Irisin promotes growth, migration and matrix formation in human periodontal ligament cells

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Abbreviations: Primary human periodontal ligament cells (hPDLCs), primary human osteoblasts (hOBs), vascular endothelial growth factor A (VEGFA), tumor necrosis factor alpha (TNFα)

Abstract

Objective: The objective of the study was to examine the effect of irisin on human periodontal ligament cells (hPDLCs) growth, migration and osteogenic behaviour *in vitro*.

Materials and Methods: Primary hPDLCs and human osteoblasts (hOBs), used as positive controls, were cultured with irisin (10 and 100 ng/ml), and effect on cell proliferation was evaluated with 5-bromo-2`-deoxyuridine incorporation at 1, 2, and 3 days, and on migration capacity was investigated by scratch assay at 2, 6, and 24 hours. Osteogenic behaviour was assessed with alkaline phosphatase activity, immunoassay at 3, 7, 14, and 21 days, and confocal laser scanning microscopy at 21 days. Mineralization was examined by Alizarin red staining at 21 days. Data were compared group wise using ANOVA tests.

Results: Irisin induced increased proliferation of primary hPDLCs and hOBs at all time points compared to untreated controls. This was confirmed by scratch assay where irisin enhanced migration of both hPDLCs and hOBs after 6 and 24 hours compared to controls. Irisin treatment promoted osteogenic behaviour of both cell types by enhancement of extracellular matrix formation. In hPDLCs irisin increased expression of type I collagen, secretion of osteoblastogenesis related proteins osteocalcin and leptin, and calcium deposition/mineralization compared to controls at 21 days. In addition, to enhance calcium deposition/mineralization in hOBs, irisin increased expression of periostin, and secretion of osteoblastogenesis related proteins osteopontin, alkaline phosphatase and osteocalcin, as compared to controls at 21 days.

Conclusions: Primary hPDLCs responded to irisin treatment with enhanced cell growth, migration, and matrix formation *in vitro*.

Keywords: Irisin, periodontal ligament, proliferation, migration, matrix formation, bone tissue engineering

Introduction

Periodontitis is defined by robust inflammation and destruction of tooth root surrounding tissues made up of bone, cementum and attachment apparatus. The ultimate goal of periodontal regeneration treatment is to regenerate lost periodontal tissues. To regenerate this elaborate tissue, several cells including cementoblasts, osteoblasts, fibroblasts, epithelial cells and periodontal ligament cells (PDLCs) are needed. Among them, PDLCs are assumed to perform an essential role in the maintenance, repair, and regeneration of the tissues that constitute the tissue attachment apparatus (Melcher 1976). Furthermore, it has been demonstrated that approximately 30 % of a population of PDLCs possess replicative and multipotency potential (Nagatomo et al., 2006), and thus could be steered to differentiate into cells with osteoblast- and cementoblast-like properties (Basdra & Komposch 1997, Ivanovski, Li, Haase, & Bartold 2001, Nagatomo et al., 2006, Gay, Chen, & MacDougall 2007). Thus, PDLCs are presumed to differentiate into not only fibroblasts but also hard tissue forming cells, such as osteoblasts or cementoblasts.

Agents stimulating proliferation and differentiation of PDLCs into osteoblast-like cells are of interest for periodontal tissue regeneration. Such agents could lead to enhanced tissue regeneration by guiding the PDLC population to produce lost bone tissue. Regarding the differentiation of PDLCs into osteoblast-like cells, the use of irisin as a stimulating agent could be of interest. Irisin is a circulating hormone-like myokine, which is secreted as a product of fibronectin type III domain-containing protein 5 from skeletal muscle in response to exercise (Boström et al., 2012). It has a significant role in the metabolic processes, and can inhibit the development of neurodegenerative diseases, type II diabetes, obesity and insulin resistance (Chen, Li, Liu, & Jia 2016). Besides metabolic potential, irisin plays an important role in bone remodelling via αV/β5 integrin receptors (Kim et al., 2018). It has been shown that very low dose irisin injections, given intermittently, improve bone mineral density and strength in mice

(Colaianni et al., 2015, Colaianni et al., 2017). These effects are consistent with *in vitro* studies showing that irisin can enhance osteoblast proliferation (Qiao et al., 2016) and differentiation (Colaianni et al., 2014, Qiao et al., 2016). In addition to induce enhancement of bone formation, irisin has been found to increase angiogenesis (Wu et al., 2015, Fu et al., 2016), which is critical in achieving successful tissue regeneration.

