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Expression of an active Gαs mutant in skeletal stem cells is sufficient and necessary for fibrous dysplasia initiation and maintenance

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Fibrous dysplasia (FD) is a disease caused by postzygotic activating mutations of GNAS (R201C and R201H) that encode the α-subunit of the Gs stimulatory protein. FD is characterized by the development of areas of abnormal fibroosseous tissue in the bones, resulting in skeletal deformities, fractures, and pain. Despite the well-defined genetic alterations underlying FD, whether GNAS activation is sufficient for FD initiation and the molecular and cellular consequences of GNAS mutations remains largely unresolved, and there are no currently available targeted therapeutic options for FD. Here, we have developed a conditional tetranycyclic ( Tet)-inducible animal model expressing the GαsR201C in the skeletal stem cell (SSC) lineage ( Tet-GαsR201C/Pttn1-Cre/LSL-rTA-ires-cre-GFP mice), which develops typical FD bone lesions in both embryos and adult mice in less than 2 weeks following doxycycline (Dox) administration. Conditional GαsR201C expression promoted PKA activation and proliferation of SSCs along the osteogenic lineage but halted their differentiation to mature osteoblasts. Rather, as is seen clinically, areas of woven bone admixed with fibrous tissue were formed. GαsR201C caused the concomitant expression of receptor activator of nuclear factor kappa-B ligand (Rankl) that led to marked osteoclastogenesis and bone resorption. GαsR201C expression ablation by Dox withdrawal resulted in FD-like lesion regression, supporting the rationale for Gαs-targeted drugs to attempt FD cure. This model, which develops FD-like lesions that can form rapidly and revert on cessation of mutant Gαs expression, provides an opportunity to identify the molecular mechanism underlying FD initiation and progression and accelerate the development of new treatment options.

Significance

We generated a novel conditional inducible mouse model of fibrous dysplasia (FD) by expressing mutant GNAS in skeletal stem cells (SSCs) in a temporally controlled and tissue-specific fashion. Typical FD bone lesions developed rapidly in mutant embryos and postnatal mice. GNAS promoted PKA activation and proliferation of SSCs along the osteogenic lineage but impaired their differentiation to mature osteoblasts and triggered increased osteoclastogenesis and bone resorption. FD lesions reverted on cessation of GNAS expression. Thus, GNAS mutation is sufficient and necessary for FD initiation and maintenance. This model provides a valuable opportunity to identify the molecular mechanism underlying FD progression and accelerate the development of more effective treatment options.

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The authors declare no conflict of interest.

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that multiple additional events subsequent to $G\alpha_{s}$ mutant expression and aberrant cell proliferation may contribute to FD, which in turn may need to be targeted for therapeutic intervention in this disease. Indeed, the precise nature of the FD/MAS initiating cells, as well as whether $G\alpha_{s}$ mutants are strictly necessary once FD/MAS is established, is at present unknown (16).

We show here that the combinatorial use of Cre-mediated conditional and tetracycline (Tet)-inducible systems resulting in the expression of mutant $G\alpha_{s}$ in SSCs is sufficient to recapitulate the clinical complexities of FD/MAS in less than 2 wk after mutant $G\alpha_{s}$ induction. We also observed that reducing $G\alpha_{s}$ expression by doxycycline (Dox) withdrawal resulted in clinical improvement of the affected mice in 4 mo, supporting the central tenet that stopping the expression or activity of $G\alpha_{s}$ will halt FD progression and promote the regression of preexisting FD lesions. The use of this genetically defined inducible FD mouse model with a rapid onset of disease progression may provide an opportunity to understand FD development at the molecular and cellular level and accelerate the development and evaluation of new FD treatments.

Results

Development of Conditional and Doxycycline-Inducible Mice Expressing $G\alpha_{s}^{R201C}$ in Skeletal Stem Cell Lineage Results in FD-Like Lesions. As part of our effort to study how $G$ proteins control stem cell fate and contribute to FD lesion formation, we developed conditional transgenic mice expressing $G\alpha_{s}$-activating mutants under the control of Tet-responsive elements (Tet-Ge $G\alpha_{s}^{R201C}$), which was rapidly induced after Dox treatment. We have recently shown that these Tet-$G\alpha_{s}^{R201C}$ mice, when crossed with cytokertin 5 promoter (K5rtTA) mice, developed a robust phenotype in a tissue-specific and induced rapid stem cell differentiation (17).

