, Ariotti N, Tian T, Hancock JF, Kloog Y. K-ras nanoclustering is subverted by overexpression of the scaffold protein galectin-3. Cancer Res. 2008; 68:6608-6616.
179. Bhagatji P, Leventis R, Rich R, Lin CJ, Silvius JR. Multiple cellular proteins modulate the dynamics of K-ras association with the plasma membrane. Biophys J. 2010; 99:3327-3335.
180. Wu KL, Huang EY, Jhu EW, Huang YH, Su WH, Chuang PC, Yang KD. Overexpression of galectin-3 enhances migration of colon cancer cells related to activation of the K-Ras-Raf-Erk1/2 pathway. J Gastroenterol. 2013; 48:350-359.
181. Song S, Ji B, Ramachandran V, Wang H, Hafley M, Logsdon C, Bresalier RS. Overexpressed galectin-3 in pancreatic cancer induces cell proliferation and invasion by binding Ras and activating Ras signaling. PLoS One. 2012; 7:e42699.
182. Levy R, Grafi-Cohen M, Kraiem Z, Kloog Y. Galectin-3 promotes chronic activation of K-Ras and differentiation block in malignant thyroid carcinomas. Mol Cancer Ther. 2010; 9:2208-2219.
183. Ruvolo PP, Ruvolo VR, Benton CB, AlRawi A, Burks JK, Schober W, Rolke J, Tidmarsh G, Hail N, Jr., Eric Davis R, Andreeff M. Combination of galectin inhibitor GCS-100 and BH3 mimetics eliminates both p53 wild type and p53 null AML cells. Biochim Biophys Acta. 2016; 1863:562-571.
184. Hossein G, Keshavarz M, Ahmadi S, Naderi N. Synergistic effects of PectaSol-C modified citrus pectin an inhibitor of Galectin-3 and paclitaxel on apoptosis of human SKOV-3 ovarian cancer cells. Asian Pac J Cancer Prev. 2013; 14:7561-7568.
185. Inohara H, Raz A. Effects of natural complex carbohydrate (citrus pectin) on murine melanoma cell properties related to galectin-3 functions. Glycoconj J. 1994; 11:527-532.
186. Jiang J, Eliaz I, Sliva D. Synergistic and additive effects of modified citrus pectin with two polybotanical compounds, in the suppression of invasive behavior of human breast and prostate cancer cells. Integr Cancer Ther. 2013; 12:145-152.
187. Liu FT, Hsu DK, Zuberi RI, Hill PN, Shenhav A, Kuwabara I, Chen SS. Modulation of functional properties of galectin-3 by monoclonal antibodies binding to the non-lectin domains. Biochemistry. 1996; 35:6073-6079.
188. Liu HY, Huang ZL, Yang GH, Lu WQ, Yu NR. Inhibitory effect of modified citrus pectin on liver metastases in a mouse colon cancer model. World J Gastroenterol. 2008; 14:7386-7391.
189. Nangia-Makker P, Hogan V, Honjo Y, Baccarini S, Tait L, Bresalier R, Raz A. Inhibition of human cancer cell growth and metastasis in nude mice by oral intake of modified citrus pectin. J Natl Cancer Inst. 2002; 94:1854-1862.
190. Pienta KJ, Naik H, Akhtar A, Yamazaki K, Replogle TS, Lehr J, Donat TL, Tait L, Hogan V, Raz A. Inhibition of spontaneous metastasis in a rat prostate cancer model by oral administration of modified citrus pectin. J Natl Cancer Inst. 1995; 87:348-353.

(Received November 24, 2016; Revised February 2, 2017; Accepted February 3, 2017)

Mini-Review

DOI: 10.5582/bst.2016.01266

Research progress on the direct antiviral drugs for hepatitis C virus

Jianjun Gao*, Chuanxia Ju

Department of Pharmacology, Qingdao University School of Pharmacy, Qingdao, Shandong Province, China.

Summary **Hepatitis C, caused by the hepatitis C virus (HCV) that attacks the liver and leads to inflammation, is a severe threat to human health. Pegylated interferon α (INF-α) and ribavirin based therapy was once the standard therapy for HCV infection. However, it is suboptimal in efficacy and poorly tolerated in some patients. In the last five years, four classes of direct antiviral drugs (NAAs) that target non-structural proteins (NS) of the virus including NS3/NS4A, NS5A, and NS5B have been developed and opened a new era in HCV treatment as they are more effective and tolerable than the INF-α and ribavirin combination regimen. Importantly, the newly introduced multiple NAAs combination therapy makes it possible to eradicate all genotypes of HCV. We review recent progress on the research and development of DAAs in the present article.**

Keywords: HCV, interferon, ribavirin, NAA, NS3/NS4A, NS5A, NS5B

1. Introduction

Hepatitis C virus infection (HCV), which occurs most commonly in Africa and Central and East Asia, is a significant public health problem with approximately 130-200 million people around the world infected at present (*1-4*). Acute HCV infection is rarely associated with life-threatening disease and about 15-45% of infected persons spontaneously clear the virus within 6 months of infection without any treatment (*5*). The remaining 55-85% of persons will develop chronic HCV infection, which is recognized as one of the major causes of chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC) (*5-7*). Specifically, the risk of liver cirrhosis is between 15-30% within 20 years in people with chronic infection (*5*). According to the data disclosed by World Health Organization (WHO), approximately 700,000 people die each year from hepatitis C-related liver diseases (*5*).

The goal of hepatitis C treatment is to obtain sustained virological response at 12 weeks (SVR12) which is recognized as the measure of treatment success and defined as undetectable HCV RNA in the blood at the end of treatment and again 12 weeks following treatment end (*8,9*). Multiple evidences support that antivirus treatment helps improve hepatic histology, prevents or delays the occurrence of liver cirrhosis, and decreases the incidence of HCC (*9,10*). Interferon α (IFN-α) with established clinical efficacy has long been used for treatment of HCV infection. Combination of pegylated IFN-α and ribavirin, a nucleoside analogue, was once the standard therapy, which cured approximately half of treated patients, but caused frequent and sometimes life-threatening adverse reactions (*11*). In the recent five years, new antiviral drugs, called direct antiviral agents (DAAs), have been developed and revolutionized the treatment of HCV because they are much more effective, safer and better-tolerated than the older therapies (*8*). The progress of DAAs research and development is reviewed in the present article.

2. Gene structure and genotypes of HCV

HCV, a positive-sense single-stranded RNA virus of the family Flaviviridae, consists of a core of genetic material (RNA), surrounded by an icosahedral protective shell of protein, and is further encased in a lipid (fatty) envelope of cellular origin (*12*). The genome of HCV consists of a single open reading frame that is translated to produce a single protein product. This single product is then proteolytically processed by viral and cellular proteases into 10 smaller proteins which are grouped

Released online in J-STAGE as advance publication January 19, 2017.

**Address correspondence to:*
Dr. Jianjun Gao, Department of Pharmacology, Qingdao University School of Pharmacy, 38 Dengzhou Road, Qingdao 266021, Shandong Province, China.
E-mail: gaojj@qdu.edu.cn

into structural proteins including Core protein, envelope glycoproteins E1 and E2, and p7 and non-structural proteins (NS) including NS2, NS3, NS4A, NS4B, NS5A, and NS5B (*13*). The structural proteins form the skeletal structure of the virus particle while non-structural proteins act as enzymes or regulatory factors that play critical roles in virus replication. Among the non-structural proteins, NS3 functions as a serine protease and forms a heterodimeric complex with NS4A that acts as a cofactor of the proteinase (*13*). NS5A is a hydrophilic phosphoprotein which plays an important role in viral replication, modulation of cell signaling pathways, and the interferon response (*14*). The NS5B protein is the viral RNA dependent RNA polymerase, which catalyzes the polymerization of ribonucleoside triphosphates (rNTP) during RNA replication (*15-17*). Given the critical role of NS3/NS4A, NS5A, and NS5B in the viral life cycle, they are currently focused on as major targets in development of DAAs.

A naturally high rate of genetic mutation due to low fidelity of RNA replication generates genetic diversity of HCV (*18*). Based on genetic differences between HCV isolates, the HCV species is classified into six genotypes (1-6) with several subtypes within each genotype. Genotype 1, which includes subtype 1a and 1b seen in almost all cases (31% and 68%, respectively), constitutes 40-50% of all HCV infections and is the most prevalent genotype worldwide (*19*). The next most prevalent genotype is genotype 3 (about 30%), followed by genotypes 2 and 4 (about 10% each), genotype 6 (about 5%), and genotype 5 (less than 1%) (*19*). Mixed genotype infections are also seen in a small proportion of patients (*19*). Among all subtypes of HCV, subtypes 1a, 1b, 2a, and 3a are globally distributed and are considered "epidemic subtypes" (*8*). Genotype is clinically important in determining potential response to medications. For example, genotypes 1 and 4 are less responsive to IFN-α-based treatment than are the other genotypes (2 , 3, 5, and 6) (*20*), which leads to difficulties in clearing HCV of these two genotypes. On the other hand, the latest developed DAAs combination regimens have the potential to eradicate all genotypes of HCV, hence enlarging the patient population who may benefit.

3. Direct antiviral drugs for HCV

Thus far, four classes of DAAs have been developed based on three targets. They are protease inhibitors targeting NS3/NS4, nucleotide analogues and non-nucleosides targeting NS5B, and NS5A phosphoprotein inhibitors (Table 1). Because the antivirus efficacy of individual DAAs usually depends on HCV genotype, genotypic subtype and disease severity (*e.g.*, cirrhosis) and many DAAs are prone to induce HCV resistance when used alone, combination regimens including two, three or even four DAAs, without pegylated IFN-α, have been developed and have become the new standard of care for HCV.

3.1. *Sofosbuvir and combination therapies*

Sofosbuvir, a nucleotide analogue targeting NS5B, was firstly approved by the US Food and Drug Administration (FDA) in 2013 for treatment of HCV genotypes 2 and 3 in combination with ribavirin or treatment-naive patients with HCV genotypes 1 and 4 in combination with ribavirin and pegylate IFN-α (*21*). Sofosbuvir is now used as the backbone for several interferon-free regimens. The combination of sofosbuvir with the NS5A inhibitor ledipasvir as a fixed-dose, once-daily, single-tablet regimen was approved by FDA in 2014. It approaches but does not achieve pan-genotypic status, with efficacy demonstrated against HCV genotype 1, 4, 5, and 6 infections (*8*). Evidence is lacking for this regimen in genotype 2, and it has only limited activity against genotype 3 without the addition of ribavirin (*8*). Given this reason, sofosbuvir plus ledipasvir is not recommended for use in genotypes 2 and 3. On the other hand, the combination of sofosbuvir with another NS5A inhibitor velpatasvir as a once-daily, single-tablet, pangenotypic regimen was recently approved for the treatment of adults with chronic HCV genotypes 1-6 in the USA, EU and Canada (*22*). In the phase III ASTRAL trials, once-daily oral sofosbuvir plus velpatasvir for 12 weeks provided high rates of SVR12 in treatment-naive and -experienced patients with chronic HCV genotype 1-6 infection (*22*). This

Table 1. The direct antiviral drugs (DAAs) developed for hepatitis C virus (HCV)

DAAs (target)	Combined agents (target)	HCV genotype	Status	*Ref.*
Sofosbuvir (NS5B)	Ribavirin	1, 4	Approved	*21*
	Ribavirin and IFN-α	2, 3	Approved	*21*
	Ledipasvir (NS5A)	1, 4, 5, 6	Approved	*8*
	Velpatasvir (NS5A)	1, 2, 3, 4, 5, 6	Approved	*22*
	Velpatasvir (NS5A) + Voxilaprevir (NS3/4A)	1, 2, 3, 4, 5, 6	Phase III	*27*
Daclatasvir (NS5A)	IFN-α + Ribavirin	1, 3, 4	Approved	*28*
	Asunaprevir (NS3/4A)	1	Approved	*30*
	Sofosbuvir (NS5B)	1, 2, 3, 4	Approved	*32*
Simeprevir (NS3/4A)	IFN-α + Ribavirin	1, 4	Approved	*34*
	Sofosbuvir (NS5B)	1	Approved	*38*
	Samatasvir (NS5A)	1, 4	Phase II	*39*
Dasabuvir (NS5B)	Ombitasvir (NS5A) + Paritaprevir (NS3/4A) + Ritonavir (P450 3A4)	1	Approved	*40*

regimen represents a valuable treatment option in adults with chronic HCV genotype 1-6 infection.

The combination of sofosbuvir, velpatasvir, and voxilaprevir, an investigational NS3/4A protease inhibitor, as a fixed-dose, once-daily regimen (SOF/VEL/VOX) is currently being evaluated for its efficacy and safety in treatment of genotypes 1-6 chronic HCV infection. Thus far, four phase 3 clinical studies including POLARIS-1, POLARIS-2, POLARIS-3, and POLARIS-4 on this regimen have been conducted. The POLARIS-1 and POLARIS-4 studies aimed to evaluate the combination regimen in patients who experienced previous NAAs treatment. The POLARIS-1 study enrolled patients who failed prior treatment with an NS5A inhibitor (*23*). The POLARIS-4 study enrolled patients who failed prior treatment with a DAA that was not an NS5A inhibitor, most with either an NS5B inhibitor alone (73%) or an NS5B inhibitor and an NS3/4A protease inhibitor (25%) (*24*). The POLARIS-2 and POLARIS-3 studies were designed to evaluate the SOF/VEL/VOX regimen in patients without previous NAAs treatment. The POLARIS-2 study enrolled patients with genotype 1-6 HCV infection with or without compensated cirrhosis (*25*), while the POLARIS-3 study enrolled patients with genotype 3 HCV infection, all of whom had compensated cirrhosis (*26*). In October 2016, results of these clinical studies were announced by the sponsor company Gilead (*27*). According to their report, the POLARIS-1, POLARIS-3, and POLARIS-4 studies met their respective pre-specified primary endpoints for patients receiving SOF/VEL/VOX. The POLARIS-2 study did not meet its primary endpoint; with a pre-specified 5% margin, the SVR12 rate for patients receiving treatment with SOF/VEL/VOX for eight weeks was not statistically non-inferior to the SVR12 rate for patients receiving SOF/VEL for 12 weeks. Results of these studies suggest that combining three potent antivirals with different mechanisms of action provided high cure rates for patients who have failed other highly effective oral DAA regimens.

3.2. *Daclatasvir and combination therapies*

Daclatasvir, an inhibitor of NS5A, was approved for treatment of HCV genotypes 1, 3, and 4 only in combination with interferons and ribavirin and also with some other DAAs such as asunaprevir, beclubavir, and sofosbuvir to overcome drug resistance, increase antiviral efficacy and decrease side effects (*28*). Asunaprevir, which is a NS3/4A inhibitor, has shown high treatment efficacy when used in combination with daclatasvir in patients with HCV genotype 1 (*29, 30*). Currently, the combination of the asunaprevir and daclatasvir regimen has been mainly used in patients with HCV genotype 1 infection in Japan. Subtype 1a has less been evaluated, and it seems that this regimen is appropriate only for HCV subtype 1b (*31*). The addition of beclubavir, an inhibitor of NS5B, to the daclatasvir/asunaprevir regimen was found to improve the SVR rate in HCV-infected patients (*31*). This regimen has been mainly used for the treatment of patients infected with HCV genotype 1 (subtypes 1a and 1b) and 4. The daclatasvir and sofosbuvir combination regimen was approved by the European medicine agency for treatment of genotype 1-4 infections in 2014, followed by the FDA for treatment of genotypes 1 and 3 (*32*). According to several meta-analyses that analyzed the efficacy of this regimen on different genotypes, daclatasvir/sofosbuvir with and without ribavirin can be considered as a highly useful treatment option in treatment-naïve or treatment-experienced patients with genotype 1 and 3 infections with and without cirrhosis (*31,33*). The ability of daclatasvir to combine with other DAAs for treatment of patients with HCV infections under different conditions makes it a good choice for HCV treatment.

3.3. *Simeprevir and combination therapies*

Simeprevir is a NS3/4A inhibitor that was approved in recent years for treatment of chronic HCV genotype 1 or genotype 4 infection in treatment-naive or previously treated adults, including those with HIV coinfection, in combination with pegylated IFN-α and ribavirin and with other NAAs such as sofosbuvir (*34-37*). It is not recommended in patients in whom prior treatment with a regimen that included simeprevir or any other HCV protease inhibitor failed and in patients with moderate or severe hepatic impairment (Child-Pugh class B or C) (*34*). Screening for presence of NS3 Q80K polymorphism is strongly recommended prior to initiation of the regimen consisting of simeprevir, pegylated IFN-α, and ribavirin (*34*). An alternative therapy may be considered in patients infected with HCV genotype 1a containing Q80K polymorphism (*34*). The regimen containing simeprevir and sofosbuvir is used in treatment-naive or previously interferon-treated patients with HCV genotype 1, with or without cirrhosis (*38*). Duration of this regimen is 24 weeks and 12 weeks, respectively, for patients with or without cirrhosis (*38*). Screening of NS3 Q80K polymorphism may also be instructive prior to initiation of simeprevir in conjunction with sofosbuvir (*34*). Combination of simeprevir and samatasvir, an investigational NS5A inhibitor, is currently undergoing phase 2 clinical trials for evaluation of its efficacy in treatment of chronic HCV infection (*39*).

3.4. *Ombitasvir/paritaprevir/ritonavir plus dasabuvir*

The regimen containing a fixed-dose combination of ombitasvir, paritaprevir, and ritonavir and dasabuvir (marketed as Viekira Pak™) was approved by FDA

and other regulatory agencies for the treatment of genotype 1 chronic HCV infection, including those with compensated cirrhosis (*40*). This regimen, also called the "3D" regimen, consists of three DAAs targeting different non-structural proteins, *i.e.* ombitasvir inhibiting NS5A, paritaprevir inhibiting NS3/NS4A, and dasabuvir, a non-nucleoside inhibiting NS5B. The regimen also consists of a cytochrome P450 3A4 inhibitor ritonavir, which can increase the plasma concentration of paritaprevir (*41*). Based on the results of the clinical trials, this regimen is suitable for treating patients infected with hepatitis C genotype 1b (*42,43*). It may be possible to use this combination to treat patients infected with genotype 1a if they have not previously been treated and do not have cirrhosis, but the addition of ribavirin is needed to maximize the response (*42*). While the five-drug regimen is very effective, it will require careful selection of patients and checking product information to avoid drugs that either interact or are contraindicated (*44*). As three new drugs are involved in this regimen, it should be cautioned that unforeseen problems may emerge in the future.

4. Conclusion

Chronic hepatitis C has been a great healthcare concern because of its high prevalence worldwide. Treatment with pegylated IFN-α and ribavirin was once the standard therapy, but poor tolerability and suboptimal response rates increased the likelihood of therapeutic failure. Introduction of DAAs opened a new era in HCV treatment with the possibility of interferon-free therapy, SVR rates approaching 100%, reduced duration of therapy, and improved tolerability. In this aspect, DAAs of the same or different classes have been introduced one after another in close succession in the last five years. Each class of DAAs targets a specific viral protein such as NS3/4A, NS5A and NS5B. The combination of DAAs with different mechanisms reduces the risk of occurrence of HCV resistance and enlarges the patient population who may benefit. Nevertheless, many unknowns still remain with the new drugs combination therapy such as potential drug-drug interactions, which warrant further in-depth studies in the future.

Acknowledgements

This project is supported by the Natural Science Foundation of China (81503094), China Postdoctoral Science Foundation (2016M600524), and Qingdao Postdoctoral Applied Research Project (2016072; Jianjun Gao, Qingdao University).

References

1. Elsebai MF, Mocan A, Atanasov AG. Cynaropicrin: A comprehensive research review and therapeutic potential as an anti-hepatitis C virus agent. Front Pharmacol. 2016; 7:472.
2. Gravitz L. Introduction: a smouldering public-health crisis. Nature. 2011; 474:S2-4.
3. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. Hepatology. 2013; 57:1333-1342.
4. Zhou M, Li H, Ji Y, Ma Y, Hou F, Yuan P. Hepatitis C virus infection in the general population: A large community-based study in Mianyang, West China. Biosci Trends. 2015; 9:97-103.
5. Fact sheet of hepatitis C. World Health Organization. *http://www.who.int/mediacentre/factsheets/fs164/en/* (accessed December 1, 2016).
6. Xia JF, Gao JJ, Inagaki Y, Kokudo N, Nakata M, Tang W. Flavonoids as potential anti-hepatocellular carcinoma agents: recent approaches using HepG2 cell line. Drug Discov Ther. 2013; 7:1-8.
7. Zhong Y, Liu B, Deng M, Xu R. Adjuvant systemic drug therapy and recurrence of hepatocellular carcinoma following curative resection. Drug Discov Ther. 2013; 7:164-166.
8. Hezode C. Pan-genotypic treatment regimens for hepatitis C virus: Advantages and disadvantages in high- and low-income regions. J Viral Hepat. 2016. DOI: 10.1111/jvh.12635.
9. Xia J, Inagaki Y, Song P, Sawakami T, Kokudo N, Hasegawa K, Sakamoto Y, Tang W. Advance in studies on traditional Chinese medicines to treat infection with the hepatitis B virus and hepatitis C virus. Biosci Trends. 2016; 10:327-336.
10. Kaneko J, Sugawara Y, Yamaguchi T, Harada N, Akamatsu N, Ishizawa T, Aoki T, Sakamoto Y, Hasegawa K, Tamura S, Tanaka T, Kokudo N. Telaprevir-based triple therapy for hepatitis C null responders among living donor liver transplant recipients. Biosci Trends. 2014; 8:339-345.
11. Chen H, Yao Y, Wang Y, Zhou H, Xu T, Liu J, Wang G, Zhang Y, Chen X, Liu Q, Huang P, Yu R. Polymorphisms of HLA-DM on treatment response to interferon/ribavirin in patients with chronic hepatitis C virus type 1 infection. Int J Environ Res Public Health. 2016; 13:E1030.
12. Op De Beeck A, Dubuisson J. Topology of hepatitis C virus envelope glycoproteins. Rev Med Virol. 2003; 13:233-241.
13. Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. Nature. 2005; 436:933-938.
14. Gupta G, Qin H, Song J. Intrinsically unstructured domain 3 of hepatitis C Virus NS5A forms a "fuzzy complex" with VAPB-MSP domain which carries ALS-causing mutations. PloS one. 2012; 7:e39261.
15. Jin Z, Leveque V, Ma H, Johnson KA, Klumpp K. Assembly, purification, and pre-steady-state kinetic analysis of active RNA-dependent RNA polymerase elongation complex. J Biol Chem. 2012; 287:10674-10683.
16. Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nat Rev Microbiol. 2007; 5:453-463.
17. Rigat K, Wang Y, Hudyma TW, Ding M, Zheng X, Gentles RG, Beno BR, Gao M, Roberts SB. Ligand-induced changes in hepatitis C virus NS5B polymerase structure. Antiviral Res. 2010; 88:197-206.
18. Kliemann DA, Tovo CV, da Veiga AB, de Mattos AA,

Wood C. Polymorphisms and resistance mutations of hepatitis C virus on sequences in the European hepatitis C virus database. World J Gastroenterol. 2016; 22:8910-8917.
19. Messina JP, Humphreys I, Flaxman A, Brown A, Cooke GS, Pybus OG, Barnes E. Global distribution and prevalence of hepatitis C virus genotypes. Hepatology. 2015; 61:77-87.
20. Simmonds P, Bukh J, Combet C, *et al*. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology. 2005; 42:962-973.
21. McConachie SM, Wilhelm SM, Kale-Pradhan PB. New direct-acting antivirals in hepatitis C therapy: a review of sofosbuvir, ledipasvir, daclatasvir, simeprevir, paritaprevir, ombitasvir and dasabuvir. Expert Rev Clin Pharmacol. 2016; 9:287-302.
22. Lee R, Kottilil S, Wilson E. Sofosbuvir/velpatasvir: a pangenotypic drug to simplify HCV therapy. Hepatol Int. 2016. DOI: 10.1007/s12072-016-9776-8.
23. *https://www.clinicaltrials.gov/ct2/show/NCT02607735?term=SOF%2FVEL%2FVOX&rank=4* (accessed December 21, 2016).
24. https://www.clinicaltrials.gov/ct2/show/NCT02639247?term=SOF%2FVEL%2FVOX&rank=2 (accessed December 21, 2016).
25. *https://www.clinicaltrials.gov/ct2/show/NCT02607800?term=SOF%2FVEL%2FVOX&rank=3* (accessed December 21, 2016).
26. *https://www.clinicaltrials.gov/ct2/show/NCT02639338?term=SOF%2FVEL%2FVOX&rank=1* (accessed December 21, 2016).
27. *http://investors.gilead.com/phoenix.zhtml?c=69964&p=irol-newsArticle&ID=2213438* (accessed December 21, 2016).
28. Asselah T. NS5A inhibitors: a new breakthrough for the treatment of chronic hepatitis C. J Hepatol. 2011; 54:1069-1072.
29. McPhee F, Sheaffer AK, Friborg J, *et al*. Preclinical profile and characterization of the hepatitis C virus NS3 protease inhibitor asunaprevir (BMS-650032). Antimicrob Agents Chemother. 2012; 56:5387-5396.
30. Lok AS, Gardiner DF, Lawitz E, *et al*. Preliminary study of two antiviral agents for hepatitis C genotype 1. N Engl J Med. 2012; 366:216-224.
31. Alavian SM, Rezaee-Zavareh MS. Daclatasvir-based treatment regimens for hepatitis C virus infection: A systematic review and meta-analysis. Hepat Mon. 2016; 16:e41077.
32. Alavian SM, Hajarizadeh B, Bagheri Lankarani K, *et al*. Recommendations for the clinical management of hepatitis C in Iran: A consensus-based national guideline. Hepat Mon. 2016; 16:e40959.
33. WHO. Guidelines approved by the Guidelines Review Committee. Guidelines for the Screening Care and Treatment of Persons with Chronic Hepatitis C Infection: Updated Version. World Health Organization. 2015.
34. Olysio (simeprevir) capsules prescribing information. Titusville, NJ; 2016.
35. Forns X, Lawitz E, Zeuzem S, *et al*. Simeprevir with peginterferon and ribavirin leads to high rates of SVR in patients with HCV genotype 1 who relapsed after previous therapy: a phase 3 trial. Gastroenterology. 2014; 146:1669-1679 e1663.
36. Manns M, Marcellin P, Poordad F, *et al*. Simeprevir with pegylated interferon alfa 2a or 2b plus ribavirin in treatment-naive patients with chronic hepatitis C virus genotype 1 infection (QUEST-2): a randomised, double-blind, placebo-controlled phase 3 trial. Lancet. 2014; 384:414-426.
37. Lawitz E, Sulkowski MS, Ghalib R, *et al*. Simeprevir plus sofosbuvir, with or without ribavirin, to treat chronic infection with hepatitis C virus genotype 1 in non-responders to pegylated interferon and ribavirin and treatment-naive patients: the COSMOS randomised study. Lancet. 2014; 384:1756-1765.
38. American Association for the Study of Liver Diseases (AASLD). Recommendations for testing, managing, and treating hepatitis C. *http://www.aasld.org/* (accessed November 21, 2016).
39. *https://www.clinicaltrials.gov/ct2/show/NCT01852604?term=simeprevir++samatasvir&rank=3* (accessed December 1, 2016).
40. Safety information – Viekira Pak (ombitasvir, paritaprevir, and ritonavir tablets; dasabuvir tablets), copackaged for oral use. *www.fda.gov* (accessed December 21, 2016).
41. Chamorro-de-Vega E, Gimenez-Manzorro A, Rodriguez-Gonzalez CG, *et al*. Effectiveness and safety of ombitasvir-paritaprevir/ritonavir and dasabuvir with or without ribavirin for HCV genotype 1 infection for 12 weeks under routine clinical practice. Ann Pharmacother. 2016; 50:901-908.
42. Ferenci P, Bernstein D, Lalezari J, *et al*. ABT-450/r-ombitasvir and dasabuvir with or without ribavirin for HCV. N Engl J Med. 2014; 370:1983-1992.
43. Andreone P, Colombo MG, Enejosa JV, Koksal I, Ferenci P, Maieron A, Mullhaupt B, Horsmans Y, Weiland O, Reesink HW, Rodrigues L, Jr., Hu YB, Podsadecki T, Bernstein B. ABT-450, ritonavir, ombitasvir, and dasabuvir achieves 97% and 100% sustained virologic response with or without ribavirin in treatment-experienced patients with HCV genotype 1b infection. Gastroenterology. 2014; 147:359-365 e351.
44. Paritaprevir/ritonavir/ombitasvir plus dasabuvir with ribavirin for chronic hepatitis C. Aust Prescr. 2016; 39:141-143.

(Received December 5, 2016; Revised December 26, 2016; Accepted December 28, 2016)

Mini-Review

DOI: 10.5582/bst.2016.01216

Progress in understanding the molecular functions of DDX3Y (DBY) in male germ cell development and maintenance

Alexei A. Kotov[1], Oxana M. Olenkina[1], Baira K. Godneeva[1,2], Vladimir E. Adashev[1,2], Ludmila V. Olenina[1,*]

[1] *Laboratory of Biochemical Genetics of Animals, Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia;*
[2] *Department of Biology, Moscow State University, Moscow, Russia.*

Summary **Human *DDX3* paralogs are housed on the X chromosome (*DDX3X*) as well as in the non-recombining region Yq11 of the Y-chromosome (*DDX3Y* or *DBY*). A gene encoding RNA helicase DDX3Y is located in the *AZoospermia Factor a* (*AZFa*) region of the Y-chromosome and expressed only in male germ cells. Deletions encompassing the *DDX3Y* gene lead to azoospermia and cause Sertoli Cell-Only Syndrome (SCOS) in humans. SCOS is characterized by a complete germ cell lack with preservation of somatic Sertoli cells. This review summarizes current advances in the study of DDX3Y functions in maintenance and development of early male germ cells. Data obtained from a mouse xenotransplantation model reveals that DDX3Y expression is enough to drive germ cell differentiation of *AZFa*-deleted human induced pluripotent stem cells (iPSCs) and for activation of the specific set of germline developmental genes. Results achieved using the testes of *Drosophila* demonstrate that DDX3Y homolog Belle is required cell-autonomously for mitotic progression and survival of germline stem cells and spermatogonia as the upstream regulator of mitotic cyclin expression.**

Keywords: Sertoli cell-only syndrome, DDX3Y, spermatogenesis, germline stem cells, cyclin B

1. Introduction

The most common cases of human male infertility which are caused by a failure of sperm production are associated with microdeletions in the *AZoospermia Factor* (*AZF*) region in the long arm of the Y chromosome (*1-4*). This region contains three loci that are termed *AZFa*, *AZFb*, and *AZFc* with corresponding deletions linked to male infertility (*3,5*) (Figure 1). It has been shown that the majority of the Y chromosome deletions originates *de novo* and putatively occurs in the father's germline (*6*). Since only a limited part of the Y chromosome pairs with the X chromosome and the *AZF* is located in the non-recombining region of the Y chromosome, the mechanism of emergence of the Y deletions is still unclear. It has been proposed that the Y deletions are the consequence of illegitimate intra-chromosomal meiotic recombination between highly repetitive sequences adjacent to the *AZF* loci (7,8).

The specific infertility phenotypes are associated with deletions in each locus (*3,9*). The *AZFa* deletions cause a complete absence of germ cells in the testis seminiferous tubules with preservation of somatic Sertoli cells (the so-called Sertoli Cell-Only Syndrome; SCOS) (*10,11*). This is the most severe azoospermia phenotype of the three deletion loci. The *AZFb* deletions lead to spermatocyte maturation arrest as well as the azoospermia phenotype. The *AZFc* deletions allow limited sperm production and thus are linked with hypospermatogenesis. The *AZFa* contains only two expressed genes both with X homologues that escape inactivation: *ubiquitin specific peptidase 9*, *Y-linked* (*USP9Y*) and *DEAD-box helicase 3*, *Y-linked* (*DDX3Y* or *DBY*). Both genes were initially considered as candidates for the male fertility factor. *USP9Y* encodes an ubiquitin-specific protease that promotes intracellular

Released online in J-STAGE as advance publication February 12, 2017.

*Address correspondence to:
Dr. Ludmila V. Olenina, Laboratory of Biochemical Genetics of Animals, Institute of Molecular Genetics RAS, 2 Kurchatov Sq., Moscow, 123182 Russia.
E-mail: olenina_ludmila@mail.ru

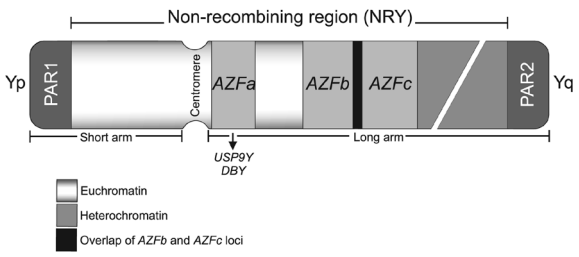

Figure 1. Representation of the *AZF* loci on the Y chromosome. PAR1 and PAR2 (shown in dark grey regions at the ends of the chromosome) are pseudoautosomal regions 1 and 2 of the Y chromosome. Outside the PARs *AZF* (*AZoospermia Factor*) loci are depicted in light grey and located in the non-recombining region (NRY). Yp and Yq designate the short and long chromosome arms of the chromosome, respectively. Adapted from (*2-4,15*) with modifications.

cleavage of ubiquitin molecules from ubiquitinated proteins (*3,9*). However, it was shown that USP9Y did not provide essential functions during spermatogenesis; mutations in *USP9Y* including deletion mutations were found to be transmittable in fertile patients (*12-14*). A second gene in the *AZFa*, *DDX3Y*, appears to represent the major spermatogenesis gene of this region (*15,16*). DDX3Y encodes an RNA helicase of the large DDX3 subfamily of the DEAD-box family of proteins, which are found from yeasts to humans. DDX3 helicases generally function in ATP-dependent RNA unwinding, strand annealing and remodeling of ribonucleoprotein complexes. They take part in most aspects of cellular RNA metabolism: mRNA nuclear export, transcriptional regulation, translation initiation, and also in cell cycle control and programmed cell death (*17-19*). The product of X-chromosomal homologue, DDX3X, is found ubiquitously in different human tissues, whereas translation of DDX3Y is restricted to male germ cells (*20*). DDX3X and DDX3Y share 91.7% homology in their amino acid sequence. Both proteins are expressed in the testes; however, DDX3Y is found predominantly at premeiotic stages in spermatogonial cells, whereas DDX3X is detected later in spermatids (*15,16,20*). This divergent expression pattern arises due to complex transcriptional and translational control mechanisms (*21,22*) and it seems to predetermine presumed specific functions of DDX3Y in premeiotic spermatogenesis steps. Testis-specific *DDX3Y* transcription initiates from a distal promoter that takes place within upstream Y-specific minisatellite *MSY2* repeats (*21*). This distal promoter region for *DDX3Y* on the Y chromosome appears to be conserved in primates; however, it is not active upstream of the mouse *Ddx3y* gene (*21*).

It has been shown that mouse homologue Ddx3y is expressed widely in all analyzed tissues (*23*). In contrast to humans mouse Ddx3y protein is found to be dispensable for spermatogenesis (*24*), but *Ddx3y* mRNA is retained in mouse spermatozoa and appears to contribute to early zygotic development (*25*). The mouse *Ddx3y* gene also has an X-chromosomal homologue *Ddx3x* (*26*). It is supposed that molecular functions of Ddx3y protein may be carried out by Ddx3x representing the same expression pattern in mouse testes (*23*). The third mouse DDX3 homologue PL10 (D1Pas1) is expressed only in the testes (*23,27,28*). The *PL10* gene is located on chromosome 1 and its expression is detected in pachytene spermatocytes and postmeiotic haploid spermatids (*23*). Thus, in spite of some similarities, the functional significance of DDX3 genes in spermatogenesis of humans and mice is quite different.

Progress in understanding the molecular functions of DDX3Y (DBY) in human male germ cell maintenance is challenging, because DDX3Y expression appears to be required for early stages of testis development in human fetal germ cells (prospermatogonia). Expression of DDX3Y during the second and third trimester of fetal testis development suggests its role in early germ cell differentiation and amplification and indicates that in individuals with a failure in DDX3Y expression germ cell loss may occur prenatally or during pre-pubertal development (*16,20*). Thus, specific functions of DDX3Y in germline development and maintenance are currently underinvestigated. In this review we focus on recently published studies that expand our understanding of the functions of DDX3Y as an essential spermatogenesis regulator.

2. DDX3Y genetically complements the *AZFa* deletion and restores germ cell development and transcription program

Mammalian spermatogenesis occurs in the seminiferous tubules of the testes. The seminiferous tubules are surrounded by the basement membrane, which isolates the tubules from the interstitial space containing myoid cells, Leydig cells and blood vessels (Figure 2). The seminiferous epithelium is composed of somatic Sertoli cells and developing germ cells at different stages of differentiation. Large undivided Sertoli cells are attached to the basement membrane, but they spread their cytoplasmic extensions towards the lumen of the tubules and surround multiple germ cells at all stages of differentiation. Spermatogonial stem cells (SSCs) originate from gonocytes (prospermatogonia), which in turn arise from primordial germ cells (PGCs) during fetal development. In the postnatal testes, SSCs are located on the basement membrane of the seminiferous tubules (Figure 2). SSCs undergo self-renewing and differentiating divisions to maintain continuous sperm production throughout male adulthood. In humans, the SSC pool is composed of A_{dark} and A_{pale} type spermatogonia. A_{dark} cells are reserved SSCs and they do not usually enter active mitotic divisions. On the contrary, A_{pale} spermatogonia are mitotically active and undergo one-two transit amplifying divisions generating type B spermatogonia. Type B spermatogonia give rise

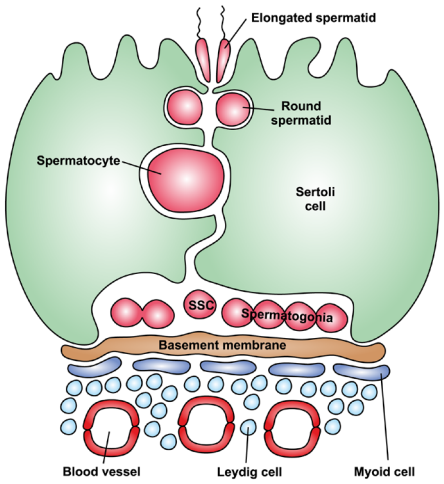

Figure 2. Scheme of human spermatogenesis. Simplified scheme of human spermatogenesis was adapted from (*30,31*) with modifications. The seminiferous tubules of the testes are surrounded by the basement membrane, which isolates the tubules from the interstitial space containing myoid cells, Leydig cells and blood vessels. The seminiferous epithelium is composed of somatic Sertoli cells and germ cells at different stages of differentiation. Spermatogonial stem cells (SSCs) are located on the basement membrane. Sertoli cells are also attached to the basement membrane, but they extend towards the lumen of the tubules and contact with germ cells at all stages of differentiation. SSCs undergo self-renewing and differentiating divisions to provide germ cells of the subsequent stage, type B spermatogonia. Type B spermatogonia give rise to primary spermatocytes. Then primary spermatocytes enter the first meiotic division to yield secondary spermatocytes, which divide into haploid spermatids migrating towards the lumen. Finally, mature spermatozoa are released into the lumen of the seminiferous tubules.

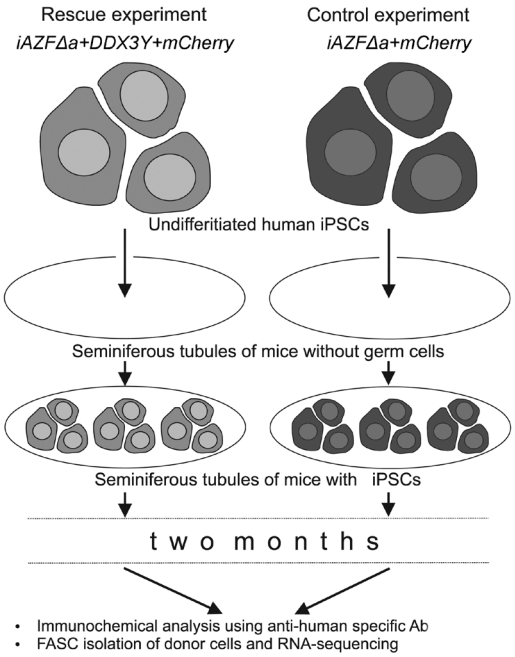

Figure 3. General scheme of the xenotransplantation assay of Ramathal *et al*. (*32*). Cell suspensions of human iPSCs with the *AZFa* deletion (*AZFΔa*) carrying corresponding transgene constructs are transplanted into the testis of busulfan-treated infertile nude mice. Two months following the transplantation, the testes are recovered and the seminiferous tubules are dissected for subsequent analysis.

to primary spermatocytes. Primary spermatocytes enter the first meiotic division and divide into secondary spermatocytes, which in turn divide into haploid spermatids, which migrate towards the lumen where terminally differentiated spermatozoa are released into the lumen (*29-31*).

To examine the functions of the *AZFa* gene *DDX3Y* in germ cell development and maintenance Ramathal and coworkers (*32*) used a mouse xenotransplantation model established previously. Mouse seminiferous tubules proved to be potent for induction of germ cell formation from human induced pluripotent stem cells (iPSCs) (*30,33*). It was shown that undifferentiated human iPSCs after being transplanted into mouse seminiferous tubules undergo differentiation to germ-cell-like cells (GCLCs) located close to the basement membrane and expressing cell-specific markers of PGCs.

Ramathal and coworkers used the iPSC line with the *AZFa* deletion (*AZFΔa*) that was genetically complemented with the *DDX3Y* gene (*32*). For this study transgene constructs carrying either FLAG-tagged *DDX3Y* with the *mCherry* marker gene (rescue lines) or *mCherry* marker alone (mutant control lines) were integrated in human iPSCs that harbored *AZFΔa*. The authors transplanted the undifferentiated iPSCs from the rescue and control lines into mouse seminiferous tubules of immunodeficient mice with endogenous spermatogenesis drug-eliminated in advance (Figure 3). After two months the post-transplantation xenografts were analyzed by immunohistochemistry to reveal human germ-cell like cells (GCLCs) using marker antibodies against NuMA (human cell-specific protein), Vasa (basic germline marker) and DDX3Y. Whereas the *AZFΔa* mutant line did not have cells that were positive for DDX3Y, all NuMA-positive GCLCs from the rescue lines were DDX3Y-positive and the majority of DDX3Y-positive GCLCs were also VASA-positive. The tubules, which were transplanted with the rescue lines, contained significantly more VASA-positive GCLCs in comparison with the tubules with *AZFΔa* mutant line xenografts. Human spermatogonia-specific marker proteins DAZ1 and UTF1 were not expressed in mouse seminiferous tubules transplanted with *AZFΔa* mutant iPSCs. However, these proteins were found in a subset of GCLCs from the *DDX3Y*-rescued lines. Thus, the authors have detected a quantitative enhancement in GCLC formation as well as germ cell differentiation in all the rescue lines.

The authors succeeded in purifying the germ cell fractions from mouse xenografts and subsequent sorting of donor cells positive for mCherry. Analysis of the transcriptome by RNA-Seq reveals that the transcription profile of DDX3Y-rescued GCLCs is significantly different from the *AZFΔa* mutant GCLCs, which exhibit a profile closely resembling undifferentiated

donor iPSCs. 248 transcripts were found to be differentially expressed between undifferentiated iPSCs, mutant GCLCs and rescue GCLCs. A large subset of transcripts which were up-regulated in iPSCs and mutant GCLCs were absent or significantly down-regulated in *DDX3Y*-rescued GCLCs. Note that the upregulated set of non-complemented *AZFΔa* GCLCs was found to be strongly enriched in pluripotency genes (including *POU5F1*, *LIN28A*, *SOX2 NANOG* and *MYC*) in contrast to *DDX3Y*-complemented GCLCs. A group of genes that specifically are up-regulated in the rescue GCLCs includes zinc-finger transcription factors, RNA-metabolism modulators, cell cycle and cell-cell communication genes. Thus, *DDX3Y*-complemented GCLCs in the mouse xenograft system are able to differentiate to the prospermatogonial stage and maintain a specific transcriptional program that is similar to that of PGCs and prospermatogonia.

3. *Drosophila homologue* of DDX3Y RNA-helicase Belle provides essential cell-autonomous functions in the male germline

In another recently published paper the testes of *Drosophila* were used as a relatively simplified model to study DDX3 functions in spermatogenesis (*34*). For *Drosophila*, effective approaches have been developed to turn on or off the expression of a selected gene in specific tissues or cells. Among them are mosaic clonal analysis (*35*); inducible RNAi-mediated gene knockdowns that could be ectopically induced in certain cells using *GAL4-UAS* system and tissue-specific promoters (*36,37*); and the CRISPR RNA/Cas9 system allowing precise gene engineering (*38*). All of them can be successfully applied for the study of spermatogenesis. Another great advantage of *Drosophila* testes as a model system consists in the possibility of studying germline stem cells and other testis cells *in situ*, using immunofluorescence confocal microscopy (*39*).

The apical testis tip of an adult fly is occupied by a somatic niche structure called the hub (Figure 4), which supports two adjacent stem cell populations: germline stem cells (GSCs) and somatic cyst stem cells (CySCs) (*40-42*). The self-renewing division of a GSC produces a new GSC and a goniablast (spermatogonium). The division of a CySC generates a self-renewing CySC and a cyst cell. A pair of somatic cyst cells encapsulates spermatogonium thus forming a functional unit of spermatogenesis – the cyst. Cyst cells are considered as mammalian Sertoli cell analogs, despite evident structural differences between the organization of the testes in *Drosophila* and humans (*41*). Spermatogonial cells within cysts undergo four rounds of mitotic amplification divisions resulting in 16-cell cysts of interconnected primary spermatocytes, each surrounded by two undivided cyst cells. Primary spermatocytes in the cyst undergo a long growth phase. Then they enter

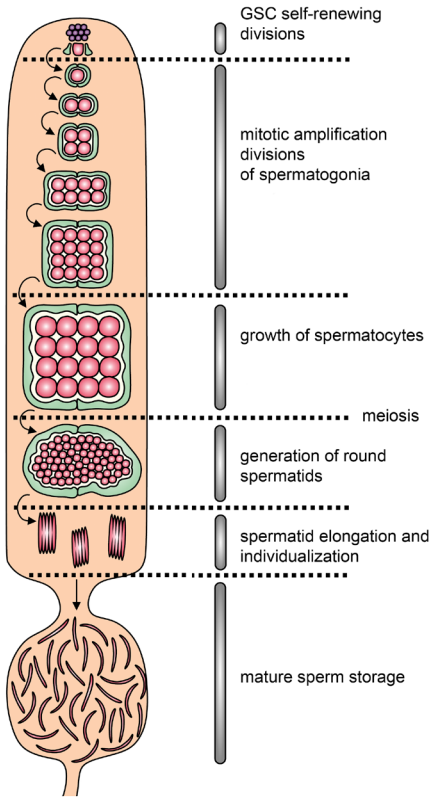

Figure 4. Representation of *Drosophila* spermatogenesis stages. At the apical testis tip germline stem cells (GSCs, red) are located adjacent to the hub (violet) and are surrounded by two somatic cyst stem cells (CySCs, green) (top of the figure). One of the daughter cells of GSC, the goniablast (red), undergoes four cell divisions to create cysts of 16 spermatogonia. Then spermatogonia switch to the spermatocyte developmental program. Mature spermatocytes synchronously enter meiosis producing 64 haploid spermatids. Finally, elongated spermatids undergo individualization at the basal end of the testis and enter the seminal vesicle, where mature sperm is stored until copulation (bottom part of the figure).

a synchronous meiotic division producing 64 haploid spermatids (Figure 4).

Drosophila RNA-helicase Belle is a single homolog of human DDX3 proteins due to the non-redundancy of protein-coding genes in the fly genome. All known *belle* null mutant alleles are lethal, suggesting that Belle has essential functions for fly viability. It was also shown that Belle was genetically required for male and female fertility; however, its molecular functions in gametogenesis had been obscure (*43*). Kotov and coworkers used flies with viable heteroallelic combinations of *belle* mutations as well as germline-specific *belle-RNAi* knockdown flies (*34*). Using immunofluorescence staining and confocal microscopy of testis preparations they found that a deficiency of *belle* led to drastic germ cell depletion in the testes of newly eclosed males, while somatic cyst cell and hub cell populations were maintained. The observed developmental disorder recapitulates the SCOS phenotype in humans carrying the *AZFa* deletions and also leads to severe azoospermia. No

GSCs were detected in the testes of *belle*$^{6/neo30}$ mutants or males with a germline *belle-RNAi* knockdown in comparison with the wild-type control (0 cells versus 9.3-9.6 cells per testis of control males). Using TUNEL assay, Kotov and coworkers found that germ cells in the *belle*$^{6/neo30}$ testes as well as in the case of a germline-specific *belle-RNAi* knockdown underwent premature cell death through apoptosis. The authors conclude that Belle is required cell-autonomously for the maintenance of early germ cells including GSCs, because *belle-RNAi* knockdown in testis somatic cells does not result in the loss of GSCs and germ cells of subsequent stages.

