S-nitrosoglutathione Reductase-dependent PPARgamma Denitrosylation Participates in MSC-derived Adipogenesis and Osteogenesis

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S-NITROSOGLUTATHIONE REDUCTASE–DEPENDENT PPARγ DENITROSYLATION PARTICIPATES IN MSC- DERIVED ADIPOGENESIS AND OSTEOGENESIS

By

Yenong Cao

A DISSERTATION

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S-NITROSOGLUTATHIONE REDUCTASE–DEPENDENT PPARΓ
DENITROSYLATION PARTICIPATES IN MSC-DERIVED ADIPOGENESIS AND
OSTEOGENESIS

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Osteogenesis and adipogenesis are two closely linked processes that share the same precursor, bone marrow mesenchymal stem cells (MSCs). MSCs are a rare population of non-hematopoietic stromal cells, present in bone marrow and almost every type of connective tissue. It is crucial to understand the signals that govern the specificity of MSC differentiation. Adipogenic transcription factor PPARγ can stimulate adipogenesis while inhibit osteogenesis. However, the role of potential regulators of PPARγ activity in this differentiation shift remains unknown.

One potential regulator is nitric oxide (NO). NO enhances adipogenesis of human preadipocytes while decreasing cell proliferation. Studies in mice with a targeted deletion of NOS1, NOS2 or NOS3 manifest distinct bone effects. These observations suggest that NO may play an important role in regulating the balance between adipocyte and osteoblast differentiation.

NO exerts its bioactivity not only by the production via nitric oxide synthases but also by the enzymatic degradation via S-nitrosylation. S-nitrosoglutathione (GSNO) exists in equilibrium with protein SNOs and can be degraded by the ubiquitously expressed GSNO reductase (GSNOR). By reducing the intracellular concentration of
GSNO, GSNOR indirectly regulates SNOs and thus serves as a key denitrosylase. Impaired denitrosylation, as manifested in GSNOR−/− mice, alters multiple stem cell behaviors, including hematopoietic stem cell number and MSCs endothelial differentiation. We have obtained GSNOR-deficient (GSNOR−/−) mice that proves to be a powerful tool to modulate endogenous SNO levels.

MSCs have been isolated and characterized from bone marrow of wild type mice and GSNOR−/− mice, which will allow us to check the MSCs behavior under different levels of NO and SNO. Our data reveal that GSNOR modulates PPARγ S-nitrosylation without affecting overall abundance of this transcription factor. Nitrosylated PPARγ has diminished transcriptional activity which is associated with reduced adipocyte differentiation and increased osteoblast formation. We further identified Cystein 139 as the target of PPARγ S-nitrosylation. Thus, S-nitrosylation participates in lineage bifurcation between adipocytes and osteoblasts. Further characterization of GSNOR−/− mice shows that they have lower body weight and bone density, with higher bone formation and bone resorption rate. Our results also suggest that GSNOR may contribute to parathyroid hormone regulation and calcium/phosphate urinary excretion, which in turn can affect the phenotype of GSNOR−/− mice. Together, these findings may provide mechanistic support for therapeutic strategies designed to offset disorders characterized by pathologic bone loss and/or excessive adipogenesis.
DEDICATION

This thesis is dedicated to my grandfather, Chuangan Zhang.
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1.1 Mesenchymal stem cells

1.1.1 Mesenchymal stem cell differentiation

Mesenchymal stem cells (MSCs) are a rare population of non-hematopoietic stromal cells, present in bone marrow and almost every type of connective tissue (1). MSCs were first described as osteoprogenitor cells from the bone marrow half a century ago (2). The term “mesenchymal stem cells” was adopted in 1990s to describe the adherent bone marrow stromal cells (3;4). Meanwhile, monoclonal antibodies were generated against human MSC surface antigens like CD105 and CD73, and cell isolation and long term culture conditions were established (5). However, comparisons between published data had been problematic due to lack of consensus in the definition of MSCs. In 2006, minimal criteria of MSCs were established by the International Society for Cellular Therapy (6) and consists of the following three aspects: 1) Adherence to plastic, 2) Positive for cell surface markers CD105, CD73 and CD90, and negative for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR, 3) Ability to differentiate into osteoblasts, adipocytes and chondrocytes. A more stringent characterization was proposed recently to assess multipotency by in vivo transplantation assays (7).

Based on its versatile differentiation capacity, MSCs has been widely used in stem cell therapy to regenerate bone, fat and heart tissues (8;9). Another major benefit of MSC therapy compared to other stem cell therapy is its immunosuppressive effects (10). However, the versatile differentiation capacity of MSCs also poses a risk of unwanted side effects of differentiation into non-target tissues. MSC differentiation is governed at
both transcriptional and post translational levels and achieving targeted and efficient MSC differentiation is one of the major focuses of regenerative medicine.

1.1.2 Differentiation and bone loss diseases

Osteoporosis, the most common type of bone disease in the aging population, is among the leading causes of morbidity in the United States. Osteoporosis is characterized by loss of bone density and bone volume, eventually leading to higher fracture rate. The cause of osteoporosis is an imbalance of osteoblast-mediated bone formation and osteoclast-mediated bone resorption (11). The upregulation of bone resorption or/and downregulation of bone formation leads to decreased bone mass (Figure 1.1). Also, it is commonly accepted that obese individuals are less likely to develop osteoporosis, partly because of the beneficial effects of weight bearing-induced mechanical tension to the bone (12). More importantly, adipokines secreted by fat tissue are crucial in bone remodeling (13). Thus, the interplay between bone and fat is important in the regulation of skeletal homeostasis. For example, the balance between adipogenesis and osteogenesis is crucial in bone health (Figure 1.1). The age-related differentiation shift of MSCs from a more osteogenic toward a primarily adipogenic lineage in the bone marrow promotes osteoporosis (14;15). Distorting this balance by increasing osteogenesis leads to progressive osseous heteroplasia (16), characterized by ectopic bone formation. Thus, it is crucial to understand the signals that govern the specificity of MSC bi-lineage differentiation into adipocytes and osteoblasts.
Figure 1.1 Bone marrow-originated cells types that are responsible for bone health.

1.2 PPARγ Signaling

1.2.1 Role of PPARγ in adipogenesis and osteogenesis

DNA binding transcriptional regulators govern the final cell lineage decision during MSC differentiation. Peroxisome proliferator-activated receptor gamma (PPARγ), a critical regulator of adipogenesis and glucose metabolism, belongs to a nuclear receptor family of ligand-inducible transcription factors. There are two major isoforms of PPARγ, PPARγ1 and PPARγ2, due to alternative splicing and differential promoter usage. PPARγ1 is expressed in multiple cells including adipocytes, haptocytes and other mesodermal lineage derived cells, while PPARγ2 is adipocyte specific. Adipogenic differentiation can be equally regulated by either isoform (17). PPARγ acts as a prime inducer of adipogenesis and it is required in adipocyte terminal differentiation. In addition, PPARγ is important to maintain mature adipocyte function and cell survival.
PPARγ null mice were embryonic lethal beyond E9.5 as a consequence of defects in extraembryonic tissues and disruption of heart and adipose tissue development (19).

PPARγ agonists, the thiazolidinediones, have been widely used to treat type II diabetes mellitus because of its effects to lower blood glucose. Major side effects of thiazolidinediones include weight gain, water retention, bone loss, and increased fracture rate, suggesting the involvement of PPARγ in skeletal metabolism (20). For example, PPARγ activation by thiazolidinediones inhibits the expression of osteogenic transcription factors in MSCs (21). In addition, rosiglitazone-induced bone loss in C57Bl/6J mice is associated with increased adipogenesis of the bone marrow (22), indicating an alternation of bone marrow milieu towards adiposity at the expense of osteogenesis upon thiazolidinediones treatment. PPARγ haploinsufficient mice had higher bone mass and bone marrow cells from these mice showed decreased adipogenesis and increased osteogenesis (23). However, the effects of PPARγ on osteogenesis are caused by primary modulation of osteogenic program by PPARγ or secondary to adipogenesis is still controversial. PPARγ also stimulates osteoclast differentiation and bone resorption (24).

1.2.2 Transcriptional and post-translational regulation of PPARγ

PPARγ can be regulated at both transcriptional and post-translational levels. In the absence of ligand binding, PPARγ forms a complex with its corepressors, such as nuclear corepressor I (NCORI) and histone deacetylases (HDACs), thus PPARγ is transcriptionally silent. Activation of PPARγ transcription includes a serial of events (25). First, the enhancer regions of PPARγ are occupied by CCAAT/enhancer-binding protein β (CEBPβ) but are not accessible. Upon adipogenic stimuli/ligand binding, levels of
CEBPβ and CEBPδ increase and cause the recruitment of a transcriptional activation complex including CEBPα and a co-activator complex. Upon activation of PPARγ by dimerization with retinoid X receptor (RXRα), it can auto-regulate its expression in coordination with CEBPα and CEBPβ. PPARγ regulates transcription via PPARγ response element (PPRE)-dependent and -independent mechanisms. As a transcription factor, PPARγ directly regulates genes harboring PPREs that are involved in adipogenesis, such as fatty acid binding protein 4 (FABP4) and CD36 (encodes fatty acid translocase).

PPARγ is also regulated at post-translational levels. The most thoroughly studied post-translational modification of PPARγ is phosphorylation. PPARγ can be phosphorylated at multiple sites and phosphorylation can either inhibit or promote the activity of PPARγ depending on the phospho-sites and the kinases (18). Mitogen activated protein kinases (MAPK) phosphorylates PPARγ2 at serine112 and inhibits its transcriptional activity (26). MAPK induced PPARγ phosphorylation can also change the subcellular localization of PPARγ via cytoplasmic-nuclear shuttling (27). In addition to MAPK, casein kinase (CK-II) induces PPARγ2 phosphorylation at serine 46 and 51 and leads to cytoplasmic localization of PPARγ and decreased transcriptional activity (28). However, cyclin dependent kinase (Cdk9) mediated PPARγ phosphorylation at serine112 increases its transcriptional activity and adipogenesis in pre-adipocytes (29). An additional PPARγ phosphorylation site is serine273 of the ligand-binding domain, regulated by Cdk5. Cdk5-mediated phosphorylation at serine273 does not affect PPARγ transcriptional activity, but alters expression of important adipokines such as adiponectin (30).
PPARγ also undergoes ubiquitination, SUMOylation and acetylation with potential interplay of these post-translational modifications. Ubiquitination regulates the stability of PPARγ but the site of ubiquitination has not been identified (31). Sumoylation of PPARγ2 at lysine 107 inhibits its transcriptional activity and this inhibitory effect can be reversed by FGF21 (32). Also, cross talk between PPARγ phosphorylation and sumoylation was observed, in which sumoylation was repressed when PPARγ phosphorylation is deficient (33).

