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Research article

Ferulic acid promotes osteogenesis of bone marrow-derived mesenchymal stem cells by inhibiting microRNA-340 to induce β-catenin expression through hypoxia

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ABSTRACT

Osteogenic differentiation is regulated through multiple signaling networks that may include responses to hypoxia. Antioxidant ferulic acid (FA) can promote hypoxia signaling by inducing hypoxic-induced factor (HIF). However, whether FA could affect osteogenesis has not been explored. We examined human bone marrow-derived mesenchymal stem cell (MSC) following FA treatment. The expression of β-catenin was measured, and candidate microRNAs that target β-catenin were studied. The involvement of hypoxia was investigated in miR-340-5p that contains hypoxia response elements (HRE) in the promoter region. Further, the osteogenic potential of FA-treated MSC was assessed by alkaline phosphatase (ALP) activity and alizarin red staining assays. Osteoblast marker gene expressions were also compared between controls and FA-treated cells. FA induced β-catenin expression in MSC. This effect is likely mediated through a derepression of β-catenin 3′-UTR inhibition by miR-340-5p. HIF-1α, which suppressed miR-340-5p promoter activation through HRE motifs, was induced by FA. The induction of β-catenin signaling by FA was consistent with an enhancement in osteogenesis of FA-treated MSC, which could be attenuated by miR-340-5p overexpression. Analysis of the signaling networks induced by FA reveals that hypoxia may promote the osteogenic program in mesenchymal stem cells via a novel microRNA pathway.

1. Introduction

Organ homeostasis needs to be dynamically maintained in order to adapt to a rapidly changing environment. The concentration of oxygen, for example, presents an environmental signal that elicits intracellular signaling important for proper tissue growth, differentiation, and function (Semenza, 2012). Hypoxia (a condition of reduced O_2 availability) can activate factors such as HIF-1α (hypoxic-induced factor-1α), a master regulator of oxygen sensing signaling (Majmundar et al., 2010; Semenza, 2009, 2012). HIF-1α is a transcription factor protein that is negatively regulated by VHL (von Hippel–Lindau protein)-dependent degradation under normoxia. Through binding hypoxia response elements (HREs) in promoter regions, a series of cellular responses to hypoxia are mediated by HIF-1α-dependent gene transcription (Semenza, 1996). Since the physiological concentration of oxygen is normally low in the connective tissues such as cartilage and bone, hypoxia is believed to be a key modulator for skeletal development and bone homeostasis. For example, a potential mechanism for hypoxia-associated bone loss could be due to enhanced osteoclast formation that depends on HIF-1α activity (Arnett et al., 2003; Bozec et al., 2008). Reciprocally, hypoxia was shown to decrease proliferation and differentiation of osteoblast cells in primary culture (Utting et al., 2006). Therefore, the well-known dependence of bone formation on the vasculature may be explained by these oxygen effects in bone cells. In contrast, hypoxia could also increase bone formation via activating angiogenesis as well as autonomous osteogenesis (Wang et al., 2007). Particularly in regenerative medicine focused on bone repair, a positive role of hypoxia in the osteogenic differentiation of mesenchymal stem cells (MSC) has been demonstrated. Human bone marrow-derived MSC cultured under hypoxia with 1% O_2 showed increased proliferation, migration, and propensity to differentiate into bone (Share, 1976). During in vitro osteogenic induction of placental-derived MSC, gene expressions of osteopontin, osteocalcin, and alkaline phosphatase (ALP) were increased by hypoxia (Gu et al., 2016). *In vivo*,

Abbreviations: FA, antioxidant ferulic acid; HIF, hypoxic-induced factor; MSC, bone marrow-derived mesenchymal stem cell; HRE, hypoxia response elements; ALP, alkaline phosphatase

