Original Article

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# The effect of His-tag and point mutation on the activity of irisin on MC3T3-E1 cells

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**Summary** **Irisin is a myokine secreted from the cleavage of fibronectin type III domain-containing protein 5 (FNDC5) and has an effect on bone formation. There are limited studies about the structure of irisin and its functional unit. In order to clarify the candidate domain responsible for irisin action, we constructed several irisin variants and tested their influence on the proliferation and osteogenesis of MC3T3-E1 cells. On the one hand, His-tag was added to the N terminal or C terminal of irisin. On the other hand, the flexible region or salt bridge site were chosen as the candidate for point mutation. Alkaline phosphatase (ALP), Runt related transcription factor 2 (Runx2) and collagen type I alpha 1 (COL1α1) were chosen to test the differentiation efficiency. We found point mutation on flexible regions, Glu-57 and Ile-107, and adding His-tag on the C-terminal of irisin did affect its action. The osteogenic potential of irisin E57K, irisin I107F and irisin$^{C\text{-}His}$ decreased about 90.1%, 88.8% and 96.6% activity of recombinant-irisin (r-irisin) ($P < 0.05$), respectively. Point mutation on the salt bridge, Arg-75, partly decreased the effect of irisin (45 ± 11.3% of r-irisin) ($P < 0.05$). N-terminal His-tag showed almost no effect (93.5 ± 25.7% of r-irisin) ($P = 0.658$). This study suggested that the flexible region of residues 55-58 and 106-108, and C-terminal of irisin are vital for its activity. Disrupting the dimerization of irisin might result in a partly reduced effect on cell differentiation.**

***Keywords:*** Irisin, MC3T3-E1 cell, osteogenesis, His-tag, point mutation

## 1. Introduction

Exercise acts as an important role on bone metabolic disorders in addition to pharmaceutical treatment. Besides mechanical interaction, it could remold bone tissue through regulating the expression of many factors such as tumor necrosis factor (*1*). Irisin is one of them and has attracted much attention since its discovery.

Irisin is composed of 112 amino acids, which can be cleaved from membrane protein human fibronectin type III domain-containing protein 5 (FNDC5) and can be detected in fat, skeletal muscle, serum and so on (*2*). Up to now, the relationship between irisin and glycolipid metabolism, muscle remodeling, non-alcohol fatty liver disease, chronic kidney disease and tumorigenesis have been reported (*3*).

Moreover, evidence suggests that irisin also contributes to bone metabolism (*4-6*). Both bone mass and bone quality of mice, with or without osteoporosis, were improved after irisin treatment (*7*). It has a direct effect on bone, including boosting bone formation and inhibiting bone absorption (*8-10*), which means it has potential in dealing with bone loss and even osteoporosis.

The concentration of irisin varies in different articles, from 0.01 to 2,000 ng/mL (*11*), which partly may be because of the detecting method. Enzyme linked immunosorbent assay (ELISA), which is the most common method, has shortages of cross reaction and inconsistent antibodies (*11,12*). Through mass

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spectrometry, Jedrychowski *et al*. reported a level of 3.6 ng/mL in human serum in the resting state, and 4.3 ng/mL after exercise (*13*). This is much lower than its dose in experiments, which suggests that irisin beyond its physiological level might have benefits.

Irisin consists of a N-terminal fibronectin III-like domain, which is attached to a flexible C-terminal structure and forms dimers (*14*). However, studies about the structure of irisin are limited and there is little known about its critical domains. His-tag is helpful in purifying or detecting protein, but it is unknown that if the activity of recombinant irisin (r-irisin) will be influenced or not by the existence and the site of the tag. Studies on irisin structure is about its synthesis, secretion and stability through mostly point mutations (*14,15*), but failed to examine its effect. In order to further understand the molecular structure of irisin, based on our previous study that r-irisin can promote the proliferation and osteogenesis of osteoblast cells (*10*), we made some changes on irisin and detected their effect on these cells.

## 2. Materials and Methods

### 2.1. *Materials*

Reagents used here included dexamethasone, ascorbic acid, beta-glycerophosphate, phenylmethanesulfonyl fluoride (PMSF) and sodium azide ($NaN_3$) (Sigma, USA). Recombinant irisin (r-irisin), which is without tag and synthsized from *E. coli*, was purchased from Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA. BCIP/NBT alkaline phosphatase color development kit, and alkaline phosphatase assay kit was purchased from Beyotime Inst Biotech, China. Fetal bovine serum (FBS) and MEM Eagle - alpha modification (α-MEM) were purchased from Gibco, USA. The bicinchoninicacie (BCA) protein assay kit was purchased from Pierce, USA. Chromogenic substrate limulus reagent kit was purchased from Solarbio, China. The pET28a(+) vector was purchased from Novagen, USA. The MC3T3-E1 cells were provided by laboratory of transplant immunology, West China Hospital, Sichuan University.

