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Osteoblast/osteocyte-specific inactivation of Stat3 decreases load-driven bone formation and accumulates reactive oxygen species

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ABSTRACT

Signal transducers and activators of transcription 3 (Stat3) is a transcription factor expressed in many cell types including osteoblasts, osteocytes, and osteoclasts. STAT3 mutations cause a rare human immunodeficiency disease that presents reduced bone mineral density and recurrent pathological fractures. To investigate the role of Stat3 in load-driven bone metabolism, two strains of osteoblast/osteocyte-selective Stat3 knockout (KO) mice were generated. Compared to age-matched littermate controls, this selective inactivation of Stat3 significantly lowered bone mineral density (7–12%, $p<0.05$) as well as ultimate force (21–34%, $p<0.01$). In ulna loading (2.50–2.75 N with 120 cycles/day at 2 Hz for 3 consecutive days), Stat3 KO mice were less responsive than littermate controls as indicated by reduction in relative mineralizing surface (rMS/BS, 47–59%, $p<0.05$) and relative bone formation rate (rBFR/BS, 64–75%, $p<0.001$). Furthermore, inactivation of Stat3 suppressed load-driven mitochondrial activity, which led to an elevated level of reactive oxygen species (ROS) in cultured primary osteoblasts. Taken together, the results support the notion that the loss-of-function mutation of Stat3 in osteoblasts and osteocytes diminishes load-driven bone formation and impairs the regulation of oxidative stress in mitochondria.

Introduction

Signal transducers and activators of transcription 3 (STAT3) is a ubiquitously expressed transcription factor, mediating cell proliferation, differentiation, and survival [1–5]. STAT3 mutations cause a rare human immunodeficiency disease, Job's Syndrome (also known as hyperimmunoglobulin E syndrome) [6,7], which is characterized by the elevated level of IgE in serum and distorted craniofacial and skeletal features. Most patients present reduced bone mineral density [8], recurrent fractures, hyperextensible joints, and scoliosis. Few studies, however, have investigated the role of STAT3 in bone metabolism.

Stat proteins are latent cytoplasmic transcription factors [2,9], and activated by cytokines including IL6, IL11, oncostatin M, leukemia inhibitory factor, cardiotrophin 1, and neurotrophin 1/B-cell stimulatory factor 3 [10,11]. These cytokines are pleiotropic, sharing a glycoprotein chain gp130 as a common signal transducer [10,12,13]. The binding of these cytokines to their receptors induces the homodimerization of gp130 and activates gp130-associated Janus kinases (JAKs) [14], which phosphorylate a tyrosine residue of Stat3 [15]. Targeted disruption of the mouse Stat3 gene leads to embryonic lethality, indicating that Stat3 is essential for early embryonic development [16].

Recent studies also suggest that Stat3 is involved in mitochondrial functions [17,18]. Stat3 regulates cell respiration and metabolism in mitochondria [17,18]. A fraction of cellular Stat3 has been found to reside within the mitochondria of mouse myocytes and hepatocytes. Without Stat3, an electron transport chain (ETC) that generates energy by oxidative phosphorylation is inhibited and reduction in mitochondrial activity leads to accumulation of ROS, but the number of mitochondria and their content of the protein that constitute complexes I and II are not altered [19–21]. On the other hand, addition of Stat3 in Stat3-null cells can restore oxidative phosphorylation, and this rescue of mitochondrial function does not require the DNA binding domain. However, expression of Stat3 with a mutation that prevents phosphorylation of serine 727 does not induce the rescue effects [17]. These data suggest that the conserved serine phosphorylation site on Stat3 is important for regulation of mitochondrial activity. Clinical evidence suggests interplay between ROS-linked oxidative stress and age-related bone loss [22–28]. Since oxidative stress is known to antagonize Wnt signaling, which is critical in load-driven bone formation [29,30], Stat3 is potentially involved in bone metabolism through regulation of anabolic genes as well as ROS-linked mitochondrial functions.