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intermittent hypoxia in rats was able to enhance bone mineral density along with increased expressions of ALP and bone morphogenetic protein-2 (BMP-2) (Oishi et al., 2016). Preconditioning rabbit bone marrow-derived MSC with hypoxia was able to improve regeneration potential of osteogenesis after transplantation (Fan et al., 2015). Mechanistically, microRNAs (miR) are recently identified as critical mediators of hypoxia signaling in the regulation of osteogenesis of mesenchymal stem cell. For example, the expression of hypoxia-inducible miR-429 (Bartoszewska et al., 2015) was shown to be increased in MC3T3-E1 cells by $CoCl_2$, a mimic of hypoxia (Huang et al., 2016). The miR-429-inhibited ZFPM2 (Zinc Finger Protein, FOG Family Member 2) induced osteogenic differentiation manifested by enhanced alkaline phosphatase activity and matrix mineralization (Huang et al., 2016). Better knowledge of such regulations for osteogenesis in the settings of hypoxia may also lead to improved understating of the therapeutic agent for promoting bone regeneration.

Ferulic acid (FA) is a hydroxycinnamic acid found in plant cell walls, and may protect cell integrity by virtue of catalyzed phenoxy radical formation (Graf, 1992; Mathew and Abraham, 2004). The applications of FA and its derivatives include industry, food, and medicine. It is believed to be an active ingredient of Chinese herbal medicine such as Angelica Sinensis Radix (ASR) (Gong et al., 2016). Over the past years, a potent antioxidant function of FA has been demonstrated as FA may serve as a chemical scavenger for free radical or other cell stress byproducts (Graf, 1992; Mathew and Abraham, 2004; Zhao and Moghadasian, 2008). Since oxidative stress plays key roles in the pathogenesis of numerous diseases including bone disorders, the therapeutic potential of FA for the treatment of bone loss have been studied. As the effects of FA include an inhibitory action to osteoclast formation and a stimulating role to osteoblast function (Lai and Yamaguchi, 2006; Sagar et al., 2016; Sassa et al., 2003), the underlying mechanisms are still unclear. Interestingly, FA may mimic some of the effects of hypoxia. An angiogenic effect of FA has been characterized to act through the induction of HIF-1α (Lin et al., 2010). Additionally, in a study of wound healing, a hydrogel composed of gelatin and FA can promote neovascularization from the host tissues via hypoxia inducible fashion (Park and Gerecht, 2014). Based on this information, we speculated that the hypoxia-mimicking effect of FA may be at least partially responsible for its positive role in bone formation. The effect of FA in MSC under osteogenic differentiation has not been examined, and we predicted a hypoxia-inducing pathway by FA may promote osteogenesis of bone progenitor cells. In the current study, we test these hypotheses by studying human bone marrow-derived MSC following FA treatment. The hypoxia-mediated signaling was then particularly focused. The results may be linked to a therapeutic potential of FA in promoting bone regeneration.

2. Methods & materials

2.1. Human bone marrow-derived MSC culturing

The protocol for using human cells was approved by the committee of The First Affiliated Hospital of Soochow University, and bone marrow cells were obtained from bone fragments of patients in The First Affiliated Hospital of Soochow University. All harvested bone marrow aspirates were diluted with low-glucose Dulbecco's modified Eagle's medium (DMEM, Thermo, Waltham, MA, USA) followed by Ficoll gradient centrifugation (1200 × g for 30 min at room temperature). The cells from the interface were collected, and washed twice in phosphate-buffered saline (PBS). Mononuclear cells were counted with a hemocytometer and plated in complete DMEM at a density of 5×10^6 cells/10 ml in 10 cm^2 tissue culture dishes. After discarding floating cells after two days, the adherent cells were continued to culture at 37 °C with 5% CO_2 in a humidified incubator. Cells were detached with 0.05% trypsin/1 mM EDTA and replated after reaching a confluence of 75–85%, and expanded cells with < 9 passages were used in the experiments. Cells were treated with vehicle controls or FA (Lin et al., 2010) (10 μM, Sigma, St. Louis, MO, USA) for 24 h. Hypoxic conditions were modeled by treating cells with $CoCl_2$ (100 μM) for 24 h.