Based on the osteogenic activity induced by irisin, the use of this molecule in periodontal tissue regeneration would be reasonable. To our knowledge, this is the first study that has examined the effect of irisin on human PDLC (hPDLC) behaviour. Thus, the aim of the study was to investigate the effect of irisin on hPDLC growth, migration and osteogenic behaviour *in vitro*.

Materials and methods

Preparation of irisin

Irisin was purchased from Adipogen (Liestal, Switzerland). One vial containing 10 µg of irisin was delivered in a freeze-dried pellet form and dissolved to a stock solution of 100 µg/ml in milliQ water. Aliquots to avoid repeated freeze-thaw cycles were prepared and stored at -20 °C until use.

Cell culture

Human periodontal ligament fibroblasts (hPDLCs) (Cambrex Bio Science, Walkersville, MD, USA) from two male donors (ages 16 and 20 years) were cultured in stromal cell basal medium supplemented with 0.1 % human fibroblastic growth factor B, 0.1 % insulin, 5 % fetal bovine serum, 0.1 % gentamicin sulphate and amphotericin-B (Lonza, Walkersville, MD, USA) in 75 cm^2 culture flasks at 37°C in a humidified atmosphere of 5% CO_2. At the time of experimental setup, the hPDLCs had reached passages 6 and 7, respectively.

Human osteoblasts (hOBs) (Cambrex Bio Science, Walkersville, MD, USA) from two male donors (ages 13 and 32 years), used as positive controls, were cultured in osteoblast culture medium supplemented with 10% foetal bovine serum, 0.1% gentamicin sulfate, amphotericin-B and ascorbic acid (Lonza, Walkersville, MD, USA) in 75 cm^2 culture flasks at 37˚C in a humidified atmosphere of 5% CO_2. At the time of experimental setup, the hOBs had reached passage 7.

To test the effect of irisin on cell viability and osteogenic capacity, hPDLCs and hOBs were seeded in 12-well tissue culture plates at a density of 3.5×10^3 cells/cm^2 and 5×10^3 cells/cm^2, respectively. After 72 h of incubation with regular medium, hPDLCs and hOBs were treated

with either 10 ng/ml irisin or 100 ng/ml irisin. The control groups consisted of hPDLCs and hOBs cultured without irisin. The hPDLCs and hOBs were maintained at 37°C in a humidified atmosphere of 5% CO_2 for up to 21 days. The culture media both with or without irisin was replaced every other day and collected for cell viability and osteogenic capacity analyses.

Cell cytotoxicity assay

The lactate dehydrogenase activity was evaluated in the culture medium collected at days 3, 7, 14 and 21 with a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. In short, a quantity of 50 µl of sample was added to 50 µl of the kit reaction mixture, and incubated for 30 min in the dark at room temperature. The absorbance of the samples was measured at 492 nm in a plate reader (Biochrom Asys Expert 96 Microplate Reader; Biochrom, Holliston, MA, USA).

The effect of irisin on cell growth and migration were evaluated by cell proliferation and wound-healing assays.

Cell proliferation assay

Cell proliferation of hPDLCs and hOBs was measured using the cell proliferation enzyme-linked immunosorbent assay kit with 5-bromo-2`-deoxyuridine (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The hPDLCs together with the hOBs were cultured in 96-well tissue culture plates at a density of $5 \times 10^3$ cells/well. 5-bromo-2`-deoxyuridine was added as a pulse at a final concentration of 10 µM 5-bromo-2`-deoxyuridine to the hPDLCs 2 hours before and to the hOBs 6 hours before the harvest at day 1, 2 and 3. The amount of incorporated 5-bromo-2`-deoxyuridine was measured at 450 nm in a plate reader (Biochrom Asys Expert 96 Microplate Reader; Biochrom, Holliston, MA, USA).

Cell migration assay

The migration capacity of hPDLCs and hOBs was evaluated in a wound-healing assay. The hPDLCs and hOBs were seeded in 12-well tissue culture plates at a density of 3.5×10^3 cells/ cm^2 and 5 x 10^3 cells/cm^2, respectively. Upon 80 % - 90 % confluence, two horizontal lines in a form of a cross were created by scraping cells with a sterile disposable 100-µl pipette tip to make a scratch wound. The cells were rinsed in phosphate-buffered saline and incubated either with cell culture medium only or with irisin (10 ng/ml and 100 ng/ml, respectively). Images of the scratches were taken at time points 0, and after 2, 6 and 24 h of incubation, using a light microscope (Olympus IX70, Tokyo, Japan) with 4-x magnification. The wound healing or closing of the scratches in defined surface areas after 2, 6 and 24 h were compared with the individual areas at time point 0 and the wound closure was calculated using Fiji software (NIH, Bethesda, MD, USA).

