In vitro effects of the myokine irisin on human adipose tissue-derived mesenchymal stem cells during proliferation and osteogenic differentiation

Cecilia Romagnoli [1], Roberto Zonefrati [2], Teresa Iantomasi [1], Maria Luisa Brandi [2]

[1] Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy
[2] F.I.R.M.O. Italian Foundation for the Research on Bone Diseases, Florence, Italy

ABSTRACT

Purpose: Irisin is a hormone-like molecule secreted from skeletal muscle in response to exercise both in mice and in humans and identified as an important effector in the crosstalk between muscle and bone. Although a number of studies report that irisin increased osteoblast differentiation in vitro and cortical bone mass *in vivo*, the models used are exclusively rodent ones. Due to the lack of reports on human cell models, the aim of our work was to investigate the *in vitro* effects of irisin on the proliferation and the osteogenic differentiation processes in human adipose tissue-derived mesenchymal stem cells (hAMSCs).

Methods: hAMSCs were obtained by enzymatic digestion and mechanical dispersion, and cultured in growth medium. Cells were exposed to 10 and 100 ng/ml irisin for the entire experimental period and refreshed every two days. The proliferation was performed in growth medium containing 2.5% fetal bovine serum, and measured by cell counting at 24-48-72 hours. Alkaline phosphatase (ALP) activity and Ca^{2+} depositions were quantified by fluorometric assay during up to 35 days of osteogenic induction.

Results: Cell proliferation assay showed that 100 ng/ml irisin significantly increased the proliferation process ($p<0.01$) vs control, with a decrease of cell doubling time from 88 to 63 hours. Osteodifferentiation with 10 and 100 ng/ml irisin showed significant increases in ALP activity vs control ($p<0.01$) after 14 days. Moreover, both tested concentrations of irisin were able to accelerate the deposition of mineralized matrix, resulting in significant increments in the production of Ca^{2+} nodules vs control after 35 days ($p<0.01$).

Conclusions: This work showed the *in vitro* effects of irisin on a human cell model of AMSCs. The preliminary results show this myokine to be an important effector on cell proliferation and during osteo-differentiation of hAMSCs, supporting the hypothesis that irisin could represent a potent new anabolic treatment to bring about gain of bone mass.

KEYWORDS

Irisin, osteogenesis, human adipose tissue-derived mesenchymal stem cells, osteoblast differentiation.

Introduction

Irisin, named after the Greek messenger goddess Iris, is a muscle tissue-secreted peptide discovered in 2012, resulting from proteolytic cleavage of fibronectin type III "domain-containing" protein 5 (FDNC5), a type III transmembrane protein highly expressed in skeletal muscle cells [1,2]. Although immunohistochemical studies have shown that irisin is found in the pancreas, liver, and stomach, it is mainly secreted from skeletal muscle [3]. Following acute bouts of exercise, FDNC5 expression is affected by regulation of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), a multispecific coactivator of transcription, which is responsible for multiple gene regulation in response to nutritional and physiological signals in tissues, and subsequently cleaved to irisin, released into the circulation [4].

Irisin was first reported by Boström *et al*. as a hormone that induces thermogenesis by acting on white adipose tissue, stimulating uncoupling protein I expression and activating the browning response [5]. More recent studies have shown that irisin has a powerful capacity to modulate bone renewal, acting as a key factor of muscle-bone crosstalk during exercise [6,7]. In fact, alongside the well-known knowledge that the mechanical loading derived from skeletal muscle primarily modulates the development and maintenance of bone mass [8], in the last decade, accumulating evidence has revealed that communication between muscle and bone also occurs in a paracrine or endocrine fashion via myokines released by muscle, and irisin is one of them [9].