Kotov and coworkers also showed that the testes of *belle*$^{6/neo30}$ third instar larvae still contained all populations of early germ cells in contrast to the testes of adult flies (*34*). This observation made it possible to propose that PGCs correctly migrated into embryonic gonads during mid-to-late embryogenesis in *belle* mutants. However, while analyzing *belle* larval testes

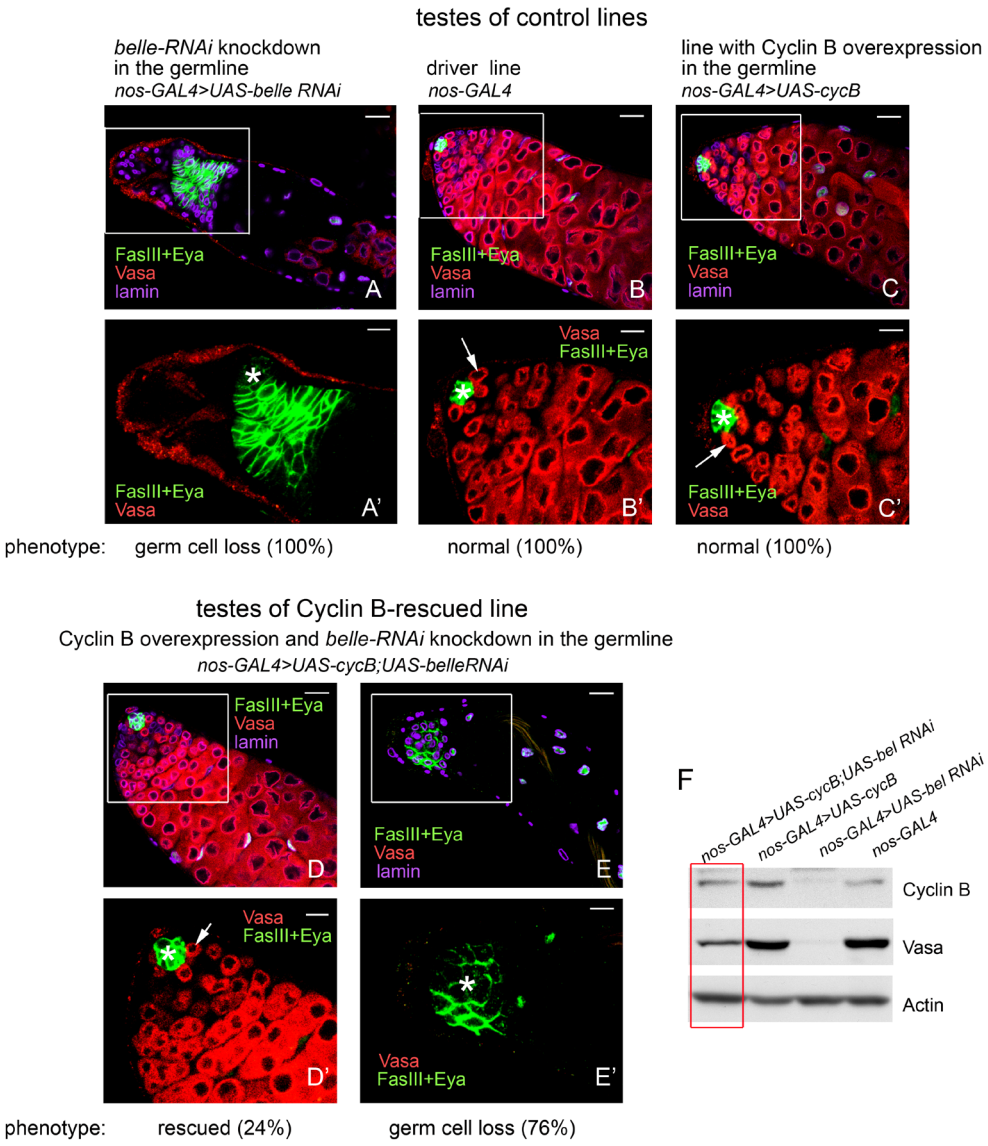

Figure 5. Transgenic overexpression of Cyclin B partially rescues spermatogenesis in the *belle-RNAi* knockdown testes. Immunofluorescent analysis of the testes of control lines **(A-C)** and Cyclin B-rescued line **(D, E)** obtained in rescue experiments. **(A-E)** Testes were stained with anti-Fas III (green, marker of the hub), anti-Eya (green, marker of mature cyst cells), anti-Vasa (red, germline marker) and anti-lamin (violet, marker of the nuclear envelope) antibodies. Scale bars are 20 μm. Bottom panels **(A'-E')** show higher magnifications of the apical tips of the testes shown in the white boxes of the corresponding top panels (without lamin staining). Where applicable, white arrows indicate one of the GSCs identified as a Vasa-stained germ cell attached to the hub (asterisks). Scale bars are 10 μm. **(D, D')** A portion of *nos-GAL4>UAS-cycB;UAS-belle RNAi* flies (24%) restores a wild-type phenotype of the testes with proper maintenance of GSCs. **(E, E')** The other portion of *nos-GAL4>UAS-cycB;UAS-belle RNAi* males has testes with reduced size that contain almost no germ cells while preserving mature cyst cells. **(F)** Western blot analysis of testis lysates from fly lines of the rescue experiment. A half-restored Vasa (germ cell marker) protein level is found in the *nos-GAL4>UAS-cycB;UAS-belle RNAi* testes (red outline). Actin stained with anti-Actin antibodies is used as a loading control. The figure was reproduced from (*34*) with permission from Elsevier.

Table 1. Functions of mitotic cyclins in the cell cycle of *Drosophila* male GSCs

Cyclin	Corresponding phase of cell cycle	Functions	Defects in male GSCs maintenance owing to the disruption of cyclin expression	*Ref.*
Cyclin A	end of S – G2 – start of M	Cyclin A in complex with either Cdc1 or Cdc2 takes part in the continuation of S phase	centrosome misorientation	(*44*)
		Maintenance of centrosome orientation in the proximity of the hub-GSC junction (centrosome orientation checkpoint)		
Cyclin B	M	Cyclin B-Cdc2 complex is responsible for entry into mitosis	G2-M transition arrest	(*34,45,46*)
			Loss of GSCs and germ cells of subsequent stages through cell death	

morphology, the authors observed a partial germ cell loss along with the presence of abnormally large early germ cells with hallmarks of G2 mitotic delay. In agreement with these observations, the authors detected a considerable decline in expression of the major mitotic cyclins, A and B, in the testes of hypomorph $belle^{EY08943/neo30}$ mutants owing to reduction of their transcription levels. The authors suggested that early germ cells including GSCs in the testes of *belle* mutants could not enter mitosis owing to an insufficient dose of mitotic cyclins. A failure to enter mitosis appears to trigger programmed cell death and leads to the germ cell loss phenotype. In support of this hypothesis, Kotov and coworkers achieved a partial but significant rescue of germ cell survival, mitotic progression (Figure 5) and male fertility by germline-specific overexpression of Cyclin B in the *belle-RNAi* knockdown testes (*34*). Overexpression of Cyclin A did not rescue the *belle-RNAi* knockdown phenotype. These results support an important role of mitotic cyclins in development and survival of early germ cells, including GSCs. Current data about the functions of major mitotic cyclins in the male GSCs cell cycle and survival are summarized in Table 1. It has been previously shown that a mutational depletion of Cyclin B led to a total lack of germ cells in the testes, and transgene overexpression of Cyclin A did not restore the germ cell loss phenotype in *cyclin B* mutants (*46*). Thus, at least one crucial function of Belle in early germ cells is the maintenance of Cyclin B level for ensuring mitotic divisions.

Overexpression of Cyclin B in the germline led to an almost complete restoration of early germ cells in the testes of $belle^{EY08943/neo30}$ mutants, but in this case male fertility was not restored (Kotov, Olenkina unpublished data), indicating additional functions of Belle during the subsequent steps of spermatogenesis. Indeed, it has been previously shown that the testes of hypomorph *belle* mutants exhibited defects in meiotic chromosome segregation and cytokinesis with a high frequency, as well as a disorganization of elongated spermatid bundles (*43*). RNA helicase Belle in flies presumably combines the functions of both human DDX3 homologues. While DDX3Y is expressed at the premeiotic stage in spermatogonia, DDX3X expression is observed later in spermatids in the human male germline (*15,16,20*). Taking into account that the understanding of the functions of DDX3X at the late stages of human spermatogenesis is rudimentary, the employment of the *Drosophila* model may contribute to elucidation of DDX3 protein functions in the maintenance of spermatogenesis in humans.

4. Conclusions

The data presented by Ramathal and coworkers clearly demonstrate that DDX3Y expression is enough for driving germ cell differentiation of *AZFa*-deleted iPSCs and for induction of germline developmental gene expression (*32*). The obtained results suggest that DDX3Y functions are required during the early developmental stage in humans that include a period from PGC specification to prospermatogonial cell development. The expression of early spermatogonia-specific marker proteins indicates that DDX3Y-rescued GCLCs are able to progress to the prospermatogonial stage and that DDX3Y regulates this transition. Taken together, these results demonstrate for the first time that DDX3Y has essential functions in early male germline development in humans.

It has previously been assumed that DDX3Y protein is involved in the control of premeiotic proliferation of germ cells (*16*). To date, regulation of the cell cycle in human spermatogonia is poorly understood (*47*). A disruption of the *Drosophila* DDX3Y homologue Belle leads to severe germ cell loss through apoptosis in the testes, while somatic cyst cells are retained intact (*34*). Considering that cyst cells in *Drosophila* testes appear to be the functional analogs of Sertoli cells of mammalian testes, this phenotype mimics that of SCOS in humans with DDX3Y expression failure as a result of a corresponding deletion. Taking into account the conserved nature of DDX3 proteins in eukaryotes, Kotov and coworkers (*34*) demonstrate that Belle is required cell-autonomously for mitotic progression and survival of GSCs and spermatogonia as the upstream regulator of mitotic cyclin expression. These results obtained using

the *Drosophila* model support the importance of the tight regulation of mitotic cyclins in the fate determination of early germ cells. Both papers discussed here provide a significant insight into the functions of DDX3Y in spermatogenesis, suggesting that DDX3Y must be considered as en essential contributor to early male germ cell development and maintenance.

Acknowledgements

We thank Anastasia Stolyarenko for help in manuscript preparation. This work was supported by the Molecular and Cellular Biology program of the Russian Academy of Sciences; by the Russian Federal Program for support of Leading Scientific Schools, grant # SS-7231.2016.4; and by the Russian Foundation for Basic Research grant # 16-34-00507 (A.A.K.).

References

1. Reijo R, Alagappan RK, Patrizio P, Page DC. Severe oligozoospermia resulting from deletions of azoospermia factor gene on Y chromosome. Lancet. 1996; 347:1290-1293.
2. Lahn BT, Page DC. Functional coherence of the human Y chromosome. Science. 1997; 278:675-680.
3. Foresta C, Moro E, Ferlin A. Y chromosome microdeletions and alterations of spermatogenesis. Endocr Rev. 2001; 22:226-239.
4. Vogt PH, Falcao CL, Hanstein R, Zimmer J. The AZF proteins. Int J Androl. 2008; 31:383-394.
5. Vogt PH, Edelmann A, Kirsch S, *et al*. Human Y chromosome azoospermia factors (AZF) mapped to different sub-regions in Yq11. Hum Mol Genet. 1996; 5:933-943.
6. Edwards RG, Bishop CE. On the origin and frequency of Y chromosome deletions responsible for severe male infertility. Mol Hum Reprod. 1997; 3:549-554.
7. Vogt PH. Genomic heterogeneity and instability of the AZF locus on the human Y chromosome. Mol Cell Endocrinol. 2004; 224:1-9.
8. Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Silber S, Oates R, Rozen S, Page DC. The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. Nat Genet. 2001; 29:279-286.
9. Navarro-Costa P, Plancha CE, Gonçalves J. Genetic dissection of the AZF regions of the human Y chromosome: Thriller or filler for male (in)fertility? J Biomed Biotechnol. 2010; 2010:936569.
10. Kamp C, Huellen K, Fernandes S, Sousa M, Schlegel PN, Mielnik A, Kleiman S, Yavetz H, Krause W, Küpker W, Johannisson R, Schulze W, Weidner W, Barros A, Vogt PH. High deletion frequency of the complete AZFa sequence in men with Sertoli-cell-only syndrome. Mol Hum Reprod. 2001; 7:987-994.
11. Lardone MC, Parodi DA, Valdevenito R, Ebensperger M, Piottante A, Madariaga M, Smith R, Pommer R, Zambrano N, Castro A. Quantification of *DDX3Y*, *RBMY1*, *DAZ* and *TSPY* mRNAs in testes of patients with severe impairment of spermatogenesis. Mol Hum Reprod. 2007; 13:705-712.
12. Foresta C, Moro E, Rossi A, Rossato M, Garolla A, Ferlin A. Role of the AZFa candidate genes in male infertility. J Endocrinol Invest. 2000; 23:646-651.
13. Krausz C, Degl'Innocenti S, Nuti F, Morelli A, Felici F, Sansone M, Varriale G, Forti G. Natural transmission of *USP9Y* gene mutations: A new perspective on the role of *AZFa* genes in male fertility. Hum Mol Genet. 2006; 15:2673-2681.
14. Luddi A, Margollicci M, Gambera L, Serafini F, Cioni M, De Leo V, Balestri P, Piomboni P. Spermatogenesis in a man with complete deletion of *USP9Y*. N Engl J Med. 2009; 360:881-885.
15. Foresta C, Ferlin A, Moro E. Deletion and expression analysis of *AZFa* genes on the human Y chromosome revealed a major role for *DBY* in male infertility. Hum Mol Genet. 2000; 9:1161-1169.
16. Gueler B, Sonne SB, Zimmer J, Hilscher B, Hilscher W, Græm N, Rajpert-De Meyts E, Vogt PH. AZFa protein DDX3Y is differentially expressed in human male germ cells during development and in testicular tumours: New evidence for phenotypic plasticity of germ cells. Hum Reprod. 2012; 27:1547-1555.
17. Schröder M. Human DEAD-box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation. Biochem Pharmacol. 2010; 79:297-306.
18. Linder P, Jankowsky E. From unwinding to clamping – the DEAD box RNA helicase family. Nat Rev Mol Cell Biol. 2011; 12:505-516.
19. Kotov AA, Akulenko NV, Kibanov MV, Olenina LV. DEAD-Box RNA helicases in animal gametogenesis. Mol Biol (Mosk). 2014; 48:16-28.
20. Ditton HJ, Zimmer J, Kamp C, Rajpert-De Meyts E, Vogt PH. The *AZFa* gene *DBY* (*DDX3Y*) is widely transcribed but the protein is limited to the male germ cells by translation control. Hum Mol Genet. 2004; 13:2333-2341.
21. Rauschendorf MA, Zimmer J, Hanstein R, Dickemann C, Vogt PH. Complex transcriptional control of the *AZFa* gene *DDX3Y* in human testis. Int J Androl. 2011; 34:84-96.
22. Rauschendorf MA, Zimmer J, Ohnmacht C, Vogt PH. DDX3X, the X homologue of *AZFa* gene *DDX3Y*, expresses a complex pattern of transcript variants only in the male germ line. Mol Hum Reprod. 2014; 20:1208-1222.
23. Vong QP, Li Y, Lau YF, Dym M, Rennert OM, Chan WY. Structural characterization and expression studies of *Dby* and its homologs in the mouse. J Androl. 2006; 27:653-661.
24. Mazeyrat S, Saut N, Grigoriev V, Mahadevaiah SK, Ojarikre OA, Rattigan A, Bishop C, Eicher EM, Mitchell MJ, Burgoyne PS. A Y-encoded subunit of the translation initiation factor Eif2 is essential for mouse spermatogenesis. Nat Genet. 2001; 29:49-53.
25. Yao CJ, Xu WJ, Gong XL, Zhou Y, Yan ZQ, Zhu ZJ, Wang ZX, Li QL, Guo XB, Wang LY, Ma D, Qiao ZD. The role of *Dby* mRNA in early development of male mouse zygotes. Asian J Androl. 2010; 12:567-577.
26. Mazeyrat S, Saut N, Sargent CA, Grimmond S, Longepied G, Ehrmann IE, Ellis PS, Greenfield A, Affara NA, Mitchell MJ. The mouse Y chromosome interval necessary for spermatogonial proliferation is gene dense with syntenic homology to the human AZFa

region. Hum Mol Genet. 1998; 7:1713-1724.
27. Leroy P, Alzari P, Sassoon D, Wolgemuth D, Fellous M. The protein encoded by a murine male germ cell-specific transcript is a putative ATP-dependent RNA helicase. Cell. 1989; 57:549-559.
28. Session DR, Lee GS, Wolgemuth DJ. Characterization of *D1Pas1*, a mouse autosomal homologue of the human AZFa region *DBY*, as a nuclear protein in spermatogenic cells. Fertil Steril. 2001; 76:804-811.
29. Phillips BT, Gassei K, Orwig KE. Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond B Biol Sci. 2010; 365:1663-1678.
30. Valli H, Phillips BT, Shetty G, Byrne JA, Clark AT, Meistrich ML, Orwig KE. Germline stem cells: Toward the regeneration of spermatogenesis. Fertil Steril. 2014; 101:3-13.
31. Greenspan LJ, de Cuevas M, Matunis E. Genetics of gonadal stem cell renewal. Annu Rev Cell Dev Biol. 2015; 31:291-315.
32. Ramathal C, Angulo B, Sukhwani M, Cui J, Durruthy-Durruthy J, Fang F, Schanes P, Turek PJ, Orwig KE, Reijo Pera R. *DDX3Y* gene rescue of a Y chromosome *AZFa* deletion restores germ cell formation and transcriptional programs. Sci Rep. 2015; 5:15041.
33. Ramathal C, Durruthy-Durruthy J, Sukhwani M, Arakaki JE, Turek PJ, Orwig KE, Reijo Pera RA. Fate of iPSCs derived from azoospermic and fertile men following xenotransplantation to murine seminiferous tubules. Cell Rep. 2014; 7:1284-1297.
34. Kotov AA, Olenkina OM, Kibanov MV, Olenina LV. RNA helicase Belle (DDX3) is essential for male germline stem cell maintenance and division in *Drosophila*. Biochim Biophys Acta. 2016; 1863:1093-1105.
35. Xu T, Rubin GM. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development. 1993; 117:1223-1237.
36. Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature. 2007; 448:151-156.
37. White-Cooper H. Tissue, cell type and stage-specific ectopic gene expression and RNAi induction in the *Drosophila* testis. Spermatogenesis. 2012; 2:11-22.
38. Gratz SJ, Cummings AM, Nguyen JN, Hamm DC, Donohue LK, Harrison MM, Wildonger J, O'Connor-Giles KM. Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. Genetics. 2013; 194:1029-1035.
39. Kibanov MV, Kotov AA, Olenina LV. Multicolor fluorescence imaging of whole-mount *Drosophila* testes for studying spermatogenesis. Anal Biochem. 2013; 436:55-64.
40. White-Cooper H. Molecular mechanisms of gene regulation during *Drosophila* spermatogenesis. Reproduction. 2010; 139:11-21.
41. Spradling A, Fuller MT, Braun RE, Yoshida S. Germline stem cells. Cold Spring Harb Perspect Biol. 2011; 3:a002642.
42. Matunis EL, Stine RR, de Cuevas M. Recent advances in *Drosophila* male germline stem cell biology. Spermatogenesis. 2012; 2:137-144.
43. Johnstone O, Deuring R, Bock R, Linder P, Fuller MT, Lasko P. Belle is a *Drosophila* DEAD-box protein required for viability and in the germ line. Dev Biol. 2005; 277:92-101.
44. Yuan H, Chiang CY, Cheng J, Salzmann V, Yamashita YM. Regulation of cyclin A localization downstream of Par-1 function is critical for the centrosome orientation checkpoint in *Drosophila* male germline stem cells. Dev Biol. 2012; 361:57-67.
45. Monk AC, Siddall NA, Volk T, Fraser B, Quinn LM, McLaughlin EA, Hime GR. How is required for stem cell maintenance in the *Drosophila* testis and for the onset of transit-amplifying divisions. Cell Stem Cell. 2010; 6:348-360.
46. Wang Z, Lin H. The division of *Drosophila* germline stem cells and their precursors requires a specific cyclin. Curr Biol. 2005; 15:328-333.
47. Wolgemuth DJ, Manterola M, Vasileva A. Role of cyclins in controlling progression of mammalian spermatogenesis. Int J Dev Biol. 2013; 57:159-168.

(Received November 17, 2016; Revised January 19, 2017; Accepted January 25, 2017)

Original Article

DOI: 10.5582/bst.2016.01183

End-of-life care bonus promoting end-of-life care in nursing homes: An 11-year retrospective longitudinal prefecture-wide study in Japan

Sho Nishiguchi[1,*], Nagisa Sugaya[1], Kentaro Sakamaki[2], Shunsaku Mizushima[1]

[1] *Department of Epidemiology and Public Health, Graduate School of Medicine, Yokohama City University, Yokohama, Japan;*
[2] *Department of Biostatistics, Yokohama City University School of Medicine, Yokohama, Japan.*

Summary **The end-of-life (EOL) care bonus introduced by the Japanese government works as a financial incentive and framework of quality preservation, including advance care planning, for EOL care among nursing home residents. This study aims to clarify the effects of the EOL care bonus in promoting EOL care in nursing homes. A longitudinal observational study using a questionnaire was conducted. We invited 378 nursing homes in Kanagawa prefecture in Japan, a region with a rapidly aging population, to participate in the study. The outcome was the number of residents dying in nursing homes from 2004 to 2014. In a linear mixed model, fixed-effect factors included year established, unit care, regional elderly population rate and hospital beds, adjacent affiliated hospital, full-time physician on site, physician's support during off-time, basic EOL care policy, usage of the EOL care bonus, EOL care conference, and staff experience of EOL care. A total of 237 nursing home facilities responded (62.7%). The linear mixed model showed that the availability of the EOL care bonus (coefficient 3.1, 95 % CI 0.67-5.51, $p = 0.012$) and years of usage of the EOL care bonus ($p < 0.001$) were significantly associated with increased numbers of residents dying in nursing homes. Our analysis revealed that the EOL care bonus has the potential to increase the number of residents receiving EOL care in nursing homes over several years. EOL care conferences, physician support for emergency care during off-time, and the presence of an adjacent affiliated hospital may also increase the number of residents receiving EOL care in nursing homes. These results suggest that a government financial incentive may contribute to effective EOL care among nursing home residents in other developed countries with rapidly aging populations.**

Keywords: End-of-life care, nursing home, end-of-life care bonus, Japan, advance care planning, financial incentive

1. Introduction

In aging societies, end-of-life (EOL) care is an important issue, especially in nursing home settings (*1*). A nursing home is internationally defined as a facility with a domestic-style environment that provides 24-hour functional support for frail elderly residents who require assistance due to losing capacity and having complex health needs, including dementia (*2,3*). Due to the aging populations in many developed countries, the demand for nursing home care is increasing. However, nursing home residents in Japan, the United States, the United Kingdom, and many other developed countries are transferred involuntarily to a hospital at their EOL, against their living wills (*4-11*). In a Japanese nationwide study in 2006, among nursing home residents who were dying, fewer residents were dying in nursing homes (30.9%) compared with residents who were dying in a hospital (43.8%) (*12*).

Released online in J-STAGE as advance publication January 30, 2017.

**Address correspondence to:*
Dr. Sho Nishiguchi, Department of Epidemiology and Public Health, Graduate School of Medicine, Yokohama City University, Yokohama, Fukuura 3-9, Yokohama City, Kanagawa Prefecture, 236-0004, Japan.
E-mail: sanazen@hotmaill.co.jp

Based on the fact that many residents were dying in hospitals against their wishes, taking into account the quality and quantity of EOL care in Japanese nursing homes, the EOL care bonus was established in 2006 (*13,14*). The primary role of the bonus is as a financial incentive from the Japanese government. A bonus payment is made per diem consisting of 800 Yen ($7US) between 29 to 3 days before the date of death, 6,800 Yen ($62) 2 to 1 days before the date of death, and 12,800 Yen ($116) on the date of death. The bonus can be retrospectively billed for 30 days up to a total of 48,000 Yen ($436) per resident by the government to the facility (*13*). The secondary role of the bonus is as a framework for quality preservation, for EOL care in nursing homes. Facilities must meet the following five eligibility criteria (*13*) to receive the bonus: employing a full-time registered nurse onsite, having a 24-hour call system for nurses, having a basic policy for EOL care that needs to be explained to residents and/or their families on admission, organizing training courses on EOL for the staff, and having an individual room for EOL care. In addition, residents must also meet the following three criteria: diagnosed with an incurable condition by a physician, have an EOL care planning provision in place with consent from the resident or the resident's family, and an explanation of, and the family's consent to EOL care must have been given/acquired for the duration of care (*13*).

A previous study in Japan showed that facility characteristics that are related to dying in nursing homes, included policies of providing EOL care, physicians being based in home care supporting clinics, and location in a region with more nursing home beds or fewer hospital beds (*15,16*). In other literature, the EOL care bonus by the Japanese government had been just introduced (*17,18*); however, there have been no studies concerning how the EOL care bonus has affected the promotion of EOL care in nursing homes. If it is revealed that the bonus by the Japanese government promotes EOL care in nursing homes, it may contribute to effective EOL care in nursing homes in other developed countries with a rapidly aging population. Therefore, the present study was conducted to identify whether the EOL care bonus promotes EOL care in nursing homes, by using retrospective longitudinal data.

2. Materials and Methods

2.1. *Setting and subjects*

Data were collected from the Kanagawa prefecture: a region with a population of 9 million, an elderly population rate of 21.5% in 2012 (*19*), and is estimated to have the highest increase in elderly population rate over the next 20 years (*20*). All of the residents of 378 nursing homes in Kanagawa prefecture were included, based on the Long-term Care Insurance Services Informational Publication System (*21*) as of November 2015. We requested the cooperation of directors of the Health and Welfare Departments in Yokohama city, Kawasaki city, Yokosuka city, and Sagamihara city, Kanagawa prefecture.

2.2. *Measures and questionnaire*

We conducted a pilot survey in order to draft an appropriate questionnaire for the main prefecture-wide survey. The pilot survey was conducted in August 2014 and included visits to two nursing homes and a questionnaire for the managers of 14 nursing homes. Based on the pilot survey, we mailed the revised questionnaire to the managers of all 378 nursing homes, divided five times for each jurisdictional area in the prefecture. The questionnaires were distributed between November 2015 and January 2016. After repeated requests by fax and phone in less responsive areas, the completed questionnaires were returned to our research office by the beginning of May 2016.

The questionnaire was based on a previous study (*15*) and gathered information relating to the number of residents who were discharged and the characteristics of the facility, such as staffing and the nature of EOL care. Residents who were discharged during the 11 fiscal years from 2004 to 2014, were divided into three discharge categories: due to death, transferred to a hospital, or other reasons. Those residents, due to death and residents dying in a nursing home were categorized as receiving EOL care in a nursing home.

2.3. *Ethical considerations*

The study was approved by the institutional review boards of the university (No. A140522015, approved on 24th July 2014). Nursing home facilities' consent was implied by the return of the questionnaires.

2.4. *Statistical analysis*

A multi-level analysis was conducted using a linear mixed-effects model. The dependent variable was the number of EOL care residents dying per 100 beds in a nursing home. Fixed-effect factors included the year the facility was established, adoption of unit care, the elderly population rate in the region of the facility, number of hospital beds in the region of the facility, the presence of an adjacent affiliated hospital, the presence of a full-time physician on site, physician's support during off-time, basic policy for EOL care, usage of the EOL care bonus, conferences for EOL care, nurses' experience of EOL care, and caregivers' experience of EOL care (Table 1). The year established was compared by dividing it into before and after the introduction of the EOL care bonus in 2006. Adoption of unit care required the facility to have all single rooms with a common living and

Table 1. Facility characteristics of nursing homes

Items	Total (n = 237)
Outline of facility	
Year established	
Before 2005	154 (65.0 %)
After 2006	80 (33.8 %)
Unknown	3 (1.3 %)
Unit care	
Yes	104 (43.9 %)
No	131 (55.3 %)
Unknown	2 (0.8 %)
Individual room (median, IQR)*	23, 4 - 88
Yes	207 (87.3 %)
No	26 (11.0 %)
Unknown	4 (1.7 %)
Region of facility	
Elderly population rate (median, IQR)	19.6, 17.2 - 22.3 %
Number of hospital beds per 10 million population (median, IQR)	766, 746 - 857
Adjacent affiliated hospital	
Yes	12 (5.1 %)
No	223 (94.1 %)
Unknown	2 (0.8 %)
Staffing	
Full-time physician on site	
Yes	14 (5.9 %)
No	221 (93.2 %)
Unknown	2 (0.8 %)
Physician's support for emergency care during off-time	
Only support by calling	119 (50.2 %)
No support	67 (28.3 %)
Full-time support	49 (20.7 %)
Unknown	2 (0.8 %)
Full-time nurse on site* (mean ± SD)	3.3 ± 1.5
Yes	231 (97.5 %)
No	1 (0.4 %)
Unknown	5 (2.1 %)
EOL care	
Basic policy for EOL care (on 2015)	
Providing EOL care in the nursing home	154 (65.0 %)
Transfer to a hospital	57 (24.1 %)
No explicit policy	24 (10.1 %)
Unknown	2 (0.8 %)
Usage of EOL care bonus (on 2015)	
Yes	143 (60.3 %)
To be prepared	38 (16.0 %)
No	55 (23.2 %)
Unknown	1 (0.4 %)
Preference documented*	
Yes	162 (68.4 %)
No	57 (24.1 %)
Unknown	18 (7.6 %)
Explanation of EOL care on nursing home admission*	
Yes	189 (79.7 %)
Yes, but not always	22 (9.3 %)
No	20 (8.4 %)
Unknown	6 (2.5 %)
Training course of EOL care*	
Yes	208 (87.8 %)
No	28 (11.8 %)
Unknown	1 (0.4 %)
Conference for EOL care (in 2015)	
Yes	157 (66.2 %)
No	63 (26.6 %)
Unknown	17 (7.2 %)
Nurses' experience of EOL care	
No	40 (16.9 %)
1-4 times	27 (11.4 %)
≥ 5 times	159 (67.1 %)
Unknown	11 (46.4 %)
Caregivers' experience of EOL care	
No	38 (16.0 %)
1-4 times	61 (25.7 %)
≥ 5 times	125 (52.7 %)
Unknown	13 (5.5 %)

End-of-life care bonus includes the factors marked *. EOL: end-of-life; IQR: interquartile range; SD: standard deviation.

dining room per 10 beds. It is desirable that the EOL care conference includes the physician, nurse, caregiver, care manager, social worker, resident (if possible), and resident's family. For every area in a prefecture, the elderly population rate and number of hospital beds were calculated in every fiscal year using local government data (*22,23*). The basic policy, usage of the EOL care bonus, and usage of the conferences for EOL care were additionally examined for every year measured. Because the facility criteria of the EOL care bonus included the presence of individual rooms, the presence of a full-time nurse on site, documentation of the resident or family preference for EOL care, an explanation of EOL care on nursing home admission, and an EOL training course, these factors were excluded from the multi-level analysis. All data analyses were performed using the SPSS software version J21 (IBM, Tokyo, Japan). A p value of < 0.05 was considered statistically significant for all analyses.

3. Results

Among all 378 nursing home facilities in the prefecture, 237 facilities (62.7%) responded by mail, fax, or email. However, the remaining 141 facilities (37.3%) did not return the questionnaire. Among residents who were discharged during the 11 fiscal years from 2004 to 2014, the death group increased annually from 68.4% to 71.9%, the transferal to hospital group decreased annually from 28.7% to 24.6%, and the other reasons group had an unremarkable change of about 3.0%.

3.1.1. *Facility characteristics*

The national government subsidized 230 facilities (97.0%), 5 (2.1%) facilities were subsidized by the local government, and the funding status of 2 (0.8%) facilities was unknown. Many facilities were established around 2006 upon the initiation of the EOL care bonus (median, interquartile range [IQR]: 2002, 1992-2008). The mean number of beds was 89.2 (standard deviation [SD]: 34.6). Individual rooms were adopted by 237 (87.3%) nursing homes and the median was 30 (IQR: 8-98) rooms.

3.1.2. *Region of facility*

Based on Kanagawa prefecture government data, we conducted a descriptive analysis that revealed the regional characteristics of all local municipalities. At the municipal level, there were large differences in elderly population rates ranging from 12.9% to 26.0% in 2004 (*22*). During the 11-year period, rates in all local municipalities increased (mean, SD: 1.5, 0.2 times), and ultimately reached from 19% to 37.4% in 2014. However, the number of hospital beds in all local municipalities did not obviously increase (mean, SD: 1.0, 0.2 times) (*23*).

3.1.3. *Staffing*

The mean number of caregiver staff was 48.5 (SD: 20.3) persons, and the mean number of full-time caregivers on site was 33.5 (SD: 14.9) persons. The mean number of nursing staff was 6.4 (SD: 2.5) persons, and the number of full-time nurses on site was 3.3 (SD: 1.5) persons. One hundred ninety-three facilities (81.4%) were on call during the night. Physicians in 111 (46.8%) facilities worked once every week.

3.2. *EOL care*

Most facilities provided EOL care in the nursing home (65.0%) and used the EOL care bonus (60.3%) (Table 1). Regarding the EOL care policy if a patient or the family wanted the patient to die at the nursing home, there were 201 (84.8%) acceptable facilities, 9 (3.8%) were acceptable only with family cooperation, and 26 (11.0%) facilities were not acceptable.

3.3. *Trends for residents dying in nursing homes*

During the period from the initiation of the EOL care bonus in 2006 until 2014, the mean annual number of residents receiving EOL care in nursing homes increased at a rate that was proportional to the progressive increase in the annual mean usage rates of the EOL care bonus (Figure 1). However, there were differences in each fiscal year between the number of residents dying in nursing homes and the number of residents who adopted the EOL care bonus (Figure 1). In addition, among the dying residents, the ratio of residents who died in a hospital progressively decreased from 2004 to 2014 (Figure 2). However, the ratio of residents who died in nursing homes progressively increased and the predominant

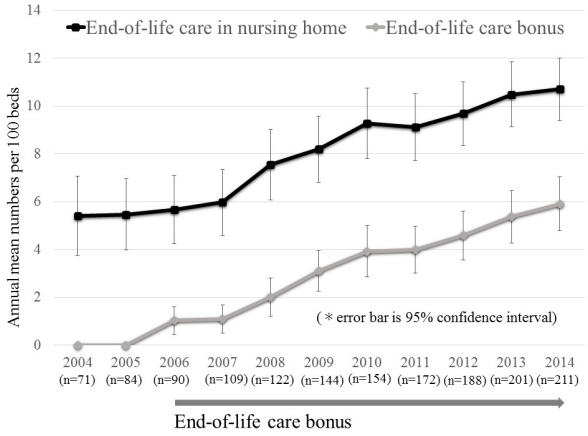

Figure 1. Trends of end-of-life care in nursing homes. The trends are presented for the facilities that reported the number of residents dying in nursing homes and the number of residents who adopted the EOL care bonus. The numbers of residents per 100 beds progressively increased from the initiation of the EOL care bonus in 2006.

location where the resident died switched from hospitals to nursing homes after the initiation of the EOL care bonus.

3.4. *The effect of the EOL care bonus in the linear mixed model*

The linear mixed model showed that the availability of the EOL care bonus (coefficient 3.1, 95% confidence interval [CI] 0.67-5.51, $p = 0.012$) and the years of usage of the EOL care bonus (each coefficient in Table 2, $p < 0.001$) were significant factors associated with increasing numbers of residents dying in nursing homes (Table 2). During the years of usage of the bonus, the coefficients increased yearly for 6 years from the onset of usage of the bonus (Table 2).

In the fixed-effect factors, apart from EOL care bonus availability and years of usage, presence of an adjacent affiliated hospital (coefficient 3.2, 95% CI 0.12-6.26, $p < 0.001$), full-time physician support for emergency care during off-time (coefficient 5.4, 95% CI 2.76-8.05, $p < 0.001$), and EOL care conferences (coefficient 2.0, 95% CI 1.00-3.01, $p < 0.001$) were significant factors associated with increasing numbers of residents dying in nursing homes (Table 2).

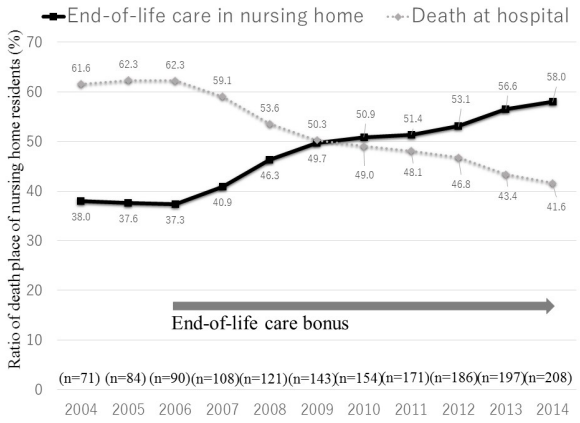

Figure 2. Ratio of number of deaths in nursing homes and hospitals after the initiation of the end-of-life care bonus. This figure presents the trends for the facilities that reported both the numbers of residents dying in nursing homes and in hospitals. The ratio of the residents dying in nursing homes increased and showed an inversion phenomenon compared with those dying in hospitals.

4. Discussion

4.1. *Summary and interpretation of the findings*

In the results of the trends in Figure 1, the number of residents receiving EOL care in nursing homes had progressively increased after the initiation of the EOL care bonus. However, because one-third of the nursing homes in the area were established recently, as described in Table 1, this might have affected the increase in the number of residents receiving EOL care in nursing homes. Additionally, the results of the ratio of death place of nursing home residents suggests that provision of EOL

Table 2. Results of multi-level mixed-effects linear regression

Items	Coefficient	95 % CI	*p* value
Time			0.104
Outline of facility			
Year established before 2006	2.4	(- 0.50-5.38)	0.103
Unit care	- 0.5	(- 3.12-2.12)	0.705
Region of facility			
Elderly population rate >75th quartile	0.6	(- 1.17-2.44)	0.225
Number of hospital beds per 10 million population >75th quartile	1.2	(- 0.79-3.28)	0.386
Adjacent to affiliated hospital	3.2	(0.12-6.26)	0.042*
Staffing			
Full time physician on site	0.2	(- 3.71-4.08)	0.926
Full time physician support for emergency care during off-time	5.4	(2.76-8.05)	< 0.001*
EOL care			
Basic policy of providing EOL care in nursing home	0.2	(- 1.29-1.77)	0.76
Availability of EOL care bonus	3.1	(0.67-5.51)	0.012*
Years of usage of EOL care bonus (year)			< 0.001*
0			
1	- 0.5	(- 2.93-1.88)	
2	0.9	(- 1.67-3.38)	
3	1.4	(- 1.15-3.93)	
4	2.0	(- 0.58-4.67)	
5	3.5	(0.84-6.19)	
6	4.6	(1.80-7.34)	
7	3.4	(0.42-6.34)	
8	5.9	(2.74-9.00)	
9	3.0	(- 0.24-6.27)	
Conference for end-of-life care	2.0	(1.00-3.01)	< 0.001*
Nurses' experience of EOL care ≥5 times	1.9	(- 0.58-4.34)	0.128
Caregivers' experience of EOL care ≥5 times	0.1	(- 2.43-2.70)	0.075

The mixed-effects model analysis included all the factors except for EOL bonus criteria in Table 1. EOL: end-of-life.

care in nursing homes progressively increased compared to those transferred and deaths in a hospital from 2004 to 2014 (Figure 2). Both results of trends showed that not only did the numbers increase, but the ratio of residents receiving EOL care in nursing homes also increased after the initiation of the EOL care bonus.

The results of the linear mixed model showed that both the availability of the EOL care bonus and the number of years the EOL care bonus had been used were significant factors associated with increasing numbers of residents receiving EOL care in nursing homes. Over the 6-year period that the EOL care bonus was used, numbers of residents receiving EOL care in nursing homes experienced a consecutive year-on-year increase. In addition, EOL care conferences, physician support for emergency care during off-time, and the presence of an adjacent affiliated hospital were also significant factors associated with numbers of residents receiving EOL care in nursing homes.

Therefore, our analysis revealed that the EOL care bonus has the potential to increase the number of residents receiving EOL care in nursing homes over several years. EOL care conferences, physician support for emergency care during off-time, and the presence of an adjacent affiliated hospital may also increase the number of residents receiving EOL care in nursing homes.

4.2. *Comparison with other studies*

Due to the cooperation of the local government's directors, the questionnaire response rate (62.7%) was higher than what would be expected, compared with a general survey study; thus, the study has high internal validity. In addition, this prefecture was the region with the most rapid increase in the elderly population rate (*20*), and with the lowest number of hospital beds in Japan (*24*). Therefore, the role of nursing homes in the region is important for EOL care in frail elderly people. Furthermore, the results can help provide comprehensive EOL care in regions with rapidly aging populations.

In the present study, only the presence of an adjacent affiliated hospital was consistent with the findings of previous studies (*15,16,25,26*). These studies showed that facility determinants of dying in nursing homes were the existence of a basic policy for EOL care, a physician based in a home care supporting clinic, location in a rural region, being adjacent to an affiliated hospital, being government-owned, location in regions with more nursing home beds, and location in regions with fewer hospital beds. However, EOL care in nursing homes was not associated with other factors in multi-levels analysis (Table 2) or in crude models (data not shown). Our study analyzed facility factors associated with the number of EOL care residents in their facilities. Thus, the study design did not assess resident factors known to be associated with EOL care in nursing homes, including having an end-stage disease, not having pneumonia as the cause of death, the process of decision-making in their EOL (defined as "advance care planning") among residents or the family, ethnicity, race, lower activities of daily living, lower cognitive status, older age, and full-time physician support (*15,24,27-30*). To the best of our knowledge, there have been no original articles about the EOL care bonus by a national government. Therefore, this study was the first multi-level analysis using a linear mixed model about the effects of an EOL care bonus by a national government.

4.3. *Possible reasons related to EOL care in nursing homes by the EOL care bonus*

According to us, the reason for increasing numbers of residents receiving EOL care in nursing homes was reflected by the two roles of the EOL care bonus: financial incentive and framework for quality preservation. The financial incentive by government might be attractive for nursing home managers. We speculated that assuring the framework for quality preservation of EOL care due to eligibility criteria of the bonus might make the implementation of EOL care in nursing homes easier, which could not have been achieved before the availability of the EOL care bonus.

The eligibility criteria for the bonus include the process of decision-making during EOL, defined as advance care planning (ACP), as one of the factors preserving the quality of EOL care in nursing homes. Since most elderly people who require a discussion about their EOL care have insufficient ability to make decisions (*31*), ACP is important (*32-36*). In nursing home settings, it is also effective in fulfilling residents' wishes for their EOL care and avoiding undesired medical treatment (*11,37*); however, ACP had been not widespread among nursing homes in developed countries including Japan (*11,38,39*). Therefore, we speculate that assuring the framework for quality preservation, including ACP, by the bonus might contribute to increasing the number of residents receiving EOL care in nursing homes.

4.4. *Areas for future research*

Based on the present study, we propose three future research areas. First, it is valuable to clarify the two roles of the EOL care bonus, *i.e.*, financial incentive and framework for quality preservation including ACP, as having an effect on EOL care in nursing homes, as speculated above. Second, if the quality preservation by the bonus will contribute to increasing the number of residents receiving EOL care in nursing homes, a study is needed to assess the outcomes regarding satisfaction of the resident or their families with the EOL care bonus. Third, we propose other nationwide prospective studies demonstrate how an EOL care bonus contributes

to reducing undesired transfers to hospitals and a widespread framework of quality preservation for EOL care including ACP. EOL care bonuses provided by national governments might improve care and reduce unnecessary complications and expenditures on preventable hospitalizations of nursing home residents in other developed countries.

4.5. *Limitations of the study*

The present study has several limitations. First, due to the nature of a longitudinal study using a questionnaire, there was a considerable amount of missing data, especially in the fiscal years predating the earliest years. Second, nursing homes that were assertive about providing EOL care in their facilities might have been more likely to respond to our questionnaires, which may have resulted in socially desirable responses, meaning that there may have been a bias in which facilities consented to participate. Third, the present study was undertaken in one prefecture only, and there may have been a bias in the selection of facilities. Fourth, the results of the study cannot explain the causal relationships due to the retrospective design. Therefore, prospective studies in other developed countries are needed.

5. Conclusions

Our analysis revealed that EOL care bonus has the potential to increase the number of residents receiving EOL care in nursing homes over several years. Owing to the increasing number, the EOL care bonus might improve the quality of EOL care in nursing homes, due to eligibility criteria. As a result, an EOL care bonus might promote quantity and quality of EOL care in nursing homes in an aging society. In addition, EOL care conferences, full-time physician support for emergent care during off-time, and being adjacent to an affiliated hospital may also contribute to EOL care in nursing homes.

Acknowledgements

This work was supported in part by the Uehiro Foundation on Ethics and Education [grant number A-046, 2015] and also in part by the Kanagawa Public Health Association [2014] for the pilot survey. We thank the directors of the Health and Welfare Departments in Yokohama city, Kawasaki city, Yokosuka city, and Sagamihara city, Kanagawa prefecture, as well as all the participating nursing homes for their contribution.

References

1. Teno JM, Gruneir A, Schwartz Z, Nanda A, Wetle T. Change in end-of-life care for Medicare beneficiaries: Site of death, place of care, and health care transitions in 2000, 2005, and 2009. JAMA. 2013; 309:470-477.
2. Sanford A. Orrell M, Tolson D, *et al.* An international definition for "nursing home". J Am Med Dir Assoc. 2015; 16:181-184.
3. Onder G, Carpenter I, Finne-Soveri H, Gindin J, Frijters D, Henrard JC, Nikolaus T, Topinkova E, Tosato M, Liperoti R, Landi F, Bernabei R. Assessment of nursing home residents in Europe: The Services and Health for Elderly in Long TERm care (SHELTER) study. BMC Health Serv Res. 2012; 12:5.
4. Akabayashi A, Slingsby BT, Kai I. Perspectives on advance directives in Japanese society: A population-based questionnaire survey. BMC Med Ethics. 2003; 4:E5.
5. Miyata H, Shiraishi H, Kai I. Survey of the general public's attitudes toward advance directives in Japan: how to respect patients' preferences. BMC Med Ethics. 2006; 7:E11.
6. Ministry of Health, Labor and Welfare, Japan. Long-term care service facilities and office survey 2006. http://*www.dbtk.mhlw.go.jp/IPPAN/ippan/sk_o_DI* (accessed January 28, 2009).
7. Saliba D, Kington R, Buchanan J, Bell R, Wang M, Lee M, Herbst M, Lee D, Sur D, Rubenstein L. Appropriateness of the decision to transfer nursing facility residents to the hospital. J Am Geriatr Soc. 2000; 48:154-163.
8. Temkin-Greener H, Zheng NT, Xing J, Mukamel DB. Site of death among nursing home residents in the United States: Changing patterns, 2003-2007. J Am Med Dir Assoc. 2013; 14:741-748.
9. Long SO. Cultural scripts for a good death in Japan and the United States: Similarities and differences. Soc Sci Med. 2004; 58:913-928.
10. Hunt KJ, Shlomo N, Addington-Hall J. End-of-life care and achieving preferences for place of death in England: Results of a population-based survey using the VOICES-SF questionnaire. Palliat Med. 2014; 28:412-421.
11. Morin L, Johnell K, Van den Block L, Aubry R. Discussing end-of-life issues in nursing homes: A nationwide study in France. Age Ageing. 2016; 45:395-402.
12. Mitsubishi research institute. Study report on securing quality of facility service in special nursing home for the aged (2007). *http://www.mri.co.jp/project_related/hansen/uploadfiles/HLUkouseih18_2.pdf* (accessed August 28, 2016). (in Japanese)
13. Ministry of health, labor and welfare, Japan. social security commission on long-term care reimbursement No. 39 (*2006*). *http://www.mhlw.go.jp/shingi/2006/01/dl/s0126-9c2.pdf* (accessed August 28, 2016). (in Japanese)
14. Ministry of health, labor and welfare, Japan. Notification No. 126 (2006). *http://www.mhlw.go.jp/seisakunitsuite/bunya/hukushi_kaigo/seikatsuhogo/tannokyuuin/02_hourei_04.html* (accessed August 28, 2016). (in Japanese)
15. Ikegami N, Ikezaki S. Japan's policy of promoting end-of-life care in nursing homes: Impact on facility and resident characteristics associated with the site of death. Health Policy. 2012; 105:303-311.
16. Nakanishi M, Niimura J, Endo K, Nishida A. Regional Supply of Nursing Home and Hospital Beds Determine Discharge Destination of Nursing Home Residents in Japan. J Am Med Dir Assoc. 2016; 17:672.e1-5.
17. Takezako Y, Ishikawa S, Kajii E. Advance directives in Japanese nursing homes. J Pain Symptom Manage. 2013;

45:63-70 e7.
18. Ikegami N, Ikezaki S. Nursing homes and end-of-life care in Japan. J Am Med Dir Assoc. 2013; 14:718-723.
19. Cabinet office, Japan. Aged Society White Paper (2012). *http://www8.cao.go.jp/kourei/whitepaper/w-2013/zenbun/s1_1_2.html* (accessed August 28, 2016). (in Japanese)
20. Ministry of health, labour and welfare, Japan. The seventh publication of data showing the current situation in the elderly Japanese population (2009). *http://www8.cao.go.jp/kourei/whitepaper/w-2009/gaiyou/pdf/1s3s.pdf* (accessed August 28, 2016). (in Japanese)
21. Kanagawa prefecture, local government in Japan. *http://www.rakuraku.or.jp/kaigo/w20/wpJTop.aspx* (accessed August 28, 2016). (in Japanese)
22. Kanagawa prefecture, local government in Japan. Kanagawa Prefecture Age-specific demographic survey (2015). *http://www.pref.kanagawa.jp/cnt/f11000* (accessed August 28, 2016). (in Japanese)
23. Kanagawa prefecture, local government in Japan. Annual report on hygiene (2015). *http://www.pref.kanagawa.jp/cnt/f6872/p22052.html* (accessed August 28, 2016). (in Japanese)
24. Kanagawa prefecture, local government of Japan. Number of medical facilities (2014). *http://www.pref.kanagawa.jp/cnt/f450232/p613941.html* (accessed August 28, 2016). (in Japanese)
25. Levy CR, Fish R, Kramer AM. Site of death in the hospital versus nursing home of Medicare skilled nursing facility residents admitted under Medicare's Part A Benefit. J Am Geriatr Soc. 2004; 52:1247-1254.
26. Houttekier D, Cohen J, Surkyn J, Deliens L. Study of recent and future trends in place of death in Belgium using death certificate data: A shift from hospitals to care homes. BMC Public Health. 2011; 11:228.
27. Levy C, Hutt E, Pointer L. Site of death among veterans living in Veterans Affairs nursing homes. J Am Med Dir Assoc. 2012; 13:199-201.
28. Houttekier D, Cohen J, Bilsen J, Addington-Hall J, Onwuteaka-Philipsen B, Deliens L. Place of death in metropolitan regions: Metropolitan versus non-metropolitan variation in place of death in Belgium, The Netherlands and England. Health Place. 2010; 16:132-139.
29. Takezako Y, Tamiya N, Kajii E. The nursing home versus the hospital as the place of dying for nursing home residents in Japan. Health Policy. 2007; 81:280-288.
30. Motiwala S, Croxford R, Guerriere DN, Coyte PC. Predictors of place of death for seniors in Ontario: A population-based cohort analysis. Can J Aging. 2006; 25:363-371.
31. Bedell SE, Pelle D, Maher PL, Cleary PD. Do-not-resuscitate orders for critically ill patients in the hospital. How are they used and what is their impact? JAMA. 1986; 256:233-237.
32. Silveira MJ, Kim SY, Langa KM. Advance directives and outcomes of surrogate decision making before death. N Engl J Med. 2010; 362:1211-1218.
33. Robinson L, Dickinson C, Rousseau N, Beyer F, Clark A, Hughes J, Howel D, Exley C. A systematic review of the effectiveness of advance care planning interventions for people with cognitive impairment and dementia. Age Ageing. 2012; 41:263-269.
34. Detering KM, Hancock AD, Reade MC, Silvester W. The impact of advance care planning on end of life care in elderly patients: Randomised controlled trial. BMJ. 2010; 340:c1345.
35. Brinkman-Stoppelenburg A, Rietjens JA, van der Heide A. The effects of advance care planning on end-of-life care: A systematic review. Palliat Med. 2014; 28:1000-1025.
36. Weathers E, O'Caoimh R, Cornally N, Fitzgerald C, Kearns T, Coffey A, Daly E, O'Sullivan R, McGlade C, Molloy DW. Advance care planning: A systematic review of randomised controlled trials conducted with older adults. Maturitas. 2016; 91:101-109.
37. Martin RS, Hayes B, Gregorevic K, Lim WK. The effects of advance care planning interventions on nursing home residents: A systematic review. J Am Med Dir Assoc. 2016; 17:284-293.
38. Matsui M, Braun KL, Karel H. Comparison of end-of-life preferences between Japanese elders in the United States and Japan. J Transcult Nurs. 2008; 19:167-174.
39. Ni P, Zhou J, Wang ZX, Nie R, Phillips J, Mao J. Advance directive and end-of-life care preferences among nursing home residents in Wuhan, China: A cross-sectional study. J Am Med Dir Assoc. 2014; 15:751-756.

