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All-trans retinoic acid modulates bone morphogenic protein 9-induced osteogenesis and adipogenesis of preadipocytes through BMP/Smad and Wnt/β-catenin signaling pathways

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ABSTRACT

It is known that excessive adipogenesis contributes to osteoporosis, suggesting that trans-differentiation of adipogenic committed preadipocytes into osteoblasts may be a potential therapeutical approach for osteoporosis. We explored whether bone morphogenic protein 9 (BMP9) could induce 3T3-L1 preadipocytes to trans-differentiate into osteoblasts. BMP9 effectively increased expression of osteogenic markers and promoted mineralization in preadipocytes. However, BMP9 also led to adipogenic differentiation of preadipocytes, as evidenced by increased lipid accumulation and up-regulation of adipogenic transcription factors. In order to regulate the switch between osetogenesis and adipogenesis, we evaluated the effect of all-trans retinoic acid (ATRA) on BMP9-induced differentiation of preadipocytes. We found that ATRA enhanced BMP9-induced osteogenic differentiation and blocked BMP9-induced adipogenic differentiation both *in vitro* and *in vivo*. Mechanistically, ATRA was shown to elevate BMP9 expression and activate BMP/Smad signaling. Additionally, BMP9 and ATRA exerted a synergistic effect on activation of Wnt/β-catenin signaling. Knockdown of β-catenin abolished the stimulatory effect of ATRA on BMP9-induced alkaline phosphatase activity and reversed the inhibitory effect of ATRA on BMP9-induced adipogenesis in preadipocytes. Furthermore, ATRA and BMP9 synergistically repressed glycogen synthase kinase 3β (GSK3β) activity and promoted Akt phosphorylation, and inhibited expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) that antagonizes phosphatidylinositol-3-kinase (PI3K) function, suggesting that Wnt/β-catenin signaling was activated at least partly through PI3K/Akt/GSK3β pathway. Collectively, ATRA mediated BMP9-induced osteogenic or adipogenic differentiation of 3T3-L1 preadipocytes by BMP/Smad and Wnt/β-catenin signaling. The combination of BMP9 and ATRA may be explored as an effective therapeutic strategy for osteoporosis.

1. Introduction

The low bone mass and increased marrow adipose tissue can be simultaneously observed in conditions leading to osteoporosis (Wronski et al., 1986; Justesen et al., 2001; Sottile et al., 2004), suggesting that reduced bone formation is associated with excess fat content in osteoporotic population (Verma et al., 2002). Adipocytes and osteoblasts share a common bone marrow progenitor – multipotential mesenchymal stem cells (MSCs) (Pittenger et al., 1999). Osteogenic or adipogenic differentiation of MSCs is influenced by specific groups of transcription factors. Runt-related transcription factor 2 (Runx2) and Osterix (Osx) are the main determinants of osteogenesis, while peroxisome proliferators activated receptor-γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs) promote adipogenesis of MSCs (Zhang et al., 2012). This process is complex *in vivo*, suggesting that adipocytes may be generated at the expense of osteoblasts during the process of osteoporosis. This shift of MSCs differentiation to adipocyte lineage may contribute to the increase in adipogenesis and decrease in osteogenesis that coincide with bone loss. Given the close association between adipocytes and osteoblasts, increasing adipogenesis in bone marrow may play an important role in the development and deterioration of osteoporosis.

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Differentiation of MSCs to adipocytes involves two phases consisted of the commitment of MSCs to preadipocytes and the process of terminal adipogenic differentiation during which preadipocytes mature into adipocytes. Therefore, trans-differentiation of preadipocytes into osteoblasts may be a potential therapeutic strategy of osteoporosis with which to both prevent excessive marrow adipogenesis and divert committed preadipocytes to become more osteoblastic with a resulting increase in bone cells. Currently, increasing evidences have indicated a large degree of plasticity between preadipocytes and osteoblasts. A previous study showed that bone marrow adipocytes can differentiate in the osteogenic direction, which primarily requires an intermediate step to induce morphological change into fibroblast-like preadipocytes (Park et al., 1999). It was also found that subcutaneous preadipocytes are able to differentiate into bone-forming osteoblasts (Justesen et al., 2004). However, the mechanism underlying trans-differentiation of preadipocytes into osteoblasts is complex and remains to be thoroughly elucidated. It has been reported that 3T3-F442A preadipocytes express bone morphogenetic protein (BMP) receptors and that BMP2 plays a critical role in the process during which 3T3-F442A preadipocytes undergo commitment to osteoblastic lineage (Ji et al., 2000; Skillington et al., 2002). Moreover, overexpression of Runx2, the downstream target of BMP2, is effective for trans-differentiation of preadipocytes into fully differentiated osteoblasts (Takahashi, 2011). Liu et al. also showed that platelet-rich plasma stimulates osteogenic differentiation of 3T3-L1 preadipocytes partially through BMP2 signaling (Liu et al., 2011). Taken together, these findings indicate that adipogenic progenitor cells can revert to osteoblast lineage in response to specific extracellular signal, such as BMP signaling.