### 2.2. *The plasmid construction and protein purification*

The whole FNDC5 sequences were amplified from mouse cDNA with the following primer: 5'-G AGTCGCCATGCCCCCAGG (forward) -3', 5'-GCTGCTCAGAGCAAGCACTG-3' (Reverse) and was subcloned into pTeasy vector (Transgen biotech, China) and confirmed by direct sequencing. Six histidines were added on its terminal. For the N-his tag irisin ($irisin^{N\text{-}His}$), the only irisin sequence was amplified from this plasmid with NdeI and XhoI recognizing sequence tag primers: 5'-GGAATTCCATATGGACAG CCCCTCAGCCCCT-3' (forward), 5'-GGCTCGAGT TATTACTCCTTCATGGTCACCTC-3' (Reverse), and ligated the fragment with pET28a(+) vector at NdeI and XhoI sites. For the $irisin^{C\text{-}His}$ the NcoI sites were chosen instead from NdeI with reverse primer: 5'-G GCTCGAGCTCCTTCATGGTCACCTCATCTTT-3' , and the PCR product was subcloned into pET28a(+) vector at NcoI and XhoI sites. After identification by DNA Sequencing, the plasmids were transformed into Rosetta (DE3) *E. coli*.

The plasmids containing point mutations were generated with the Fast mutagenesis System (Transgen biotech, China). Briefly, the primers containing the point mutation with 15-20 bp 5" and 10 bp 3" overlap region were designed, then PCRed the pET28a- $irisin^{N\text{-}His}$ plasmid with each pair of primers individually. The aa mutation numbers are those for FNDC5, starting with the signal peptide. The pair of oligonucleotides for PCR of $irisin^{N\text{-}His}$ E57K were (forward)5'-TGG GAT GTC CTG GAG GAT AAA GTG GTC ATT G-3', (reverse)5'-T ATC CTC CAG GAC ATC CCA GCT CAG CAC GGC-3'. The pair $irisin^{N\text{-}His}$ R75E were (forward)5'-AAG GAT GTG CGG ATG CTC GAG TTC ATT CAG G-3', (reverse)5'-TC GAG CAT CCG CAC ATC CTT CTT CTG CTG AG-3'. The pair of $irisin^{N\text{-}His}$ I107F were (forward) 5'-CAT GTG CAG GCC ATC TCC TTC CAG GGA CAG-3', (reverse) 5'-A GGA GAT GGC CTG CAC ATG GAC GAT ATA TTC-3'. All plasmids were identified by direct sequencing and transformed into Rosetta (DE3) *E. coli*.

The *E. coli* were cultured at 37ºC respectively in LB overnight, then the overnight cultures were diluted 1:100 and cultured under the same conditions continupusly. When the $OD_{600}$ of the culture reached 0.6-0.8, 0.15mM IPTG was added and cultured at 25ºC for 3 hours to induce the expression and translation of irisin protein. The protein was purified using a Ni-sepharose 6FF column and dialyzed with PBS (pH7.4) solution. The concentration of the recombinant irisin was measured with Pierce BCA protein Assay Kit. The chromogenic substrate limulus reagent kit was used to detect the endotoxin.

### 2.3. *Cell culture and treatment*

The mouse osteoblastic MC3T3-E1 cells were grown in α-MEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. All the cells were cultured in a humidified atmosphere of 95% air and 5% $CO_2$ at 37ºC. The culture medium was changed every 2-3 days.

The cells were divided into seven groups and treated with different r-irisin proteins at a concentration of 20nm, they were: a blank control group (con), $irisin^{N\text{-}His}$, $irisin^{C\text{-}His}$, irisin E57K, irisin R75E, irisin I107F and a commercial r-irisin.

### 2.4. *Cell proliferation detection*

The cells were plated into a 96-well plate at a density

of $1.5 \times 10^4$ cells/cm$^2$. The medium was added with the different irisin proteins after cell adherence. At 0, 24, 48 and 72 hours of culturing, cell counting kit (CCK-8) solution was added to each well and incubated for 2 hours, then the absorbance was measured at 450 nm. All experiments were performed in four replicates.

