Original Article

Epithelial or mesenchymal: Where to draw the line?

Jianyuan Chai[1,2,*], Cristina Modak[1], Wasim Mouazzen[1], Reinier Narvaez[1], Jennifer Pham[1]

[1] *VA Long Beach Healthcare System, Long Beach, CA, USA;*
[2] *Department of Medicine, University of California, Irvine, CA, USA.*

Summary **Epithelial and mesenchymal cells represent two of the main differentiated cell types in all vertebrates. However, their distinction is not always absolutely clear. Dozens of molecules have been used as markers for each cell type, while emerging evidence questions their validity. The aim of this study was to compare the molecular phenotype of these two cell types. Twenty-two commonly used molecular markers were evaluated by quantitative PCR and immunofluorescence in six lines of human and rat epithelial cells and fibroblasts. The epithelial cells were also examined for their responses to TGFβ1 stimulation. All of the "markers" tested were found in both epithelial and mesenchymal cells. Some epithelial markers, such as CLDN5, OCLN, DSG1 and TJP1, were expressed even higher in fibroblasts than in epithelial cells. In comparison, mesenchymal markers showed more fidelity, but CDH2 and MMP9 were still significantly higher in epithelial cells than in mesenchymal cells. Furthermore, TGFβ1 up-regulated epithelial markers CTNNB1 and CTNND1, but suppressed mesenchymal markers such as S100A4, FGF1 and FGF2. In conclusion, no gene expression is cell-type restricted. Although some of these "markers" are expressed more in one cell type than in the other or differently localized, none of them shows a consistent pattern across species to make them universal markers. Nonetheless, some molecules appear to be better markers than others for a specific cell type. The information provided here is expected to serve as a reference for both basic and clinical researchers in the fields of epithelial-mesenchymal transition, molecular cell typing and cancer diagnosis.**

Keywords: Epithelial cell, mesenchymal cell, EMT, molecular marker

1. Introduction

Epithelial and mesenchymal cells represent two of the main cell types in all vertebrates. Their distinctions in morphology and cellular organization have already been recognized in the late 19th century. Epithelial cells are commonly characterized by their: (*i*) strong intercellular connection; (*ii*) keratin-based cytoskeleton; and (*iii*) distinct cell polarity. In contrast, mesenchymal cells have loose or no intercellular connections among them, contain a highly developed cytoskeletal structure with vimentin as an intermediate filament, and have no clear distinction in either apical, lateral or basal side when they are in rest. However, more and more studies found that these two types of cells are not as rigid as they were thought; and both epithelial and mesenchymal cells show some degree of cell plasticity *in vitro* and *in vivo*, namely, they are interchangeable. This phenomenon is called epithelial-mesenchymal transition (EMT) or in reverse, mesenchymal-epithelial transition (MET).

Although the awareness of EMT and MET phenomena can be dated back as early as 1908 in Lillie's embryological book 'The Development of the Chick' (*1*), their detailed description was not established until 1982 by Greenburg and Hay (*2*). During the 1990s, EMT gained more recognition as a possibly important mechanism in malignant and chronic progression of fibrotic disorders and cancer (*3-7*) and it has been studied extensively ever since. It was during these early years of extensive EMT studies that transforming growth factor beta 1 (TGFβ1) was reported as an inducer of EMT in normal mammary epithelial cells (*8*). Since then, even though other

**Address correspondence to:*
Dr. Jianyuan Chai, Dept Research (09-151), VA Long Beach Healthcare System, 5901 E. Seventh Street, Long Beach, CA 90822, USA.
e-mail: Jianyuan.chai@va.gov

molecules, particularly growth factors and extra cellular matrix molecules, have also been reported to induce or facilitate EMT, TGFβ1 is one of the most recognized inducers of EMT both *in vitro* and *in vivo*, particularly in embryonic development and cancer progression, as well as in epithelial tissue injury (*9*). Through these early benchmark studies, it is now understood that EMT and MET are two essential processes in embryonic development and morphogenesis to facilitate tissue differentiation and organ formation, and these cellular capabilities are kept very well by most vertebrates throughout their adulthood as key mechanisms for wound healing and tissue repair. Although these differentiated cells are not as much versatile as stem cells, epithelial and mesenchymal cells by inter-conversion can compensate for stem cell shortage in tissue repair. Inadvertently, these beautiful features of differentiated cells are sometimes hijacked by "evil forces" when they are deregulated (*3*). As mentioned previously, fibrosis is a typical example in which EMT is overwhelmed and, as a result, normal tissue structure is replaced by excessive mesenchymal cells and their products (*4*). An even worse case, which has been attracting more attention in recent years, is cancer, in which some genetically altered epithelial cells have acquired mesenchymal features to move around in our body, invade our healthy organs and break down their normal functions (*3,5,6*).

In order to prevent unnecessary EMT or MET, it is often required to detect cell type changes as early and precisely as possible. Many molecules have been claimed and used as markers for either epithelial cells or mesenchymal cells. However, an increasing amount of evidence suggests that none of these markers seem to be perfect. This study is to compare the molecular phenotype of epithelial cells with mesenchymal cells in the resting state, to evaluate the molecular markers that are commonly used nowadays to distinguish epithelial cells from mesenchymal cells, and to explore the uniqueness of these two cell types. To provide a positive control for our studies, we also examined these markers in epithelial cells in response to TGFβ1, the most recognized EMT-inducing agent (*9*).

2. Materials and Methods

2.1. *Cell culture*

Three human and three rat cell lines were compared in this study: human esophageal squamous epithelial cells (Het-1A), human gastric adenocarcinoma epithelial cells (AGS), human colon fibroblast (CCD-18Co), rat gastric mucosal epithelial cells (RGM1), rat intestinal epithelial cells (IEC-6), and rat embryonic fibroblast (Rat2). All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) except RGM1 which was obtained from Riken Cell Bank (Tsukuba, Japan). Culture media used for each cell line are: Het-1A – Bronchial Epithelial Growth Medium with bullets and 10% FBS (Lonza, Walkersville, MD, USA), AGS – RPMI 1640 with 10% FBS (Lonza), CCD-18Co – Eagle's Minimum Essential Medium (ATCC) with 10% FBS, RGM1 – Dulbecco's Modified Eagle's Medium/F12 with 10% FBS (Lonza), IEC-6 – Dulbecco's Modified Eagle's Medium (Lonza) supplemented with 0.1 units/mL bovine insulin (Sigma-Aldrich, Saint Louis, MI, USA) and 10% FBS, and Rat2 – Dulbecco's Modified Eagle's Medium with 10% FBS. For all the experiments in this study, comparable cell density was insured by plating equal number of cells and confirming by microscopic visualization prior to serum starvation. All cells were serum starved for 5 h before experiments. Where applicable, after serum starvation, cells were treated with either vehicle (control) or recombinant TGFβ1 (Invitrogen, Camarillo, CA, USA) at 5 ng/mL for 24 h.

2.2. *Real-time PCR*

Cells were cultured in 6-well plates till desired confluence. Total RNA was extracted from the cells using RNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Reverse transcription was done in MyCycler (Bio-Rad, Hercules, CA, USA) following the protocol: 25°C/10 min – 55°C/30 min – 85°C/5 min – 4°C/∞. All of the reagents for reverse transcription, including transcriptor reverse transcriptase, PCR nucleotide mix, protector RNase inhibitor, and random primer p[dN]6, were purchased from Roche (Mannheim, Germany). Real-time PCR was performed in iCycler (Bio-Rad) following two-step program, using SYBR Green master mix and specific primers from SABiosciences (Frederick, MD). C_t readings from epithelial samples were compared against the C_t value from fibroblast within the same species. Data were generated from at least 5 independent experiments and were analyzed according to $\Delta\Delta C_t$ method.

2.3. *Fluorescence microscopy*

Cells were cultured till desired confluence on microscopic cover slips that were pre-coated with 5% rat tail type I collagen (BD Biosciences, San Diego, CA, USA) in 0.02 N acetic acid. Cells were fixed for 10 min in either 4% paraformaldehyde (for membrane-associated antigens) or cold methanol (for cytoplasmic antigens). For cytoplasmic antigens, cells were also permeabilized in cold acetone for 5 min after fixation. Before a primary antibody was applied, cells were incubated in serum-free Protein Block (Dako, Carpentaria, CA, USA) for 30 min to eliminate possible non-specific antibody binding. To determine the specificity of each primary antibody, the primary antibody in control cover slips was replaced

with a pre-immune serum. The primary antibodies used for cell staining include: E-cadherin (1:500), N-cadherin (1:500), desmoglein (1:500), p120 catenin (1:500), and γ-catenin (1:500) all from BD Biosciences; fibronectin (1:200), S100A4 (1:100), vimentin (1:100), cytokeratin-14 (1:200), acidic cytokeratin (1:50), non-muscle myosin heavy chain (1:500), and β-catenin (1:500) all from Abcam (Cambridge, MA, USA); cytokeratin-18 (1:200) from Enzo (Plymouth Meeting, PA, USA); smooth muscle α-actin (1:400) from Sigma-Aldrich; occludin (1:100) and ZO-1 (1:100) from Invitrogen. FITC-conjugated secondary antibodies were purchased from Abcam and diluted in PBS according to the manufacturer's recommendation. Nuclei were counter-stained with either propidium iodide or DAPI (Invitrogen). To show actin polymerization, cells were fixed in 3.7% formaldehyde (Sigma-Aldrich) for 10 min, permeabilized in 0.1% Triton X-100 (Thermo Fisher Scientific, Pittsburg, PA, USA) for 5 min, and incubated with Oregon Green 488 phalloidin (Invitrogen) for 20 min.