Initial studies on bone have demonstrated that irisin stim-

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Contact
Maria Luisa Brandi; marialuisa.brandi@unifi.it; marialuisa@marialuisabrandi.it
F.I.R.M.O. Italian Foundation for the Research on Bone Diseases,
Via San Gallo 123, 50129 Florence, Italy
Phone: +39 055 2336663

ulates the *in vitro* osteoblast differentiation of murine bone marrow stem cells [10], and promotes proliferation and osteoinduction via the mitogen-activated protein kinase (MAPK) signaling pathway [11] and by activating the bone morphogenetic protein (BMP)/small mothers against decapentaplegic (SMAD) pathway [12,13]. Moreover, it has been shown that irisin administration to young male mice at a weekly dose of 100 µg/kg for 28 days is able to lead to a significant increase in cortical bone mass and strength, as well as cortical tissue mineral density, periosteal circumference, and bending strength [14], and prevents unloading-induced bone loss *in vivo* [15].

Although a number of reports highlight the capacity of irisin to promote bone regeneration, the models used are exclusively rodent ones. Due to the very limited availability of studies on human models, the purpose of this work was to investigate the *in vitro* effects of the myokine irisin on the proliferation and the osteogenic differentiation processes in a clonal cell line of human adipose tissue-derived mesenchymal stem cells (hAMSCs).

Materials and methods

hAMSC cultures

An hAMSC line was isolated from a small fragment of human subcutaneous adipose tissue in Prof. Brandi's lab before the 2000s. The biopsy was obtained during general surgery performed at AOU-Careggi, Firenze (Italy), and sent, completely anonymously, to the laboratory. Culture conditions, cell cloning, soft agar assay, and phenotype characterization were carried out as previously described [16].

Treatment with irisin

Different concentrations of irisin (Sigma-Aldrich, Saint Luis, MO, USA, #SRP6284) were used in the experiments in order to evaluate the effects of the myokine on hAMSC proliferation and osteogenic differentiation. All the experiments were performed using 10 and 100 ng/ml irisin, added to the media.

Proliferation analysis, alkaline phosphatase (ALP) assay, and mineralization of hAMSCs

Proliferation analysis, ALP assay, and mineralization of hAMSCs were performed as previously described [17].

Statistical analysis

For proliferation analysis, statistical processing was performed during the "log phase" of the growth curves using the linearity test and the parallelism test of the linear regressions of the obtained growth curves. For assays of ALP and mineralized calcium deposits the experiments were carried out in quadruplicate and each experiment repeated three times. All data were expressed as means ± SD and statistical significance was determined by one-way ANOVA analysis with Bonferroni's multiple comparison test, using GraphPad Prism Software (San Diego, CA, USA). $P \leq 0.05$ was considered statistically significant.

Results

Characterization of the hAMSC-L2 finite clonal line

The plastic-adherent hAMSC population derived from human adipose tissue was cloned at passage 3. Teen clones were obtained and, among them, clone hAMSC-L2 was randomly selected for subsequent characterization.

The hAMSC-L2 cell line showed an elongated and fusiform, fibroblast-like shape, with a great number of cellular processes (Figure 1A). The hAMSC-L2 line did not show growth in soft agar after 4 weeks in culture, demonstrating no malignant transformation in cells (Figure 1B).

Afterwards, in order to verify their multipotency, both of the cell lines, hAMSCs and hAMSC-L2, were differentiated toward adipogenic, chondrogenic, and osteogenic phenotypes.

hAMSC-L2 induction with adipogenic medium (AM) for 21 days resulted in an expanded cell morphology and the accumulation of multiple intracellular lipid-filled droplets in a significant fraction of the cells (Figure 2A).

Figure 1 Representative images of the hAMSC-L2 clonal cell line grown on a dish (A) and on soft agar (B). Phase contrast microscopy. Objective 10x, scale bar 200 µm.