Osteogenic behaviour

Osteogenic behaviour of cells was evaluated by alkaline phosphatase activity assay, immunoassay, Alizarin red staining and immunocytochemistry.

Alkaline phosphatase activity assay

The ability of alkaline phosphatase to hydrolyze P-nitrophenyl phosphate substrates (Sigma-Aldrich, St. Louis, MO, USA) into the yellow end-product, p-nitrophenol, was used to quantify the alkaline phosphatase activity in the medium after 3, 7, 14 and 21 days of culture. A quantity of 25 µl of sample was incubated with 100 µl P-nitrophenyl phosphate for 30 min in the dark at room temperature and subsequently 50 µl of 3 M NaOH was added to stop the reaction. The absorbance was measured at 405 nm in a plate reader (Biochrom Asys Expert 96 Microplate Reader; Biochrom, Holliston, MA, USA) and the alkaline phosphatase activity was quantified

using a standard curve based on calf intestinal alkaline phosphatase (Promega, Madison, WI, USA).

Immunoassay: Quantification of secreted proteins

Multianalyte profiling of protein levels in the medium was performed on the Luminex 200 system (Luminex, Austin, TX, USA) employing xMAP (multi-analyte profiling) technology. Acquired fluorescence data were analyzed by the xPONENT 3.1 software (Luminex, Austin, TX, USA). The amount of specific factors in the medium after 3, 7, 14 and 21 days of culture was measured using the human bone (HBNMAG-51K) and the human cytokine/chemokine (HCYTOMAG-60K) panels (Millipore, Billerica, MA, USA). All analyses were performed according to the manufacturer`s protocols.

Mineralization assay: Alizarin red staining

After 21 days of culture, hPDLCs and hOBs with or without irisin were washed with phosphate-buffered saline three times and fixed in 95 % ethanol for 30 min, stained with 1 % alizarin red for 5 minutes, and washed with milliQ water, as described previously (Dahl 1952). The Alizarin red staining for irisin (10 ng/ml and 100 ng/ml, respectively) and control groups was captured at 10-x magnification using a light microscope (Olympus IX70, Tokyo, Japan). For this experiment, an additional control group containing irisin in the medium with no cells was also tested to allow for the visualization of false positive results due to possible interaction between irisin and the Alizarin red staining.

To quantify mineralization, the Alizarin red deposition was extracted with 10 % cetyl pyridinium chloride (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 2 hours, and measured at 562 nm in a plate reader (Biochrom Asys Expert 96 Microplate Reader; Biochrom, Holliston, MA, USA).

Immunocytochemistry and confocal laser scanning microscopy

After 21 days of culture, hPDLCs and hOBs with or without irisin were fixed in 4% paraformaldehyde for 15 min and subsequently stored in phosphate-buffered saline. Fixed cells were permeabilized with 0.1 % Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline for 5 minutes. After washing once in phosphate-buffered saline, hPDLCs and hOBs were blocked with 10 % normal goat serum (Abcam, Cambridge, United Kingdom) diluted in phosphate-buffered saline for 1 hour, and incubated with mouse monoclonal anti-collagen type I (ab90395) and rabbit polyclonal anti-periostin (ab14041) primary antibodies (Abcam, Cambridge, United Kingdom) at 1:300 in 2 % normal goat serum overnight at 4°C. After washing three times in phosphate-buffered saline, hPDLCs and hOBs were incubated with Alexa 488-conjugated goat anti-mouse and Alexa 568-conjugated goat anti-rabbit secondary antibodies (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) at 1:500 in 4 % normal goat serum for 1 hour at room temperature. After washing twice in phosphate-buffered saline, hPDLCs and hOBs were incubated with Hoechst stain (Sigma-Aldrich, St. Louis, MO, USA) at 1µg/ml in phosphate-buffered saline for 10 minutes at room temperature, and mounted with Mowiol® 4-88 (Sigma-Aldrich, St. Louis, MO, USA) to glass slides and left in the dark at room temperature ready for microscope analysis.