2.5. *qPCR*

Cells were seeded into 6-well plates at a density of $1.5 \times 10^4$/cm$^2$ and cultured in osteogenic medium composed of 10%FBS, 0.1 uM dexamethasone, 10mM β-glycerophosphate and 50 ug/mL ascorbic acid. After culturing for 3 or 14 days, cells were harvested for total RNA extraction with Trizol (Ambion, USA). According to manufacturer`s instructions, the cDNA was synthesized by Revert Aid First Strand cDNA synthesis Kit (Thermo, EU). SYBR Green Master Mix (Applied Biosystems, USA) was used to perform real-time PCR on Applied Biosystems 7500 Real-time PCR system. The 2−Δctmethod was used to calculate the relative gene expressions of alkaline phosphatase (ALP), Runt related transcription factor 2 (Runx2) and collagen type I alpha 1(COL1α1). The primers of each target gene are listed in Table 1.

2.6. *ALP detection*

Cells were plated into 6-well plates at a density of $1.5 \times 10^4$/cm$^2$ and grouped as described above in two replicates. All cells were cultured in osteogenic medium and the medium was changed every 3 days. After culture for 14days, the cells were fixed or harvested for ALP staining or quantification, respectively.

For ALP staining, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes, then rinsed with deionized water and stained to recognize ALP following the instruction of BCIP/NBT alkaline phosphatase color development kit. Images of each group were taken.

For ALP quantification, cells were washed with PBS, then lysed and collected with buffer comprised of 20 mM pH8.0 Tris-HCl, 150 mM NaCl, 1% TritonX-100, 0.02% $NaN_3$ and 1 mM PMSF. The collections were centrifuged and the supernatant was used to detect the ALP activity through alkaline phosphatase assay kit. After being normalized to the total protein content quantified by BCA protein assay kit, the ALP activity was standardized and compared with each other.

2.7. *Statistical analysis*

Data are presented as mean ± standard deviation (SD) and analyzed with SPSS22.0. Comparisons among two groups were evaluated by two-tailed student's *t* test. Comparisons among multiple groups were evaluated by one-way analysis of variance (ANOVA) and Turkey's test. $P < 3.35$ was considered as significant.

## 3. Results

3.1. *Detecting the irisin with tag or point mutation*

The recombinant plasmids were confirmed by direct DNA sequencing. The concentration of irisin$^{N\text{-}His}$, irisin$^{C\text{-}His}$, irisinE57K, irisin R75E, and irisin I107F were 0.413 ug/uL, 0.367 ug/uL, 0.179 ug/uL, 0.341 ug/uL and 0.691 ug/uL, respectively. The endotoxin of each of them was 5.71 EU/mL, 5.09 EU/mL, 5.27 EU/mL, 4.92 EU/mL, 5.39 EU/mL and 6.59 EU/mL, respectively.

3.2. *The effect of irisin with tag on the proliferation and differentiation of MC3T3-E1 cells*

Compared with the blank control group, cells cultured with irisin$^{N\text{-}His}$ or r-irisin were significantly more after 48 hours, while no difference was found between the two groups. irisin$^{C\text{-}His}$ had no effect on cell proliferation (Figure 1A).

MC-3T3 E1 cells were cultured with osteogenic medium. Three days later, irisin$^{N\text{-}His}$ and r-irisin increased the expression of ALP about 82% and 95% (1.82 ± 0.29 *vs*. 1.00 ± 0.10, 1.95 ± 0.25 *vs*. 1.00 ± 0.10, respectively). The expression of Col1α1 increased about 52% and 70% (1.52 ± 0.24 *vs*. 1.00 ± 0.12, 1.70 ± 0.20 *vs*. 1.00 ± 0.12). The expression of Runx2 increased about 64% and 77% (1.64 ± 0.13 *vs*. 1.00 ± 0.13, 1.77 ± 0.09 *vs*. 1.00 ± 0.13) (Figure 1B). After culture for 14 days, irisin$^{N\text{-}His}$ and r-irisin increased the expression of ALP about 140% and 157% (2.40 ± 0.44 *vs*. 1.00 ± 0.22, 2.57 ± 0.28 *vs*. 1.00 ± 0.22, respectively). The expression of Col1α1 increased about 253% and 273% (3.53 ± 0.40 *vs*. 1.00 ± 0.18, 3.73 ± 0.60 *vs*. 1.00 ± 0.18). The expression of Runx2 increased about 279% and 351% (3.79 ± 0.42 *vs*. 1.00 ± 0.25, 4.51 ± 0.49 *vs*. 1.00 ± 0.25). No significant differences of osteogenic-related markers were found between the irisin$^{C\text{-}His}$ group and the control (Figure 1C).