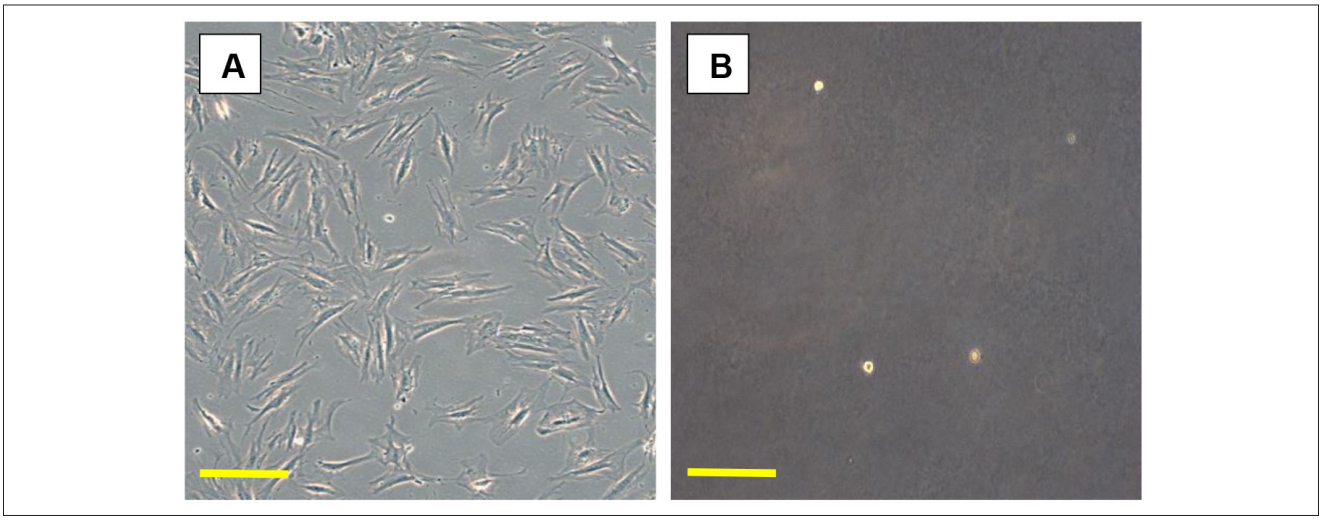
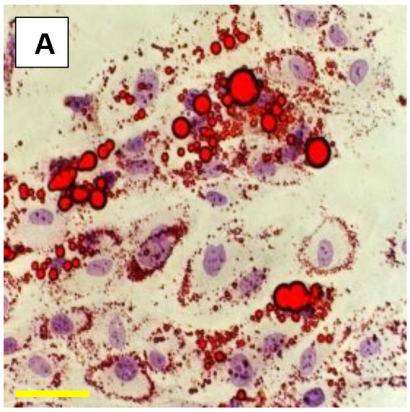
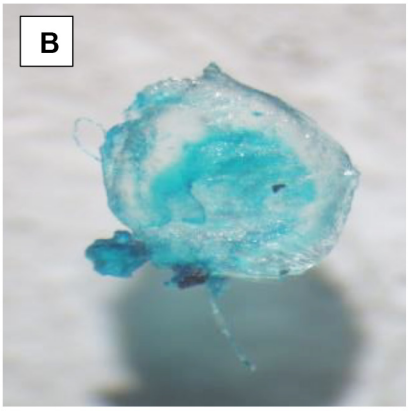


Figure 2 Multipotentiality of hAMSC-L2 clonal cell line.

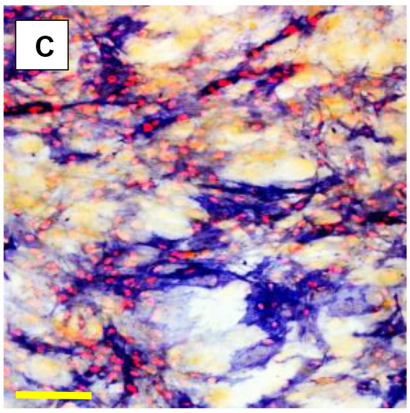
(A) Representative image of Oil Red O staining of hAMSC-L2 after 21 days of adipogenic induction; the intracellular droplets of lipids are stained in red, and nuclei counterstained with Mayer's acid hemalum in blue. Images acquired in brightfield microscopy. Objective 40x, scale bar 50 µm.

