Growth differentiation factor 11 is a protective factor for osteoblastogenesis by targeting PPARγ

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1. Introduction

Osteoporosis is a skeletal disorder characterized by bone loss, which results in architectural deterioration of the skeleton, compromised bone strength and an increased risk of fragility fractures (Canalis et al., 2007). Bone is a dynamic tissue that constantly remodels by balancing osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Under physiological conditions, formation and resorption are tightly coupled, thereby maintaining skeletal homeostasis. Under pathological conditions such as osteoporosis or bone metastasis of cancer, the simultaneously decreased formation and increased resorption lead to the uncoupling of remodeling and bone loss (Canalis et al., 2007; Rachner et al., 2011; Zaidi, 2007). Osteoblasts are differentiated from bone marrow mesenchymal stem cells (MSCs), which can also differentiate into adipocytes, depending on both extracellular milieu and intracellular signaling (Akune et al., 2004; Karsenty et al., 2009; Kawai and Rosen, 2010; Lazarenko et al., 2007). For instance, osteoclasts are differentiated from macrophage precursors in the hematopoietic lineage in response to the cytokine Receptor Activator of NF-κB Ligand (RANKL), depending on the ratio of RANKL to osteoprotegerin (OPC), a RANKL decoy receptor that inhibits osteoclast differentiation (Novack and Teitelbaum, 2008).

Osteoporosis is an aging-related disease, and aging is one of the main risk factors for osteoporosis (Tanishima and Morio, 2013). Osteoporotic fractures are highly prevalent in older persons having catastrophic consequences in their quality of life and increasing disability and mortality in this population (Gates and Das, 2011). The mechanisms of osteoporosis in older persons are unique in terms of cellular changes and response to osteoporosis treatment. Therefore, it is meaningful to identify the factors that change with aging and affect the development of osteoporosis.

Growth differentiation factor (GDF11), a member of the TGF-β superfamily, is a circulating factor in young mice that declines with age (Loffredo et al., 2013). Restoring systemic GDF11 levels reverses age-related dysfunction in mouse heart (Loffredo et al., 2013), skeletal muscle (Sinha et al., 2014), and brain (Shi and Liu, 2011; Katsimpardi et al., 2014). However, it still remains elusive whether GDF11 could also reverse age-related disorders in other tissues, such as the bone. Here we identify GDF11 as a factor changes with aging and osteoporosis, and affects the osteoblast–adipocyte differentiation balance by promoting PPAR-γ SUMOylation and inactivation.

2. Materials and methods

2.1. Patients

The plasma were collected from women patients with osteoporosis (n = 9; average age = 60.3), old healthy women donors (n = 8; average age = 61.2) and young women donors (n = 8; average age = 33.1). The plasma were stored at −80 °C before investigation. None of the patients or donors were treated with any drug before plasma collection. A written form of informed consent was obtained from all patients and...
donors, and the study was approved by the Clinical Research Ethics Committee of Gongli Hospital of Pudong New Area (Shanghai, China).

2.2. Osteoporosis model in mice

Osteoporosis in 8–10 weeks old female C57BL/6 mice (from VITAL RIVER) was induced by ovariectomy as described previously (Chang et al., 2009). The plasma and bone composition were harvested and studied 6 weeks after surgery. The plasma were stored at —80 °C before investigation. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Gongli Hospital of Pudong New Area (Shanghai, China).

2.3. Enzyme linked immunosorbent assay (ELISA)

Human and mouse plasma were collected and stored for ELISA assay. The ELISA kits for detecting human and mouse GDF11 were purchased from Huzhan (Shanghai, China), and standard experiments were performed in accordance to the guidance.

2.4. Quantitative real-time PCR (q-PCR)

Total RNAs were extracted from bone marrows or MSCs with TRIzol. 1 μg RNA was subjected for synthesis of cDNA with a One Step RT-PCR Kit (TaKaRa). q-PCR was performed with the SYBR Green (TaKaRa) detecting method on an ABI-7500 RT-PCR system (Applied Biosystems). GAPDH was used as housekeeping gene. The primers used in this study were listed in Table 1.