3. Results

3.1. *Epithelial markers*

The most prominent feature of epithelial cells is their cohesion and tendency to form continuous cell layers regardless of *in vitro* or *in vivo*. Adjacent epithelial cells are connected to one another through multiple intercellular locks, including tight junctions, adherens junctions, desmosomes and gap junctions, which in turn link to the intracellular cytoskeleton. For this reason, the molecules constituting these membranous structures are commonly used as markers to identify epithelial cells and therefore, they were carefully examined one-by-one in this study.

Tight junctions (TJ) are located in the most apical lateral regions of epithelial cells and thus they are also indicators for cell polarity. The main components of a TJ include transmembrane proteins claudin (23 members known in human), occludin, junctional adhesion molecule (JAM-A, -B, and -C) and intracellular adaptor protein zona occludin (ZO-1, -2, and -3) which connects a TJ to the actin cytoskeleton (*10*).

At the transcriptional level, all TJ components were expressed not only in epithelial cells, but also detectable in mesenchymal cell lines, even though TGFβ1 treatment significantly knocked down expression of most genes in most cell lines (Figures 1 and 2). Nonetheless, claudin-5 (CLDN5), one of the most common claudins in gastrointestinal epithelial cells (*11*), was expressed at much higher levels in human epithelial cells than in human fibroblasts, consistent with what would be expected for a TJ molecule (Figure 1). In rat cell lines, however, it was just the opposite where Rat2 fibroblasts expressed ~2-fold more claudin-5 than the two rat epithelial cell lines (Figure 2). Rat2 fibroblasts also expressed higher levels of occludin (OCLN) than either IEC-6 or RGM1 epithelial cells (Figure 2). Interestingly, in human AGS cells, which expressed a great amount of OCLN, TGFβ1 did not inhibit the gene expression, as it did in all the other cases, but actually promoted it. Finally, the level of TJP1, the gene coding for the first identified TJ protein ZO-1, was found significantly higher in CCD-18Co human fibroblasts than in either Het-1A or AGS human epithelial cells (Figure 1). Immunocytochemical staining confirmed expression of all TJ proteins in both epithelial and mesenchymal cell lines and did not reveal any consistent shift in localization between these two cell types, with the exception of ZO-1 which was exclusively localized to the membrane in all epithelial cells, but showed additional cytoplasmic staining in the two mesenchymal cell lines (Figure 3).

Adherens junctions (AJ) are specialized sub-apical structures that form stable cell-cell contacts in essentially all types of tissue (*12*). Molecules that constitute an AJ include transmembrane protein E-cadherin and intracellular adaptors α-, β-, and δ-catenin (p120), which in turn provide anchorage to the actin cytoskeleton.

Among AJ components, E-cadherin expression is commonly considered a hallmark of epithelial cells. Its expression is mainly controlled at the transcriptional level, seemingly, CDH1, the gene coding for E-cadherin, is normally inactivated in mesenchymal cells (*13*). While this was confirmed for human CCD-18Co fibroblast cells both at the mRNA and protein level (Figures 1 and 3), Rat2 fibroblasts, which appeared to have only small amounts of CDH1 mRNA compared to IEC-6 epithelial cells, still expressed ~10-fold higher levels compared to RGM1 epithelial cells (Figure 2) and stained clearly positive for E-cadherin protein at the membrane (Figure 3). Catenins were not unique to epithelial cells either. Both β-catenin (CTNNB1) and δ-catenin (CTNND1) were expressed at considerable amounts in all cell lines and were even significantly higher in Rat2 fibroblasts than in RGM1 epithelial cells (Figure 2). Interestingly, TGFβ1 treatment promoted CTNNB1 and CTNND1 expression in all epithelial cell lines (Figures 1 and 2).

Furthermore, their proteins were localized predominantly to the cell membrane in all cell lines except the cancerous epithelial cell line – AGS – in which β-catenin was nuclear localized (Figure 3). Rat2 fibroblasts also showed some nuclear staining of β-catenin but still retained strong, defined membrane localization as well (Figure 3). Even though TGFβ1 treatment induced nuclear localization also in human Het-1A and rat RGM1 cells, both rat epithelial cell lines still exhibited strong membrane localization (Supplementary Figure).

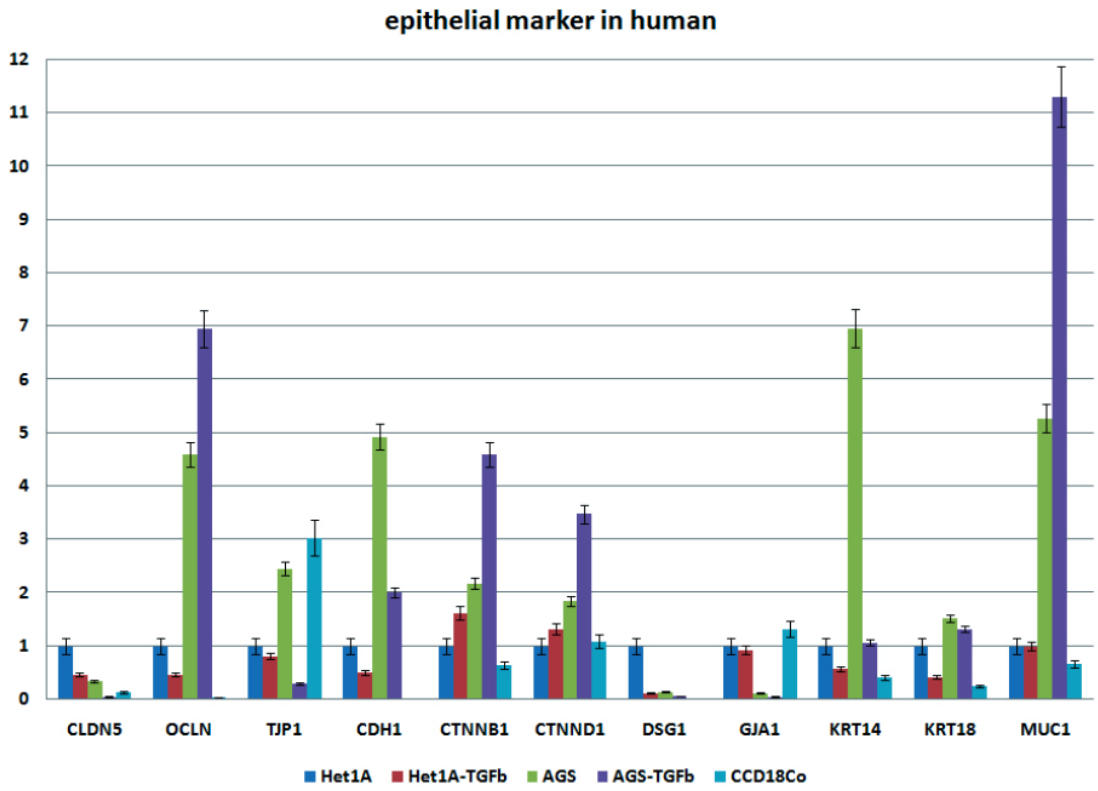


Figure 1. mRNA expression of epithelial markers in human cell lines. Three human cell lines, including esophageal squamous epithelial cells (Het-1A), gastric adenocarcinoma cells (AGS) and colon fibroblasts (CCD-18Co), were examined in their resting state and TGFβ1-induced EMT state. Non-treated Het-1A epithelial cells were set as a standard in the graph and the rest of the cells were compared against it. Results represent averages from at least three independent experiments with error bars representing standard error. $p < 0.01$.