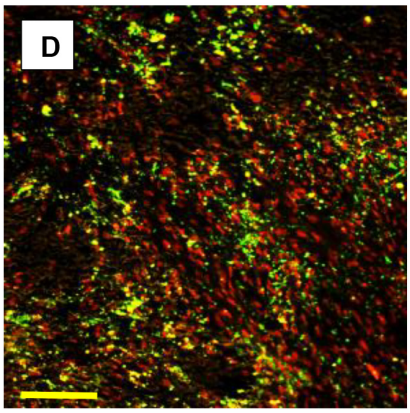




(B) Representative image of Alcian Blue staining of 3D high density pellet of hAMSC-L2 after 21 days of chondrogenic induction; stereomicroscopy image with original magnification 16x.

(C) Representative image of Fast Red Violet BB staining after 14 days of osteogenic induction; ALP+ cells are stained in blue and nuclei counterstained with ethidium iodide in conventional red. Composite image acquired in brightfield and epifluorescence microscopy. Objective 10x, scale bar 200 µm.





(D) Representative image of calcein staining after 28 days in OM; mineralized calcium deposits are stained in fluorescent green and nuclei counterstained with ethidium iodide in conventional red color. Image acquired in epifluorescence microscopy. Objective 10x, scale bar 200 µm.

Chondrogenic differentiation of hAMSC-L2 was observed after 21 days in chondrogenic medium (CM) with the formation of a 3D high density pellet, which was found to be positive to Alcian Blue, highlighting the presence of glycoproteins, which are characteristic of hyaline cartilaginous tissue (Figure 2B).

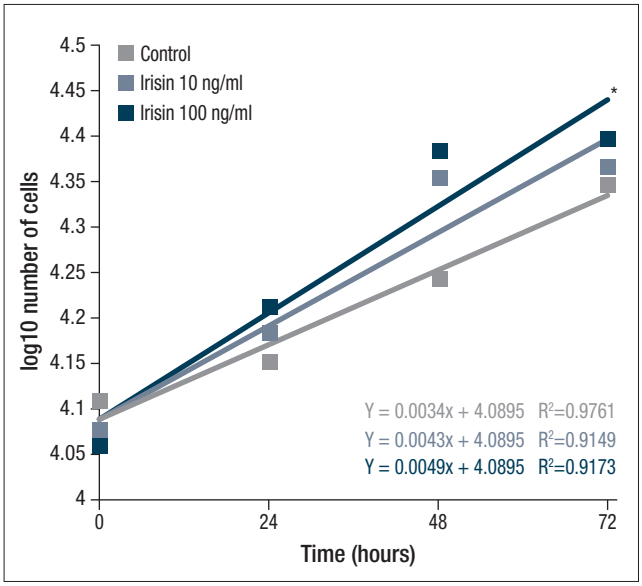
Osteogenic induction of the hAMSC-L2 line was assessed with osteogenic medium (OM) up to 21 days and observed during the entire period of study, monitoring the ALP expression and the production of mineralized calcium deposits. Specifically, after 14 days, cells were almost all strongly positive for ALP, with only a few, scattered, negative cells (Figure 2C). Osteoblast activity was shown by the production of an osteoid matrix by hAMSC-L2 cell line cultured in OM, obtaining deposition of a mineralized matrix at 28 days after induction, in a time-dependent manner. The area of matrix formation coincided with that of calcein staining (Figure 2D). The same behavior, using AM, CM, or OM, was confirmed in the heterogeneous hAMSC population before cell cloning (data not shown).

Proliferation process of hAMSC-L2 in the presence of irisin

We evaluated the effects of the myokine irisin at the concentrations of 10 and 100 ng/ml on the proliferation process of the hAMSC-L2 finite clonal cell line.

Figure 3 shows a significant increase in proliferation when hAMSC-L2 cells were treated with irisin at the concentration of 100 ng/ml, with respect to the control group (*p≤0.01), and with a decrease in the cell doubling time from 88 to 63 hours. On the other hand, no significant results were obtained using the lower concentration of irisin, 10 ng/ml, although the trend was the same with the duplication time reduced from 88 to 70 hours (Figure 3).