2.5. Ex vivo bone marrow differentiation

Osteoblasts were differentiated from marrow MSCs as described (Wei et al., 2011a, 2011b; Krum et al., 2008). Briefly, cells were cultured for 3–7 d in MSC medium (Mouse Mesen Cult Proliferation Kit; Stem Cell Technologies), then differentiated in α-MEM (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 5 mM l-glutamine, and 100 μg/mL ascorbic acid (mineralization medium) (GPA mixture) for 9 d. Mature osteoblasts were identified as (alkaline phosphatase-positive) ALP + cells by using fast red violet LB salt. For adipocyte differentiation, bone marrow MSCs were cultured for 3–7 d in MSC medium and differentiated in α-MEM containing 10% FBS, dexamethasone (1 μM), 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), and insulin (5 μg/mL) (DMEM mixture) for 2 d and then in α-MEM containing 10% FBS and insulin (5 μg/mL) for an additional 4 d. Mature adipocytes were identified as (oil red O/RO+ ) cells. Osteoblasts were differentiated as described (Wan et al., 2007; Wei et al., 2010). For treatment, rosiglitazone (1 μM; Sigma) and GDF11 (0.5 μg/mL; Abnova) were used as indicated in figure legends.

2.6. Immunoprecipitation and western blot

MSCs were lysed with cell lysis buffer (Beyotime). Immunoprecipitation and western blot analysis were done using standard protocols as described previously (Lu et al., 2011). Briefly, cell lysis was subjected for pre-clear with 30 μL Dynabeads® Protein A (Invitrogen), BSA and related IgG antibody (Santa Cruz) for 3 h at 4 °C and was then subjected for immunoprecipitation with antibodies against IgG, PPAR-γ or SUMO-1 for overnight 4 °C. Next, 50 μL Dynabeads® Protein A was added and the samples were incubated for overnight at 4 °C. Finally, the immunoprecipitates were washed with lysis buffer for 5 times. The samples were then boiled for 5 min. Total proteins and immunoprecipitates were applied to SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes, followed by blocking in the buffer containing 5% fat-free dry milk. The membranes were then probed with indicated antibodies overnight, and then washed and incubated with HRP-conjugated secondary antibodies (Zhongshanjinqiao) for 1.5 h and finally visualized using Chemiluminescent ECL reagent (Vigorous Biotechnology). The following antibodies were used: Anti-GAPDH (Cell Signaling Technology), anti–PPAR-γ (Cell Signaling Technology), anti–SUMO-1 (Santa Cruz), anti–p–PPAR-γ (Ser 112) (Santa Cruz), anti–p–PPAR-γ (Ser 273) (Bioboss).

2.7. Luciferase assay

To generate the luciferase reporter vectors, an IRES was amplified from pMSCV-PIG and cloned into the BgIII site of pGL3–Basic (Promega). ACC and FABP4 promoter fragments (Kim et al., 2013) were amplified from human genomic DNA and cloned into the Xhol site. 24 h before transfection, 7 × 104 cells were plated per well in a 24-well plate. pGL3 constructs (100 ng) plus 1 ng of the Renilla luciferase plasmid pRL–SV40 (Promega) were transfected using FuGENE 6 (Roche). 24 h after transfection, luciferase assays were performed using the dual luciferase reporter assay system (Promega). For each condition tested, the luciferase activity was normalized to the activity produced from empty vector.