1.3 Nitric oxide signaling

1.3.1 Nitric oxide and S-nitrosylation

Nitric oxide (NO) is an important intercellular signaling molecule. NO is synthesized by three nitric oxide synthases: neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3) from L-arginine, oxygen and NADPH. NO exerts its function through multiple pathways: the classical soluble guanylyl cyclase-cyclic GMP (sGC-cGMP) pathway and the non-sGC-cGMP pathways such as S-nitrosylation. The biological function of NO was initially ascribed to the activation of sGC through formation of a heme-nitrosyl, and subsequent production of cGMP as a second messenger. Another major bioactivity of NO is S-nitrosylation, the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine, forming S-nitrosothiols (SNOs) (34). NO can react with intracellular antioxidant glutathione (GSH) to form S-nitrosoglutathione (GSNO). GSNO exists in equilibrium with protein SNOs and can be degraded by the ubiquitously expressed GSNO reductase (GSNOR), also known as alcohol dehydrogenase 5 (ADH5). By reducing the intracellular concentration of GSNO, GSNOR indirectly regulates SNOs and thus serves as a key denitrosylase (Figure 1.2).
Figure 1.2 GSNOR mediated NO signaling. Nitric oxide (NO) is a free radical signaling molecule which is synthesized by nitric oxide synthase (NOS). NO then undergoes metabolism to form S-nitrosoglutathione (GSNO). GSNO can be degraded by S-nitrosoglutathione reductase (GSNOR), or it can also undergo S-nitrosylation to form protein-SNOs (S-nitrosylated proteins). The knockdown of GSNOR can lead to the accumulation of GSNO, thus the increase of S-nitrosylation.

Impaired denitrosylation, as manifested in GSNOR\(^{-/-}\) mice, alters multiple stem cell characteristics, including lower hematopoietic stem cell number and decreased MSC endothelial differentiation (35;36). As for the intact animal, GSNOR\(^{-/-}\) mice were more vulnerable to endotoxic shock (37), had higher incidents of hepatic cancer (38) and deficiency in lymphocyte development (39), but were protective from myocardial injury (36) and asthma (40) compared to wild type mice. However, the role of GSNOR mediated S-nitrosylation in bone and fat homeostasis was not characterized before.

S-nitrosylation can modulate gene expression by direct modification of various transcription factors, including HIF-1(36), NF-κB (41) and estrogen receptor (42). SNOs
inhibit the DNA-binding activity of the zinc-finger transcription factors, such as vitamin D receptor (VDR) and RXR (43). It has been proposed that this inhibition is due to the S-nitrosylation of a thiolate group within the zinc finger. This posttranslational modification changes the coordination of zinc ion with cysteines, thus disrupting the zinc-finger structure (44). PPARγ can be S-nitrosylated in murine mesangial cells (45). However, the functional aspect of PPARγ S-nitrosylation is still unknown.

1.3.2 The role of NO in adipogenesis and osteogenesis

NO enhances adipogenesis of human preadipocytes while decreasing cell proliferation (46). Adipogenic differentiation of mesenchymal cells is also inhibited by the suppression of NO production (47). In addition, NO is a crucial regulator of osteoblast proliferation and differentiation. Studies in mice with a targeted deletion of NOS1, NOS2 or NOS3 manifest distinct bone effects. NOS1−/− mice have higher bone mass with decreased bone remodeling in the absence of defects in osteoblast proliferation or differentiation, suggesting a non-cell-autonomous mechanism (48). NOS2 modulates cytokine secretion and thereby affects osteoblast differentiation (49). NOS2 deficiency also causes impaired fracture healing in a mice model of femoral midshaft osteotomy (50). Osteoblasts from NOS3−/− mice have lower proliferation and alkaline phosphatase expression, consistent with decreased bone mass (51). NO is also a key osteoblast-osteoclast coupling factor with a potent inhibitory effect on bone resorption (52). Nitroglycerin, an NO donor commonly used for angina pectoris, relieves bone loss (53;54). These observations suggest that NO plays an important role in regulating the balance between adipocyte and osteoblast differentiation, and eventually mediating bone health.
1.4 Basics of skeletal homeostasis

1.4.1 Bone remodeling

Bone remodeling happens to replace old or damaged bone in order to maintain a healthy skeletal state. Bone remodeling starts with activation of bone resorption by osteoclasts. First, osteoclasts originated from hematopoietic progenitors are recruited to the remodeling site and excavate the calcified matrix. Then osteoblasts differentiated from MSCs assemble at the cavity and bone formation begins. With the progression of bone formation, some osteoblasts embed into the matrix and become osteocytes. Bone formation terminates when the cavity is refilled (11). An essential component of a balanced remodeling is that the amount of newly generated bone is the same as the amount removed, which is referred to as coupling.

1.4.2 Hormonal regulation of bone

Bone is the largest reservoir of minerals such as calcium and phosphate in the body, thus it plays a pivotal part in mineral homeostasis by coupling bone formation and bone resorption. Parathyroid hormone (PTH), secreted by parathyroid gland, is an important hormone to regulate calcium homeostasis. In response to hypocalcemia, PTH secretion is enhanced in order to restore a normal level of calcium. PTH increases bone efflux of calcium and phosphate, thus serves as a potential contributor to bone loss seen in patients with hyperparathyroidism. PTH also enhances the calcium reabsorption at the expense of phosphate reabsorption in the kidney. In addition, calcium and phosphate absorption are increased in small intestine in response to PTH. As a consequence, PTH restores the calcium homeostasis in coordination with bone, kidney and small intestine (55).
PTH is also a stimulator of osteoclast differentiation and bone resorption. However, PTH is clinically used to treat severe bone loss due to its distinct effects based on the duration of treatment. Intermittent administration of PTH can stimulate bone formation via reactivation of osteoblast differentiation and inhibition of osteoblast apoptosis (56). Intermittent treatment of PTH also inhibits adipogenesis of human bone marrow cells while enhancing osteoblast formation (57). Continuous PTH infusion, on the other hand, enhances bone resorption and bone loss through activation of osteoclasts (58).

Another important hormone that regulates calcium and phosphate homeostasis is fibroblast growth factor (FGF23). FGF23 rises dramatically in osteomalacia (soft bone caused by defective bone mineralization secondary to inadequate phosphate and calcium) and mineral/bone disorder in chronic kidney disease (59). FGF23 is secreted by osteocytes (mature bone cells derived from osteoblasts). As a phosphaturic hormone, FGF23 decreases renal tubular phosphate reabsorption. It can also indirectly affect calcium reabsorption in the kidney by modulating the levels of Klotho, a FGF23 coreceptor (55;60). A recent report showed that FGF23 directly promotes renal calcium reabsorption through the TRPV5 channel (61). FGF23 also inhibits the parathyroid gland secretion of PTH (55). The interplay between FGF23 and PTH is crucial in the homeostasis of bone mineral metabolism.
Chapter 2. Methods

2.1 Mice

GSNOR\(^{-/-}\) mice were generated as previously described (37). WT C57BL/6J mice were purchased from the Jackson Laboratories. Two month-old male mice were used in this study. Mice were fed standard normal chow containing 4% fat ad libitum unless stated otherwise. For the rosiglitazone study, mice were fed with rosiglitazone mixed chow (Harlan, 150 mg rosiglitazone/kg chow) for 1 week.

2.2 Bone analyses

Fat mass, lean mass, and BMD were measured by dual-energy x-ray absorptiometry (DEXA). \(\mu\)CT was done as described (62). Quantitative histomorphometric analysis was conducted in a blinded fashion with the OsteoMeasure morphometry system (Osteometrics). To label mineralization deposition, sequential subcutaneous injections of 12 mg/ml calcein (Sigma-Aldrich; 20 mg/kg body weight) and 6 mg/ml demeclocycline (Sigma-Aldrich; 15 mg/kg body weight) in 2% sodium bicarbonate solution were performed. Calcein and demeclocycline were injected 9 days and 2 days, respectively, before the mice were euthanized. Static parameters of bone formation and resorption were measured in a defined area between 181 \(\mu\)m and 725 \(\mu\)m from the growth plate. For dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured in unstained sections under ultraviolet light, using a B-2A set long-pass filter consisting of an excitation filter ranging from 450 to 490 nm, a barrier filter at 515 nm, and a dichroic mirror at 500 nm. BFR was calculated. The terminology and units used are those recommended by the
2.3 Plasma and Urine parameters

24-hour urine was collected from individual mice housed in a metabolic chamber (Tecniplast). Mice were given two days to adapt to the metabolic cage and 24h urine was collected on the third day, every 12 hours. Serum was collected from these mice by cardiac puncture followed by centrifugation of the blood at 1500 g for 20 minutes. Concentrations of plasma and urinary inorganic calcium and phosphate were determined by Vitros Dry Slide Chemistry Analyzer (Ortho-Clinical Diagnostics Inc.). Ionized calcium was measured using VetScan i-STAT 1 handheld analyzer (Abaxis Inc.). Concentration of urine creatinine was determined by Vitros Dry Slide Chemistry Analyzer. Plasma FGF23 concentrations were measured using the FGF-23 ELISA Kit (Immutopics Inc.). Plasma PTH concentrations were measured using the PTH ELISA kit (Immutopics Inc.).

2.4 Three point bending test

The left femurs and tibias were tested in an Instron testing machine (Model 3344, Instron Corp.). Fresh-frozen bones were thawed to room temperature (22°C). For the femurs, the anterior cortex at the middiaphysis was placed in compression and the posterior cortex in tension during the test; for the tibias, the posterolateral cortex at the middiaphysis was placed in compression and the anteromedial cortex in tension. The lower support points were separated by an extent of 50% of the femoral length (femur length/2) and by 63% of the tibial length (tibia length/1.6). A constant displacement rate of 0.03 mm/s was applied until the bone fractured. Fracture was taken as complete loss of
load-carrying ability. To stabilize the specimen, a small preload (5% of the average maximal load) was applied before actual testing. During the bending test, load-displacement data were collected by a computerized data-acquisition system at a sampling rate of 80 Hz. The biomechanical properties evaluated were the maximum load (a measure of the maximum force that the bone withstood before fracture [N]), resilience (a measure of the ability of a bone to suffer elastic deformity [J]), Young’s modulus (mPa), and stiffness (N/mm) (which are measures of the extrinsic rigidity of the bone tissue), and ductility (a measure of the ability of bone to resist to the propagation of cracks).

