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Research Article

Assessing the osteoblast transcriptome in a model of enhanced bone formation due to constitutive G_s–G protein signaling in osteoblasts

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ABSTRACT

G protein-coupled receptor (GPCR) signaling in osteoblasts (OBs) is an important regulator of bone formation. We previously described a mouse model expressing Rs1, an engineered constitutively active G_s-coupled GPCR, under the control of the 2.3 kb Col I promoter. These mice showed a dramatic age-dependent increase in trabecular bone of femurs. Here, we further evaluated the effects of enhanced G_s signaling in OBs on intramembranous bone formation by examining calvariae of 1- and 9-week-old Col1(2.3)/Rs1 mice and characterized the *in vivo* gene expression specifically occurring in osteoblasts with activated G_s G protein-coupled receptor signaling, at the cellular level rather than in a whole bone. Rs1 calvariae displayed a dramatic increase in bone volume with partial loss of cortical structure. By immunohistochemistry, Osterix was detected in cells throughout the inter-trabecular space while Osteocalcin was expressed predominantly in cells along bone surfaces, suggesting the role of paracrine mediators secreted from OBs driven by 2.3 kb Col I promoter could influence early OB commitment, differentiation, and/or proliferation. Gene expression analysis of calvarial OBs revealed that genes affected by Rs1 signaling include those encoding proteins important for cell differentiation, cytokines and growth factors, angiogenesis, coagulation, and energy metabolism. The set of G_s-GPCRs and other GPCRs that may contribute to the observed skeletal phenotype and candidate paracrine mediators of the

Abbreviations: GPCR, G protein-coupled receptor; OBs, osteoblasts; TRAP, tartrate-resistant acid phosphatase; FTIR, Fourier transform infrared; SRμCT, synchrotron radiation micro-computed tomography; GFP, green fluorescent protein; qPCR, quantitative real-time polymerase chain reaction; Ct, cycle threshold; FACS, fluorescent activated cell sorting; PCM, primary culture medium; SDM, secondary osteogenic differentiation medium; BMSCs, bone marrow stromal cells; VK, Von Kossa; CRE, cyclic AMP responsive elements

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effect of G_s signaling in OBs were also determined. Our results identify novel detailed *in vivo* cellular changes of the anabolic response of the skeleton to G_s signaling in mature OBs.

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Introduction

Osteoblasts (OBs) play a key role in control of skeletal homeostasis by influencing the initiation and extent of bone resorption and bone formation through complex mechanisms that are only partially understood. A number of factors contribute to this complexity. Fortunately, considerable progress has been made over the past few decades in defining the molecular events associated with the transition of committed osteoprogenitors to fully differentiated, post-proliferative OBs and osteocytes [1,2]. In particular, the roles of transcription factors, such as Runx2 and Osterix, in the progression of OB differentiation are well documented [3,4]. Much less clear are the roles of extrinsic factors, such as hormones, cytokines, and other elements of the skeletal microenvironment, in regulating OB commitment, differentiation, and function. Moreover, very little is known about how such extrinsic factors regulate OBs in different anatomic components.