Type I collagen- and periostin-expressing cells were imaged using a Leica confocal laser scanning microscope (Leica TCS SP8; Leica Microsystems, Wetzlar, Germany). The cells were excited with the 488 and 568 nm laser lines. To allow type I collagen and periostin quantification, imaging settings were kept constant during individual assays of both irisin and control groups. The type I collagen and periostin expression for both irisin and control groups were determined for three parallels per donor of hPDLCs and hOBs with 5 images per sample,

and the images were analyzed for type I collagen and periostin fluorescence intensity quantification using Fiji software (NIH, Bethesda, MD, USA).

Statistics

For each of the cell types tested (hPDLCs and hOBs) the data obtained after cytotoxicity, proliferation, wound-healing, alkaline phosphatase activity, protein secretion to cell culture media, Alizarin red staining and immunocytochemistry quantification analyses at each time point were compared between the groups (both irisin and control groups) using Holm-Sidak test following a parametric one-way analysis of variance (ANOVA). Where the equal variance and/or the normality test failed, a Kruskal-Wallis one-way ANOVA on ranks (Dunn's method) was performed (SigmaPlot 14.0; Systat Software, San Jose, CA, USA). A probability of ≤ 0.05 was considered significant. No = 4 were assayed in parallel for each group, cell type, donor, and time point, all the listed analyses, except for proliferation (No = 6) and Alizarin red staining (No = 3).

Results

1. Effect of irisin on cell cytotoxicity

Neither of the irisin concentrations caused a significant increase in the lactate dehydrogenase activity in the culture medium from hPDLCs and hOBs compared to untreated cells used as controls (Figure 1).

2. Effect of irisin on cell proliferation and migration

Irisin administration induced a significant increase in cell proliferation of hPDLCs after 1, 2 and 3 days ($p = 0.007$, $p = 0.001$, and $p = 0.001$, respectively for 10 ng/ml; and $p = 0.001$, $p = 0.003$, and $p = 0.027$, respectively for 100 ng/ml) (Figure 1). The lower irisin concentration caused a slightly greater increase in cell proliferation than the higher concentration in hPDLCs at days 2 and 3 ($p = 0.039$, and $p = 0.008$, respectively). Irisin administration to hOBs resulted also in enhanced proliferation ($p = 0.006$, $p = 0.001$, and $p = 0.003$, respectively for 10 ng/ml; and $p = 0.001$, $p = 0.004$, and $p = 0.001$, respectively for 100 ng/ml), however in contrast to the observation in hPDLCs, the highest irisin concentration caused a slightly greater increase in cell proliferation than the lower concentration at day 2 ($p = 0.034$) in hOBs (Figure 1).

Both the high and low dosage of irisin enhanced the migration of hPDLCs in a wound-healing assay after 6 and 24 hours compared to untreated control ($p = 0.021$, and $p = 0.001$, respectively for 10 ng/ml, and $p = 0.002$, and $p = 0.001$, respectively for 100 ng/ml) (Figure 2). Similarly, irisin treatment also enhanced the migration of hOBs in a wound-healing assay after 2, 6 and 24 hours compared to control ($p < 0{,}001$ for 10 ng/ml after 2 hours, $p = 0.001$ and $p < 0{,}001$, respectively for 10 ng/ml and 100 ng/ml after 6 hours, $p < 0{,}001$ and $p < 0{,}001$, respectively for 10 ng/ml and 100 ng/ml after 24 hours) (Figure 2). Nevertheless, hOBs with irisin had a lower migration activity compared to hPDLCs with irisin (Figure 2).

3. Effect of irisin on osteogenic behaviour

Osteogenic effect of irisin in hPDLCs and hOBs was evaluated by quantification of a selection of osteoblast-related factors in the cell culture media, and immunocytochemistry and Alizarin red staining of the cells.

Effect of irisin on osteoblast differentiation markers

Treatment with irisin facilitated extracellular matrix formation in hPDLCs by increased type I collagen deposition. At 21 days, hPDLCs treated with both irisin concentrations demonstrated significantly enhanced deposition of type I collagen, as visualized by confocal laser scanning microscopy imaging and fluorescence intensity quantification, compared to control ($p = 0.009$ for 10 ng/ml, and $p = 0.023$ for 100 ng/ml) (Figure 3). The same effects were not observed in hOBs exposed to irisin; these cells had the same levels of type I collagen deposition as the untreated control (Figure 4).