(Received October 5, 2016; Revised December 17, 2016; Accepted January 8, 2017)

Original Article

DOI: 10.5582/bst.2016.01195

Trends and characteristics of all-cause mortality among HIV-infected inpatients during the HAART era (2006-2015) in Shanghai, China

Yongjia Ji[1,§], Zhenyan Wang[1,§], Jiayin Shen[1,§], Jun Chen[1], Junyang Yang[1], Tangkai Qi[1], Wei Song[1], Yang Tang[1], Li Liu[1], Yinzhong Shen[1], Renfang Zhang[1], Hongzhou Lu[1,2,3,*]

[1] *Department of Infectious Diseases, Shanghai Public Health Clinical Center, Fudan University, Shanghai, China;*
[2] *Department of Infectious Diseases, Huashan Hospital Affiliated to Fudan University, Shanghai, China;*
[3] *Department of Internal Medicine, Shanghai Medical College, Fudan University, Shanghai, China.*

Summary **Globally, the overall mortality rate among HIV-infected patients has significantly declined during the HAART era. Deaths among HIV-infected inpatients need to be characterized in order to formulate intervention strategies to further improve medical care for this population and their prognosis. In the current study, deaths among HIV-infected inpatients from 2006 to 2015 at a medical center for HIV infection and AIDS patient care in Shanghai, China were retrospectively analyzed. Trends in mortality rates and the proportion of deaths caused by AIDS or non-AIDS-related illnesses were evaluated. A bivariate analysis was performed to identify the demographic and clinical factors associated with AIDS or non-AIDS-related deaths among HIV-infected inpatients. Among 6,473 HIV-infected patients who were discharged from 2006 to 2015, 326 deaths (5.04%) were identified. The yearly mortality rate declined significantly over time ($\chi^2 = 34.41$, $p < 0.001$). Results revealed that most deaths were attributed to AIDS-related illnesses (76.9 %, 233/303), and the proportion of causes of death did not change significantly over time ($\chi^2 = 13.847$, $p = 0.127$). Bivariate analysis identified characteristic factors associated with AIDS-related mortality. Compared to patients who died of non-AIDS illnesses, patients who died of AIDS-related illnesses had a CD4+ T cell count lower than 50 cells/μL (OR 4.587, 2.377-8.850) and fewer liver (OR 0.391, 0.177-0.866) or renal comorbidities (OR 0.188, 0.067-0.523) on admission. Results indicated that the overall in-hospital mortality rate among HIV-infected patients has declined over the past decade. However, AIDS-related illnesses were still the major causes of deaths among HIV-infected inpatients, suggesting that further efforts are needed to improve AIDS care in China.**

Keywords: Hospital mortality, HIV infection, AIDS, antiretroviral therapy

1. Introduction

Highly active antiretroviral therapy (HAART) has led to a dramatic decline in mortality among people living with human immunodeficiency virus (HIV) infection (PLWH) or acquired immune deficiency syndrome (AIDS) (*1*), with improved life expectancy approaching that of the general population (*2*). As a large multinational study from prior to 1995 to 2006 indicated, the excess mortality rate among HIV-infected individuals decreased from 40.8 to 6.1 per 1000 person-years (*3*).

In the current HAART era, AIDS is more like a chronic manageable disease rather than a rapidly progressing and fatal one (*2*). Therefore, the focus of HIV/AIDS care has been shifting from inpatient medical treatment to outpatient-based chronic disease management. However, the hospitalization rate and inpatient mortality rate remained higher in PLWH despite increasing accessibility to HAART in comparison to

Released online in J-STAGE as advance publication January 30, 2017.

[§]These authors contributed equally to this works.

**Address correspondence to:*
Dr. Hongzhou Lu, Department of Infectious Diseases, Shanghai Public Health Clinical Center, Fudan University, No. 2901 Caolang Road, Shanghai, China.
E-mail: luhongzhou@fudan.edu.cn

either the general population or HIV-negative individuals (*4-6*).

Deaths among HIV-infected inpatients during the HAART era need to be characterized in order to formulate intervention strategies to improve medical care and further reduce mortality in this population. Recent studies examining deaths of HIV-infected inpatients in the United States revealed that the in-hospital mortality rate declined significantly and that major causes of deaths had shifted to non-AIDS-related diseases (*4,7*). In settings with limited medical resources such as sub-Saharan Africa, the in-hospital mortality rate among HIV-infected adults remained high and AIDS-related illnesses still caused most deaths (*8*). To the extent known, the mortality trends or the causes of death among Chinese HIV-infected inpatients have seldom been reported during the HAART era. The current study retrospectively reviewed the outcomes, calculated the mortality, and analyzed the causes of death in HIV-infected inpatients at a medical center for HIV infection and AIDS in Shanghai over the past decade (2006-2015).

2. Materials and Methods

2.1. *Subjects*

This study was conducted at the Shanghai Clinical Center for Public Health (SCCPH), a tertiary care hospital affiliated with Fudan University. The SCCPH is the only designated medical institution that has provided outpatient and inpatient care for PLWH in Shanghai since the 1990s. Currently, about 6,000 HIV-infected patients in Shanghai are being regularly followed by the SCCPH. In 2015, more than 1,300 HIV-infected patients were admitted for AIDS or non-AIDS-related illnesses. All deaths of HIV-infected inpatients at the SCCPH from January 1, 2006 to December 31, 2015 were retrospectively reviewed. This study was approved by ethics committee of the SCCPH.

2.2. *Data collection*

A standardized data collection tool was used to abstract demographic characteristics (*i.e*. age, gender, duration of hospital stay), medical comorbidities besides AIDS-related diseases (cardio-cerebrovascular disease including hypertension, congestive heart failure, myocardial infarction, and stroke; diabetes mellitus; pulmonary disease including chronic obstructive pulmonary disease and asthma; liver disease including chronic hepatitis B or C and liver cirrhosis; kidney dysfunction defined by serum creatinine > 1.5 mg/dL; and anemia defined as hemoglobin < 100 g/L; thrombocytopenia defined as a blood platelet count < 100×10^9/L), a history of HAART administration, and the CD4+ T cell count on admission.

Two HIV-trained clinicians independently identified the causes of deaths based on clinical, laboratory, radiological, and microbiologic data through a comprehensive review of medical records. If there was disagreement over the cause of death, the final determination was made by consensus between the two clinicians. The causes of deaths were classified as AIDS-related or non-AIDS-related illnesses based on published definitions. Death caused by one of the Centers for Disease Control and Prevention (CDC) category C diseases was defined as AIDS-related death (*9,10*). Non-AIDS illnesses included non-AIDS infections, cardiovascular disease, liver disease, non-AIDS malignancy, renal disease, gastrointestinal hemorrhage, and trauma. Infectious etiologies not on the list of AIDS-defining conditions were classified as non-AIDS infection, e.g. sepsis and non-recurrent bacterial pneumonia (*4*). Cardiovascular disease was deemed to cause death if patients had one of the following clinical manifestations: cardiac arrest, ischemic or hemorrhagic stroke, congestive heart failure, myocardial infarction, or cardiac arrhythmia. Death from liver disease was deemed to occur when one of the following manifestations occurred with underlying liver disease: coagulopathy, bleeding esophageal varices, hepatic encephalopathy, hepatorenal syndrome, or spontaneous bacterial peritonitis. Death from renal disease was defined as death from primary renal failure. Deaths caused by gastrointestinal bleeding without underlying liver disease were attributed to gastrointestinal hemorrhage.

2.3. *Statistical analysis*

Statistical analysis was performed using SPSS version 19.0 for Windows. Overall or yearly mortality rates were calculated as the percentage of deaths overall or yearly among patients who were diagnosed with an HIV infection or AIDS and discharged (*4,7*). The chi-squared test was used to compare categorical variables, and group comparisons of quantitative variables were performed using a parametric (analysis of variance) or nonparametric technique (Wilcoxon test) in accordance with the distribution of the variable. Bivariate analysis was used to determine factors associated with AIDS versus non-AIDS-related deaths in the cohort.

3. Results

3.1. *Mortality among HIV-infected inpatients*

In total, there were 326 in-hospital deaths among 6,473 HIV-infected patients discharged from the SCCPH from 2006 to 2015 (Figure 1), with an overall mortality rate of 5.04% (326/6,473). Notably, the mortality rate among HIV-infected inpatients ($\chi^2 = 34.41$, $p < 0.001$) tended to decline during this period, in which the yearly inpatient mortality rates were 12.33% (9/73), 8.84% (16/181), 8.2% (25/305), 5.74% (24/418), 6.67% (37/555), 4.42% (31/701), 5.44% (46/845), 4.49%

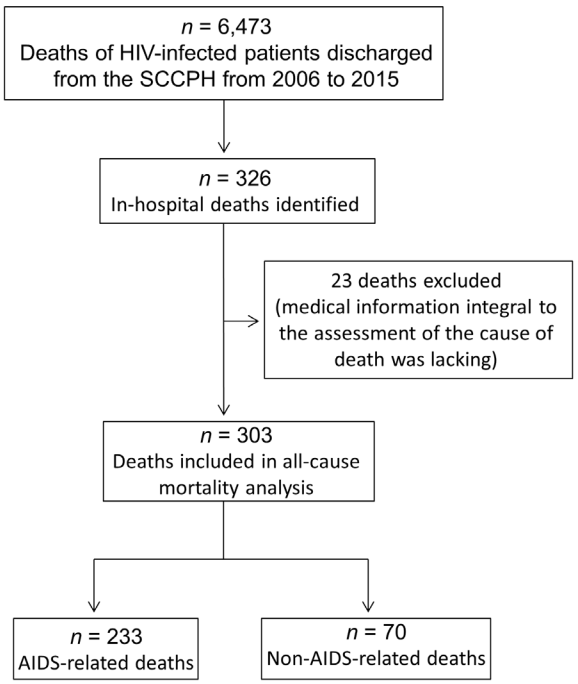

Figure 1. Flow for identifying the study population. Among 6,473 HIV-infected patients who were discharged from the SCCPH from 2006 to 2015, there were 326 deaths. In 23 deaths, medical information integral to the assessment of the cause of death was lacking, so those deaths were excluded. The remaining 303 deaths were included in an all-cause mortality analysis. Of those deaths, 233 were caused by AIDS-related illnesses (76.9%) while the remaining 70 were non-AIDS-related.

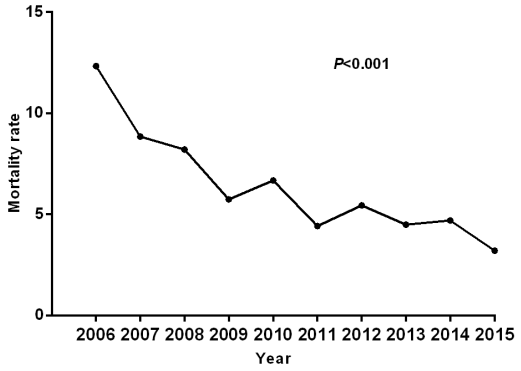

Figure 2. Yearly mortality rates among HIV-infected inpatients from 2006-2015. The yearly inpatient mortality rate declined from 12.33% in 2006 to 3.20% in 2015.

(42/935), 4.70% (54/1,148), and 3.20% (42/1,312), respectively (Figure 2).

3.2. *Analysis of causes of death*

Out of 326 deaths of HIV-infected patients, 23 lacked medical information integral to the assessment of the cause of death, so those deaths were excluded from further analysis (Figure 1). The remaining 303 deaths were included in the all-cause mortality analysis, and the demographic and clinical characteristics of these deaths are summarized in Table 1. The vast majority

Table 1. Demographics and clinical characteristics of HIV-infected inpatients who died from 2006 to 2015

Items	Total $n = 303$	2006 $n = 9$	2007 $n = 15$	2008 $n = 22$	2009 $n = 23$	2010 $n = 33$	2011 $n = 29$	2012 $n = 42$	2013 $n = 40$	2014 $n = 49$	2015 $n = 41$	p value
Age, Years, median (interquartile range)	48 (37-56)	44 (28-57)	45 (40-56)	49 (39-52)	50 (37-57)	47 (41-54)	49 (37-55)	51 (36-56)	52 (39-60)	50 (33-60)	44 (34-53)	0.372
Male gender N (%)	274 (90.4)	7 (77.8)	14 (93.3)	19 (86.4)	21 (91.3)	31 (93.9)	26 (89.7)	38 (90.5)	36 (90.0)	44 (89.8)	38 (92.7)	0.964
CD4+ T cell count/mm^3, median (interquartile range)	20 (7-66)	34 (16-91)	73 (16-298)	6 (3-63)	10 (5-24)	27 (3-52)	19 (10-43)	21 (9-59)	14 (7-106)	28 (9-67)	18 (7-56)	0.166
Hospital stay, Days, median (interquartile range)	15 (7-30)	21 (5-27)	23 (12-53)	12 (7-31)	17 (9-35)	22 (11-52)	16 (8-28)	14 (6-29)	21 (11-35)	13 (6-27)	9 (5-22)	0.257
On ART N (%)	62 (20.5)	0 (0)	5 (33.3)	2 (9.1)	4 (17.4)	9 (27.3)	6 (20.7)	11 (26.2)	8 (20.0)	9 (18.4)	8 (19.5)	0.724
Duration of ART, Months, median (interquartile range)	12 (4-18)	--	5 (5-17)	48 (12-84)	5 (3-9)	6 (2-18)	8 (1-15)	6 (2-12)	20 (12-42)	18 (12-60)	5 (4-11)	0.230
Comorbidities												
Cardia-cerebrovascular disease N (%)[a]	31 (10.2)	0 (0)	2 (13.3)	5 (22.7)	4 (17.4)	4 (12.1)	1 (3.4)	1 (2.4)	7 (17.5)	6 (12.2)	1 (2.4)	0.070
Pulmonary disease N (%)[b]	4 (1.3%)	0 (0)	0 (0)	0 (0)	1 (4.3)	0 (0)	1 (3.4)	0 (0)	0 (0)	2 (4.1)	0 (0)	0.514
Liver disease N (%)[c]	50 (16.5%)	5 (55.6)	5 (33.3)	3 (13.6)	6 (26.1)	7 (21.2)	2 (6.9)	7 (16.7)	5 (12.5)	3 (6.1)	7 (17.1)	0.011
Kidney dysfunction N (%)[d]	28 (9.2)	1 (11.1)	1 (6.67)	1 (4.5)	1 (4.3)	3 (9.1)	1 (3.4)	2 (4.8)	2 (5.0)	6 (12.2)	10 (24.4)	0.064
Diabetes mellitus N (%)	23 (7.6)	0 (0)	0 (0)	3 (13.6)	1 (4.3)	2 (6.1)	3 (10.3)	2 (4.8)	6 (15.0)	3 (6.1)	3 (7.3)	0.570
Anemia N (%)[e]	101 (33.3)	2 (22.2)	9 (60.0)	4 (18.2)	9 (39.1)	9 (27.3)	9 (31.0)	17 (40.5)	12 (30.0)	13 (26.5)	17 (41.5)	0.217
Thrombocytopenia N (%)[f]	72 (23.8)	4 (44.4)	4 (26.7)	3 (13.6)	8 (34.8)	6 (18.2)	5 (17.2)	10 (23.8)	8 (20.0)	11 (22.4)	13 (31.7)	0.532

[a]Cardia-cerebrovascular disease including hypertension, congestive heart failure, myocardial infarction, and stroke. [b]Pulmonary disease including chronic obstructive pulmonary disease and asthma. [c]Liver disease including chronic hepatitis B or C and liver cirrhosis. [d]Kidney dysfunction defined as serum creatinine > 1.5 mg/dL. [e]Anemia defined as hemoglobin < 100g/L. [f]Thrombocytopenia defined as thrombocytes < 100×10^9/L.

Table 2. Causes of death among HIV-infected inpatients from 2006 to 2015

Cause of death	Number of patients $n = 303$ (%)
AIDS-related death	233 (76.9)
Pneumocystis jirovecii pneumonia	80 (26.4)
NTM/MTB infection[a]	56 (18.5)
AIDS-related encephalopathy[b]	47 (15.5)
Recurrent bacterial pneumonia	31 (10.2)
AIDS-related malignancy[c]	18 (5.9)
Wasting syndrome	1 (0.4)
Non-AIDS-related death	70 (23.1)
Sepsis	18 (5.9)
Liver disease	11 (3.6)
Non-recurrent bacterial pneumonia	9 (3.0)
Gastrointestinal hemorrhage	8 (2.6)
Non-AIDS-related malignancy	7 (2.3)
Cardiovascular disease	6 (2.0)
Renal disease	5 (1.7)
Trauma	2 (0.7)
Other[d]	4 (1.3)

[a]Nontuberculous mycobacteria or mycobacterium tuberculosis infection. [b]AIDS-related encephalopathy including progressive multifocal leukoencephalopathy, cryptococcal meningitis, cerebral toxoplasmosis, and CMV encephalitis. [c]AIDS-related malignancy including Kaposi sarcoma and non-Hodgkin's lymphoma. [d]Included bacterial meningitis, acute myelitis, neurological disorders, and malaria.

of these patients were male (90.4%, 274/303), with a median age of 48 years (IQR 37-56 years). Of those patients, 62 (62/303, 20.5%) were taking HAART when admitted. The median CD4+ T cell count on admission was 20 cells/μL (IQR 7-66 cells/μL) and the duration of hospitalization was 15 days (IQR 7-30 days). Variables other than liver comorbidities on admission did not change significantly over the 10-year period.

All causes of the 303 deaths that were analyzed are summarized in Table 2. Major causes of death were AIDS-related illnesses (76.9%, 233/303), with Pneumocystis pneumonia (PCP) (26.4 %, 80/303) and recurrent bacterial pneumonia (10.2%, 31/303) as the two most common causes followed by nontuberculous mycobacteria (NTM) or Mycobacterium tuberculosis (MTB) infection (18.5%, 56/303), and AIDS-related encephalopathy (15.5%, 47/303) including progressive multifocal leukoencephalopathy, cryptococcal meningitis, cerebral toxoplasmosis, and CMV encephalitis. Other less frequent AIDS-related causes of death were AIDS-related malignancy (5.9%, 18/303) (*i.e.* Kaposi sarcoma and non-Hodgkin's lymphoma) and wasting syndrome due to AIDS (0.4%, 1/303). A point worth noting is that non-AIDS-related illnesses caused roughly 1/4 (23.1%, 70/303) of the inpatient deaths at the SCCPH. Sepsis (5.9%, 18/303) and liver diseases (3.6%, 11/303) were the two major non-AIDS-related causes of death. Other common causes were non-recurrent bacterial pneumonia (3%, 9/303), gastrointestinal hemorrhage (2.6%, 8/303), non-AIDS-related malignancy (2.3%, 7/303), cardiovascular disease (2%, 6/303), renal disease (1.7%, 5/303), and trauma (0.7%, 2/303). The remaining non-AIDS-related deaths was caused by other comorbidities

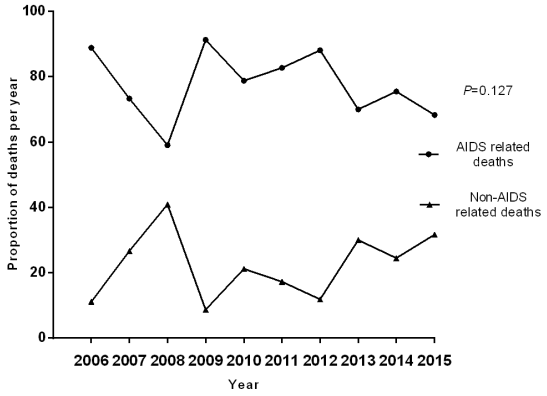

Figure 3. Trends in deaths caused by AIDS versus non–AIDS illnesses from 2006-2015. The proportion of AIDS-related and non-AIDS-related deaths did not differ significantly over time.

(1.3%, 4/303) such as bacterial meningitis, acute myelitis, a neurological disorder, and malaria. As shown in Figure 3, the proportion of AIDS-related or non-AIDS-related deaths did not change significantly over time ($\chi^2 = 13.847$, $p = 0.127$).

3.3. *Characteristic factors associated with AIDS-related or non-AIDS-related deaths*

Compared to patients who died from non-AIDS-related illness, patients who died from AIDS were younger (median age: 47 *vs*. 51 years, $p = 0.017$), they had a lower CD4+ T cell count (median 17 *vs*. 69 cells/μL, $p < 0.001$), and they were less likely to be receiving HAART (16.3% *vs*. 34.3%, $p = 0.001$) on admission. In contrast, comorbidities on admission including kidney dysfunction (27.1% *vs*. 3.9%, $p < 0.001$), cardia-cerebrovascular disease (21.4% *vs*. 6.9%, $p < 0.001$), diabetes mellitus (15.7% *vs*. 5.2%, $p = 0.003$), anemia (51.4% *vs*. 27.9%, $p < 0.001$), thrombocytopenia (35.7% *vs*. 20.2%, $p = 0.007$), and liver disease (24.3% *vs*. 14.2%, $p = 0.045$) were more common among non-AIDS-related deaths (Table 3).

The factors associated with the cause of death were further analyzed using multivariate logistic regression (Table 3). Factors including a CD4+ T cell count < 50 cells/μL (OR: 4.587; 95% CI: 2.387-8.815) and fewer comorbidities such as liver (OR: 0.402; 95% CI: 0.182-0.886) and kidney comorbidities (OR: 0.192; 95% CI: 0.069-0.535) on admission were independently associated with AIDS-related deaths.

4. Discussion

A comprehensive awareness of the deaths among HIV-infected inpatients is believed to be essential to improving the outcomes and quality of medical care provided by medical personnel. However, a nationwide study of the details of deaths among HIV-infected inpatients in China still needs to be conducted.

Table 3. Univariate and multivariate analysis of clinical characteristics and comorbidities associated with AIDS-related deaths

Items	AIDS-related deaths, *n* = 233	Non-AIDS-related deaths, *n* = 70	Univariate analysis[a]		Multivariate analysis[b]	
			p value	OR (95% CI)	*p* value	OR (95% CI)
Clinical characteristics						
Male gender	211 (90.6)	63 (90.0)	0.889	1.006 (0.921-1.099)	0.777	0.853 (0.284-2.5621)
Age > 60 y	36 (15.5)	19 (27.1)	0.026	0.491 (0.260-0.926)	0.385	1.407 (0.652-3.036)
CD4+ T cell < 50 cells/mm^3	183 (78.5)	26 (37.1)	< 0.001	2.115 (1.548-2.829)	< 0.001	4.587 (2.377-8.850)
On HAART	38 (16.3)	24 (34.3)	0.001	0.476 (0.308-0.735)	0.475	0.766 (0.370-1.589)
Comorbidities						
Cardia-cerebrovascular disease	16 (6.9)	15 (21.4)	< 0.001	0.320 (0.167-0.615)	0.310	0.600 (0.224-1.606)
Pulmonary disease	2 (0.9)	2 (2.9)	0.199	0.300 (0.043-2.094)	0.280	0.280 (0.028-2.820)
Liver disease	33 (14.2)	17 (24.3)	0.045	0.583 (0.347-0.982)	0.021	0.391 (0.177-0.866)
Kidney dysfunction	9 (3.9)	19 (27.1)	< 0.001	0.142 (0.067-0.300)	0.001	0.188 (0.067-0.523)
Diabetes mellitus	12 (5.2)	11 (15.7)	0.003	0.328 (0.151-0.710)	0.158	0.445 (0.145-1.368)
Anemia	65 (27.9)	36 (51.4)	< 0.001	0.542 (0.399-1.147)	0.119	0.579 (0.291-1.151)
Thrombocytopenia	47 (20.2)	25 (35.7)	0.007	0.565 (0.377-0.847)	0.389	0.720 (0.341-1.520)

[a] χ^2 test. [b]Multivariate logistic regression.

The current study investigated the mortality rate, the underlying causes, and the possible factors correlated with different causes of death among HIV-infected inpatients at the SCCPH. Since the SCCPH is the most influential medical center for HIV/AIDS patient care in Eastern China, the findings of this study may partially depict the outcomes for HIV-infected patients admitted for inpatient care during the HAART era in China, and especially those with access to vast medical resources.

Results revealed an overall mortality rate of 5.04% for HIV-infected inpatients at the SCCPH from 2006 to 2015. This rate was higher than that reported in Western countries during the HAART era (2-3%) (*4,5*), but it was significantly lower than that reported in sub-Saharan Africa (*8*). Therefore, this difference presumably reflects an association between socio-economic status and the outcomes of HIV infection to some extent.

Importantly, a marked decline in the yearly mortality rate (from 12.33% in 2006 to 3.2% in 2015) among HIV-infected inpatients was noted in the current study, and a similar trend has been noted in the West. There are several potential explanations for this trend. First and foremost, increased accessibility to HAART due to promotion of the National Free Antiretroviral Therapy Program (NFATP) since 2004 has been crucial to improving outcomes for HIV-infected inpatients (*11,12*). Second, the efforts of both government and social groups have raised public awareness of HIV infection over the last decade (*13*) and the coverage of HIV screening and surveillance has expanded, especially among high-risk populations. One recent study found that testing for HIV increased more than two-fold among men who have sex with men (MSM) from 2008 to 2012 in Nanjing, the capital of Jiangsu Province in Eastern China, while testing programs for MSM were promoted (*14*). Moreover, the SCCPH is the designated medical facility providing inpatient care for HIV-infected patients in Shanghai and Eastern China, so medical personnel at the SCCPH have gained experience managing HIV infection and AIDS-related illnesses. This may explain why the survival of HIV-infected inpatients has improved during the HAART era, as a study previously reported (*15*).

The current results revealed that the deaths of HIV-infected patients admitted to the SCCPH were mainly caused by AIDS-related illnesses. Results also revealed no significant change in the proportion of causes of death due to AIDS or non-AIDS-related illnesses over the last decade. Similar results were reported in another study investigating outcomes for HIV-infected patients after discharge from a hospital in Beijing, the capital of China (*16*). However, the major causes of death among HIV-infected inpatients in Western countries have shifted to non-AIDS-related illnesses during the HAART era, and the proportion of non-AIDS-related deaths has increased over time (*4,7*). These differences might be correlated with the current status of the HIV epidemic in China. A cross-sectional study conducted in 10 provinces of mainland China from 2009 to 2010 revealed that more than 70% of newly diagnosed cases of HIV infection were categorized as late presentation (defined as a CD4 count ≤ 200 cells/μL at HIV diagnosis), and the study found that late presentation was associated with increased AIDS-related morbidity and mortality (*17*). Similarly, another recent study also reported that most Chinese HIV-infected patients (72.6%) had a concurrent AIDS diagnosis at the time of HIV diagnosis or developed AIDS within 1 year after HIV diagnosis (*18*). In contrast, studies conducted in the West reported that no more than 30% of HIV-infected individuals presented with CD4+ T cells < 200/μL (*19*). The current study also found that most deceased HIV-infected inpatients, and especially those who died of AIDS-related illnesses, had a low CD4+ T cell count (< 50 cell/μL) and were not receiving HAART when admitted. These factors are the two biggest risk factors for developing AIDS-related illnesses such as opportunistic infections and malignancies (*20,21*). Despite the increasing

accessibility to HAART and increased public awareness of HIV infection, further efforts are needed to improve AIDS care and management in China.

Similar to findings from recent studies (*4,7*), the current results revealed that a lower CD4+ T cell count (< 50/μL) was a characteristic of mortality due to AIDS-related illness, and liver or kidney comorbidities on admission were more common in patients who died of non-AIDS-related illnesses. Consequently, the current study has highlighted the need for physicians to pay closer attention to these clinical features and the need to optimize decision-making accordingly.

Admittedly, this study had several limitations. First, it was an observational and retrospective study, so the possibility of bias in assigning causes of death by reviewing medical records cannot be ruled out. Second, this study did not include a proper control group such as HIV-infected inpatients who survived or HIV-negative inpatients who died in hospital, which would otherwise substantiate the current conclusions. The findings of this study may not fully depict the overall outcomes for HIV-infected inpatients in China, and especially those in regions with different socioeconomic levels and medical resources. Further prospective studies involving HIV-infected inpatients at multiple centers need to be conducted in order to better ascertain the outcomes for and characteristics of these patients in China during the HAART era.

5. Conclusion

Although the yearly overall mortality rate among HIV-infected inpatients tended to decline, this study found that the major causes of deaths were attributed to AIDS-related illnesses over the past decade. In light of those circumstances, further efforts are needed to improve HIV surveillance and AIDS care in China.

Acknowledgements

The authors wish to thank the staff of the Infectious Diseases Department at the SCCPH for their dedication and hard work. This study was supported by the National Natural Science Foundation of China (NSFC No. 81571977 and No. 31500697), the Shanghai Pujiang Program (No. 15PJ1407300), and the Program to Support Medical Science of the Shanghai Science and Technology Committee (No. 16411960400). The authors have no conflicts of interest to disclose.

References

1. Weber R, Ruppik M, Rickenbach M, Spoerri A, Furrer H, Battegay M, Cavassini M, Calmy A, Bernasconi E, Schmid P, Flepp M, Kowalska J, Ledergerber B, Swiss HIVCS. Decreasing mortality and changing patterns of causes of death in the Swiss HIV Cohort Study. HIV Medicine. 2013; 14:195-207.
2. Nakagawa F, Lodwick RK, Smith CJ, Smith R, Cambiano V, Lundgren JD, Delpech V, Phillips AN. Projected life expectancy of people with HIV according to timing of diagnosis. Aids. 2012; 26:335-343.
3. Bhaskaran K, Hamouda O, Sannes M, Boufassa F, Johnson AM, Lambert PC, Porter K, Collaboration C. Changes in the risk of death after HIV seroconversion compared with mortality in the general population. JAMA. 2008; 300:51-59.
4. Kim JH, Psevdos G, Jr., Gonzalez E, Singh S, Kilayko MC, Sharp V. All-cause mortality in hospitalized HIV-infected patients at an acute tertiary care hospital with a comprehensive outpatient HIV care program in New York City in the era of highly active antiretroviral therapy (HAART). Infection. 2013; 41:545-551.
5. Crum-Cianflone NF, Grandits G, Echols S, Ganesan A, Landrum M, Weintrob A, Barthel R, Agan B, Infectious Disease Clinical Research P. Trends and causes of hospitalizations among HIV-infected persons during the late HAART era: What is the impact of CD4 counts and HAART use? J Acquir Immune Defic Syndr. 2010; 54:248-257.
6. Buchacz K, Baker RK, Moorman AC, Richardson JT, Wood KC, Holmberg SD, Brooks JT, Investigators HIVOS. Rates of hospitalizations and associated diagnoses in a large multisite cohort of HIV patients in the United States, 1994-2005. Aids. 2008; 22:1345-1354.
7. Cowell A, Shenoi SV, Kyriakides TC, Friedland G, Barakat LA. Trends in hospital deaths among human immunodeficiency virus-infected patients during the antiretroviral therapy era, 1995 to 2011. J Hosp Med. 2015; 10:608-614.
8. Wajanga BM, Webster LE, Peck RN, Downs JA, Mate K, Smart LR, Fitzgerald DW. Inpatient mortality of HIV-infected adults in sub-Saharan Africa and possible interventions: A mixed methods review. BMC Health Serv Res. 2014; 14:627.
9. Buehler M, Berkelman MRL. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR Recomm Rep. 1990.
10. Kowalska JD, Mocroft A, Ledergerber B, Florence E, Ristola M, Begovac J, Sambatakou H, Pedersen C, Lundgren JD, Kirk O, EuroSIDA Study G. A standardized algorithm for determining the underlying cause of death in HIV infection as AIDS or non-AIDS related: Results from the EuroSIDA study. HIV Clin Trials. 2011; 12:109-117.
11. Zhu H, Napravnik S, Eron JJ, Cole SR, Ma Y, Wohl DA, Dou Z, Zhang Y, Liu Z, Zhao D, Yu L, Liu X, Cohen MS, Zhang F. Decreasing excess mortality of HIV-infected patients initiating antiretroviral therapy: Comparison with mortality in general population in China, 2003-2009. J Acquir Immune Defic Syndr. 2013; 63:e150-157.
12. Zhang F, Dou Z, Yu L, Xu J, Jiao JH, Wang N, Ma Y, Zhao Y, Zhao H, Chen RY. The effect of highly active antiretroviral therapy on mortality among HIV-infected former plasma donors in China. Clin Infect Dis. 2008; 47:825-833.
13. Shang H, Xu J, Han X, Spero Li J, Arledge KC, Zhang L. HIV prevention: Bring safe sex to China. Nature. 2012; 485:576-577.
14. Yan H, Li J, Raymond HF, Huan X, Guan W, Hu H, Yang H, McFarland W, Wei C. Increased HIV testing among men who have sex with men from 2008 to 2012, Nanjing,

China. PloS one. 2016; 11:e0154466.
15. Kitahata MM, Koepsell TD, Deyo RA, Maxwell CL, Dodge WT, Wagner EH. Physicians' experience with the acquired immunodeficiency syndrome as a factor in patients' survival. N Engl J Med. 1996; 334:701-706.
16. Xiao J, Du S, Tian Y, Su W, Yang D, Zhao H. Causes of death among patients infected with HIV at a tertiary care hospital in China: An observational cohort study. AIDS Res Hum Retroviruses. 2016.
17. Shen Y, Lu H, Wang Z, Qi T, Wang J. Analysis of the immunologic status of a newly diagnosed HIV positive population in China. BMC Infect Dis. 2013; 13:429.
18. Dai SY, Liu JJ, Fan YG, Shan GS, Zhang HB, Li MQ, Ye DQ. Prevalence and factors associated with late HIV diagnosis. J Med Virol. 2015; 87:970-977.
19. Hall HI, Halverson J, Wilson DP, Suligoi B, Diez M, Le Vu S, Tang T, McDonald A, Camoni L, Semaille C, Archibald C. Late diagnosis and entry to care after diagnosis of human immunodeficiency virus infection: A country comparison. PloS one. 2013; 8:e77763.
20. Crowe SM, Carlin JB, Stewart KI, Lucas CR, Hoy JF. Predictive value of CD4 lymphocyte numbers for the development of opportunistic infections and malignancies in HIV-infected persons. J Acquir Immune Defic Syndr. 1991; 4:770-776.
21. Low A, Gavriilidis G, Larke N, MR BL, Drouin O, Stover J, Muhe L, Easterbrook P. Incidence of opportunistic infections and the impact of antiretroviral therapy among HIV-infected adults in low- and middle-income countries: A systematic review and meta-analysis. Clin Infect Dis. 2016; 62:1595-603.

(Received October 26, 2016; Revised December 20, 2016; Accepted January 11, 2017)

Original Article

DOI: 10.5582/bst.2016.01171

The immune dysfunction in ankylosing spondylitis patients

Zhongliang Duan[1,2,§], Yuyan Gui[1,2,3,§], Cui Li[1,2], Jing Lin[1,2], Hans-Jürgen Gober[4], Juanxiu Qin[5], Dajin Li[1,2,3], Ling Wang[1,2,3,6,*]

[1] *Obstetrics and Gynecology Hospital, Fudan University, Shanghai, China;*
[2] *The Academy of Integrative Medicine of Fudan University, Shanghai, China;*
[3] *Laboratory for Reproductive Immunology, Hospital & Institute of Obstetrics and Gynecology, IBS, Fudan University Shanghai Medical College, Shanghai, China;*
[4] *Department of Pharmacy, Kepler University Clinic, NeuromedCampus, Linz, Austria;*
[5] *Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China;*
[6] *Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai, China.*

Summary **Ankylosing spondylitis (AS) is a spinal arthritic disease that is often associated with human leukocyte antigen (HLA)-B27, while only part of HLA-B27 carriers become AS patients. T cells have been reported to play an important role in the pathology of AS. T-cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3) and programmed death-1 (PD-1) have been known to negatively regulate the immune response. In this study, we used flow cytometry to analyze the immunological differences of peripheral blood from 21 patients with AS, 22 cases who didn't have AS but were found to be HLA-B27 positive (HLA-B27+ group), and 16 normal healthy individuals (Healthy group). The level of $CD4^+$, $CD8^+$ T cells, and Treg of each group was observed. The expression of Tim-3 and PD-1 and the production of IFN-γ, IL-6, TNF-α, IL-4, and IL-10 were examined as well. We found that the percentage of Treg in AS group was lower than that of healthy group. The expression of PD-1 on $CD8^+$ T cells and Tim-3 on $CD4^+$ T cells was lower in the AS group. AS group had lower IL-10 production by $CD4^+$ T cells and higher IL-6 production by $CD8^+$ T cells. The results of HLA-B27+ group were similar to that of the healthy group. These data suggested that patients with AS had an impairment in the ability to negatively regulate the immune response, which might be related to the etiology of AS. To further investigate the roles of Tim-3 and PD-1 on $CD4^+$ T cells and $CD8^+$ T cells, we tested IFN-γ and IL-10 production by Tim-3^+, Tim-3^-, PD-1^+, and PD-1^- $CD4^+$ and $CD8^+$ T cells from AS patients. We found that Tim-3^+CD4^+, Tim-3^+CD8^+ and PD-1^+CD8^+ T cells produced more IL-10 than other subsets. In conclusion, there is a dysfunction of T cells in AS that is associated with PD-1 and Tim-3.**

Keywords: PD-1, Tim-3, IL-6, IL-10, $CD4^+$ T cell, $CD8^+$ T cell, Ankylosing spondylitis

1. Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease whose etiology remains unclear (*1*). The treatment takes a long time and requires control of the associated inflammation. It often affects sacroiliac joints, the spine and peripheral joints (*2*).

Recent studies perceive AS as an autoimmune disorder that is associated with human leukocyte antigen (HLA)-B27 (*3*) and T cells (*4*). T cells appear to take part in maintaining immune tolerance in humans, whose dysfunction leads to diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), recurrence spontaneous abortion (RSA) and some inflammatory bowel diseases (*5-8*). The alternations of T cells found in AS patients include increased frequency of peripheral Th2 lymphocytes (*9*) and Th17 lymphocytes (*10-11*). $CD4^+$ T cells may play an important role in the pathology of AS, so as $CD8^+$ T cells (*12*). Moreover, it is noteworthy

Released online in J-STAGE as advance publication February 7, 2017.

[§]These authors contributed equally to this works.

**Address correspondence to:*
Dr. Ling Wang, Obstetrics & Gynecology Hospital, Fudan University, 413 Zhaozhou Road, Shanghai 200011, China.
E-mail: Dr.wangling@fudan.edu.cn

that spondyloarthropathies can be recognized by the presence of $CD4^+$ T cells and HLA-B27 reactive $CD4^+$ T cells may be related to these disorders (*13*). Of interest, only part of the HLA-B27 positive carriers become AS patients.

In addition to T cells, the inhibitory co-stimulatory molecules PD-1 and Tim-3 have also been a hot topic in AS research. Programmed death-1 (PD-1), first reported in 1992, belongs to immunoglobulin (Ig) superfamily (*14*) and can be found on the surface of peripheral T cells, B cells, NK cells, dendritic cells, and monocytes (*15*). PD-1 maintains immune tolerance and protects tissues from autoimmune attack by acting with its ligand (PD-L) (*16*). T-cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3), often expressed in Th1, Th17, and $CD8^+$ T cells (*17*), has been known to downregulate immune responses and control inflammation (*18*). PD-1 and Tim-3 are often found to have unusual expression in chronic infections and autoimmune diseases, including HIV infection (*19*), tuberculosis (*19*), sepsis (*20*), and feto-maternal immune regulation (*21*).

In the course of immunity and defense, the delicate balance between IFN-γ and IL-10 is crucial. IFN-γ is an activator of T cells, monocytes, and neutrophils, which drives the clearance of pathogens, while IL-10 acts to hinder this process (*22-24*).

To explore the etiology and pathology of ankylosing spondylitis, we designed this experiment to make a further step in understanding the influence of immune tolerance in AS. AS patients, HLA-B27 positive and HLA-B27 negative normal healthy subjects were recruited. The level of $CD4^+$, $CD8^+$ T cells, and Treg of each group was observed by flow cytometry. The expression of Tim-3 and PD-1 was examined and the production of IFN-γ, IL-6, TNF-α, IL-4, and IL-10 was tested as well. To further investigate the roles of Tim-3 and PD-1 on $CD4^+$ T cells and $CD8^+$ T cells, we tested IFN-γ and IL-10 production by $Tim\text{-}3^+$, $Tim\text{-}3^-$, $PD\text{-}1^+$, and $PD\text{-}1^-$ $CD4^+$ and $CD8^+$ T cells from AS patients.

Our study aimed at investigating the frequency change of T cells in AS patients and determining the expression and production of these inhibitory co-stimulatory molecules and regulatory molecules in the development of AS. Most importantly, it was of clinical significance to make a comparison of AS patients, HLA-B27 positive, and HLA-B27 negative normal healthy subjects, which offered a new perspective to have an overall examination of the screening index HLA-B27.

2. Materials and Methods

2.1. *Reagents*

Flow cytometry antibodies CD4-FITC, IL-6-PE, CD8-FITC, TNF-α-PE/cy7, IL-10-BV421, Tim-3-PE, IL-4-APC, PD-1-APC, CD25-PE, CD127-APC, IFN-γ-PE/cy7, as well as Fix/Perm Buffer, were purchased from Biolegend (San Diego, CA, USA).

2.2. *Patients and controls*

Table 1 showed the information of all the studied subjects. A total of 21 AS cases (14 men and 7 women) were included in this study, along with 22 cases (14 men and 8 women) who didn't have AS but were found to be HLA-B27 positive (HLA-B27+) in a conventional medical examination, and 16 healthy controls (11 men and 5 women) who were selected from Ren Ji Hospital of Shanghai Jiao Tong University. The AS patients, aged 16-63 years, were diagnosed by the modified New York Criteria (*25*). Disease activity was evaluated using the Bath AS Disease Activity Index (BASDAI) (*26*), with the 21 patients' BASDAI Score > 4. There was no significant difference in percentage of gender and age among three groups.

2.3. *Flow cytometry (FCM)*

Peripheral blood (PB) was harvested in the presence of EDTA-K_2. One aliquot of PB was incubated with the monoclonal antibody CD4-FITC for 30 minutes, after which red blood cell lysis buffer was added. The sample was washed with PBS, then placed in Fix/Perm Buffer for 40 minutes to lyse the white blood cells, and then washed again with PBS. The antibodies IL-4-APC, IL-6-PE, and TNF-α-PE/cy7 were added and incubated for 30 minutes, and the sample was then washed with PBS.

Table 1. Clinical characteristics of AS, HLA-B27+, and Healthy groups

Items	AS (n = 21)	HLA-B27+ (n = 22)	Healthy (n = 16)
Age			
Range (years)	16-63	17-64	20-62
Mean ± SD	37.0 ± 9.8	38.3 ± 14.0	34.6 ± 10.1
Gender, n (%) male	14 (66.67)	14 (63.64)	11 (68.75)
Clinical features			
HLA-B27 positive	all	all	none
BASDAI	> 4	NA	NA

NA: not available.

A second aliquot of PB was incubated with monoclonal antibodies against CD4, PD-1, and Tim-3 for 30 minutes, after which red blood cell lysis buffer was added. The sample was washed with PBS, then placed in Fix/Perm Buffer for 40 minutes to lyse the white blood cells and washed again with PBS. Antibodies against IFN-γ and IL-10 were added and incubated for 30 minutes, and the sample was then washed with PBS.

Another aliquot of PB was treated similarly as above, except CD8 antibody used instead of CD4 antibody, to test IL-4, IL-6, TNF-α, PD-1, and Tim-3 on it.

Last aliquot of PB was stained by CD4-FITC, CD25-PE, and CD127-APC to test Treg.

Approximately 100,000 stained cells in each sample were analyzed by a CyAN ADP flow cytometer (Beckman Coulter, Brea, CA, USA).

2.4. *Statistical analysis*

All values were expressed as the mean ± SD. Data was analyzed with SPSS ver.19 and Prism 5. Demographic data among the groups was compared by the Chi-square test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. *AS patients showed lower Tim-3 on CD4$^+$ T cells*

The percentage of CD4$^+$ T cells was of no significant difference among three groups (Figure 1B). Compared with the healthy group (1.39 ± 0.69%), the AS group (0.94 ± 0.47%) showed notably lower ($p < 0.05$) Tim-3$^+$/CD4$^+$ (Figure 2A). However, the percentage of PD-1$^+$/CD4$^+$ was not significantly different among three groups (Healthy, 32.67 ± 6.27%; HLA-B27+, 37.75 ± 19.25% *vs*. AS, 32.78 ± 12.18%) (Figure 2B). These data suggested the decline of Tim-3 expression on CD4$^+$ T cells in AS patients while CD4$^+$ T cells and PD-1 remained unchanged.

3.2. *AS patients showed lower PD-1 on CD8$^+$ T cells, while Tim-3 is unchanged*

The percentage of CD8$^+$ T cells in the three groups was also analyzed by flow cytometry, and no significant difference was found among these groups (Figure 1D). We found obviously lower expression of PD-1$^+$/CD8$^+$ in the AS group (22.04 ± 8.25%) than the healthy group (35.92 ± 6.21%) and the HLA-B27+ group (31.93 ± 13.16%) (Figure 2D). However, the percentage of Tim-3$^+$/CD8$^+$ was not significantly different (10.91 ± 3.74, 10.27 ± 4.85 *vs*. 11.48 ± 5.01) (Figure 2C). The results suggested the downregulation of PD-1 expression on CD8$^+$ T cells in AS patients while CD8$^+$ T cells and Tim-3 remained unchanged.

3.3. *Expression of IL-10 in CD4$^+$ T cells was lower in AS patients*

To further explore the downstream changes in AS develoment, we examined IFN-γ, IL-6, TNF-α, IL-4, and IL-10 expression to analyze the intracellular events. We found that there was no significant

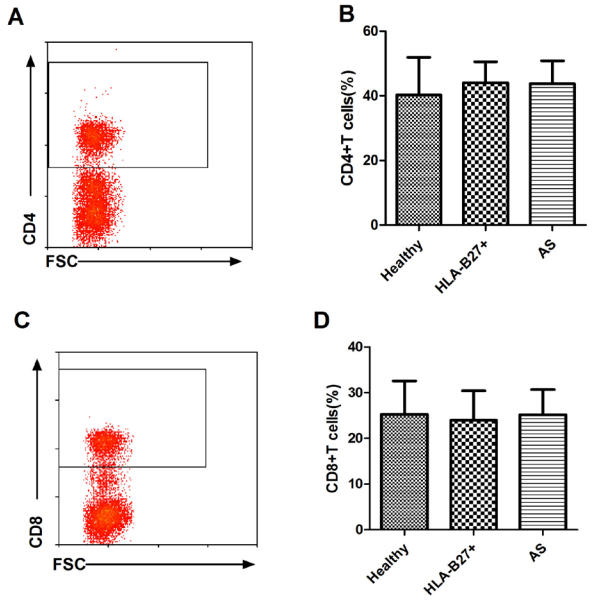

Figure 1. The percentage of CD4$^+$ and CD8$^+$ T cells in different groups. Samples were analyzed by flow cytometry with antibodies against CD4 and CD8 marking the T cell surface and with gating for CD4- and CD8-positive lymphocytes. Data was presented as the mean ± SD. The analysis compared the AS group ($n = 21$) with the healthy group ($n = 16$) and the HLA-B27+ group ($n = 22$).

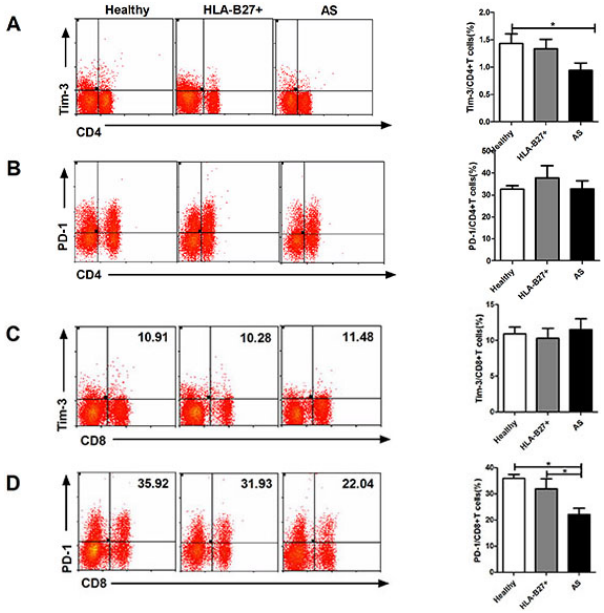

Figure 2. The expression of Tim-3 on CD4$^+$ and PD-1 on CD8$^+$ T cells in AS patients. Blood samples from the AS group ($n = 21$), the healthy group ($n = 16$) and the HLA-B27+ group ($n = 22$) were analyzed by flow cytometry. **(A, B)** CD4$^+$ T cells positive for Tim-3 and PD-1, analyzed by flow cytometry. **(C, D)** CD8$^+$ T cells positive for Tim-3 and PD-1, analyzed by flow cytometry. Data was presented as the mean ± SD. $^*p < 0.05$.

difference in the expression of IFN-γ/CD4$^+$ among three groups (healthy, 86.56 ± 13.12%; HLA-B27+, 91.53 ± 6.50%; and AS, 85.55 ± 8.69%). Similar results were obtained for IL-6, TNF-α, and IL-4 in CD4$^+$ T cells. However, IL-10/CD4$^+$ showed a decrease in the AS group. The results in Healthy, HLA-B27+, and AS group were 1.92 ± 0.94%, 1.79 ± 0.87%, and 0.91 ± 0.51%, respectively (Figure 3).