One of the most important mechanisms for cellular response to extrinsic factors is through G protein signaling. G protein-coupled receptors (GPCRs) are highly relevant to OB differentiation and function, as evidenced by the anabolic skeletal response to parathyroid hormone (PTH) and prostaglandin E [5,6], two agents that act on GPCRs presented on OB lineage cells. Genetic studies in human and mice further support the key role played by G protein signaling [7–9]. We described a number of transgenic mouse models in which G protein coupled signaling has been manipulated *in vivo* in OBs that express the 2.3 kb Col I promoter. These models include OB-specific expression of an engineered constitutively active G_s-coupled receptor, Rs1, and OB-specific expression of pertussis toxin (PTX) to block G_i signaling; both of which demonstrate an anabolic bone phenotype [10,11]. Mice expressing Rs1, showed an increase in bone accrual within the skull and in femur size, assessed by the whole body microCT analysis, with a dramatic age-dependent increase in trabecular bone with features resembling fibrous dysplasia. At 9 weeks of age, the male and female mutant mice showed dramatic increases (380%) in whole-body areal bone mineral density, as determined by dual-energy x-ray absorptiometry scanning. Histological assessment of femoral bones indicated that there was an increase in OB lineage cells, especially immature OBs, indicated by an expansion of cells expressing early OB markers, Runx2 and Osterix. Increased osteoclast activity was suggested by the large number of tartrate-resistant acid phosphatase (TRAP)-positive regions adjacent to the trabeculi within the lesions. Bone tissue quality, mineralization, composition, and maturity, of calvariae and femurs in Rs1 mice were also assessed by the complementary techniques of Fourier transform infrared (FTIR) spectroscopy and synchrotron radiation micro-computed tomography (SRμCT) [12]. We demonstrated that mineral-to-matrix ratio and cross-link ratio were significantly lower in 6- and 15-week mutant bones. No differences in FTIR spectroscopic parameters were detected between the two anatomic sites despite the different bone-formation processes (endochondral *vs.* intramembranous). Tissue mineral density was also significantly lower in 3- and 9-week transgenic femoral diaphyses. The results indicate that continuous G_s activation in mature OBs lead to deposition of immature bone tissue with reduced mineralization.

The ability of Rs1 signaling in mature OBs to drive expansion of immature OBs could be mediated by paracrine factors, secreted from mature OBs that influence early OB commitment, differentiation, and/or proliferation. However, little is known about the *in vivo* cellular basis of the skeletal changes in response to enhanced G_s signaling in mature OBs.

In this study, we determined how G_s signaling in mature OBs affects bone formation by examining the Rs1 calvarial bone phenotype at 1 and 9 weeks of age and investigated the effect of enhanced OB G_s signaling on the OB transcriptome by examining alterations in gene expression *in vivo* in OBs from calvariae of 1-week-old Rs1 mice, compared to controls. The functionally related, differentially expressed genes, the G_s- and G_i-GPCRs, and candidate paracrine mediators that may contribute to the observed skeletal phenotype of the effect of G_s signaling in OBs were determined.

Methods

Transgenic mice

To examine the influence of G_s signaling in OBs, we used a mutated version of the G_s-coupled 5HT-4 serotonin receptor (Rs1) that has constitutive G_s signaling activity [13], together with the tetracycline-regulated system (Tet-off) to regulate the expression of Rs1 *in vivo*. The Col1(2.3)-tTA/TetO-Rs1 double transgenic mice (abbreviated Col1(2.3)/Rs1) were generated by crossing mice carrying the heterozygous TetO-Rs1 transgene with mice carrying the homozygous Col1(2.3)-tTA transgene. Endochondral bones of these mutant mice were well characterized as described [10,14,15].

In this study, we utilized green fluorescent protein (GFP)-based reporters to identify Rs1 expressing cells for investigating OB transcriptome analysis. We co-expressed a histone-GFP marker in OBs *in vivo* alone (abbreviated Col1(2.3)/GFP) or with Rs1 in triple transgenic mice (abbreviated Col1(2.3)/GFP/Rs1). This was accomplished by generating mice harboring a TetO-histone-GFP gene (mouse line Tg (TetO-HIST1H2BJ/GFP) 47Efu/J, Jackson Laboratory) with a TetO-Rs1 gene. Double transgenic mice were then crossed with mice homozygous for the 2.3 Col1-tTA genes to examine the skeletal effects of G_s signaling in mature OBs. Both transgenes driven by TetO were expressed in OBs only when the 2.3 Col1-tTA transgene was present. In this approach, 25% of the progeny were controls (express only histone-GFP) and 25% were experimental (express both histone-GFP and Rs1). All animals were maintained in the FVB/N background. Mice were on regular chow (LabDiet 5053; PMI Nutrition, St. Louise, MO) without doxycycline administration to allow transgene expression in experimental mice since conception. All protocols were approved by the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center.