Deposition of periostin, which among multiple functions is also shown to regulate extracellular matrix formation (Norris et al., 2007), was enhanced in hOBs treated with irisin. After 21 days of culture hOBs treated with both irisin concentrations displayed significantly increased deposition of periostin compared to control ($p = 0.026$ for 10 ng/ml, and $p = 0.011$ for 100 ng/ml) (Figure 4). In contrast, hPDLCs treated with 100 ng/ml irisin showed a significantly decreased deposition of periostin compared to control ($p = 0.035$) (Figure 3).

The amount of osteopontin in medium from hPDLCs with both irisin concentrations was similar to control throughout cell culture period (Figure 5), whereas hOBs demonstrated increased secretion of osteopontin compared to control at 21 days ($p = 0.001$ for 100 ng/ml) (Figure 5).

The secretion of osteocalcin was significantly increased from hPDLCs with both irisin concentrations compared to control at 21 days ($p = 0.010$ for 10 ng/ml, and $p = 0.003$ for 100

ng/ml) (Figure 5). In medium from hOBs incubated with the highest concentration of irisin the secretion of osteocalcin was decreased compared to control for days 7 and 14 ($p = 0.013$, and $p = 0.003$, respectively), and thereafter at 21 days significantly increased compared to control ($p = 0.025$) (Figure 5).

The content of vascular endothelial growth factor A (VEGFA) was significantly increased from hPDLCs with 10 ng/ml irisin compared to control at 14 days ($p = 0.017$) (Figure 5). Also in medium from hOBs with 100 ng/ml irisin the amount of VEGFA was significantly enhanced compared to control at days 3 and 21 ($p = 0.015$, and $p = 0.028$, respectively) (Figure 5).

The secretion of leptin, which is known to facilitate osteoblastogenesis via peripheral pathway (Turner et al., 2013), was significantly increased from hPDLCs with 100 ng/ml irisin at days 3 and 21 ($p = 0.007$, and $p = 0.024$, respectively) (Figure 5). Conversely, secretion of leptin from hOBs with both irisin concentrations was similar to control at days 3, 7, 14, and significantly decreased at day 21 from hOBs with 100 ng/ml irisin compared to control ($p = 0.021$) (Figure 5).

Excretion of interleukin 6 was significantly decreased from hPDLCs with 10 ng/ml irisin compared to control at 21 days ($p = 0.004$), and from hPDLCs with 100 ng/ml irisin compared to control at days 3 and 7 ($p = 0.001$, and $p = 0.001$, respectively) (Figure 6). From hOBs treated with both irisin concentrations the secretion of interleukin 6 was significantly increased compared to control at 21 days ($p = 0.005$ for 10 ng/ml, and $p = 0.027$ for 100 ng/ml) (Figure 6).

The secretion of Dickkopf-1 from hPDLCs and hOBs with both irisin concentrations was similar to controls throughout cell culture period (Figure 6). Similarly, the quantity of tumor necrosis factor alpha (TNFα) from hPDLCs with both irisin concentrations was similar to control throughout cell culture period (Figure 6). On the other hand, secretion of TNFα from

hOBs with both irisin concentrations demonstrated a decreasing-increasing-decreasing trend compared to control. At 3 and 21 days, hOBs with 100 ng/ml irisin had a significantly lower secretion of TNFα compared to control ($p = 0.011$, and $p = 0.007$, respectively) (Figure 6). Additionally, secretion of sclerostin, was significantly decreased from hPDLCs with 10 ng/ml irisin compared to control at days 14 and 21 ($p = 0.030$, and $p = 0.030$, respectively), and from hOBs with both irisin concentrations compared to control at 21 days ($p = 0.019$) (Figure 6).

Effect of irisin on alkaline phosphatase activity

The alkaline phosphatase activity in the culture medium from hPDLCs with both irisin concentrations was similar to control throughout cell culture period (Figure 7). But, the alkaline phosphatase activity from hOBs with 100 ng/ml irisin was significantly increased compared to control at 21 days of culture ($p = 0.001$) (Figure 7).

Effect of irisin on mineralization

After 21 days of culture, mineralization was significantly increased from hPDLCs and hOBs with both irisin concentrations compared to control according to quantification of Alizarin red staining ($p = 0.009$, and $p = 0.001$, respectively for 10 ng/ml in hPDLCs and hOBs; and $p = 0.001$, and $p = 0.006$, respectively for 100 ng/ml in hPDLCs and hOBs) (Figure 8). In both cell types, hPDLCs and hOBs, the highest irisin concentration caused a more marked staining compared to the lower irisin concentration (Figure 8).

Discussion

To the authors best knowledge this is the first time to demonstrate that irisin treatment promoted hPDLC growth and migration together with enhanced extracellular matrix formation. Further, in hOBs, used as positive controls, irisin increased growth, migration and extracellular matrix formation.

Effect of irisin on cell growth and migration

The study demonstrated that irisin enhanced proliferation of hPDLCs and hOBs, as observed by 5-bromo-2`-deoxyuridine incorporation. Besides stimulated cell growth, irisin also accelerated migration of hPDLCs and hOBs, as assessed by scratch assay as a simplified *in vitro* wound healing assay. The higher migration/wound healing capacity of irisin treated hPDLCs as compared to irisin treated hOBs could partly be due to a shorter cell doubling time of hPDLCs as reported by the manufacturers. Interestingly, the deposition of periostin, which is known to enhance PDLC proliferation and migration (Wu et al., 2018), was decreased in hPDLCs treated with higher irisin concentration when compared to control at day 21. Contrarily, hOBs treated with both low and high irisin concentrations showed an increased expression of periostin when compared to control after 21 days of culture. Enhanced periostin accumulation in hOBs was expected as it has multiple functions in bone tissue, including promotion of osteoblast proliferation (Merle & Garnero 2012).

Previously, the proliferative effect of irisin on hPDLCs and hOBs has not been tested. However, it has been demonstrated that irisin at a concentration of 100 ng/ml enhances proliferation of primary rat osteoblasts and mouse osteoblastic cell line, MC3T3-E1 cells, compared to untreated cells utilized as control (Qiao et al., 2016).

Effect of irisin on osteoblast differentiation

Several osteoblast-like characteristics were identified for hPDLCs treated with irisin including increased deposition of type I collagen, enhanced secretion of osteoblastogenesis related proteins, like osteocalcin and leptin, and angiogenesis related protein VEGFA. Further, indication for mineralization ability was demonstrated by elevated Alizarin red staining.

The structure and function of periodontal tissues are closely related to occlusal function. The periodontal ligament is a functionally important tissue in tooth support and regulation of the alveolar bone remodelling (MCCulloch, Lekic, & McKee 2000). These properties of periodontal ligament are mainly derived from type I collagen, the most abundant extracellular matrix component (Butler, Birkedal-Hansen, Beegle, Taylor, & Chung 1975). In this study, hPDLCs treated with irisin at both low and high concentrations demonstrated increased deposition of type I collagen compared to control at 21 days. In contrast, the expression of periostin was reduced in hPDLCs treated with the highest irisin concentration as compared to control at day 21. Periostin has been found to form a complex with type I collagen (Norris et al., 2007) and thereby enhance its assembly into extracellular matrix (Egbert et al., 2014, Kudo & Kii 2018). Irisin treatment induced an increase in type I collagen and although the binding site for collagens on periostin has not been identified (Kudo & Kii 2018), it cannot be excluded that the co-localization and possible assembly of these proteins may mask or occupy antibody-binding epitopes on periostin giving a false negative result. However, irisin induced an enhanced mineralization of hPDLCs, and a reduction in periostin, previously identified as a negative regulator of mineralization (Zhou et al., 2015), support this observation. The hOBs treated with both low and high irisin concentrations had an increased expression of periostin, however the levels of type I collagen were similar to control after 21 days of culture. This is in agreement with a previous study hypothesizing that periostin is necessary in bone to retard premature deposition of mineralization nodules on type I collagen before it is

modified/remodelled (Fortunati et al., 2010). Further, the expression of type I collagen can be also induced by mechanical cyclic stretch in osteoblasts (Wang, Jia, Gilbert, & Woo 2003). Therefore, periodontal regeneration therapy with occlusal function induced irisin could be advantageous.

Angiogenesis plays a critical role in bone remodelling. The development of vasculature and circulation is critical for the homeostasis and regeneration of bone, without which, the tissue would degenerate and die (Schmid, Wallkamm, Hämmerle, Gogolewski, & Lang 1997). VEGFA is known to regulate osteoblast differentiation (Mayr-Wohlfart et al., 2002) as well as increase vascularization during bone formation (Schmid, Wallkamm, Hämmerle, Gogolewski, & Lang 1997). The hPDLCs treated with irisin at a concentration of 10 ng/ml demonstrated increased secretion of VEGFA when compared to control at 14 days.