**Table 1. Primer pairs used for qPCR**

| Target gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| ALP | TGACCTTCTCTCCTCCATCC | CTTCCTGGGAGTCTCATCCT |
| Runx2 | CCGTGGCCTTCAAGGTTGT | TTCATAACAGCGGAGGCATTT |
| COL1α1 | GCTCCTCTTAGGGGCCACT | CCACGTCTCACCATTGGGG |
| GAPDH | TGCACCACCAACTGCTTAG | GGATGCAGGGATGATGTTC |

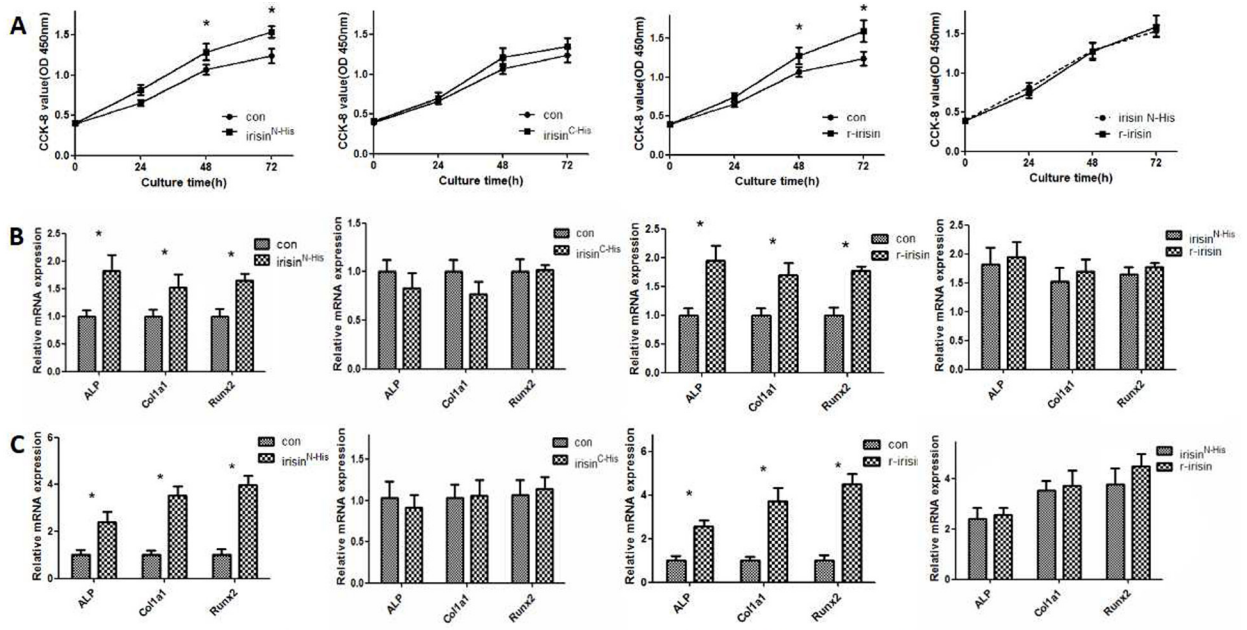


**Figure 1. Effects of irisin with tag on the proliferation and differentiation of MC3T3-E1 cells. (A)** At 0, 24, 48 and 72 hours of culture, cell counting kit (CCK-8) was applied to detect proliferation. **(B-C)** MC3T3-E1 cells were cultured with osteogenic medium and irisin with tags. After culture for 3 days **(B)** and 14 days **(C)**, the cells were harvested and used for real-time RT-PCR. The mRNA expression levels are shown relative to the values of the control.