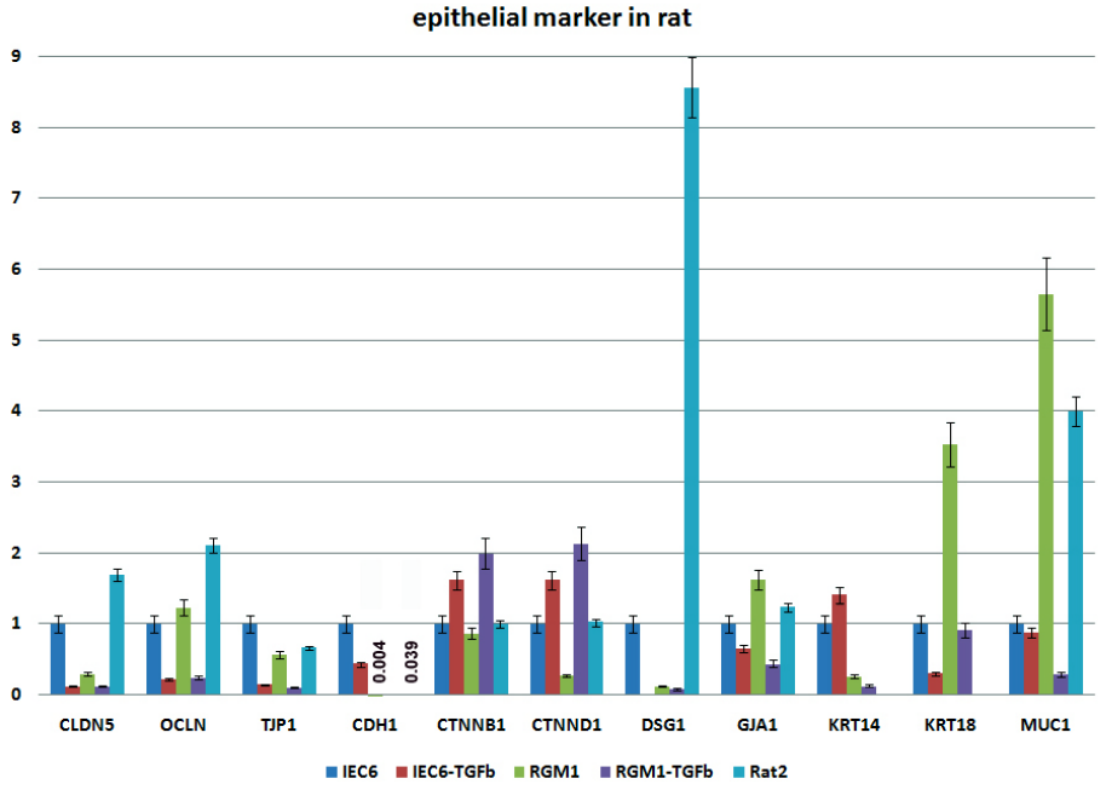


Figure 2. mRNA expression of epithelial markers in rat cell lines. Three rat cells lines, including intestinal columnar epithelial cells (IEC-6), gastric mucosal columnar epithelial cells (RGM1) and embryonic fibroblasts (Rat2), were examined in their resting state and TGFβ1-induced EMT state. Non-treated IEC-6 epithelial cells were set as a standard in the graph and the rest of the cells were compared against IEC-6. Results represent averages from at least three independent experiments with error bars representing standard error. $p < 0.01$.

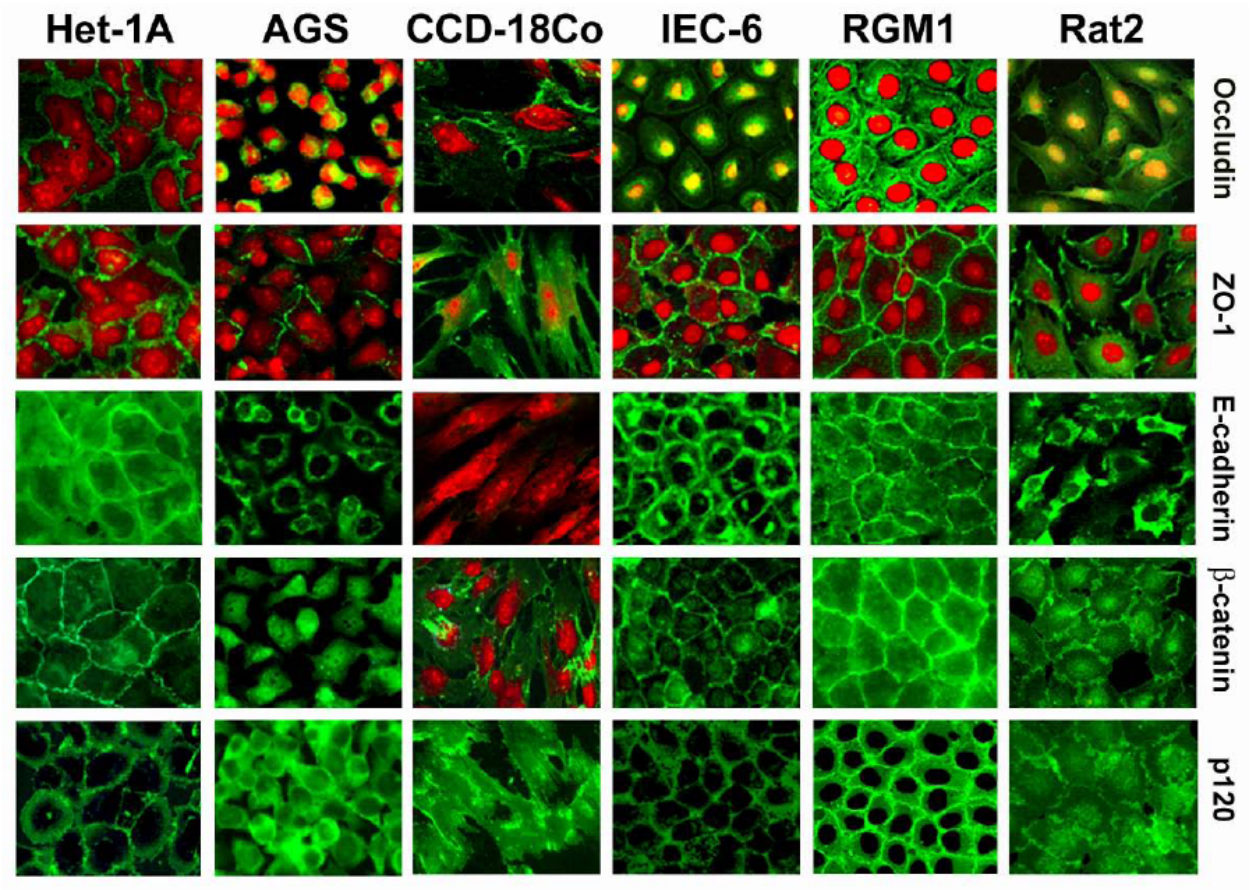


Figure 3. Immunofluorescence detection of protein localization of epithelial markers in human (Het-1A, AGS, CCD-18Co) and rat cell lines (IEC-6, RGM1, Rat2). Localization patterns of putative epithelial markers Occludin, ZO-1, E-cadherin, β-catenin and p120 were evaluated as described in the methods section. Nuclei were counterstained with propidium iodide (red) as needed.

Desmosomes are like buttons joining the lateral edges of adjacent epithelial cells through cadherin molecules desmoglein and desmocollin which link with cytokeratin fibers through desmosomal plaque proteins such as desmoplakin and γ-catenin (Plakoglobin).

Although desmoglein (DSG1) was not expressed in human CCD-18Co fibroblasts (Figure 1), its expression in Rat2 fibroblasts was found extremely higher than in either rat epithelial cell line (Figure 2). Immunofluorescence cell staining showed strong membrane localization of γ-catenin in both epithelial and mesenchymal cell lines (Figure 4). Similar results were obtained for desmoglein, with no clear distinction in localization patterns between epithelial and mesenchymal cells. On the other hand, TGFβ1 had a suppressive effect on desmosomes components (Figures 1 and 2).

Gap junctions are channel-like structures each composed of two connexons which connect across the intercellular space and regulate trafficking of small molecules (< 1 kDa) between adjacent cells. Each connexon is a pore through the cell membrane and is formed by a ring of six connexin proteins (> 20 isoforms identified). Connexins are expressed by virtually all types of cells, including fibroblasts, except sperms and erythrocytes (*14-17*).

Our data showed expression of connexin-43 (GJA1) in all six cell lines examined. Moreover, its mRNA level in CCD-18Co fibroblasts was significantly higher than in either Het-1A or AGS epithelial cells (Figure 1). It was also slightly higher in Rat2 fibroblasts than in IEC-6 epithelial cells (Figure 2). TGFβ1 had an inhibitory effect on GJA1.

Keratins are intermediate filament proteins found in the epithelial tissue. There are at least 20 different keratin isoforms in two groups found together in pairs which characterize the type of epithelium: the low weight, acidic type I keratins and the high weight, basic or neutral type II. Thus this specific keratin fingerprint is commonly used to classify all epithelia upon their cytokeratin expression profile.