2.5 Bone marrow mesenchymal stem cells collection and characterization

All cells were maintained under a 37°C/5% CO₂ atmosphere. MSCs were grown with 20% (vol/vol) FBS (Atlanta Biologicals), 1% (vol/vol) penicillin and streptomycin, and αMEM (Invitrogen). BMMNCs were maintained with 10% (vol/vol) FBS, 1% (vol/vol) penicillin and streptomycin, and αMEM (Invitrogen). HEK-293T cells were cultured with 10% (vol/vol) FBS, 1% (vol/vol) penicillin and streptomycin, and DMEM (Invitrogen).

2.5.1 Cell collection

Femur and tibia were dissected, cleaned off muscle and cut off two ends. MSCs were collected by flushing the bone marrow with MSC medium (with antibiotics P/S) through a 40μm cell filter (Figure 2.1A). Then the cell suspension was centrifuged and treated with red blood cell lysis buffer (Sigma) for 10 minutes with vortex every 2 minutes. Cells were centrifuged again and plated in a 25mm flask and the medium was changed after 3 days in culture to let the cells fully attached to the culture flask. It usually
takes 1-2 months for the primary culture to reach confluency and ready for the first passage. During that period of time, change half of the culture medium every 2-3 days.

2.5.2 Cell characterization

Cells were characterized by FACS analysis of CD34, CD45, CD90, CD73, CD105, Sca-1 and Lin (35) (Figure 2.1B). Also, cells were able to differentiate into adipocytes, osteoblasts and chondrocytes measured by Oil red O staining, Alizarin red S staining and Alcian blue staining respectively.

Figure 2.1 Mesenchymal stem cell collection and characterization. (A) Collection of MSCs from bone marrow of mice. (B) Flow cytometry characterization of MSCs (35).

2.5.3 FACS analysis

MSCs were trypsinized with 0.05% Trypsin for 5 minutes in the incubator. Cell pellets were collected and resuspended with PBS to wash. Cell suspension was
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centrifuged again and resuspended with 1ml FACS buffer (PBS+1% BSA+5% FBS). Cells were counted and divided into 1 million cells per FACS tube. 1 million cells/sample were centrifuged again and resuspended in 100µl of FACS buffer. Antibody (1.5-2µl) was added and the mixture was incubated on ice for an hour and protected from light (vortex every 10 minutes). After an hour, added PBS to top, centrifuged and washed with 3ml PBS again. Cell pellet was resuspended in 400µl of PBS and sent for FACS analysis.

2.6 Ex vivo bone marrow MSC differentiation

MSCs generated from WT and GSNOR+/− mice were cultured for 14 days in adipogenic differentiation medium (containing 1 μM dexamethasone, 10 μg/ml insulin, 100 μM, indomethacin, and 0.5 μM 1-methyl-3-isobuthyxanthine in 10% FBS 1% P/S DMEM medium, start differentiation when 100% confluence) or osteogenic differentiation medium (containing 100 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerophosphate in 10% FBS 1% P/S alpha-MEM medium, supplements added to medium freshly, start treatment when 50%-70% confluence). At the end of differentiation, fat-droplet formation in adipogenic differentiation was stained and quantified by ORO staining, and calcium formation in osteogenic differentiation was analyzed by ALS staining. Baseline staining served as negative control.

2.7 Oil Red O and Alizarin Red S staining and quantification

For ORO staining, cells were rinsed with PBS and fixed with 10% formalin for 30-60 minutes. After fixation, cells were rinsed with H2O and could be stored in the fridge for future staining. ORO staining working solution was prepared freshly by mix three parts of ORO staining stock solution (300mg of Oil Red O powder in 100mL 99% isopropanol, stable for 1 year) with two parts of DI water. The mixture was incubated for
10 minutes at room temperature and filtered by Whatman filter paper. Each well was rinsed with H2O and incubated with 60% isopropanol for 5 minutes. Then cells were incubated with ORO working solution for 20 minutes. Then stain was rinsed off carefully with H2O. For ALS staining, cells were first rinsed with PBS and fixed with 70% ethanol for 30-60 minutes. After fixation, cells were rinsed with H2O and could be stored in the fridge for future staining. Calcium deposition staining was done by incubation with 40mM alizarin red-S, pH 4.2 for 5 minutes. Rinse off stain very gently with H2O, as calcium deposition layer may detach from the bottom of 12-well cell culture plate. ORO quantification was performed using ImageJ (63). Pictures first underwent “split channels” to generate a clear contrast. Then the contrast between positive and negative staining was adjusted evenly using “threshold.” The percentages of positive staining areas were automatically produced by the “analyze particles” function. ALS quantification was conducted by absorbing the dye with 10% cetylpyridinium chloride buffered with 10 mM sodium phosphate (pH 7) and measuring absorption at 550 nm with a spectrophotometer.

2.8 In vivo osteogenic differentiation

Approximately 2.0 × 10^6 MSCs were loaded with GelFoam (5 mm × 5 mm × 5 mm; Pfizer) as a carrier by squeezing the GelFoam sponge and putting it into the 50µl cell suspension for 20 minutes in the cell culture incubator to let the cells fully absorbed into the GelFoam. Then, GelFoam was subcutaneously implanted into the dorsal surfaces of 2- to 4-month-old female NOD-SCID mice as described previously (64). Briefly, an under skin pocket was created surgically and GelFoam was put into the pocket. At 7 weeks after implantation, the implants were harvested, fixed in formalin and H&E staining of the histological sections was analyzed using ImageJ.
2.9 RNA extraction and qRT-PCR

MSC RNA was extracted using the RNasy kit (QIAGEN) according to the manufacturer’s instructions. RNA from WAT was extracted using the RNAeasy Lipid Tissue Mini Kit (QIAGEN). Gene expression was determined using TaqMan Gene Expression Assays. First-strand cDNA was prepared using a high-capacity cDNA reverse transcriptase kit (Applied Biosciences, Life Technologies). Reverse-transcribed cDNA was used for quantitative real-time PCR (RT-PCR) using pre-designed TaqMan probes for mouse adipogenic markers (Pparg, adiponectin, Fabp4, Cd36, Cebpα, Cebpβ, and Cebpδ) and for osteogenic markers (Runx2, osteopontin, and osteocalcin) as well as the internal control Gusb. The actual number of transcripts was calculated by ΔCt. Fold changes were calculated by the ΔΔCt method using endogenous controls for normalization (65).

2.10 Western blot

For protein extraction, MSCs were homogenized in RIPA buffer with protease inhibitors. The cell lysates were centrifuged at 14,000 g for 15 minutes at 4°C, and the supernatant was boiled in 4× sample buffer and analyzed by SDS-PAGE (Invitrogen), as described in the manufacturer’s protocol. For primary Ab against PPARγ (Santa Cruz Biotechnology Inc., clone H100), an HRP-conjugated secondary Ab (Promega) was used.

2.11 SNO-RAC to measure S-nitrosylation

SNO-RAC assay was performed in the dark as described (66). Basically, SNO-RAC contains three steps: 1) block the free thiols by methylthionylation, 2) reduce the SNOs to thiols with ascorbate, 3) capture the newly-formed thiols with thiol reactive resin (Figure 2.2). Dark brown tubes were used and experiments were conducted in the dark room when possible. MSCs were homogenized in HEN buffer (250 mM Hepes, 1
mM EDTA, and 0.1 mM neocuproine, pH 7.7). Free cysteine residues were blocked with MMTS in the presence of SDS for 20 minutes with frequent vortex. Then the protein lysate was incubated with 100% acetone at -20 degree for 20 minutes and centrifuged at 15000g for 20 minutes. The pellet was washed with 70% acetone and resuspended in HENS buffer. A thorough resuspension combining vortex and pipetting could yield the best results. 10 µl of input samples were taken from each tube. Thiol reactive resin (Sigma-Aldrich) was activated by incubating with water for half an hour. Sodium ascorbate (Sigma-Aldrich, at a final concentration of 20mM) was added and omission of ascorbate served as a negative control. Protein lysate was incubated with thiol-reactive resin (Sigma-Aldrich) for 3-5 hours in the dark. After incubation, the resin-protein complex was washed with HEN buffer for five times. Resin-captured proteins were eluted using 50 µl elution buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 100 mM β-mercaptoethanol) at room temperature with frequent agitation and heated at 95°C for 5 minutes in non-reducing SDS–PAGE loading buffer. The expression of PPARγ was determined by Western blot analysis as mentioned above.

![Figure 2.2 Illustration of SNO-RAC assay. Pictures adapted from reference (66).](image)
2.12 Chromatin Immunoprecipitation (ChIP)

ChIP *Fabp4* promoter sequence was provided by F. Picard (Université Laval, Quebec, Canada). WT MSCs were treated with increasing concentrations of GSNO for 5 hours and then harvested for ChIP analysis, as previously described (Millipore EZ-ChIP kit Catalog number 17-371). Briefly, cells were crosslinked in formaldehyde and sonicated to generate 200-1000 bp pieces. Then DNA-bound proteins were enriched with antibody incubation. Crosslinks of DNA were reversed and DNA was detected by standard PCR or quantitative real time PCR. Chromatin was immunoprecipitated with an anti-PPARγ Ab (Santa Cruz Biotechnology Inc., clone H100). RT-PCR amplification of the immunoenriched DNA samples was performed using primers for the *Fabp4* promoter (5′-ATGTCACAGGCATCTTATCCACC-3′ and 5′-AACCCTGCAAAAGAGACAGAGG-3′) and detected by agarose gel electrophoresis and RT-PCR.

2.13 Luciferase assay

The PPRE luciferase construct and PPARγ overexpression construct were obtained from Addgene (originally provided by B. Spiegelman, Harvard Medical School, Boston, Massachusetts, USA). Luciferase assay was performed according to the protocol of the Dual-Luciferase Reporter Assay System (Promega). Briefly, 293T cells were cotransfected with PPRE construct, renilla reporter and either empty plasmid or PPARγ overexpression vector. After 24 hours of transfection, cells were lysed for luminescence detection. The PPRE sequence was from the Spiegelman group: PPRE × 3 (5′-GTCGACAGGGACCAGGACAAAGGTCACGTTCGGGAGTCGAC, 3 copies). Cells were lysed in 200 μl Passive Lysis Buffer (Promega). Duplicate 20 μl samples were
mixed with 100 μl of Luciferase Assay Reagent (Promega). Luciferase activity was measured using a SIRIUS luminometer (Berthold Detection System, v3.1). Luminescence in each sample was normalized to Renilla luciferase activity.