Figure 3 Graphical representation in linear regression of the kinetics of growth for the hAMSC-L2 line cultured in Coon's medium + FBS 2.5% in the presence of irisin at the concentrations of 10 and 100 ng/ml. The equation and the R^2 value are reported for each straight line. Values are the means of triplicate. *p≤0.01 versus untreated control group.



ALP enzymatic activity in hAMSC-L2 treated with irisin

Spectrofluorometric assay was performed in hAMSC-L2 cultivated in OM and treated or not treated with irisin at the concentrations of 10 and 100 ng/ml, in order to assess ALP activity at specific experimental times, from 0 to 35 days of osteoinduction.

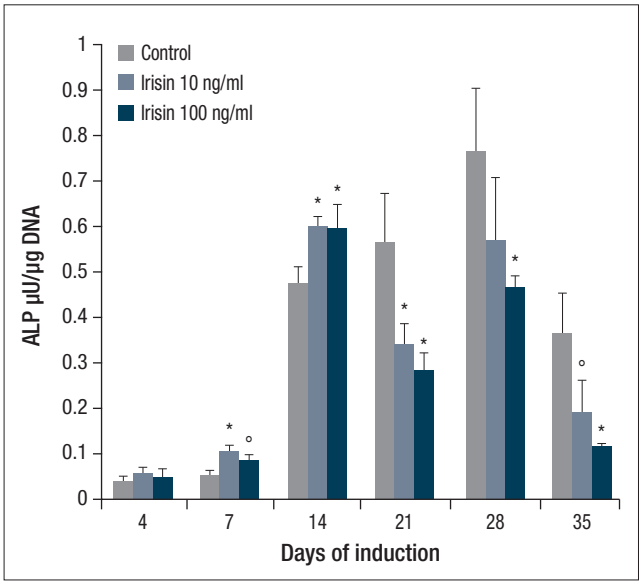
The results showed that, compared with the untreated control, irisin at both of the tested concentrations was able to stimulate ALP, leading to significant increases in the enzymatic activity of hAMSC-L2 after 7 and 14 days of osteogenic induction (Figure 4). Thereafter, after 21 days, ALP values measured in the presence of irisin were significantly lower than those detected in untreated cells, in which ALP continues to grow up to 28 days after induction, before decreasing.

Mineralized calcium deposits in hAMSC-L2 treated with irisin

The formation of mineralized extracellular matrix was assessed by spectofluorometric assay, quantifying the production of mineralized calcium deposits in hAMSC-L2, cultured or not cultured with irisin 10 or 100 ng/ml, at different experimental time points, from 0 to 35 days of osteoinduction.