Paired-related homeobox 1 (Prrx1)-Cre mice, expressing Cre recombinase under the control of Prrx1-derived enhancer, are frequently used to study bone development, as it is expressed in the early limb bud mesenchyme and in a subset of craniofacial mesenchymal cells (18, 19), as well as in postnatal SSCs in limb and craniofacial bones (20, 21). LSL-rtTA-IRES-GFP is known as a “linker” mouse, as it only expresses rtTA and GFP proteins after the expression of Cre recombinase that eliminates a Stop-STOP-Stop (LSL) cassette in the Prrx1-expressing cells, resulting in conditional and cell type-specific expression of rtTA and GFP simultaneously (22).

To investigate whether expression of $G\alpha_{s}^{R201C}$ in bone progenitor (stem) cells is sufficient to initiate FD, we crossed Tet-Ge $G\alpha_{s}^{R201C}$ mice with double transgenic Prrx1-Cre and linker mice (Fig. L1). All pups were born healthy with no obvious difference in either size or behavior. Specific expression of $G\alpha_{s}^{R201C}$ transgene in bone tissues was confirmed by quantitative PCR (qPCR) analysis and Glu-Glu (EE) tag immunoblotting and found associated with phosphorylation of cAMP-responsive element-binding protein (CREB), downstream signaling of $G\alpha_{s}$ (Fig. 1 B and C) and mutant ($G\alpha_{s}^{R201C}$, Tet-Ge/$G\alpha_{s}^{R201C}$/Prrx1-Cre) mice. This finding is consistent with the markedly elevated intracellular cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) phosphorylation observed in Dox-treated $G\alpha_{s}^{R201C}$ bone marrow stromal cell (BMSC) cultures (Fig. 1 D and E). Breeding $G\alpha_{s}^{R201C}$ mice with double-fluorescent Cre reporter mice (ROS426 mT/mG) confirmed the skeletal-specific lineage of the Cre-recombination event in the limbs, showing green Cre activation and red unrecruined tissues in an adult Dox-treated mouse (Fig. 1 F and Fig. SI 1). Indeed, we confirmed that $G\alpha_{s}^{R201C}$ is not expressed in other nonosseous tissues, such as muscle and skin. FD-like lesions initiated specifically from growth plates within limb bones in $G\alpha_{s}^{R201C}$ mice (Fig. 1 F). These data support the efficient and tissue-specific activation of the $G\alpha_{s}$ signaling pathway.

$G\alpha_{s}^{R201C}$ Expression During Embryogenesis Results in FD-Like Lesions. To investigate the early embryogenic mutation events that give rise to FD, we initiated Dox administration to female mice (6 g/kg in the diet) during gestation from embryonic day 4.5 to investigate the effect of $G\alpha_{s}^{R201C}$ on bone development during embryogenesis. $G\alpha_{s}^{R201C}$ pups were born following a Mendelian distribution with obviously dysmorphic limbs compared with their littermates (Fig. 2 A). Whole-mount staining showed clear limb deformity and marked lytic changes along with impaired mineralization in calvarial sutures of the skull (Fig. 2 B). Histological analysis of forelimbs demonstrated skull vault osteolysis in $G\alpha_{s}^{R201C}$ mice with predominance of chondroid matrix and reduced endochondral ossification (Fig. 2 C). In addition, abnormal undermineralized trabeculae embedded within a dense fibrocortical matrix, a distinctive histological feature of FD, were already present at this stage (23). These findings suggest that activating $G\alpha_{s}$ mutation could initiate FD early lesions during embryonic bone development.