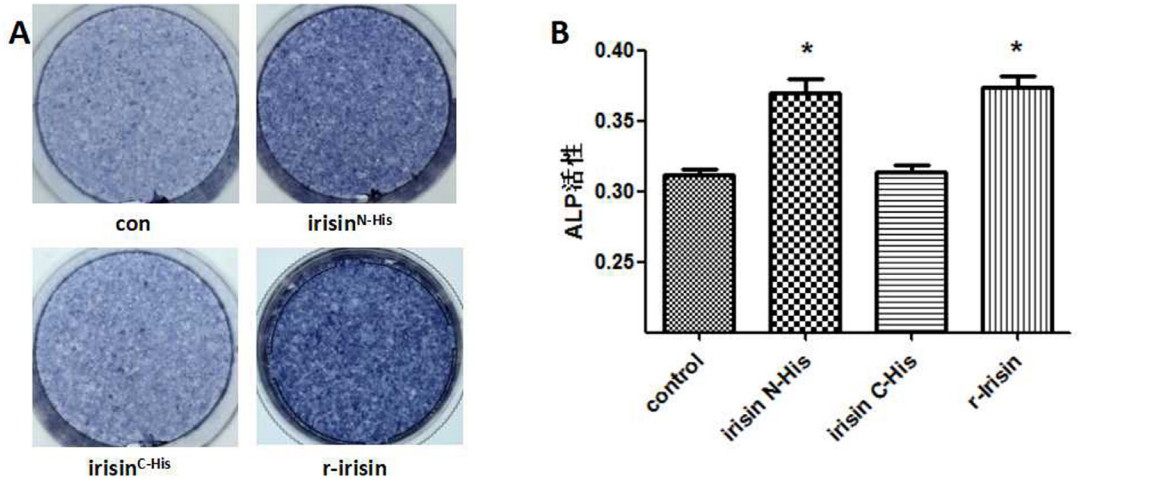


**Figure 2 Effects of irisin with tag on the osteoblastic potential of MC3T3-E1 cells.** After culture for 14 days, the cells were fixed and stained to recognize ALP following the instruction of BCIP/NBT alkaline phosphatase color development kit **(A)**. The cells were washed and collected to detect the ALP activity through alkaline phosphatase assay kit **(B)**.

**Table 2. The ALP activity of MC3T3-E1 cells cultured with irisin with tag for 14 days. Data are shown as mean ± SD**

| Items | Fold r-irisin (ALP activity) | *P* value |
|---|---|---|
| irisin$^{N\text{-}His}$ | 0.935 ± 0.257 | 0.658 |
| irisin$^{C\text{-}His}$ | 0.034 ± 0.134 | < 0.01 |
| r-irisin | 1.000 ± 0.210 | – |

As to ALP staining and ALP quantification, the expression of ALP in irisin$^{N\text{-}His}$ or r-irisin group was higher than that in the blank control group (Figure 2). After eliminating the initial differentiation result revealed by the control group, a similar ALP activity between irisin$^{N\text{-}His}$ and r-irisin group was found. The ALP activity of irisin$^{N\text{-}His}$ group was about 93.5% of that in the r-irisin group, and the ALP activity of irisin$^{C\text{-}His}$ group was about 3.4% of that in the r-irisin group (Table 2).

### 3.3. *The effect of irisin with point mutation on the proliferation and differentiation of MC3T3-E1 cells*

Compared with the blank control group, cells cultured with irisin E57K was significantly more after 48 hours, and irisin R75E group showed this effect after 72 hours. No significant difference was discovered among irisin E57K, irisin R75E and r-irisin. Irisin I107F had no

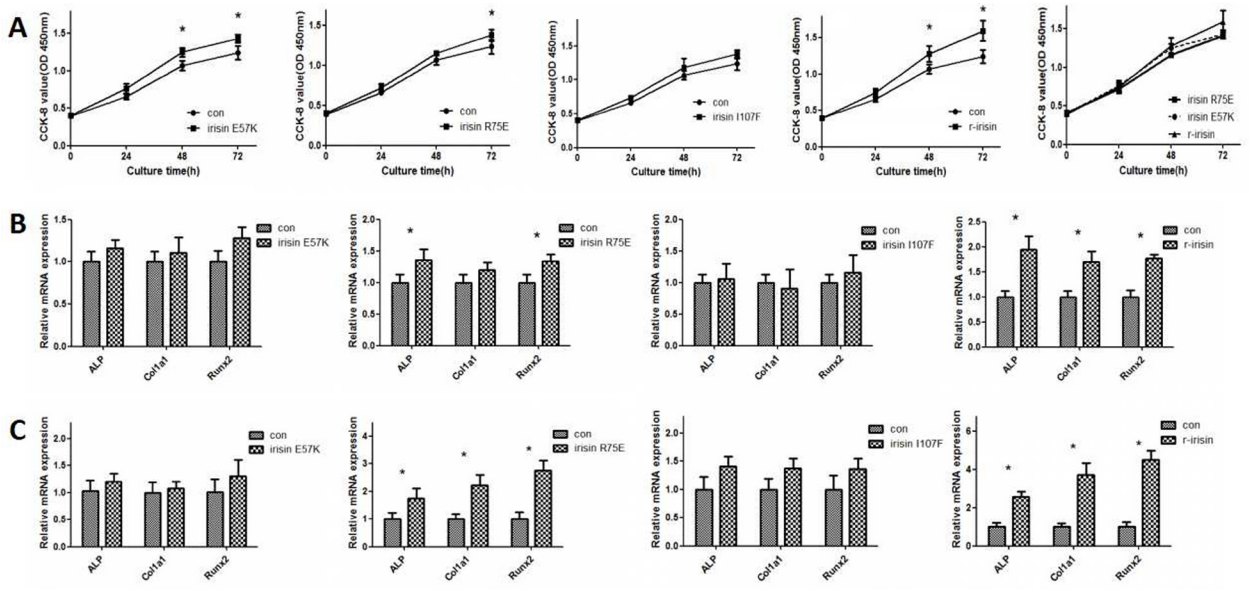


**Figure 3. Effects of irisin with point mutation on the proliferation and differentiation of MC3T3-E1 cells. (A)** At 0, 24, 48 and 72 hours of culture, cell counting kit (CCK-8) was applied to detect the proliferation. **(B-C)** MC3T3-E1 cells were cultured with osteogenic medium and irisin with point mutation. After culture for 3 days **(B)** and 14 days **(C)**, the cells were harvested and used for real-time RT-PCR. The mRNA expression levels are shown relative to the values of the control.