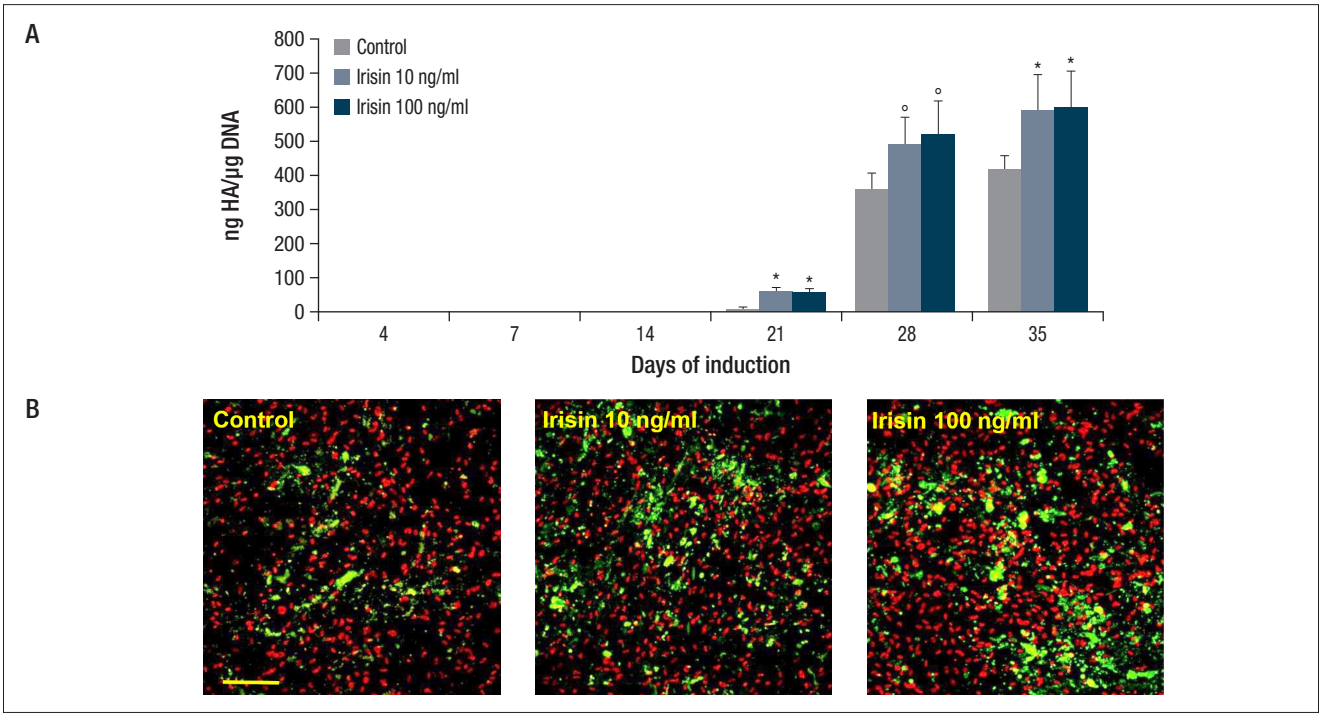
Figure 5A shows that the calcium content increased in a time-dependent manner in hAMSC-L2 cultured in OM, and both of the tested concentrations of the myokine (10 and 100 ng/ml) caused significant increases in the production of mineralized calcium nodules starting from 21 days of osteogenic induction, reaching a maximum after 35 days, compared with the untreated control. This indicates that irisin is able to promote the deposition of an abundant mineralized matrix.

Figure 4 Quantitative analysis of ALP enzyme activity in hAMSC-L2 cultured in OM, and treated or not treated with irisin, from 4 to 35 days of osteoinduction. ALP was evaluated by spectrofluorimetric assay and the values are the mean ± SD of three independent experiments. *p <0.01 and °p<0.05 compared to the respective untreated control.



Quantitative data reporting mineralization and deposition of calcium nodules of hAMSC-L2, after 28 days of osteoinduction in the presence of irisin treatments, were supported by epifluorescence microscopy observations. As shown in figure 5B, Ca^{2+} mineral deposits were increased in samples treated with 10 and 100 ng/ml of irisin concentrations, with respect to the control group treated in OM.

Figure 5 Quantitative analysis of the production of mineralized calcium nodules in hAMSC-L2 cultured in OM, and treated or not treated with irisin, from 4 to 35 days of osteoinduction. Mineralization was evaluated by spectrofluorimetric assay and the values are at the mean ± SD of three independent experiments. *p <0.01 and °p<0.05 compared with the respective untreated control (**A**). Representative fluorescence observations of calcium nodules produced by hAMSC-L2 after 28 days of osteogenic induction in the presence of different concentrations of irisin. Calcium nodules are stained in green with calcein and nuclei are stained in red with ethidium bromide. Objective 10x, scale bar 200 µm (**B**).



Discussion

Muscle and bone are two individual compartments that are anatomically and biochemically related to each other and constitute a single functional musculoskeletal unit [18]. The importance of skeletal muscle as an endocrine tissue able to produce and secrete, after contraction, a myriad of cytokines, called myokines, has made it possible to explain and understand in depth its close relationship with bone [7,9].

The identification of irisin as a key messenger in the crosstalk between muscle and bone, able to promote bone regeneration, makes this myokine attractive from a therapeutic perspective, for those conditions where there is a loss of bone [3].

Even though the literature contains extensive evidence of the ability of irisin to promote bone formation, acting on osteoblast differentiation, studies in the literature are performed exclusively on animal models, both *in vitro* and *in vivo* [10-12,14,15,19,20].