In our study, no keratins were found in Rat2 fibroblasts, but CCD-18Co fibroblasts expressed both keratin-14 (KRT14) and -18 (KRT18) at the transcriptional level, even though they were overall relatively low (Figure 1). On the other hand, cell staining for acidic keratins (A-keratin) showed positive only in epithelial cells (Figure 4). In addition, consistent with keratins being epithelial markers, TGFβ1 treatment significantly reduced expression of both keratins in

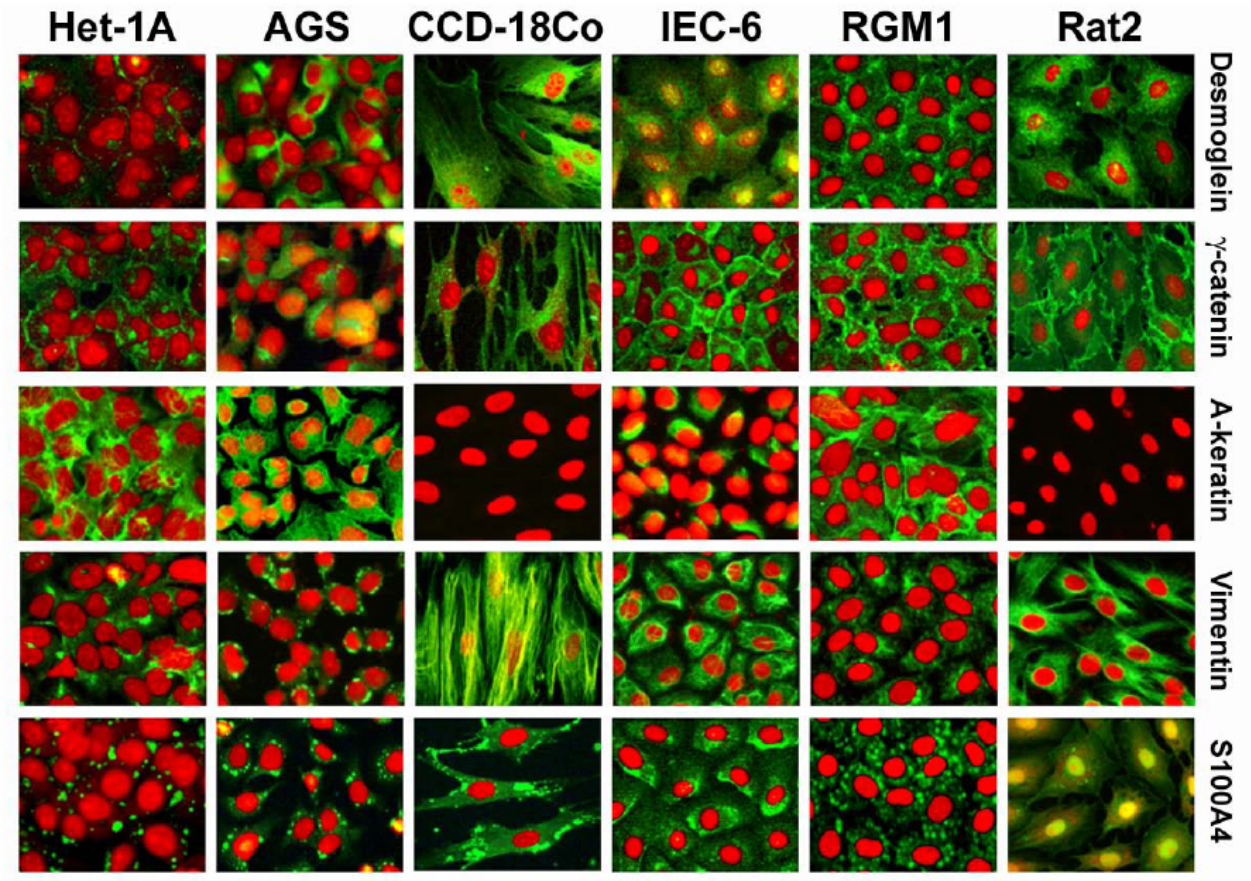


Figure 4. Immunofluorescence detection of protein localization of epithelial and mesenchymal markers in human (Het-1A, AGS, CCD-18Co) and rat cell lines (IEC-6, RGM1, Rat2). Localization patterns of putative epithelial markers (desmoglein, γ-catenin, and acidic keratin) and mesenchymal markers (vimentin and S100A4) were evaluated as described in the methods section. Nuclei were counterstained with propidium iodide (red) as needed.

most epithelial cell lines, with the exception of KRT14 in IEC6 cells (Figures 1 and 2).

Mucins are glycoproteins produced and mostly secreted by epithelial cells to protect themselves against pathogens from outside. At least 19 isoforms have been found in human, some of which are transmembrane proteins, for example, mucin-1. Mucin-1 is localized to the apical side of the epithelium and its cytoplasmic domain binds to the actin cytoskeleton, and therefore mucin-1 is also used as an epithelial polarity marker.

In our study, mucin-1 (MUC1) expression was detectable in all six cell lines examined, with no consistent trend difference between epithelial and mesenchymal cells (Figures 1 and 2). For instance, in Rat2 fibroblasts MUC1 was expressed significantly higher than in IEC-6 epithelial cells but lower than in RGM1 epithelial cells. Moreover, its expression in AGS cells was significantly increased in response to TGFβ1.

3.2. *Mesenchymal markers*

Mesenchymal cells are known for having advanced cytoskeletal structure, high motility, and for producing more extracellular matrix proteins compared to epithelial cells.

Vimentin is an intermediate filament protein that has been widely used as a molecular marker for mesenchymal cells. However, numerous data have shown that vimentin can also be expressed in epithelial cells involved in physiological processes requiring epithelial cell migration, such as placentation and wound healing.

In our study, vimentin (VIM) mRNA expression was found in all the cell lines except AGS, although it was significantly higher in both human and rat fibroblasts than in epithelial cells within the same species (Figures 5 and 6). Its protein expression was also detected in all the epithelial cells including AGS cells (Figure 4), but it did not show typical filamentous phenotype in either Het-1A or AGS as seen in fibroblasts. On the other hand, all rat cell lines expressed high levels of vimentin protein independent of cell type (Figure 4). TGFβ1 increased VIM expression in all epithelial cells (Figures 5 and 6), which displayed a more typical filamentous phenotype as well (Supplementary Figure).

S100A4, also known as fibroblast-specific protein (FSP1), was once described as an absolute marker for fibroblasts (*18*). Any cells that express S100A4 protein and/or show S100A4 promoter activity were classified as fibroblasts. However, more and more evidence

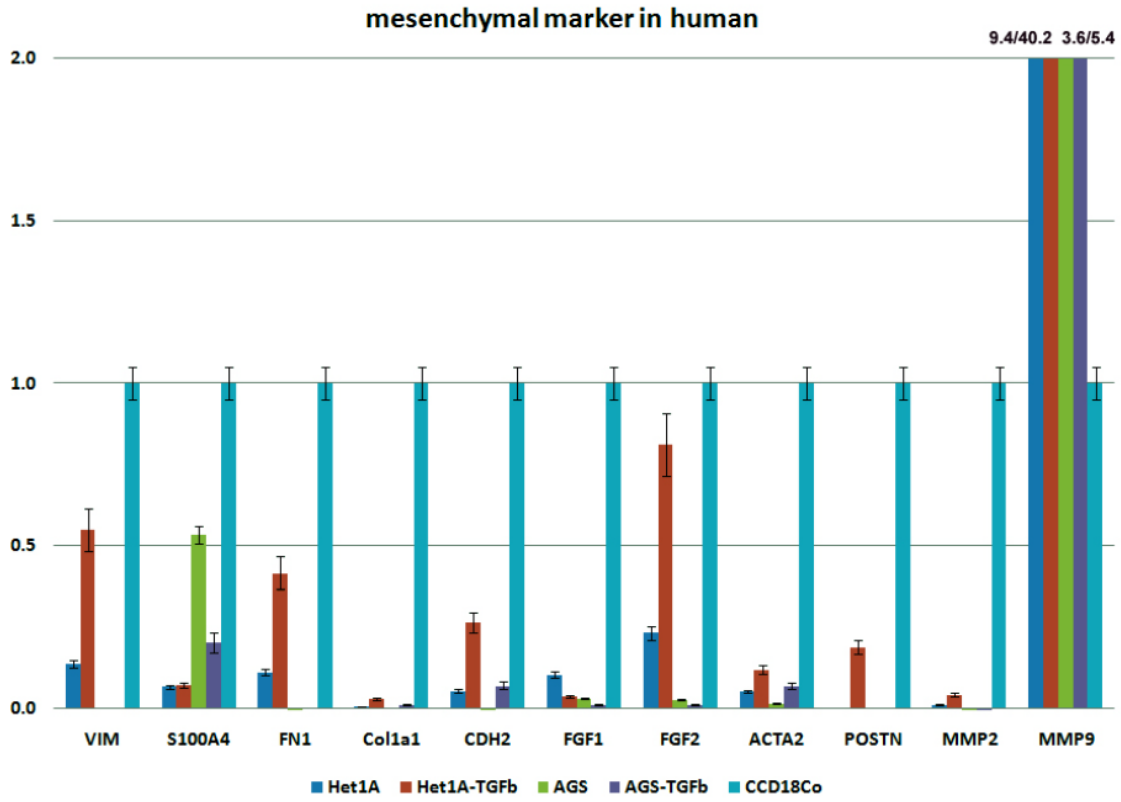


Figure 5. mRNA expression of mesenchymal markers in human cell lines. Three human cell lines (Het-1A, AGS and CCD-18Co) were examined in their resting state and TGFβ1-induced EMT state. Non-treated CCD-18Co fibroblasts were set as a standard in the graph and the rest of the cells were compared against it. Results represent averages from at least three independent experiments with error bars representing standard error. $p < 0.01$.