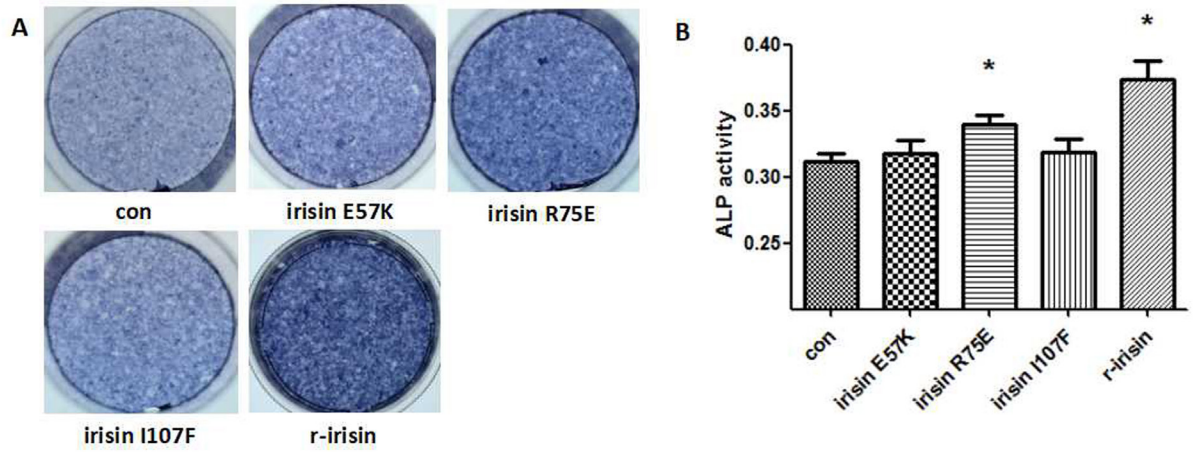


**Figure 4. Effects of irisin with point mutation on the osteoblastic potential of MC3T3-E1 cells.** After culture for 14 days, the cells were fixed and stained to recognize ALP following the instruction of BCIP/NBT alkaline phosphatase color development kit **(A)**. The cells were washed and collected to detect the ALP activity through alkaline phosphatase assay kit **(B)**.

effect on cell proliferation (Figure 3A).

When culturing in osteogenic medium, the mRNA expression of ALP and Runx2 were elevated by irisin R75E after 3 days (1.36 ± 0.16 *vs*. 1.00 ± 0.10, 1.34 ± 0.11 *vs*. 1.00 ± 0.13) (Figure 3B). The expression of ALP, Col1α1 and Runx2 were about 74%, 122% and 175% more than the control, respectively (1.74 ± 0.37 *vs*. 1.00 ± 0.22, 2.22 ± 0.38 *vs*. 1.00 ± 0.18, 2.75 ± 0.37 *vs*. 1.00 ± 0.25), after culturing for 14 days. No significant differences of osteogenic-related markers were found among irisin E57K, irisin I107F group and blank-control group (Figure 3C).

The ALP staining showed a higher expression of ALP in irisin R75E group, but less than the r-irisin group. While such elevation was not observed in irisin I107F or irisin E57K group (Figure 4). When the initial differentiation result, which was revealed by the blank control group, was eliminated, these three groups all revealed decreased ALP activity than the r-irisin group. The ALP activity of irisin R75E group was about 45.0% of that in the r-irisin group. The numbers were 9.9% and 11.2% for the irisin E57K group and irisin I107F group, respectively (Table 3).