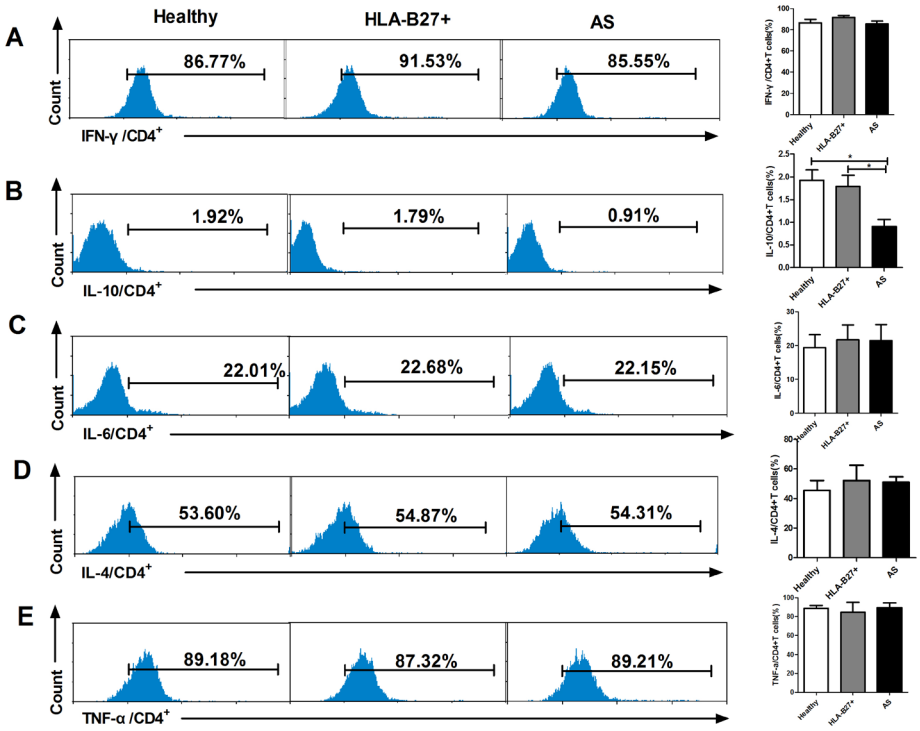

Figure 3. The production of cytokines in CD4$^+$ T cells in different groups. CD4$^+$ T cells were analyzed by flow cytometry for the production of IFN-γ, IL-6, TNF-α, IL-4, and IL-10. Data was analyzed using Prism 5 **(B)**.*$p < 0.05$, AS ($n = 21$), Healthy ($n = 16$) and HLA-B27+ ($n = 22$).

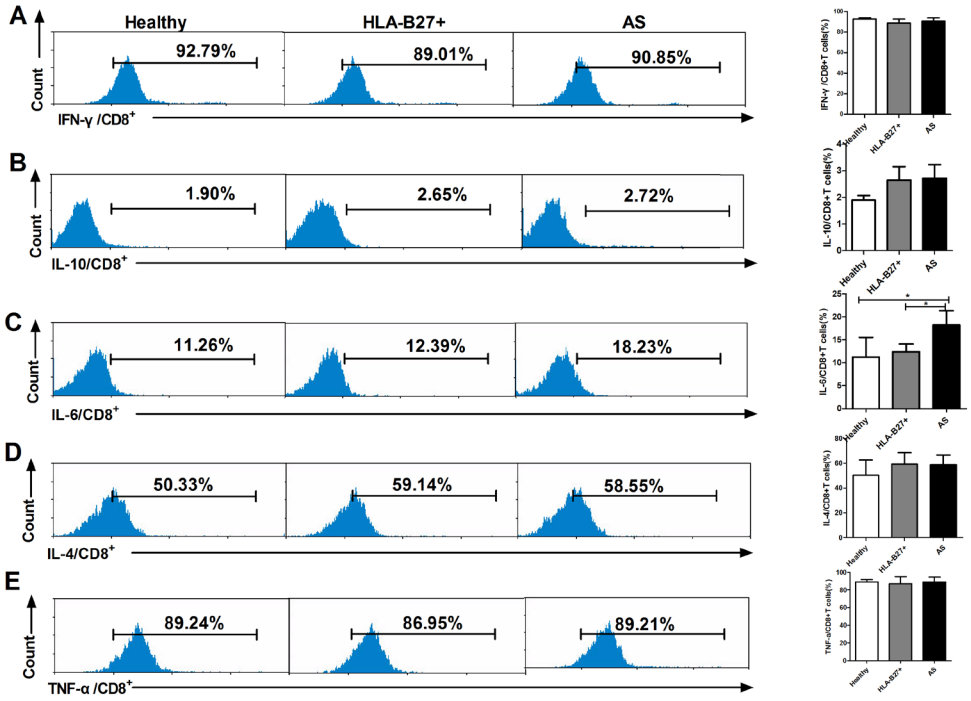

Figure 4. The production of cytokines in CD8$^+$ T cells in different groups. The production of IFN-γ, IL-10, TNF-α, and IL-4 in CD8$^+$ T cells did not differ among three groups, while IL-6 was higher in AS patients. Data was analyzed using Prism 5. *$p < 0.05$, AS ($n = 21$), Healthy ($n = 16$) and HLA-B27+ ($n = 22$)

3.4. *Expression of IL-6 in CD8$^+$ T cells was higher in AS patients*

We also tested the expression of IFN-γ, IL-6, TNF-α, IL-4, and IL-10 in CD8$^+$ T cells. The frequency of IL-6/CD8$^+$ cells in the AS group (18.23 ± 0.94%) was higher than the healthy group (11.26 ± 1.28%) and HLA-B27+ group (12.39 ± 0.63%). However, other cytokines showed no significant differences among three groups (Figure 4). These results suggested higher expression of IL-6/CD8$^+$ in AS patients.

3.5. *Tim-3$^+$ T cells from AS patients produced more IL-10*

To further investigate the roles of Tim-3 and PD-1 on CD4$^+$ T cells and CD8$^+$ T cells, we tested IFN-γ and IL-10 production by Tim-3$^+$CD4$^+$, Tim-3$^-$CD4$^+$, Tim-3$^+$CD8$^+$, Tim-3$^-$CD8$^+$, PD-1$^+$CD4$^+$, PD-1$^-$CD4$^+$, PD-1$^+$CD8$^+$ and PD-1$^-$CD8$^+$ T cells from AS patients. We found that Tim-3$^+$CD4$^+$, Tim-3$^+$CD8$^+$ and PD-1$^+$CD8$^+$ T cells produced more IL-10 than other subsets of T cells (Figure 5). There was no obvious difference in IFN-γ production. These data suggested that PD-1 and Tim-3 might influence the production of IL-10.

3.6. *AS patients showed lower percentage of Treg*

Table 2 showed the percentage of PBMC in AS, HLA-B27+, and Healthy groups. The percentage of CD4$^+$CD25$^+$CD127^{low} T cells in these three groups was also analyzed (Figure 6). We found that the percent of CD4$^+$CD25$^+$CD127^{low}/CD4$^+$ in AS patient (2.70 ± 0.80%) was significantly lower than the healthy group (3.47 ± 0.83%) ($p < 0.05$), but there was no significant difference compared to HLA-B27+ group (2.96 ± 0.35%) ($p > 0.05$).

4. Discussion

Ankylosing spondylitis is a spinal arthritic disease that is often associated with HLA-B27 (*27-29*); however, fewer than 5% of HLA-B27 carriers become AS patients (*30-31*), which leaves over a lot of questions. We started from the immunological differences among AS patients and both HLA-B27 positive and negative healthy controls to further explore the immune regulation in the development of AS, with focus on Tim-3 and PD-1.

We first determined the total levels of CD4, CD8 and Treg lymphocytes among three groups. The results turned out that there was no significant difference in the expression of either CD4$^+$ T cells or CD8$^+$ T cells, while AS patients showed lower percentage of Treg. Our finding of CD4$^+$ T cells is similar to the results of Aktas *et al*. (*32*) but in contrast to the research of Chen *et al*.

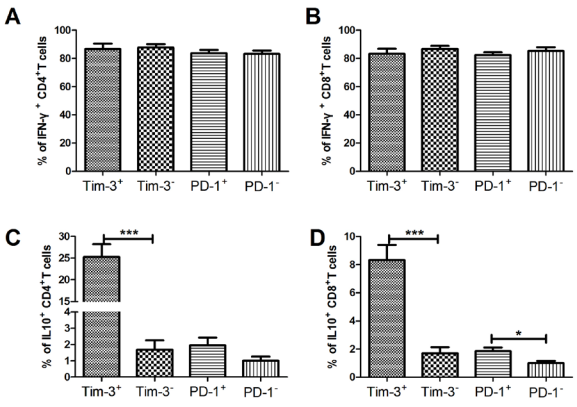

Figure 5. Tim-3$^+$ and PD-1$^+$ T cells produced more IL-10 than Tim-3$^-$ and PD-1$^-$ T cells. We examined IFN-γ and IL-10 production by Tim-3$^+$CD4$^+$, Tim-3$^-$CD4$^+$, Tim-3$^+$CD8$^+$, Tim-3$^-$CD8$^+$, PD-1$^+$CD4$^+$, PD-1$^-$CD4$^+$, PD-1$^+$CD8$^+$, and PD-1$^-$CD8$^+$T cells from AS patients ($n = 21$). Tim-3$^+$CD4$^+$, Tim-3$^+$CD8$^+$ and PD-1$^+$CD8$^+$ T cells produced more IL-10 than other subsets of T cells. $^*p < 0.05$.

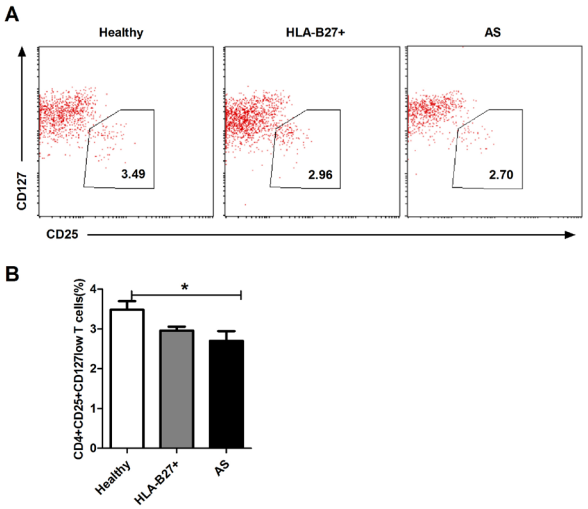

Figure 6. The expression of CD4$^+$CD25$^+$CD127^{low} T cells in AS patients. The percentage of CD4$^+$CD25$^+$CD127^{low} T cells in three groups was analyzed by flow cytometry. We found that it was significantly lower than healthy group ($p < 0.05$), but of no significant difference than HLA-B27+ group. Data was analyzed using Prism 5. $^*p < 0.05$, AS ($n = 21$), Healthy ($n = 16$) and HLA-B27+ ($n = 22$).

Table 2. Percentage of PBMC in AS, HLA-B27+, and Healthy groups

Items	AS ($n = 21$)	p value	HLA-B27+ ($n = 22$)	p value	Healthy ($n = 16$)
CD3$^+$ T cells	63.21 ± 7.46	0.42	57.85 ± 9.16	0.38	60.42 ± 10.52
CD4$^+$ T cells	40.33 ± 2.53	0.29	44.04 ± 1.40	0.20	43.76 ± 1.72
CD8$^+$ T cells	25.18 ± 1.37	0.96	24.00 ± 1.37	0.54	25.29 ± 1.59
CD4$^+$25$^+$127^{low} T cells	2.70 ± 0.80	0.03	2.96 ± 0.35	0.33	3.47 ± 0.83

p value: *vs.* Healthy group.

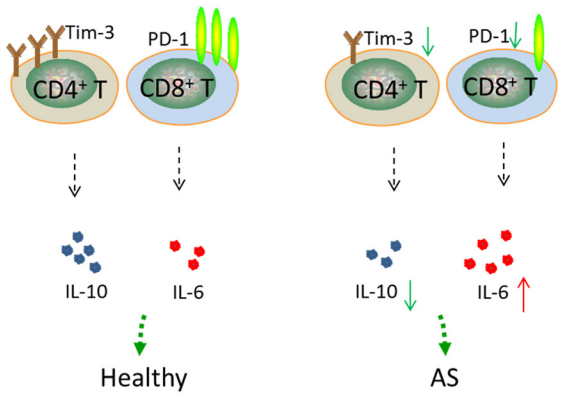

Figure 7. Schematic diagram of PD-1 and Tim-3 signals in AS patients. The expression of Tim-3 on $CD4^+$ T cells and PD-1 on $CD8^+$ T cells in AS was lower, accompanied by inadequate production of cytokines IL-10, which suggested poor inflammation inhibitory. The number of PD-1 on $CD8^+$ T cells in AS was decreased while their production of cytokines IL-6 was higher, which possibly contributed to the occurrence of AS.

(*33*). We speculated that it was due to the small sample and different severity of disease. The C-reactive protein of AS patients in Chen's report was 1.84 ± 0.41mg/dL, while we didn't detect it. The level of $CD8^+$ T cells, which recognize MHC 1 molecules directly to defend against infections, was also not higher in the AS patients. This results indicated that changes of number in CD4 and CD8 lymphocytes are not associated with the occurrence of AS.

Tim-3 and PD-1 take part in control negative regulation of immune responses in many diseases (*34*), establishing the balance between the activation and inhibition of T cells, with a tendency towards the latter. We found that the expression of Tim-3 on $CD4^+$ T cells and PD-1 on $CD8^+$ T cells was notably lower in AS, which implied that Tim-3 and PD-1 on T cells could affect the disease.

Cytokine analysis showed that IFN-γ, IL-6, TNF-α, and IL-4 levels in $CD4^+$ T cells were not altered, but IL-10 level in $CD4^+$ T cells was lower in the AS group. As a classic immuno-regulatory and anti-inflammatory cytokine, IL-10 had decreased expression in $CD4^+$ T cells which might correlate with AS. Meanwhile, IL-6 level in $CD8^+$ T cells was obviously higher in the AS group than the other two control groups. Proinflammatory cytokine IL-6 is known to induce the activation of osteoclasts (*35*), which might closely relate to the bone metabolism associated with AS.

Taken together, our study showed that the dysfunction of immune suppression played an important role in AS. A paradigm that illustrated the possible mechanism of Tim-3 and PD-1 signals in regulation of the immune response in AS was proposed (Figure 7).

Our study did not succeed in finding the immunological differences between the HLA-B27 positive and negative healthy groups, which almost shared the same expression levels of the tested markers. This might explain the reason why less than 5% of HLA-B27 positive individuals develop AS (i.e., most HLA-B27 positive individuals do not have downregulated Tim-3 and PD-1 expression). $Tim\text{-}3^+CD4^+$, $Tim\text{-}3^+CD8^+$, and $PD\text{-}1^+CD8^+$ T cells were found to produce more IL-10 than other subsets of cells ($Tim\text{-}3^-CD4^+$, $Tim\text{-}3^-CD8^+$, and $PD\text{-}1^-CD8^+$ T cells). Therefore, we hypothesized that Tim-3 and PD-1 were involved in the development of AS through the regulation of IL-10 production. The role of Tim-3 and PD-1 on $CD4^+$ and $CD8^+$ T cells should be investigated further to examine the pathogenesis of AS.

Our findings provided a new way to think about the diagnosis and treatment of AS. Current pharmacological therapies and physiotherapies, such as regular exercise or spa therapy (*36*), mainly relieve the symptoms of AS (*36*). Dorothea *et al*. reported that body hyperthermia treatment could increase IL-10 and toll-like receptor 4 expression which were believed to play an active role in the curative effect (*37*). In recent studies, blockade of PD-1 or Tim-3 molecules to restore the balance of immune regulation has been a hot topic (*23,38*). The development of this blockade as a way to ameliorate AS is the basis of our ongoing work.

Acknowledgements

This work was supported by the National Natural Science Foundation of China No. 31571196 (to Ling Wang), the Science and Technology Commission of Shanghai Municipality 2015 YIXUEYINGDAO project No. 15401932200 (to Ling Wang), the FY2008 JSPS Postdoctoral Fellowship for Foreign Researchers P08471 (to Ling Wang), the National Natural Science Foundation of China No. 30801502 (to Ling Wang), the Shanghai Pujiang Program No. 11PJ1401900 (to Ling Wang), the National Natural Science Foundation of China No. 81401171 (to Xuemin Qiu), Development Project of Shanghai Peak Disciplines-Integrated Chinese and Western Medicine, and the Program for Outstanding Medical Academic Leader (to Dajin Li).

References

1. DiCarlo EF, Kahn LB. Inflammatory diseases of the bones and joints. Semin Diagn Pathol. 2011; 28:53-64.
2. Bakland G, Nossent HC. Epidemiology of spondyloarthritis: A review. Curr Rheumatol Rep. 2013; 15:351.
3. Khan MA. HLA-B27 and its pathogenic role. J Clin Rheumatol. 2008; 14:50-52.
4. Zhou L, Zhang Y, Xu H, Hu L, Zhang C, Sun L, Xie Y, Lu H, Zhang Z, Hu W, Lin X. Decreased programmed death-1 expression on the T cells of patients with ankylosing spondylitis. Am J Med Sci. 2015; 349:488-492.
5. Scalapino KJ, Tang Q, Bluestone JA, Bonyhadi ML, Daikh DI. Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer

of *ex vivo* expanded regulatory T cells. J Immunol. 2006; 177:1451-1459.
6. Morgan ME, Flierman R, van Duivenvoorde LM, Witteveen HJ, van Ewijk W, van Laar JM, de Vries RR, Toes RE. Effective treatment of collagen-induced arthritis by adoptive transfer of $CD25^+$ regulatory T cells. Arthritis Rheum. 2005; 52:2212-2221.
7. Yuan MM, Du MR, Wang MY, Duan ZL, Meng Y, Jin LP, Li MQ, Li DJ. Combination of $CD4^+CD25^+CD127^-$ regulatory T cells with MLC-BE and BE-Ab2: An efficient evaluation of the therapy of paternal lymphocyte induced immunization in unexplained recurrent spontaneous abortion patients. Int J Clin Exp Pathol. 2015; 8:4022-4032.
8. Mottet C, Uhlig HH, Powrie F. Cutting edge: Cure of colitis by $CD4^+CD25^+$ regulatory T cells. J Immunol. 2003; 170:3939-3943.
9. Yang PT, Kasai H, Zhao LJ, Xiao WG, Tanabe F, Ito M. Increased CCR4 expression on circulating $CD4^+$ T cells in ankylosing spondylitis, rheumatoid arthritis and systemic lupus erythematosus. Clin Exp Immunol. 2004; 138:342-347.
10. Jandus C, Bioley G, Rivals JP, Dudler J, Speiser D, Romero P. Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. Arthritis Rheum. 2008; 58:2307-2317.
11. Baeten D, Sieper J, Braun J, Baraliakos X, Dougados M, Emery P, Deodhar A, Porter B, Martin R, Andersson M, Mpofu S, Richards HB. Secukinumab, an Interleukin-17A Inhibitor, in Ankylosing Spondylitis. N Engl J Med. 2015; 373:2534-2548.
12. Chen M, Zhang L, Ren Y, Zhang K, Yang Y, Fang Y, Yan X, Peng D, Gao C, Li S. Defective Function of $CD24^+CD38^+$ Regulatory B Cells in Ankylosing Spondylitis. DNA Cell Biol. 2016; 35:88-95.
13. Boyle LH, Goodall JC, Gaston JS. The recognition of abnormal forms of HLA-B27 by $CD4^+$ T cells. Curr Mol Med. 2004; 4:51-58.
14. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. Embo J. 1992; 11:3887-3895.
15. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008;26:677-704
16. Luan Y, Chai D, Peng J, Ma S, Wang M, Ma H, Li X, Fu S, Pan X, Wang X, Qin S, Xu T. A fully human monoclonal antibody targeting PD-L1 with potent anti-tumor activity. Int Immunopharmacol. 2016; 31:248-256.
17. Han S, Feng S, Xu L, Shi W, Wang X, Wang H, Yu C, Dong T, Xu M, Liang G. Tim-3 on peripheral $CD4^+$ and $CD8^+$ T cells is involved in the development of glioma. DNA Cell Biol. 2014; 33:245-250.
18. Wang M, Ji B, Wang J, Cheng X, Zhou Q, Zhou J, Cao C, Guo Q. Tim-3 polymorphism downregulates gene expression and is involved in the susceptibility to ankylosing spondylitis. DNA Cell Biol. 2014; 33:723-728.
19. Sada-Ovalle I, Ocana-Guzman R, Perez-Patrigeon S, Chavez-Galan L, Sierra-Madero J, Torre-Bouscoulet L, Addo MM. Tim-3 blocking rescue macrophage and T cell function against Mycobacterium tuberculosis infection in HIV+ patients. J Int AIDS Soc. 2015; 18:20078.
20. Gao DN, Yang ZX, Qi QH. Roles of PD-1, Tim-3 and CTLA-4 in immunoregulation in regulatory T cells among patients with sepsis. Int J Clin Exp Med. 2015; 8:18998-19005.
21. Meggyes M, Lajko A, Palkovics T, Totsimon A, Illes Z, Szereday L, Miko E. Feto-maternal immune regulation by TIM-3/galectin-9 pathway and PD-1 molecule in mice at day 14.5 of pregnancy. Placenta. 2015; 36:1153-1160.
22. Cope A, Le Friec G, Cardone J, Kemper C. The Th1 life cycle: Molecular control of IFN-γ to IL-10 switching. Trends Immunol. 2011; 32:278-286.
23. Markwick LJ, Riva A, Ryan JM, *et al*. Blockade of PD1 and TIM3 restores innate and adaptive immunity in patients with acute alcoholic hepatitis. Gastroenterology. 2015; 148:590-602.
24. Ciccia F, Accardo-Palumbo A, Giardina A, *et al*. Expansion of intestinal $CD4^+CD25^{high}$ Treg cells in patients with ankylosing spondylitis: A putative role for interleukin-10 in preventing intestinal Th17 response. Arthritis Rheum. 2010; 62:3625-3634.
25. van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. Arthritis Rheum. 1984; 27:361-368.
26. Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: The Bath Ankylosing Spondylitis Disease Activity Index. J Rheumatol. 1994; 21:2286-2291.
27. Smith JA. Update on ankylosing spondylitis: Current concepts in pathogenesis. Curr Allergy Asthma Rep. 2015; 15:489.
28. Reveille JD. An update on the contribution of the MHC to AS susceptibility. Clin Rheumatol. 2014; 33:749-757.
29. Colbert RA, Tran TM, Layh-Schmitt G. HLA-B27 misfolding and ankylosing spondylitis. Mol Immunol. 2014; 57:44-51.
30. Brown MA, Kennedy LG, MacGregor AJ, Darke C, Duncan E, Shatford JL, Taylor A, Calin A, Wordsworth P. Susceptibility to ankylosing spondylitis in twins: The role of genes, HLA, and the environment. Arthritis Rheum. 1997; 40:1823-1828.
31. Cortes A, Hadler J, Pointon JP, *et al*. Identification of multiple risk variants for ankylosing spondylitis through high-density genotyping of immune-related loci. Nat Genet. 2013; 45:730-738.
32. Aktas CE, Cosan F, Kucuksezer UC, Bilgic S, Cagatay Y, Gul A, Deniz G. Behcet's disease: Immunological relevance with arthritis of ankylosing spondylitis. Rheumatol Int. 2013; 33:733-741.
33. Chen MH, Chen WS, Lee HT, Tsai CY, Chou CT. Inverse correlation of programmed death 1 (PD-1) expression in T cells to the spinal radiologic changes in Taiwanese patients with ankylosing spondylitis. Clin Rheumatol. 2011; 30:1181-1187.
34. Lee KA, Shin KS, Kim GY, Song YC, Bae EA, Kim IK, Koh CH, Kang CY. Characterization of age-associated exhausted CD8 T cells defined by increased expression of Tim-3 and PD-1. Aging Cell. 2016; 15:291-300.
35. de Menezes EG, Machado AA, Barbosa FJ, de Paula FJ, Navarro AM. Bone metabolism dysfunction mediated by the increase of proinflammatory cytokines in chronic HIV infection. J Bone Miner Metab. 2016. doi:10.1007/s00774-016-0749-8
36. Braun J, van den Berg R, Baraliakos X, *et al*. 2010 update of the ASAS/EULAR recommendations for the management of ankylosing spondylitis. Ann Rheum Dis. 2011; 70:896-904.

37. Zauner D, Quehenberger F, Hermann J, Dejaco C, Stradner MH, Stojakovic T, Angerer H, Rinner B, Graninger WB. Whole body hyperthermia treatment increases interleukin 10 and toll-like receptor 4 expression in patients with ankylosing spondylitis: A pilot study. Int J Hyperthermia. 2014; 30:393-401.
38. Wang SC, Li YH, Piao HL, Hong XW, Zhang D, Xu YY, Tao Y, Wang Y, Yuan MM, Li DJ, Du MR. PD-1 and Tim-3 pathways are associated with regulatory $CD8^+$ T-cell function in decidua and maintenance of normal pregnancy. Cell Death Dis. 2015; 6:e1738.

(Received September 7, 2016; Revised December 26, 2016; Accepted January 31, 2017)

Original Article

DOI: 10.5582/bst.2016.01224

Knocking down TCF8 inhibits high glucose- and angiotensin II-induced epithelial to mesenchymal transition in podocytes

Changhuan Bai[1,2], Shumei Liang[3], Yunshan Wang[4], Bo Jiao[1,*]

[1] *Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, Ji'nan, Shandong, China;*
[2] *Department of Pharmacy, The Second People's Hospital of Liaocheng, Liaocheng, Shandong, China;*
[3] *Department of Obstetrics and Gynecology, Shandong Provincial Hospital Affiliated to Shandong University, Ji'nan, Shandong, China;*
[4] *Weihai International Biotechnology Research and Development Center, Shandong University, Weihai, Shandong, China.*

Summary **Epithelial to mesenchymal transition (EMT) is a physiological phenomenon in mammalian embryogenesis by which epithelial cells become mesenchymal stem cells. Studies have indicated that an inappropriate EMT plays a key role in a variety of pathogenic processes such as embryonic development and tumor metastasis. Moreover, recent studies have indicated EMT also plays an important role in renal fibrosis. In the current study, glucose and angiotensin II promoted EMT in podocytes as well as changes in the cellular morphology of podocytes. A high concentration of glucose and angiotensin II also promoted podocyte movement and migration. Moreover, a high concentration of glucose and angiotensin II promoted TCF8 expression. Inhibiting TCF8 expression with siRNA reversed EMT in podocytes in the presence of a high concentration of glucose and angiotensin. Inhibiting TCF8 expression also reversed changes in cellular morphology and podocyte movement and migration. Therefore, glucose and angiotensin II may promote EMT in podocytes via TCF8.**

Keywords: Epithelial to mesenchymal transition (EMT), podocyte, glucose, angiotensin II, TCF8

1. Introduction

In the kidneys, podocytes are attached to the outside of the glomerular basement membrane. The glomerular basement membrane plays an important role in maintaining the normal structure and function of the glomerulus *via* the actin cytoskeleton of podocytes and specific expression of protein molecules (*1*). Nephrin, WT1, and synaptopodin are phenotypic markers of mature podocytes (*2*). Podocytes have long processes that interdigitate and that are separated by very narrow spaces (30-40 nm) that are bridged by a membrane called the slit diaphragm (*3*). This diaphragm allows water and small solute molecules to pass through but it filters out plasma proteins and other macromolecules, and this property is the main determinant of the permeability of the renal glomerular filtration barrier (*1*). Therefore, podocytes play an important role in protecting the integrity of the glomerular filtration barrier, adjusting the ultrafiltration coefficient, and maintaining the normal structure of the glomerular basement membrane (*4*). Ultrastructural changes in podocytes and reduced expression of associated molecules is closely related to renal impairment and can lead to glomerulosclerosis and renal interstitial fibrosis (*5*).

The epithelial-mesenchymal transition (EMT) is a process by which polarized epithelial cells undergo numerous biochemical changes to obtain a mesenchymal cell phenotype (*6*). EMT is also involved in tumor migration and invasion (*7*). Podocytes are epithelial cells, so when they are affected by different conditions (such as TGF-β, a high concentration of glucose, doxorubicin, or some other stimuli), EMT may occur (*8-10*). When EMT occurs, epithelial phenotypic markers such as E-cadherin, P-cadherin, nephrin, podocalyxin, and synaptopodin are downregulated, and mesenchymal phenotypic markers

Released online in J-STAGE as advance publication January 22, 2017.

**Address correspondence to:*
Dr. Bo Jiao, Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, 44 West Culture Road, Ji'nan, Shandong, China.
E-mail: jiaob@sdu.edu.cn

such as desmin, FSP-1, MMP-9, ILK, fibronectin, vimentin, α-SMA, collagen I, and snail are upregulated (*11*). EMT is considered to be the key factor for an impaired kidney producing myofibroblasts in renal fibrosis, which may lead to renal interstitial fibrosis (*12*). Podocytes, which are also known as glomerular visceral epithelial cells, are cells affected by different types of injury. EMT in podocytes will result in damage to the glomerular filtration barrier and proteinuria; in severe cases, it may lead to podocyte detachment from the glomerular basement membrane and apoptosis, causing a decrease in the number of podocytes, thereby aggravating proteinuria and glomerular sclerosis (*13,14*). EMT in podocytes is a key starting point for studying the mechanisms by which proteinuria and renal fibrosis develop (*15*).

The aim of the current study was to examine EMT in podocytes in the presence of a high concentration of glucose and angiotensin II. This study examined the effects of a high concentration of glucose and angiotensin II on EMT and the expression of TCF8 in podocytes. This study also examined the effects of downregulating TCF8 on EMT in podocytes.

2. Materials and Methods

2.1. *Cell culture and reagents*

The podocyte line used in this study was donated by Prof. Ding Jie (Peking University First Hospital, Beijing, China). E-cadherin, α-catenin, N-cadherin, vimentin, TCF8, and β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. *qRT-PCR*

Total RNA was extracted from cell lines using the TRIZOL reagent (Invitrogen). Reverse transcription (RT) was performed using the Thermoscript RT System (Invitrogen). Hotstart PCR conditions were as follows: 45 s at 94°C, 30 s at 55°C, and 1 min at 72°C for 28-30 cycles (for TCF8) or 26 cycles (for glyceraldehyde 3-phosphate dehydrogenase (GAPDH)). The primers used in the study were: TCF8: sense 5'-GCACAACCAAGTGCAGAAGA-3' and antisense 5'-CATT TGCAGATTGAGGCTGA-3'; GAPDH: sense 5'-TGCCTCCTGCACCACCAACT-3' and antisense 5'-CCCGTTCAGCTCAGGGATGA-3'.

2.3. *Plasmid construction and transfection*

For TCF8 RNA interference, control and TCF8 shRNA plasmids (#1: CCGGGCTGCCAATAAGCAAACGAT TCTCGAGAATCGTTTGCTTATTGGCAGCTTTTT; #2: CCGGGCTGTTGTTCTGCCAACAGTTCTCGA GAACTGTTGGCAGAACAACAGCTTTTT; #3: CC GGCGGCGCAATAACGTTACAAATCTCGAGATT TGTAACGTTATTGCGCCGTTTTT) were purchased from Sigma-Aldrich Biotechnology (Sigma-Aldrich, CA, USA) and were used to transfect podocytes to establish a cell line with knocked-down TCF8. The transfection efficiency of TCF8 was verified with Western blotting and quantitative reverse transcription PCR (qRT-PCR) analyses.

2.4. *Western blot assay*

Equal amounts of protein were separated using SDS polyacrylamide gels and were electrotransferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were immunoblotted overnight at 4°C with primary antibodies, followed by their respective secondary antibodies. β-actin was used as the loading control.

2.5. *Cell invasion and motility assay*

Cell invasion was measured in Matrigel (Sigma, St. Louis, MO, USA)-coated Transwell inserts (6.5 mm, Costar, NY, USA) containing polycarbonate filters with 8-μm pores as previously described. The inserts were coated with 50 μL of 1 mg/mL Matrigel matrix in accordance with the manufacturer's recommendations. 2×10^5 cells in 200 μL of serum-free medium were plated in the upper chamber, and 600 μL of medium with 10% fatal bovine serum was added to the lower well. After incubation for 24 h, top cells were removed and bottom cells were counted. Cells that migrated to the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. For each membrane, five random fields were counted at ×10 magnification. The mean was calculated and data were expressed as the mean ± S.D. from three independent experiments done in triplicate. Motility assays were similar to invasion assays with Matrigel except that the Transwell inserts were not coated with Matrigel.

3. Results

3.1. *A high concentration of glucose and angiotensin II promoted EMT in podocytes*

Previous studies reported that a high concentration of glucose and angiotensin II may promote EMT in kidney epithelial cells (*16,17*). The current study examined whether those substances would have the same effect on podocytes. Podocytes were treated with 0, 10, 20, and 30 mM of glucose. As shown in Figures 1A and 1B, the expression of the epithelial marker molecules E-cadherin and α-catenin decreased with an increase in the glucose concentration. As shown in Figures 1C and 1D, the expression of the mesenchymal marker molecules N-cadherin and vimentin increased with an increase in the glucose concentration. Podocytes were then treated

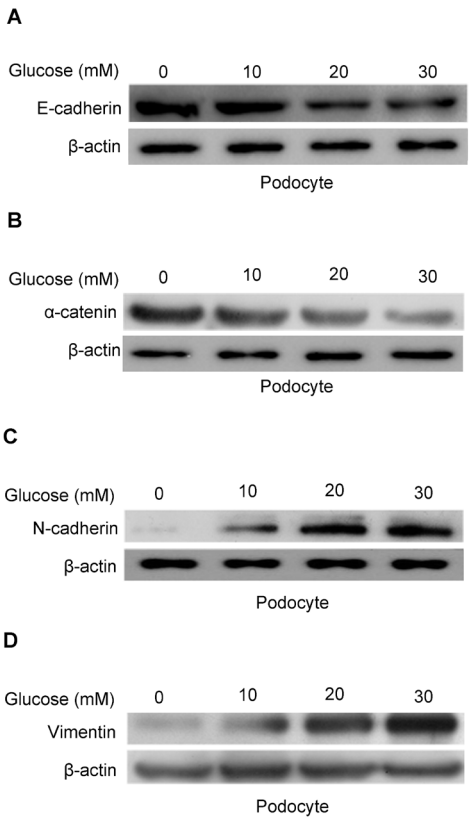

Figure 1. A high concentration of glucose inhibited the expression of epithelial biomarkers (E-cadherin and α-catenin) and promoted the expression of mesenchymal biomarkers (N-cadherin and vimentin) in podocytes. (A) Podocytes were incubated with 0-30 mM of glucose for 24 h and the expression of E-cadherin was measured with Western blotting. **(B)** Podocytes were incubated with 0-30 mM of glucose for 24 h and the expression of α-catenin was measured with Western blotting. **(C)** Podocytes were incubated with 0-30 mM of glucose for 24 h and the expression of N-cadherin was measured with Western blotting. **(D)** Podocytes were incubated with 0-30 mM of glucose for 24 h and the expression of vimentin was measured with Western blotting.

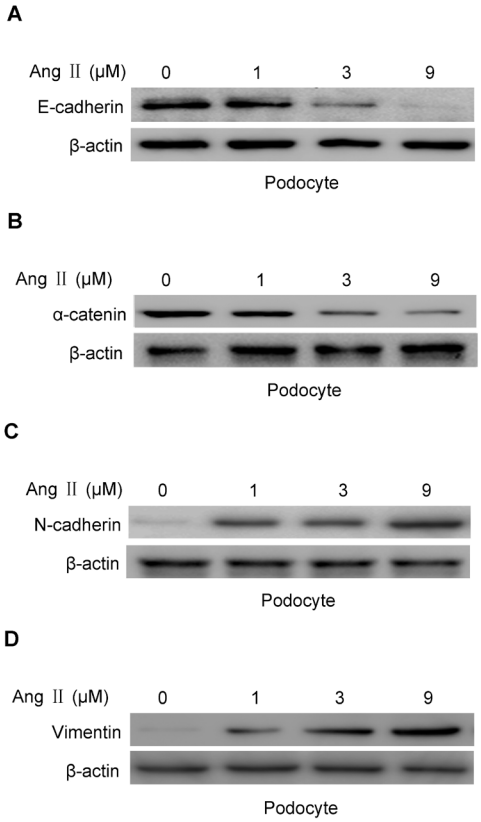

Figure 2. Angiotensin II inhibited the expression of epithelial biomarkers (E-cadherin and α-catenin) and promoted the expression of mesenchymal biomarkers (N-cadherin and vimentin) in podocytes. (A) Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and the expression of E-cadherin was measured with Western blotting. **(B)** Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and the expression of α-catenin was measured with Western blotting. **(C)** Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and the expression of N-cadherin was measured with Western blotting. **(D)** Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and the expression of vimentin was measured with Western blotting.

with 0, 3, 6, and 9 μM of angiotensin II. As shown in Figures 2A and 2B, the expression of the epithelial marker molecules E-cadherin and α-catenin decreased with an increase in the angiotensin II concentration. As shown in Figures 2C and 2D, the expression of the mesenchymal marker molecules N-cadherin and vimentin increased with an increase in the angiotensin II concentration. Results indicated that a high concentration of glucose and angiotensin II promoted EMT in podocytes.

3.2. *A high concentration of glucose and angiotensin II changed podocyte morphology and promoted podocyte migration and invasion*

The current study examined the effects of a high concentration of glucose and angiotensin on podocytes. As shown in Figures 3A and 3B, podocytes displayed morphological changes when stimulated with 30 mM of glucose or 9 μM of angiotensin II. As shown in Figures 3C-3F, a high concentration of glucose and angiotensin promoted podocyte cell migration and invasion. The number of migrating and invading podocytes in the presence of 30 mM of glucose or 9 μM of angiotensin II was about twice that in the presence of 0 mM of glucose or angiotensin II.

3.3. *A high concentration of glucose and angiotensin II promoted TCF8 expression*

TCF8 is a transcription factor that plays an important role in EMT (*6*). The current study examined whether a high concentration of glucose and angiotensin II affects TCF8. Podocytes were treated with 0, 10, 20, and 30 mM of glucose. As shown in Figure 4A, the expression of TCF8 increased with an increase in the glucose concentration. Podocytes were then treated with 0, 3, 6, and 9 μM of angiotensin II. As shown in Figure 4B, the expression of TCF8 increased with an increase in the angiotensin II concentration. Results indicated that a high concentration of glucose and angiotensin II promoted TCF8 expression. TCF8 expression was

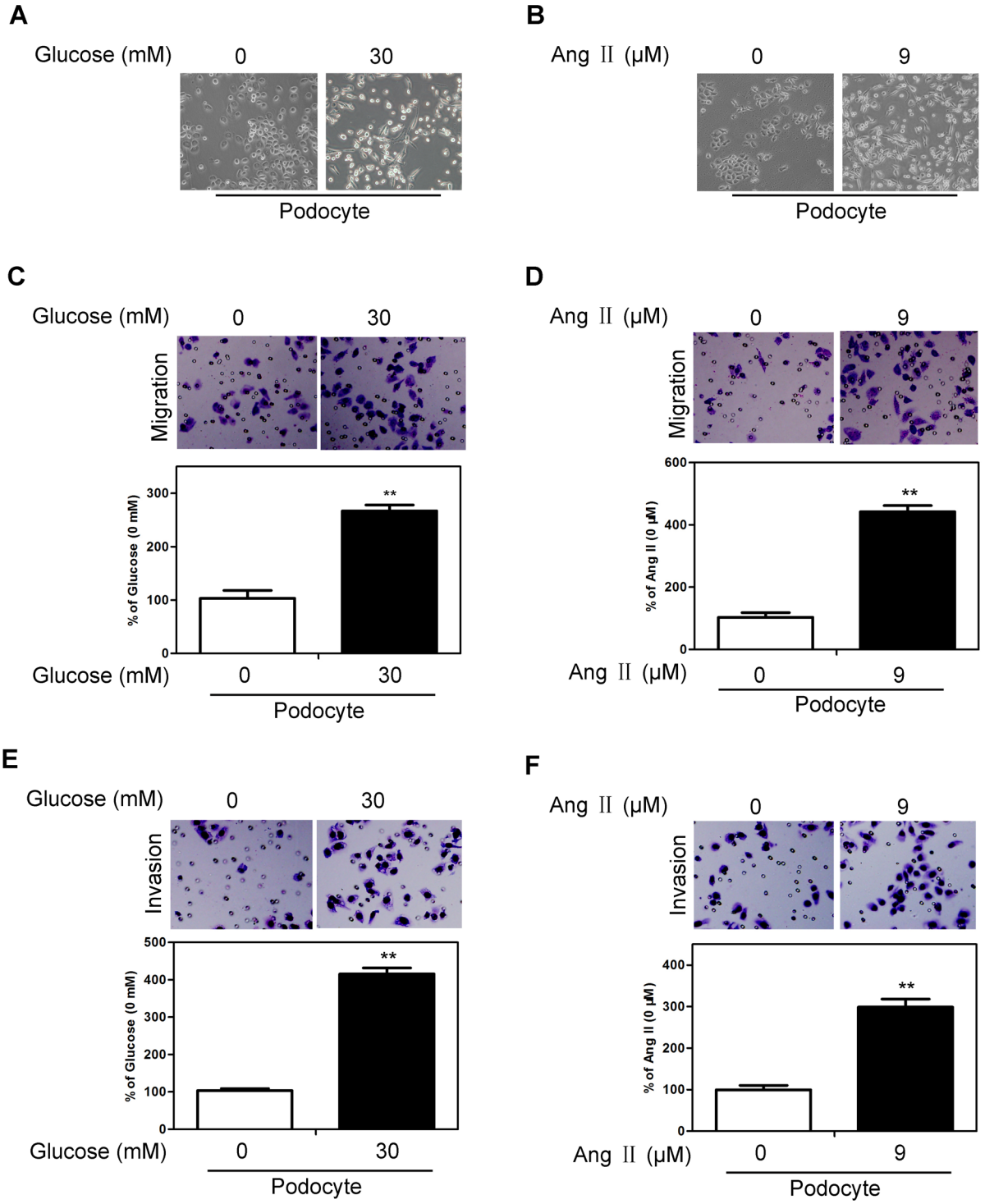

Figure 3. A high concentration of glucose or angiotensin II promoted changes in the cell morphology of podocytes as well as podocyte migration and invasion. (A) Changes in the cell morphology of podocytes in the presence of 0 and 30 mM of glucose. **(B)** Changes in the cell morphology of podocytes in the presence of 0 and 9 μM of angiotensin II. **(C)** The migration of podocytes in the presence of 0 and 30 mM of glucose was measured with a transwell assay. **(D)** The migration of podocytes in the presence of 0 and 9 μM of angiotensin II was measured with a transwell assay. **(E)** Invasion by podocytes in the presence of 0 and 30 mM of glucose was measured with a Matrigel assay. **(F)** Invasion by podocytes in the presence of 0 and 9 μM of angiotensin II was measured with a Matrigel assay.

suppressed using antisense RNA. Three TCF8 antisense RNA sequences were transfected into podocytes. As shown in Figures 4C and 4D, TCF8 expression was down-regulated according to Western blotting and qRT-PCR.

3.4. *Silencing TCF8 reversed EMT that was induced with a high concentration of glucose or angiotensin II*

As shown in Figures 5A and 5B, the expression of the epithelial marker molecules E-cadherin and α-catenin

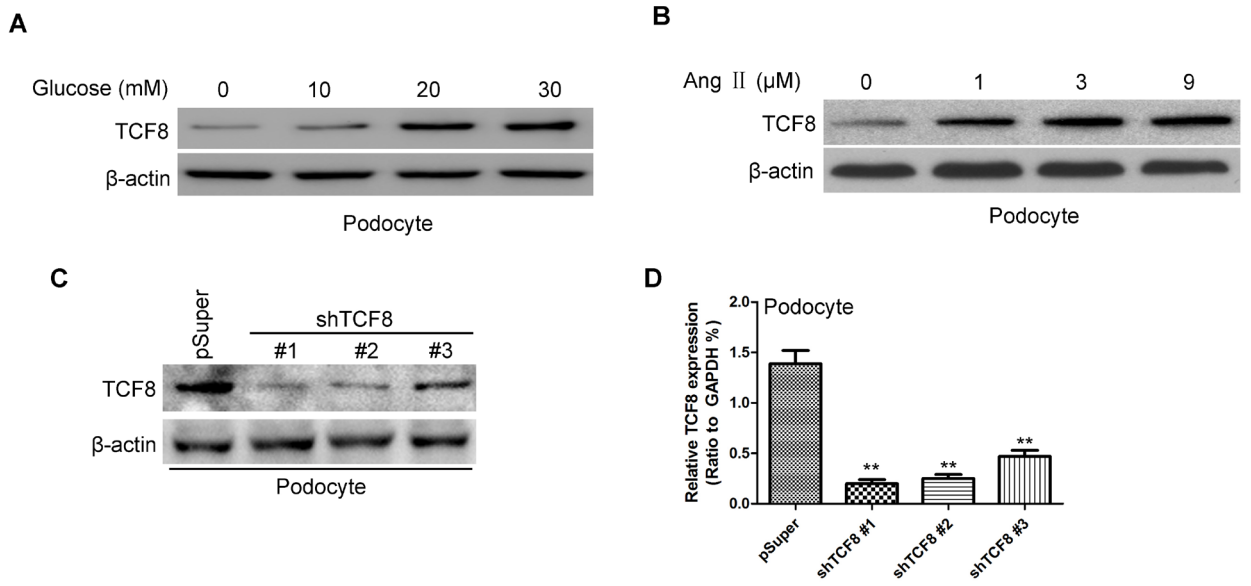

Figure 4. A high concentration of glucose or angiotensin II promoted the expression of TCF8 in podocytes. (A) Podocytes were incubated with 0-30 mM of glucose for 24 h and TCF8 expression was measured with Western blotting. **(B)** Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and TCF8 expression was measured with Western blotting. **(C)** Expression of TCF8 protein after three TCF8 antisense RNA sequences were transfected into podocytes was assayed using Western blotting. **(D)** Expression of TCF8 mRNA after three TCF8 antisense RNA sequences were transfected into podocytes was assayed using qRT-PCR.

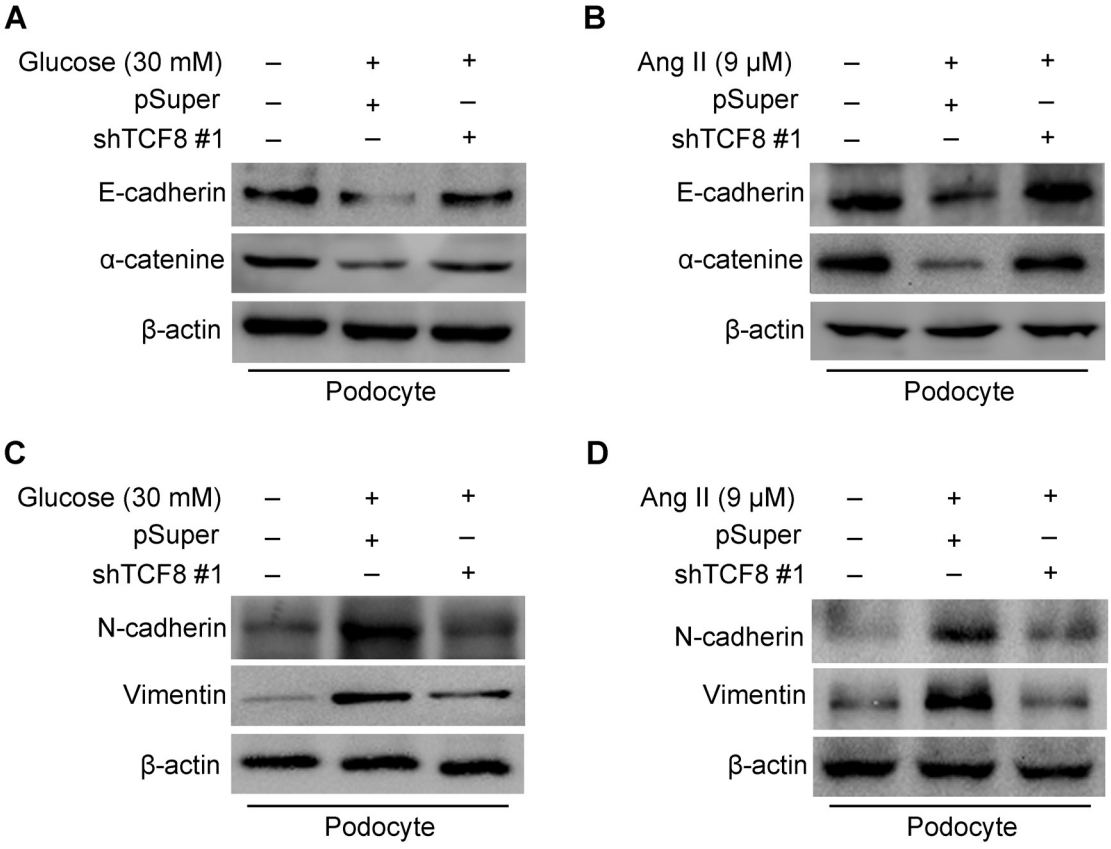

Figure 5. Silencing TCF8 expression in podocytes reversed expression of EMT biomarkers induced with glucose and angiotensin II. (A) Podocytes with silenced TCF8 were incubated with 0 or 30 mM of glucose for 24 h and expression of the epithelial biomarkers E-cadherin and α-catenin was measured with Western blotting. **(B)** Podocytes with silenced TCF8 were incubated with 0 or 9 μM of angiotensin II for 24 h and expression of the epithelial biomarkers E-cadherin and α-catenin expression was measured with Western blotting. **(C)** Podocytes with silenced TCF8 were incubated with 0 or 30 mM of glucose for 24 h and expression of the mesenchymal biomarkers N-cadherin and vimentin was measured with Western blotting. **(D)** Podocytes with silenced TCF8 were incubated with 0 or 9 μM of angiotensin II for 24 h and expression of the mesenchymal biomarkers N-cadherin and vimentin was measured with Western blotting.

decreased with an increase in the glucose or angiotensin II concentration, but this phenomenon was reversed in podocytes with silenced TCF8. As shown in Figures 5C and 5D, the expression of the mesenchymal marker molecules N-cadherin and vimentin increased with an increase in the glucose or angiotensin II concentration,

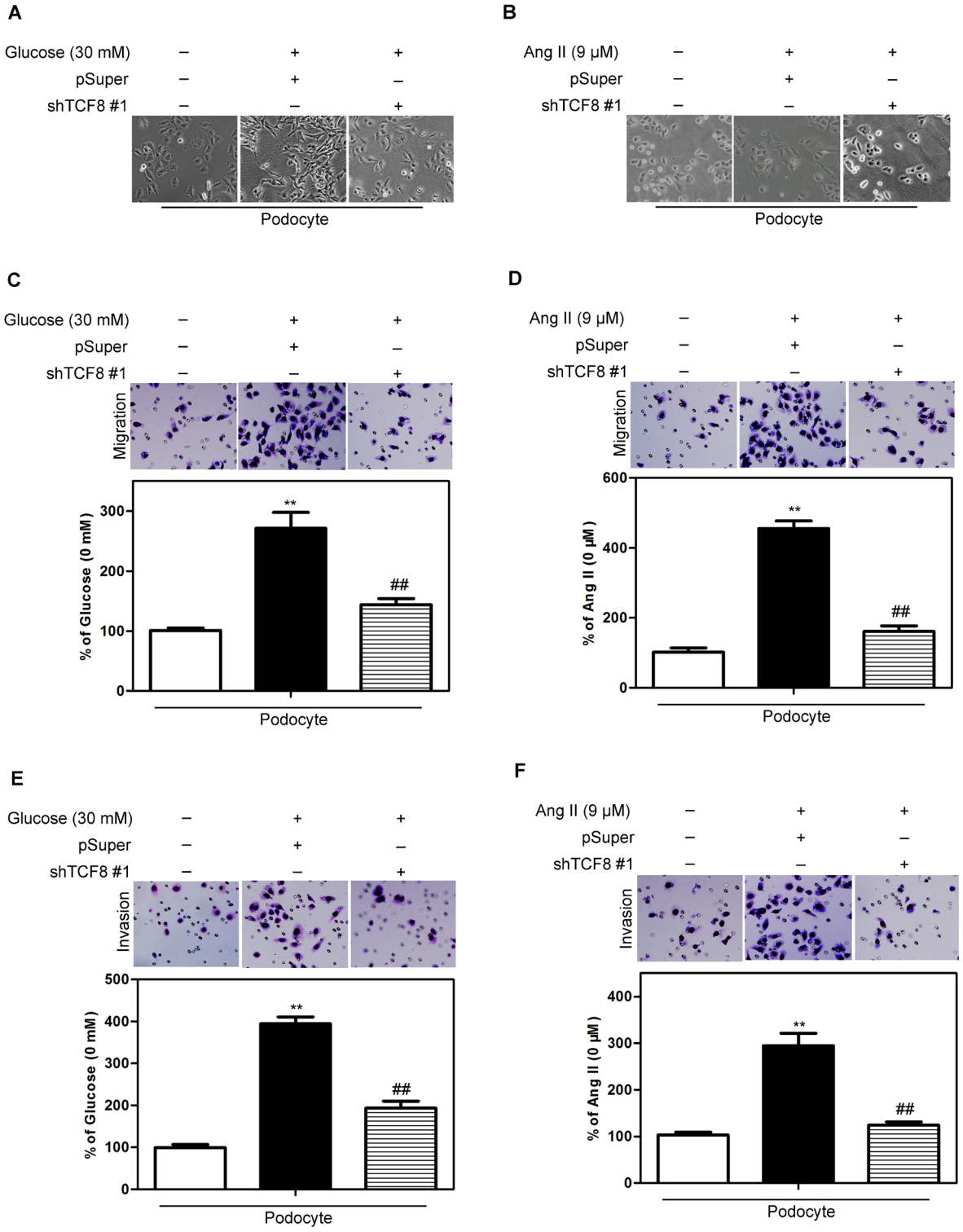

Figure 6. Silencing TCF8 expression reversed changes in the cell morphology of podocytes and podocyte migration and invasion induced with a high concentration of glucose or angiotensin II. (A) Normal podocytes and changes in in the cell morphology of podocytes with silenced TCF8 in the presence of 0 and 30 mM of glucose. **(B)** Normal podocytes and changes in the cell morphology of podocytes with silenced TCF8 in the presence of 0 and 9 μM of angiotensin II. **(C)** Migration of normal podocytes and podocytes with silenced TCF8 in the presence of 0 and 30 mM of glucose. **(D)** Migration of normal podocytes and podocytes with silenced TCF8 in the presence of 0 and 9 μM of angiotensin II. **(E)** Invasion by normal podocytes and podocytes with silenced TCF8 in the presence of 0 and 30 mM of glucose. **(F)** Invasion by normal podocytes and podocytes with silenced TCF8 in the presence of 0 and 9 μM of angiotensin II.

but this phenomenon was reversed in podocytes with silenced TCF8. Results indicated that silencing TCF8 reversed EMT that was induced with a high concentration of glucose or angiotensin II.

