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Constitutive stimulatory G protein activity in limb mesenchyme impairs bone growth

Anara Karaca [a,1], Vijayram Reddy Malladi [a,1], Yan Zhu [a,1], Olta Tafaj [a], Elena Paltrinieri [a], Joy Y. Wu [a,b], Qing He [a], Murat Bastepe [a,*]

[a] Endocrine Unit, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA
[b] Division of Endocrinology, Stanford University School of Medicine, Stanford, CA 94305, USA

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ABSTRACT

GNAS mutations leading to constitutively active stimulatory G protein alpha-subunit (Gsα) cause different tumors, fibrous dysplasia of bone, and McCune-Albright syndrome, which are typically not associated with short stature. Enhanced signaling of the parathyroid hormone/parathyroid hormone-related peptide receptor, which couples to multiple G proteins including Gsα, leads to short bones with delayed endochondral ossification. It has remained unknown whether constitutive Gsα activity also impairs bone growth. Here we generated mice expressing a constitutively active Gsα mutant (Gsα-R201H) conditionally upon Cre recombinase (cGsα^{R201H} mice). Gsα-R201H was expressed in cultured bone marrow stromal cells from cGsα^{R201H} mice upon adenoviral-Cre transduction. When crossed with mice in which Cre is expressed in a tamoxifen-regulatable fashion (CAGGCre-ER™), tamoxifen injection resulted in mosaic expression of the transgene in double mutant offspring. We then crossed the cGsα^{R201H} mice with Prx1-Cre mice, in which Cre is expressed in early limb-bud mesenchyme. The double mutant offspring displayed short limbs at birth, with narrow hypertrophic chondrocyte zones in growth plates and delayed formation of secondary ossification center. Consistent with enhanced Gsα signaling, bone marrow stromal cells from these mice demonstrated increased levels of c-fos mRNA. Our findings indicate that constitutive Gsα activity during limb development disrupts endochondral ossification and bone growth. Given that Gsα haploinsufficiency also leads to short bones, as in patients with Albright's hereditary osteodystrophy, these results suggest that a tight control of Gsα activity is essential for normal growth plate physiology.

1. Introduction

The alpha-subunit of the stimulatory G protein (Gsα), which is essential for the actions of many hormones, stimulates adenylyl cyclase to generate the ubiquitous intracellular second messenger cAMP [1–5]. Heterozygous inactivating mutations in the gene encoding Gsα (*GNAS*) cause multiple human disorders characterized by hormone resistance and skeletal and developmental abnormalities (e.g. Albright's hereditary osteodystrophy and pseudohypoparathyroidism) [6–8]. *GNAS* mutations that cause constitutive activation of Gsα, on the other hand, are found in many different benign and malignant tumors and cause McCune-Albright syndrome (MAS), a disorder characterized by fibrous dysplasia of bone (FD), hyperpigmented skin lesions, and hyperactivity of multiple endocrine organs [9–12].

The stimulatory G protein signaling downstream of parathyroid hormone-related peptide (PTHrP, encoded by *PTHLH*) is an important regulator of endochondral bone formation, acting via the cAMP/protein kinase A (PKA) pathway [13]. Accordingly, inactivating mutations of *GNAS*, *PTHLH*, *PDE4D* (encoding cAMP phosphodiesterase type 4D), and *PRKAR1A* (encoding type 1α regulatory subunit of PKA), cause disorders characterized by short stature and brachdactyly type E [7,8,14–18]. Interestingly, patients who carry activating mutations of the parathyroid hormone/PTHrP receptor (PTHR) (i.e. Jansen's metaphyseal chondrodysplasia) are also short with disproportionately short limbs [19]. Thus, it appears that both inactivation and hyperactivation of the PTHrP/Gsα/cAMP/PKA signaling pathway disrupts long bone growth. However, the PTHR couples to additional heterotrimeric G proteins and G protein-independent pathways [20], some of which are known to play roles in growth plate physiology based on mouse studies [21,22]. It is thus conceivable that these additional signaling pathways contribute, fully or partially, to the bone growth phenotypes associated

Abbreviations: Gsα, stimulatory G protein alpha-subunit; MAS, McCune-Albright Syndrome; FD, fibrous dysplasia of bone; CNC, Carney-Complex; PTHrP, PTH-related peptide; PTHR, PTH/PTHrP receptor; BMSCs, bone marrow stromal cells.