**Table 3. The ALP activity of MC3T3-E1 cells cultured with irisin with point mutation for 14 days. Data are shown as mean ± SD**

| Items | Fold r-irisin (ALP activity) | *P* value |
|---|---|---|
| irisin E57K | 0.099 ± 0.152 | < 0.01 |
| irisin R75E | 0.450 ± 0.113 | < 0.05 |
| irisin I107F | 0.112 ± 0.160 | < 0.01 |
| r-irisin | 1.000 ± 0.210 | – |

## 4. Discussion

In this study, we conducted 6XHis-tag or point mutation on irisin and detected their influence on the proliferation and differentiation of MC3T3-E1 cells. We found that irisin$^{N\text{-}His}$, irisin E57K and irisin R75E could boost cell proliferation. irisin$^{N\text{-}His}$ and irisin R75E had a positive effect on cell differentiation, and irisin$^{N\text{-}His}$ had the most similar effect as wild type irisin, while irisin R75E had a weaker effect than r-irisin.

Irisin has two glycosyl sites which make irisin a glycoprotein. In order to exclude the interference of glycosylation, prokaryotic cell *E. coli* was chosen to synthesize irisin in this study. Mineralized nodule could be observed in MC3T3-E1 cells after culture in induced medium for 14 days (*16*). So we detected the expression of Runx2, Col1α1 and ALP at the 3$^{rd}$ and 14$^{th}$ day of induced differentiation.

Our experiment showed that the N-terminal his-tag did not influence the effect of irisin on promoting the proliferation and differentiation of osteoblasts, but when the His-tag was on the C terminal, the protein almost completely lost its function. This suggested that the C-terminal might play a critical role. Another study found that His-tag on the C-terminal did not influence the effect of irisin on increasing oxygen consumption of cadiomyoblasts (*17*). The different cell might partly explain the difference. It needs to be mentioned that irisin$^{N\text{-}His}$ used in this study contains 18 amino acid residues before the irisin sequence, and they are MGSHHHHHHSSGLVPRGS; and irisin$^{C\text{-}His}$ has 8 residues (LEHHHHHH) following the C-terminal.

Structure analysis by crystallization suggests that irisin is composed of a β-sandwich with three β-strands on one side and four on the other, which is shown below: βA(35-43) -βB(46-52) -βC(60-69) -βC'(73-81) -βE(85-91) -βF(95-106) -βG(108-116). The flexible loops (residues 55-58 and 106-108) are considered as possible candidates to interact with other proteins (*14*). So the 57$^{th}$ and 107$^{th}$ amino acids were chosen for point mutations. We replaced the acidic glutamic, the 57$^{th}$ amino acid located between ligand βB and βC, with a basic lysine. The 107$^{th}$ amino acid, isoleucine, was replaced by a larger amino acid, phenylalanine. Our results showed that the irisin I107F did not influence the proliferation and differentiation of osteoblast cells. The irisin E57K had a significant effect on cell proliferation but failed to influence cell differentiation. This proved that residues 106-108 and 55-58 are very critical for irisin, and the pathways involved in cell proliferation and differentiation activated by irisin might be different.

Schumacher *et al*. reported that the salt bridges between Arg-75 and Glu-79 were important for maintaining irisin dimers (*14*). We used the glutamine instead of Arg-75 to interfere with formation of the salt bridge, and found that the osteogenic activity of the protein still exists but is weaker. This indicated that the salt bridge also contributes to the protein activity.

The endotoxin mixed in protein product from *E. coli* could induce inflammation and influence cell activity (*18-20*). The concentration of endotoxin in every protein sample was below the minimum concentration reported to exert an effect on osteoblasts (*21*). However, the candidate sites chosen to be changed were limited in this study and there is still a problem about identifying the receptor for irisin. Future research focusing on these might be helpful in biochemical or pharmacological exploration.

In conclusion, this research reported the influence of 6XHis-tag and point mutants of irisin on the proliferation and differentiation of osteoblasts. The flexible region of residues 55-58 and 106-108, and C-terminal of irisin are vital for its activity, and disrupting the dimerization of irisin might result in a reduced effect.

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## References

1. Schon HT, Weiskirchen R. Exercise-Induced Release of Pharmacologically Active Substances and Their Relevance for Therapy of Hepatic Injury. Front Pharmacol. 2016; 7:283.
2. Erickson HP. Irisin and FNDC5 in retrospect: An exercise hormone or a transmembrane receptor? Adipocyte. 2013; 2:289.
3. Chen N, Li Q, Liu J, Jia S. Irisin, an exercise-induced myokine as a metabolic regulator: An updated narrative review. Diabetes Metab Res Rev. 2016; 32:51-59.
4. Singhal V, Lawson EA, Ackerman KE, Fazeli PK, Clarke H, Lee H, Eddy K, Marengi DA, Derrico NP, Bouxsein ML, Misra M. Irisin levels are lower in young amenorrheic athletes compared with eumenorrheic athletes and non-athletes and are associated with bone density and strength estimates. PLoS One. 2014; 9:e100218.
5. Palermo A, Strollo R, Maddaloni E, Tuccinardi D, D'Onofrio L, Briganti SI, Defeudis G, De Pascalis M, Lazzaro MC, Colleluori G, Manfrini S, Pozzilli P, Napoli N. Irisin is associated with osteoporotic fractures independently of bone mineral density, body composition or daily physical activity. Clin Endocrinol (Oxf). 2015; 82:615-619.
6. Engin-Ustun Y, Caglayan EK, Gocmen AY, Polat MF. Postmenopausal Osteoporosis Is Associated with Serum Chemerin and Irisin but Not with Apolipoprotein M Levels. J Menopausal Med. 2016; 22:76-79.
7. Free patents online. IRISIN FOR CARE AND PREVENTION OF OSTEOPOROSIS. European Patent EP3081228. *http://www.freepatentsonline.com/EP3081228.html* (accessed September 19, 2018).
8. Colaianni G, Cuscito C, Mongelli T, Oranger A, Mori G,