3.5. *Silencing TCF8 reversed changes in podocyte morphology and podocyte migration and invasion induced with a high concentration of glucose or angiotensin II*

As shown in Figures 6A and 6B, morphological changes induced with a high concentration of glucose or angiotensin II were reversed in podocytes with silenced TCF8. As shown in Figures 6C and 6D, a higher rate of cell migration was induced with a high concentration of glucose or angiotensin II, but this phenomenon was reversed in podocytes with silenced TCF8. As shown in Figures 6E and 6F, a higher rate of cell invasion was induced with a high concentration of glucose or angiotensin II, but this phenomenon was reversed in podocytes with silenced TCF8. In conclusion, silencing TCF8 reversed changes in podocyte morphology and podocyte migration and invasion induced with a high concentration of glucose or angiotensin II.

4. Discussion

Podocyte injury is a key factor for proteinuria in some kidney diseases (*2*). Proteinuria was assumed to be the result of podocyte depletion due to apoptosis (*2*). However, a recent study has indicated that the podocyte count did not change significantly when microalbuminuria was evident in diabetic rats (*18*). In addition, podocyte detachment and apoptosis lag far behind the onset of proteinuria. Whether initial podocyte injury leads to the onset of proteinuria is uncertain (*3*). Emerging evidence has indicated that podocytes can undergo EMT when exposed to specific pathological conditions, and this may explain the onset of proteinuria. A study has described the phenotypic and morphological changes in diseased podocytes as an EMT (*19*).

Mounting evidence has revealed that glucose and angiotensin II are two key mediators associated with progressive renal injury in chronic kidney disease, and specifically in hypertensive and diabetic nephropathy (in which progressive renal fibrosis is a major determinant of clinical outcomes) (*16*). The current study characterized the role of TCF8 in EMT in podocytes. EMT markers were detected in the presence of a high concentration of glucose or angiotensin II. Results indicated that a high concentration of glucose or angiotensin II promoted EMT in podocytes, and results also indicated that a high concentration of glucose and angiotensin II caused changes in podocyte morphology and they promoted podocyte migration and invasion. Moreover, results indicated that TCF8 is significantly overexpressed in the presence of a high concentration of glucose or angiotensin II. Silencing TCF8 reversed EMT in podocytes and changes in podocyte morphology, and it also reversed the increase in podocyte migration and invasion. Collectively, these findings are the first to suggest that TCF8 is involved in EMT in podocytes.

Recent studies have indicated that EMTs are not limited to renal epithelial cells but that EMTs also occur in endothelial cells and podocytes (*20*). EMT in podocytes may be reversible before podocytes decrease and die off. Podocytes may have biological characteristics causing an EMT under pathological conditions, and an EMT could conceivably result. Podocytes may undergo some changes such as cell detachment, apoptosis, and an EMT when subjected to a harmful stimulus, and the severity and duration of those changes may depend on the specific injury. However, there are numerous unknowns regarding the mechanism of EMT in podocytes and how an EMT in podocytes leads to glomerular disease.

TCF8, also known as ZEB1 or δ EF1, is located on the short arm of human chromosome 10 (*21*). TCF8 is characterized by a homeodomain flanked by two zinc finger domains, and TCF8 encodes 1108 amino acids. An important molecular event in EMT is the downregulation of E-cadherin. TCF8 can bind with E-cadherin promoter and inhibit the expression of E-cadherin. TCF8 has been found to be closely related to EMT in tumors, but its role in EMT in podocytes has yet to be elucidated.

In conclusion, the current results revealed that TCF8 was generally overexpressed in podocytes in the presence of a high concentration of glucose or angiotensin II and that TCF8 was involved in EMT. These results indicate that TCF8 plays a key role in promoted EMT in podocytes and that TCF8 may be an effective novel therapeutic target for the management of glomerular disease.

References

1. May CJ, Saleem M, Welsh GI. Podocyte dedifferentiation: A specialized process for a specialized cell. Front Endocrinol (Lausanne). 2014; 5:148.
2. Ilatovskaya DV, Levchenko V, Lowing A, Shuyskiy LS, Palygin O, Staruschenko A. Podocyte injury in diabetic nephropathy: Implications of angiotensin II-dependent activation of TRPC channels. Sci Rep. 2015; 5:17637.
3. Harindhanavudhi T, Parks A, Mauer M, Caramori ML. Podocyte structural parameters do not predict progression to diabetic nephropathy in normoalbuminuric type 1 diabetic patients. Am J Nephrol. 2015; 41:277-283.
4. Du M, Wang Q, Li W, Ma X, Wu L, Guo F, Zhao S, Huang F, Wang H, Qin G. Overexpression of FOXO1 ameliorates the podocyte epithelial-mesenchymal transition induced by high glucose in vitro and in vivo. Biochem Biophys Res Commun. 2016; 471:416-422.
5. Liu Y. New insights into epithelial-mesenchymal transition in kidney fibrosis. J Am Soc Nephrol. 2010;

21:212-222.
6. Wang Y, Wen M, Kwon Y, *et al*. CUL4A induces epithelial-mesenchymal transition and promotes cancer metastasis by regulating ZEB1 expression. Cancer Res. 2014; 74:520-531.
7. Ding Y, Li X, Hong D, Jiang L, He Y, Fang H. Silence of MACC1 decreases cell migration and invasion in human malignant melanoma through inhibiting the EMT. Biosci Trends. 2016; 5:258-264.
8. Kumar PA, Welsh GI, Raghu G, Menon RK, Saleem MA, Reddy GB. Carboxymethyl lysine induces EMT in podocytes through transcription factor ZEB2: Implications for podocyte depletion and proteinuria in diabetes mellitus. Arch Biochem Biophys. 2016; 590:10-19.
9. Zhao L, Wang X, Sun L, Nie H, Liu X, Chen Z, Guan G. Critical role of serum response factor in podocyte epithelial-mesenchymal transition of diabetic nephropathy. Diab Vasc Dis Res. 2016; 13:81-92.
10. Dai H, Zhang Y, Yuan L, Wu J, Ma L, Shi H. CTGF mediates high-glucose induced epithelial-mesenchymal transition through activation of beta-catenin in podocytes. Ren Fail. 2016; 16:1-6.
11. Sun LN, Chen ZX, Liu XC, Liu HY, Guan GJ, Liu G. Curcumin ameliorates epithelial-to-mesenchymal transition of podocytes in vivo and in vitro via regulating caveolin-1. Biomed Pharmacother. 2014; 68:1079-1088.
12. Chen T, Zheng LY, Xiao W, Gui D, Wang X, Wang N. Emodin ameliorates high glucose induced-podocyte epithelial-mesenchymal transition in-vitro and in-vivo. Cell Physiol Biochem. 2015; 35:1425-1436.
13. Li G, Li CX, Xia M, Ritter JK, Gehr TW, Boini K, Li PL. Enhanced epithelial-to-mesenchymal transition associated with lysosome dysfunction in podocytes: Role of p62/Sequestosome 1 as a signaling hub. Cell Physiol Biochem. 2015; 35:1773-1786.
14. Zhang C, Xia M, Boini KM, Li CX, Abais JM, Li XX, Laperle LA, Li PL. Epithelial-to-mesenchymal transition in podocytes mediated by activation of NADPH oxidase in hyperhomocysteinemia. Pflugers Arch. 2011; 462:455-467.
15. Jung E, Kim J, Ho Kim S, Kim S, Cho MH. Gemigliptin improves renal function and attenuates podocyte injury in mice with diabetic nephropathy. Eur J Pharmacol. 2015; 761:116-124.
16. Yang F, Huang XR, Chung AC, Hou CC, Lai KN, Lan HY. Essential role for Smad3 in angiotensin II-induced tubular epithelial-mesenchymal transition. J Pathol. 2010; 221:390-401.
17. Tang R, Yang C, Tao JL, You YK, An N, Li SM, Wu HL, Liu HF. Epithelial-mesenchymal transdifferentiation of renal tubular epithelial cells induced by urinary proteins requires the activation of PKC-alpha and betaI isozymes. Cell Biol Int. 2011; 35:953-959.
18. Jha JC, Thallas-Bonke V, Banal C, Gray SP, Chow BS, Ramm G, Quaggin SE, Cooper ME, Schmidt HH, Jandeleit-Dahm KA. Podocyte-specific Nox4 deletion affords renoprotection in a mouse model of diabetic nephropathy. Diabetologia. 2016; 59:379-389.
19. Loeffler I, Wolf G. Epithelial-to-Mesenchymal Transition in Diabetic Nephropathy: Fact or Fiction? Cells. 2015; 4:631-652.
20. Ziyadeh FN, Wolf G. Pathogenesis of the podocytopathy and proteinuria in diabetic glomerulopathy. Curr Diabetes Rev. 2008; 4:39-45.
21. Zhan HX, Wang Y, Li C, Xu JW, Zhou B, Zhu JK, Han HF, Wang L, Wang YS, Hu SY. LincRNA-ROR promotes invasion, metastasis and tumor growth in pancreatic cancer through activating ZEB1 pathway. Cancer Lett. 2016; 374:261-271.

(Received November 29, 2016; Revised December 27, 2016; Accepted December 31, 2016)

Original Article

DOI: 10.5582/bst.2016.01217

An ethanol extract of *Aster yomena* (Kitam.) Honda inhibits lipopolysaccharide-induced inflammatory responses in murine RAW 264.7 macrophages

Hye-Joo Kang[1,2,§], Ji-Suk Jeong[3,§], No-Jin Park[4], Geun-Bae Go[4], Sung Ok Kim[5], Cheol Park[6], Byung Woo Kim[7], Su-Hyun Hong[1,*], Yung Hyun Choi[1,2,*]

[1] *Department of Biochemistry, Dongeui University College of Korean Medicine, Busan, Korea;*
[2] *DAnti-Aging Research Center & Blue-Bio Industry RIC, Dongeui University, Busan, Korea;*
[3] *Gurye Wild Flower Institute, Gurye, Korea;*
[4] *Gurye-gun Agricultural Center, Gurye, Korea;*
[5] *Department of Food Science & Biotechnology, College of Engineering, Kyungsung University, Busan, Korea;*
[6] *Department of Molecular Biology, College of Natural Sciences & Human Ecology, Dongeui University, Busan, Korea;*
[7] *Department of Life Science and Biotechnology, College of Natural Sciences & Human Ecology, Dongeui University, Busan, Korea.*

Summary ***Aster yomena* (Kitam.) Honda has been widely used as a traditional herbal medicine for centuries to treat cough, asthma, insect bites, etc. Recent reports indicate that *A. yomena* possesses a wide spectrum of pharmacological activities; however, few experiments have described its anti-inflammatory properties. The present study examined the anti-inflammatory effects of an ethanol extract of *A. yomena* leaves (EEAY) on lipopolysaccharide (LPS)-stimulated murine RAW 264.7 macrophages. Treatment with EEAY significantly reduced the secretion of pro-inflammatory molecules, such as nitric oxide and interleukin-1β, in LPS-stimulated RAW 264.7 cells, without incurring any significant cytotoxicity. These protective effects were accompanied by a marked reduction in the expression of regulatory genes at the transcription level. Treatment with EEAY also inhibited the DNA-binding activity of nuclear factor-κB (NF-κB) by suppression of nuclear translocation of NF-κB and by degradation of the inhibitor of NF-κB; these effects were associated with suppression of the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase signaling pathways. The EEAY treatment also potently suppressed LPS-induced toll like receptor (TLR) 4 expression and attenuated the binding of LPS to the macrophage cell surface. In addition, EEAY treatment markedly inhibited LPS-induced accumulation of intracellular reactive oxygen species in RAW 264.7 macrophages. Therefore, the inhibitory effects of EEAY on LPS-stimulated inflammatory responses in RAW 264.7 macrophages were apparently associated with suppression of the TLR-mediated NF-κB signaling pathway. More work is needed to fully understand the critical role and clinical usefulness of EEAY treatment, but the findings of the present study provide some insights into the potential of EEAY as a therapeutic agent for treatment of inflammatory disorders.**

Keywords: *Aster yomena* (Kitam.) Honda, macrophages, anti-inflammation, NF-κB TLR4

Released online in J-STAGE as advance publication February 7, 2017.

[§]These authors contributed equally to this works.
**Address correspondence to:*
Drs. Yung Hyun Choi and Su-Hyun Hong, Department of Biochemistry, College of Korean Medicine, Dongeui University, Busan 47227, Korea.
E-mail: choiyh@deu.ac.kr (Choi YH) and hongsh@deu.ac.kr (Hong SH)

1. Introduction

Inflammation is one of the first protective responses of the body and involves activation of immune system processes. The inflammatory response is a highly regulated self-limiting process for identifying and destroying invading pathogens and restoring normal tissue structure and function (*1,2*). However, in many

diseases, an excessive inflammatory response is a very common and important basic pathologic process (*2,3*). Inflammatory stimulants, such as the lipopolysaccharide (LPS) endotoxins of Gram-negative bacteria, activate macrophages through Toll-like receptor 4 (TLR4), a member of the TLR family. These macrophages, together with neutrophils and dendritic cells, act as the first cellular mediators of the inflammatory process (*4-6*).

TLR4 activation by LPS triggers activation of several intracellular signaling pathways, including phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs). This further activation ultimately allows nuclear translocation of nuclear factor κB (NF-κB), a dimer of p65 and p50 subunits, which, in turn, promotes secretion of pro-inflammatory mediators, including nitric oxide (NO), and pro-inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) (*7-9*). Overproduction of these pro-inflammatory molecules eventually leads to deleterious consequences associated with pathogenesis of many inflammatory diseases (*9,10*).

Another important component of inflammation is oxidative stress, which reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify these reactive intermediates (*11,12*). Overproduction of ROS by activated macrophages is an important contributor to the manifestation of inflammation (*13,14*), and ROS are also involved in the production of inflammatory cytokines in LPS-stimulated macrophages (*15*). Suppression of production of inflammatory factors by blocking macrophage activation can be viewed as a valuable therapeutic approach for relieving progression of inflammatory disorders (*16,17*). Development of anti-inflammatory agents that can prevent activation of LPS signaling and its downstream molecules, is therefore a necessary prerequisite for prevention and treatment of various inflammatory diseases.

One well recognized source of anti-inflammatory compounds are the botanical materials used as traditional medicines in many cultures. One of these sources is *Aster yomena* (Kitam.) Honda, an edible vegetable and perennial herb that belongs to the Asteraceae family. This plant is widely distributed in Asia and has been used in traditional medicine for the treatment of various diseases, such as cough, asthma, and insect bites (*18*). Previous studies have indicated that leaf extracts and compounds from this plant possess many pharmacological properties, including anti-microbial (*19,21*), antioxidant (*21,22*), anti-coagulant (*23*), and peroxynitrite-scavenging (*24*) activities. A recent study indicated that some of the phenolic compounds produced by *A. yomena* were potent inhibitors of IL-6 production in TNF-α stimulated osteoblast cells, a clear indication of their anti-inflammatory potential (*25*). However, to the best of our knowledge, the molecular mechanisms involved in this anti-inflammatory action have remained elusive. Therefore, the present study was conducted as part of our on-going research program that seeks out novel anti-inflammatory active substances from traditional medicinal resources. Here, we investigated an ethanol extract of *A. yomena* leaves (EEAY) for its anti-inflammatory action in LPS-stimulated RAW 264.7 macrophage cells.

2. Materials and Methods

2.1. *Preparation of the EEAY*

The dried leaves of *A. yomena* were obtained from Gurye Wild Flower Institute (Gurye, Republic of Korea) and authenticated by Professor S.H. Hong, Department of Biochemistry, Dongeui University College of Korean Medicine (Busan, Republic of Korea). The leaves (50 mg) were cut into small pieces, ground into a fine powder, and then soaked in 70% ethanol (500 mL) for 2 days. The extracted liquid was filtered twice through Whatman No. 3 filter paper to remove any insoluble materials and then concentrated using a rotary evaporator (Rikakikai Co., Ltd., Tokyo, Japan) as previously described (*26*). The concentrated extract (EEAY) was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, MO, USA) to a final concentration of 200 mg/mL (extract stock solution) and was subsequently diluted with cell culture medium to the desired concentration prior to use.

2.2. *Cell culture*

The murine macrophage RAW 264.7 cell line was obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Daegu, Republic of Korea) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin (WelGENE Inc.).

2.3. *Cell viability assay*

Cell viability was determined with a colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co.) assay. In brief, RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and incubated at 37°C for 24 h. Cells were treated with various concentrations of EEAY for 24 h or pretreated with EEAY for 1 h before stimulation with 100 ng/mL LPS for 24 h. After incubation, the medium was discarded, and MTT solution (5 mg/mL in phosphate-buffered saline, PBS) was added to each well and incubated for another 3 h at 37°C. The medium was removed and DMSO was added to dissolve the formazan dye. The optical density was then read at 560 nm using a

microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) to determine cell viability (*27*).

2.4. *Measurement of NO production in RAW 264.7 macrophages*

The production of NO in culture supernatants was assayed using Griess reagent (Sigma-Aldrich Chemical Co.). In brief, cells were pretreated with various concentrations of EEAY for 1 h and stimulated with LPS for 24 h. The supernatant was then collected and mixed with the same volume of Griess reagent for 10 min at room temperature in the dark. Absorbance was measured at 540 nm with a microplate reader, and NO concentrations were calculated by using a standard curve generated with known concentrations of sodium nitrite (*28*). Fresh culture medium was used as the blank in all experiments.

2.5. *Measurement of IL-1β production in RAW 264.7 macrophages*

Inhibitory efficacy of EEAY on the production of IL-1β was measured by culturing cells under the same conditions used for the NO measurement assay. Levels of IL-1β concentrations in the culture media were determined with a selective enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions (*29*).

2.6. *RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions and then reverse transcribed using an M-MLV reverse transcriptase kit (BioNEER, Daejeon, Republic of Korea) to produce cDNAs. RT-generated cDNAs encoding inducible NO synthase (iNOS) and IL-1β genes were amplified by PCR using the desired primers (BioNEER). The PCR reaction was initiated at 94°C for 2 min, followed by 31 cycles of 94°C for 30 sec, 30 sec annealing temperature, 72°C for 30 sec, and a final extension step at 72°C for 5 min. Following amplification, PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide (EtBr, Sigma-Aldrich Chemical Co.), and visualized by ultraviolet illumination. In a parallel experiment, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

2.7. *Protein extraction and Western blot analysis*

Cell pellets were collected and resuspended in extraction lysis buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol], for 30 min at 4°C. In a parallel experiment, nuclear and cytosolic proteins were separated using NE-PER nuclear and cytosolic extraction reagents (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's protocol. Protein concentration in the cell lysate was determined using a DC™ Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of protein from each sample were separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidine fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were incubated overnight at 4°C with the corresponding primary antibodies purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Cell Signaling Technology, Inc. (Boston, MA, USA). Membranes were then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Amersham Co., Arlington Heights, IL, USA) at room temperature for 2 h. Using an enhanced chemiluminescence (ECL, Amersham Co.) detection system, immunoreactive bands were monitored and exposed to X-ray film.

2.8. *Electrophoretic mobility assay (EMSA)*

The EMSA was performed using the nuclear extract. Synthetic complementary NF-κB binding oligonucleotides (Santa Cruz Biotechnology, Inc.) were 3'-biotinylated using a biotin 3'-end DNA labeling kit (Pierce Biotechnology), according to the manufacturer's instructions, and annealed for 30 min at room temperature. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5X Tris borate/EDTA before being transferred onto a positively charged nylon membrane (Hybond™N^+) in 0.5X Tris borate/EDTA at 100 V for 30 min. The transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2. Horseradish peroxidase-conjugated streptavidin was used according to the manufacturer's instructions to detect the transferred DNA.

2.9. *Immunofluorescence assay*

NF-κB p65 nuclear translocalization was detected by an immunofluorescence assay. For this study, RAW 246.7 cells were pretreated with EEAY for 1 h and then stimulated with LPS for 1 h. The cells were fixed with 3.7% paraformaldehyde in PBS for 10 min at 4°C, permeabilized with 0.4% Triton X-100 in PBS for 10 min, and blocked with 5% bovine serum albumin for 1 h. Cells were probed with anti-p65 NF-κB antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C and then incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 2

h at room temperature. The position of the cell nucleus was determined with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemical Co.) solution (1 mg/mL) for 15 min. After washing with PBS, fluorescence was visualized using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Cells were also stimulated with Alexa Fluor 488-conjugated LPS (100 ng/mL, AF-LPS; Invitrogen Life Technologies) for 30 min in the presence or absence of EEAY to assay for LPS/TLR4 complex formation. Cells were fixed, stained with anti-TLR4 antibody for 90 min at 4˚C, and then incubated with secondary antibodies conjugated with Alexa Fluor 594 (Invitrogen Life Technologies) for 1 h. Stained cells were observed under a fluorescence microscope.

2.10. *Measurement of ROS generation in RAW 264.7 macrophages*

The ROS levels in RAW 264.7 cells were measured by washing the cells twice with PBS and then lysing them with 1% Triton X-100 in PBS for 10 min at 37°C. Cells were then stained with 10 μM 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR, USA) for 20 min at room temperature in the dark. Green fluorescence emitted by DCF was recorded at 515 nm using a flow cytometer (Becton Dickinson, San Jose, CA, USA) (*30*). Images showing the generation of intracellular ROS were also obtained using a fluorescence microscope.

2.11. *Statistical analysis*

All data are presented as mean ± standard deviation (SD). Significant differences among groups were determined using the unpaired Student's *t*-test. A value of $p < 0.05$ was accepted as an indication of statistical significance. All the figures shown here reflect data obtained from at least three independent experiments.

3. Results

3.1. *Cytotoxic effects of EEAY and LPS on RAW 264.7 macrophages*

The possibility of cytotoxicity caused by EEAY treatment was excluded by treating RAW 264.7 cells with various concentrations of EEAY for 24 h. The MTT assay showed no cytotoxicity at concentrations up to 500 μg/mL EEAY in the presence or absence of 100 ng/mL LPS (Figure 1). We therefore selected 300 μg/mL EEAY as the maximum concentration for further experiments using RAW 264.7 cells.

3.2. *EEAY inhibits LPS-induced NO and IL-1β production in RAW 264.7 macrophages*

Inhibitory properties of EEAY were tested on the LPS-induced production of NO and IL-1β as a representative pro-inflammatory mediator and cytokine, respectively (*8,9*), in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of EEAY for 1 h and then stimulated with 100 ng/mL LPS for another 24 h. The levels of NO and IL-1β in the culture medium were determined by the Griess reaction assay and ELISA, respectively. As shown in Figure 2A and B, stimulation

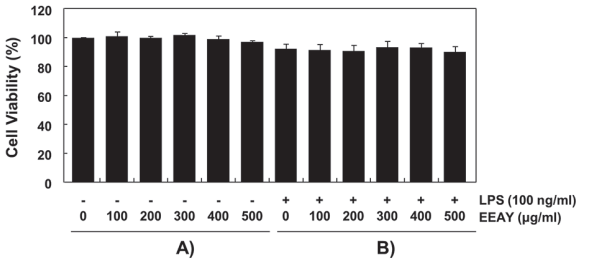

Figure 1. Effect of an ethanol extract of *Aster yomena* leaves (EEAY) on the cell viability of RAW 264.7 macrophages. Cells were treated with various concentrations of EEAY for 24 h **(A)** or pretreated with the indicated concentrations of EEAY for 1 h prior to lipopolysaccharide (LPS) (100 ng/mL) treatment for 24 h **(B)**. Cell viability was assessed with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay, and the results are expressed as the percentage of surviving cells over control cells (no addition of EEAY and LPS).

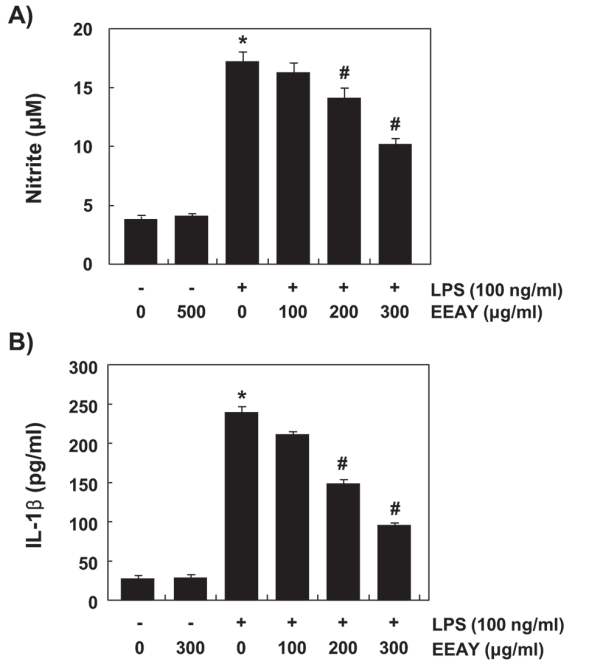

Figure 2. Inhibition of NO and IL-1β production by an ethanol extract of *Aster yomena* leaves (EEAY) in LPS-stimulated RAW 264.7 macrophages. Cells were pretreated with the indicated concentrations of EEAY for 1 h prior to incubation with 100 ng/mL LPS for 24 h. Levels of NO **(A)** and IL-1β **(B)** in culture media were measured by Griess assay and a commercial ELISA kit, respectively. Each value indicates the mean ± SD and is representative of the results obtained from three independent experiments ($^{*}p < 0.05$ compared to the control; $^{\#}p < 0.05$ compared to cells cultured with 100 ng/mL LPS).

with LPS markedly induced the production of NO and IL-1β when compared to cells not stimulated with LPS; however, EEAY significantly inhibited NO and IL-1β secretion in a concentration-dependent manner.

3.3. *EEAY suppresses LPS-induced iNOS and IL-1β expressions at the protein and mRNA levels in RAW 264.7 macrophages*

We subsequently used RT-PCR and Western blot analysis to investigate the possible relationship between the inhibitory effects of EEAY on NO and IL-1β production and regulation of expression of their synthesis enzymes. As indicated in Figure 3A and B, EEAY effectively inhibited protein and mRNA expression of iNOS and IL-1β in LPS-stimulated RAW 264.7 cells. Therefore, EEAY appeared to suppress NO and IL-1β production in LPS-stimulated RAW 264.7 cells by reducing expression of their encoding genes at the transcriptional level.

3.4. *EEAY blocks LPS-induced NF-κB activation in RAW 264.7 macrophages*

Active NF-κB translocates to the nucleus, where it activates its target genes including iNOS and IL-1β by binding to their promoter regions (*31,32*). We used immunoblotting of cytoplasmic and nuclear extracts to investigate the possibility that EEAY attenuates LPS-induced activation of the NF-κB signaling pathway. EEAY pretreatment inhibited nuclear accumulation of the NF-κB p65 subunit, as well as IκBα degradation, in LPS-stimulated RAW 264.7 cells (Figure 4A). Immunocytochemistry analysis also indicated that NF-κB p65 was normally sequestered in cytoplasm, whereas robust nuclear accumulation of NF-κB p65 was observed in RAW 264.7 microglial cells following stimulation with LPS. However, LPS-mediated nuclear translocation of NF-κB was effectively abolished by pretreatment with EEAY (Figure 4B). In agreement with the immunoblotting data, DNA-binding activity of NF-κB was markedly increased in response to LPS treatment, whereas pretreatment of the cells with EEAY significantly reduced DNA-binding activity of NF-κB (Figure 4C). These data indicate that EEAY inhibits LPS-induced NF-κB activation by attenuating the IκBα degradation.

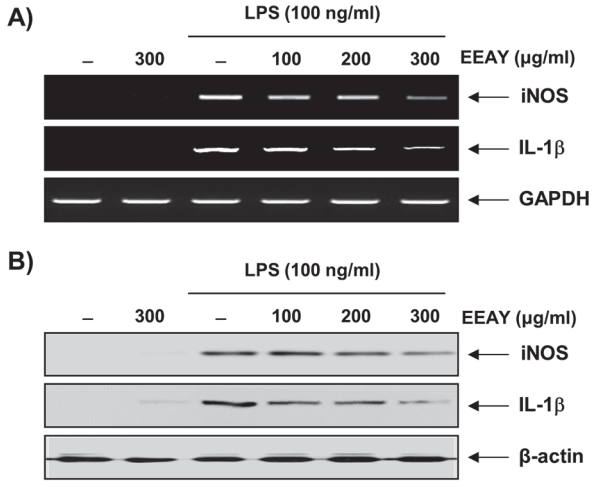

Figure 3. Suppression of iNOS and IL-1β expression by an ethanol extract of *Aster yomena* leaves (EEAY) in LPS-stimulated RAW 264.7 macrophages. Cells were pretreated with different concentrations of EEAY for 1 h, followed by stimulation with 100 ng/mL LPS for 24 h. **(A)** Total RNAs were prepared for RT-PCR analysis of the iNOS and IL-1β mRNA expression using the indicated primers. **(B)** Cell lysates were prepared for Western blot analysis with antibodies specific for murine iNOS and COX-2, and an enhanced chemiluminescence (ECL) detection system. Experiments were repeated three times and similar results were obtained. GAPDH and β-actin were used as internal controls for the RT-PCR and Western blot analysis, respectively.

3.5. *EEAY attenuates LPS-induced activation of PI3K/Akt and MAPK signaling pathways in RAW 264.7 macrophages*

LPS-induced NF-κB activation has a known association with activation of the PI3K/Akt and MAPK signaling pathways (*7-9*). Therefore, we examined the effect of EEAY on LPS-induced activation of Akt, a downstream kinase of PI3K, and three kinases of the MAPK pathway: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. As shown in Figure 5, the level of unphosphorylated Akt was unaffected by either LPS or EEAY treatment, whereas phosphorylation of Akt showed a marked increase within 30 min following stimulation with LPS. Stimulation of the cells with LPS induced rapid activation of ERK, JNK, and p38 MAPK, with peak levels of each phosphorylated MAPK occurring 30 min after addition of LPS; the unphosphorylated forms were unaltered. However, pretreatment with EEAY resulted in significant blockage of LPS-induced phosphorylation of Akt, as well as of the MAPKs. These results revealed that EEAY is capable of disrupting the PI3K/Akt and MAPK signal transduction pathways that are activated by LPS in RAW 264.7 cells and that EEAY might be involved in inhibition of inflammatory mediator release.

3.6. *EEAY inhibits LPS-induced TLR4 expression and the interaction between LPS and TLR4 in RAW 264.7 macrophages*

We next assessed the effects of EEAY on the LPS-activated TLR4 signaling pathway. As indicated in Figure 6A, the expression of TLR4 protein was markedly up-regulated in LPS-treated RAW 264.7 cells compared with untreated cells. However, pretreatment of the cells with EEAY induced a concentration-dependent inhibition of this increased expression of TLR4. We also used AF-conjugated LPS to examine the influence of EEAY on LPS binding to TLR4 on the RAW 264.7 macrophage

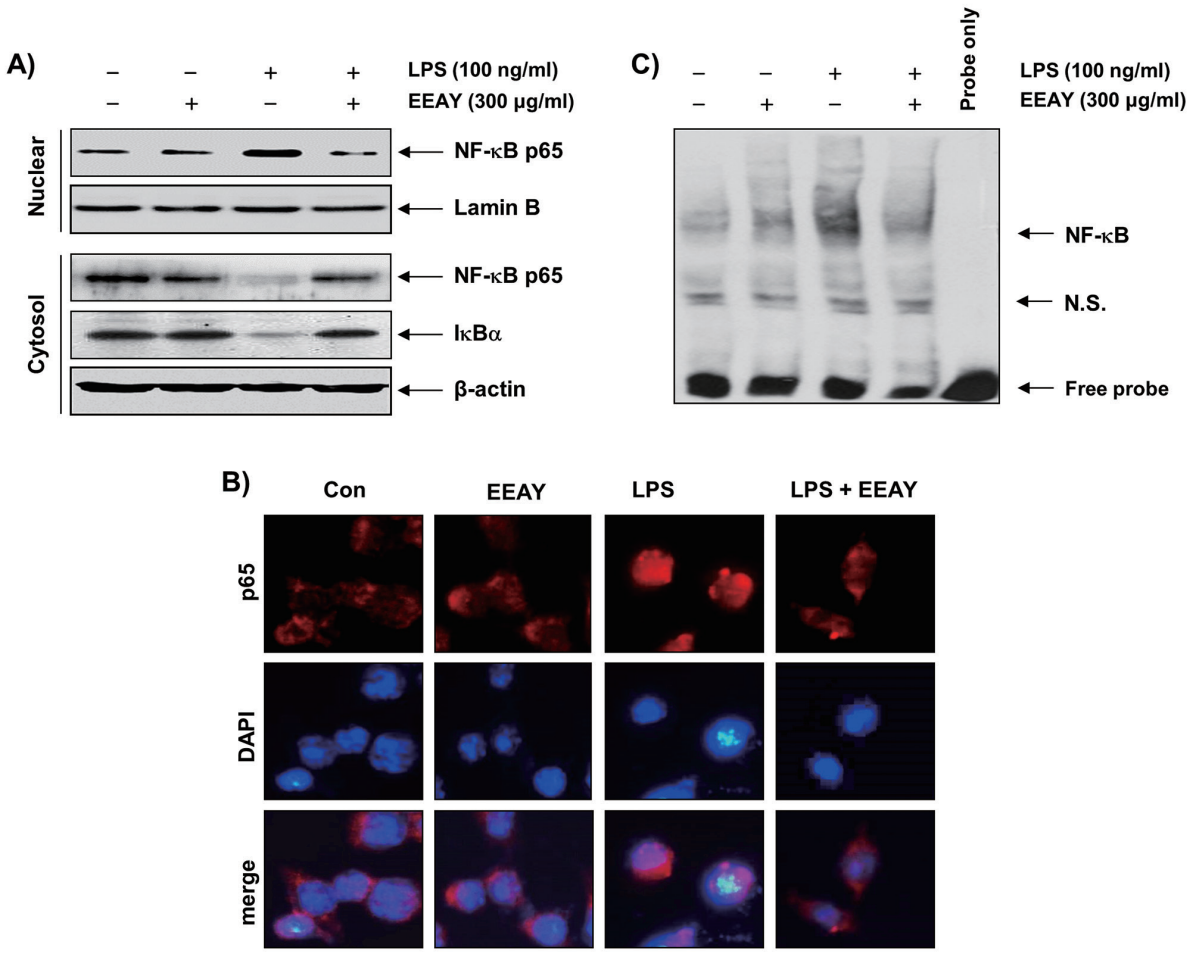

Figure 4. Inhibition of NF-κB activation by an ethanol extract of *Aster yomena* leaves (EEAY) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. (A) Cells were preincubated with 300 μg/mL EEAY for 1 h before 100 ng/mL LPS treatment for 30 min. Nuclear and cytosolic proteins were prepared for Western blot analysis using anti-NF-κB p65 and anti-IκB-α antibodies, and an enhanced chemiluminescence (ECL) detection system. Lamin B and β-actin were used as internal controls for the nuclear and cytosolic fractions, respectively. **(B)** Cells were pretreated with 300 μg/mL EEAY for 1 h before stimulation with LPS for 30 min. Nuclear extracts were subsequently assayed for NF-κB activity by an electrophoretic mobility assay (EMSA). **(C)** Cells were pretreated with 100 μg/mL EEAY for 1 h before 100 ng/mL LPS treatment. After 30 min of incubation, the localization of NF-κB p65 was visualized with fluorescence microscopy after immunofluorescence staining with anti-NF-κB p65 antibody and a fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG antibody (green). Cells were also stained with DAPI to visualize the nuclei (blue). Results are representative of those obtained from three independent experiments.

surface. As indicated in Figure 6B, treatment of cells with AF-LPS alone resulted in increased fluorescence of AF-LPS and TLR4 on the macrophage surface when compared with the control group. However, this binding of AF-LPS to the membrane of RAW 264.7 cells was blocked by pretreatment with EEAY, as indicated by attenuation of the fluorescence intensity. Therefore, EEAY might block LPS-induced activation of the TLR4 signaling pathway by suppression of LPS binding to the cell surface.

3.7. *EEAY reduces LPS-induced accumulation of ROS in RAW 264.7 macrophages*

Oxidative stress is partially responsible for the initiation of inflammation (*11,14*). Therefore, we used DCF-DA staining to examine the effects of EEAY on LPS-induced generation of ROS in RAW 264.7 cells. The flow cytometry results indicated an accumulation of intracellular ROS at 1 h, and the ROS levels continued to increase up to 3 h following LPS treatment (Figure 7A). However, this increase in LPS-stimulated ROS production was markedly attenuated by pretreatment with EEAY (Figure 7B). The positive control, generated by treatment with the ROS scavenger N-acetyl-l-cysteine (NAC), also showed effective attenuation of LPS-induced ROS generation, and EEAY itself did not contribute to ROS generation. Taken together, the results suggest that the antioxidant properties of EEAY may be responsible for its anti-inflammatory properties in RAW 264.7 cells.

4. Discussion

When cells are infected by Gram-negative bacteria, LPS acts as a prototypical ligand of the membrane-bound

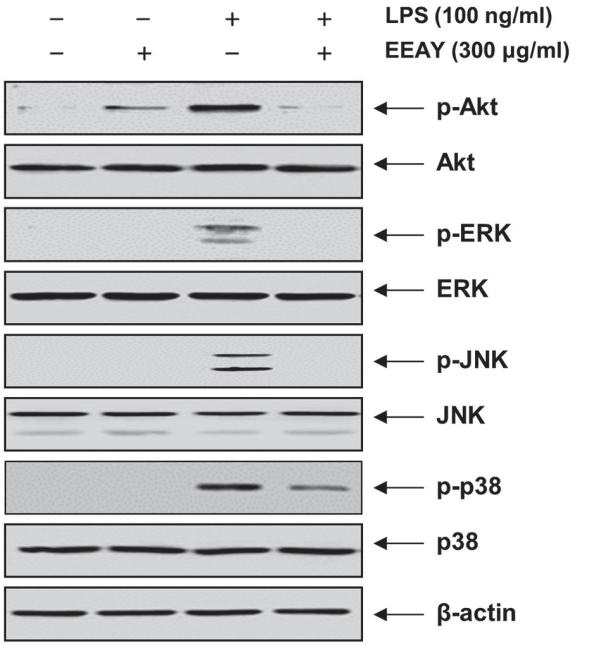

Figure 5. Effect of an ethanol extract of *Aster yomena* leaves (EEAY) on lipopolysaccharide (LPS)-induced phosphorylation of Akt and MAPKs in RAW 264.7 macrophages. Cells were pretreated with 300 μg/ml EEAY for 1 h prior to exposure to LPS for 30 min, and total proteins were isolated. The proteins were subjected to SDS-polyacrylamide gels, followed by Western blot analysis using the indicated antibodies, and an enhanced chemiluminescence (ECL) detection system. β-actin was used as the internal control. The experiments were repeated three times and similar results were obtained.

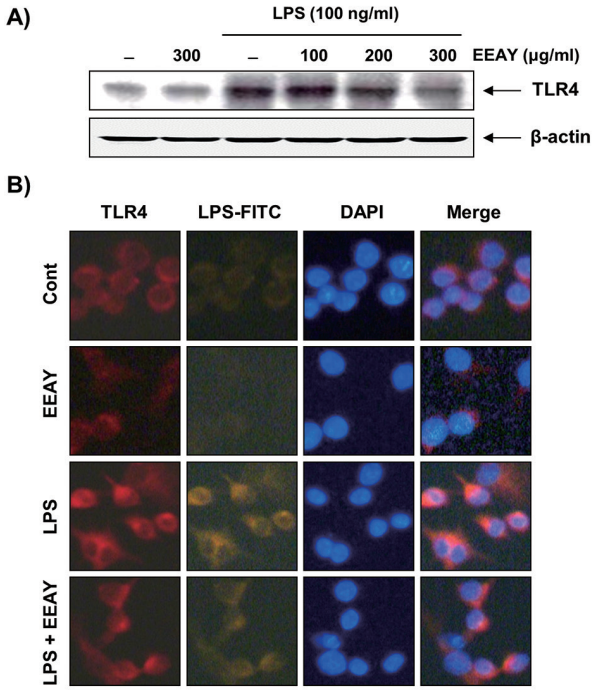

Figure 6. Attenuation of lipopolysaccharide (LPS)-induced TLR4 expression, and interaction between LPS and TLR4 by an ethanol extract of *Aster yomena* leaves (EEAY) in LPS-stimulated RAW 264.7 macrophages. (A) Cells were pretreated with the indicated concentrations of EEAY for 1 h prior to LPS treatment, and total protein was isolated at 6 h after LPS treatment. Levels of TLR4 protein were assessed by Western blot analysis using anti-TLR4 antibody, and an enhanced chemiluminescence (ECL) detection system. β-actin was used as the internal control. **(B)** Cells were incubated with Alexa Fluor 488-conjugated LPS (AF-LPS) for 1 h in the absence or presence of 300 μg/mL EEAY, and then distribution of AF-LPS and TLR4 was detected by a fluorescence microscopy.

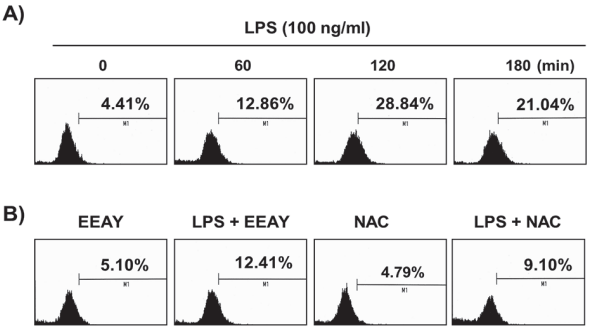

Figure 7. Effect of an ethanol extract of *Aster yomena* leaves (EEAY) on LPS-induced reactive oxygen species (ROS) production in RAW 264.7 macrophages. RAW 264.7 cells were treated with 100 ng/mL lipopolysaccharide (LPS) for the indicated time **(A)** or pre-incubated with or without 300 μg/mL EEAY or 10 mM NAC for 1 h and then stimulated with 100 ng/mL LPS for 2 h **(B)**. Cells were incubated with 10 μM 2',7'-dichlorofluorescein diacetate (DCF-DA) for 30 min at 37°C. Cells were collected and dichlorofluorescein (DCF) fluorescence was measured by flow cytometry. Values were represented as means ± SD of two independent experiments.

TLR4 pattern recognition receptor in macrophages (*5,6*). The activation of the TLR4 pathway triggers intracellular signaling pathways that culminate in the activation of several signaling molecules, such as NF-κB, PI3K/Akt, and MAPKs (*8,9*). This activation of macrophages then triggers inflammation through the production and release of pro-inflammatory mediators and cytokines, like NO and IL-1β, respectively, which play a central role in initiating and sustaining the inflammatory response (*4,6*). Stimulation of macrophages with LPS increases expression of the iNOS gene and promotes excessive NO release *via* decomposition of L-arginine. iNOS is also activated in response to inflammatory stimuli such as cytokines, IL, and bacterial endotoxin (*33,34*). In addition to its role in NO release, IL-1β also serves as one of the major pro-inflammatory cytokines released following LPS stimulation of macrophages, and its excessive production has been linked to development of chronic inflammatory diseases (*35,36*). In the present study, EEAY pretreatment prevented the LPS-stimulated production of NO and IL-1β in RAW 264.7 macrophages by potent inhibition of iNOS and IL-1β expression at both the protein and mRNA levels. This inhibition occurred without cytotoxicity, supporting EEAY as a promising target for inhibiting the early steps in inflammatory pathways.

Transcription factor NF-κB plays a significant role in the LPS-induced expression of many inflammation-related enzymes and cytokines (*31,32*). NF-κB is normally retained in the cytoplasm due to its association

with its endogenous inhibitor, IκB-α, which renders NF-κB inactive. Once activated by inflammatory stimuli, such as LPS, IκB-α is rapidly phosphorylated and degraded by an ubiquitin-proteasome pathway. Thereafter, NF-κB translocates to the nucleus, where it binds to target gene promoters and drives transcriptional activation of various pro-inflammatory genes (*6,32*). Therefore, agents that effectively modulate NF-κB activation are promising candidates for treatment of various inflammatory diseases. Our results revealed that LPS stimulation resulted in nuclear translocation of NF-κB p65 in RAW 264.7 macrophages; however, EEAY attenuated this nuclear translocation by inhibiting LPS-induced IκB-α degradation. In addition, EEAY suppressed binding of NF-κB to DNA. Consequently, inactivation of the NF-κB signaling pathway by EEAY might lead to down-regulation of pro-inflammatory factors and a consequent reduction in their release. This would prevent inflammation and might be the molecular mechanism underlying the anti-inflammatory effects of EEAY.

One response to inflammatory stimuli is the phosphorylation of intracellular signaling molecules belonging to the PI3K/Akt and MAPK signaling pathways; this often accelerates production of pro-inflammatory mediators and cytokines (*8,9*). The rate of phosphorylation of these molecules, which act as upstream regulators of NF-κB, is also well recognized to increase in LPS-stimulated macrophages, so that phosphorylation plays a critical role in progression of inflammation (*31,37*). The effects of EEAY on LPS-induced phosphorylation of Akt and MAPKs were therefore examined to further explore mechanisms underlying its anti-inflammatory effects. Immunoblotting experiments confirmed that EEAY treatment attenuated the extent of Akt phosphorylation, indicating inactivation of the PI3K/Akt signaling pathway. EEAY treatment also down-regulated LPS-triggered activation of all three MAPKs (ERK, JNK, and p38 MAPK) studied in the RAW 264.7 macrophages. Therefore, EEAY suppressed PI3K/Akt and MAPK signaling pathways in LPS-treated RAW 264.7 macrophages, and then inactivated activation of the NF-κB signaling pathway to prevent activation of inflammation-related genes.

Many studies have indicated that the TLR family of pattern recognition receptors includes central mediators of the inflammatory response. Accumulating evidence now indicates that TLR4 specifically mediates signaling induced by LPS (*37,38*). A first step in the LPS/TLR4-mediated inflammatory signaling pathway is binding of LPS to TLR4, in conjunction with MYD88 adapters at the plasma membrane; this then initiates intracellular signaling cascades (*4,5*). Activation of TLR4 by LPS induces phosphorylation of PI3K/Akt and MAPK signaling molecules, as well as the IB kinase complex, eventually resulting in activation of NF-κB (*40,41*). Therefore, expression levels of TLR4 are significant as they enable optimal LPS responsiveness. In the present study, we found that TLR4 protein expression was highly stimulated by LPS and concentration-dependently inhibited by EEAY. Treatment with EEAY also markedly reduced formation of the complexes of LPS with TLR4, indicating that EEAY treatment could disrupt association of TLR4 with its adaptors, leading to inactivation of TLR4 in LPS-stimulated RAW 264.7 macrophages. Therefore, EEAY could inhibit an initial step of the intracellular signaling cascades by blocking the TLR4 signaling pathway and subsequently suppressing activation of NF-κB.

