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The cytoskeletal regulatory scaffold protein GIT2 modulates mesenchymal stem cell differentiation and osteoblastogenesis

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ABSTRACT

G protein-coupled receptor kinase interacting protein 2 (GIT2) is a signaling scaffold protein involved in the regulation of cytoskeletal structure, membrane trafficking, and G protein-coupled receptor internalization. Since dynamic cytoskeletal reorganization plays key roles both in osteoblast differentiation and in the maintenance of osteoclast polarity during bone resorption, we hypothesized that skeletal physiology would be altered in GIT2$^{-/-}$ mice. We found that adult GIT2$^{-/-}$ mice have decreased bone mineral density and bone volume in both the trabecular and cortical compartments. This osteopenia was associated with decreased numbers of mature osteoblasts, diminished osteoblastic activity, and increased marrow adiposity, suggesting a defect in osteoblast maturation. *In vitro*, mesenchymal stem cells derived from GIT2$^{-/-}$ mice exhibited impaired differentiation into osteoblasts and increased adipocyte differentiation, consistent with a role for GIT2 in mesenchymal stem cell fate determination. Despite elevated osteoclast inducing cytokines and osteoclast numbers, GIT2$^{-/-}$ mice also exhibit impaired bone resorption, consistent with a further role for GIT2 in regulating osteoclast function. Collectively, these findings underscore the importance of the cytoskeleton in both osteoblast and osteoclast function and demonstrate that GIT2 plays essential roles in skeletal metabolism, affecting both bone formation and bone resorption *in vivo*.

1. Introduction

GPCRs exert their effects on bone and calcium metabolism through complex molecular mechanisms involving diverse downstream signaling events and multiple signaling complexes. Many GPCR-interacting proteins recruited during signal initiation and termination have been identified, including G proteins, arrestins and clathrin. Among the first of these proteins to be discovered were the GRKs, which are crucial for receptor phosphorylation and internalization. A search for proteins that interact with GRKs led to the identification of GITs [1].

GITs are signaling scaffold proteins comprising a family of ARF-GAPs. In cells, GITs exist in a oligomeric complexes with PIX/Cool proteins [2]. PIX/Cool proteins are GEFs for the cytoskeletal regulatory small GTPases, Rac1 and Cdc42. GIT/PIX complexes are known to function as scaffolds for a variety of signaling proteins, including GRKs, PAKs, FAK, the MEK1-ERK1/2 mitogen-activated proteins kinases, and phospholipase Cγ [3]. The 2 GIT family members, GIT1 and GIT2, are highly expressed in neurons, vascular smooth muscle, endothelial cells and bone. In addition to their roles as regulators of GPCR internalization and resensitization [1,4], *in vitro*, GIT proteins have been investigated for their participation in focal adhesion dynamics, cell migration [5,6], and as scaffolding proteins directing the spatial localization of signaling molecules such as MEK1 and ERK1/2 [7]. *In vivo*, GIT expression has been shown to regulate emotional function [8,9] vascular development [10] and

Abbreviations: GPCR, G protein-coupled receptor; GRK, GPCR kinase; GIT, GRK-interacting protein; ARF-GAPs, ADP-ribosylation factor GTPase-activating proteins; GEF, guanine nucleotide exchange factor; MSC, mesenchymal stem cell; qCT, quantitative micro-computed tomography; WT, wild type; GIT2$^{-/-}$, mice lacking GIT2; DEXA, dual-energy X-ray absorptiometry; BV/TV, bone volume/total volume; DPD, deoxypyridinoline; LPL, lipoprotein lipase; aP2, adipocyte lipid binding protein 2; RUNX2, runt-related transcription factor 2; Col1a, collagen1a; PKL, paxillin-kinase-linker; FAK, focal adhesion kinase; BMD, bone mineral density; Tb, trabecular bone; SEM, standard error of the mean; qCT, quantitative micro-computed tomography; CSA, cross sectional area; Oc.S, osteoclast/bone surface; Ob.S, osteoblast/bone surface; Cool, cloned-out of library; PAK, p21-activated kinase; PIX, PAK-interactive exchange factor; ERK, extracellular-signal regulated kinase; MEK, mitogen activated ERK kinase; c-Src, cellular-Src; OB, osteoblast; OC, osteoclast; PKL/GIT2, paxillin-kinase-linker; OSX, osterix.

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mitochondrial biogenesis [11]. Little, however, is known about the role of GIT proteins in bone physiology.

Bone density is the product of complex interactions between OBs, which form new bone, and OCs, which resorb bone. OB differentiation and function is highly dependent upon cell adhesion and cytoskeletal organization. Recent studies have shown that extracellular cues, derived from interactions with the extracellular matrix and transmitted via cytoskeletal tension, direct MSC differentiation to either OB or adipocyte lineages [12–16], suggesting that proteins involved in regulating actin cytoskeletal dynamics, like GITs, may play a key role in the fate determination of MSCs. The contribution, however, of GIT proteins to OB lineage commitment has not been established.