Rapid Onset of FD-Like Lesions in the Postnatal Skeleton of $G\alpha_{s}^{R201C}$ Mice. In human, FD typically originates during childhood (12). To better assess the postnatal onset and evolution of the FD lesions, mice were treated with Dox (0.05 g/L in drinking water) at the weaning stage (21 d old) and evaluated visually daily for signs of bone lesions. Although we did not observe any significant abnormalities in ion homeostasis, such as blood phosphate, calcium, potassium, and magnesium, within 1 wk of Dox treatment, most limbs of $G\alpha_{s}^{R201C}$ mice showed progressive swelling and limb deformity, with FD-like lesions starting at the growth plates (Fig. 2 S2). By 2 wk, all limbs of $G\alpha_{s}^{R201C}$ mice exhibited FD-like lesions, as judged by the visible swelling and confirmation by radiological and histological analyses; littermates appeared normal (Fig. 3 A and B). X-ray imaging revealed a FD-resembling “ground glass” appearance in limb bones, with distinct hyperostosis and deformation (Fig. 3 C). Whole-mount skeletal staining revealed expansive bone deformities and undermineralization of the bone lesions, also typical of human FD (Fig. 3 D). Furthermore, $\mu$CT imaging confirmed these lytic changes and revealed spontaneous microfractures in limb bones (Fig. 3 E). These bone deformities and fractures led to limping behavior found in $G\alpha_{s}^{R201C}$ mice. Consistent with a common location of FD lesions in humans, defects in calvarial sutures in skull were also observed by $\mu$CT imaging (Fig. 3 F). Analysis of $\mu$CT scans of limb bone revealed reduced bone volume, suggesting increased bone resorption (Fig. 3 G).

After 2 wk of Dox administration, H&E staining revealed marked deformities in limb bones of $G\alpha_{s}^{R201C}$ mice with diffuse boundaries of cortical, trabecular, and bone marrow compartments. Normal bone structures were substituted by unstructured fibroosseous tissues, composed of immature, undermineralized woven bone, blended into a fibrocortical matrix of collagen (Fig. 3 A and B), which diminished, or absent hematopoietic and adipose areas of bone marrow, all distinctive features of FD (Fig. S3 A–D). The irregular trabecular pattern was reminiscent of the classic “Chinese characters” feature of FD histology (Fig. S3 A). Sirius red staining showed a widespread disorganized pattern of woven bone and green birefringent collagen-rich fibrosis in $G\alpha_{s}^{R201C}$ mice as seen under polarized light (Fig. 4 C and D). As in human FD, lesions did not show signs of malignant transformation. Additionally, consistent with human FD, $G\alpha_{s}^{R201C}$ mice showed prominent lesion osteoid, in association with severe mineralization defect, as revealed by Toluidine Blue (Fig. 4 E) and von Kossa staining (Fig. 4 F) of undecalcified in pmethyl methacrylate (PMMA)-embedded limb sections. Histomorphometric analysis of these areas demonstrated elevated numbers of osteoblasts and osteoclasts, and a marked increase in osteoid volume compared with control mice (Fig. 4 G), suggesting increased bone remodeling and defective mineralization. These ectopic intracortical remodeling sites mimic the tunneling resorption pattern in the bones found in human FD and hyperparathyroidism, to which FD is related pathophysiologically ($G\alpha_{s}$-stimulated cAMP signaling by excess PTH) (2, 26). In conclusion, $G\alpha_{s}^{R201C}$ mice develop classical histological features of FD lesions.
**Expression in Postnatal Skeletal Progenitors Arrest Their Osteogenic Terminal Differentiation.** FD is characterized histologically by the presence of abundant fibroblast-like cells with pre-osteoblastic characteristics in fibrotic areas. As is typical of FD, the islands of dysplastic, woven bone in lesions are not rimmed by mature osteoblasts (23–25). The fibroblast-like FD cells express early osteogenic markers, as assessed immunohistochemically, including the runt-related transcription factor 2 (Runx2), osteonectin (Ocn), and alkaline phosphatase (Alp) (Fig. 5A). However, the late osteogenic marker, osteocalcin (Ocn), was poorly expressed (Fig. 5A). In contrast, only the calcified structures and abnormal osteoblasts on the surface of the woven bone showed moderate expression of Ocn. These data support the concept that the FD fibroblast-like cells are osteogenic precursors and that GαsR201C impairs osteogenic differentiation of immature osteogenic cells into osteoblasts (27).

To assess the effect of GαsR201C on osteogenic differentiation, we took advantage of the EE tag to assess by double-labeling immunofluorescence coexpression of GαsR201C with various markers of osteogenic differentiation. During FD lesion progression, GαsR201C was highly expressed with early osteogenic markers, as evidenced by colocalization of EE tag with the early osteogenic markers Runx2, Ocn, Alp, and Collagen I (Col1) (Fig. 5B). Again, the mature osteoblast marker, Ocn was rarely observed within FD-like lesions.
but was evident in the cells in the mature mineralized bone (Fig. 5B). Paralleling the finding of a mineralization defect observed histologically, BMSCs isolated from G\(_{\alpha_s}\)R201C mice showed a severe impaired mineralization capacity upon expression of G\(_{\alpha_s}\)R201C, which induced by Dox (Fig. S4A). Furthermore, we observed highly proliferative (27) activity and relatively low apoptotic rates of the fibroblast-liked cells demonstrated by 5-ethynyl-2′-deoxyuridine (EdU) proliferation assay and anti-caspase3 staining, respectively (Fig. 5C and D).