ROS accumulation and the compensatory response from the endogenous antioxidant network results in a redox imbalance that causes oxidative stress (*12,15*). Overwhelming generation of ROS is strongly associated with many other pathological conditions, including inflammation. Chronic inflammation causes an amplification in inflammatory signals in macrophages through activation of the NF-κB signaling pathway and over-expression of inflammation-associated genes (*41,42*). The inflammatory mediators and cytokines also promote an influx of macrophages that, in turn, accelerate intracellular accumulation of ROS (*11,14*). In addition, ROS, acting as a secondary messenger, participates in the TLR4-mediated inflammatory signaling pathway (*43*). Therefore, the profound ability of EEAY to suppress ROS in LPS-stimulated RAW 264.7 macrophages might be attributable to its ability to scavenge free radicals. The EEAY-mediated inhibition of ROS generation might also potentially inhibit the intracellular signaling cascade-dependent expression of pro-inflammatory mediators and cytokines, thereby explaining EEAY‘s strong anti-inflammatory properties.

In conclusion, the results presented here demonstrate that EEAY exerts potent anti-inflammatory effects in RAW 264.7 macrophages. Treatment of LPS-stimulated RAW 264.7 macrophages with EEAY significantly attenuated production of NO and IL-1β by reducing their corresponding gene expression. Anti-inflammatory effects of EEAY were mediated by suppression of NF-κB activation and subsequent attenuation of PI3K/Akt and MAPK signaling pathways. The ability of EEAY to inhibit the inflammatory response was associated, at least partially, with suppression of activation of TLR4 and a reduction of intracellular ROS production. The results of this study support use of EEAY as an alternative candidate for safe and effective treatment of inflammatory diseases.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) grant funded by the Korea government (2015R1A2A2A01004633 and 2016R1C1B2016529) and Blue-Bio Industry Regional Innovation Center

(RIC08-06-07) at Dongeui University as a RIC program under Ministry of Trade, Industry & Energy and Busan city.

References

1. Conti B, Tabarean I, Andrei C, Bartfai T. Cytokines and fever. Front Biosci. 2004; 9:1433-1449.
2. Freire MO, Van Dyke TE. Natural resolution of inflammation. Periodontol 2000. 2013; 63:149-164.
3. Amin AR, Attur M, Abramson SB. Nitric oxide synthase and cyclooxygenases: Distribution, regulation, and intervention in arthritis. Curr Opin Rheumatol. 1999; 11:202-209.
4. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature. 2000; 406:782-787.
5. Laird MH, Rhee SH, Perkins DJ, Medvedev AE, Piao W, Fenton MJ, Vogel SN. TLR4/MyD88/PI3K interactions regulate TLR4 signaling. J Leukoc Biol. 2009; 85:966-977.
6. Nikaido, H., Molecular basis of bacterial outer membrane permeability revisited Microbiol Mol Biol Rev. 2003; 67:593-656.
7. Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS Jr, Mayo MW. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-κB. Mol Cell Biol. 2000; 20:1626-1638.
8. Kim SH, Smith CJ, Van Eldik LJ. Importance of MAPK pathways for microglial pro-inflammatory cytokine IL-1 beta production. Neurobiol Aging. 2004; 25:431-439.
9. Muralidharan S, Mandrekar P. Cellular stress response and innate immune signaling: Integrating pathways in host defense and inflammation. J Leukoc Biol. 2013; 94:1167-1184.
10. McDaniel ML, Kwon G, Hill JR, Marshall CA, Corbett JA. Cytokines and nitric oxide in islet inflammation and diabetes. Proc Soc Exp Biol Med. 1996; 211:24-32.
11. Brüne B, Dehne N, Grossmann N, Jung M, Namgaladze D, Schmid T, von Knethen A, Weigert A. Redox control of inflammation in macrophages. Antioxid Redox Signal. 2013; 19:595-637.
12. Yoon SY, Hong GH, Kwon HS, Park S, Park SY, Shin B, Kim TB, Moon HB, Cho YS. S-adenosylmethionine reduces airway inflammation and fibrosis in a murine model of chronic severe asthma *via* suppression of oxidative stress. Exp Mol Med. 2016; 48:e236.
13. Varga A, Budai MM, Milesz S, Bácsi A, Tőzsér J, Benkő S. Ragweed pollen extract intensifies lipopolysaccharide-induced priming of NLRP3 inflammasome in human macrophages. Immunology. 2013; 138:392-401
14. Mills EL, O'Neill LA. Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal. Eur J Immunol. 2016; 46:13-21.
15. Haddad JJ, Land SC. Redox signaling-mediated regulation of lipopolysaccharide-induced proinflammatory cytokine biosynthesis in alveolar epithelial cells. Antioxid Redox Signal. 2002; 4:179-193.
16. Cunha TM, Verri WA Jr, Schivo IR, Napimoga MH, Parada CA, Poole S, Teixeira MM, Ferreira SH, Cunha FQ. Crucial role of neutrophils in the development of mechanical inflammatory hypernociception. J Leukoc Biol. 2008; 83:824-832.
17. Zhang L, Wang CC. Inflammatory response of macrophages in infection. Hepatobiliary Pancreat Dis Int. 2014; 13:138-152.
18. Sim JH, Lee HS, Lee S, Park DE, Oh K, Hwang KA, Kang HR, Ye SK, Kim HR. Anti-asthmatic activities of an ethanol extract of *Aster yomena* in an ovalbumin-induced murine asthma model. J Med Food. 2014; 17:606-611.
19. Park SH, Eom AH. Effects of mycorrhizal and endophytic fungi on plant community: A microcosm study. Mycobiology. 2007; 35:186-190.
20. Yun J, Lee H, Ko HJ, Woo ER, Lee DG. Fungicidal effect of isoquercitrin *via* inducing membrane disturbance. Biochim Biophys Acta. 2015; 1848:695-701.
21. Heo BG, Park YS, Chon SU, Lee SY, Cho JY, Gorinstein S. Antioxidant activity and cytotoxicity of methanol extracts from aerial parts of Korean salad plants. Biofactors. 2007; 30:79-89.
22. Lee W, Woo ER, Lee DG. Phytol has antibacterial property by inducing oxidative stress response in *Pseudomonas aeruginosa*. Free Radic Res. 2016; 50:1309-1318.
23. Choi JH, Kim DW, Park SE, Choi BS, Sapkota K, Kim S, Kim SJ. Novel thrombolytic protease from edible and medicinal plant *Aster yomena* (Kitam.) Honda with anticoagulant activity: Purification and partial characterization. J Biosci Bioeng. 2014; 118:372-377.
24. Nugroho A, Kim KH, Lee KR, Alam MB, Choi JS, Kim WB, Park HJ. Qualitative and quantitative determination of the caffeoylquinic acids on the Korean mountainous vegetables used for chwinamul and their peroxynitrite-scavenging effect. Arch Pharm Res. 2009; 32:1361-1367.
25. Kim AR, Jin Q, Jin HG, Ko HJ, Woo ER. Phenolic compounds with IL-6 inhibitory activity from *Aster yomena*. Arch Pharm Res. 2014; 37:845-851.
26. Park C, Jeong JS, Jeong JW, Kim YJ, Jung YK, Go GB, Kim SO, Kim GY, Hong SH, Yoo YH, Choi YH. Ethanol extract of *Kalopanax septemlobus* leaf induces caspase-dependent apoptosis associated with activation of AMPK in human hepatocellular carcinoma cells. Int J Oncol. 2016; 48:261-270.
27. Oh K, Moon HG, Lee DS, Yoo YB. Tissue transglutaminase-interleukin-6 axis facilitates peritoneal tumor spreading and metastasis of human ovarian cancer cells. Lab Anim Res. 2015; 31:188-197.
28. Lee H, Pyo MJ, Bae SK, Heo Y, Kim CG, Kang C, Kim E. Improved therapeutic profiles of PLA2-free bee venom prepared by ultrafiltration method. Toxicol Res. 2015; 31:33-40.
29. Wang L, Xu ML, Liu J, Wang Y, Hu JH, Wang MH. *Sonchus asper* extract inhibits LPS-induced oxidative stress and pro-inflammatory cytokine production in RAW264.7 macrophages. Nutr Res Pract. 2015; 9:579-585.
30. Eom SA, Kim DW, Shin MJ, Ahn EH, Chung SY, Sohn EJ, Jo HS, Jeon SJ, Kim DS, Kwon HY, Cho SW, Han KH, Park J, Eum WS, Choi SY. Protective effects of PEP-1-catalase on stress-induced cellular toxicity and MPTP-induced Parkinson's disease. BMB Rep. 2015; 48:395-400.
31. Kolyada AY, Madias NE. Transcriptional regulation of the human iNOS gene by IL-1beta in endothelial cells. Mol Med. 2001; 7:329-343.
32. Rigoglou S, Papavassiliou AG. The NF-κB signalling pathway in osteoarthritis. Int J Biochem Cell Biol. 2013; 45:2580-2584.
33. Joubert J, Malan SF. Novel nitric oxide synthase

inhibitors: A patent review. Expert Opin Ther Pat. 2011; 21:537-560.
34. Uehara EU, Shida Bde S, de Brito CA. Role of nitric oxide in immune responses against viruses: Beyond microbicidal activity. Inflamm Res. 2015; 64:845-852.
35. Dinarello CA. A clinical perspective of IL-1β as the gatekeeper of inflammation. A clinical perspective of IL-1β as the gatekeeper of inflammation. Eur J Immunol. 2011; 41:1203-1217
36. Palomo J, Dietrich D, Martin P, Palmer G, Gabay C. The interleukin (IL)-1 cytokine family – Balance between agonists and antagonists in inflammatory diseases. Cytokine. 2015; 76:25-37.
37. Li Q, Verma IM. NF-κB regulation in the immune system. Nat Rev Immunol. 2002; 2:725-734.
38. Yu H, Ha T, Liu L, Wang X, Gao M, Kelley J, Kao R, Williams D, Li C. Scavenger receptor A (SR-A) is required for LPS-induced TLR4 mediated NF-κB activation in macrophages. Biochim Biophys Acta. 2012; 1823:1192-1198.
39. Frobøse H, Rønn SG, Heding PE, Mendoza H, Cohen P, Mandrup-Poulsen T, Billestrup N. Suppressor of cytokine signaling-3 inhibits interleukin-1 signaling by targeting the TRAF-6/TAK1 complex. Mol Endocrinol. 2006; 20:1587-1596.
40. Jawan B, Kao YH, Goto S, Pan MC, Lin YC, Hsu LW, Nakano T, Lai CY, Sun CK, Cheng YF, Tai MH, Eng HL, Wang CS, Huang CJ, Lin CR, Chen CL. Propofol pretreatment attenuates LPS-induced granulocyte-macrophage colony-stimulating factor production in cultured hepatocytes by suppressing MAPK/ERK activity and NF-κB translocation. Toxicol Appl Pharmacol. 2008; 229:362-373.
41. Kauppinen A, Suuronen T, Ojala J, Kaarniranta K, Salminen A. Antagonistic crosstalk between NF-κB and SIRT1 in the regulation of inflammation and metabolic disorders. Cell Signal. 2013; 25:1939-1948.
42. Tan HY, Wang N, Li S, Hong M, Wang X, Feng Y. The reactive oxygen species in macrophage polarization: Reflecting its dual role in progression and treatment of human diseases. Oxid Med Cell Longev. 2016; 2016:2795090.
43. Yang FL, Yang YL, Liao PC, Chou JC, Tsai KC, Yang AS, Sheu F, Lin TL, Hsieh PF, Wang JT, Hua KF, Wu SH. Structure and immunological characterization of the capsular polysaccharide of a pyrogenic liver abscess caused by *Klebsiella pneumoniae*: Activation of macrophages through Toll-like receptor 4. J Biol Chem. 2011; 286:21041-21051.

(Received November 20, 2016; Revised January 2, 2017; Accepted January 11, 2017)

Original Article

DOI: 10.5582/bst.2016.01211

Yangjing Capsule extract promotes proliferation of GC-1 spg cells *via* up-regulated POU3F1 pathway

Baofang Jin[1,*], Bin Cai[1,*], Dalin Sun[1], Xindong Zhang[2], Yugui Cui[3], Weimin Deng[1], Chao Gao[3]

[1] *Andrology Department of Integrative Medicine, Zhongda Hospital, School of Medicine, Southeast University, Nanjing, China;*
[2] *Reproductive Medicine Center, the Affiliated Drum Tower Hospital, Medical College, Nanjing University, Nanjing, China;*
[3] *State Key Laboratory of Reproductive Medicine, Clinical Center of Reproductive Medicine, First Affiliated Hospital, Nanjing Medical University, Nanjing, China.*

Summary **As is similar to glial cell line-derived neurotrophic factor (GDNF), the Yangjing Capsule (YC) extract could also lead to proliferation of spermatogonial stem cells (SSCs) by stimulating the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway; however, the regulatory effect of YC extract on the expression of POU3F1 still remains unknown. The objective of this study is to determine whether the transcription factor POU3F1 is up-regulated by YC extract through the PI3K/AKT signaling pathway to regulate SSCs survival and proliferation. Cultured GC-1 spermatogonial (spg) cells were treated with 0.01, 0.1, and 1 mg/mL YC extract for 48 h. Cell viability was analyzed using MTT assay, while POU3F1 expression was quantitatively detected using real time-polymerase chain reaction and Western blot analysis. POU3F1, GDNF family receptor alpha1 (GFRα1) short interfering ribonucleic acid (siRNA), and LY294002 (PI3K inhibitor) were applied as blockers to explore the underlying pathway. After 48 h treatment with YC extract, GC-1 spg cells proliferated and POU3F1 expression was significantly increased in a dose-dependent manner. POU3F1 siRNA partially blocked those effects of YC extract. Both GFRα1 siRNA and LY294002, as upstream blockers, reduced POU3F1 expression induced by YC extract. The conclusion is that YC extract promotes proliferation of GC-1 spg cells *via* up-regulation of POU3F1. The potential mechanism is that YC extract triggers the activation of the PI3K/AKT pathway and then up-regulates POU3F1 expression.**

Keywords: Yangjing Capsule (YC), glial cell line-derived neurotrophic factor (GDNF), spermatogonial stem cells (SSCs), POU3F1

1. Introduction

Spermatogonial stem cells (SSCs) are essential for maintaining male fertility as well as species continuity and provide the foundation for spermatogenesis (*1*). The SSCs are the only stem cells in the body that undergo self-renewal throughout the lifetime and transmit genetic information to subsequent generations (*2,3*). Similar to other tissue-specific stem cell populations, the SSCs also maintain tissue homeostasis by retaining the capacity for self-renewal and differentiation (*4*), and their fate decisions are controlled by intrinsic molecular pathways which are activated by extrinsic signals such as growth factor stimuli. Self-renewal and differentiation of rodent SSCs including mouse (*5*), rat (*6,7*), and hamster (*8*), depend on the response to the growth factor glial cell line-derived neurotrophic factor (GDNF). By binding to the glycosylphosphatidylinositol-anchored cell surface molecule of GDNF family receptor alpha1 (GFRα1), GDNF is able to trigger the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway and up-regulate expression of POU3F1 and eventually lead to survival and proliferation of SSCs (*9-11*) (Figure 1).

The POU domain transcription factors, also known as the octamer (OCT)-binding family of transcription

Released online in J-STAGE as advance publication January 31, 2017.

*Address correspondence to:
Drs Baofang Jin and Bin Cai, Andrology Department of Integrative Medicine, Zhongda Hospital, School of Medicine, Southeast University, Nanjing 210009, China.
E-mail: hexiking@126.com (Jin BF); caibin394395@126.com (Cai B)

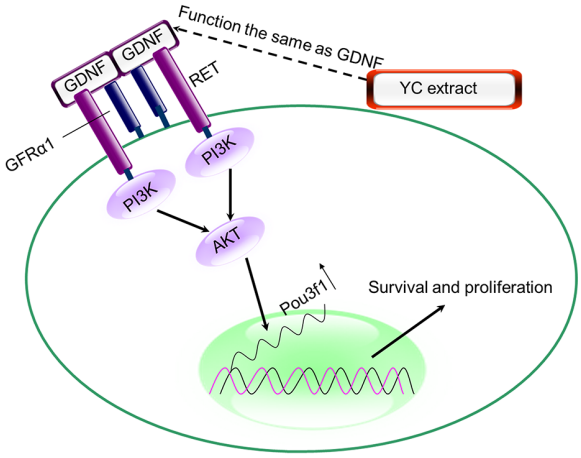

Figure 1. Mechanisms of GDNF and YC extract promoting the proliferation of spermatogonial stem cells (SSCs). GDNF binds with GFRα1 through RET tyrosine kinase to activate PI3K/AKT intracellular signaling pathway and up-regulate the transcription factor POU3F1, and thus leads to SSCs proliferation. The YC extract triggers the activation of PI3K/AKT pathway and then up-regulates POU3F1 expression similar to GDNF.

factors, have played diverse roles in cellular processes and stem cell functions (*12*). POU3F1 (also known as OCT6, TST-1) is a POU-subclass homeobox transcription factor, and it is expressed in testis as well as in brain. In previous studies, POU3F1 has been examined as a regulator of neural cell development (*13-15*). Targeted disruption of POU3F1 expression in mice caused neonatal lethality in which pups displayed abnormal myelination of the axon sheath (*13*). POU3F1 was then first recognized as a candidate involved in SSCs when it was shown to be highly expressed in rat germ cells with enriched SSC activity (*16*). *In vivo*, POU3F1 is expressed by all proliferating spermatogonia (*17*), one previous large-scale microarray analyses revealed that POU3F1 gene expression was regulated by GDNF in cultured SSCs containing $THY1^+$ germ cell populations (*18*). In another study, it was demonstrated that SSCs were induced by the GDNF-PI3K-AKT-POU3F1 pathway in mouse $Thy1^+$ spermatogonial cell cultures (*10*). Moreover, reduction of POU3F1 gene expression by short interfering ribonucleic acid (siRNA) treatment resulted in apoptosis in cultured germ cell populations, and further transplantation analyses revealed impaired SSCs activity *in vitro*. Hence, POU3F1 has been examined as an important intrinsic regulator of GDNF-induced survival and self-renewal of SSCs (*10*).

Yangjing Capsule (YC), a Traditional Chinese Medicine (TCM) formula, primarily contains 11 kinds of herbs (Herba Epimedii Brevicornus, Placenta Hominis, Concha Ostreae, Radix Angelicae Sinensis, Hirudo, Semen Astragali Complanati, Rhizoma Polygonati Sibirici, Radix Rehmanniae preparata, Semen Vaccariae Segetalis, Radix Astragali Mongolici and Semen Litchi). Our team has conducted clinical and experimental studies on YC extract for nearly ten years. It could significantly improve sperm density, vitality, and DNA integrity in infertile males (*19,20*). Moreover, after YC extract therapy, noticeable sperm was found in the semen of patients' with azoospermia who suffered from DAZ gene deletion (*21*). Further investigations indicated that spermatogenesis could be enhanced by YC extract several ways. The YC extract stimulated mouse Leydig tumor cells to secrete testosterone which motivated peritubular myoid cells to produce factors influencing SSCs maintenance (*22,23*). The YC extract could also contribute to proliferation of SSCs by motivating the PI3K/AKT pathway, revealing the same functionality as GDNF (*24*). However, the regulatory effect of YC extract on expression of POU3F1 still remains unknown. Thus, the objective of the present study is to determine whether the transcription factor POU3F1 is up-regulated by YC extract through the PI3K/AKT signaling pathway to regulate SSCs survival and proliferation.

2. Materials and Methods

2.1. *Chemicals*

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and lyophilized trypsin-ethylenediaminetetraacetic acid were purchased from GIBCO BRL (Grand Island, NY, USA). 3-[4,5-Dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide (MTT), diethyl pyrocarbonate, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), ammonium peroxodisulphate, and Tris-hydrochloride were obtained from Sigma (St. Louis, MO, USA). The recombinant murine GDNF was purchased from Pepro Tech (Rocky Hill, NJ, USA). TRIzol reagent, PrimeScript RT Master Mix, and SYBR Green PCR Master Mix reagent kits were obtained from TaKaRa (TaKaRa Biotechnology, Dalian, China). The primers were synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA). The whole protein extraction kits were purchased from KeyGen (KeyGen Biotech. Co. Ltd., Nanjing, China). The goat polyclonal anti-POU3F1 and rabbit polyclonal anti-GFRα1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit monoclonal anti-AKT (phospho S473) was purchased from Abcam (Cambridge, MA, USA). The mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from BioWorld (St. Louis Park, MN, USA). Enhanced Chemiluminescence was obtained from Amersham Biosciences (Uppsala, Sweden).

2.2. *Preparation of YC extract*

The YC consists of 11 traditional Chinese drugs: 13.3%

Yinyanghuo (Herba Epimedii Brevicornus), 13.3% Wangbuliuxing (Semen Vaccariae Segetalis), 13.3% Muli (Concha Ostreae (calcined)), 10% Danggui (Radix Angelicae Sinensis), 10% Huangqi (Radix Astragali Mongolici), 6.7% Shayuanzi (Semen Astragali Complanati), 6.7% Ziheche (Placenta Hominis), 6.7% Huangjing (Rhizoma Polygonati Sibirici), 6.7% Lizhihe (Semen Litchi), 6.7% Shuizhi (Hirudo), and 6.7% Shudihuang (Radix Rehmanniae Preparata). The YC extract was prepared based on the methods described by Kao (*25*) *et al*. and Hu (*26*) *et al*. The content of the YC (3.33 g, equivalent to 10 g of crude drug) was extracted with 333 mL of double distilled water and subsequently subjected to ultrasonic extraction for 45 min. The supernatant was collected and the residue was dissolved and extracted in a similar manner. The two solutions were combined and centrifuged at 13,000 g for 30 min at 4°C to collect the supernatant, which was concentrated to 100 mL with a rotary evaporator at 60°C. The final concentration of the YC extract corresponded to 100 mg/mL of the crude herbal dose. A radioimmunoassay (RIA) confirmed that there was no GDNF in the YC extract, and RIA was performed to avoid the influence of GDNF on the GC-1 spg cells. The pH of the extract was adjusted to 7.0, and the extract was sterilized by filtration on a super clean bench and stored at -80°C for use.

2.3. *Cell culture and treatment*

The mouse GC-1 spermatogonial (spg) cell line was obtained from ATCC (CRL-2053). The GC-1 spg cells were cultured in DMEM medium supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin, and then incubated in a 5% CO_2 incubator at 37°C. The YC extract was diluted with DMEM without serum to make various concentrations. In the clinical setting, the YC was administered to treat male infertility at a dose of 2 pills three times a day. This dosage was equivalent to 9 g of crude drug. Considering the mean volume of one adult as approximately 0.06 m^3, the distribution of medicine can be estimated as 0.15 mg/mL. Hence, the concentration of 0.1 mg/mL was chosen as the middle dose to feed the cells. Then, this dose was adjusted 10-fold to investigate a range of concentrations corresponding to 0.01, 0.1, and 1 mg/mL of the crude herbal dose.

2.4. *SiRNA transfection and PI3K inhibition of GC-1 spg cells*

The GC-1 spg cells were treated at a density of 2×10^5/well in 6-well plates with 0, 0.01, 0.1, and 1 mg/mL YC extract and 20 ng/mL GDNF (used as positive control), respectively. After 48 h, the cells were collected for messenger RNA and protein analysis to detect the expression of POU3F1 using quantitative real time-polymerase chain reaction (RT-PCR) and Western blots. To explore the underlying signaling mechanism, POU3F1, GFRα1 siRNA, and PI3K inhibitor (LY294002) were carried out. For POU3F1 knockdown, 21-nucleotide siRNA sequences (sense: 5'-CCC UCU ACG GUA ACG UGU UTT -3' and antisense: 3'-TTG GGA GAU GCC AUU GCA CAA -5') targeting mouse POU3F1 sequence (CCC TCT ACG GTA ACG TGT T) were designed using BLOCK-iT RNA interference (RNAi) Designer (Invitrogen, Carlsbad, CA, USA) and synthesized by Invitrogen. For GFRα1 knockdown, 21-nucleotide siRNA sequences (sense: 5'-GCC CUC ACA GGC UUC UGU UTT-3' and antisense: 3'-TTC GGG AGU GUC CGA AGA CAA-5') targeting mouse GFRα1 sequence (GCC CTC ACA GGC TTC TGT T) were also designed and synthesized by Invitrogen (*27*). The Stealth RNAi negative control was obtained from Invitrogen, which was used as a control for monitoring nonsequence-specific effects. Before transfection, GC-1 spg cells were seeded in 6-well plates at a density of 1×10^5/well for 24 h and then POU3F1, GFRα1 siRNA, and Stealth RNAi negative control were transfected into GC-1 spg cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 24 h after transfection, the cells were treated with 1 mg/mL YC extract for 48 h and then harvested for quantitative RT-PCR and Western blot analyses. For PI3K inhibition, GC-1 spg cells were exposed to 25 μM LY294002 (targeting the ATP-binding site of the PI3K) for abrogating PI3K activation. Two hours later, 1 mg/mL YC extract was added. After 48 h, the cells were harvested for quantitative RT-PCR and Western blot analyses.

2.5. *MTT assay of cell proliferation*

The cells were seeded in 96-well plates and treated with 0 and 1 mg/mL YC extract, 20 ng/mL GDNF, 1 mg/mL YC extract with 25 pmol/mL control siRNA, and 25 pmol/mL POU3F1 siRNA, respectively (n = 5) for 48 h. 20μL of MTT (5 mg/mL) was added to each well and incubated for 4 h before it was discarded. Then the obtained purple-blue MTT formazan precipitate was dissolved in 100μL DMSO. The absorbance (OD) was measured at 490 nm and the proliferation ratio (%) was calculated using the formula: (average OD treatment group/average OD control group-1) × 100%.

2.6. *RNA isolation and quantitative RT-PCR*

Cells at a density of 2×10^5/well were plated in 6-well plates for 48 h. The total RNA was extracted using TRIzol reagent and measured using spectrometry at an OD 260/280. Later, extracted RNAs were reversibly transcribed into cDNA in a total volume of 20 μl with PrimeScript RTMaster Mix. All of the RT-PCR reactions were performed with a CFX96 RT-PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green PCR Master Mix (Bio-Rad

Laboratories, Hercules, CA, USA). GAPDH was used as the internal control. Primer sequences were designed and synthesized by Invitrogen as follows: GAPDH, sense: 5'-AGG TTG TCT CCT GCG ACT TCA-3' and antisense: 5'-GGG TGG TCC AGG GTT TCT TAC T-3'; POU3F1, sense: 5'- TAC CGC GAA GTG CAG AAG C -3' and antisense: 5'- CGT GGG TAG CCA TTG AGG G -3'; and GFRα1, sense: 5'-AGA AGC AGT TTC ACC CAG-3' and antisense: 5' ATC ATC ACC ACC ACC ATC-3'. Reactions were performed at 94°C for 2 min followed by 40 cycles at 94°C for 30 s, 55°C for 15 s, and 70°C for 1 min. The final extension was carried out for 5 min at 72°C. A melting curve analysis was performed to confirm the products. The relative abundance of the target mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. The data were expressed as the percentage of control (100%)

2.7. *Protein extraction and western blot analysis*

Cells were seeded in 6-well plates at a density of 2×10^5/well for 48 h. The cells were harvested, washed three times with precooled phosphate-buffered saline, and treated with cell lysis buffer for Western blot analysis. After centrifugation at 12,000 g at 4°C for 15 min, the supernatants were collected and stored at -80°C until analysis. The concentrations of protein were measured using the Bio-Rad Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were normalized to 60 μg/lane and separated on 12% SDS-polyacrylamide gel electrophoresis, and subsequently transferred to nitrocellulose membranes. After treatment with blocking solution (5% skim milk powder in Tris-buffered saline) at 37°C for 1 h, the membranes were incubated overnight with the primary antibodies goat polyclonal anti-POU3F1 (1:200 dilution), rabbit monoclonal anti-pAKT (1:5000 dilution), rabbit polyclonal anti-GFRα1 (1:400 dilution), or mouse monoclonal anti-GAPDH (1:3000 dilution) at 4°C. After washing with Tris buffered saline with Tween 20 three times, the membranes were incubated with HRP-conjugated secondary antibodies (1:3000 dilution) at 37°C for 1 h and examined using enhanced chemiluminescence. The relative protein levels in each sample were normalized to those of GAPDH to standardize for variations in loading. Densitometric analyses of the scanned immunoblotting images were performed using a Quantity One image system (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. *Statistical analysis*

All data were analyzed using a SPSS (Version 19.0) statistical package and presented as mean ± standard deviation for three independent experiments. One-way analysis of variance was used to analyze the differences between groups, followed by Dunnett's *t*-test. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. *Effects of the YC extract on the expression of POU3F1 mRNA and protein*

As shown in Figure 2A, the expression of POU3F1 mRNA increased significantly after exposure to 0.01, 0.1, and 1 mg/mL YC extract and 20 ng/mL GDNF ($p = 0.083$, $p < 0.001$, $p < 0.001$ and $p < 0.001$ respectively). As shown in Figure 2B, expression of POU3F1 protein also increased evidently under the same conditions. The YC extract enhanced the expression of POU3F1 mRNA and protein in a dose-dependent manner with a maximal effect observed at a 1 mg/mL concentration. Hence, a YC extract dose of 1 mg/mL was selected for further experiments.

3.2. *POU3F1 knockdown blocks YC extract induced proliferation of GC-1 spg cells*

To investigate the transcriptional level mechanism by which YC extract can induce proliferation of GC-1 spg cells, a POU3F1 knockdown was performed.

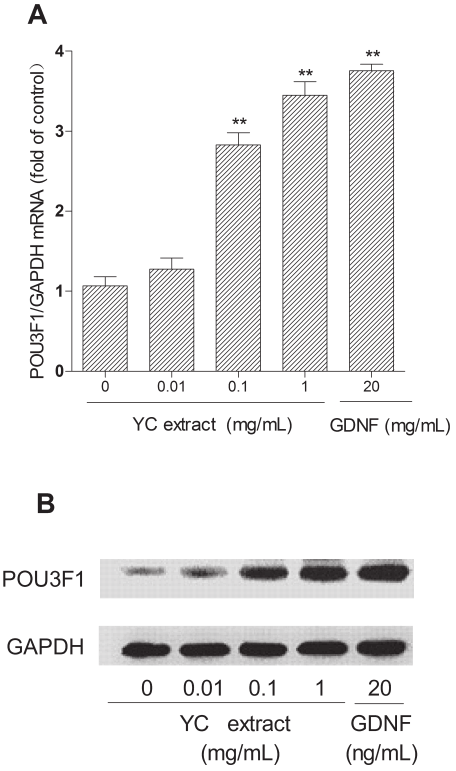

Figure 2. Effects of the YC extract on the expression of POU3F1 mRNA and protein in GC-1 spg cells. The GC-1 spg cells were treated with 0.01, 0.1, and 1 mg/ml YC extract or 20 ng/ml GDNF for 48 h. The expression of mRNA was detected using quantitative RT-PCR. The expression of protein was detected using Western blot analysis. The data are expressed as percentage of control (100%). **$p < 0.01$ compared with control group. Data are presented as means (SD) from three independent experiments and representative bands are shown.

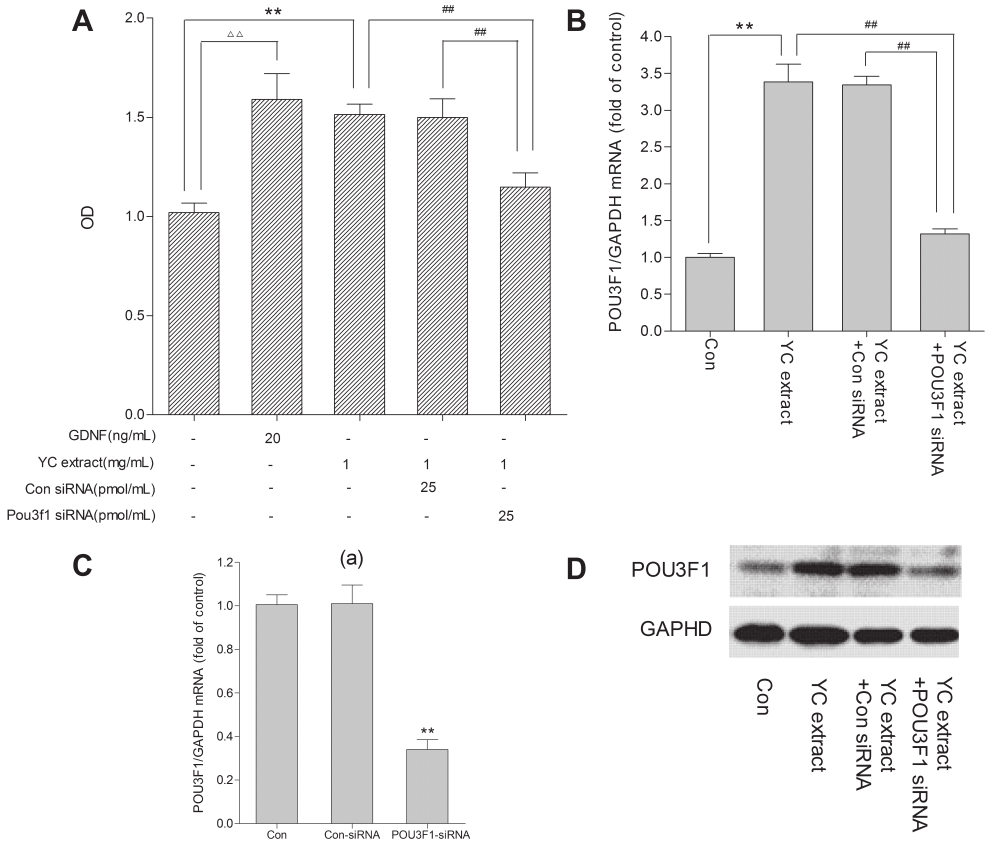

Figure 3. POU3F1 knockdown blocked YC extract induced proliferation of GC-1 spg cells. (A) The GC-1 cells were exposed to control blank, 20 ng/mL GDNF, 1 mg/mL YC extract, and 1 mg/mL YC extract with 25 pmol/mL control siRNA or POU3F1 siRNA. $^{**}p < 0.01$ compared with control group, $^{\Delta\Delta}p < 0.01$ compared with control group, $^{\#\#}p < 0.01$ compared with YC extract and POU3F1 siRNA group, $n = 5$. **(B)**, **(D)** POU3F1 knockdown blocked YC extract induced upregulation of POU3F1 expression. The GC-1 cells were exposed to control blank, 1 mg/mL YC extract, and 1 mg/mL YC extract with 25 pmol/mL control siRNA or POU3F1 siRNA. $^{**}p < 0.01$ compared with control group. $^{\#\#}p < 0.01$ compared with YC extract with POU3F1 siRNA group. **(C)** The expression of POU3F1 mRNA was reduced by 66% in POU3F1 siRNA-treated cultures compared with those treated with nontargeting control siRNA at 36 h after transfection. Data are presented as means (SD) from three independent experiments and representative bands are shown.

As shown in Figure 3A, YC extract and GDNF significantly stimulated cell proliferation ($p < 0.001$ for both). However, POU3F1 siRNA (25 pmol/mL) could evidently reduce the stimulative effect of YC extract by 24% ($p < 0.001$). As shown in Figures 3B and 3D, POU3F1 siRNA could abolish the upregulation of POU3F1 expression at levels of mRNA and protein induced by YC extract with a 61% interference efficiency ($p < 0.001$).

3.3. *PI3K inhibition blocks YC extract induced Up-regulation of POU3F1 expression*

To explore whether the YC extract induced proliferation of GC-1 spg cells *via* PI3K/AKT signaling pathway, LY294002, a PI3K inhibitor, was added 2 h before the YC extract treatment. As shown in Figure 4A, LY294002 could abrogate the upregulatory expression of POU3F1 mRNA induced by YC extract, with a 64% interference efficiency ($p < 0.001$). As shown in Figure 4B, the POU3F1 protein levels also down-regulated remarkably with similarity to POU3F1 mRNA. Meanwhile, the upregulation of pAKT protein expression induced by YC extract, an evidence for PI3K/AKT pathway activation, was also suppressed by LY294002.

3.4. *PI3K inhibition blocks YC extract induced Up-regulation of POU3F1 expression*

Considering the membrane receptor GFRα1's participation in the YC extract mediated pathway, a GFRα1 knockdown experiment was conducted to confirm whether down-regulated GFRα1 had an effect on POU3F1 expression. As shown in Figures 5A and 5D, GFRα1 siRNA significantly inhibited the upregulation of YC extract induced POU3F1 mRNA and protein expression by 58% ($p < 0.001$ for both). Correspondingly, the upregulation of GFRα1 and pAKT expression induced by YC extract were also suppressed by GFRα1 siRNA (Figure 5B and 5D) ($p < 0.001$ for both). So far, an unambiguous signaling pathway

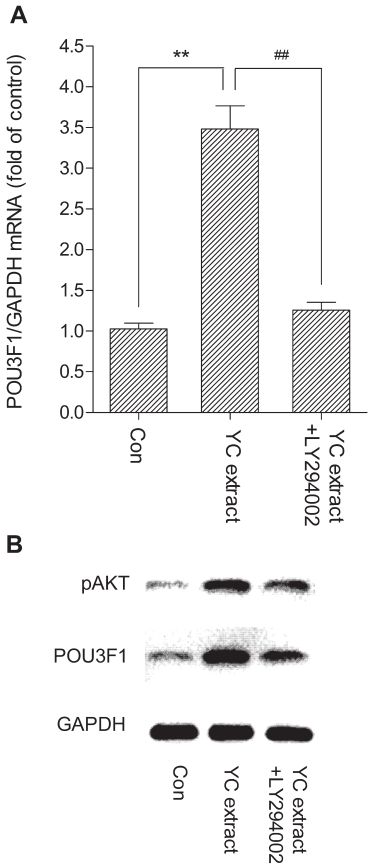

Figure 4. PI3K inhibition blocked the YC extract induced up-regulation of POU3F1 expression. The GC-1 cells were exposed to control blank, 1 mg/mL YC extract, and 1 mg/mL YC extract with 25 μM LY294002. $^{**}p < 0.01$ compared with control group. $^{\#\#}p < 0.01$ compared with YC extract group. Data are presented as means (SD) from three independent experiments and representative bands are shown.

behind the YC extract induced proliferation of GC-1 spg cells at the cytomembrane (GFRα1), cytoplasm (PI3K/AKT), and intra-nuclear transcriptional levels (POU3F1) were confirmed in this study.

4. Discussion

At present, functional assay systems shed new light on studying the SSCs population and enable use of these cells for applications in animal transgenesis and medicine *(28)*. Comprehension of SSCs at the molecular level provides an opportunity to explore TCM mechanism of action on SSCs. Interestingly, the GC-1 spg cell line has served as a convenient tool to critically study pathways regulating the SSCs fate decisions and survival. As stated previously, the YC extract can lead to proliferation of GC-1 spg cells by activating the PI3K/AKT pathway, sharing the same functionality as GDNF, while the downstream signaling mechanism is poorly understood in consideration of POU3F1 as a significant transcription factor regulated by GDNF. In the present study, the role of POU3F1 in YC extract mediated proliferation of SSCs was examined.

The GDNF, secreted by Sertoli cells and peritubular myoid cells, is the major paracrine factor specifically responsible for the maintenance and self-renewal of SSCs *in vivo* (*29,30*). Other signaling pathways like insulin-like growth factor-1 receptor (IGF-1R) (*31*) mediated and fibroblast growth factor 2 (FGF2) (*32*) mediated pathways also play a role. The GDNF binding to GFRα1 induces RET activation that further leads to the activation

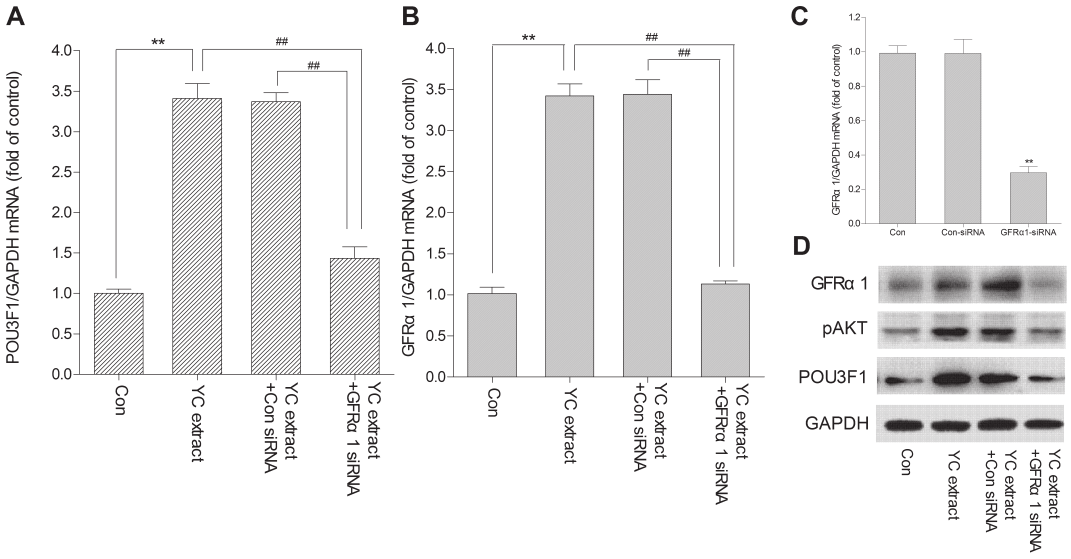

Figure 5. GFRα1 knockdown blocked the YC extract induced up-regulation of POU3F1 and pAKT expression. (A), (B) The GC-1 cells were exposed to control blank, 1 mg/mL YC extract, and 1 mg/mL YC extract with 25 pmol/mL control siRNA or GFRα1 siRNA. $^{**}p < 0.01$ compared with control group. $^{\#\#}p < 0.01$ compared with YC extract with GFRα1 siRNA group. **(A)**, **(D)** GFRα1 knockdown blocked YC extract induced upregulation of POU3F1 expression. **(B)**, **(D)** Correspondingly, the upregulation of GFRα1 and pAKT expression induced by YC extract were also suppressed by GFRα1 siRNA. **(C)**Treatment with GFRα1 siRNA resulted in 70% reduction of gene expression compared with nontargeting control siRNA at 36 h after transfection. Data are presented as means (SD) from three independent experiments and representative bands are shown.

Table 1. Primary active ingredients and functions of Chinese herbs

Chinese herbs	Active ingredients	Functions	*Ref.*
Placenta Hominis	hCG	Promoted the proliferation of SSCs after transplantation.	(*42*)
	GnRH	Promoted the release of FSH and LH by pituitary thereby stimulating spermatogenesis	(*45*)
Herba Epimedii Brevicornus	Total flavonoids	Increased testicular weights, sperm counts and sperm motility. Restored oxidative damage by up-regulating the expression of antioxidant enzymes (SOD3 and GPX1).	(*46*)
	Icariin	Increased testosterone levels by up-regulation the expression of PBR and StAR. Increased FSHR and claudin-11 mRNA expression in Sertoli cells.	(*47*)
Concha Ostreae	Zinc	Promoted the proliferation of SSCs, the progression spermatogenesis and sperm motility.	(*43*)
	Selenium	Promoted the proliferation of SSCs. Protected against oxidative damage to spermatozoa throughout the process of sperm maturation. Selenoproteins served as structural components of mature spermatozoa.	(*44*) (*48*)
Hirudo	Hirudin	Improved testicular microcirculation.	(*49*)
Radix Angelicae Sinensis	Ferulic acid	Improved testicular microcirculation.	

SOD3: superoxide dismutase 3; GPX1: glutathione peroxidase 1; PBR: peripheral type benzodiazepine receptor; StAR: steroidogenic acute regulatory protein; hCG:human chorionic gonadotropin; GnRH: Gonadotropin-releasing hormone; FSH: follicle stimulating hormone; FSHR: follicle stimulating hormone receptor; LH: luteinizing hormone.

of the PI3K/AKT signaling pathway which is required for spermatogonial self-renewal in mice (*11,33*). Further research showed that POU3F1 was an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse SSCs (*10*). Based on the comparable effects of YC extract and GDNF, it was speculated that POU3F1 also played a key role in YC extract's mediated biological effects. In this study we blocked three key signal factors GFRα1 (cytomembrane level), PI3K(cytoplasm level), POU3F1(intra-nuclear transcriptional level) in the GDNF induced signaling pathway to test whether the YC extract went through the same pathway.

To investigate the role of POU3F1 in the proliferation of GC-1 spg cells induced by YC extract, siRNA mediated POU3F1 knockdown was used in this study. As shown in Figure 3, POU3F1 siRNA (25 pmol/mL) almost entirely abolished the proliferative effect of GC-1 cells induced by YC extract. Correspondingly, POU3F1 knockdown also abrogated the elevated expression of POU3F1 mRNA and protein induced by YC extract (Figure 3). On the basis of these results, it was inferred that YC extract exerts biological effects partly *via* POU3F1.

To further explore the YC extract mediated signaling mechanism, the GC-1 cells were treated with 25 μM LY294002 to inhibit the PI3K pathway. In previous studies, it was found that the PI3K pathway played a central role in the GDNF induced self-renewal of SSCs (*33,34*). The binding of GDNF to GFRα1 triggers the PI3K/AKT pathway and eventually leads to self-renewal of SSCs *via* up-regulated expression of POU3F1. As expected, pAKT protein and POU3F1 expression were increased evidently after YC extract treatment, confirming the PI3K/AKT pathway activation (Figure 4). Moreover, LY294002 markedly down-regulated the expression of pAKT and POU3F1, indicating that PI3K/AKT were the explicit signaling molecules of YC extract induced up-regulation of POU3F1 expression.

Furthermore, GFRα1 siRNA was used to interrupt the binding of YC extract to GFRα1, to observe whether GFRα1 expression and downstream pAKT and POU3F1 were restrained. The GFRα1 is expressed in all stages of type A spermatogonia (*35*), and it is a necessary component of the GFRα1/RET complex. The RET alone is unable to bind with GDNF unless it is co-expressed with the GFRα1 receptor. The association of GDNF with RET tyrosine kinase is mediated by GFRα1, and GDNF cannot induce RET autophosphorylation in the cells that lack GFRα1 expression (*27*). A previous study (*24*) revealed that both GFRα1 siRNA and LY294002 could markedly abrogate the stimulative effect of YC extract. As shown in Figure 5, GFRα1 knockdown almost entirely abrogated the elevated expression of GFRα1 mRNA and protein induced by YC extract. Accordingly, the up-regulated pAKT protein, POU3F1 mRNA and protein expression also dropped down after GFRα1 siRNA treatment. Conclusively, it could be inferred that POU3F1, GFRα1, and PI3K played crucial roles in the promotion of GC-1 spg cells self-renewal induced by YC extract.

To date, some medicines have been found with definite effects on the proliferation of SSCs. For example, natural drugs like Petasites Japonicas butanol (*36*), Rhodiola Sachalinensis polysaccharides (*37*), Lycium Bararum polysaccharides (*38*) and Astragalus (*39*), others like growth factors (*40*), follicle stimulating hormone (FSH) (*41*), human chorionic gonadotropin (hCG) (*42*), Zinc (*43*) and Selenium (*44*), all have proliferative effects. In comparison with biomonomers of natural drugs and other chemicals, sophisticated compounds of TCM have various effects made by a variety of chemical components, multi-linked and multi-targeted in the body. As a sophisticated compound, the YC extract contains 11

Chinese medicinal herbs with multitudes of monomers, including hCG, Zinc and Selenium, which show direct evidence of the proliferative effect on SSCs. The primary active ingredients of the YC extract are listed in the Table1. It may promote proliferation of GC-1 spg cells two ways. One way is through the hypothalamic-pituitary-testicular axis that works. Placenta Hominis contains gonadotropin-releasing hormone (GnRH) and hCG. GnRH can promote the release of FSH and luteinizing hormone (LH) by pituitary thereby stimulating spermatogenesis (*45*). hCG exerts the same function as LH (*42*). Another way is through the testicular microenvironment. Total flavonoids of Herba Epimedii Brevicornus can restore oxidative damage in the testis (*46*). Icariin has testosterone mimetic properties and can significantly increase testosterone levels (*47*). Zinc and Selenium are essential trace elements for the maintenance of SSCs, the progression of spermatogenesis, and the regulation of sperm motility (*43,48*). Hirudin and ferulic acid can improve testicular microcirculation and metabolic function (*49*).

5. Conclusions

In summary, it was concluded that YC extract could up-regulate POU3F1 expression, partly *via* GFRα1, by triggering the activation of the PI3K/AKT pathway and finally lead to self-renewal of SSCs. The present study findings provide evidence of molecular biology mechanism of TCM to further guide the clinical treatment of male infertility. In addition, most of the transcription factors (POU3F1, BCL6B, ETV5, ID4, and LHX1) that have been identified as promoting SSCs self-renewal are up-regulated by GDNF (*12*). Since GDNF is crucial for promoting SSCs self-renewal and YC extract possesses similar functionality compared to GDNF, the above mentioned transcription factors could be responsible for coordinating the action of YC extract in SSCs. In future research, it will be meaningful to conduct a microarray analysis on cultures of SSCs induced by YC extract to gain a more comprehensive list of GDNF regulated genes. Furthermore, as YC extract consists of multiple components, further studies will be focused on excavating the primary effective biomonomer.

Acknowledgements

This work was supported by the Chinese National Natural Science Foundation (No. 81273760, No. 81302969) and a Postgraduate Innovation Program issued by Jiangsu Province Educational Ministry. The authors would like to thank the State Key Laboratory of Reproductive Medicine, the Clinical Center of Reproductive Medicine, and the Nanjing Medical University for providing laboratory and technological assistance.