2.14 Mutagenesis

Cysteine-to-serine mutations were constructed by GENEWIZ. HEK-293T cells were transfected with WT and mutant PPARγ by Lipofectamine 2000. 24 hours after transfection, cell lysates were collected and SNO-RAC was performed as described above. Cell lysates were treated with 100 μM GSNO for 10 minutes prior to SNO-RAC.

2.15 Immunofluorescence

Cells were fixed in 4% paraformaldehyde, permeabilized, and blocked with 0.3% TritonX-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 min. Cells were then incubated with primary antibodies: rabbit PPARγ mAb (Cell Signaling) overnight at 4°C and then with anti-rabbit 568-conjugated secondary antibodies (Invitrogen) for 1 h in the dark at room temperature. Nuclear labeling was obtained with DAPI. The slides were mounted in Prolong Gold anti-fade reagent (Invitrogen). Images were obtained using a Zeiss LSM-710 confocal microscope (Analytical Imaging Core Facility, University of Miami). A negative control (PBS containing 1% BSA and 10% donkey serum with no primary antibody) was included in every experiment.

2.16 Osteoclast differentiation

Bone marrow mononuclear cells (BMMNCs) were generated by culturing bone marrow cells with mouse colony stimulating factor (25ng/ml M-CSF, Sigma). BMMNCs
were cultured in osteoclastogenic differentiation medium (containing 25ng/ml M-CSF and 100ng/ml RANKL, Millipore) for 6 days and TRAP staining was conducted using the TRAP kit (Sigma) according to the manufacturer’s instructions. The number of TRAP positive osteoclasts was counted using Image J software (National Institutes of Health).

2.17 Statistical analysis

Comparisons of 2 groups were performed using 2-tailed, unpaired Student’s t test and presented as mean ± SEM. Means of more than 2 groups were compared by 1-way ANOVA or by 2-way ANOVA when 2 conditions were involved. Bonferroni’s post hoc tests were applied when appropriate. P < 0.05 was considered significant.
Chapter 3 Loss of GSNOR function hinders adipogenic differentiation and enhances osteogenic differentiation of MSCs

3.1 GSNOR−/− MSCs has decreased adipogenesis

3.1.1 Comparison of the adipogenic differentiation ability of WT and GSNOR−/− MSCs

MSCs were isolated and characterized as described in the method section and were subjected to adipocyte differentiation conditions in vitro for 14 days. GSNOR−/− cells grown in adipogenic medium had significantly less oil red O (ORO) staining, indicating a lower propensity to form fat droplets than WT cells (Figure 3.1).

**Figure 3.1 Adipogenic differentiation of WT and GSNOR−/− MSCs.** Oil Red O (ORO) staining and quantification of WT and GSNOR−/− cells. Scale Bar: 100µm. *** P<0.001; n=6.

Expression of the adipogenic marker *Pparg* increased in both WT and GSNOR−/− cells following differentiation, but this increase was attenuated in GSNOR−/− cells (Figure 3.2A). Expression of multiple adipogenic transcription factors, such as CCAAT/enhancer-binding proteinβ (*Cebpb*), an upstream regulator of PPARγ, was decreased in GSNOR−/− MSCs (Figure 3.2A) as was expression of the downstream adipogenic effector of PPARγ, fatty acid–binding protein 4 (*Fabp4*) (Figure 3.2A). CD36, a membrane-bound fatty acid translocase and downstream target of PPARγ, was also markedly decreased (87-fold) in GSNOR−/− MSCs (Figure 3.2B). These results indicate
that both the upstream and downstream of PPARγ signaling were suppressed in GSNOR−/− MSCs following adipogenic differentiation.

Figure 3.2 Adipogenic gene expression of WT and GSNOR−/− MSCs. (A) Expression of adipogenic genes in WT and GSNOR−/− MSCs grown in adipogenic medium. n=5-7. *** P<0.001, * P<0.05 compared to WT. (B) Expression of CD36 in WT and GSNOR−/− MSCs grown in adipogenic medium. n=6. * P<0.05.

3.1.2 The effects of GSNOR inhibitor on adipogenesis

To further probe the impact of GSNOR signaling, we incubated GSNOR−/− and WT MSCs with the GSNOR inhibitor 4-[[2-[(2-cyanophenyl)methyl]thio]-4-oxothieno-[3,2d] pyrimidin-3(4H)-yl]methyl]-benzoic acid (50 μM and 100 μM) (67). This inhibitor can exclude GSNOR substrate GSNO from its binding site in GSNOR through noncompetitive or uncompetitive binding and thus cause the accumulation of SNOs. It can selectively inhibit GSNOR among other alcohol dehydrogenases (67). GSNOR−/− cells were not affected by the inhibitor. In contrast, WT MSCs exhibited significantly decreased expression of Pparg and Fabp4, mimicking the knockout genotype (Figure 3.3 A,B). Furthermore, the GSNOR inhibitor decreased ORO staining in WT cells, mimicked the inhibited fat droplet formation phenotypes in GSNOR−/− cells (Figure 3.4A,B).
3.1.3 The effects of NO donor and NOS inhibitor on adipogenesis

To further study the effects of NO synthesis on adipogenesis, cells were treated with NO donor and NOS inhibitor during 2 weeks of differentiation. GSNO can serve as a NO donor or a GSH donor and induce multiple responses including S-nitrosylation, glutathionylation, tyrosine nitration and heme-NO formation. Treatment with the NO donor GSNO (100µM) inhibited fat-droplet formation in WT cells without affecting Pparg expression (Figure 3.5). Surprisingly, fat droplet formation in WT cells was also
reduced after treating with the pan-NOS inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 30 μM) (Figure 3.5 A,B), suggesting that a physiological level of NO is crucial to maintain adipogenic capacity. Adipogenic differentiation of GSNOR−/− cells was not affected by treatment with 30 μM L-NAME (Figure 3.5). Together, these results indicate that loss of GSNOR function inhibits adipogenic differentiation of MSCs.

Figure 3.5 The effects of NO donor and inhibitor on MSC adipogenic differentiation. (A) Oil Red O staining of WT BMMSCs grown in adipogenic medium and treated with NO donor GSNO (100μM), or pan-NOS inhibitor L-NAME (30μM) for 2 weeks and GSNOR−/− BMMSCs treated with L-NAME. Scale Bar: 100μm. (B) Oil Red O staining was quantified and normalized to baseline staining. (C) Expression of PPARγ after drug treatment. n=3 per group (A-C). * P<0.05, ** P<0.01, *** P<0.001 compared to WT.
3.2 GSNOR<sup>−/−</sup> MSCs has increased osteogenesis

3.2.1 Comparison of the osteogenic differentiation ability of WT and GSNOR<sup>−/−</sup> MSCs

We next examined the role of GSNOR in osteogenic differentiation of MSCs. WT and GSNOR<sup>−/−</sup> MSCs were grown in osteogenic medium for 2 weeks, and Alizarin red-S (ALS) staining, indicative of calcium deposition, was quantified. GSNOR<sup>−/−</sup> cells produced significantly more calcium compared with WT cells (Figure 3.6A) and showed increased expression of the late osteogenic marker osteocalcin (Bglap) (Figure 3.6B). However, expression of the early osteogenic markers Runx2 and osteopontin (Spp1) was not different between WT and GSNOR<sup>−/−</sup> cells at this time point (Figure 3.6 C,D), likely due to osteogenic differentiation having already reached the late mineralization stage (Figure 3.6A).

![Figure 3.6 Osteogenic differentiation of WT and GSNOR<sup>−/−</sup> MSCs. (A) Whole well scan of Alizarin Red-S (ALS) staining of WT and GSNOR<sup>−/−</sup> MSCs. ALS staining was quantified. ** P<0.01; n=6. (B) Expression of Bglap (Osteocalcin) in MSCs grown in osteogenic medium. * P<0.05; n=6. (C, D) Expression of Runx2 (C), Spp1 (Osteopontin, D). n=6.](image-url)
Therefore, we examined early osteogenic markers in MSCs prior to differentiation and found higher expression levels of Runx2 (Figure 3.7A), Spp1 (Figure 3.7B), and Bglap (Figure 3.7C) in GSNOR$^{-/-}$ cells compared with WT.

![Image of bar charts](image)

**Figure 3.7 Osteogenic gene expression at baseline.** Expression of Runx2 (A), Spp1 (Osteopontin, B) and Bglap (Osteocalcin, C) at baseline in the absence of osteogenic supplements. * P<0.05, n=6.

### 3.2.2 The effects of GSNOR inhibitor on osteogenesis

Treatment of WT MSCs with the GSNOR inhibitor (100µM) significantly increased osteogenic differentiation as measured by ALS staining, while no effect was seen in GSNOR$^{-/-}$ cells (Figure 3.8A). It suggests that the effects of GSNOR deficiency in the GSNOR$^{-/-}$ cells could not be further enhanced by GSNOR inhibitor treatment. We also tested the effects of GSNOR inhibitor on the osteogenic genes expression. GSNOR inhibitor significantly increased the expression of Bglap but not Spp1 and Runx2 (early osteogenic markers) in WT cells (Figure 3.8B-D).
3.2.3 The effects of NO donor and NOS inhibitor on osteogenesis

We further tested the effects of NO donor and NOS inhibitor on osteogenesis. Osteogenic differentiation of GSNOR$^{-/-}$ MSCs was reduced by NOS inhibitor L-NAME as measured by Alizarin Red S staining and Bglap expression, but osteogenic differentiation of WT cells was not affected by either L-NAME or GSNO (Figure 3.9).
Figure 3.9 The effects of NO donor and inhibitor on MSC osteogenic differentiation. (A) WT BMMSCs were grown in osteogenic medium and treated with NO donor GSNO (100µM), or pan-NOS inhibitor L-NAME (30µM) for 2 weeks. GSNOR−/− BMMSCs were treated with L-NAME. Alizarin Red-S staining was performed and quantified. (B) Cells were treated similarly in A and expression of Bglap assessed by quantitative real-time RT-PCR analysis. n=3 per group. * P<0.05 compared to WT, # P<0.05 compared to GSNOR−/−.