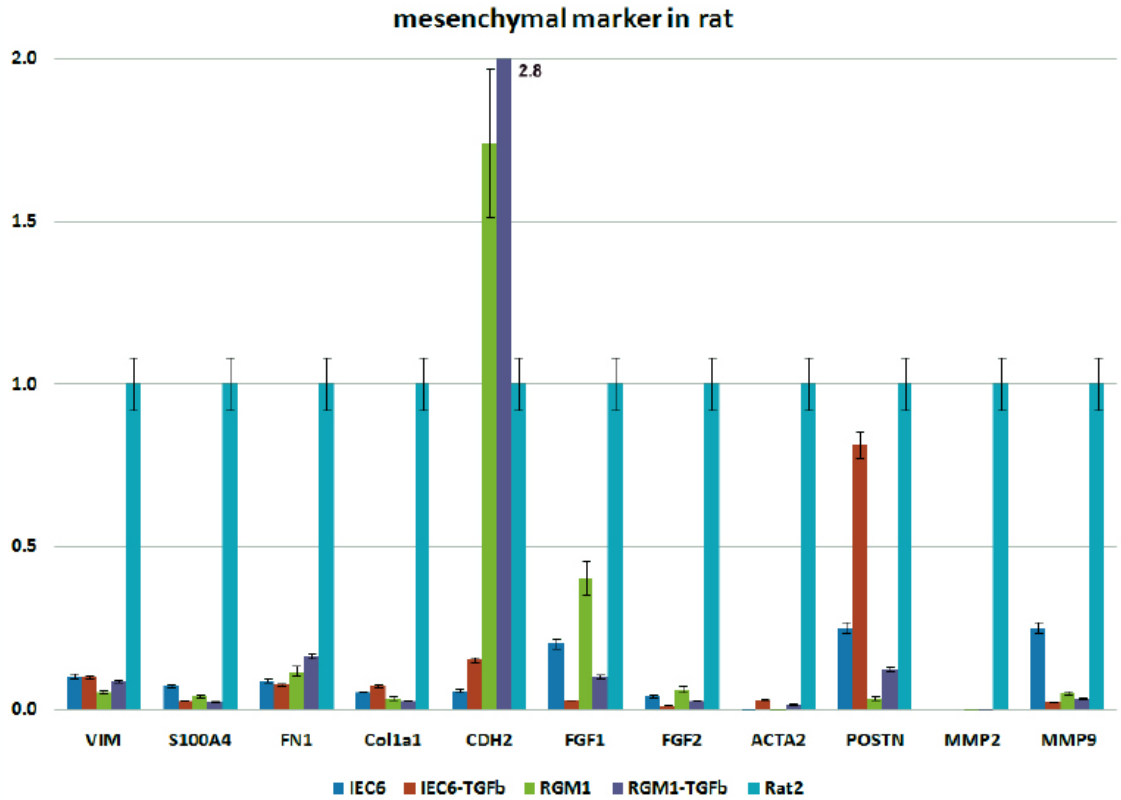


Figure 6. mRNA expression of mesenchymal markers in rat cell lines. Three rat cells lines (IEC-6, RGM1 and Rat2) were examined in their resting state and TGFβ1-induced EMT state. Non-treated Rat2 fibroblasts were set as a standard in the graph and the rest of the cells were compared against it. Results represent averages from at least three independent experiments with error bars representing standard error. $p < 0.01$.

shows that S100A4 is also expressed in other cell types, including blood cells, platelets, endothelial cells, smooth muscle cells, cardiomyocytes, astrocytes, and biliary epithelial cells (*19*).

In our study, S100A4 mRNA expression was found in all the cell lines that were studied, even though expression levels were relatively low for most epithelial cell lines, with the exception of AGS cancer cells, which expressed considerable amounts, albeit still lower than in fibroblasts (Figures 5 and 6). Surprisingly, TGFβ1 strongly inhibited S100A4 expression in 3 out of 4 epithelial cell lines, with the exception of Het-1A cells, raising more questions about the eligibility of S100A4 as a mesenchymal marker. S100A4 protein expression was also clearly detected in all the six cell lines, with rat epithelial RGM1 cells expressing comparable amounts to fibroblasts (Figure 4).

Fibronectin exists in 2 forms, plasma fibronectin and cellular fibronectin. Plasma fibronectin is synthesized by hepatocytes and represents about 1% of serum protein, while cellular fibronectin is made by many different cell types, including fibroblasts, chondrocytes, endothelial cells, macrophages, as well as certain epithelial cells (*20*). The protein is deposited in the extracellular matrix as highly insoluble fibronectin filaments.

In our study, cellular fibronectin (FN1) was expressed in all the cell lines examined, although it was significantly higher in mesenchymal cells than in epithelial cells (Figures 5 and 6). Cell staining confirmed that fibronectin protein was produced and secreted by five out of six cell lines with no clear distinction in expression pattern (Figure 7). Its expression in AGS cells was not detectable, and TGFβ1 treatment, which promoted FN1 expression in most other epithelial cell lines, did not induce it either.

Collagens are the main component of connective tissue and also the main protein of the extracellular matrix that supports other tissues. They are primarily synthesized by fibroblasts. Among 29 types of collagens known so far, type I collagen is the most abundant one in the human body.

In this study, we examined expression of COL1A1 – the gene coding for the α-subunit of type I collagen. Although all six cell lines expressed COL1A1, it was drastically higher in mesenchymal cells than in any epithelial cells (Figures 5 and 6). TGFβ1 treatment modestly promoted expression in most epithelial cell

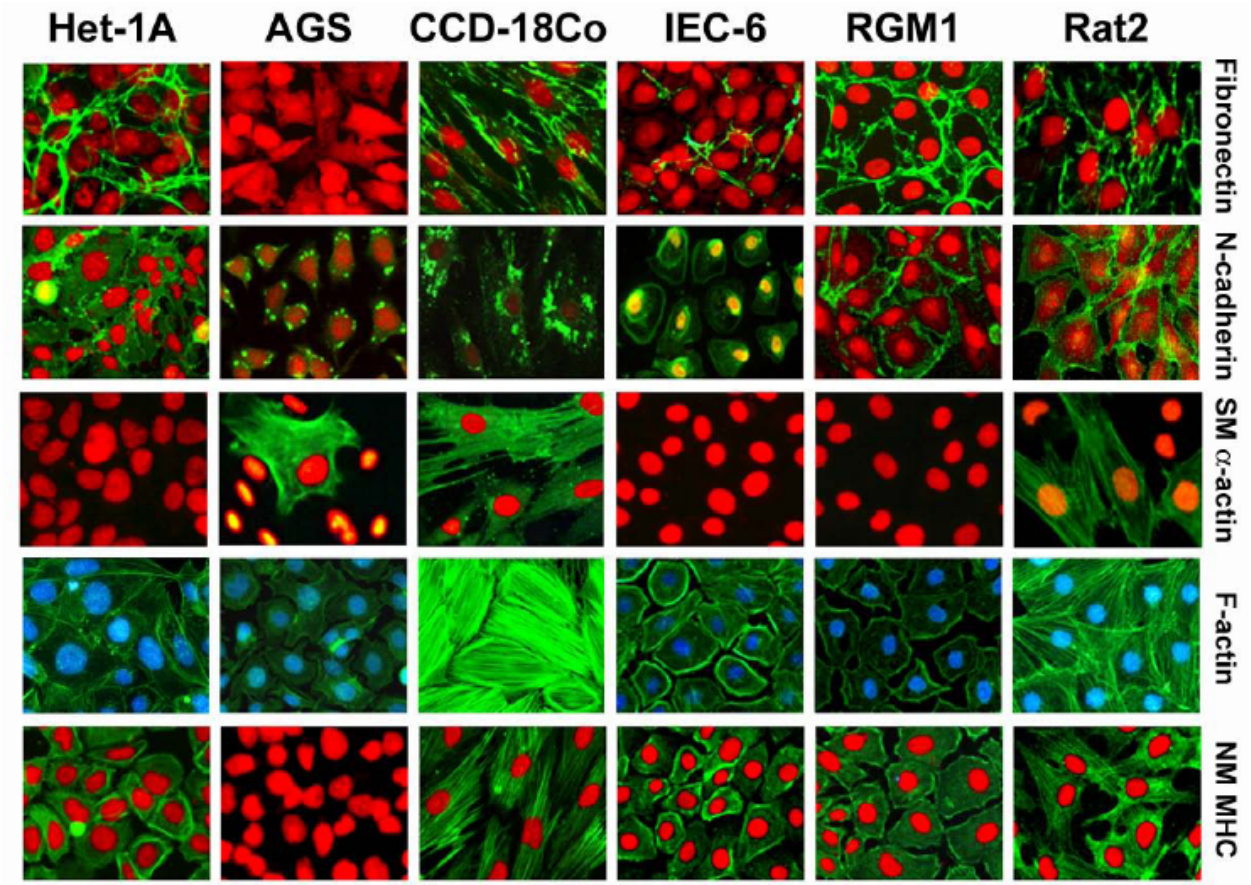


Figure 7. Immunofluorescence detection of protein localization of mesenchymal markers in human (Het-1A, AGS, CCD-18Co) and rat cell lines (IEC-6, RGM1, Rat2). Localization patterns of putative mesenchymal markers (fibronectin, N-cadherin and SM α-actin) were evaluated as described in the methods section. Nuclei were counterstained with either propidium iodide (red) or DAPI (blue) as needed. F-actin and non-muscle myosin heavy chain (NM MHC) were stained to confirm that all the non-treated epithelial cells were not undergoing EMT.

lines (Figures 5 and 6).