References

1. Brinster RL. Male germline stem cells: from mice to men. Science. 2007; 316:404-405.
2. Brinster RL. Germline stem cell transplantation and transgenesis. Science. 2002; 296:2174-2176.
3. de Rooij DG. Proliferation and differentiation of spermatogonial stem cells. Reproduction. 2001; 121:347-354.
4. Caires KC, de Avila J, Mclean DJ. Endocrine regulation of spermatogonial stem cells in the seminiferous epithelium of adult mice. Biores Open Access. 2012; 1:222-230.
5. Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A. 2004; 101:16489-16494.
6. Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE, Garbers DL. Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. Proc Natl Acad Sci U S A. 2005; 102:17430-17435.
7. Ryu BY, Kubota H, Avarbock MR, Brinster RL. Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc Natl Acad Sci U S A. 2005; 102:14302-14307.
8. Kanatsu-Shinohara M, Muneto T, Lee J, Takenaka M, Chuma S, Nakatsuji N, Horiuchi T, Shinohara T. Long-term culture of male germline stem cells from hamster testes. Biol Reprod. 2008; 78:611-617.
9. Goertz MJ, Wu Z, Gallardo TD, Hamra FK, Castrillon DH. Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest. 2011; 121:3456-3466.
10. Wu X, Oatley JM, Oatley MJ, Kaucher AV, Avarbock MR, Brinster RL. The POU domain transcription factor POU3F1 is an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse spermatogonial stem cells. Biol Reprod. 2010; 82:1103-1111.
11. Oatley JM, Avarbock MR, Brinster RL. Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J Biol Chem. 2007; 282:25842-25851.
12. Song H, Wilkinson MF. Transcriptional control of spermatogonial maintenance and differentiation. Semin Cell Dev Biol. 2014; 30:14-26.
13. Ghazvini M, Mandemakers W, Jaegle M, Piirsoo M, Driegen S, Koutsourakis M, Smit X, Grosveld F, Meijer D. A cell type-specific allele of the POU gene Oct-6 reveals Schwann cell autonomous function in nerve development and regeneration. EMBO J. 2002; 21:4612-4620.
14. Suzuki N, Rohdewohld H, Neuman T, Gruss P, Scholer HR. Oct-6: a POU transcription factor expressed in embryonal stem cells and in the developing brain. EMBO J. 1990; 9:3723-3732.
15. He X, Treacy MN, Simmons DM, Ingraham HA, Swanson LW, Rosenfeld MG. Expression of a large family of POU-domain regulatory genes in mammalian brain development. Nature. 1989; 340:35-41.
16. Hamra FK, Schultz N, Chapman KM, Grellhesl DM, Cronkhite JT, Hammer RE, Garbers DL. Defining the

spermatogonial stem cell. Dev Biol. 2004; 269:393-410.
17. Lee J, Kanatsu-Shinohara M, Morimoto H, Kazuki Y, Takashima S, Oshimura M, Toyokuni S, Shinohara T. Genetic reconstruction of mouse spermatogonial stem cell self-renewal *in vitro* by Ras-cyclin D2 activation. Cell Stem Cell. 2009; 5:76-86.
18. Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL. Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci U S A. 2006; 103:9524-9529.
19. Jin BF, Huang YF, Yang XY, Shang XJ, Lu XH, Wang XL. Clinical observation on treatment of oligospermatism with Yangjing capsule. Journal of Nanjing TCM University. 2006; 22:286-289. (in Chinese)
20. Jin BF, Huang YF, Xia XY, Wang XL, Yang XY, Zhou ZM. Effect of Yangjing capsule and Xinxibao on sperm DNA integrity of patients with male infertility. Chinese Journal of Andrology. 2006; 20:45-49. (in Chinese)
21. Jin BF, Yang XY, Liu JY, Huang YF, Wang XL, Xu FS. Integrated treatment for azoospermia caused by radiotherapy after surgical treatment of spermatocytoma: a case report. National Journal of Andrology. 2006; 12:836-838. (in Chinese)
22. Gu YL, Zhang XD, Sun DL, Zhao HL, Cai B, Gao C, Gao L, Cui YG, Tang ZA, Jin BF. The Stimulative Effect of Yangjing Capsule on Testosterone Synthesis through Nur77 Pathway in Leydig Cells. Evid Based Complement Alternat Med. 2015; 2015:408686.
23. Chen LY, Brown PR, Willis WB, Eddy EM. Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance. Endocrinology. 2014; 155:4964-4974.
24. Wang ZQ, Jin BF, Zhang XD, Cui YG, Sun DL, Gao C. Yangjing Capsule Extract Promotes Proliferation of GC-1 Spg Cells. Evid Based Complement Alternat Med. 2014; 2014:640857.
25. Kao ST, Yeh CC, Hsieh CC, Yang MD, Lee MR, Liu HS, Lin JG. The Chinese medicine Bu-Zhong-Yi-Qi-Tang inhibited proliferation of hepatoma cell lines by inducing apoptosis *via* G0/G1 arrest. Life Sci. 2001; 69:1485-1496.
26. Hu B, An HM, Shen KP, DU Q. Effects of Tenglong Buzhong Decoction on proliferation and apoptosis of human colon carcinoma cell line LS174T. Zhong Xi Yi Jie He Xue Bao. 2010; 8:575-580. (in Chinese)
27. He Z, Jiang J, Hofmann MC, Dym M. Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation *via* the inactivation of RET tyrosine kinase. Biol Reprod. 2007; 77:723-733.
28. Kanatsu-Shinohara M, Shinohara T. Spermatogonial stem cell self-renewal and development. Annu Rev Cell Dev Biol. 2013; 29:163-187.
29. Chen LY, Willis WD, Eddy EM. Targeting the Gdnf Gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. Proc Natl Acad Sci U S A. 2016; 113:1829-1834.
30. Chen SR, Liu YX. Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling. Reproduction. 2015; 149:159-167.
31. Wang S, Wang X, Wu Y, Han C. IGF-1R signaling is essential for the proliferation of cultured mouse spermatogonial stem cells by promoting the G2/M progression of the cell cycle. Stem Cells Dev. 2015; 24:471-483.
32. Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K, Ogonuki N, Jijiwa M, Takahashi M, Ogura A, Shinohara T. Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Reports. 2015; 4:489-502.
33. Lee J, Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, Toyokuni S, Kimura T, Nakano T, Ogura A, Shinohara T. Akt mediates self-renewal division of mouse spermatogonial stem cells. Development. 2007; 134:1853-1859.
34. Braydich-Stolle L, Kostereva N, Dym M, Hofmann MC. Role of Src family kinases and N-Myc in spermatogonial stem cell proliferation. Dev Biol. 2007; 304:34-45.
35. Grasso M, Fuso A, Dovere L, de Rooij DG, Stefanini M, Boitani C, Vicini E. Distribution of GFRA1-expressing spermatogonia in adult mouse testis. Reproduction. 2012; 143:325-332.
36. Kang HR, Lee YA, Kim YH, Lee DG, Kim BJ, Kim KJ, Kim BG, Oh MG, Han CK, Lee S, Ryu BY. Petasites japonicus Stimulates the Proliferation of Mouse Spermatogonial Stem Cells. PLoS One. 2015; 10:e133077.
37. Yu DD, Geng GX, Zhang CJ, Liu YW, Hu JH, Cao HL, Li QW. Effects of Rhodiola polysaccharides on proliferation of spermatogonial stem cells *in vitro*. Acta Ecologiae Animalis Domastici. 2013; 34:54-57. (in Chinese)
38. Liu HL. Effect of Lycium Bararum polysaccharides on mouse spermatogonial stem cells cultured *in vitro*. Journal of Anhui Agri. 2012; 40:1490-1492. (in Chinese)
39. Yu RJ, Hong Y, Liu Q, Zhang K, Wang WJ. Effects of Astragalus injection on proliferation of rat spermatogonial stem cells. Journal of Jiangxi University of TCM. 2010; 22:67-69. (in Chinese)
40. Guo XH, Lin Y, Wang YL, Zheng LW. Growth factors promote the survival and proliferation of human spermatogonium stem cells *in vitro*. National Journal of Andrology. 2008; 14:876-878. (in Chinese)
41. Nobrega RH, Morais RD, Crespo D, de Waal PP, de Franca LR, Schulz RW, Bogerd J. Fsh Stimulates Spermatogonial Proliferation and Differentiation in Zebrafish *via* Igf3. Endocrinology. 2015; 156:3804-3817.
42. Akhondi MM, Najar RA, Jeddi-Tehrani M, Sadeghi MR, Zarnani AH, Rabbani H, Salehkhou S, Eini L, Hoseinzadeh F, Heidari M. The effect of human chorionic gonadotropin treatment on recipient mouse germ cell proliferation following spermatogonial stem cell transplantation of neonatal donor mice. Avicenna J Med Biotechnol. 2010; 2:23-35.
43. Yamaguchi S, Miura C, Kikuchi K, Celino FT, Agusa T, Tanabe S, Miura T. Zinc is an essential trace element for spermatogenesis. Proc Natl Acad Sci U S A. 2009; 106:10859-10864.
44. Shi L, Zhao H, Ren Y, Yao X, Song R, Yue W. Effects of different levels of dietary selenium on the proliferation of spermatogonial stem cells and antioxidant status in testis of roosters. Anim Reprod Sci. 2014; 149:266-272.
45. Zheng JJ, Mao JF, Wu XY, Wang X, Liu ZX, Huang BK. Effect of pulsatile GnRH therapy on pituitary-testicular axis function in male patients with pituitary stalk interruption syndrome. National Medical Journal of China. 2016; 96:1668-1672. (in Chinese)
46. Yuan D, Wang H, He H, Jia L, He Y, Wang T, Zeng X, Li Y, Li S, Zhang C. Protective effects of total flavonoids

from Epimedium on the male mouse reproductive system against cyclophosphamide-induced oxidative injury by up-regulating the expressions of SOD3 and GPX1. Phytother Res. 2014; 28:88-97.
47. Chen M, Hao J, Yang Q, Li G. Effects of icariin on reproductive functions in male rats. Molecules. 2014; 19:9502-9514.
48. Ahsan U, Kamran Z, Raza I, Ahmad S, Babar W, Riaz MH, Iqbal Z. Role of selenium in male reproduction - a review. Anim Reprod Sci. 2014; 146:55-62.
49. Guan GY, Tian BQ, Zheng XM, Li SW. Effect of angelica sinensis ligustici leech mixture on the spermatogenesis of experimental varicocele in rats. Medical Journal of Wuhan University. 2007; 28:576-579. (in Chinese)

(Received November 9, 2016; Revised December 27, 2016; Accepted January 11, 2017)

Commentary

DOI: 10.5582/bst.2016.01249

Progress of research on microRNAs with diagnostic value in asbestos exposure: A call for method standardization

Luigina Micolucci[1,2,*], Maria Rita Rippo[2], Fabiola Olivieri[2,3], Antonio Domenico Procopio[2,3]

[1] *Computational Pathology Unit, Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Ancona, Italy;*
[2] *Laboratory of Experimental Pathology, Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Ancona, Italy;*
[3] *Center of Clinical Pathology and Innovative Therapy, Italian National Research Center on Aging (INRCA-IRCCS), Ancona, Italy.*

Summary **Malignant mesothelioma (MM) is an insidious, lethal asbestos-related cancer that is poorly responsive to current treatments. Specific and sensitive biomarkers providing early MM diagnosis in exposed subjects, who are at high-risk of developing it, are sorely needed. MicroRNAs (miRNAs) are endogenous, non-coding, small RNAs with a well-established diagnostic role in cancer and pollution exposure. In a recent systematic review and qualitative meta-analysis followed by a functional investigation, we examined all the available data on the miRNA biomarkers involved in asbestos exposure and MM pathways. This invited commentary aims to provide an insightful critique into the state of the art of the research into clinically relevant miRNA biomarkers, highlighting the strengths and weaknesses of current research efforts in this field. It also reviews the suggestions advanced to improve biomarker development productivity and the translation of research results into clinical practice, stressing that multicenter multidisciplinary studies adopting standardized methods and protocol sharing are the key to move from the workbench to the clinic.**

Keywords: Asbestos, mesothelioma, microRNA, mesomiRs, molecular pathological epidemiology

1. Introduction

Asbestos and asbestos-like fibers are naturally occurring crystalline silicates whose exceptional physicochemical properties have led to their extensive use in innumerable industrial applications worldwide (*1,2*). Occupational or environmental exposure to asbestos is associated to the development of asbestos-related diseases (ARDs) through accumulation of asbestos fibers and bodies in the lungs (*2*).

ARDs are characterized by a slow onset and an insidious course. They induce a range of non-malignant inflammatory diseases (asbestosis) due to formation of plaques in the pleura and to permanent fibrosis, which promote carcinogenesis (*3*). Malignant ARDs include bronchogenic carcinoma and mesothelioma of the pleura (80-90%), peritoneum (10-15%), and other mesothelial surfaces (< 5%) (*4*). MM is rare in the general population, but common in exposed cohorts. It is a lethal cancer characterized by considerable latency (≥ 30-60 years) (*5*), poor prognosis and quality of life, and unresponsiveness to currently available treatments. Symptoms are non-specific and the differential diagnosis (by pleural biopsy) is complex, invasive, and often late (*6*). The success and applicability of current multimodal therapeutic protocols depend on tumor stage, patient performance status and co-morbidities (*7*). Patients with advanced, unresectable, and poorly differentiated disease as well as co-morbidities have a worse prognosis (*7*). Increased treatment effectiveness through patient-tailored care and management depend on monitoring exposed subjects and early disease detection. The discovery and validation of MM-specific, non-invasive biomarkers, a goal that has been pursued for more than 20 years (*8*), would enable disease detection at the asymptomatic stage. Recent studies have found that microRNAs (miRNAs) play an

Released online in J-STAGE as advance publication January 30, 2017.

*Address correspondence to:
Dr. Luigina Micolucci, Computational Pathology Unit, Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, via Tronto 10 – 60126, Ancona, Italy.
E-mail: l.micolucci@univpm.it; luigina.micolucci@gmail.com

important role in MM biology and have the potential to be employed both as biomarkers and as therapeutic targets (*9*). They are short, non-coding RNAs with a key role as post-transcriptional regulators in physiological and pathological processes; they interact with target mRNAs in a sequence-specific manner, and are differentially expressed in several diseases including cancer onset and progression. High-quality miRNAs are tissue-specific and easy to extract from tissue, cells, and body fluids (*10,11*); circulating cell-free miRNAs are highly stable because they are bound to specific carriers, such as microvesicles, Argonaute proteins, and high-density lipoproteins (*12-14*). These evidence have led to suggest a role for them as clinical molecular markers. Given the invasive nature of MM diagnosis and differential diagnosis from other cancers or benign proliferations, a variety of technological approaches and study designs have been applied over the past 10 years to test the value of miRNAs as non-invasive MM biomarkers. However, although a myriad putative miRNAs having diagnostic/prognostic relevance have been identified, the translation of research findings to clinical practice has met with limited success.

2. Diagnostic value of miRNAs in asbestos exposure: state of the art

In 2015, our group undertook a systematic review to collect and analyze the best evidence on the question (*15*). The miRNAs reported to have diagnostic potential since the earliest studies were comprehensively reviewed in an effort to find an evidence-based consensus on their biomarker potential in asbestos-exposed subjects and MM patients. Secondary data analysis has huge possibilities to identify high-quality evidence in these datasets and to provide guidance when the literature is inconsistent and studies disagree. The results of our work, the first systematic review and qualitative/quantitative meta-analysis on the issue, suggested that miRNAs may indeed play a key role in the diagnosis of asbestos exposure and ARDs and through it also improve prognosis and survival. A systematic search of the major biomedical databases for miRNA expression signatures related to asbestos exposure and MM provided a number of promising candidates, which were subjected to functional and bioinformatic analysis to assess their biomarker potential (*15*). The evidence-based picture thus obtained highlighted some major strengths and weaknesses of miRNA research in the field.

2.1. *Strengths of MM-miRNA research*

The literature search found a number of promising miRNAs, designated as "mesomiRs" (MM-associated miRNAs), with early diagnostic potential (*15*). In particular, two signatures, one found in blood and another in tissue, are expressed differently in asbestos-exposed subjects *vs*. MM patients:

i) the circulating miRNAs miR-126-3p, miR-103a-3p, and miR-625-3p were seen to provide a particularly promising multi-marker panel in combination with mesothelin and/or fibulin-3 for early, non-invasive diagnosis and screening of high-risk, exposed subjects (*15*); circulating miRNAs have ideal biomarker value, because they are non-invasive, stable, not expensive to test, and vary little in the general population.

ii) the tissue miRNAs that have been described most consistently (miR-16-5p, miR-126-3p, miR-143-3p, miR-145-5p, miR-192-5p, miR-193a-3p, miR-200b-3p, miR-203a-3p, and miR-652-3p) were pooled into a meta-signature that was found to have diagnostic value (*15*).

Application of these two miRNA panels, which are endowed with high sensitivity and specificity, has the potential to supply a more accurate assessment of the likelihood of MM development by asbestos-exposed subjects compared with other biomarkers; it may even allow to assess patients, the rate of cancer progression, and prognosis based on relative miRNA expression (*15*). The performance of the two biomarker panels should also be assessed in terms of surveillance of high-risk patients and early MM detection, so that adjuvant - systemic or targeted - therapies can be instituted at an earlier time point. This work should be supplemented by validation studies carried out in the population at risk, using a sensitive detection method and large cohorts of patients and controls. These studies would enable miRNA research to be translated into clinical practice. The evaluation of biomarker panels, rather than single molecules, provides the conceptual framework for defining the status of biological systems in health and disease and falls into the sphere of molecular pathological epidemiology (MPE). This novel discipline, which has been defined as "epidemiology of molecular pathology and heterogeneity of disease" (*16*), straddles traditional pathology and epidemiology and assesses how particular exposures influence disease risk through the search for and evaluation of molecular pathological markers also in relation to exogenous (*e.g.* exposure) and endogenous factors (*17,18*). Under the umbrella of the "the unique disease principle" (*19*), MPE is intertwined with precision medicine (*16,20*) and is the research branch capable of identifying potential biomarkers for the new frontier of personalized medicine (*21*).

Analysis of the meta-dataset suggested to us that there could be a correlation between deregulated circulating and tissue miRNAs and the pathogenic process triggered by asbestos exposure (*15*). These miRNA pools should be further evaluated not only as diagnostic instruments, but also as possible therapeutic targets by assessing their molecular role. Another key task, beside the evaluation of their up- or down-regulation, is the validation of their targets and regulators, which would clarify how miRNAs

induce or repress critical pathways involved in the carcinogenesis triggered by asbestos exposure.

2.2. *Weaknesses of MM-miRNA research*

An interesting consequence of the evaluation of the works collected in the meta-analysis was the insight we gained into the problems that hamper the translation of research findings into clinical applications. In particular, the lack of study design and method standardization seriously hampers the reproducibility of results obtained in different laboratories, magnifying inconsistencies. Major obstacles were identified in the pre-analysis, intra-analysis, and post-analysis stages. Pre-analytical factors include:

i) patient selection bias, represented by high interindividual variability in exposure levels and genetics (MM subtypes, MM stage, benign proliferation, rate of cancer progression) and by the method adopted to assess asbestos exposure.

ii) sample availability, especially in rare diseases like MM.

iii) lack of standardization in sample collection, handling, and storage (MM samples included fresh/frozen biopsy specimens; formalin-fixed paraffin-embedded (FFPE) tissue; macro-dissected tissue; laser-captured micro-dissected tissue; tissue collected after treatment; plasma, serum, and blood cell fraction, and cell lines).

iv) control sample inconsistency: FFPE biopsies of healthy pleura tissue, patient-matched non-neoplastic pleura, lung, pericardium, healthy lung from asbestos-exposed subjects, specimens from a range of cancers, non-neoplastic proliferations, plasma/serum from healthy or exposed subjects, blood cell fraction of healthy/exposed subjects, immortalized cell lines, and normal human mesothelial cell cultures. In addition, some studies comparing MM isotypes did not envisage a control group of normal samples (*15*).

Analytical factors include:

i) different performance of platforms and variability within and across the analytical methods applied for the discovery and quantification of novel biomarkers. MiRNA quantification approaches were also widely different, including real-time quantitative PCR, qRT-PCR array, microarray, in situ hybridization-based assays, and variants thereof.

ii) limited sample size, statistically underpowered datasets impairing robustness of evidence.

iii) lack of appropriate reference standards and quality control.

Obstacles in the post-analysis phase include different qRT-PCR normalization methods and statistical approaches, poor study design, particularly in the validation phase (which is often missing or is conducted in the same patient cohort used for screening), and finally the failure to report important preanalytical issues related to specimen provenance and biomarker assay protocols (*15*).

We hope that this overview of the strengths of the approaches that have been applied to discover promising miRNAs and of the limitations that hinder the progress of biomarker research toward clinical validation of results may induce a greater focus of research efforts.

3. A call to action to turn the biomarker potential of miRNAs into a clinical reality

We share the view that all investigations should begin with a clearly defined, appropriately designed study that eventually confers clinical benefits on patients (*22*). As regards MM, the poor prognosis and quality of life of patients and the lack of an effective cure urge strong and effective action. Finding biomarkers capable of non-invasive diagnosis or of early disease prediction in high-risk subjects would have enormous implications (*15*). However, the search for biomarkers is a complex process whose steps include verification, validation, demonstration of analytical validity, evaluation of clinical value and, ultimately, assessment of clinical effectiveness (*23*). The major limitations hampering the translation of basic biomarker research into useful clinical assays are reported in Figure 1. Our conclusions are in line with those reached by the National Biomarker Development Alliance (NBDA) after a two-year review and consultation process. This unique trans-sector alliance - which is dedicated to solve the problems hindering biomarker research and discovery to accelerate their clinical application - has identified a number of difficulties affecting the whole biomarker research pipeline, from discovery to clinical validation and regulatory approval (*24,25*). According to the NBDA, key shortcomings of biomarker research translation also include the lack of common standards for data reporting and exchange, for database design and interoperability, for longitudinal integration of discovery and clinical development data, and for the integration of molecular profiling data into electronic medical records. A further weakness is the present reliance on isolated facilities endowed with high technical specialization, whereas coherent systems-based approaches that reflect the "multidimensional technical, clinical and regulatory complexities required to validate a new generation of multiplex molecular diagnostics" would ultimately provide better results (*24*).

These considerations provide a further call to action, to plan well-designed studies for the rapid validation of miRNAs with biomarker potential, alone or combined with mesothelin or fibulin-3, and to test their clinical value in high-risk individuals. In parallel, further basic research work should be aimed to investigate the molecular pathways that are regulated by aberrantly expressed miRNAs. Experimental methods, patient

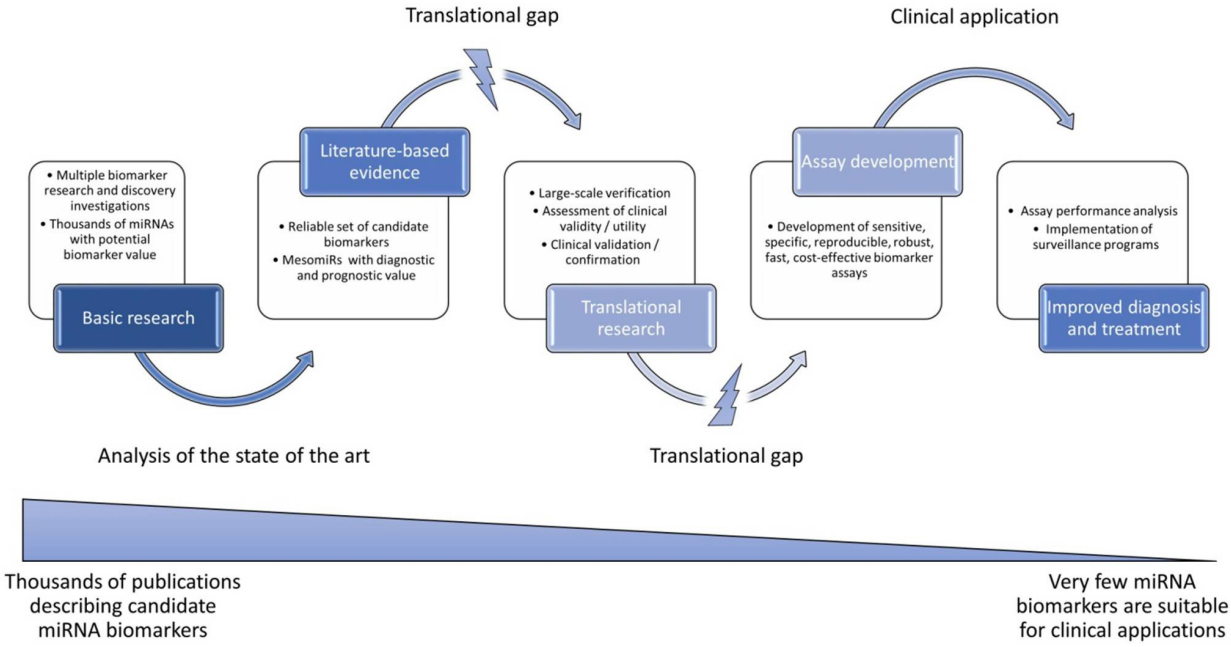

Figure 1. The figure illustrates the problems and gaps in the translation of basic biomarker research into useful clinical assays. According to the NBDA, "*...biomarker research and discovery is a modular, highly interdependent process that requires a systems-based, end-to-end approach to ensure seamless transfer of candidate markers across a series of modules from early discovery to clinical validation and their final regulatory approval*" (*25*). This approach, together with the establishment of broadly accepted standards, "*can dramatically reduce the number of candidate biomarkers eligible to move forward to development*" (*25*).

populations, sample type, and specimen handling and storage protocols should carefully be defined and standardized because they can make the difference between success and failure. Shared quality control guidelines for pre- and post-analytical steps and their documentation would also considerably enhance the definition and refinement of robust miRNA biomarkers. However, such studies, especially those involving rare diseases, take several years, require large samples, and are generally not feasible by single laboratories, both in terms of specimen availability and of resources.

Joint research programs are all the more critical when investigating rare diseases, since they expand sample size and increase statistical power. Success in biomarker research and discovery "demands integration of multidisciplinary expertise and trans-sector collaboration between academia, clinical medicine regulators, industry, payers and patients" (*24*). Future efforts should thus be directed at developing and coordinating transnational research efforts where researchers, clinicians, public health experts, funders, and politicians join forces. This will also help *i*) prevent duplication of efforts and waste of money and time by ensuring efficient use of resources; *ii*) maximize the reliability of the data obtained; *iii*) improve early diagnosis, monitoring, and prognosis; and *iv*) hone treatment strategies. Ideally, highly specialized biomarker research teams investigating miRNAs, their targets and regulators, and related functions could be convened under the NBDA umbrella to lay down the standards, best practices, and guidelines required to set up a systems-based approach.

4. Conclusion

The peculiarities of ARDs, especially the latency of their onset and their distribution, involve that a large number of asbestos-exposed individuals worldwide are still to become MM patients. It is therefore essential to accelerate the search for novel, effective tools and strategies to prevent, diagnose, detect early, and cure these diseases. A multicenter, multidisciplinary and, critically, closely regulated, integrated and standardized systems-based approach would be the method ensuring the fastest return.

Acknowledgements

The authors are grateful to Word Designs for the language revision (www.silviamodena.com).

References

1. Frank AL, Joshi TK. The global spread of asbestos. Ann Glob Heal. 2014; 80:257-262.
2. IARC. Arsenic, metals, fibres, and dusts. IARC Monogr Eval Carcinog Risks Hum. 2012; 100:11-465.
3. Norbet C, Joseph A, Rossi SS, Bhalla S, Gutierrez FR. Asbestos-Related Lung Disease: A Pictorial Review. Curr Probl Diagn Radiol. 2015; 44:371-382.
4. Delgermaa V, Takahashi K, Park E-K, Le GV, Hara T,

Sorahan T. Global mesothelioma deaths reported to the World Health Organization between 1994 and 2008. Bull World Health Organ. 2011; 89:716-724.
5. Yang H, Testa JR, Carbone M. Mesothelioma epidemiology, carcinogenesis, and pathogenesis. Curr Treat Options Oncol. 2008; 9:147-157.
6. Husain AN, Colby T, Ordonez N, *et al*. Guidelines for pathologic diagnosis of malignant mesothelioma: 2012 update of the consensus statement from the International Mesothelioma Interest Group. Arch Pathol Lab Med. 2013; 137:647-667.
7. Opitz I. Management of malignant pleural mesothelioma-The European experience. J Thorac Dis. 2014; 6:S238-252.
8. Greillier L, Baas P, Welch JJ, Hasan B, Passioukov A. Biomarkers for malignant pleural mesothelioma: Current status. Mol Diagn Ther. 2008; 12:375-390.
9. Reid G. MicroRNAs in mesothelioma: From tumour suppressors and biomarkers to therapeutic targets. J Thorac Dis. 2015; 7:1031-1040.
10. Shen J, Stass SA, Jiang F. MicroRNAs as potential biomarkers in human solid tumors. Cancer Lett. 2013; 329:125-136.
11. Nair VS, Pritchard CC, Tewari M, Ioannidis JPA. Design and Analysis for Studying microRNAs in Human Disease: A Primer on -Omic Technologies. Am J Epidemiol. 2014; 180:140-152.
12. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. Nucleic Acids Res. 2011; 39:7223-7233.
13. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A. 2011; 108:5003-5008.
14. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol. 2011; 13:423-433.
15. Micolucci L, Akhtar MM, Olivieri F, Rippo MR, Procopio AD. Diagnostic value of microRNAs in asbestos exposure and malignant mesothelioma: systematic review and qualitative meta-analysis. Oncotarget. 2016; 7:58606-58637.
16. Ogino S, Lochhead P, Giovannucci E, Meyerhardt JA, Fuchs CS, Chan AT. Discovery of colorectal cancer PIK3CA mutation as potential predictive biomarker: Power and promise of molecular pathological epidemiology. Oncogene. 2014; 33:2949-2955.
17. Hamada T, Keum N, Nishihara R, Ogino S. Molecular pathological epidemiology: new developing frontiers of big data science to study etiologies and pathogenesis. J Gastroenterol. 2016. doi: 10.1007/s00535-016-1272-3.
18. Ogino S, Chan AT, Fuchs CS, Giovannucci E. Molecular pathological epidemiology of colorectal neoplasia: An emerging transdisciplinary and interdisciplinary field. Gut. 2011; 60:397-411.
19. Ogino S, Lochhead P, Chan AT, Nishihara R, Cho E, Wolpin BM, Meyerhardt JA, Meissner A, Schernhammer ES, Fuchs CS, Giovannucci E. Molecular pathological epidemiology of epigenetics: Emerging integrative science to analyze environment, host, and disease. Mod Pathol. 2013; 26:465-484.
20. National Research Council (US) Committee on A Framework for Developing a New Taxonomy of Disease. Toward Precision Medicine. Building a Knowledge Network for Biomedical Research and a New Taxonomy of Disease. Washington (DC): National Academies Press (US); 2011. doi: 10.17226/13284.
21. Lu Y-F, Goldstein DB, Angrist M, Cavalleri G. Personalized Medicine and Human Genetic Diversity. Cold Spring Harb Perspect Med. 2014; 4:a008581.
22. Brockmöller J, Tzvetkov M V. Pharmacogenetics: data, concepts and tools to improve drug discovery and drug treatment. Eur J Clin Pharmacol. 2008; 64:133-157.
23. Byron SA, Van Keuren-Jensen KR, Engelthaler DM, Carpten JD, Craig DW. Translating RNA sequencing into clinical diagnostics: opportunities and challenges. Nat Rev Genet. 2016; 17:257-271.
24. Poste G, Compton CC, Barker AD. The national biomarker development alliance: confronting the poor productivity of biomarker research and development. Expert Rev Mol Diagn. 2015; 15:211-218.
25. Barker AD, Compton CC, Poste G. The National Biomarker Development Alliance accelerating the translation of biomarkers to the clinic. Biomark Med. 2014; 8:873-876.

(Received December 28, 2016; Revised January 17, 2017; Accepted January 25, 2017)

Commentary

DOI: 10.5582/bst.2016.01221

Intesive fludarabine-high dose cytarabine-idarubicin combination as induction therapy with risk-adapted consolidation may improve treatment efficacy in younger Acute Myeloid Leukemia (AML) patients: Rationales, evidences and future perspectives

Fabio Guolo*, Paola Minetto, Marino Clavio, Maurizio Miglino, Roberto Massimo Lemoli, Marco Gobbi

Clinic of Hematology, Department of Internal Medicine (DiMI), University of Genoa, IRCCS AOU San Martino-IST, Genoa, Italy.

Summary **Acute Myeloid Leukemia (AML) is the commonest form of leukemia in the adults, with an incidence of 3-4 cases per 100,000 people/year. After the first description of the effective cytarabine + antracycline (3+7) induction regimen, in the last 3 decades, no effective targeted drug has been included in the standard treatment of AML. Many efforts of modifying 3+7 adding a third drug or increasing the dose of anthracycline, cytarabine or both did not lead to substantial improvements, mainly due to increased toxicity. Many in vitro and in vivo evidences suggested that fludarabine may increase efficacy of cytarabine through a synergistic effect. Considering the continuous improvements in supportive care and management of infectious complications the feasibility of more intensive induction strategies have increased and a renewed interest in fludarabine-containing induction strategies arose. The recent MRC AML 15 trial has shown that a fludarabine-containing induction, FLAG-Ida, resulted superior to conventional 3+7 in terms of complete remission rates, relapse incidence and survival, although only a minority of patients could complete the whole planned consolidation program due to an excessive hematological toxicity. Our group recently published a 10-year experience with a fludarabine-containing induction that slightly differed from the MRC one and resulted in good efficacy and higher feasibility. In this commentary we review the major evidences supporting the employ of a fludarabine-containing induction in AML, and discuss the future perspectives.**

Keywords: Acute myeloid leukemia (AML), fludarabine, high dose cytarabine

Acute myeloid leukemia (AML) has an incidence of 3-4 cases per 100,000 people/year and is the commonest form of acute leukemia in the adults (*1,2*).

The development of AML is a multi-step process linked to the progressive accumulation of mutations in a multipotent stem cell. According to a hierarchical model, different mutations occur during leukemogenesis, with founder mutations usually affecting genes involved in the epigenetic machinery (*1,2*).

Intensive induction chemotherapy followed by a consolidation treatment for patients achieving hematological complete remission represents the backbone of AML treatment (*3*).

In the last three decades no effective new drugs have been introduced for AML treatment, with the exception of gemtuzumab-ozogamicin, whose potential benefit for AML patients has not been completely elucidated (*1*).

Standard induction therapy for younger AML patients is still based on a combination of daunorubicin and cytarabine. The rational for testing alternative

Released online in J-STAGE as advance publication January 24, 2017.

**Address correspondence to:*
Dr. Fabio Guolo, Clinic of Hematology, Department of Internal Medicine (DiMI), University of Genoa, IRCCS AOU San Martino-IST, largo Rosanna Benzi 10, 16132, Genoa, Italy.
E-mail: fabio.guolo21@gmail.com

regimens including higher dosage cytarabine (Ara-C), a different anthracycline as idarubicin or a third drug such as and fludarabine and etoposide is to try to reduce the rate of treatment failure. Idarubicin is the anthracycline derivative that is less sensitive to P-glycoprotein (PgP) and more toxic to multi-drug resistance (MDR) cells (*4*). Fludarabine inhibits various cellular metabolic pathways such as DNA, RNA and protein synthesis (*5*). It has been demonstrated that the combination of Fludarabine with Cytarabine results in a synergistic effect on myeloid cells (*6*): *in vitro* studies have shown that fludarabine is able to enhance the concentration of Ara-C triphosphate (Ara-CTP), the active metabolite of Ara-C, in leukemic blasts (*7*) and to inhibit DNA repair mechanisms, providing a rationale for combination therapy with DNA-damaging agents (idarubicin and mitoxantrone) (*8*). The combination of fludarabine plus cytarabine (± anthracycline) provided interesting results when it was first tested on relapsed and refractory AML patients (*9*). Furthermore, fludarabine is reported to be toxic to MDR cells (*5,6*), with the potential of counteract the poor prognostic value of Pgp expression (*10*).

In vitro, the efficacy of fludarabine-cytarabine combination has been improved by adding granulocyte colony stimulating factor (G-CSF) that seems to be able to recruit quiescent leukemic cells into the S-phase of the cell cycle, thus rendering them more sensitive to cycle-specific drugs. G-CSF may also increase the formation of the active metabolite of fludarabine, F-Ara-ATP, and Ara-CTP (*11*).

However, the first studies designed to improve standard 3+7 induction either by increasing or modifying the anthracycline or cytarabine dosing failed to produce better results, probably because of an excess of toxicity (*12,13*). In 1991 our group started investigating induction strategies based on fludarabine-containing regimens in relapsed/refractory and high-risk patients (*14,15*). We showed that a regimen including only one cycle of fludarabine, cytarabine, idarubicin and G-CSF (FLAG-Ida) was effective, well tolerated and improved the feasibility of stem cell transplantation in younger, untreated, de novo AML patients (*16*). Moreover, the continuous improvements in supportive care and management of infectious complications contributed to increase the feasibility of more intensive inductions (*17*). In 2004, we modified the original schedule by omitting G-CSF priming (FLAI), adding a second induction course with cytarabine and idarubicin in order to increase efficacy, and we improved the risk-oriented consolidation (*18*).

In the recent MRC AML15, standard 3+7 with or without etoposide was compared to a fludarabine containing induction which consisted in two identical courses of FLAG-Ida (*19*). CR rate after the first course, relapse risk and survival were better in the FLAG-Ida arm, however, due to higher myelosuppression, only a minority of patients were able to complete the whole planned consolidation therapy (*19*). However, it has to be noted that even for patients who received only the two FLAG-Ida courses without any consolidation, outcome was equivalent to patients receiving 3+7 double induction plus the full planned high dose Ara-C consolidation (*19*).

On the contrary, according to our FLAI-5 induction, fludarabine is administered only in the first course only and idarubicin dose is increased from 10 mg/sqm to 12 mg/sqm in the second course. This strategy may reduces the myelosuppression and the incomplete haematological recovery rate, allowing the majority of patients to complete the scheduled therapy in a timely manner, without jeopardizing the efficacy (*18*).

Consolidation therapy, comprehending allogeneic stem cell transplantation (allo-SCT) or chemotherapy alone, for patients achieving hematological complete remission is fundamental in order to prevent leukemia relapse (*1,7,20*). In the MRC trial, as well as in our experience, consolidation therapy following fludarabine containing induction did indeed improve outcome(*18,19*), however, the concern of therapy-related toxicity is not negligible.

The consolidation strategy is generally chosen after the evaluation of disease-related factors (*i.e.* risk assessment according to clinical and biological features at diagnosis and response to induction) and patient-related factors (*i.e.* performance status, comorbidities, infectious complications) (*1,7,20*) and the correct identification of patients who may benefit from early allo-SCT consolidation is fundamental in order to maximize treatment efficacy (*21*).

Allo-SCT increases the chance of disease cure, through the immunological control of residual disease eventually surviving after the intensive conditioning regimen (*7,20*). In the last decade a deep reduction in transplant related mortality has been achieved thanks to the development of new strategies to prevent Graft versus Host Disease, and through a better management of early and late transplant related complications (*22*). The use of unconventional hematopoietic stem cells sources, as matched unrelated donors and cord blood and the introduction of new transplant procedures designed for the utilization of haploidentical donors have largely increased the feasibility of allo-SCT for patients lacking a sibling matched donor.(*22-24*) Moreover reduced-intensity conditioning regimens allowed older or frail patients in hematological remission to benefit from the transplant associated immunological control of leukemia (*7,20*).

In poor-risk AML patients according to standard risk classification allo-SCT in CR1 is widely recommended. On the contrary the role of allo-SCT for patients belonging to intermediate-risk groups is still matter of debate, considering the therapy-related mortality (TRM) and long term complications related to Graft

versus Host Disease and immunosuppression (*1,7,20*). As a general indication, according to ELN, allo-SCT should be performed in CR1 if an advantage in LFS of at least 10% can be deduced from the individual risk of relapse and non-relapse mortality (*20*). The prognostic role of pre-transplant minimal residual disease (MRD) assessment before allo-SCT in predicting relapse risk and overall survival, have been reported by several groups (*25*). A deep MRD evaluation through high sensitivity techniques as multicolor-flow cytometry or real- time PCR, should be routinely employed to improve patients risk stratification, identifying those who may benefit from a more aggressive consolidation strategy (*25*).

Following the landmark study by the CALGB (*26*), high-doses of Cytarabine (HD-AR-C) have become the standard consolidation chemotherapy for younger patients not undergoing allo-SCT. However, since different studies led to conflicting results, a consensus has not been reached about the usefulness of higher versus intermediate doses of Cytarabine and the number of consolidation cycles which have to be administered (*1,7*). The possible explanation of these discrepancies is that cytarabine was given in the context of different drug combinations, with different infusion rate and following different inductions schedules. The recent MRC trial, who applied two or three courses of higher dose Cytarabine (3 g/sqm bid days 1,3,5) showed that overall consolidation chemotherapy did improve survival, but there was no significant difference between two or three cycles (*19*). In our experience we applied consolidation chemotherapy with lower doses of Cytarabine (2 g/sqm daily days 1 to 4) and confirmed that survival was improved by consolidation chemotherapy in patients not undergoing allo-SCT in first CR; however, we found that 3 or more cycles were better than 2 (*18*). This discrepancy with the MRC trial may be explained by the difference in the Cytarabine dose, with an higher number of cycles needed in our experience to reach the same cumulative dose (*18,19*).

The timing and the intensity of consolidation therapy in the next year will probably be guided by MRD assessment: many groups have highlighted the importance of MRD clearance in AML and how the persistence of MRD is strongly linked to relapse risk. However there is still no consensus on how and when MRD should be assessed and which therapeutic decision should be taken after MRD evaluation (*25*).

Therefore, until specific MRD-driven strategies will be developed, after intensive fludarabine-containing regimen, two or three courses of high dose Cytarabine should be the standard consolidation, at least for younger patients not undergoing allo-SCT.

As a future perspective, the good results of more intensive induction strategies such as FLAI could be further improved with the addition of innovative therapies. Following the observation that the synergistic effect observed with the combination fludarabine-cytarabine could be mostly related to an increased availability of ara-CTP into the leukemic blasts, a modification on cytarabine chemical structure was attempted. Elacytarabine is the elaidic acid ester derivative of cytarabine, designed to enter cells independently of nucleoside transporters. Phase I/II trials were encouraging although subsequent clinical studies did not confirm superiority of elacytarabine as monotherapy in relapsed/refractory AML, compared with standard of care (*27*). Perhaps, elacytarabine should be further tested in earlier stage of disease and in combination with fludarabine or other conventional AML drugs. Many groups have shown that low-dose Gemtuzumab Ozogamicin can be safely incorporated in modern induction therapy and may improve the outcome, at least for non high-risk patients (*28*). Moreover, recent evidence suggest that the addition of the multi-kinase inhibitor midostaurin to conventional 3+7 induction improves the outcome in FLT-ITD mutated AMLs (*29*). Since fludarabine containing induction seems to partially overwhelm the negative prognostic impact of FLT3-ITD, at least when concomitant NPM1 mutation is present (*30*), there is a strong rationale for incorporating midostaurin in fludarabine containing regimen as well.

In conclusion, thanks to the great improvement in the supportive care, nowadays more intensive fludarabine-containing induction represents the most effective therapy at least for younger AML patients. A risk-oriented strategy is of great importance in order to maximize the adherence to therapy and to tailor consolidation to the individual risk, minimizing treatment related-toxicity. In the next years, the incorporation of targeted molecules and the implementation of minimal residual disease-driven choices will further contribute to improve the overall good results achieved.

References

1. Döhner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. N Engl J Med. 2015; 373:1136-1152.
2. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, Bloomfield CD, Cazzola M, Vardiman JW. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016; 127:2391-2405.
3. Döhner H, Estey EH, Amadori S, *et al*. European LeukemiaNet. Diagnosis and management of acute myeloid leukemia in adults: Recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115:453-474.
4. Berman E, McBride M. Comparative cellular pharmacology of daunorubicin and idarubicin in human multidrag-resistant leukemia cells. Blood. 1992;79:3267-3272.
5. Michieli M, Damiani D, Ermacora A, Masolini P, Raspadori D, Visani G, Scheper RJ, Baccarani M.

P-glycoprotein, lung resistance-related protein and multidrug resistance associated protein in de novo acute non lymphocytic leukemias: Biological and clinical implications. Br J Haematol. 1999;104:328-335.
6. Michelutti A, Michieli M, Damiani D, Melli C, Ermacora A, Grimaz S, Candoni A, Russo D, Fanin R, Baccarani M. Effect of fludarabine and arabinosylcytosine on multidrug resistant cells. Haematologica. 1997; 82:143-147.
7. Gandhi V, Estey E, Keating MJ, Plunkett W Fludarabine potentiates metabolism of cytarabine in patients with acute myelogenous leukemia during therapy. J Clin Oncol. 1993; 11:116-124.
8. Plunkett W, Gandhi V, Huang P, Robertson LE, Yang LY, Gregoire V, Estey E, Keating MJ. Fludarabine: Pharmacokinetics, mechanisms of action, and rationales for combination therapies. Sem Oncol. 1993; 20:2-12.
9. Estey E, Plunkett W, Gandhi V, Rios MB, Kantarjian H, Keating MJ. Fludarabine and Arabinosylcytosine therapy of refractory and relapsed acute myelogenous leukaemia. Leuk Lymphoma. 1993;9:343-350.
10. Russo D, Priccolo G, Michieli MG, *et al*. Fludarabine, arabinosyl cytosine and idarubicin (FLAI) for remission induction in poor-risk acute myeloid leukaemia. Leuk Lymphoma. 2001;40:335-343.
11. Estey E, Thall P, Andreeff M, Beran M, Kantarjian H, O'Brien S, Escudier S, Robertson LE, Koller C, Kornblau S, Pierce S, Freireich E, Deisseroth A, Keating M. Use of granulocyte colony-stimulating factor before, during, and after Fludarabine plus cytarabine induction therapy of newly diagnosed acute myelogenous leukemia or myelodisplastic syndromes: Comparison with Fludarabine plus cytarabine without granulocyte colony-stimulating factor. J Clin Oncol. 1994; 12:671-678.
12. Burnett AK, Russell NH, Hills RK, Kell J, Cavenagh J, Kjeldsen L, McMullin MF, Cahalin P, Dennis M, Friis L, Thomas IF, Milligan D, Clark RE; UK NCRI AML Study Group. A randomized comparison of daunorubicin 90 mg/m^2 *vs* 60 mg/m^2 in AML induction: Results from the UK NCRI AML17 trial in 1206 patients. Blood. 2015; 125:3878-385.
13. The AML Collaborative Group. A systematic collaborative overview of randomized trials comparing Idarubicin with daunorubicin (or other anthracyclines) as induction therapy for acute myeloid leukaemia. Br J Haematol. 1998; 103:100-109.
14. Clavio M, Carrara P, Miglino M, Pierri I, Canepa L, Balleari E, Gatti AM, Cerri R, Celesti L, Vallebella E, Sessarego M, Patrone F, Ghio R, Damasio E, Gobbi M. High efficacy of fludarabine-containing therapy (FLAG-FLANG) in poor risk acute myeloid leukemia. Haematologica. 1996; 81:513-520.
15. Clavio M, Gatto S, Beltrami G, Cerri R, Carrara P, Pierri I, Canepa L, Miglino M, Balleari E, Masoudi B, Damasio E, Ghio R, Sessarego M, Gobbi M. First line therapy with fludarabine combinations in 42 patients with either post myelodysplastic syndrome or therapy related acute myeloid leukaemia. Leuk Lymphoma. 2001; 40:305-313.
16. Clavio M, Gatto S, Beltrami G, *et al*. Fludarabine, ARA-C, idarubicin and G-CSF (FLAG-Ida), high dose ARA-C and early stem cell transplant. A feasable and effective therapeutic strategy for *de novo* AML patients. J Exp Clin Cancer Res. 2002;21:481-487.
17. Othus M, Kantarjian H, Petersdorf S, Ravandi F, Godwin J, Cortes J, Pierce S, Erba H, Faderl S, Appelbaum FR, Estey E. Declining rates of treatment-related mortality in patients with newly diagnosed AML given 'intense' induction regimens: A report from SWOG and MD Anderson. Leukemia. 2014; 28:289-292.
18. Guolo F, Minetto P, Clavio M, *et al*. High feasibility and antileukemic efficacy of fludarabine, cytarabine, and idarubicin (FLAI) induction followed by risk-oriented consolidation: A critical review of a 10-year, single-center experience in younger, non M3 AML patients. Am J Hematol. 2016;91:755-762.
19. Burnett AK, Russell NH, Hills RK, Hunter AE, Kjeldsen L, Yin J, Gibson BE, Wheatley K, Milligan D. Optimization of chemotherapy for younger patients with acute myeloid leukemia: Results of the medical research council AML 15 trial. J Clin Oncol. 2013; 31:3360-3368.
20. Schlenk RF. Post-remission therapy for acute myeloid leukemia. Haematologica 2014; 99:1663-1670.
21. Cornelissen JJ, Gratwohl A, Schlenk RF, Sierra J, Bornhäuser M, Juliusson G, Råcil Z, Rowe JM, Russell N, Mohty M, Löwenberg B, Socié G, Niederwieser D, Ossenkoppele GJ. The European LeukemiaNet AML Working Party consensus statement on allogeneic HSCT for patients with AML in remission: An integrated-risk adapted approach. Nat Rev Clin Oncol. 2012; 9:579-590.
22. Ballen KK, Koreth J, Chen YB, Dey BR, Spitzer TR. Selection of optimal alternative graft source: Mismatched unrelated donor, umbilical cord blood, or haploidentical transplant. Blood. 2012; 119:1972-1980.
23. Raiola AM, Dominietto A, Di Grazia C, Lamparelli T, Gualandi F, Ibatici A, Bregante S, Van Lint MT, Varaldo R, Ghiso A, Gobbi M, Carella AM, Signori A, Galaverna F, Bacigalupo A. Unmanipulatedhaploidentical transplants compared with other alternative donors and matched sibling grafts. Biol Blood Marrow Transplant. 2014; 20:1573-1579.
24. Bacigalupo A, Dominietto A, Ghiso A, *et al*. Unmanipulatedhaploidentical bone marrow transplantation and post-transplant cyclophosphamide for hematologic malignanices following a myeloablative conditioning: An update. Bone Marrow Transplant 2015; 50 Suppl2:S37-9.
25. Araki D, Wood BL, Othus M, Radich JP, Halpern AB, Zhou Y, Mielcarek M, Estey EH, Appelbaum FR, Walter RB. Allogeneic hematopoietic cell transplantation for acute myeloid leukemia: Time to move toward a minimal residual disease-based definition of complete remission? J Clin Oncol. 2016;34:329-336.
26. Bloomfield CD, Lawrence D, Byrd JC, Carroll A, Pettenati MJ, Tantravahi R, Patil SR, Davey FR, Berg DT, Schiffer CA, Arthur DC, Mayer RJ. Frequency of prolonged remission duration after high-dose cytarabine intensification in acute myeloid leukemia varies by cytogenetic subtype. Cancer Res. 1998;58:4173-4179.
27. Roboz GJ, Rosenblat T, Arellano M, *et al*. International randomized phase III study of elacytarabine versus investigator choice in patients with relapsed/refractory acute myeloid leukemia. J Clin Oncol. 2014; 32:1919-1926.
28. Burnett A, Cavenagh J, Russell N, Hills R, Kell J, Jones G, Nielsen OJ, Khwaja A, Thomas I, Clark R; UK NCRI AML Study Group. Defining the dose of Gemtuzumab ozogamicin in combination with induction chemotherapy in acute myeloid leukemia: A comparison of 3 mg/m2 with 6 mg/m2 in the NCRI AML17 Trial. Haematologica. 2016; 101:724-731.

29. Gallogly MM, Lazarus HM. Midostaurin: An emerging treatment for acute myeloid leukemia patients. J Blood Med. 2016; 7:73-83.
30. Guolo F, Minetto P, Clavio M, *et al*. Fludarabine-containing induction increases minimal residual disease clearance and improves survival in AML patients with concomitant NPM1 and FLT3-ITD mutations (abstract). 2015; 100439. *http://learningcenter.ehaweb.org/eha/2015/20th/100439/fabio.guolo.fludarabine-containing.induction.increases.minimal.residual.html?f=m1 (*accessed November 23, 2016).