Brunetti G, Colucci S, Cinti S, Grano M. Irisin enhances osteoblast differentiation in vitro. Int J Endocrinol. 2014; 2014:902186.
9. Graziana C, Concetta C, Teresa M, Paolo P, Cinzia B, Peng L, Ping L, Loris S, Mariasevera DC, Giorgio M. The myokine irisin increases cortical bone mass. Proc Natl Acad Sci U S A. 2015; 112:12157-12162.
10. Qiao X, Ying N, Ma Y, Yan C, Ran C, Yin W, Ying H, Xu W, Xu L. Corrigendum: Irisin promotes osteoblast proliferation and differentiation *via* activating the MAP kinase signaling pathways. Sci Rep. 2016; 6:18732.
11. Albrecht E, Norheim F, Thiede B, Holen T, Ohashi T, Schering L, Lee S, Brenmoehl J, Thomas S, Drevon C, Erickson H, Maak S. Irisin – A myth rather than an exercise-inducible myokine. Sci Rep. 2015; 5:8889.
12. Boström P, Wu J, Jedrychowski M P, *et al*. A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature, 2013; 481:463-468.
13. Jedrychowski MP, Wrann CD, Paulo JA, Gerber KK, Szpyt J, Robinson MM, Nair KS, Gygi SP, Spiegelman BM. Detection and Quantitation of Circulating Human Irisin by Tandem Mass Spectrometry. Cell Metab. 2015; 22:734.
14. Schumacher MA, Chinnam N, Ohashi T, Shah RS, Erickson HP. The Structure of Irisin Reveals a Novel Intersubunit β-Sheet Fibronectin Type III (FNIII) Dimer: IMPLICATIONS FOR RECEPTOR ACTIVATION*. J Biol Chem. 2013; 288:33738-33744.
15. Nie YW, Liu DJ. N-glycosylation is required for FDNC5 stabilization and irisin secretion. Biochem J. 2017; 474:3167.
16. Lei Z, Gui-ying X, Lan X, Wei X, Zhi J, Shi-liang W. Establishment of an osteoblast model by inducing MC3T3-E1 subclone 14 in vitro. Chin J Hemorh. 2012; 22:16-18.
17. Xie C, Zhang Y, Tran TDN, Wang H, Li S, George EV, Zhuang H, Zhang P, Kandel A, Lai Y. Irisin Controls Growth, Intracellular $Ca^{2+}$ Signals, and Mitochondrial Thermogenesis in Cardiomyoblasts. Plos One. 2015; 10:e0136816.
18. Bandow K, Maeda A, Kakimoto K, Kusuyama J, Shamoto M, Ohnishi T, Matsuguchi T. Molecular mechanisms of the inhibitory effect of lipopolysaccharide (LPS) on osteoblast differentiation. Biochem Biophys Res Commun. 2010; 402:755-761.
19. Nakao J, Fujii Y, Kusuyama J, Bandow K, Kakimoto K, Ohnishi T, Matsuguchi T. Low-intensity pulsed ultrasound (LIPUS) inhibits LPS-induced inflammatory responses of osteoblasts through TLR4-MyD88 dissociation. Bone. 2014; 58:17-25.
20. Wen-jv F, De-zhi Z, Xing Z, Yang G, Zhi-yang Y. Primary experimental study on the influence of LPS on osteoblast apoptosis in vitro. J Cell Mol Med. 2014; 24:8-12.
21. Guo C, Yuan L, Wang JG, Wang F, Yang XK, Zhang FH, Song JL, Ma XY, Cheng Q, Song GH. Lipopolysaccharide (LPS) induces the apoptosis and inhibits osteoblast differentiation through JNK pathway in MC3T3-E1 cells. Inflammation. 2014; 37:621.

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