2.8. Statistical analyses

All values are expressed as the mean ± SEM. Statistical differences between two groups were determined using Student’s t test. Two way ANOVA was used to analyze differences among groups more than two. Statistical analysis was performed using GraphPad Prism 6. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. GDF11 is decreased during aging and osteoporosis

To investigate the potential role of the aging-related factor GDF11 in the development of osteoporosis, we first tested the expression pattern of GDF11 in human patients with osteoporosis and in mouse osteoporosis model (ovariectomy). We found that the serum level of GDF11 was much lower in osteoporosis compared to normal age-matched donors (Fig. 1A). Similarly, the serum level of GDF11 in mice with osteoporosis was significantly decreased compared to normal mice (Fig. 1B). As GDF11 is expressed widely (McPherron et al., 1999), we also tested the mRNA level of GDF11 in the bone marrow cells of mice with osteoporosis. The GDF11 mRNA level in the bone marrow was significantly down-regulated in mice with osteoporosis (Fig. 1C). As osteoporosis is an aging-related disease, we wanted to know whether the level of GDF11 changed with age. Interestingly, the results indicated that serum levels of GDF11 were markedly reduced in old human donors and in old mice compared to normal young donors and young mice respectively (Fig. 1D–E). Furthermore, GDF11 mRNA level was decreased in bone marrow of old mice compared to young ones (Fig. 1F).

3.2. GDF11 induces osteoblastogenesis

The decline in GDF11 expression in osteoporosis implicates that GDF11 may contribute to osteoblastogenesis. To test whether GDF11

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<th>Table 1: Primers used for quantitative real-time PCR.</th>
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<td><strong>Name</strong></td>
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<tr>
<td>GDF11</td>
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<tr>
<td>Osteonectin</td>
</tr>
<tr>
<td>Coll1a1</td>
</tr>
<tr>
<td>Adiponectin</td>
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<td>Cxcl12</td>
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**Gene expression**

For gene expression analysis, total RNAs were extracted from bone marrows or MSCs with TRIzol. 1 μg RNA was subjected for synthesis of cDNA with a One Step RT-PCR Kit (TaKaRa). q-PCR was performed with the SYBR Green (TaKaRa) detecting method on an ABI-7500 RT-PCR system (Applied Biosystems). GAPDH was used as housekeeping gene. The primers used in this study were listed in Table 1.
alters the osteoblastogenesis balance, we performed ex vivo bone marrow differentiation assays under conditions that favored osteoblast formation. In the osteoblast differentiation assays, GDF11 promoted the development of alkaline phosphatase-positive (ALP+) cells (Fig. 2A), indicating that the GDF11 facilitates osteoblast development. Furthermore, we also found that GDF11 treatment could elevate the mRNA levels of osteoblast-specific genes, including osterix, osteocalcin, and cal1a1 (Fig. 2B–D). Taken together, GDF11 contributes to osteoblastogenesis ex vivo.

3.3. GDF11 inhibits adipogenesis

Osteoblasts are differentiated from bone MSCs, which can also differentiate into adipocytes, depending on both extracellular milieu and intracellular signaling (Akune et al., 2004; Karsenty et al., 2009; Kawai and Rosen, 2010). Another interesting question we wanted to know was whether GDF11 affected the differentiation of MSCs into adipocytes. Therefore, we performed ex vivo adipocyte differentiation assay with DMI (dexamethasone, IBMX, insulin) mixture. We found that GDF11 treatment significantly inhibited adipogenesis induced by DMI mixture (Fig. 3A). In addition, GDF11 also reduced the expression of adipocyte-specific genes, including adiponectin, fatty acid binding protein 4 (FABP4), and acetyl-CoA carboxylase (ACC; Fig. 3B–D).