(Received November 24, 2016; Revised January 12, 2017; Accepted January 16, 2017)

Commentary

DOI: 10.5582/bst.2016.01225

To a better understanding of the giardial ENTH protein function

Constanza Feliziani*, María C. Touz

Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC – CONICET – Universidad Nacional de Córdoba, Córdoba, Argentina.

Summary **Epsin *N*-terminal homology (ENTH) domains are present at the N-terminus of either the epsin or epsin-related (epsinR) proteins. These proteins have been involved in clathrin-mediated trafficking and are critical for membrane deformation at the site of vesicle budding. While more than one type of these proteins have been described in many eukaryotic cells, the protozoa parasite *Giardia lamblia* contains only one member of this ENTH-protein family. In the last two years, four works have been published showing that this giardial protein might play diverse functions. This commentary gives a brief overview on the current status of the particular characteristics and functions of this unique protein.**

Keywords: *Giardia lamblia*, epsin

1. Introduction

A feature that defines eukaryotic cells is the compartmentalization of their cytoplasm in different membrane-associated organelles. In order to maintain these compartments, cells have developed mechanisms to ensure that specific proteins are targeted to particular organelles (*1*). Thus, subcellular compartmentalization became an essential feature in these organisms, allowing the correct interrelation of certain intracellular components and enabling reactions to occur efficiently and orderly. To connect all compartments proteins and lipids are trapped into transport vesicles, which are made of "coatomers", specific for a particular compartment. Clathrin-Coated Vesicles (CCVs) are transport vesicles that have been extensively characterized in a wide variety of eukaryotic cells and mediate the delivery of cargo molecules to the endosomal/lysosomal compartments. These vesicles are covered by a layer that is composed of scaffold proteins, clathrin and several oligomeric and monomeric adapter proteins (APs: Adaptor Proteins). The APs binds clathrin and also recognize specific classification signals that are present in the cytosolic domains of transmembrane proteins, producing accumulation of these proteins in the CCVs.

Giardia lamblia is a parasite of great importance not only because it is the cause of a very frequent disease, giardiasis, but also because it shows clear evidence of reductive evolution (*2*). In this scenario, *G. lamblia* presents an atypical endomembranous system (*3,4*), characterized by the presence of ER and lysosome-like peripheral vacuoles (PVs) and by the absence of other organelles characteristic of higher eukaryotes such as endosomes, lysosomes, mitochondria, peroxisomes, and a morphologically recognizable Golgi apparatus. The simplicity of the giardial subcellular organization makes this parasite an excellent model to study the basis of different cellular processes in eukaryotes, such as vesicular protein trafficking. The very existence of CCVs in *Giardia* is controversial since typical clathrin-coated cages or clathrin budding have never been observed in this parasite. However, there is increasing amount of fresh data suggesting that *Giardia* utilizes both conserved and non-conserved mechanisms for protein delivery via clathrin vesicles. Without a morphologically discernible Golgi apparatus, the anterograde vacuolar protein trafficking seems to start at the ER through the action of AP-1 and clathrin while receptor-mediated endocytosis involves the participation of AP-2 and clathrin. Last year, the presence of a monomeric adaptor protein containing an ENTH domain (see below) was discovered. Here, we showed and discussed all the findings about GlENTHp

Released online in J-STAGE as advance publication January 24, 2017.

**Address correspondence to:*
Dr. Constanza Feliziani, Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC – CONICET – Universidad Nacional de Córdoba, Friuli 2434, Córdoba, Argentina.
E-mail: cfeliziani@immf.uncor.edu

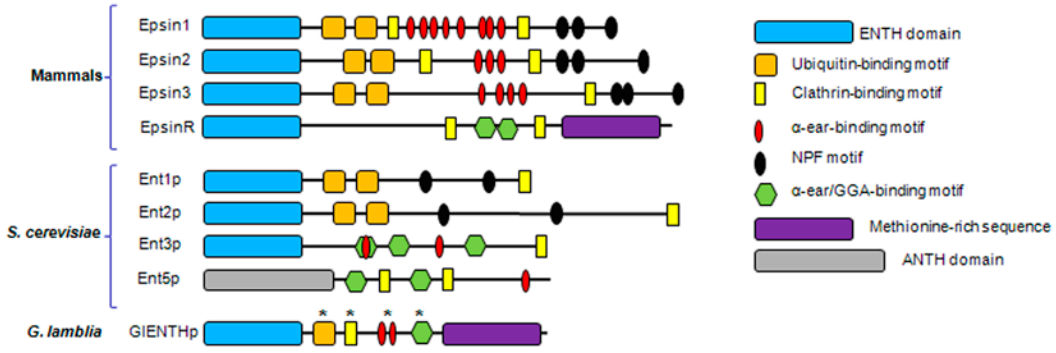

Figure 1. Schematic representation of E/ANTH proteins of mammals, *S. cerevisiae* and *G. lamblia*. The ENTH or ANTH domain is depicted in the N-terminus. Specific motifs within their C-terminal region are also represented. NPF motifs bind to Eps15 homology (EH) domain-containing proteins. Asterisks in GlENTHp denote no defined localization or presence of the motifs.

(for *Giardia lamblia ENTH protein*), focussing on its function and origin.

2. The multiple function of GlENTHp

The ENTH/ANTH/VHS superfamily are composed by proteins that possesses either an ENTH (Epsin N-terminal homology), an ANTH (AP180 N-terminal homology) or a VHS (Vps27, Hrs and STAM) domain at their N-terminus and have been identified in proteins that participate in clathrin-mediated budding in mammalian cells (*5*). Among the ENTH family, the epsin subfamily are composed by classical epsin proteins and the epsin-related (epsinR) protein that play a role in clathrin-mediated endocytosis or Golgi-to-endosome protein trafficking, respectively (*6,7*). The ENTH module binds phosphoinositides (PIs), with epsin preferring $PI4,5P_2$ while epsinR favouring PI4P binding. At their C-terminal these proteins contain several short motifs that mediate interactions with endocytic proteins and are characteristic for each protein (Figure 1) (*8*).

In Feliziani *et al*. 2015, we presented our first studies on GlENTHp (GL50803_3256), which possesses the highly conserved ENTH domain at its N-terminal region (*9*). Because, only one member of the family is present in *Giardia*, we wondered whether it might function as a classical epsin or as an epsinR-like protein in this parasite. Analysis of its secondary and tertiary structure showed that the only conserved motif between GlENTHp and other members of this family was the ENTH domain but possesses a methionine-rich sequence with unknown function that is only observed in the epsinR (*10*). Besides the lack of other conserved motifs like the ubiquitin-interacting motif (UIM), clathrin-binding motifs and the α-ear (AP-2)-binding or γ-ear (AP-1)-binding motifs, we found that GlENTHp is able to interact with proteins involved in CCVs formation, besides specific PIs. By immunofluorescence assay (IFA) and confocal microscopy, we showed that GlENTHp localized in the cytosol and somehow in the nuclei. Similarly to classical epsin-type adaptors, it was associated with the giardial αAP-2, clathrin heavy chain, ubiquitin and it was able to interact with $PI3,4,5P_3$, which was observed at the plasma membrane (PM) of the parasite. We also found that GlENTHp played an active role in receptor-mediated endocytosis of low-density lipoproteins. On the other hand, a direct association was found with the γAP-1 and PI4P, present in the *Giardia* ER-sorting sites, and was implicated in the trafficking of the soluble acid phosphatase from the ER to the PVs, suggesting that GlENTHp could also carry out a role of epsinR in the parasite. When the PVs were ultrastructural analyzed in transgenic cells over-expressing GlENTHp, we observed that these oval-shaped vacuoles lost their heterogeneous electron-density when compared with *wild-type* cells. Conversely, enhance electron-density of the PVs was detected in cells in which the GlENTHp expression was either reduced or replaced by a mutant that is unable to bind PIs (*9*). These studies suggest that GlENTHp would be involved in the transport of material to and/or from the PVs, playing an active role in the maturation of these vacuoles. The alteration of the GlENTHp function caused a severe defects in cell growth, which correlated with more electron-dense PVs, reinforcing the idea that GlENTHp could be implicated in the preservation of the PV homeostasis and cell survival. Several evidences support the idea that the proteins involved in endocytosis may also play a role in the nucleus (*11,12*). Surprisingly, we found an increase of the GlENTHp mutant in one of the nuclei, when compared with endogenous GlENTHp. This result was correlated with an increase nuclear localization of GlENTHp, but not of the mutant, when the cells were treated with LY294002 to reduce the synthesis of $PI3,4,5P_3$ and PI4P. These results suggest that GlENTHp might continuously move in and out of the nuclei depending on its ability to bind PIs. Further analyses are necessary to disclose how the mechanism of GlENTHp trafficking through the nuclear membrane takes place and what the meaning of this behavior is.

At the time we were performing this analysis, another group published new results about GlENTHp (*13*). Surprisingly, they found that GlENTHp localized in the giardial ventral disk, a microtubule structure and the primary organelle of attachment to the cell host (*13*). One explanation of this differential behavior of the protein might be just a technical problem since the cytosolic and the membrane distribution of GlENTHp was demonstrated by biochemical and cellular analysis in our work. Moreover, many other differences were reported in this Short Communication, like the partial PI binding specificity of GlENTHp and the lack of binding to any endosomal components, including clathrin. Because we demonstrated each finding utilizing more than one approach, we are confident that we were able to disclose the native function of GlENTHp.

In conclusion, significant steps have been made in the functional characterization of GlENTHp, disclosing that not only AP-1 and AP-2 (as we previously envisioned) are involved in the clathrin-mediated traffic in *Giardia*, but the action of the monomeric GlENTHp is also required. Conversely to AP-1, which was implied in the ER-to-PV protein trafficking or to AP-2, which participates in receptor-mediated endocytosis, GlENTHp seems to play a critical role in both pathways, depending on the specific requirement of the parasite. This is another evidence that *Giardia* might use the same protein to perform multiple functions, making the studies in this parasite increasingly fascinating.

3. Evolutionary origin

The members of the ENTH family are highly conserved, even among organisms as diverse as humans, *Xenopus*, *Drosophila*, *Arabidopsis*, yeasts, and *T. brucei*. However, there are differences in the number of proteins that are members of this family in each species. Thus, while yeast *S. cerevisiae* and most vertebrates (including primates, rodents and zebrafish) contain at least two paralogs of the ENTH family, only one member of this family is present in organisms such as *Toxoplasma gondii, Plasmodium Falciparum and Giardia lamblia* (*14*). In *Saccharomyces cerevisiae* it has been characterized two classical epsin homologues, Ent1p and Ent2p, which are essential proteins with redundant functions, and two epsinR orthologues, Ent3p and Ent5p (*15,16*). In an elegant report, De Craene and co-workers performed *in silico* analysis of this ENTH/ANTH/VHS superfamily, consisting of proteins from 84 genomes representative of the different eukaryotic taxa. They found the ENTH domain of *Giardia* (member of the Excavata taxa), is the unique representative of the ENTH/ANTH/VHS function, thus suggesting that multiple tasks might be performed by the unique ENTH-containing protein (*14*). This statement encouraged us to analyze whether GlENTHp could be a key player in the evolution of the ENTH family by testing its role in *S. cerevisiae*.

In Feliziani *et al*. 2016, we showed that neither the overexpression of GlENTHp nor of the gENTH alone rescued the lethal phenotype in the *ent1Δent2Δ* cells (*17*). It was shown that the essential function of the ENTH motif resided in the conserved Y100 and T104 residues (*18*). Because gENTH domain lacks the conserved T within its sequence, we were wonder whether it might be the reason for the incapacity of GlENTHp to rescue the lethal *ent1Δent2Δ* cells. To test this, we performed complementary assays now using the $GlENTH_{N107Yp}$ and $gENTH_{N107Y}$ mutants. The results showed that none of the mutants were able to complement the *ent1Δent2Δ* cells, suggesting that the presence of the Y/T residues in conserved sites was not sufficient for GlENTHp to supplement the function of Ent1/2p in *S. cerevisiae*. Through functional assays, we evaluated the location of the pheromone a factor receptor Ste3 and the polymerization of the actin-binding protein ABP1 in *wild-type* cells by overexpressing GlENTHp. We found that GlENTHp was not able to act as a dominant negative protein of Ent1/2p by interfering with its normal function. At this point, we concluded that GlENTHp was unable to replace the function of Ent1/2p in fungi, most likely due to the limited degree of homology between sequences of these proteins beyond the ENTH domain. Also, it is possible that the preference of GlENTHp, for $PI3,4,5P_3$ and PI4P over $PI4,5P_2$, altered its interaction with the appropriate target at the PM, resulting in rescue failure in *ent1Δent2Δ* cells. But can GlENTHp be able to perform Ent3/5p functions in *S. cerevisiae* instead? To evaluate this possibility, we assessed deficiencies in the transport of fluorescence carboxypeptidase S, GFP-Cps1 (*19*), and alteration of α-factor maturation (*20*) in *ent3Δent5Δ* cells (*21*). The results showed that the expression of GlENTHp neither re-established the localization of GFP-Cps1 nor restored the maturation of the α-factor, being unable to perform the function of Ent3/5p. To assess whether GlENTHp could act as a dominant negative protein of the Ent3/5p, the localization of Cps1 and maturation of α-factor was again analyzed but now using *wild-type* cells overexpressing GlENTHp. By performing fluorescence microscopy assays in living cells and halo bioassays, we found that overexpression of GlENTHp interfered with the function of epsinR-type proteins in yeasts, evidenced by changes in the route of GFP-Cps1 and by alterations in the maturation of the α-factor. The interaction of Ent3/5p with PI4P has been reported to be crucial for their localization and function (*22*). Thus, we decided to test whether the dominant negative effect of GlENTHp overexpression was related to a change in the localization of Ent3/5p by overexpressing GlENTHp, gENTH and the mutant that is unable to bind PIs. Only when GlENTHp was

over-expressed in cells expressing Ent3p or Ent5p, a change in the localization of Ent3/5p was observed, suggesting that GlENTHp physically interfered with the Ent3/5p recruitment to the *trans*-Golgi network (TGN). In spite of lacking the canonical domains of interaction, we decided to test whether GlENTHp interacted with clathrin, the adapter subunit γAP-1 (APL4) and/or the ADP-ribosylation factor-binding proteins GGA1 and GGA2 (*15*), by performing FRET experiments in live yeast cells. The results showed that GlENTHp interacts with APL4, GGA1 and GGA2. Based on the results obtained, we proposed that GLENTHp transiently interacts with PI4P at the TGN membrane, blocking the binding of GGAs to the membrane and thus preventing the GGA2p/Ent3p binding. GlENTHp could also compete directly with Ent3/5p for the binding to AP-1 and GGAs but not clathrin, modifying the packaging of these proteins in the CCVs. These finding probably explains the clear misslocalization and missfunction of Ent3/5p when GlENTHp is overexpressed. Taken together, our results reinforce the idea that although there is a conserved sorting mechanism between *S. cerevisiae* and *G. lamblia*, a higher degree of similarity in the protein sequence is necessary to replicate the protein function in a different organism.

4. Conclusions

Although clathrin-mediated vesicular trafficking is one of the most conserved through the eukaryotic evolution and involves common regulators, it is more and more evident that it shows distinct particularities depending on the requirements of each cell type. In this sense, the finding that *Giardia* is able to utilize GlENTHp as a monomeric adaptor to fulfill two different clathrin-mediated mechanisms, point emphasis on the behavior of organisms that undergo reductive evolution. More studies are required to disclose other putative functions of GlENTHp performed beyond the cytoplasm. This, at the end, will contribute to understand the plasticity of this parasite to adapt to different environment thorough its evolution.

Acknowledgements

This research was supported by the Argentine Agencia Nacional para la Promoción de la Ciencia y Tecnología (FONCyT- PICT2013-1122). The authors would like to thank Dr. Andrea Rópolo for her insightful suggestions and comments.

References

1. Munro S. Organelle identity and the organization of membrane traffic. Nat Cell Biol. 2004; 6:469-472.
2. Sogin ML, Gunderson JH, Elwood HJ, Alonso RA, Peattie DA. Phylogenetic meaning of the kingdom concept: An unusual ribosomal RNA from *Giardia lamblia*. Science. 1989; 243:75-77.
3. Gillin FD, Reiner DS, McCaffery JM. Cell biology of the primitive eukaryote *Giardia lamblia*. Annu Rev Microbiol. 1996; 50:679-705.
4. Lujan HD, Touz MC. Protein trafficking in *Giardia lamblia*. Cell Microbiol. 2003; 5:427-434.
5. Itoh T, De Camilli P. BAR, F-BAR (EFC) and ENTH/ANTH domains in the regulation of membrane-cytosol interfaces and membrane curvature. Biochim Biophys Acta. 2006; 1761:897-912.
6. Ford MG, Mills IG, Peter BJ, Vallis Y, Praefcke GJ, Evans PR, McMahon HT. Curvature of clathrin-coated pits driven by epsin. Nature. 2002; 419:361-366.
7. Mills IG, Praefcke GJ, Vallis Y, Peter BJ, Olesen LE, Gallop JL, Butler PJ, Evans PR, McMahon HT. EpsinR: An AP1/clathrin interacting protein involved in vesicle trafficking. J Cell Biol. 2003; 160:213-222.
8. Maldonado-Baez L, Wendland B. Endocytic adaptors: Recruiters, coordinators and regulators. Trends Cell Biol. 2006; 16:505-513.
9. Feliziani C, Zamponi N, Gottig N, Ropolo AS, Lanfredi-Rangel A, Touz MC. The giardial ENTH protein participates in lysosomal protein trafficking and endocytosis. Biochim Biophys Acta. 2015; 1854:646-659.
10. Kalthoff C, Groos S, Kohl R, Mahrhold S, Ungewickell EJ. Clint: A Novel Clathrin-binding ENTH-Domain Protein at the Golgi. Mol Biol Cell. 2002; 13:4060-4073.
11. Vecchi M, Polo S, Poupon V, van de Loo JW, Benmerah A, Di Fiore PP. Nucleocytoplasmic shuttling of endocytic proteins. J Cell Biol. 2001; 153:1511-1517.
12. Hyman J, Chen H, Di Fiore PP, De Camilli P, Brunger AT. Epsin 1 undergoes nucleocytosolic shuttling and its eps15 interactor NH_2-terminal homology (ENTH) domain, structurally similar to *Armadillo* and HEAT repeats, interacts with the transcription factor promyelocytic leukemia Zn^2+ finger protein (PLZF). J Cell Biol. 2000; 149:537-546.
13. Ebneter JA, Hehl AB. The single epsin homolog in *Giardia lamblia* localizes to the ventral disk of trophozoites and is not associated with clathrin membrane coats. Mol Biochem Parasitol. 2014; 197:24-27.
14. De Craene JO, Ripp R, Lecompte O, Thompson JD, Poch O, Friant S. Evolutionary analysis of the ENTH/ANTH/VHS protein superfamily reveals a coevolution between membrane trafficking and metabolism. BMC Genomics. 2012; 13:297.
15. Duncan MC, Costaguta G, Payne GS. Yeast epsin-related proteins required for Golgi-endosome traffic define a gamma-adaptin ear-binding motif. Nat Cell Biol. 2003; 5:77-81.
16. Duncan MC, Payne GS. ENTH/ANTH domains expand to the Golgi. Trends Cell Biol. 2003; 13:211-215.
17. Wendland B, Steece KE, Emr SD. Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis. EMBO J. 1999; 18:4383-4393.
18. Aguilar RC, Longhi SA, Shaw JD, Yeh LY, Kim S, Schon A, Freire E, Hsu A, McCormick WK, Watson HA, Wendland B. Epsin N-terminal homology domains perform an essential function regulating Cdc42 through binding Cdc42 GTPase-activating proteins. Proc Natl Acad Sci U S A. 2006; 103:4116-4121.
19. Eugster A, Pecheur EI, Michel F, Winsor B, Letourneur F, Friant S. Ent5p is required with Ent3p and Vps27p

for ubiquitin-dependent protein sorting into the multivesicular body. Mol Biol Cell. 2004; 15:3031-3041.
20. Payne GS, Schekman R. Clathrin: A role in the intracellular retention of a Golgi membrane protein. Science. 1989; 245:1358-1365.
21. Copic A, Starr TL, Schekman R. Ent3p and Ent5p exhibit cargo-specific functions in trafficking proteins between the trans-Golgi network and the endosomes in yeast. Mol Biol Cell. 2007; 18:1803-1815.
22. Demmel L, Gravert M, Ercan E, Habermann B, Muller-Reichert T, Kukhtina V, Haucke V, Baust T, Sohrmann M, Kalaidzidis Y, Klose C, Beck M, Peter M, Walch-Solimena C. The clathrin adaptor Gga2p is a phosphatidylinositol 4-phosphate effector at the Golgi exit. Mol Biol Cell. 2008; 19:1991-2002

(Received November 29, 2016; Revised January 13, 2017; Accepted January 16, 2017)

News

DOI: 10.5582/bst.2017.01040

Global concern regarding the fifth epidemic of human infection with avian influenza A (H7N9) virus in China

Yinzhong Shen, Hongzhou Lu*

Shanghai Public Health Clinical Center, Fudan University, Shanghai, China.

Summary **Since the first outbreak of human infection with avian influenza A (H7N9) virus was identified in 2013, five seasonal outbreaks have occurred in China. The fifth outbreak started earlier than usual. A sudden increase in cases of human infection with avian influenza A (H7N9) virus has been reported in China since September 2016, and the number of cases reported in this season is exceeding that reported in previous seasons. This increase in the number of new cases of H7N9 infection has caused domestic and international concern. This paper summarizes the current prevalence of H7N9 in China and it also discusses measures that China has taken to control this outbreak. This paper also describes steps China must take in the future. This paper can serve as a reference for prevention and control of H7N9 outbreaks around the world.**

Keywords: Avian influenza A (H7N9) virus, outbreak, China

The H7N9 subtype of avian influenza A virus was not known to have the capacity to infect humans until March 31, 2013. Novel reassortant avian influenza A (H7N9) viruses are associated with severe and fatal respiratory diseases in humans, and most patients who are confirmed to be infected with H7N9 virus are critically ill (*1,2*). Since the first outbreak of human infection with avian influenza A (H7N9) virus was identified in 2013, five seasonal outbreaks have occurred in China. The fifth outbreak started earlier than usual. A sudden increase in cases of human infection with avian influenza A (H7N9) virus has been reported in China since September 2016, and the number of cases reported in this season is exceeding that reported in previous seasons (*3*). This increase in the number of new cases of H7N9 infection has caused domestic and international concern.

According to Disease Outbreak News issued by the World Health Organization (WHO) on February 22, 2017, a total of 1223 laboratory-confirmed cases of human infection with avian influenza A (H7N9) virus had been reported since early 2013 (*3*). The number of human cases developing since October 1, 2016 accounted for nearly one-third of all human cases of H7N9 infection reported since 2013 (*3*). These cases include five cases reported in Taiwan, 20 cases reported in Hong Kong, one case reported in Macau, two cases reported in Canada, and one case reported in Malaysia (*3*). As of February 23, 2017, at least 425 cases had been reported during the fifth outbreak in China, which began in October and spiked suddenly in December (*4*). According to the National Health and Family Planning Commission (NHFPC) of China, cases of H7N9 infection have been reported in 16 provinces since January 2017 (*5*). According to data released by the NHFPC, 192 cases of H7N9 infection (including 79 deaths) were reported in January 2017 (*6*). Most cases occurred around the Yangtze and Pearl river deltas.

A surveillance report (*7*) suggested that most cases in the fifth outbreak were still highly sporadically distributed without any epidemiological links; the main characteristics remained the same and the genetic characteristics of viral strains that were isolated during this outbreak were similar to characteristics of strains involved in earlier outbreaks. However, several notable features of the fifth outbreak are (*7*): *i*) a quarter of the infected individuals were farmers; *ii*) most of the infected individuals lived in urban areas and reported

Released online in J-STAGE as advance publication February 28, 2017.

**Address correspondence to:*
Dr. Hongzhou Lu, Department of Infectious Diseases, Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, China.
E-mail: luhongzhou@fudan.edu.cn

exposure to live poultry; and *iii*) only 5% of infected individuals received oseltamivir within 48 hours of the onset of symptoms.

Obstacles to the control of this outbreaks in China are: *i*) live poultry markets and the live poultry trade; *ii*) the public's preference for fresh food markets; *iii*) raising free-range chickens, particularly in rural areas; *iv*) the public's limited knowledge about H7N9; *v*) delays in early identification and diagnosis; and *vi*) continuing isolation of H7N9 from animals and their surroundings.

The situation has prompted China to enhance prevention and control measures. On February 22, 2017, China's premier, Li Keqiang, convened a meeting to take steps to control outbreaks and Premier Li asked provincial officials in affected areas to close live poultry markets as soon as possible (*8*). The state has asked all poultry markets to thoroughly disinfect their premises. China will implement a new model to help upgrade the poultry industry; this model involves "raising livestock on a larger scale, centralized slaughter, cold chain transport, and sale of chilled meat." On January 24, 2017, the NHFPC issued an updated guidance (2017 version) on clinical management of H7N9 (*9*), and the NHFPC has trained doctors in screening and early diagnosis of the infection and treatment of critically ill patients. China needs to continue enhancing strict H7N9 surveillance, to coordinate multiple sectors to control infection, and to initiate national health preparedness. Health education and public campaigns should be enhanced to increase public awareness of the disease. Strategies to facilitate rapid identification of cases and early antiviral treatment need to be promptly implemented.

References

1. Gao R, Cao B, Hu Y, *et al*. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med. 2013; 368:1888-1897.
2. Li Q, Zhou L, Zhou M, *et al*. Epidemiology of human infections with avian influenza A(H7N9) virus in China. N Engl J Med. 2014; 370:520-532.
3. World Health Organization. Human infection with avian influenza A(H7N9) virus – China. *http://www.who.int/csr/don/22-february-2017-ah7n9-china/en* (accessed February 25, 2017).
4. H7N9 surge in China prompts high-level call for market closures. *http://www.cidrap.umn.edu/news-perspective/2017/02/h7n9-surge-china-prompts-high-level-call-market-closures* (accessed February 25, 2017).
5. National Health and Family Planning Commission of China. A resolute determination to prevent and control the prevalence of H7N9: China's joint working body on prevention and control of influenza A (H7N9) meets by national video conference to further enhance measures to prevent and control the prevalence of H7N9. *http://www.nhfpc.gov.cn/zhuz/xwfb/201702/5dac5466ce244083b86daa43b3968ed6.shtmltm* (accessed February 25, 2017, in Chinese).
6. Bureau of Disease Control and Prevention, National Health and Family Planning Commission of China. General prevalence of notifiable infectious diseases in January 2017. *http://www.nhfpc.gov.cn/jkj/s3578/201702/f1e4cfe184e44f80ae57d0954c3d5fce.shtml* (accessed February 25, 2017, in Chinese).
7. Zhou L, Ren R, Yang L, Bao C, Wu J, Wang D, Li C, Xiang N, Wang Y, Li D, Sui H, Shu Y, Feng Z, Li Q, Ni D. Sudden increase in human infection with avian influenza A(H7N9) virus in China, September-December 2016. Western Pac Surveill Response J. 2017. doi:10.5365/wpsar.2017.8.1.001. *http://ojs.wpro.who.int/ojs/index.php/wpsar/article/view/521/733* (accessed February 25, 2017).
8. National Health and Family Planning Commission of China. China takes measures to further enhance and implement joint prevention and control of H7N9 outbreaks; the prevalence of H7N9 in China has tended to be stable. *http://www.nhfpc.gov.cn/zhuz/xwfb/201702/c07c7cf6555b4e11a0be02dd82195fa1.shtml* (accessed February 25, 2017, in Chinese).
9. National Health and Family Planning Commission of China. Notice of the National Health and Family Planning Commission on medical management of human infection with avian influenza A (H7N9) virus. *http://www.nhfpc.gov.cn/yzygj/s3593g/201701/2dbdbc6e82dd4fdfa57508499f61cdfc.shtml* (accessed February 25, 2017, in Chinese).

(Received February 25, 2017; Accepted February 27, 2017)

News

DOI: 10.5582/bst.2017.01041

China is closely monitoring an increase in infection with avian influenza A (H7N9) virus

Qi Tang[1], Meiying Shao[2], Lingzhong Xu[1,*]

[1] *Department of Social Medicine and Health Service Management, School of Public Health, Shandong University, Ji'nan, Shandong, China;*
[2] *Division of Pain Management, Qilu Hospital, Shandong University, Ji'nan, Shandong, China.*

Summary **The fifth outbreak of human infection with avian influenza A (H7N9) virus has struck far and wide in China. The number of cases of infection with the avian influenza A (H7N9) suddenly increased in 2013-2014, but the number of cases reported this winter has exceeded the number reported in all previous seasons. Given this situation, the National Health and Family Planning Commission issued updated Chinese guidelines (2017 version) on diagnosis and treatment of infection with the avian influenza A (H7N9) virus on January 24, 2017. In addition, the Chinese Government closed many live poultry markets in urban and rural areas in a number of provinces and the Government has taken proactive measures to surveil, respond to, and prevent potential pandemics involving the avian influenza A (H7N9) virus.**

Keywords: Avian influenza A (H7N9) virus, outbreak, guideline

Influenza pandemic is a serious threat to public health in today's world. Because of the influenza virus' great adaptability and ready variation, frequent outbreaks of avian influenza A (H7N9) over the past 5 years have attracted interest in controlling human influenza pandemics in the near future. However, the fifth outbreak of human infection with avian influenza A (H7N9) virus has more serious than previous outbreaks over the past few years. Given these concerns, China is closely monitoring an increase in the number of cases of infection with the avian influenza A (H7N9) virus.

1. The avian influenza A (H7N9) virus became seriously prevalent after October 1, 2016

The fifth outbreak of human infection with avian influenza A (H7N9) virus struck far and wide in China. On January 9, 2017, 106 human cases of infection with avian influenza A (H7N9) were reported to the National Health and Family Planning Commission (NHFPC) (*1*). Fifty-two of those cases were reported from Jiangsu, 21 were reported from Zhejiang, 14 were reported from Anhui, 14 were reported from Guangdong, 2 were reported from Shanghai, 2 were reported from Fujian, and 1 was reported from Hunan. At the time of reporting, there were and 57 cases of a severe infection and 35 deaths.

According to the World Health Organization (WHO), more than a thousand cases of infection with the avian influenza A (H7N9) virus have been reported in China over the past four years, with a mortality rate of 39% (*2*). The avian influenza A (H7N9) virus is mainly prevalent in coastal areas and especially active in Zhejiang, Guangdong, and Jiangsu provinces (Figure 1). The number cases of infection with the avian influenza A (H7N9) virus suddenly increased in 2013-2014, but the number of cases reported this winter has exceeded the number reported in all previous seasons (Figure 2). According to the data, the number of cases from October 1, 2016 to the present accounts for nearly one-third of all cases of infection with the avian influenza A (H7N9) virus reported since 2013 (*3*). However, the specific reasons for the outbreak of the avian influenza A (H7N9) virus this winter are not yet clear.

Released online in J-STAGE as advance publication February 28, 2017.

**Address correspondence to:*
Dr. Lingzhong Xu, Department of Social Medicine and Health Service Management, School of Public Health, Shandong University, Mailbox No. 110, 44, Wenhuaxi Road, Ji'nan, 250012, Shandong, China.
E-mail: lzxu@sdu.edu.cn

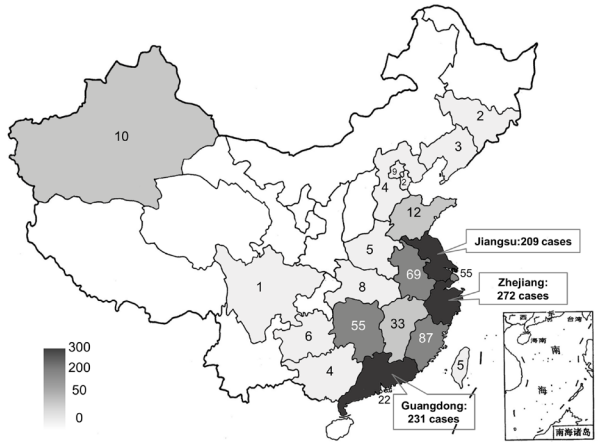

Figure 1. Distribution of the cumulative number of cases of infection with the avian influenza A (H7N9) virus in China since January 1, 2013. According to the World Health Organization (WHO), more than a thousand cases of infection with the avian influenza A (H7N9) virus have been reported in China over the past four years, with a mortality rate of 39% (*2*).The avian influenza A (H7N9) virus is mainly prevalent in the coastal areas, and especially active in Zhejiang (272 cases), Guangdong (231 cases), and Jiangsu provinces (209 cases).

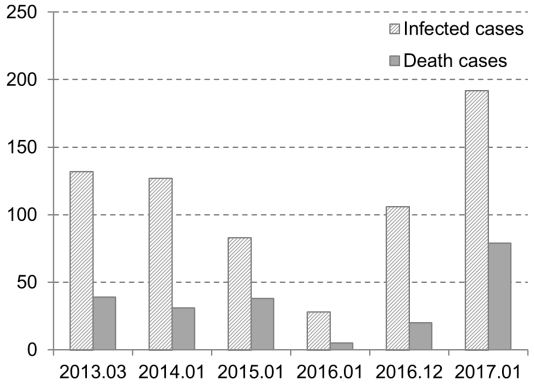

Figure 2. Cases of infection with the avian influenza A (H7N9) virus and deaths in China from March 2013 to January 2017. The number of cases of infection with the avian influenza A (H7N9) virus and deaths in China has increased each year. A point worth mentioning is that the number of cases suddenly increased in 2013-2014, but the number of cases reported this winter has exceeded the number reported in all previous seasons. According to the data, the number of cases from October 1, 2016 to the present has accounted for nearly one-third of all reported cases of infection with the avian influenza A (H7N9) virus since 2013.

2. Chinese guidelines (2017 version) on infection with the avian influenza A (H7N9) virus have been updated

On January 24, 2017, the NHFPC issued updated Chinese guidelines (2017 version) on diagnosis and treatment of infection with the avian influenza A (H7N9) virus (*4*). These guidelines have 8 sections covering etiology, epidemiology, pathogenesis and pathology, clinical features, diagnosis and differential diagnosis, treatment, prevention and control of iatrogenic infection, and criteria for lifting quarantines.

A point worth mentioning is that the guidelines focus on indicated drugs and drug use. Western drugs that are approved include oseltamivir, oseltamivir, and zanamivir while traditional Chinese medicines that are approved include Shufeng Jiedu capsules, Lianhua Qingwen capsules, and Jinlian Qingfei effervescent tablets. The guidelines emphasize drug selection, recommended dosages, eligible patients, and drug efficacy, further standardizing the clinical management of and response to infection with the avian influenza A (H7N9) virus (*5*).

3. Reducing the risk of exposure to infected poultry

An important aspect of the 80 cases reported in January is that affected individuals were exposed to poultry or a live poultry market. Exposure to infected live or dead poultry or direct or indirect exposure to contaminated surroundings, via settings such as live meat markets, appears to be a primary risk factor for human infection with the avian influenza A (H7N9) virus. The whole process of slaughtering, plucking, and handling carcasses of infected poultry and preparing poultry for consumption could be a risk factor.

In order to prevent and control outbreaks, the NHFPC and representatives from the ministries of agriculture, industry, and commerce conducted joint investigations in Jiangsu, Zhejiang, Anhui, and Guangdong provinces where cases were more prevalent. Government officials have jointly supervised, inspected, and guided local surveillance, medical treatment, and prevention and control and they have promoted control measures with a focus on management of live poultry markets and inter-regional transportation. As a specific example of those measures, many provinces and cities in China have closed live poultry markets. The latest to be closed is in Wuhan, Hubei Province. Twenty-two cases of infection with the avian influenza A (H7N9) virus have occurred in Hubei Province this year, and nearly half of those cases occurred in Wuhan. Given this fact, the city closed 445 live poultry markets and 1,016 stalls and it culled 26,000 poultry. Earlier, Zhejiang Province closed every live poultry market, the City of Xinghua, Jiangsu Province suspended the city's live poultry trade, and the City of Anqing, Wangjiang County, and Xuancheng Jing County in Anhui Province closed every live poultry market in urban and rural areas (*6*).

4. Focusing on surveillance and preventing potential pandemics

The ongoing prevalence of avian influenza A (H7N9) viruses is a public health concern since these viruses often caused severe diseases and these viruses have

the potential to mutate to become more transmissible between humans. In addition, influenza pandemics are unpredictable but recurring events that can have health, economic, and social consequences worldwide (*7*). Once an avian or zoonotic influenza virus emerges with the ability to cause sustained human-to-human transmission, the human population has little to no immunity from the virus. Whether avian and other zoonotic influenza viruses that are currently prevalent will cause a future pandemic is unknown. However, what is important is to work with the government to continue monitoring various avian and other zoonotic influenza viruses that have caused infections in both animal and human populations.

Given these concerns, outbreaks of the avian influenza A (H7N9) virus pose a real challenge to China's emergency management, and specific measures are being considered more than before. The Chinese Government has emphasized prevention and control of the avian influenza A (H7N9) virus and the Government has made a significant effort by taking proactive measures (*8*). *i*) The NHFPC has enhanced the surveillance of outbreaks and it conducts timely risk assessments and studies to ascertain any changes in epidemiology (*9*); *ii*) The NHFPC asked local health and family planning commissions to implement measures to effectively control sources of outbreaks and to minimize the number of people affected; *iii*) Early diagnosis and treatment of cases of infection with the avian influenza A (H7N9) virus have been enhanced, treatment of cases of severe infections has been enhanced, and cases of severe infections and deaths have been reduced; *iv*) Relevant prefectures in Jiangsu Province closed live poultry markets in late December 2016 and Zhejiang, Guangdong, and Anhui provinces have enhanced regulation of live poultry markets; and *v*) Campaigns have been initiated to inform the public of risks and information has been shared with the public.

References

1. National Health and Family Planning Commission (NHFPC). General prevalence of notifiable infectious Diseases in January. *http://www.nhfpc.gov.cn/jkj/s3578/201702/f1e4cfe184e44f80ae57d0954c3d5fce.shtml* (accessed February 18, 2017, in Chinese).
2. World Health Organization. Emergencies preparedness, response – China. *http://www.who.int/csr/don/archive/country/chn/en/* (accessed February 17, 2017, in Chinese).
3. World Health Organization. Human infection with avian influenza A(H7N9) virus – China. *http://www.who.int/csr/don/20-february-2017-ah7n9-china/zh/* (accessed February 18, 2017).
4. National Health and Family Planning Commission (NHFPC). Notice of the National Health and Family Planning Commission on medical management of human infection with avian influenza A (H7N9) virus. *http://www.nhfpc.gov.cn/yzygj/s3593g/201701/2dbdbc6e82dd4fdfa57508499f61cdfc.shtml* (accessed February 21, 2017, in Chinese).
5. Tao Z, Yang Y, Shi W, Xue M, Yang W, Song Z, Yao C, Yin J, Shi D, Zhang Y, Cai Y, Tong C, Yuan Y. Complementary and alternative medicine is expected to make greater contribution in controlling the prevalence of influenza. Biosci Trends. 2013; 7:253-256.
6. Phoenix Bulletin. Wuhan has closed 445 live poultry trading markets. *http://hb.ifeng.com/a/20170215/5387816_0.shtml* (accessed February 17, 2017, in Chinese).
7. Li Q, Zhou L, Zhou M, *et al*. Epidemiology of human infections with avian influenza A(H7N9) virus in China. N Engl J Med. 2014; 370:520-32.
8. World Health Organization. Human infection with avian influenza A(H7N9) virus – China. *http://www.who.int/csr/don/17-january-2017-ah7n9-china/zh/* (accessed February 17, 2017).
9. United Nations News Center. China is closely monitoring an increase in cases of infection with the avian influenza A (H7N9) virus. *http://www.un.org/chinese/News/story.asp?newsID=27588* (accessed February 17, 2017, in Chinese).

(Received February 23, 2017; Revised February 26, 2017; Accepted February 27, 2017)

BST
BioScience Trends

Guide for Authors

1. Scope of Articles

BioScience Trends is an international peer-reviewed journal. BioScience Trends devotes to publishing the latest and most exciting advances in scientific research. Articles cover fields of life science such as biochemistry, molecular biology, clinical research, public health, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of 100 references. Mini reviews are also accepted.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 2,000 words in length (excluding references).

Case Reports should be detailed reports of the symptoms, signs, diagnosis, treatment, and follow-up of an individual patient. Case reports may contain a demographic profile of the patient but usually describe an unusual or novel occurrence. Unreported or unusual side effects or adverse interactions involving medications will also be considered. Case Reports should not exceed 3,000 words in length (excluding references).

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in BioScience Trends in the last 6 months or issues of general interest. Letters should not exceed 800 words in length and may contain a maximum of 10 references.

3. Editorial Policies

Ethics: BioScience Trends requires that authors of reports of investigations in humans or animals indicate that those studies were formally approved by a relevant ethics committee or review board.

Conflict of Interest: All authors are required to disclose any actual or potential conflict of interest including financial interests or relationships with other people or organizations that might raise questions of bias in the work reported. If no conflict of interest exists for each author, please state "There is no conflict of interest to disclose".

Submission Declaration: When a manuscript is considered for submission to BioScience Trends, the authors should confirm that 1) no part of this manuscript is currently under consideration for publication elsewhere; 2) this manuscript does not contain the same information in whole or in part as manuscripts that have been published, accepted, or are under review elsewhere, except in the form of an abstract, a letter to the editor, or part of a published lecture or academic thesis; 3) authorization for publication has been obtained from the authors' employer or institution; and 4) all contributing authors have agreed to submit this manuscript.

Cover Letter: The manuscript must be accompanied by a cover letter signed by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For example of Cover Letter, please visit http://www.biosciencetrends.com/downcentre.php (Download Centre).

Copyright: A signed JOURNAL PUBLISHING AGREEMENT (JPA) form must be provided by post, fax, or as a scanned file before acceptance of the article. Only forms with a hand-written signature are accepted. This copyright will ensure the widest possible dissemination of information. A form facilitating transfer of copyright can be downloaded by clicking the appropriate link and can be returned to the e-mail address or fax number noted on the form (Please visit Download Centre). Please note that your manuscript will not proceed to the next step in publication until the JPA Form is received. In addition, if excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

Suggested Reviewers: A list of up to 3 reviewers who are qualified to assess the scientific merit of the study is welcomed. Reviewer information including names, affiliations, addresses, and e-mail should be provided at the same time the manuscript is submitted online. Please do not suggest reviewers with known conflicts of interest, including participants or anyone with a stake in the proposed research; anyone from the same institution; former students, advisors, or research collaborators (within the last three years); or close personal contacts. Please note that the Editor-in-Chief may accept one or more of the proposed reviewers or may request a review by other qualified persons.

Language Editing: Manuscripts prepared by authors whose native language is not English should have their work proofread by a native English speaker before submission. If not, this might delay the publication of your manuscript in BioScience Trends.

The Editing Support Organization can provide English proofreading, Japanese-English translation, and Chinese-English translation services to authors who want to publish in BioScience Trends and need assistance before submitting a manuscript. Authors can visit this organization directly at http://www.iacmhr.com/iac-eso/support.php?lang=en. IAC-ESO was established to facilitate manuscript preparation by researchers whose native language is not English and to help edit works intended for international academic journals.

4. Manuscript Preparation

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g.* DNA). Single words should not be abbreviated.

Title Page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; and 5) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the manuscript; if no conflict of

interest exists for each author, please state "There is no conflict of interest to disclose"). Please visit Download Centre and refer to the title page of the manuscript sample.

Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For article types including Original Article, Brief Report, Review, Policy Forum, and Case Report, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For News and Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Key words or phrases that do not occur in the title should be included in the Abstract page.

Introduction: The introduction should be a concise statement of the basis for the study and its scientific context.

Materials and Methods: The description should be brief but with sufficient detail to enable others to reproduce the experiments. Procedures that have been published previously should not be described in detail but appropriate references should simply be cited. Only new and significant modifications of previously published procedures require complete description. Names of products and manufacturers with their locations (city and state/country) should be given and sources of animals and cell lines should always be indicated. All clinical investigations must have been conducted in accordance with Declaration of Helsinki principles. All human and animal studies must have been approved by the appropriate institutional review board(s) and a specific declaration of approval must be made within this section.

Results: The description of the experimental results should be succinct but in sufficient detail to allow the experiments to be analyzed and interpreted by an independent reader. If necessary, subheadings may be used for an orderly presentation. All figures and tables must be referred to in the text.

Discussion: The data should be interpreted concisely without repeating material already presented in the Results section. Speculation is permissible, but it must be well-founded, and discussion of the wider implications of the findings is encouraged. Conclusions derived from the study should be included in this section.

Acknowledgments: All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al.* Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. Examples are given below:

Example 1 (Sample journal reference):
Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

Example 2 (Sample journal reference with more than 15 authors):
Darby S, Hill D, Auvinen A, *et al.* Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

Example 3 (Sample book reference):
Shalev AY. Post-traumatic stress disorder: diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):
Ministry of Health, Labour and Welfare of Japan. Dietary reference intakes for Japanese. *http://www.mhlw.go.jp/houdou/2004/11/h1122-2a.html* (accessed June 14, 2010).

Tables: All tables should be prepared in Microsoft Word or Excel and should be arranged at the end of the manuscript after the References section. Please note that tables should not in image format. All tables should have a concise title and should be numbered consecutively with Arabic numerals. If necessary, additional information should be given below the table.

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be understood without referring to the text. Symbols used in figures must be explained.

Figure Preparation: All figures should be clear and cited in numerical order in the text. Figures must fit a one- or two-column format on the journal page: 8.3 cm (3.3 in.) wide for a single column, 17.3 cm (6.8 in.) wide for a double column; maximum height: 24.0 cm (9.5 in.). Please make sure that the symbols and numbers appeared in the figures should be clear. Please make sure that artwork files are in an acceptable format (TIFF or JPEG) at minimum resolution (600 dpi for illustrations, graphs, and annotated artwork, and 300 dpi for micrographs and photographs). Please provide all figures as separate files. Please note that low-resolution images are one of the leading causes of article resubmission and schedule delays. All color figures will be reproduced in full color in the online edition of the journal at no cost to authors.

Units and Symbols: Units and symbols conforming to the International System of Units (SI) should be used for physicochemical quantities. Solidus notation (*e.g.* mg/kg, mg/mL, mol/mm^2/min) should be used. Please refer to the SI Guide www.bipm.org/en/si/ for standard units.

Supplemental data: Supplemental data might be useful for supporting and enhancing your scientific research and BioScience Trends accepts the submission of these materials which will be only published online alongside the electronic version of your article. Supplemental files (figures, tables, and other text materials) should be prepared according to the above guidelines, numbered in Arabic numerals (*e.g.*, Figure S1, Figure S2, and Table S1, Table S2) and referred to in the text. All figures and tables should have titles and legends. All figure legends, tables and supplemental text materials should be placed at the end of the paper. Please note all of these supplemental data should be provided at the time of initial submission and note that the editors reserve the right to limit the size and length of Supplemental Data.

5. Submission Checklist

The Submission Checklist will be useful during the final checking of a manuscript prior to sending it to BioScience Trends for review. Please visit Download Centre and download the Submission Checklist file.

6. Online Submission

Manuscripts should be submitted to BioScience Trends online at http://www.biosciencetrends.com. The manuscript file should be smaller than 5 MB in size. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail at office@biosciencetrends.com.

7. Accepted Manuscripts

Proofs: Galley proofs in PDF format will be sent to the corresponding author via e-mail. Corrections must be returned to the editor (proof-editing@biosciencetrends.com) within 3 working days.

Offprints: Authors will be provided with electronic offprints of their article. Paper offprints can be ordered at prices quoted on the order form that accompanies the proofs.

Page Charge: Page charges will be levied on all manuscripts accepted for publication in BioScience Trends ($140 per page for black white pages; $340 per page for color pages). Under exceptional circumstances, the author(s) may apply to the editorial office for a waiver of the publication charges at the time of submission.

(*Revised February 2013*)

Editorial and Head Office:
Pearl City Koishikawa 603
2-4-5 Kasuga, Bunkyo-ku
Tokyo 112-0003 Japan
Tel: +81-3-5840-8764
Fax: +81-3-5840-8765
E-mail: office@biosciencetrends.com

BST
BioScience Trends

JOURNAL PUBLISHING AGREEMENT (JPA)

Manuscript No.:

Title:

Corresponding Author:

The International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) is pleased to accept the above article for publication in BioScience Trends. The International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) reserves all rights to the published article. Your written acceptance of this JOURNAL PUBLISHING AGREEMENT is required before the article can be published. Please read this form carefully and sign it if you agree to its terms. The signed JOURNAL PUBLISHING AGREEMENT should be sent to the BioScience Trends office (Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku, Tokyo 112-0003, Japan; E-mail: **office@biosciencetrends.com**; Tel: **+81-3-5840-8764**; Fax: **+81-3-5840-8765**).

1. Authorship Criteria

As the corresponding author, I certify on behalf of all of the authors that:
1) The article is an original work and does not involve fraud, fabrication, or plagiarism.
2) The article has not been published previously and is not currently under consideration for publication elsewhere. If accepted by BioScience Trends, the article will not be submitted for publication to any other journal.
3) The article contains no libelous or other unlawful statements and does not contain any materials that infringes upon individual privacy or proprietary rights or any statutory copyright.
4) I have obtained written permission from copyright owners for any excerpts from copyrighted works that are included and have credited the sources in my article.
5) All authors have made significant contributions to the study including the conception and design of this work, the analysis of the data, and the writing of the manuscript.
6) All authors have reviewed this manuscript and take responsibility for its content and approve its publication.
7) I have informed all of the authors of the terms of this publishing agreement and I am signing on their behalf as their agent.

2. Copyright Transfer Agreement

I hereby assign and transfer to IACMHR Co., Ltd. all exclusive rights of copyright ownership to the above work in the journal BioScience Trends, including but not limited to the right 1) to publish, republish, derivate, distribute, transmit, sell, and otherwise use the work and other related material worldwide, in whole or in part, in all languages, in electronic, printed, or any other forms of media now known or hereafter developed and the right 2) to authorize or license third parties to do any of the above.

I understand that these exclusive rights will become the property of IACMHR Co., Ltd., from the date the article is accepted for publication in the journal BioScience Trends. I also understand that IACMHR Co., Ltd. as a copyright owner has sole authority to license and permit reproductions of the article.

I understand that except for copyright, other proprietary rights related to the Work (*e.g.* patent or other rights to any process or procedure) shall be retained by the authors. To reproduce any text, figures, tables, or illustrations from this Work in future works of their own, the authors must obtain written permission from IACMHR Co., Ltd.; such permission cannot be unreasonably withheld by IACMHR Co., Ltd.

3. Conflict of Interest Disclosure

I confirm that all funding sources supporting the work and all institutions or people who contributed to the work but who do not meet the criteria for authors are acknowledged. I also confirm that all commercial affiliations, stock ownership, equity interests, or patent-licensing arrangements that could be considered to pose a financial conflict of interest in connection with the article have been disclosed.

Corresponding Author's Name (Signature):

Date: