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Estrogen receptor signaling in the ferutinin-induced osteoblastic differentiation of human amniotic fluid stem cells

M. Zavatti [a,*], M. Guida [b], T. Maraldi [a], F. Beretti [a], L. Bertoni [a], G.B. La Sala [c], A. De Pol [a]

[a] Department of Surgical, Medical, Dental and Morphological Sciences with Interest in Transplants, Oncology and Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy
[b] EURAC Research, Center for Biomedicine, Bolzano, Italy
[c] Unit of Obstetrics and Ginecology, IRCCS-Arcispedale Santa Maria Nuova, Reggio Emilia, Italy

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ABSTRACT

Aims: Ferutinin is a diaucane sesquiterpene with a high estrogenic activity. Since ferutinin is able to enhance osteoblastic differentiation of human amniotic fluid stem cells (hAFSCs), the aim of this study was to evaluate the role of the estrogen receptors α (ERα) and G-protein coupled receptor 30 (GPR30) in ferutinin-mediated osteoblastic differentiation. Moreover, it was investigated if MEK/ERK and PI3K/Akt signaling pathways are involved in ferutinin-induced effects.
Main methods: hAFSCs were cultured in a standard medium or in an osteoblastic medium for 14 or 21 days and ferutinin was added at 10^{-8} M. Immunofluorescence techniques and Western-blot 21analysis were used to study estrogen receptors and signaling pathways.
Key findings: In both undifferentiated and differentiated hAFSCs we identified ERα and GPR30 with a nuclear or cytoplasmatic localization, respectively. The presence of ferutinin in the osteoblastic medium leads to an increase in ERα expression. To dissect the role of estrogen receptors, MPP and G15 were used to selectively block ERα and GPR30, respectively. Notably, ferutinin enhanced osteoblastic differentiation in cells challenged with G15. Ferutinin was able to increase ERK and Akt phosphorylations with a different timing activation. These phosphorylations were antagonized by PD0325901, a MEK inhibitor, and wortmannin, a PI3K inhibitor. Both MPP and G15 inhibited the ferutinin-induced MEK/ERK and PI3K/Akt pathway activations. In the osteoblastic condition, PD0325901, but not wortmannin, reduced the expression of OPN and RUNX-2, whereas ferutinin abrogated the down-modulation triggered by PD0325901.
Significance: PI3K/Akt pathways seems to mediate the enhancement of hAFSCs osteoblastic differentiation triggered by ferutinin through ERα.

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1. Introduction

Ferutinin (jaeschkeanadiol *p*-hydroxybenzoate) is a daucane phytoestrogen found in the plants of *Ferula* genus (Umbelliferae). It has been demonstrated that ferutinin has an estrogenic activity because it is able to bind estrogen receptors [1,2]. In vitro experiments showed a higher affinity for estrogen receptor (ER) α ($IC_{50} = 33.1$ nM) than for ERβ ($IC_{50} = 180.5$ nM) [2]. Moreover, ferutinin has an important role in bone metabolism since it is able to prevent and to treat osteoporosis induced by estrogen deficiency in ovariectomized rats [3]. The effects on bone mass exerted by ferutinin are comparable to those exerted by estradiol benzoate [4,5]. Recently, we demonstrated that ferutinin, through ERα, enhances bone reconstruction, when orally administered in rats with a calvarias critical size bone defect, filled with a collagen type 1 and human amniotic fluid stem cells (hAFSCs) construct [6]. This construct leads to an approximately 70% bone reconstruction showing that ferutinin could act, as a healing promoting factor, on hAFSCs inducing osteogenic differentiation [6,7]. Among stem cells, hAFSCs attracted the interest of researchers because they are placed midway between embryonic and adult stem cells, are easily to obtain without ethical problems and can be maintained in culture without difficulties [8,9]. hAFSCs are able to differentiate toward osteogenic lineage under suitable conditions such as the presence of ascorbic acid that stimulates extracellular matrix (ECM) synthesis, dexamethasone that stimulates cellular differentiation and β-glycerophosphate that promotes ECM mineralization [10]. In our laboratory, the osteogenic potential of hAFSCs was also demonstrated in vitro and in vivo on three-dimensional surface of poly-D,L-lactic acid, fibroin, or collagen [11,12]. Regarding the effects of ferutinin on osteogenic differentiation, no studies were reported in literature about the mechanism of action of this phytoestrogen. It is well known that mitogen-activated protein kinase

* Corresponding author at: Department of Surgical, Medical, Dental and Morphological Sciences with Interest in Transplants, Oncology and Regenerative Medicine, University of Modena and Reggio Emilia, Via Del Pozzo 71, 41124 Modena, Italy.
E-mail address: manuela.zavatti@unimore.it (M. Zavatti).

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(MAPK) signaling pathways are involved in cell survival, proliferation and differentiation [13,14] and in particular ERK signaling pathway stimulates osteogenic differentiation of stem cells [15]. Many phytoestrogens exerted their stimulating effects on osteoblastic differentiation via ERK signaling. Resveratrol stimulates human bone marrow-derived mesenchymal stem cells (BMSCs) proliferation and osteoblastic differentiation through an ER-dependent mechanism and coupling to ERK1/2 activation [16]. Xanthoumol stimulates osteoblastic differentiation by activation of RUNX-2 via a mechanism related to the p38 MAPK and ERK signaling pathways in MC3T3-E1 and C2C12 cells [17]. Quercetin promotes proliferation, osteogenic differentiation and angiogenic factor secretion of BMSCs through ERK and p38 signaling pathways [18]. Also the PI3-kinase-Akt pathways [19,20] is an important player of the osteogenic network as demonstrated by Mukherjee and Rotwein [21]. They showed that Akt activity is required for all stages of osteoblast differentiation (lineage commitment, early differentiation and maturation). Among phytoestrogens, icariin exerts its osteogenic effect on rat BMSCs activating the PI3K-Akt signaling pathways [22]. Puerarin, an isoflavone glycoside from *Pueraria lobate* (Willd.), stimulates bone formation through activation of PI3K/Akt pathways in rat calvarial osteoblasts [23].

The current study was carried out to explain a possible molecular mechanism of ferutinin-induced osteoblastic differentiation of hAFSCs, through ERα and GPR30, evaluating the role of the MEK/ERK and PI3K/Akt signaling pathways.

2. Materials and methods

2.1. Cell culture and treatments

Supernumerary amniocentesis samples were provided by the Laboratorio di Citogenetica, Arcispedale Santa Maria Nuova (Reggio Emilia, Italy). All samples were collected with informed consent of patients according to Italian law and ethical committee guidelines (Protocol n° 2015/0,004,362 of 02/24/2015). Amniotic fluid stem cells (AFSCs) were isolated according to De Coppi et al. [8] and Maraldi et al. [11]. Briefly, human amniocentesis cultures were harvested by trypsinization and subjected to c-Kit immunoselection by MACS® technology (Miltenyi Biotec, Cologne, Germany) [11]. c-Kit positive cells were subcultured routinely at 1:6 dilution and not allowed to expand beyond a 80% of confluence.

AFSCs were grown in a minimum essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all reagents, EuroClone, Milan, Italy) at 37 °C and 5% CO_2. Ferutinin (Indena SpA, Milan, Italy) was solubilized in dimethyl sulfoxide (DMSO) and added to the culture medium at the concentration of 10^{-8} M.

In the ERK and Akt pathways studies ferutinin was added to cell medium for different time periods (5′, 15′, 30′, 1 h or 2 h) whereas the MEK inhibitor PD0325901 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 10 μM and the PI3Kinase inhibitor wortmannin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 100 nM were added 1 h prior to ferutinin. The ERα antagonist methyl-piperidino-pyrazole hydrate (MPP) (Sigma Aldrich, St Louis, MO, USA) and the GPR30 antagonist 4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline (G15) (Tocris Bioscience, Bristol, UK) were used at the concentration of 1 μM, 1 h prior to ferutinin.

2.2. Osteoblastic differentiation

Cells were seeded approximately 3000 cells/cm^2 on culture dishes. When over 80% of confluence was reached, the standard medium was replaced with the osteoblastic one consisting of αMEM with 10% FBS, 100 μM 2P-ascorbic acid, 100 nM dexamethasone, 10 mM β-glyrocerophosphate (all from Sigma-Aldrich, St Louis, MO, USA), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin [24]. The culture medium was refreshed every 3–4 days until 21 days of culture. In the osteogenic conditions ferutinin was used at the concentration of 10^{-8} M, MPP and G15 at 1 μM.

To analyze signaling pathways during osteoblastic differentiation, AFSCs were pretreated with PD0325901 10 μM or wortmannin 100 nM for 1 h and then incubated with ferutinin for 14 days.

2.3. Western blot analysis

Whole cell lysates obtained from AFSCs at two different conditions of culture (osteogenic or not) were harvested, washed with phosphate buffered saline (PBS), and gently lysed on ice for 10 min in hypotonic lysis buffer (20 mM Tris-HCl, pH 7, containing 1% Nonidet P40, 150 mM NaCl, 10% Glycerol, 10 mM EDTA, 20 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM Na_3VO_4, and freshly added Sigma-Aldrich Protease Inhibitor Cocktail). After sonication, lysates were cleared by centrifugation for 15 min at 14,000 g in a refrigerated centrifuge. The supernatants were collected and the protein concentrations were determined by Bradford assay. 60 μg of protein for each sample were separated by 8 or 10% SDS page and then transferred to PVDF membrane. The Western blot technique was performed as described previously [25]. Briefly, the membranes were blocked with 3% dry milk and 2% bovine serum albumin (BSA) in Tris-buffered saline–Tween 20 (0.1%) (TBS-T) for 30 min. Blots were incubated overnight at 4 °C with one of the following primary antibodies (Abs), diluted 1:700 in TBS-T + 2% BSA and 3% milk: mouse *anti*-ERα (Millipore, Billerica, MA, USA), rabbit *anti*-GPR30 (Genetex, Irvine, CA, USA), rabbit anti pERK (DB Biotech, Kosice, Slovakia, EU), rabbit *anti*-pAKT(s473) and rabbit anti-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Runx2 and mouse *anti*-Osteopontin (Abcam, Cambridge, UK), goat *anti*-Akt1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing three times in blocking buffer, membranes were incubated with peroxidase-labelled *anti*-rabbit, anti-mouse or anti-goat secondary Abs diluted 1:3000, for 1 h at room temperature. All membranes were visualized using ECL (enhanced chemioluminescence, Amersham, UK). Goat *anti*-βactin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-ERK1/2 or goat anti-Akt1/2 were used as protein loading control.

Densitometry was performed on three independent experiments by ImageJ Data Analyzer software. The relative expression of each protein was quantified and normalized to the corresponding protein loading control.

2.4. Immunofluorescence and confocal microscopy

Monolayer cells were fixed in 4% paraformaldehyde in PBS at pH 7.4 for 20 min and then processed for subsequent immunofluorescence or histological staining.

Fixed monolayer cells were permeabilized with 0.1% TritonX-100 in PBS for 5 min to detect nuclear and cytosolic markers. Permeabilized and not permeabilized samples were washed three times with PBS and then blocked with 3% BSA in PBS for 30 min at RT. Samples were incubated with the primary antibodies diluted 1:50 in PBS containing 3% BSA: mouse *anti*-ERα and rabbit anti-Osteocalcin (OCN) (Millipore, Billerica, MA, USA), rabbit anti-GPR30 (Genetex, Irvine, CA, USA), mouse *anti*-OPN (Abcam, Cambridge, UK), rabbit *anti*-Runx2, for 1 h at RT.

After washing with PBS 3% BSA, the samples were incubated for 1 h at RT with the secondary antibody diluted 1:100 in PBS 3% BSA: goat anti-mouse Alexa fluor® 488, donkey *anti*-rabbit-Alexa fluor® 647 (Life Technologies, Carlsbad, CA, USA). After washing in PBS, samples were stained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) in H_2O for 5 min and then mounted with anti-fading medium, 0.21 M DABCO (1,4-diazabicyclo[2.2.2]octane) and 90% glycerol in 0.02 M Tris, pH 8.0. Negative controls consisted of samples not incubated with the primary antibody.

The multi-labeling immunofluorescence experiments were carried out avoiding cross-reactions between primary and secondary antibodies.

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