3.2.4 MSC-induced bone regeneration in vivo

Next, we assessed the impact of GSNOR on MSC differentiation in vivo and compared the in vivo bone regeneration capacity of WT and GSNOR−/− MSCs following subcutaneous implantation of cells within a GelFoam plug (ref (64) and Figure 3.10A). After 7 weeks, cell implants were removed and H&E staining was performed to visualize the bone formation. GSNOR−/− MSCs implants formed a hard, bony structure while WT MSCs implants still maintained a gel-like structure (Figure 3.10 B).

Figure 3.10 MSC-based bone regeneration in vivo. (A) Scheme for assessing MSC-based bone regeneration in vivo. (B) Pictures of implants upon removal after 7 weeks.
H&E staining quantification of bone area showed that MSC-mediated bone formation was significantly greater in GSNOR<sup>−/−</sup> cell implants (Figure 3.11A,B). These data demonstrate that the enhanced osteogenic differentiation capacity of GSNOR<sup>−/−</sup> MSCs directly causes higher bone regeneration in vivo. Taken together, these results suggest that increased SNO bioavailability enhances the osteogenic potential of MSCs and that GSNOR<sup>−/−</sup> MSCs are more committed toward the osteoblast lineage.

**Figure 3.11 Bone regeneration staining and quantification.** (A) H&E staining of tissue samples from NOD-SCID mice after subcutaneous implantation with WT or GSNOR<sup>−/−</sup> MSCs following the protocol described in Figure 3.10 (A). H&E staining was quantified (B). Formation of bone (B) and connective tissue (CT) around Gelfoam (G) are indicated. Scale bar: 500µm. n=3. (B) Semi-quantitative analysis of new bone formation. n=3.
Chapter 4. Loss of GSNOR function alters body composition and bone remodeling

4.1 Characterization of the body composition of GSNOR\textsuperscript{−/−} mice

We further sought to address the impact of altered lineage bifurcation of GSNOR\textsuperscript{−/−} MSCs on the phenotype of intact animals. Two-month old male GSNOR\textsuperscript{−/−} mice were smaller and weighed less than corresponding WT mice (Figure 4.1A,B). Over the next 11 months, GSNOR\textsuperscript{+} mice maintained this relatively lower body weight (Figure 4.1B). Food intake was not altered in GSNOR\textsuperscript{−/−} mice (Figure 4.1C). GSNOR\textsuperscript{−/−} mice had a lower percentage of fat mass (Figure 4.1D) and a higher percentage of lean mass, but lower lean mass weight (Figure 4.1E).

Figure 4.1 Body weight, food intake and body composition of WT and GSNOR\textsuperscript{−/−} mice. (A,B) Whole body morphology and body weight of male WT and GSNOR\textsuperscript{−/−} mice. ** P<0.01 ***P<0.001; n=6. (C) Food intake of WT and GSNOR\textsuperscript{−/−} mice measured in metabolic cage. n=6. (D, E) Percent and weight of fat mass (D), percent and weight of lean mass (E) of 2-month-old male mice were measured by dual energy X-ray densitometry. n=6.

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We also measured adipocyte size, since weight gain is caused largely by adipocyte hypertrophy, a process that contributes to the enlargement of adipose tissue (25). GSNOR−/− epididymal adipocytes were, on average, significantly smaller than WT adipocytes (Figure 4.2).

![Image]

**Figure 4.2 Adipocyte surface area quantification.** Adipose tissue was stained with H&E and adipocyte surface area was quantified. Scale bar: 100µm. n=3.

In contrast to MSCs, expression of *Pparg* and its downstream target *Fabp4* were unchanged in GSNOR−/− white adipose tissue (WAT) (Figure 4.3). Interestingly, expression of *Cebpd*, an upstream regulator of PPARγ, was increased in GSNOR−/− WAT, but that of *Cebpb* was not (Figure 4.3). Together, these results show that intact GSNOR−/− animals have a phenotype consistent with impaired adipogenesis.
Figure 4.3 Adipogenic genes expression in white adipose tissue (WAT). WT n=5 and GSNOR<sup>−/−</sup> n=4, * P<0.01 compared to WT.

4.2 Characterization of the bone phenotypes of GSNOR<sup>+/−</sup> mice

4.2.1 Bone formation

We next performed histological and μCT analyses of bone samples to assess bone formation and resorption in intact animals, testing the prediction that the GSNOR<sup>−/−</sup> mice exhibit increased bone formation. Calcein double staining labeled the bone formation surfaces during two calcein injections. Analysis of calcein double labeling by measuring the distance between two calcein labels demonstrated a higher dynamic of bone formation rate (BFR) in GSNOR<sup>−/−</sup> mice relative to WT (Figure 4.4A). Consistent with these histological findings, parameters of bone formation and mineralization were increased in GSNOR<sup>−/−</sup> mice (Figure 4.4B,C and Table 4.1), including osteoblast surfaces per bone surface (Ob.S/BS), mineralization apposition rate (MAR), and BFR/BS.
Figure 4.4 Staining of mineralization front and histomorphometric analysis of bone formation and mineralization in distal femur. (A) Calcein double labeling of the distal femur, the mineralization front was stained in green. Scale bar: 200µm. n=3. (B,C) Quantification of histomorphometric analysis of osteoblast content (B) and mineralization (C) in the femur. WT n=8 and GSNOR^{−/−} n=6.

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Table 4.1 Histomorphometric analysis of femur from WT and GSNOR^{−/−} mice. n=8 (WT) and n=6 (GSNOR^{−/−}).
4.2.2 Bone mineral density

Surprisingly and paradoxically, GSNOR<sup>−/−</sup> mice had lower overall bone mineral density (BMD) compared with WT mice (Figure 4.5A). μCT imaging of the vertebrae and femur demonstrated that the GSNOR<sup>−/−</sup> mice had reduced amounts of trabecular bone compared with WT mice (Figure 4.5B).

Figure 4.5 Bone mineral density and representative images of vertebra and femur. (A) Whole body bone mineral density (BMD). n=6. (B) Representative images of the vertebra and femur. n=3.

More specifically, GSNOR<sup>−/−</sup> mice had significantly less trabecular bone volume per total volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N; Figure 4.6A-C). Trabecular separation (Tb.Sp), a measurement of average thickness of the marrow cavities, was higher in GSNOR<sup>−/−</sup> mice (Figure 4.6D). BMD of cortical bone was also significantly reduced (Table 4.2).

Figure 4.6 Micro CT analysis of bone volume. Quantification of μCT analysis of bone volume (A), trabecular thickness (B), trabecular number (C) and trabecular separation (D). ** P<0.01, *** P<0.001, WT n=5 and GSNOR<sup>−/−</sup> n=4.
Table 4.2 µCT analysis of femur from WT and GSNOR<sup>−/−</sup> mice. n=5 (WT) and n=4 (GSNOR<sup>−/−</sup>).

4.2.3 Bone resorption

Bone histomorphometric analysis of the femur indicated that GSNOR<sup>−/−</sup> mice had significantly increased osteoclast surfaces (Oc.S/BS) and eroded surfaces (ES/BS), indicative of increased bone resorption (Figure 4.7 and Table 4.1). The increased rate of bone formation and bone resorption seen in GSNOR<sup>−/−</sup> mice indicated higher bone turnover, with a net result of lower bone volume and reduced BMD.

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<td>0.0023±8.029x10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td>0.0012±0.00016</td>
<td>0.0003</td>
</tr>
<tr>
<td>Tb. Th (μm)</td>
<td>56.30±1.267</td>
<td>48.32±1.299</td>
<td>0.0034</td>
</tr>
<tr>
<td>Tb. Sp (μm)</td>
<td>224.1±4.870</td>
<td>289.3±15.80</td>
<td>0.0033</td>
</tr>
<tr>
<td>BMD</td>
<td>1.667±0.020</td>
<td>1.593±0.004</td>
<td>0.0148</td>
</tr>
</tbody>
</table>

Figure 4.7 Histomorphometric analysis of bone resorption. Quantification of histomorphometric analysis of osteoclast content in the femur. WT n=8 and GSNOR<sup>−/−</sup> n=6.
To address the paradoxical findings of overall BMD in the context of increased osteoblast formation, we assessed the impact of GSNOR deficiency on osteoclasts. First, we verified our findings of increased bone resorption by measuring osteoclast number per bone perimeter (N.OC/B.Pm) in GSNOR<sup>−/−</sup> mice (Table 4.1). We obtained bone marrow mononuclear cells (BMMNCs) from WT and GSNOR<sup>−/−</sup> mice and grew them in osteoclast differentiation medium for 6 days.

Osteoclast differentiation was characterized by the presence of tartrate-resistant acid phosphatase–positive (TRAP-positive) cells containing more than 3 nuclei. The number of TRAP-positive osteoclasts was significantly increased in cultures of GSNOR<sup>−/−</sup> compared with WT BMMNCs (Figure 4.8), suggesting that GSNOR loss of function promotes osteoclastogenesis. This result is consistent with the enhanced bone resorption phenotype observed in GSNOR<sup>−/−</sup> mice.

**Figure 4.8** GSNOR<sup>−/−</sup> BMMNCs have enhanced osteoclast differentiation *in vitro*. (A) TRAP staining of WT and GSNOR<sup>−/−</sup> cells. Scale Bar: 100µm. (B) The number of TRAP-positive osteoclasts was quantitated.  n=3, * P< 0.05 compared to WT. Statistical significance was determined by unpaired student’s t-test (2-tailed) and presented as mean ± SEM.
4.2.4 Bone strength

To evaluate whether morphological phenotypes of the femur in GSNOR\(^{-/-}\) mice actually caused mechanical fragility, we performed a 3-point bending test. In GSNOR\(^{-/-}\) mice, maximum load and stiffness were both decreased (Figure 4.9A,B), indicating mechanical weakness in the femur of GSNOR\(^{-/-}\) mice. In addition, Young’s module and resiliency were lower in GSNOR\(^{-/-}\) mice (Figure 4.9C,D).