Taken together, these findings support the role of skeletal progenitors as FD lesion initiators, in which activating mutations of G\(_{\alpha_s}\) promote their proliferation in an expansive, dysregulated fashion. The excess woven bone matrix synthetized by these SSC-derived progenitors may fail to mineralize properly due to an arrested differentiation and abnormal matrix expression.

**G\(_{\alpha_s}\)R201C-Expressing Cells Induce Osteoclastogenesis and Increase Bone Resorption.** Human skeletal progenitors harboring G\(_{\alpha_s}\) mutants express receptor activator of nuclear factor kappa-B ligand (Rankl), which leads to increased bone resorption of dysplastic bone in FD (28). In a rapid response to Dox administration, differentiation of osteoclasts was significantly stimulated surrounding areas of woven bone in the limbs of G\(_{\alpha_s}\)R201C mice, as evidenced by an increased number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells, pointing to increased osteoclastogenesis and bone resorption (Fig. S2A). After 2 wk of G\(_{\alpha_s}\)R201C induction, numerous resorbing osteoclasts with lacunae were prominent in FD-like lesions (Fig. 6A), which is consistent with the lytic areas observed by X-ray imaging and \(\mu\)CT reconstruction (Fig. 3C, E, and F). Consistently, increased serum level of tartrate-resistant acid phosphatase 5b (TracP 5b) was
found in Gαs R201C mice (Fig. 6B). To explore the mechanism of elevated osteoclast differentiation, we measured the expression of Rankl and its decoy receptor, Osteoprotegerin (Opg) by qPCR analysis. Rankl and Rankl/Opg values were significantly increased in Gαs R201C mice (Fig. 6C and Fig. S4B). As expected, the circulating level of Rankl was also remarkably elevated in Gαs R201C mice (Fig. 6D). The excess Rankl was secreted by FD-initiating Gαs R201C cells, as evidenced by colocalization with EE tag (Fig. 6E). The association of Rankl with osteoclasts, indicated by cathepsin K (Ctsk) staining (Fig. 6F), further supports the notion that the maturation of osteoclasts was stimulated by Rankl- and GNAS-expressing cells within FD-like lesions (29).

Reducing Gαs R201C Expression Results in Clinical Improvement of the Preexisting FD Bone Lesions. To test whether reducing the expression of activated Gαs will result in a regression of the FD-like lesions, Dox administration was discontinued after 2 wk, at a time when FD lesions were readily evident. Mice were followed for an additional 4 mo (Fig. 7A). μCT examination revealed mice had slightly thickened limbs of Gαs R201C, but without porous-like, lytic phenotype or fractures (Fig. 7B). Surprisingly, there were no significant differences in the bone volume between Gαs R201C in control mice at this stage (Fig. 7C). As seen on H&E staining, the limbs of Gαs R201C mice exhibited a dramatic recovery, with dense cortical bone and proper enclosed diaphyseal marrow cavity. We noticed that the remineralized cortical bone, recovered from previous widespread fibrous tissue, was fused together, narrowing the marrow cavity compared with littermate controls (Fig. 7D). Hematopoietic cells were able to repopulate the marrow spaces at the recovered lesional sites with increased numbers of adipocytes (black arrows and star area) (Fig. 7D). Collagen-rich fibrocellular matrix and multinucleated giant cells were not present at this stage. Von Kossa staining of undecalcified limb sections show irregular shaped undermineralized woven bone (white arrows), which are surrounded by excess osteoid (black arrows) in Gαs R201C mice (Fig. 7E). Osteoclastic resorption in preexisting FD lesions was dramatically reduced, as demonstrated by minimal TRAP staining (Fig. 7F). We also observed significant remineralization in the cranial area as seen on μCT examination. The skull of Gαs R201C mice showed a normal morphology with healed sutures (Fig. 7G). The significant clinical improvement of limbs and skulls in mice that were previously affected with FD after the discontinuation of Gαs R201C expression, indicates that stopping the expression or activity of Gαs R201C mutants halts FD progression and may promote the regression of preexisting FD lesions.