N-cadherin is normally found in neural tissue, retina, endothelial cells, fibroblasts, osteoblasts, mesothelium, myocytes, limb cartilage, oocytes, spermatids and Sertoli cells. Switch from E-cadherin to N-cadherin expression in epithelial cells is often considered a sign of EMT (*21*). While this switch is an integral part of several processes during normal development, aberrant expression of N-cadherin by cancer cells contributes to their invasiveness and metastasis in various tissues, including breast, thyroid, bladder, prostate and pancreas (*22,23*), making this a widely used marker for EMT and cancer studies.

In our study, mRNA expression of N-cadherin (CDH2) was found in all the cells examined except AGS, even though its level in Het-1A cells was also very low compared to its expression in CCD-18Co fibroblasts (Figure 5). On the contrary, CDH2 expression in RGM1 epithelial cells was found significantly higher than in Rat2 fibroblasts (Figure 6). TGFβ1 promoted CDH2 expressions in all the epithelial cell lines. Cell staining showed positive membrane localization in all six cell lines, including AGS (Figure 7).

Fibroblast growth factors (FGFs) are a group of over 25 small mitogenic proteins for fibroblasts. Acidic FGF (FGF1) and basic FGF (FGF2) are the prototypes of the FGF family.

In our study, both FGF1 and FGF2 were identified in all cell lines examined; moreover, TGFβ1 had an inhibitory effect on FGF1 in all the epithelial cells and also knocked down FGF2 in three out of four epithelial cell lines (Figures 5 and 6).

Smooth muscle α-actin (SM α-actin) is traditionally used as a marker to distinguish myofibroblasts from regular fibroblasts (*21*).

At the transcriptional level, our data showed that SM α-actin (ACTA2) was expressed in every cell line examined (Figures 5 and 6), although it was significantly higher in mesenchymal cells than in any epithelial cell lines. Cell staining showed that 40-60% of CCD-18Co and Rat2 cells were positive for SM α-actin. In epithelial cells, while there was still detectable signal, it was weak and not in filamentous phenotype as seen in mesenchymal cells (Figure 7). TGFβ1 increased ACTA2 expressions in all the epithelial cells.

Periostin (POSTN), also known as osteoblast-specific factor 2, is a secreted, homodimeric protein synthesized by mesenchymal cells such as smooth muscle cells, fibroblasts and osteoblasts, as well as in the periosteum and periodontal ligament. Recent clinical evidence has revealed that periostin is involved in the development of various tumors, such as breast, lung, colon, pancreatic, and ovarian cancers where it promotes EMT, invasion, and metastasis (*25*).

In our study, POSTN was not detectable in AGS cells, but it was expressed in all the remaining epithelial cells (Figures 5 and 6). Its expression was greatly up-regulated by TGFβ1 in all the epithelial cells.

Matrix metalloproteinases (MMPs) are a group of over 25 proteases, either secreted or membrane-associated, that rely on metal ions for their catalytic activity. Collectively MMPs are capable of degrading all kinds of extracellular matrix components, therefore, their expression level is critical to embryonic development, tissue remodeling and cancer metastasis. Mesenchymal cells are the main source of MMPs and, for this reason, expression of MMPs in epithelial cells is often considered an indicator of EMT (*26,27*).

Most MMPs are tightly regulated at the transcriptional level. In our study, we measured expression of MMP2 (gelatinase A) and MMP9 (gelatinase B), which are closely correlated with EMT and cancer metastasis (*28*). MMP2 expression was undetectable in IEC-6 cells and was still minimal in the rest of the epithelial cell lines compared to its level in the fibroblasts of the same species (Figures 5 and 6). The expression of MMP9, however, was significantly higher in both human epithelial cell lines than in CCD-18Co fibroblasts (Figure 5). TGFβ1 had a positive effect on both MMPs.

When epithelial cells are in the process of EMT, they do not only express mesenchymal markers, but also increase their mobility, which can be reflected by formation of actin and myosin stress fibers. To confirm that the epithelial cell lines used in this study were not undergoing EMT when examined in their rest phase, which could have otherwise explained improper expression of some putative mesenchymal markers, we stained the cells for filamentous actin (F-actin) and nonmuscle myosin heavy chain (NM-MHC). In both CCD-18Co and Rat2 fibroblasts, extensive actin and myosin stress fibers were found. In contrast, actin and myosin in the epithelial cells only displayed cortical distribution (Figure 7), while AGS cells did not express NM-MHC at all. In contrast, when the same epithelial cell lines were subjected to TGFβ1 treatment to induce EMT, they all displayed strong actin and, to a lesser extent, myosin stress fibers (Supplementary Figure), suggesting that all the epithelial cells used in this study were not undergoing EMT when the experiments were conducted.

4. Discussion

Dozens of molecules have been commonly used by both basic scientists and clinical researchers as markers to distinguish epithelial and mesenchymal cells for different applications, including molecular cell typing and cancer diagnosis. However, emerging evidence questions the validity of these molecular identifiers. Among epithelial markers, Claudin-5 has been identified in cardiomyocytes (*29*); JAM-A and JAM-C have been reported in fibroblasts derived from various tissues including derma, lung, cornea, and embryo

(*30*); ZO-1 is not only associated with cell membrane of corneal fibroblasts, but it can also translocate to the nucleus to serve as a transcription cofactor in case of corneal injury (*31*); Mucin-1 is also expressed in myofibroblasts (*32*); and all four catenins (α, β, γ, and δ) have been documented in fibroblasts (*33,34*). In addition, all of the desmosome proteins are expressed in periodontal ligament fibroblasts (*35*), dental pulp fibroblasts (*36*) and other non-epithelial cells. For example, desmoplakin interacts with cytokeratin filaments in epithelial cells, but also binds to desmin in cardiomyocytes and to vimentin in fibroblasts. E-cadherin expression is commonly considered as a gold seal of epithelial cells. Modulation of E-cadherin expression levels has been vastly used as a key theme of epithelial plasticity and cancer metastasis. However, E-cadherin expression is still maintained in most differentiated tumors, including carcinomas of the skin, head and neck, esophagus, breast, lung, liver, colon, and prostate. Furthermore, our study has shown that E-cadherin is not only expressed in rat embryonic fibroblasts, but its level in those cells is consistently higher compared to rat gastric epithelial cells. Similarly, keratin subtype expression patterns are commonly used as markers to identify different types of epithelial malignancies, as the keratin profile tends to remain constant when an epithelium undergoes malignant transformation. However, keratins have been also found in non-epithelial cells (*37*). In our study, KRT-14 transcription was found in human colon fibroblasts, even though its expression level was significantly lower than that of both human epithelial lines examined.

For mesenchymal cells, as Hay once said (*38*), "There are, in fact, no specific biochemical markers by which we can define the mesenchyme". Among the mesenchymal markers that we tested, vimentin is regularly expressed in ocular epithelial cells together along with cytokeratin (*39,40*); S100A4 is reported in biliary epithelial cells (*41*); Periostin has been detected at high expression levels in both endometrial (*42*) and mammary epithelial cells (*43*); and fibronectin is not only made by fibroblasts, but also produced by many other cell types including certain epithelial cells (*20*). N-Cadherin is another commonly used mesenchymal marker in EMT studies and the switch in expression from E-cadherin to N-cadherin is considered a hallmark of EMT progression and, to some extent, cancer cell invasion (*21,23*). However, several types of epithelial cells express N-cadherin naturally and the E/N switch in cancer cell invasion is not always followed. For example, N-cadherin is required to maintain corneal limbal epithelial progenitor cells (*44*). Moreover, ovarian surface epithelial cells normally express N-cadherin, but switch to E-cadherin during progression to the neoplastic state (*45*), suggesting that an N/E switch rather than E/N switch might play a role in the initiation of ovarian carcinogenesis (*23*). On the other hand, N-cadherin and E-cadherin are concurrently expressed in the epithelial cells of intra-hepatic bile ducts in normal liver (*46*).