![Figure 4.9 Bone strength of WT and GSNOR\(^{-/-}\) mice. (A, B, C, D) Femur maximum load (A), stiffness (B), Young’s module (C) and resiliency (D). n=4.](image)

4.3 GSNOR\(^{-/-}\) mice has altered calcium and phosphate homeostasis

4.3.1 GSNOR\(^{-/-}\) mice has higher urinary excretion of calcium and phosphate

To gain additional insights into the paradoxical phenotype of enhanced bone resorption in the face of increased bone formation, we assessed calcium and phosphate homeostasis in the GSNOR\(^{-/-}\) mice, as extracellular calcium and phosphate are required for matrix mineralization and the maintenance of normal bone structure. As shown previously, GSNOR\(^{-/-}\) mice have lower BMD and a higher mineralization rate (Figure 4.4, 4.5). We first examined levels of parathyroid hormone (PTH), a key regulator of calcium and phosphate efflux from bone; indeed, PTH levels were higher in GSNOR\(^{-/-}\) mice (Figure 4.10).
Figure 4.10 PTH levels of WT and GSNOR<sup>−/−</sup> mice. **P<0.01. n=7.

Despite the elevated levels of PTH, both total and ionized calcium serum levels were not altered in GSNOR<sup>−/−</sup> mice (Figure 4.11A,B). Serum phosphate levels were also equivalent in GSNOR<sup>−/−</sup> and WT mice (Figure 4.11C).

We further measured the urinary excretion of calcium and phosphate. The urinary phosphate/creatinine ratio was higher in GSNOR<sup>−/−</sup> mice (Figure 4.12A), consistent with elevated PTH levels. Paradoxically, GSNOR<sup>−/−</sup> mice also had an increased urine calcium/creatinine ratio (Figure 4.12B), counter to the expected effect of elevated PTH. FGF23, a phosphaturic hormone secreted by osteocytes to enhance urinary calcium reabsorption (21, 22), was also increased in GSNOR<sup>−/−</sup> mice (Figure 4.12C). We attributed the increased urine calcium/creatinine ratio to the indirect effects of FGF23 to
inhibit PTH. Thus, elevations of urinary calcium and phosphate excretion have the potential to contribute to the low bone mass phenotype of GSNOR\(^{-/-}\) mice.

![Graph and Figures]

**Fig 4.12 Urinary calcium and phosphate levels and serum FGF23 levels.** (A) Urine phosphate/urine creatinine ratio. n=5-6. (B) Urine calcium/urine creatinine ratio. (C) Serum FGF23 level. Statistical significance was determined by t-test and presented as mean ± SEM. n=5-7. * P<0.05, ** P<0.01 compared to WT.

### 4.3.2 The effect of GSNOR inhibitor on calcium and phosphate homeostasis

To test the association of the changes in calcium and phosphate metabolism to alterations in PPAR\(\gamma\) in the GSNOR\(^{-/-}\) mice, we treated mice with the PPAR\(\gamma\) agonist rosiglitazone. While 1 week of rosiglitazone treatment did not alter the serum total calcium, ionized calcium, serum phosphate levels, or urinary calcium/creatinine ratio (Figure 4.13A-C) in either mouse strain, rosiglitazone did increase the urinary phosphate/creatinine ratio in WT but not in GSNOR\(^{-/-}\) mice (Figure 4.13D). Importantly, rosiglitazone reduced elevated PTH levels toward normal, yet further increased FGF23 levels in the GSNOR\(^{-/-}\) mice (Figure 4.13 F and G). Additional experiments to measure PTH in fasting mice revealed similar findings (Figure 4.13 H). Our results suggest that GSNOR deficiency alters the balance of PTH-FGF23 crosstalk, contributing to the high bone turnover and bone loss.
GSNOR\textsuperscript{−/−} mice have altered calcium and phosphate hormonal regulation in response to rosiglitazone. (A, B) Serum calcium level (A) and ionized calcium (iCa) level (B) in 2-month-old male WT and GSNOR\textsuperscript{−/−} mice. (C) Serum phosphate level in 2-month-old male WT and GSNOR\textsuperscript{−/−} mice. (D) Urine phosphate/urine creatinine ratio. (E) Urine calcium/urine creatinine ratio. (F) Serum FGF23 level. (G) Serum PTH level. (H) Fasting serum PTH level. Statistical significance was determined by two-way ANOVA and presented as mean ± SEM. n=5-7. * P<0.05 compared to WT, # P<0.05 compared to GSNOR\textsuperscript{−/−}, ### P<0.001 compared to GSNOR\textsuperscript{−/−} analyzed by Bonferroni’s multiple comparison test.
Chapter 5. S-nitrosylation of PPARγ decreases transcriptional activity of PPARγ

5.1 S-nitrosylation of PPARγ leads to decreased transcriptional activity

5.1.1 S-nitrosylation of PPARγ is enhanced in GSNOR<sup>−/−</sup> mice

We next evaluated the potential mechanism of impaired adipogenesis and enhanced osteogenesis seen in GSNOR<sup>−/−</sup> MSCs. PPARγ is a transcriptional master regulator of adipogenic differentiation and stimulates adipogenesis while inhibiting osteogenesis (23). We hypothesized that GSNOR loss of function increased PPARγ S-nitrosylation and decreased PPARγ function under basal conditions similarly to what occurs when PPARγ is S-nitrosylated in murine mesangial cells (45), consistent with PPARγ being a regulator of adipogenesis and osteogenesis. To test this hypothesis, we performed SNO resin-assisted capture (SNO-RAC) assays on undifferentiated MSCs from WT and GSNOR<sup>−/−</sup> mice (68). S-nitrosylated proteins were captured by thiol-reactive resin, and PPARγ S-nitrosylation status was assessed by immunoblotting. Although, as previously mentioned, *Pparg* mRNA expression from GSNOR<sup>−/+</sup> MSCs was higher following adipogenic differentiation (Figure 3.2), there was no difference in the protein expression of PPARγ in undifferentiated MSCs (Figure 5.1, total input lane). Importantly, GSNOR<sup>−/−</sup> MSCs demonstrated approximately 50% greater S-nitrosylation compared with WT cells under basal conditions (Figure 5.1, upper lane; the relative density ratio of S-nitrosylated PPARγ/total PPARγ in GSNOR<sup>−/−</sup> mice was 1.57-fold±0.33-fold compared with WT mice). The identification of endogenous SNO in the SNO-RAC assay was validated by the elimination of signals by UV irradiation applied prior to the assay (which cleaves the SNO bond) and the omission of ascorbate, which prevents SNOs from being reduced (69). These data demonstrate increased S-
nitrosylation of PPARγ as a potential mechanism for the decreased adipogenesis and enhanced osteogenesis of GSNOR−/− MSCs.

**Figure 5.1** GSNOR−/− MSCs have enhanced constitutive S-nitrosylation of PPARγ. SNO-PPARγ in MSCs of WT versus GSNOR−/− mice was measured by SNO-RAC assay; UV: UV light; Asc: Ascorbic Acid. Pretreatment with UV light and Omission of ascorbic acid were used as negative controls. * P<0.05; n=3. Statistical significance between two groups was determined by Student’s t-test. Representative blots show S-nitrosylated and total PPARγ. The relative ratio of S-nitrosylated PPARγ to total PPARγ in WT mice is arbitrarily defined as 1. The lanes were run on the same gel but were noncontiguous.

**5.1.2 S-nitrosylation of PPARγ decreases its transcriptional activity**

To determine whether enhanced S-nitrosylation of PPARγ affects its transcriptional activity, we used a luciferase reporter assay. 293T cells were cotransfected with firefly luciferase under the control of a PPARγ response element (PPRE) (70) plus either a plasmid encoding PPARγ or the empty vector control. Cells were treated with vehicle or the NO donor GSNO, widely used to augment S-nitrosylation in multiple cell types (71), for 5 hours (50 or 250 μM). GSNO treatment resulted in an increase of PPARγ S-nitrosylation without affecting PPARγ protein expression (Figure 5.2A,B) and also significantly decreased PPARγ luciferase activity (Figure 5.2C).
These results suggest that PPARγ transcriptional activity is decreased upon GSNO-induced S-nitrosylation. We further tested the effects of PPARγ agonist rosiglitazone on transcriptional activity of PPARγ in the presence of GSNO. Interestingly, GSNO-induced downregulation of PPARγ transcriptional activity was not rescued by rosiglitazone (Figure 5.2C), suggesting a ligand-independent mechanism.

Figure 5.2 PPARγ expression, S-nitrosylation and transcriptional activity after GSNO treatment in HEK-293T cells. (A) PPARγ protein expression in HEK-293T cells after GSNO treatment. (B) PPARγ protein S-nitrosylation in HEK-293T cells measured by SNO-RAC after GSNO treatment. Asc: Ascorbic Acid. n=3. *P<0.05 compared to no GSNO treatment control. (C) PPARγ luciferase activity in HEK-293T cells treated with GSNO in the presence or absence of rosiglitazone (Rosi, 1µM). * P<0.05 compared to PPARγ CTL, # P<0.05 compared to PPARγ Rosi; n=5.
5.1.3 Binding of PPARγ to its downstream target FABP4 is decreased upon S-nitrosylation

GSNOR−/− MSCs exhibited decreased expression of Fabp4 compared with WT MSCs following adipogenic differentiation (Figure 2.2). PPARγ is found in the cytoplasm, but nuclear translocation of PPARγ is crucial for its function (72). We tested the effects of S-nitrosylation on PPARγ localization. No difference in PPARγ nuclear localization was observed in WT and GSNOR−/− MSCs (Figure 5.3).

![Figure 5.3 Cytoplasmic and nuclear expression of PPARγ.](image)

**Figure 5.3 Cytoplasmic and nuclear expression of PPARγ.** (A) Cytoplasmic and nuclear localization of PPARγ in WT and GSNOR−/− MSCs. (B) Quantification of PPARγ localization. n=4. (Nuclear protein loading: 6.5µg, Cytoplasm protein loading: 12.5µg).
To assess whether S-nitrosylation of PPARγ affects transcription of its target gene, *Fabp4*, we performed ChIP assays. Histone 3 (H3) Ab–treated samples acted as positive controls. At baseline, the binding affinity of PPARγ for the promoter region of *Fabp4* was decreased in GSNOR−/− MSCs (Figure 5.4).