Calcification plays a major role in maintaining the rigidity of bone tissues for support and movement (Clarke 2008). Mineralization of matrix proteins was assessed by the presence of mineralizing noduli stainable by Alizarin red. In hPDLCs, incubation with irisin at both low and high concentrations significantly enhanced the cell monolayer surface covered by mineralized noduli when compared to control at 21 days. In addition, secretion of leptin was markedly elevated from hPDLCs treated with irisin at a concentration of 100 ng/ml when compared to control. Previously, leptin has been shown to facilitate osteoblastogenesis in hOBs by stimulating proliferation, de novo collagen synthesis, and mineralization (Gordeladze, Drevon, Syversen, & Reseland 2002). In addition, hPDLCs treated with irisin at both low and high concentrations demonstrated significantly enhanced secretion of osteocalcin when compared to control at 21 days, confirming that hPDLCs with irisin promote osteoblast differentiation. Osteocalcin is secreted by osteoblasts at the time of bone calcification and is hence a marker of late osteoblast differentiation (Stein, Lian, Stein, Wijnen, & Montecino 1996). Further, secretion of sclerostin, which reduces osteoblastic bone formation (Lewiecki

2014), was markedly decreased from hPDLCs with irisin at a concentration of 10 ng/ml when compared to control at 14 and 21 days.

Likewise to hPDLCs, hOBs treated with irisin expedited osteoblastogenesis when compared to control. Enhanced osteogenic behaviour of hOBs with irisin was shown by increased deposition of periostin, regulator of extracellular matrix formation (Norris et al., 2007), and secretion of alkaline phosphatase and osteopontin, proteins related to maturation and organization of bone extracellular matrix (Stein, Lian, Stein, Wijnen, & Montecino 1996). More precisely, alkaline phosphatase activity was significantly increased from hOBs with higher irisin concentration when compared to control at 21 days, and secretion of osteopontin was significantly enhanced from hOBs with irisin at both low and high concentrations when compared to control at 21 days. Nevertheless, the expression of type I collagen was not enhanced from hOBs with irisin, as shown previously (Colaianni et al., 2014). Further, secretion of osteocalcin, which is known to be related to the ordered deposition of hydroxyapatite (Stein, Lian, Stein, Wijnen, & Montecino 1996), was significantly elevated from hOBs with higher irisin concentration when compared to control at 21 days. Concomitantly, at 21 days hOBs with both low and high irisin concentrations presented a greatly decreased secretion of sclerostin, inhibitor of osteoblastic bone formation (Lewiecki 2014), and notably increased secretion of interleukin 6, stimulator of osteoblastic bone formation (Blanchard, Duplomb, Baud'huin, & Brounais 2009), when compared to control. In addition, mineralization, which was evaluated by Alizarin red staining, was enhanced for hOBs with both low and high irisin concentrations when compared to control at 21 days. Also, an indication for facilitated vascularization was observed by significantly increased secretion of VEGFA from hOBs with higher irisin concentration when compared to control at days 3 and 21. So, these findings are in agreement with previous *in vitro* studies showing that irisin can enhance OB proliferation (Qiao et al., 2016) and differentiation by up-

regulating markers related to both early and late osteoblast differentiation (Colaianni et al., 2014, Qiao et al., 2016).

In conclusion, hPDLCs responded to irisin treatment with enhanced cell growth, migration and osteogenic behaviour *in vitro*. Therefore, irisin treatment may represent a feasible strategy for regeneration of hard tissue defects in the management of periodontitis by enhancing the potential for hPDLCs guided remodelling of periodontal ligament and alveolar bone formation.

Acknowledgments

The authors thank Catherine Anne Heyward and Duarte Nunes De C Mateus for technical assistance with immunocytochemistry and confocal laser scanning microscopy.

Conflict of interest

Graziana Colaianni and Maria Grano are name inventors of the European Patent No EP3081228B1, titled “Irisin for care and prevention of osteoporosis”.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Figure legends

Figure 1. Cell cytotoxicity assay (A, B). Lactate dehydrogenase (LDH) activity in the culture medium from hPDLCs (A) and hOBs (B) with 10 ng/ml and 100 ng/ml irisin is shown in percentage of controls at 1, 2 and 3 days. Values represent the mean (+ SD) of four parallels per each group, cell type, donor, and time point. Statistical analysis (Holm-Sidak test following ANOVA): (*) $p \leq 0.05$ versus control. Cell proliferation assay (C, D). DNA synthesis (5-bromo-2`-deoxyuridine (BrdU) incorporation) in hPDLCs (C) and hOBs (D) with 10 ng/ml and 100 ng/ml irisin is shown in percentage of controls at 1, 2 and 3 days. Values represent the mean (+ SD) of six parallels per each group, cell type, donor, and time point. Statistical analysis (Holm-Sidak test following ANOVA): (*) $p \leq 0.05$ versus control.

Figure 2. Cell migration assay. Migrated hPDLC (A) and hOB (B)-surface area, expressed as a percentage of the total surface area relative to time point 0, after 2, 6, and 24 hours, with and without irisin. Wound area at time point 0, measured immediately after the creation of the scratch for each of the individual samples are defined as 100%. Values represent the mean (+ SD) of four parallels per each group, cell type, donor, and time point. Statistical analysis (Holm-Sidak test following ANOVA): (*) $p \leq 0.05$ versus control.

Figure 3. Confocal images of hPDLCs with and without irisin (Ir) immunolabeled with Hoechst (A, E, I), type I collagen (B, F, J) and periostin (C, G, K) at 21 day. Merged images of Hoechst, type I collagen and periostin (D, H, L with scalebar: 100 µm) at 21 day. The images are representative for the respective groups. Fluorescence intensity at 21 day is shown for type I collagen and periostin in hPDLCs treated with 10 ng/ml irisin and 100 ng/ml irisin in percentage of controls (M). Values represent the mean (+ SD) of three parallels per donor of hPDLCs with 5 images per sample. Statistical analysis (Holm-Sidak test following ANOVA): (*) $p \leq 0.05$ versus control (M).

Figure 4. Confocal images of hOBs with and without irisin (Ir) immunolabeled with Hoechst (A, E, I), type I collagen (B, F, J) and periostin (C, G, K) at 21 day. Merged images of Hoechst, type I collagen and periostin (D, H, L with scalebar: 100 µm) at 21 day. The images are representative for the respective groups. Fluorescence intensity at 21 day is shown for type I collagen and periostin in hOBs with and without irisin (M). Values for type I collagen represent the mean (+ SD) of three parallels per donor of hOBs with 5 images per sample. Statistical analysis (Holm-Sidak test following ANOVA): (*) $p \leq 0.05$ versus control. Values for periostin

represent the median (+ IQR) of three parallels per donor of hOBs with 5 images per sample. Statistical analysis (Kruskal-Wallis one-way ANOVA on ranks (Dunn's method)): (*) $p \leq 0.05$ versus control (M).

Figure 5. Immunoassay: Quantification of secreted proteins. Secretion of osteopontin (OPN), osteocalcin (OC), vascular endothelial growth factor A (VEGFA) and leptin (LEP) to cell culture medium from hPDLCs and hOBs with 10 ng/ml and 100 ng/ml irisin is shown in percentage of controls at 3, 7, 14 and 21 days. Values represent the mean (+ SD) of four parallels per each group, cell type, donor, and time point. Statistical analysis (Holm-Sidak test following ANOVA): (*) $p \leq 0.05$ versus control.

Figure 6. Immunoassay: Quantification of secreted proteins. Secretion of interleukin 6 (IL 6), dickkopf-1 (DKK1), tumor necrosis factor alpha (TNFa) and sclerostin (SOST) to cell culture medium from hPDLCs and hOBs with 10 ng/ml and 100 ng/ml irisin is shown in percentage of controls at 3, 7, 14 and 21 days. Values represent the mean (+ SD) of four parallels per each group, cell type, donor, and time point + SD. Statistical analysis (Holm-Sidak test following ANOVA): (*) $p \leq 0.05$ versus control.

Figure 7. Alkaline phosphatase (ALP) activity assay. ALP activity in culture medium from hPDLCs (A) and hOBs (B) with 10 ng/ml and 100 ng/ml irisin is shown in percentage of controls at 3, 7, 14 and 21 days. Values represent the mean (+ SD) of four parallels per each group, cell type, donor, and time point. Statistical analysis (Holm-Sidak test following ANOVA): (*) $p \leq 0.05$ versus control.

Figure 8. Mineralization assay – Alizarin red staining. The Alizarin red staining in hPDLCs without irisin (A), with 10 ng/ml irisin (B) and 100 ng/ml irisin (C) at 21 day; in hOBs without irisin (D), with 10 ng/ml irisin (E) and 100 ng/ml irisin (F) at 21 day. The images are representative for the respective groups. Scalebar represents 100 µm. The extracted alizarin red from hPDLCs and hOBs at 21 day (G). Values represent the mean (+ SD) of three parallels per each group, cell type, and donor. Statistical analysis (Holm-Sidak test following ANOVA): (*) $p \leq 0.05$ versus control.