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In this study, we addressed two questions: Is Stat3 necessary for induction of bone formation in response to mechanical loading? Does inactivation of Stat3 in osteoblasts and osteocytes impair mitochondrial functions and lead to accumulation of ROS? Since mechanical loading is known to upregulate expression of Stat3 [31] and activate Wnt signaling [32], we hypothesized that the inactivation of Stat3 specific to osteoblasts and osteocytes would reduce load-driven bone formation and elevate the level of ROS. Stat3 KO mice specific to osteoblasts as well as osteocytes were generated using Cre-loxP recombination by breeding the mice with Cre recombinase driven by a type I collagen promoter with the mice carrying floxed Stat3. The two loxP sites flank the exons 18–20 of the Stat3 gene [33]. Although two lines of transgenic mice using the collagen α1(I)2.3 and α1(I)3.6 fragments were established [34], herein we mainly presented data collected from the Col3.6-Cre; $Stat3^{flox/flox}$ mice since these two lines exhibited similar responses to mechanical loading. Data collected from the Col2.3-Cre;$Stat3^{flox/flox}$ mice are presented in the Supplementary Data.

Materials and methods

Experimental animals

All procedures performed in this study were in accordance with the Indiana University Animal Care and Use Committee Guidelines. The Cre recombinase transgenic mice and the floxed Stat3 mice were generated as reported previously [33,34] and kindly provided by Dr. Barbara Kream (University of Connecticut Health Center) and Dr. Xin-Yuan Fu (Department of Microbiology and Immunology, Indiana University School of Medicine), respectively. The Cre recombinase transgene is driven by the by the collagen α1(I)2.3 and α1(I)3.6 fragments (Col2.3-Cre and Col3.6-Cre), while in floxed Stat3 mice ($Stat3^{flox/flox}$) the two loxP sites flank the exons 18–20 of the Stat3 gene [33]. Conditional Stat3 knockout (KO) mice specific to osteoblasts and osteocytes were generated by breeding mice with Cre recombinase driven by the type I collagen promoter and mice with floxed Stat3. Offspring, which were heterozygous or homozygous for the Cre transgene and homozygous for the loxP sites, were used in this study together with their littermate controls.

Two lines of conditional Stat3 KO mice, Col2.3-Cre;$Stat3^{flox/flox}$ and Col3.6-Cre;$Stat3^{flox/flox}$, were established in which Stat3 was deleted using the collagen α1(I)2.3 and α1(I)3.6 fragments, respectively. A deletion of the exons 18 and 20, which encode the SH2 domain of Stat3, results in a conditional Stat3 KO in osteoblasts and osteocytes (Fig. 1).

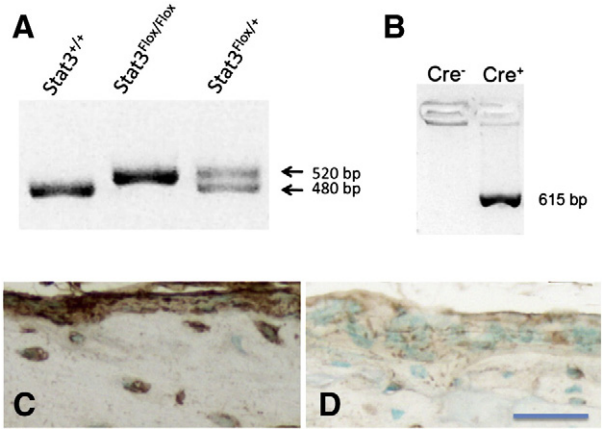

Fig. 1. Characterization of the conditional Stat3 KO mice. (A) $Stat3^{Flox}$ allele detected with a pair of PCR primers that generate 520-bp DNA fragments, and Stat3 wildtype allele corresponding to 480-bp fragments. (B) Col2.3-Cre and Col-3.6-Cre transgenes detected with the primers amplifying 615-bp fragments. (C) Immunohistochemical staining for wildtype control with Stat3 protein present in periosteal osteoblasts and osteocytes. (D) Immunohistochemical staining for KO (Col3.6-Cre;$Stat3^{flox/flox}$) mice without Stat3 protein. (Bar = 50 μm).