3.4. GDF11 promotes PPAR-γ SUMOylation and inactivation

The studies above indicate that GDF11 favors osteoblastogenesis by promoting the differentiation of osteoblasts and inhibiting the differentiation of adipocytes from bone MSCs. This phenotype implicates that GDF11 may regulate a core factor for osteoblast–adipocyte differentiation balance. PPAR-γ is a pivotal factororchestrating the balance of adipocyte–osteoblast differentiation. Rosiglitazone, the agonist of PPAR-γ, could induce the differentiation of MSCs toward adipocytes. We guessed that GDF11 may regulate the expression or activity of PPAR-γ. We firstly tested whether rosiglitazone could affect the levels of GDF11 in both plasma and bone marrow (Fig. 4A–B). We next investigated the effects of GDF11 on rosiglitazone-induced PPAR-γ expression. However, GDF11 treatment could not reduce rosiglitazone-induced PPAR-γ expression in MSCs both at mRNA level (Fig. 4C). We also found that PPAR-γ protein...
level was not affected by GDF11 treatment (Fig. 4D–E). In addition, the phosphorylation of PPAR-γ at ser112 and ser273 sites, which are important for PPAR-γ activity, was also not affected by GDF11 (Fig. 4D–E).

Interestingly, we found that GDF11 treatment elevated the SUMOylation level of PPAR-γ (Fig. 4F–G), which is known as a negative modification for PPAR-γ activity (Dutchak et al., 2012). Indeed, we found that GDF11 treatment reduced the activity of PPAR-γ as a transcription factor, because GDF11 overexpression inhibited PPAR-γ-induced activation of the promoters of ACC and FABP4 in 293T cells (Fig. 5A). Finally, we showed that GDF11 treatment reduced ACC and FABP4 expression induced by rosiglitazone in MSCs (Fig. 5B). Taken together, GDF11 inhibits PPAR-γ activity by promoting PPAR-γ SUMOylation.

4. Discussion

Here in this study, we identified GDF11 as a novel factor that changed with osteoporosis and aging. We showed that GDF11 treatment significantly promoted MSC differentiation toward osteoblasts and altered the gene expression pattern, whereas adipocyte differentiation was inhibited. Mechanistically, we found that GDF11 could promote the SUMOylation of PPAR-γ and decreased its activity as a transcriptional factor.

Among the diseases and disorders associated with advancing age, one of the most debilitating is the loss of bone mass and function leading to osteoporosis. About half of the women at age of 50 or older will suffer an osteoporotic fracture during their lifetime, causing disability, increased mortality, and financial burden (Tanishima and Morio, 2013). Factors that determine risk of osteoporosis and fractures include genetic, hormonal, and nutritional factors, and specific pharmacological therapies for immunological, and inflammatory disorders (McPherron et al., 1999). However, the circulating factors that decline with aging and affect osteoporosis remain to be found. Proteomics analysis using aptamer-based technology to quantitatively evaluate plasma samples from ten young (2 months) and ten old (23 months) revealed 13 analytes that reliably distinguished young mice from old mice (Loffredo et al., 2013).

Among these factors, GDF11 was demonstrated to reverses age-related dysfunction in mouse heart (Loffredo et al., 2013; Biesemann et al., 2014), skeletal muscle (Sinha et al., 2014), and neuron (Shi and Liu, 2011; Katsimpardi et al., 2014). Here, we found that plasma level of GDF11 declined with aging and in patients with osteoporosis. GDF11 contributes to the maintenance of the bone hemostasis in adult mice by promoting osteoblast differentiation from MSCs, and represses adipocyte differentiation.

GDF11 is a major negative regulator of skeletal muscle mass and initiates multiple metabolic changes, including enhanced insulin sensitivity. In adult heart, GDF11 regulates energy homeostasis in the heart and prevents heart failure (Loffredo et al., 2013; Biesemann et al., 2014). GDF11 represses AMP-activated kinase activation in the heart via TGF-β-activated kinase 1, thereby preventing a metabolic switch toward glycolysis and glycogen accumulation (Biesemann et al., 2014). Supplementation of systemic GDF11 levels, which normally decline with age, by heterochronic parabiosis or systemic delivery of recombinant protein, reverses functional impairments and restores genomic integrity in aged muscle stem cells (satellite cells). Increased GDF11 levels in aged mice also improve muscle structural and functional features and

Fig. 3. GDF11 inhibits adipogenesis. Bone marrow cells from C57BL/6 mice were differentiated into adipocytes ex vivo by using DMI (dexamethasone, IBMX, insulin) mixture in the presence or absence of GDF11 (50 nM). (A) Representative image showing oil red O (ORO)-stained adipocyte differentiation culture. Mature adipocytes were ORO+ (red) cells. GDF11 treatment inhibits adipocyte differentiation. (B–D) GDF11 inhibits the expression of adipocyte-specific genes. N = 5 in each group.