For that reason, the purpose of our research was to investigate the effect of the myokine irisin on human cell models. As far as we know, this is the first report of the use of hAMSCs to understand more about the influence of irisin on *in vitro* proliferation and osteodifferentiation in human cells.

In the present work, we isolated and characterized a single cell-derived clone of hAMSCs, called hAMSC-L2, to investigate the potential of a homogeneous population of MSCs, and highlight their major phenotypic traits, in order to obtain more coherent results with respect to the use of a heterogeneous subset of cells obtained from adipose tissue, as discussed by Guilak *et al*. [21].

Our results on the proliferation process show that irisin, at the higher concentration of 100 ng/ml, is able to significantly promote the proliferative activity of hAMSCs, decreasing the doubling time from 88 hours to 63 hours. Our data are in agreement with *in vitro* reports showing a significant proliferative effect of irisin in primary rat and mouse osteoblast cell lines at the same concentration of 100 ng/ml, that is close to the physiological concentration in human serum [11].

In addition, our results are in line with the findings of a previously published paper on human periodontal ligament cells, in which *in vitro* stimulation, using both 10 and 100 ng/ml of irisin, caused a great increase in cell proliferation[22]. However, in our experiments, irisin at 10 ng/ml was not able to determine a significant increase in cell replication rate, although the trend was the same as that observed with the 100 ng/ml concentration, with a reduction of the duplication time from 88 to 70 hours. On the other hand, a report by Chen *et al*. showed that only a higher concentration of irisin treatment (10 nM) induced a significant increase in the proliferation rate of primary osteoblasts from mice, while the lowest tested concentrations (1 and 5 nM) had no significant effects on osteoblast proliferation [20]. Also, this is in agreement with a study by Xue *et al*. in which concentrations of irisin from 0.1 to 20 ng/ml did not affect murine bone mesenchymal stromal cell (BMSCs) [12].

Regarding osteoblast differentiation, we found evidence that both the tested concentrations of irisin (10 and 100 ng/ml) were able to accelerate and promote osteoblast induction and differentiation, increasing ALP enzyme activity and the deposition of a mineralized extracellular matrix in our cell model.

In particular, our specific findings are in agreement with previous results in which mouse BMSCs, induced to differentiate into osteoblasts, showed significant increases in ALP expression with an irisin-dependent mechanism, highlighted by the use of an antibody direct against irisin [10]. Moreover, treatments with irisin 100 ng/ml led to significant gains in ALP gene and protein expressions and also mineralization in primary rat osteoblasts and a murine pre-osteoblastic cell line, MC3T3-E1, compared with untreated cells used as control [11]. A murine study by Colaianni *et al*. showed that incubation with irisin at the concentration of 100 ng/ml significantly increased the number of ALP-positive cells and mineralized colonies, as well as the expression of early differentiation genes, including ALP and collagen type I alpha 1 [14]. A positive effect on the ALP enzyme and on the formation of mineralized nodules after osteogenic induction was also found in BMSCs treated with irisin at the concentration of 5 ng/ml [12]. Conversely, another study performed in hPDL cells stimulated with irisin 10 and 100 ng/ml did not show any significant variations in ALP activity, with respect to the control without irisin [22]. Nevertheless, the authors reported significant increases in mineralization, evaluated with Alizarin Red staining, for both irisin concentrations when compared with control at 21 days [22].

Conclusions

Our research has highlighted, for the first time, the importance of using human *in vitro* cell models to investigate the effects of the myokine irisin, and hAMSCs were found to be an excellent tool for this type of research.

The irisin-induced proliferative and osteogenic effects suggest that irisin may be used as a possible treatment target for osteopenia and osteoporosis in the future. However, further studies will be required to identify more concentrations of irisin that are active on this type of human cell model and the signaling pathways involved, in order to tightly balance bone remodelling, and hypothesize a feasible strategy for hard tissue regeneration.

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Conflict of Interest: *The authors declare that there is no conflict of interest regarding the publication of this paper.*