GIT2 has been implicated in OC function. The nonreceptor tyrosine kinase c-Src is a known regulator of OC function, and $Src^{-/-}$ OCs exhibit impaired actin cytoskeletal organization, leading to diminished OC function and osteopetrosis. In a recent study to identify critical c-Src substrates in OCs, it was shown that Src-dependent phosphorylation and localization of GIT2 to OC sealing zones is essential for maintaining sealing zones and OC polarity for bone resorption *in vitro* [17]. There is also reported increased bone mass in $GIT1^{-/-}$ mice due to an OC defect [18].

As modulators of GPCR signaling, cytoskeletal rearrangement and cell adhesion, we hypothesized that GIT proteins are required for skeletal development and bone remodeling. To determine the contribution of the GIT family member GIT2 to bone metabolism *in vivo*, we evaluated $GIT2^{-/-}$ mice for alterations in bone formation and bone resorption. We found that the absence of GIT2 significantly decreases trabecular and cortical bone mass. Investigation of OC function confirmed impaired bone resorption, despite a marked increase in OC bone surface in $GIT2^{-/-}$ mice. The observed osteopenia in the setting of decreased bone resorption is explained by attenuated differentiation of MSC to OBs and increased adipogenesis. Together these findings highlight the complex skeletal effects of GIT2 expression, which are critical to the proliferation, differentiation and function of both OBs and OCs.

2. Materials and methods

2.1. Generation of GIT2 KO mice

The derivation of the C57B6/129 $GIT2^{-/-}$ mice was previously described [19]. All animals were treated in accordance with NIH guidelines for the care and use of animals under a protocol approved by the Duke University Institutional Animal Care and Use Committee.

2.2. Bone densitometry and quantitative computed tomography

BMD and qCT were performed as previously described [20].

2.3. Histology and histomorphometry

Quantitative histomorphometric analysis of vertebral spine trabecular bone was performed using techniques described previously [20] and data are expressed in units recommended by the American Society of Bone and Mineral Research [21].

2.4. Serum biochemistry and bone turnover markers

Serum osteocalcin, plasma PTH, urine DPD excretion and urine creatinine concentrations were measured as previously described [20]. Urine and Serum calcium measurements were determined using the Total Calcium LiquiColor colorometric assay (Stanbio Laboratory; Boerne, Texas) following the manufacturer's protocol.

2.5. Total DNA content

To control for potential viability differences between cells of different genotypes, total DNA concentration was determined. After 24 days of culture, media was aspirated and cells lysed by freeze–thaw in deionized water. DNA was stained with *Hoechst* 33258 and quantified (360 nm excitation/460 nm emission).

2.6. Statistics

All values are expressed as means ± SEM. For comparisons between two groups, statistical significance was assessed using a two-tailed unpaired *t*-test. Additional methods are described in the Supplementary section.

3. Results

3.1. Decreased bone mineral density in $GIT2^{-/-}$ mice

Adult $GIT2^{-/-}$ mice are fertile and show no gross phenotypic abnormalities, skeletal deformities or defects when compared to WT mice. To examine the contributions of GIT2 expression on bone metabolism, BMD measurements were obtained by DEXA on 15-week old male and female mice (Fig. 1A and B). Both male and female $GIT2^{-/-}$mice had significantly decreased vertebral spine and femoral shaft BMD compared to their WT counterparts.

3.2. $GIT2^{-/-}$ mice have decreased trabecular and cortical bone morphometry

To determine the effects of GIT2 expression on trabecular bone morphometry, qCT measurements of the distal femur were acquired from 15-week old male and female WT and $GIT2^{-/-}$ mice (Fig. 1C–F). $GIT2^{-/-}$ mice had a significantly decreased BV/TV, decreased trabecular number, and decreased trabecular thickness compared to WT mice (Fig. 2A–C). Cortical bone indices were examined by qCT of the mid-femoral shaft (Supplementary Fig. 1A and B). Both male and female $GIT2^{-/-}$ mice had significantly decreased cortical bone thickness and cortical bone area compared to WT mice. This loss of cortical bone mass caused a significant decrease in both periosteal circumference and CSA in the female $GIT2^{-/-}$ mice as well as significant increases in endosteal circumference and medullary area in the $GIT2^{-/-}$ male mice compared to WT (Supplementary Fig. 1C–F). As shown in Fig. 1G, the decrease in bone mass was more pronounced in the female $GIT2^{-/-}$ mice compared to the male $GIT2^{-/-}$ mice.

3.3. Effect GIT2 expression on OB number, OC number and bone remodeling

Histomorphometric analysis revealed a significant increase in Oc.S and a significant decrease in Ob.S in the $GIT2^{-/-}$ mice compared to WT mice (Fig. 2A–D). As shown in Fig. 2E and F, these histomorphometric findings were associated with a decrease in serum and urine biomarkers of bone formation and bone resorption including: 1. Urine DPD, a marker of bone resorption, and 2. Serum osteocalcin levels, a marker of anabolic bone formation. Consistent with decreased osteoblastic activity, gene expression of osteocalcin in calvarial bone of neonatal mice was significantly decreased in the $GIT2^{-/-}$ mice compared to WT mice (Supplementary Fig. 2A). Moreover, there was a significant increase in calvarial mRNA expression of the protein modulators of OC differentiation and proliferation, RANKL and OPG (Supplementary Fig. 2B and C), suggesting a compensatory response to decreased osteoblastic activity. Consistent with decreased bone resorption, urine calcium excre-