* Corresponding author at: Endocrine Unit, Massachusetts General Hospital, 50 Blossom St. Thier 10, Boston, MA 02114, USA.
E-mail address: bastepe@helix.mgh.harvard.edu (M. Bastepe).

[1] These authors contributed equally.

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with increased PTHR signaling. Moreover, patients with MAS, who carry constitutively active Gsα mutants, do not typically present with short stature or brachdactyly. Only few cases with short stature have been reported in the literature [23,24], and these may reflect some of the associated hormonal abnormalities, such as precocious puberty. In addition, patients with Carney-Complex (CNC), who have mutations in *PRKAR1A* that cause increased PKA activity, also lack short stature as a typical clinical feature [25]. Although both MAS and CNC patients can sometimes have excess growth hormone, which may mask direct effects of increased Gsα/cAMP/PKA signaling on the growth plate, the paucity of patients with short stature may suggest that the endochondral bone formation is preserved despite enhanced activation of this signaling pathway. It has therefore remained uncertain whether constitutive Gsα activity directly impairs bone growth.

In this study, we generated mice in which a constitutively active Gsα mutant (Gsα-R201H) can be expressed upon the action of Cre recombinase (cGsα^{R201H} mice). Activation of the transgene expression in early limb-bud mesenchyme resulted in short limbs with growth plate abnormalities and delayed endochondral ossification.

2. Materials and methods

2.1. Generation of the cGsα^{R201H} mice and in vivo activation of transgene expression

The HA-tagged rat Gsα-R201H cDNA, described previously [26], was cloned into pBSApBpACAGftILn [27], using *Sal*I and *Sbf*I restriction sites. This plasmid was then digested with *Asc*I and *Stu*I to obtain a 8637-bp fragment containing the transgene. After gel purification, this DNA fragment was microinjected into C57BL/6 × 129/SvJ F1-hybrid embryos (the Gene Modification Facility, Harvard University). The founders were identified by PCR using a forward primer annealing to the HA-containing portion of Gsα cDNA (5'-GACGTGCCGGATTACGCGTC-3′) and a reverse primer annealing to the native Gsα sequence (5'-TCTCAGGGTTGGCCAGCTCC-3′). The founders were crossed with wild-type C57BL/6 mice to generate F1 and F2 heterozygous offspring, which were used for analysis of transgene expression. The cGsa^{R201H} mice were crossed with either Prx1-Cre or CAGGCre-ER™ mice to activate transgene expression in developing limb mesenchyme and in widespread tissues, respectively. Single mutant (Cre-only or transgenic-only) and wild-type littermates among the offspring of these crosses did not show gross differences from one another and were used as control mice. Both males and females were used for the analyses and comparisons were made among littermates. Prx1-Cre and CAGGCre-ER™ mice were obtained from Jackson Laboratories (Stock No: 005584 and 004682, respectively). Control and double mutant offspring from cGsα^{R201H} and CAGGCre-ER™ matings were injected intraperitoneally with 75 mg/kg/daily tamoxifen (Sigma-Aldridge) from P14 through P18 for 5 consecutive days and euthanized for tissue analyses seven days later. All the mouse studies received approval from the Massachusetts General Hospital Institutional Animal Care and Use Committee.