![Figure 5.4 Binding of PPARγ to its downstream target FABP4 is decreased in GSNOR−/− MSCs. Chromatin immunoprecipitation (ChIP) analysis of PPARγ binding for the promoter region of FABP4 in WT and GSNOR−/− MSCs, * P<0.05 compared to corresponding IgG, # P<0.05 compared to WT PPARγ Ab group; n=4.](image)

Furthermore, WT MSCs treated with 500 μM GSNO for 5 hours were found to have decreased PPARγ-binding affinity for the *Fabp4* promoter region compared with vehicle-treated cells (Figure 5.5A). We further measured PPARγ S-nitrosylation after GSNO treatment in MSCs to verify the effects of GSNO. We found that this decrease in PPARγ-binding affinity in the presence of GSNO was accompanied by an increase in PPARγ S-nitrosylation without altering its expression (Figure 5.5B,C). These data are consistent with the decreased adipogenesis seen in WT cells treated with GSNO and suggest that S-nitrosylated PPARγ has decreased affinity for the promoter of FABP4.
Together, our results suggest that increased protein S-nitrosylation due to a lack of GSNOR reduces the transcriptional activity of PPARγ.

Figure 5.5 The effects of GSNO on S-nitrosylation and binding of PPARγ to its downstream target FABP4. (A) ChIP analysis of PPARγ binding to the promoter region of the FABP4 gene in WT MSCs treated with 500µM GSNO. The lanes were run on the same gel but were noncontiguous. * P<0.05 compared to IgG without GSNO treatment, # P<0.05 compared to PPARγ Ab without GSNO treatment; n=4. (B) PPARγ protein expression in WT and GSNOR−/− MSCs after GSNO treatment. (C) PPARγ protein S-nitrosylation in WT MSCs measured by SNO-RAC after GSNO treatment. Asc: Ascorbic Acid. n=3, *P<0.05 compared to no GSNO treatment control.
5.2 Mutagenesis study

5.2.1 Structure of PPARγ and identification of the potential cysteine targets of PPARγ S-nitrosylation

To determine which cysteines are S-nitrosylation targets, we generated 2 cysteine-to-alanine mutations by site-directed mutagenesis: Cys 139 single mutation and Cys 156/159 double mutation. PPARγ has 10 cysteines, and a 3D structure of PPARγ shows the 10 cysteines in red, with white arrows indicating the 3 mutated residues (Figure 5.6A). A topology map of PPARγ indicated that the 3 mutated cysteines are located within the first zinc-finger structure of the regulatory domain (Figure 5.6B).

![3D Structure of PPARγ](image)

**Figure 5.6 3D Structure and topology map of PPARγ.** (A) 3D structure of PPARγ (73). (B) Topology map of PPARγ.

Cys 139 was identified by GPS-SNO 1.0 software (Figure 5.7A) and conforms to an acid-base nitrosylation conservative motif (Figure 5.7B) (14, 29). The Cys 156/159
double mutation was first characterized by the Spiegelman group, who found that this mutation could cause diminished adipogenesis in preadipocytes (74).

**Figure 5.7 Analysis of S-nitrosylation site in PPARγ.** There are ten potential cysteine residues (Cys) in PPARγ sequence. A software, GPS-SNO 1.0, was used to predict the S-nitrosylation site (75). No cysteine was identified using high threshold and Cys139 was identified under medium threshold. (A) Identification of Cys 139 with GPS-SNO 1.0. (B) Illustration of three cysteine targets: Cys 139, Cys 156 and Cys 159 labeled in red.

5.2.2 Localization and transcriptional activity of PPARγ is decreased in the mutants

We first characterized the cellular localization of these mutants in HEK-293T cells to determine whether cysteine mutations lead to a disruption of protein 3D structure, because cysteine is crucial for disulfide bond formation. We found that all the mutants localized primarily to the nucleus of the cells in a pattern similar to that of WT PPARγ protein (Figure 5.8).
Furthermore, we measured transcriptional activity of the two PPARγ mutants. Cells over-expressed with WT PPARγ showed increased luciferase activity in response to rosiglitazone (1μM). However, the luciferase activity of the 2 PPARγ mutants was diminished and could not be rescued by rosiglitazone, possibly due to a disruption of the first zinc-finger structure (Figure 5.9).

Figure 5.8 Sub-cellular localization of wild type and two PPARγ mutants (M1, M2) in HEK-293T cells.
Figure 5.9 Transcriptional activity of PPARγ and its mutants. PPARγ luciferase activity in HEK-293T cells overexpressed with WT and mutants, in the presence or absence of the PPARγ agonist rosiglitazone (Rosi, 1μM). *P<0.05 compared to WT CTL, n=4.

5.2.3 S-nitrosylation of the PPARγ mutants

In order to test S-nitrosylation of the PPARγ mutants, we overexpressed WT and mutant PPARγ in HEK-293T cells. Cells were exposed to vehicle or GSNO, and PPARγ S-nitrosylation was measured by SNO-RAC. GSNO is widely used to augment S-nitrosylation in multiple cell types (71). While WT PPARγ showed robust S-nitrosylation induced by GSNO, mutation of Cys 139 to alanine eliminated the response to GSNO (Figure 5.10). Thus, Cys 139 is the cysteine target of PPARγ S-nitrosylation. While this result does not exclude other cysteines as potential S-nitrosylation targets, it strongly points Cys 139 as a primary site of regulation.
Figure 5.10 S-nitrosylation of PPARγ mutant 1. SNO-PPARγ in HEK-293T cells overexpressed with WT and mutant 1 (Cys 139), measured by SNO-RAC. Asc: Ascorbic Acid. Omission of ascorbic acid were used as negative controls.* P<0.05 compared to WT without GSNO treatment, n=3.

However, the double PPARγ mutation (Cys 156 and 159) exhibited an S-nitrosylation response to GSNO similar to that of WT PPARγ (Figure 5.11), indicating that double mutation did not disrupt the cysteins responsible for S-nitrosylation. These data suggest what may be a novel mechanism of PPARγ transcriptional regulation: S-nitrosylation of cysteine 139 induced alteration of the zinc-finger motif. Our data suggest that the decreased PPARγ transcriptional activity seen in GSNOR± MSCs can be attributed to higher levels of PPARγ S-nitrosylation, leading to the disruption of the first zinc-finger structure.
Figure 5.11 S-nitrosylation of PPARγ mutant 2. SNO-PPARγ in HEK-293T cells overexpressed with WT and mutant 2 (double mutant: Cys 156/159), measured by SNO-RAC. Asc: Ascorbic Acid. Omission of ascorbic acid were used to as negative controls.* P<0.05 compared to overexpression of WT PPARγ and without GSNO treatment, # P<0.05 compared to overexpression of M2 PPARγ and without GSNO treatment, n=5.
Chapter 6. Discussion

Bone marrow–derived MSCs are a common precursor to adipocytes and osteoblasts. Although lineage-fate specification is controlled by crosstalk between multiple key transcription factors, higher order regulation of this process remains poorly understood. While PPARγ (23) and NO signaling participate in fate decisions between bone and fat lineages, the interaction between these control mechanisms has not yet been established. Here, we used MSCs derived from mice deficient in the prototypic denitrosylase GSNOR to show that excessive S-nitrosylation of PPARγ contributes to diminished adipogenic and increased osteoblastic differentiation. This phenotype is associated with decreased PPARγ transcriptional activity, as illustrated by diminished binding to Fabp4. We further identified S-nitrosylation of Cys 139 as a principle mechanism of PPARγ transcriptional inhibition. Together, these findings offer insights into the regulation of cell fate decisions in adult mesenchymal tissue homeostasis and provide potential therapeutic strategies to offset disorders of bone loss.

6.1 Crosstalk between NO and PPARγ signaling

6.1.1 Advantage of GSNOR−/− mice as a model

NO regulates osteoblast and adipocyte differentiation (46;48); all 3 NOS isoforms are expressed in osteoblasts, and all except NOS1 are expressed in adipocytes (76;77). With regard to downstream effects, NO has a biphasic effect on bone, where low concentrations of NO produced by NOS3 are essential for bone formation, while high concentrations of NO produced by NOS2 are inhibitory (78;79). NOS3 appears to be the predominant NOS expressed in human adipocytes (76). In obese woman, there is a higher
expression of NOS2 and NOS3 compared to lean controls (76). NOS2/– mice have higher body weight while NOS3/– mice have similar body weight compared to WT mice (50;80). Thus, the role of NO synthesis in adipogenesis and osteogenesis may be NOS subtype and NO concentration dependent.

While WT and GSNOR/+ MSCs constitutively express NOS1 and NOS2, but not NOS3, GSNOR/– MSCs have elevated NOS1 expression but same amount of NO production compared to WT cells (35). The use of mice with a targeted deletion of GSNOR circumvents the disparities caused by varied NOS/NO amount in MSCs and allows us to define the role of S-nitrosylation in NO-regulated adipocyte and osteoblast differentiation.

6.1.2 PPARγ S-nitrosylation

The mechanism of PPARγ- and NO-signaling interaction is not well established. PPARγ stimulates NO production by enhancing NOS3 expression (81). PPARγ can also increase NOS2 activity and modulate immune reaction (82). Here, we provide what we believe to be a novel feedback regulation of PPARγ transcriptional activity by NO-mediated S-nitrosylation.

PPARγ regulates adipogenesis and osteogenesis by multiple posttranslational modifications, including phosphorylation, ubiquitination, and SUMOylation (18). Here, we show that the posttranslational modification S-nitrosylation participates fundamentally in PPARγ transcriptional activity. PPARγ regulates transcription via PPRE-dependent and -independent mechanisms. However, the transcriptional regulation of PPARγ in MSCs has heretofore been unknown. Our results reveal that S-nitrosylation of PPARγ inhibits its transcriptional activity at the Fabp4 promoter in MSCs. Our
findings in GSNOR\textsuperscript{−/−} MSCs fully recapitulate the differentiation phenotypes of bone marrow cells from mice with \textit{Pparg} haploinsufficiency (23), providing further confirmation that S-nitrosylation inhibits PPAR\textgreek{y} activity. Lower expression of CEBP\textgreek{b} observed in GSNOR\textsuperscript{−/−} MSCs indicates that events upstream of PPAR\textgreek{y} were altered, providing an additional mechanism for decreased adipogenesis.

6.2 S-nitrosylation

6.2.1 Specificity of S-nitrosylation

S-nitrosylation is primarily mediated by nitric oxide synthases (NOSs). Unlike other well-studied post-translational modifications such as phosphorylation and ubiquitination that are modulated by specific kinases and E3 ligases, S-nitrosylation was thought to be modulated by only three NO synthases. Although enzymes other than NOSs that directly induce S-nitrosylation have not been well characterized, two denitrosylases were shown to regulate S-nitrosylation. One is GSNOR that is extensively described in this paper. The other is thioredoxin/thioredoxin reductase (Trx/TR), also a disulfide reductase. Mitochondrial isoform of Trx2 regulates denitrosylation of caspase-3, thereby serves as an activation signal and promotes apoptosis (83;84). Due to the limited number of enzymes that control nitrosylation and denitrosylation, a question is raised about its specificity.