BMPs are growth factors that belong to the transforming growth factor β (TGFβ) superfamiliy. Based on analyzing 14 types of BMPs, it has been found that BMP9 is one of the most potent BMPs in inducing osteogenic differentiation of MSCs (Cheng et al., 2003). However, little information is available on the effect of BMP9 on differentiation of preadipocytes. Recently, BMP9 was shown to promote adipogenic differentiation of human white preadipocytes (Lord et al., 2010), whereas it is still unclear whether preadipocytes can be converted into osteoblasts in response to BMP9. Given the strongest osteoinductive activity of BMP9, we hypothesize that BMP9 may also effectively induce osteogenic differentiation of preadipocytes. Therefore, we evaluated the effect of BMP9 on osteogenic differentiation of preadipocytes in this study. As a cell model, we used the well established 3T3-L1 preadipocyte cell line that is used extensively in studying adipocyte biology.

Though BMP9 has the most potent osteoinductive activity, other signaling molecules are also required to enhance BMP9-induced bone formation. Retinoic acids (RAs) are derivatives of vitamin A. We have previously demonstrated the synergism between RA and BMP9 in inducing osteogenic differentiation of MSCs (Zhang et al., 2010). Moreover, RA was shown to be able to inhibit adipogenic differentiation and cooperate with BMP2 to induce osteogenic differentiation in preadipocytes (Sato et al., 1980; Skillington et al., 2002). These observations suggest the potential of RA to cooperate with BMP9 to promote osteogenesis and reverse BMP9-induced adipogenesis in preadipocytes. Thus, we also investigated the effect of all-trans-RA (ATRA), the abundant form of RA, on BMP9-induced osteogenic and adipogenic differentiation of preadipocytes. We found that BMP9 could simultaneously induce osteogenic and adipogenic differentiation of 3T3-L1 preadipocytes both *in vitro* and *in vivo*. ATRA potentiated BMP9-induced osteogenic differentiation and blocked BMP9-induced adipogenic differentiation in preadipocytes through activation of BMP/Smand and Wnt/β-catenin pathways. Lastly, our data also suggested that Wnt/β-catenin activation may be resulted from activation of phosphatidylinositol-3-kinase (PI3K)/Akt/glycogen synthase kinase 3β (GSK3β) pathway by ATRA and BMP9.

2. Materials and methods

2.1. Cell culture and chemicals

3T3-L1, HCT 116 and HEK293 cell lines were obtained from ATCC. Cell lines were maintained in the conditions described (Cheng et al., 2003; Zhang et al., 2010). ATRA was obtained from Sigma–Aldrich (Saint Louis, USA). ATRA was dissolved in DMSO and aliquots were stored in −80 °C. DMSO was used as solvent control. For cell culture treated with ATRA, the medium was changed every 3 days. Unless indicated otherwise, all chemicals were purchased from Sigma–Aldrich.

2.2. Construction of recombinant adenoviruses

Recombinant adenoviruses expressing BMP9 (AdBMP9) and small interference RNA (siRNA) targeted β-catenin (AdR-simBC) were generated previously using the AdEasy technology, as described (Tang et al., 2009; Chen et al., 2010a,b). AdBMP9 and AdR-simBC also respectively expressed GFP and RFP as a marker for monitoring infection efficiency. Adenoviruses expressing only GFP (AdGFP) and RFP (AdRFP) were used as controls.

2.3. Preparation of conditioned medium

Subconfluent HCT116 cells were infected with an optimal titer of AdBMP9. At 24 h after infection, the culture medium was changed to serum-free DMEM. BMP9 conditioned medium (BMP9-CM) was collected at 48 h after infection and used immediately.

2.4. Alkaline phosphatase (ALP) assays

ALP activity was assessed by a modified Great Escape SEAP Chemiluminescence assay (BD Clontech, Mountain View, CA) as described previously (Chen et al., 2010a,b), and/or histochemical staining assay performed with BCIP/NBT Alkaline phosphatase Color Development Kit (Beyotime, Jiangsu, China) according to manufacturer's instructions. For the bioluminescence assays, each assay condition was performed in triplicate and the results were repeated in at least three independent experiments. ALP activity was normalized by total cellular protein concentrations among the samples.

2.5. Western blotting analysis

For total protein level assay, cells were washed with cold PBS (4 °C) and lysed in 300 μl lysis buffer. For nucleus fraction protein extraction, the protein was harvested with Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China) according to manufacturer's instructions. Cell lysate was denatured by boiling and loaded onto a 10% gradient SDS–PAGE. After electrophoretic separation, proteins were transferred to polyvinylidene difluoride membrane (Millipore). Membrane was blocked with a solution containing 10 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween 20 (TBS-T) and 5% non-fat dry milk for 4 h, and probed with the primary antibody at 4 °C overnight, followed by incubation with a secondary antibody conjugated with horseradish peroxidase for 2 h. The proteins of interest were detected using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Chemical, Rockford, IL). Primary antibodies were obtained from Santa Cruz, as follows: anti-osteopontin, anti-osteocalcin, anti-Runx2, anti-Osx, anti-Dlx5, anti-PPARγ, anti-C/EBPα, anti-C/EBPβ, anti-phosphor-Smad1/5/8, anti-Smad1/5/8, anti-β-catenin, anti-BMP9,