Fig. 4. GDF11 regulates PPAR-γ SUMOylation. (A) Rosiglitazone (1 µM) reduces GDF11 serum level. (B) Rosiglitazone (1 µM) reduces GDF11 mRNA in the bone marrow of mice. (C) GDF11 (50 nM) does not change the mRNA level of PPARγ2 induced by rosiglitazone (1 µM). (D–E) Representative western blot showing GDF11 (50 nM) does not change PPAR-γ total protein and phosphorylation. (F–G) Representative western blot showing that GDF11 (50 nM) increases PPAR-γ SUMOylation. (F) Immunoprecipitation against SUMO-1. (G) Immunoprecipitation against PPAR-γ.
increased strength and endurance exercise capacity (Sinha et al., 2014). GDF11 alone can improve the cerebral vasculature and enhance neurogenesis. The identification of factors that slows down the age-dependent deterioration of the neurogenic niche in mice may constitute the basis for new methods of treating age-related neurodegenerative and neurovascular diseases (Katsimpardi et al., 2014). Here we find that PPAR-γ is a target of GDF11 in the bone hemoctasis, which may also indicate the role of GDF11 in fat metabolism.

Polymorphisms of the PPAR-γ gene are associated with osteoporosis (Harslof et al., 2011). The nuclear receptor PPAR-γ is a crucial cellular and metabolic switch that regulates many physiologic and disease processes. Emerging evidence reveals that PPAR-γ is also a key modulator of skeletal remodeling. Long-term use of rosiglitazone, a synthetic PPAR-γ agonist and a drug to treat insulin resistance, increases fracture rates among patients with diabetes. Recent studies have revealed that PPAR-γ activation not only suppresses osteoblastogenesis, but also activates osteoclastogenesis, thereby decreasing bone formation while sustaining or increasing bone resorption (Kang et al., 2007; Takada et al., 2007). The pro-osteoclastogenic effect of rosiglitazone is mediated by a transcriptional network comprised of PPAR-γ, PPAR-gamma co-activator 1α (PGC-1α) and estrogen-related receptor α (ERα), which promotes both osteoclast differentiation and mitochondrial activation (Wei et al., 2010). Therefore, PPAR-γ plays dual roles in bone homeostasis by regulating both mesenchymal and hematopoietic lineages (Wan, 2010). PPAR-γ facilitates bone loss by inhibiting osteoblastogenesis and stimulating adipogenesis from bone MSCs (Wei et al., 2012). Fibroblast growth factor (FGF21), which activates PPAR-γ by SUMOylation, regulates the antiadipogenic actions of thiazolidinediones and promotes bone loss (Wei et al., 2012). Here we identify GDF11 as a negative factor for PPAR-γ activation and function. We find that rosiglitazone could decrease the plasma level of GDF11. Reversely, GDF11 increases PPAR-γ SUMOylation and decreases its activity as a transcription factor.

In summary, we identify GDF11 as a novel negative factor for osteoporosis development and GDF11 supplement may serve as a candidate therapeutic strategy for osteoporosis.

Conflicts of Interest

The authors have no conflict of interest.

Acknowledgments

This work was supported by Discipline Leaders Project of the Shanghai Municipal Pudong New Area Health System (PWRd2012–16), National Natural Science Foundation of China (81201367), and Key Discipline Funding Project of Shanghai Municipal Pudong New Area Health Bureau (PWXz2014–09).

References