Note that Col2.3-Cre blocks Stat3 expression in mature osteoblasts and osteocytes, while Col3.6-Cre mutates Stat3 in early and mature osteoblasts as well as osteocytes. The genotype was determined by PCR as described previously [33,34].

In vivo ulna loading

To examine the role of Stat3 in load-driven bone formation, we subjected Stat3 deficient mice (Col2.3-Cre;$Stat3^{flox/flox}$ and Col3.6-Cre;$Stat3^{flox/flox}$) and littermate controls (Col2.3-Cre;$Stat3^{+/+}$ and Col3.6-Cre;$Stat3^{+/+}$ mice) to axial ulnar loading as previously described [35,36] and conducted bone histomorphometry. Using 16-week-old mice, dynamic loads (120 cycles/day at 2 Hz, with peak forces of 2.50 N for female and 2.75 N for male mice) were applied under general anesthesia with 3–5% isoflurance for three consecutive days using an electromagnetic actuator (Bose ElectroForce 3200 series; EnduraTEC). The loading forces were selected to ensure that both male and female mice experiences similar peak strains during loading. In a pilot load-strain calibration experiment using 3 mice randomly selected from each gender and strain of mouse, in situ mechanical strains achieved during loading were measured on the medial surface of the ulnae as described previously by our group [35,37]. The peak force of 2.5 N produced about ~2800 and ~2970 microstrains at the midshaft of ulnas of female control mice and conditional Stat3 KO mice, respectively. The peak force of 2.75 N produced about ~2800 and ~2940 microstrains at the midshaft of ulnas of male control mice and conditional Stat3 KO mice, respectively. The left forearms were not loaded and served as nonloaded controls. All mice were allowed normal cage activity between loading sessions and afterward. Intraperitoneal injections of calcein (30 mg/kg body weight; Sigma) and alizarin (50 mg/kg body weight; Sigma) were administered on days 5 and 11 after the first loading bout. All animals were sacrificed after 14 days.

Right (loaded) and left (nonloaded control) ulnas were processed for histomorphometry to evaluate load-induced bone formation. Femurs were used to determine bone size, bone mineral density and mechanical properties. In particular, the right distal femurs of Stat3 deficient mice (Col3.6-Cre;$Stat3^{flox/flox}$) and littermate controls (Col3.6-Cre;$Stat3^{+/+}$ mice) were sectioned and used for measurement of bone formation and resorption.

Peripheral dual-energy X-ray absorptionmetry (pixiMUS)

Bone mineral content (BMC, g) and bone mineral density (BMD, g/mm^2) of the left femurs were evaluated using peripheral dual-energy X-ray absorptiometry (pDXA; PIXIMus II; GE-Lunar Co.).

Biomechanical testing

Femurs were brought to room temperature slowly (~2 h) in a saline bath, and mechanical testing was conducted by three-point bending using a microforce materials testing machine (Vitrodyne V1000; Liveco, Inc., Burlington, VT). Loads were applied at the mid-diaphysis (10 mm apart from a pair of supports) in the anteroposterior direction. Tests were conducted at a cross-head speed of 0.2 mm/s, during which force-displacement curves were generated. We determined ultimate force (F_U; N), stiffness (S; N/mm) and work to failure (U; mJ). Note that F_U represents the strength of the bone, whereas U is a measure of the energy required to break the bone.

Histomorphometry

Bone specimens were immersed in 10% neutral buffered formalin for 48 h. The specimens were then dehydrated in graded alcohols, cleared in xylene, and embedded in methyl methacrylate. Using a diamond-embedded wire saw (Histo-saw; Delaware Diamond Knives,