In our current study we provide a detailed analysis of all of these putative epithelial and mesenchymal markers and show that none of them exhibit exclusive epithelial or mesenchymal expression, supporting the emerging evidence that none of the molecular markers commonly used to distinguish epithelial cells from mesenchymal cells is universally unique to one cell type. They are all detectable in both cell types and, while some differences in expression and/or localization were observed, these were also not consistent enough throughout the cell lines studied to classify them as universal markers. These two cell types represent two poles and there are numerous intermediate states or subtypes of cells in between. Although they are differentiated cells, they are also interchangeable depending on environmental condition. Change from epithelial state to mesenchymal state or vice versa is a dynamic process. It is a cumulative effect of different expression levels of multiple genes that ultimately sets these two phenotypes apart.

Moreover, despite the great similarity between human and rat, our results clearly indicate that there may still be some cross-species differences that make our quest for universal markers even harder. On the other hand, taking into account that the line between epithelial and mesenchymal identity is not as rigid as it is usually thought to be, we can still identify some molecules that better serve the purpose. Based on our data, keratins appear to be the most consistent epithelial marker among the different candidates tested. Both keratin-14 and -18 are absolutely omitted in Rat2 fibroblasts and are expressed at much higher levels in human epithelial cells compared to corresponding mesenchymal cells CCD-18Co, which express only moderate amounts. Consistent with their epithelial phenotype, TGFβ1 treatment, which promotes EMT, drastically reduced expression of both keratins in most epithelial cell lines. Moreover, even though keratins can be detected at the transcriptional level in CCD-18Co fibroblasts, their protein products are not found, as seen from A-keratin staining, which is exclusive to the epithelial cells in both human and rat. E-cadherin would be our next choice, due to some differences across species. E-cadherin exhibits strong epithelial preference in the human panel; it is expressed in significant amounts in both epithelial cell lines and clearly localized to the membrane but it is absolutely not expressed in human CCD-18Co cells, making it also a good epithelial marker for human cells. Its expression in Rat2 fibroblasts, on the other hand, was found to be higher than in RGM1 epithelial cells, but one should acknowledge the fact that in both these cell lines CDH1 levels are extremely low. Again, the EMT inducer TGFβ1 had a negative effect on CDH1 expression

in all cells examined, further supporting the role of E-cadherin as an epithelial marker. Finally, even though ZO-1 (TJP1) was expressed at high levels in human fibroblasts, it was mainly localized to the cytoplasm, unlike in epithelial cells in which it was exclusively localized to the membrane. So was true with rat cells. TGFβ1 treatment also led to a loss of membrane localization in most epithelial cells, supporting the notion that membrane-to-cytoplasm localization of ZO-1 could also be an informative marker for EMT.

Compared to epithelial markers, mesenchymal markers showed higher fidelity. The majority of mesenchymal markers, except CDH2 and MMP9, exhibited a clear mesenchymal preference with little expression in epithelial cells. Moreover, even though MMP9 was expressed significantly higher in both human epithelial cell lines than in human fibroblasts and RGM1 cells expressed higher levels of CDH2 compared to Rat2 cells, both of these genes were up-regulated by TGFβ1 treatment, consistent with a mesenchymal phenotype. Among all the mesenchymal markers, MMP2 exhibits the strongest distinction, with only very little expression in the epithelial lines examined. COL1A1 is next, with a similar, if not as striking, trend across both species. POSTN and FGF1 also showed very good trends within the human panel. However, while POSTN could still be considered a viable marker in rat cells, FGF2 proved a much better marker in rat cells than FGF1. Moreover, while TGFβ1 treatment promoted POSTN expression in most epithelial cell lines, it actually had a mostly negative effect on both FGF1 and FGF2, raising additional questions about the validity of either FGF as a mesenchymal marker. Vimentin also has significantly higher mRNA expression in fibroblast cells compared to epithelial cells across species but the difference in staining pattern is much more dramatic in the human panel than in the rat panel. On the other hand, although ACTA2 expression in mesenchymal cells is also found overwhelmingly higher than in epithelial cells, it might be due to myofibroblast differentiation.

In summary, despite the limited number of cell lines (mostly of gastrointestinal origin) used in this study, our data support the conclusion that there is no universal molecular marker that can be absolutely unique to either epithelial cells or mesenchymal cells, especially in the light of some differences across species. Nonetheless, among the various markers analyzed, keratins appear to be the No.1 choice for epithelial cells, while E-cadherin is next, depending on the species studied. Among the mesenchymal markers, most, but not all, of them are predominantly expressed by mesenchymal cells, providing a better range of choices. MMP2 would be the preferred marker, COL1A1 would be next. Vimentin and POSTN could also be good alternative markers, while FGFs appear to have clear species differences, where FGF1 is better as a human marker and FGF2 as a rat marker, and could not be recommended as first/sole choice. Loss of ZO-1 membrane localization and/or cytoplasmic redistribution could also be used as a mesenchymal marker, even though it would also not be recommended as a first/sole choice given other alternatives. These recommendations should however be used with caution, taking into account that they were based on data collected from immortalized cell lines. Variations may be found *in vivo* or in primary cell systems. Researchers are advised to take extra precaution when choosing molecular markers to define a cell type.

Finally, multiple molecules involved in EMT can be envisioned as targets of anti-EMT therapy to prevent or restrain invasion and metastasis of cancer cells. Given the complexity of the molecular and cellular pathways leading to EMT, a forced stimulation of MET can be a very neat approach to control EMT. In order to achieve this, however, it is imperative for us to gain a deeper understanding of this dynamic process through a detailed characterization of its different steps and components. Our study here provides ample data in that direction.

Acknowledgements

This work was supported by the Department of Veterans Affairs of the United States and the American Heart Association.

References

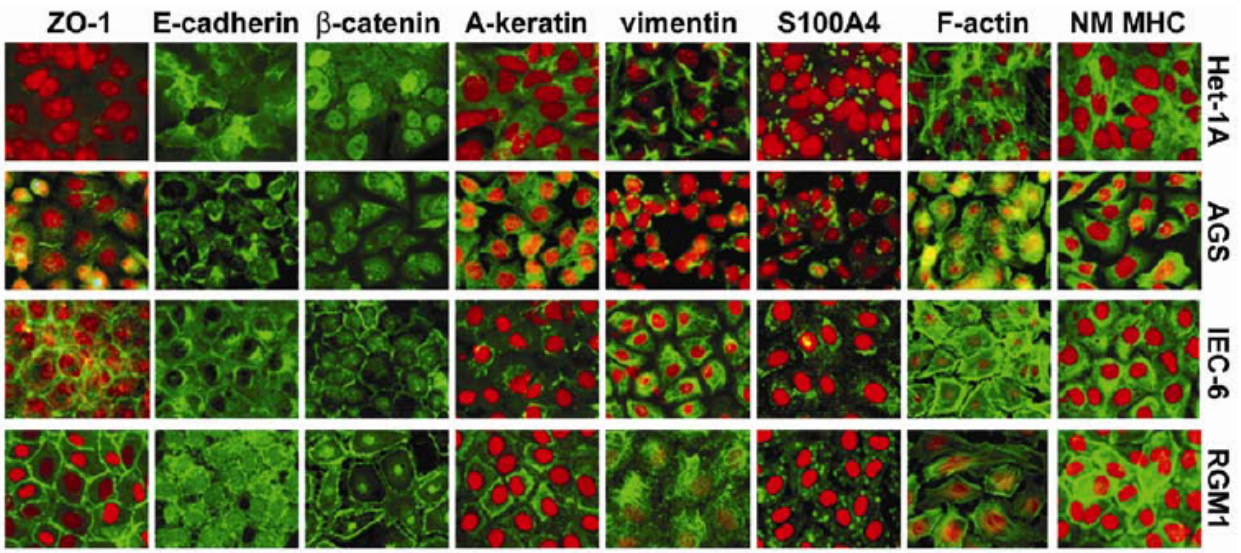
1. Lillie FR. The Development of the Chick: An Introduction to Embryology. Henry Holt and Company, New York, NY, USA, 1908.
2. Greenburg G, Hay ED. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol. 1982; 95:333-339.
3. Ruiz P, Günthert U. The cellular basis of metastasis. World J Urol. 1996; 14:141-150.
4. Radisky DC, Kenny PA, Bissell MJ. Fibrosis and cancer: do myofibroblasts come also from epithelial cells *via* EMT? J Cell Biochem. 2007; 101:830-839.
5. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002; 2:442-454.
6. Guarino M, Rubino B, Ballabio G. The role of epithelial-mesenchymal transition in cancer pathology. Pathology. 2007; 39:305-318.
7. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest. 2003; 112:1776-1784.
8. Miettinen PJ, Ebner R, Lopez AR, Derynck R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J Cell Biol. 1994; 127:2021-2036.
9. Zavadil J, Boettinger EP. TGF-β and epithelial-to-mesenchymal transitions. Oncogene. 2005; 24:5764-5774.
10. Balda MS, Matter K. Tight junctions and the regulation