2.2. MicroRNA profiling

We first searched for miRNAs that have complementary sites at the 3′-UTR of human β-catenin gene by TargetScanHuman (Lewis et al., 2005). To identify candidate microRNAs regulated by ferullic acid in MSC, a miRNA microarray containing the resultant miRNAs was generated with customized probe sets containing designed oligoes (IDT) (Liu et al., 2008). Total miRNA was isolated using mirPremier microRNA Isolation Kit (Sigma-Aldrich, St. Louis, MO, USA) from cultured cells treated with either control or FA, and was then labeled as the first strand biotin-cDNA. The hybridization steps were performed on a hybridization station (Tecan) followed by an indirect detection of streptavidin-Alexa647 conjugate (Invitrogen, Carlsbad, CA, USA). The image analysis of microarray was carried out by GenePix pro (Molecular Devices, Sunnyvale, CA, USA), and the expression profile was compared between MSC cultured in media containing FA or control.

2.3. mRNA extraction and real-time PCR

Total mRNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA), and then reverse transcribed to complementary cDNAs using Superscript II according to manufacturer's instructions (Biorad, Hercules, CA, USA). Validated primer sets were used for: *PTPRCAP* (CD45), forward, 5′-TTGAGCGACAGGAGGATGAG-3′ and reverse, 5′-GACGCCTCTCCACATTGCT − 3′; *ITGB1*(CD29), forward, 5′-CCTACTTCTGCACGATGTGATG-3′ and reverse, 5′-CCTTTGCTACGGT TGGTTACATT-3′; *THY1*(CD90), forward, 5′-ATCGCTCTCCTGCTAACA GTC-3′ and reverse, 5′-CTCGTACTGGATGGGTGAACT-3′; *ENG*(CD105), forward,5′-TGCACTTGGCCTACAATTCCA-3′ and reverse, 5′-AGCTGCC CACTCAAGGATCT-3′; *CTNNB1,* forward, 5′-AGCTTCCAGACACGCT ATCAT-3′ and reverse, 5′-CGGTACAACGAGCTGTTTCTAC-3′; *OSX,* forward,5′-CCTCTGCGGGACTCAACAAC-3′ and reverse, 5′-AGCCCAT TAGTGCTTGTAAAGG-3′; *RUNX2,* forward, 5′-TGGTTACTGTCATGGC GGGTA-3′ and reverse, 5′-TCTCAGATCGTTGAACCTTGCTA-3′; *Actin,* forward,5′-AGAGCTACGAGCTGCCTGAC-3′ and reverse, 5′-AGCACTG TGTTGGCGTACAG-3′. Triplicate PCR reactions were performed by cyber green-based system (Applied Biosystems, Waltham, MA, USA) with cycling conditions as 15 s at 95 °C, 1 min at 60 °C for 40 times. The expression levels were normalized to actin. Data are expressed as relative levels as means ± standard errors. Melting curves of all primers were shown in Supplementary Fig. S1.

2.4. Western blotting

Cells were resuspended in the lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 0.1% NP-40 alternative, 10 mM HEPES, 0.25% Na-deoxycholate, 1 mM Na_3VO_4, 0.5 mM NaF, pH = 7.4 (Protease Inhibitor Cocktail, Roche, 1 tablet/10 ml). Cell lysates after spin were quantitated by BCA protein assays, and 30 μg total protein was run on SDS-PAGE followed by transfer to PVDF membranes. The membranes were blocked by 1% BSA (bovine serum albumin, Sigma, USA), and incubated with primary antibodies overnight at 4 °C. Primary antibodies for β-catenin, HIF-1α, and GAPDH were all obtained from Abcam. HRP conjugated secondary antibodies were used to visualize bands by an ECL-based imaging system.

2.5. MicroRNA transfection and inhibition

MystiCq microRNA qPCR assay kit (MIRAP00331) was purchased from Sigma-Aldrich (St. Louis, MO, USA) to measure expression levels of miR-340-5p. The MISSION Lenti miR-340-5p mimic (HLMIR0502) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and were