The specificity of S-nitrosylation can be regulated by compartmentalization, such as subcellular proximity of proteins to NOSs. The high local concentration of NO generated by NOSs can directly react with protein thiols to form SNOs. Alternatively, S-nitrosoylation motif was discovered to convey a certain degree of specificity. There are three kinds of S-nitrosylation motif: acid-base motif, hydrophobic motif and a
combination of these two structures (34). The acid-base motif structure provides the thiolate anion with a more ionizable environment, thus making the cysteine more accessible to SNOs due to cysteine stabilization (44). The acid-base structure can either be adjacent to the cysteine in primary sequence or in proximity due to the 3D structure. Another proposed S-nitrosylation motif is the hydrophobic regions around target cysteines, like adjacent aromatic residues, increasing the thiol nucleophilicity and thus enhancing its reactivity. For example, cysteine 3635 of ryanodine receptor 1 (RYR1) locating at the hydrophobic region was identified as the only S-nitrosylation target within RYR1 (85;86).

Our data indicated that Cys 139 of PPARγ is a primary S-nitrosylation target of exogenously supplied SNO. The amino acids surrounding Cys 139 conform to an acid-base motif previously described as a conservative S-nitrosylation domain (14), while the locations of Cys 156 and Cys 159 do not conform to this motif. In the PPARγ primary sequence, Cys 139 is flanked by glutamic acid and arginine. Our finding of Cys 139, but not Cys 156 and 159, as a PPARγ S-nitrosylation target provides additional evidence of the existence of a conservative S-nitrosylation motif.

Another level of S-nitrosylation specificity is conveyed by recently identified transnitrosylases. Transnitrosylation is defined as the direct NO transfer between thiols of small molecular weight S-nitrosothiols (like GSNO) or SNO proteins to a target protein (87). For example, S-nitrosylated GAPDH was shown to be a nuclear transnitrosylase that induced S-nitrosylation of SIRT1 and HDAC2 (88). The large quantity of S-nitrosylated proteins discovered so far can serve as potential transnitrosylases to specific
target proteins and thus provide diversity and specificity to the regulation of S-nitrosylation.

6.2.2 S-nitrosylation and transcriptional regulation

NO is an important regulator of zinc-finger transcription factors (43;89). SNOs inhibit the DNA-binding activity of the zinc-finger transcription factors, such as EGR-1 and SP1 (44). For example, S-nitrosylation of transcriptional factor NF-κB at a single reactive cysteine inhibits NF-κB DNA binding and thereby gene transcription (90). It has been proposed that this inhibition is due to the S-nitrosylation of a thiolate group within the zinc finger. This posttranslational modification changes the coordination of zinc ion with cysteines, thus disrupting the zinc-finger structure (44). Our data provide further support to this theory, suggesting that in GSNOR−/− MSCs, a higher level of PPARγ S-nitrosylation leads to disruption of the first zinc-finger structure and decreased PPARγ transcriptional activity.

6.3 Future directions and clinical implications

6.3.1 Cell lineage determination

Our studies reveal a complex phenotype with regard to osteoblast differentiation of MSCs. Here, we showed that osteogenic differentiation was augmented in GSNOR−/− MSCs, and this finding was confirmed by experiments with the GSNOR inhibitor. In the intact animal, however, while there is evidence of increased bone formation, this effect is offset by augmented bone resorption due to enhanced formation of osteoclasts that arise from BMMNCs. Thus S-nitrosylation signaling participates in the formation of both MSCs-derived osteoblasts and HSCs-derived osteoclasts. PPARγ stimulates osteoclast
differentiation and bone resorption via c-Fos signaling (24). However, increased osteoclast differentiation under GSNOR deficiency is not consistent with reduced PPARγ transcriptional activity, indicating additional mechanism under the GSNOR deficiency to regulate osteoclastogenesis.

6.3.2 S-nitrosylation and its regulation of post-translational modifications

The broad impact of S-nitrosylation can be conveyed by its role to modulate other post-translational modifications (91). Several kinases such as c-Jun N terminal kinase (JNK), protein kinase C (PKC) and G-protein coupled receptor kinase-2 (GRK2) can be S-nitrosylated and leads to inhibition of the activities of these kinases (71;92;93). However, stimulatory effects of S-nitrosylation are also observed in the regulation of glucokinase (94). S-nitrosylation also modulates acetylation and thus affects gene transcription. histone deacetylase 2 (HDAC2) is regulated by S-nitrosylation at two specific cysteine residues, leading to enhanced acetylation and transcription activation (95). Interestingly, S-nitrosylation also regulates other cysteine-based post-translational modifications, such as glutathionylation, S-palmytoylation and sulfhydration (96). In general, S-nitrosylation prevents further oxidation of protein thiols because of the unfavorable structure orientation (97). The impact of PPARγ S-nitrosylation on other PPARγ post-translational modifications such as phosphorylation needs further study.

6.3.3 GSNOR in calcium homeostasis and hormonal regulation

We performed a detailed analysis of calcium/phosphate metabolism to gain additional insights into the skeletal phenotype of GSNOR-deficient animals. GSNOR+/− mice had higher serum levels of PTH and FGF23 and increased urinary loss of phosphate and calcium, which together contribute to diminished bone density. Chronic PTH
exposure increases osteoclast activity and bone resorption (58), providing additional mechanistic insights of elevated bone resorption in GSNOR$^{-/-}$ mice. Future studies will aim to establish the relative contributions of altered hormonal homeostasis and increased PPARγ S-nitrosylation to stem cell differentiation and the pathological bone loss phenotypes.

GSNOR$^{-/-}$ mice also represent an interesting model of hormonal regulation. The elevated PTH levels, accompanied by high bone turnover and upregulated FGF23, partly resemble secondary hyperparathyroidism. The elevated FGF23 and concurrent upregulation of PTH represent the impaired calcium-induced inhibition of PTH in chronic kidney diseases (98). Rosiglitazone treatment simultaneously increased FGF23 levels while reducing PTH in GSNOR$^{-/-}$ mice, but without significantly reducing urinary calcium. The differential response to rosiglitazone suggests that GSNOR loss of function alters the set point for FGF23, PTH, and calcium phosphate sensing in multiple organs, including parathyroid gland and kidney. Importantly, PPARγ agonists are known to upregulate α-klotho, a necessary coreceptor of FGF23 (99). However, the absence of hypocalcemia suggests a chronic compensation effect of high PTH or normocalcemic primary hyperparathyroidism. Further study is needed to elucidate the role of GSNOR in parathyroid function, renal reabsorption and calcium homeostasis.

In addition to PTH and FGF23, vitamin D is another crucial regulator of calcium homeostasis. Vitamin D is metabolized to 25(OH)D and then convert to its active form 1, 25 (OH)$_2$ D. The main function of 1, 25 (OH)$_2$ D is to maintain calcium concentration in the body by modulating intestinal calcium absorption (55). Most of the effects of vitamin D are exerted through vitamin D receptor (VDR). Like PPARγ, VDR is also a nuclear
receptor that forms a heterodimer with retinoid-X-receptor to propagate transcription (100;101). Thus, VDR may serve as another potential S-nitrosylation target contributing to the calcium metabolism and bone phenotypes in GSNOR<sup>−/−</sup> mice.

6.3.4 Stem cell therapy in bone diseases and aging

MSCs are a promising stem cell population that can regenerate the heart, bone and other mesoderm-derived organs. Although the gold standard in bone regeneration is still autologous bone grafting, several drawbacks persist, including availability of limited grafting material and donor-site morbidity. The bone regenerative capacity of MSCs has been tested in multiple clinical trials and FDA-approved MSC allografts are used for spine fusion and other applications (102). We showed that GSNOR<sup>−/−</sup> MSCs had enhanced bone formation capacity when transplanted subcutaneously into mice. Future study will focus on the regenerative capacity of GSNOR genetically engineered cells in a bone defect pre-clinical model.

This project also has important implications in the study of aging. 2-months-old GSNOR<sup>−/−</sup> mice had osteopenia, a key feature of aging, and the bone loss phenotype persisted until 11-months-old. Age-related loss of functional stem cells is one contributor of the aging process. Transplantation of young MSCs, but not old MSCs, to aging mice can slow down the aging related bone loss and surprisingly prolong the life span of these aged mice (103). Another future direction is to test the effects of GSNOR deficiency on age-related osteoporosis and deterioration of MSC differentiation capacity.

6.4 Summary and the working model

In summary, our data reveal that GSNOR modulates PPARγ S-nitrosylation without affecting overall abundance of this transcription factor. Nitrosylated PPARγ has
diminished transcriptional activity, which is associated with reduced adipocyte
differentiation and increased osteoblast formation. Thus, S-nitrosylation participates in
lineage bifurcation between adipocytes and osteoblasts. These findings have important
implications and offer broad insights for understanding the regulation of lineage
specification of adult precursor cells. Our results also suggest that GSNOR may
contribute to PTH regulation and calcium urinary excretion, which in turn can affect the
phenotype of GSNOR<sup>−/−</sup> mice. A working model of GSNOR<sup>−/−</sup> mice indicates that
GSNOR is a crucial regulator of stem cell differentiation and body homeostasis (Figure
6.1). Together, these findings may provide mechanistic support for therapeutic strategies
designed to offset disorders characterized by pathologic bone loss and/or excessive
adipogenesis.

![Figure 6.1 The adipose-skeletal phenotypes of mice with GSNOR deficiency.](image)

GSNOR<sup>−/−</sup> MSCs have decreased adipogenesis and increased osteoblast differentiation
due, at least in part, to increased S-nitrosylation of PPARγ. Despite increased osteoblast
formation from MSCs, GSNOR<sup>−/−</sup> mice have diminished bone density. This phenotype is
associated with increased osteoclast differentiation, and elevated urinary excretion of
both phosphate and calcium. In addition, there is elevated FGF23 and PTH in the animal.
This phenotype suggests that other endocrine or renal mechanisms may participate in the
bone loss of the GSNOR<sup>−/−</sup> mice. Parts of the artwork were derived from references (2;3).
Reference List