of gene expression. Biochim Biophys Acta. 2009; 1788:761-767.
11. Rahner C, Mitic LL, Anderson JM. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. Gastroenterology. 2001; 120:411-422.
12. Niessen CM, Gottardi CJ. Molecular components of the adherens junction. Biochim Biophys Acta. 2008; 1778:562-571.
13. van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. Cell Mol Life Sci. 2008; 65:3756-3788.
14. Zhang Y, Kanter EM, Laing JG, Aprhys C, Johns DC, Kardami E, Yamada KA. Connexin43 expression levels influence intercellular coupling and cell proliferation of native murine cardiac fibroblasts. Cell Commun Adhes. 2008; 15:289-303.
15. Follonier L, Schaub S, Meister JJ, Hinz B. Myofibroblast communication is controlled by intercellular mechanical coupling. J Cell Sci. 2008; 121:3305-3316.
16. Li G, Satyamoorthy K, Meier F, Berking C, Bogenrieder T, Herlyn M. Function and regulation of melanoma-stromal fibroblast interactions: when seeds meet soil. Oncogene. 2003; 22:3162-3171.
17. Ko JA, Yanai R, Morishige N, Takezawa T, Nishida T. Upregulation of connexin43 expression in corneal fibroblasts by corneal epithelial cells. Invest Ophthalmol Vis Sci. 2009; 50:2054-2060.
18. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG. Identification and characterization of a fibroblast marker: FSP1. J Cell Biol. 1995; 130:393-405.
19. Schneider M, Hansen JL, Sheikh SP. S100A4: a common mediator of epithelialmesenchymal transition, fibrosis and regeneration in diseases? J Mol Med. 2008; 86:507-522.
20. Walia B, Castaneda FE, Wang L, Kolachala VL, Bajaj R, Roman J, Merlin D, Gewirtz AT, Sitaraman SV. Polarized fibronectin secretion induced by adenosine regulates bacterial-epithelial interaction in human intestinal epithelial cells. Biochem J. 2004; 382:589-596.
21. Wheelock MJ, Shintani Y, Maeda M, Fukumoto Y, Johnson KR. Cadherin switching. J Cell Sci. 2008; 121:727-735.
22. Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J Cell Biol. 1999; 147:631-644.
23. Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol. 2000; 148:779-790.
24. Chai J, Norng M, Tarnawski AS, Chow J. A critical role of serum response factor in myofibroblast differentiation during experimental oesophageal ulcer healing in rats. Gut. 2007; 56:621-630.
25. Ruan K, Bao S, Ouyang G. The multifaceted role of periostin in tumorigenesis. Cell Mol Life Sci. 2009; 66:2219-2230.
26. Laffin B, Wellberg E, Kwak HI, Burghardt RC, Metz RP, Gustafson T, Schedin P, Porter WW. Loss of singleminded-2s in the mouse mammary gland induces an epithelialmesenchymal transition associated with up-regulation of slug and matrix metalloprotease 2. Mol Cell Biol. 2008; 28:1936-1946.
27. Uttamsingh S, Bao X, Nguyen KT, Bhanot M, Gong J, Chan JL, Liu F, Chu TT, Wang LH. Synergistic effect between EGF and TGF-beta1 in inducing oncogenic properties of intestinal epithelial cells. Oncogene. 2008; 27:2626-2634.
28. Troussard AA, Costello P, Yoganathan TN, Kumagai S, Roskelley CD, Dedhar S. The integrin linked kinase (ILK) induces an invasive phenotype *via* AP-1 transcription factordependent upregulation of matrix metalloproteinase 9 (MMP-9). Oncogene. 2000; 19:5444-5452.
29. Sanford JL, Edwards JD, Mays TA, Gong B, Merriam AP, Rafael-Fortney JA. Claudin-5 localizes to the lateral membranes of cardiomyocytes and is altered in utrophin/dystrophin-deficient cardiomyopathic mice. J Mol Cell Cardiol. 2005; 38:323-332.
30. Morris AP, Tawil A, Berkova Z, Wible L, Smith CW, Cunningham SA. Junctional Adhesion Molecules (JAMs) are differentially expressed in fibroblasts and co-localize with ZO-1 to adherens-like junctions. Cell Commun Adhes. 2006; 13:233-247.
31. Benezra M, Greenberg RS, Masur SK. Localization of ZO-1 in the nucleolus of corneal fibroblasts. Invest Ophthalmol Vis Sci. 2007; 48:2043-2049.
32. Kamoshida S, Tsutsumi Y. Expression of MUC-1 glycoprotein in plasma cells, follicular dendritic cells, myofibroblasts and perineurial cells: immunohistochemical analysis using three monoclonal antibodies. Pathol Int. 1998; 48:776-785.
33. Gurung A, Uddin F, Hill RP, Ferguson PC, Alman BA. Beta-catenin is a mediator of the response of fibroblasts to irradiation. Am J Pathol. 2009; 174:248-255.
34. Kim K, Daniels KJ, Hay ED. Tissue-specific expression of beta-catenin in normal mesenchyme and uveal melanomas and its effect on invasiveness. Exp Cell Res. 1998; 245:79-90.
35. Yamaoka Y, Sawa Y, Ebata N, Yoshida S, Kawasaki T. Desmosomal proteins in cultured and intact human periodontal ligament fibroblasts. Tissue Cell. 1999; 31:605-609.
36. Sawa Y, Kuroshima S, Yamaoka Y, Yoshida S. Intracellular distribution of desmoplakin in human odontoblasts. J Histochem Cytochem. 2005; 53:1099-1108.
37. Xu X, Roberts SA, Pasha TL, Zhang PJ. Undesirable cytokeratin immunoreactivity of native nonepithelial cells in sentinel lymph nodes from patients with breast carcinoma. Arch Pathol Lab Med. 2000; 124:1310-1313.
38. Hay ED. The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev Dyn. 2005; 233:706-720.
39. Kasper M, Moll R, Stosiek P, Karsten U. Patterns of cytokeratin and vimentin expression in the human eye. Histochemistry. 1988; 89:369-377.
40. FitzGerald PG. Lens intermediate filaments. Exp Eye Res. 2009; 88:165-172.
41. Díaz R, Kim JW, Hui JJ, Li Z, Swain GP, Fong KS, Csiszar K, Russo PA, Rand EB, Furth EE, Wells RG. Evidence for the epithelial to mesenchymal transition in biliary atresia fibrosis. Hum Pathol. 2008; 39:102-115.
42. Hiroi H, Momoeda M, Nakazawa F, Koizumi M, Tsutsumi R, Hosokawa Y, Osuga Y, Yano T, Tsutsumi O, Taketani Y. Expression and regulation of periostin/OSF-2 gene in rat uterus and human endometrium. Endocr J. 2008; 55:183-189.
43. Haertel-Wiesmann M, Liang Y, Fantl WJ, Williams LT. Regulation of cyclooxygenase-2 and periostin by Wnt-3

in mouse mammary epithelial cells. J Biol Chem. 2000; 275:32046-32051.
44. Higa K, Shimmura S, Miyashita H, Kato N, Ogawa Y, Kawakita T, Shimazaki J, Tsubota K. N-cadherin in the maintenance of human corneal limbal epithelial progenitor cells *in vitro*. Invest Ophthalmol Vis Sci. 2009; 50:4640-4645.
45. Patel IS, Madan P, Getsios S, Bertrand MA, MacCalman CD. Cadherin switching in ovarian cancer progression. Int J Cancer. 2003; 106:172-177.
46. Mosnier JF, Kandel C, Cazals-Hatem D, Bou-Hanna C, Gournay J, Jarry A, Laboisse CL. N-cadherin serves as diagnostic biomarker in intrahepatic and perihilar cholangiocarcinomas. Mod Pathol. 2009; 22:182-190.

(Received April 6, 2010; Accepted May 6, 2010)

Appendix



Supplementary Figure. Immunofluorescence detection of protein localization of epithelial and mesenchymal markers in TGFβ1-treated human and rat epithelial cell lines. Human (Het-1A, AGS) and rat (IEC-6, RGM1) epithelial cell lines were treated with TGFβ1 to induce EMT. Localization patterns of putative epithelial (ZO-1, E-cadherin, β-catenin and acidic keratin) and mesenchymal markers (Vimentin and S100A4) were evaluated by cell staining as described in the methods section. Nuclei were counterstained with either Propidium Iodide (red) or DAPI (blue) as needed. F-actin and NM MHC fiber-like staining was used as a positive control for EMT.