2.2. BMSC isolation, qRT-PCR analysis, and western blots

Bone marrow stromal cells (BMSCs) were isolated from femurs of 4 to 6 week-old mice and cultured in alpha-MEM medium containing 10% fetal bovine serum, as previously described [28]. After two-weeks of culture, cells were trypsinized and subcultured in 12- or 24-well plates. The following day, cells were transduced with adenovirus encoding Cre recombinase, yellow fluorescent protein (YFP), *lacZ*, or HA-tagged native Gsα (Gsα-HA). Three days after transduction, total RNA was isolated from transduced BMSCs using the RNeasy Plus Mini Kit (QIAGEN), and cDNA was synthesized with the ProtoScript II First stand cDNA synthesis kit (New England Biolabs). qRT-PCR analysis was performed with specific primers and FastStart Universal SYBR Green Master (Roche) with β-actin as a reference gene. Primer sequences for amplification of the HA-tagged Gsα-R201H were the same as those used for genotyping (see above). Primer sequences for amplifying c-fos and β-actin transcripts have been previously described [29,30]. In separate experiments, whole cell lysates were prepared three days after transduction, followed by quantification of protein concentration by using the BCA reagent (Pierce). Equal amounts of total proteins for each cell lysate were separated by 9% SDS-PAGE, blotted on nitrocellulose filters by using a semi-dry blotter, and immunoreacted to a mouse monoclonal anti-HA antibody (1:1000; Abcam). After washing and subsequent incubation with anti-mouse IgG-HRP (Santa Cruz Biologicals), immunoreactive proteins were detected through the use of enhanced chemiluminescence. β-actin immunoreactivity, detected by a monoclonal antibody (Santa Cruz Biologicals), was used subsequently to verify comparable amount of protein loading among the wells.

2.3. Skeleton staining

Skeletal preparations of cartilage and bone were stained with alcian blue/alizarin red as described [31], but with minor modifications. Briefly, following euthanasia, the neonates were skinned, eviscerated, and fixed in 95% ethanol overnight, and followed by incubation in acetone for 24 h to remove the fat. Intact skeletons were then stained overnight in alcian blue (0.3 mg/ml alcian blue 8GX, 80% ethanol, 20% acetic acid). The next day, skeletons were cleaned in 70% ethanol for 6–8 h and then transferred to 1% KOH for 24 h until mostly clear. Subsequently, the skeletons were counterstained overnight in alizarin red (50 μg/ml in 1% KOH), followed by cleaning in 1% KOH and 20% glycerol for 2 days or more.

2.4. X-gal staining and histological analyses

Adult mice were initially perfused with intracardiac administration of 2% paraformaldehyde in phosphate-buffered saline, and their organs were dissected and further fixed overnight at 4 °C. After washing with phosphate-buffered saline several times, organs were cryoprotected with 30% sucrose and embedded in the OCT compound (Tissue-Tek). Frozen sections were prepared at 12 μm on Shandon cryostat machine. Cultured BMSCs were fixed at room temperature for 20 min with 0.2% gluteraldehyde in phosphate-buffered saline containing 0.02% Nonidet-P40 and subsequently washed with phosphate-buffered saline containing 0.02% Nonidet-P40, 0.01% sodium-deoxycholate, and 2 mM MgCl2. Fixed and washed tissue sections and cultured BMSCs were incubated at 37C overnight in the X-gal staining solution, as described [32]. For light microscopy, femurs were removed from double mutant and control littermates and fixed in 4% paraformaldehyde overnight at 4 °C. Femurs of newborn and three-week-old mice were subsequently decalcified in 20% EDTA (pH 7.4) for a week or 3 weeks, respectively. Decalcified femurs were embedded in paraffin blocks by standard histological procedures, sectioned (5–6 μm thickness) at several different levels, and stained with hematoxylin and eosin (Center for Skeletal Research Histology Core, Endocrine Unit, Massachusetts General Hospital). X-gal stained BMSCs in culture dishes were analyzed by Leica MZ16 dissecting microscope and captured by using Leica DFC420 camera and Leica Application Suite software (Houston, TX). Stained slides were analyzed by Nikon Eclipse Ni microscope (Tokyo, Japan), and images captured by using Diagnostic Instruments SPOT RT-SE™ Digital Camera (Houston, TX) and SPOT software. The ImageJ software [33] was employed to measure the relative lengths of hypertrophic and columnar proliferating chondrocyte zones in distal femur growth plates.

2.5. Statistical analyses

Mean and standard errors were calculated from multiple independent qRT-PCR experiments and growth plate measurements. Statistical