Discussion

We have generated a conditional and doxycycline-inducible mouse model for FD and show that the expression in the SSC lineage of Gαs R201C results in a rapid onset of disease exclusively in the skull and long bones, the two most problematic and common areas for FD in humans. By mimicking the manifestations of human FD with this mouse model, we conclusively demonstrate that expression of Gαs-activating mutations in skeletal progenitor cells, including its SSC compartment, is sufficient and necessary to establish FD in embryos and adult mice.
We first took advantage of the tissue-specific Cre-recombinase expression driven by the Prrx1 gene to explore the effect of activating mutations of GNAS during embryogenesis. Our data show that activating mutations of Gαs in developing cranial and limb bones result in expansive bone deformities and mineralization deficiency, in contrast to the lack of effects of GαsR201C on embryonic bone development, which was reported previously (16). Instead, we demonstrated that GαsR201C mutations are sufficient to induce typical FD phenotype when expressed in skeletal progenitors. In the current study, significant bone defects were mainly

Fig. 5. The preosteogenic feature of GαsR201C mutant cells. (A) Immunohistochemistry of osteogenic markers in control and FD limb bone sections. Runx-related transcription factor 2 (Runx2), osterix (Osx), and alkaline phosphatase (Alp) are enriched in the fibroblastic-like cells within fibrous tissue (ft); Ocn is only found positive in the cells in contact with lesional trabeculae. (B) Double-labeling immunofluorescence staining show elevated expression of early osteogenic markers in Glu-Glu (EE) tag-positive GαsR201C mutant cells along the development of FD. In tissues of day 0, white dashed lines outline boundaries between the hypertrophic zone (hz) and ossification zone (oz) of growth plate or boundaries between cortical bone (cb) and bone marrow (bm). In tissues of day 2 and day 7, white dashed lines outline boundaries between FD lesions (fibrous tissue, ft) and unaffected cortical bone (cb) or bone marrow (bm). Col1; Collagen I. 5-ethynyl-2′-deoxyuridine (EdU) click staining (C) and cleaved caspase-3 immunofluorescence staining (D) showed high proliferation and relatively low apoptosis rate of GαsR201C mutant cells.
observed in calvarial sutures and bones in GαsR201C mice (Figs. 2B and 3F). However, we did not observe significant pathological changes in bones of the skull base, which is consistent with others’ finding that postnatal calvarial SSCs expressing Prrx1 reside exclusively in calvarial sutures (21). Further efforts will be devoted to the detailed analysis of FD progression and pathological manifestations in the cranial area. These data lend support to the hypothesis that a subset of embryonic bone progenitor cells may already harbor the causative mutation of FD (6). This in turn raises the possibility that FD initiating cells promoting subclinical lesions may already be present at birth, which undetected, may then progress to FD/MAS during childhood.

At the cellular level, our data suggest that GαsR201C-initiated FD development involves several distinct mechanisms. As depicted in Fig. S5, initially GαsR201C expression accelerates the proliferation of skeletal progenitor cells, including skeletal stem cells, which remain committed to early stage osteogenic lineage, as judged by the expression of Runx2, Osx, Alp, and Col1, typical of preosteoblastic cells. However, these cells cannot undergo full differentiation from preosteoblasts to mature osteoblasts. This is accompanied by impaired mineralization (30, 31) as well as robust expression and secretion of Rankl by these preosteoblastic cells that stimulate abundant osteoclastogenesis and bone resorption. Indeed, the combined overgrowth of fibroblast-like, poorly differentiated preosteoblasts, with the remarkable activation of osteoclasts and bone remodeling by GαsR201C-expressing cells may underlie the rapid formation of FD lesions upon expression of GαsR201C in SSC lineage.

To date, there is no cure for FD. The available medical treatments are only palliative, focusing on relieving symptoms. The frequent pathological fractures and pain that accompany FD may be related to abundant osteoclastogenesis and bone resorption. Indeed, the combined overgrowth of fibroblast-like, poorly differentiated preosteoblasts, with the remarkable activation of osteoclasts and bone remodeling by GαsR201C-expressing cells may underlie the rapid formation of FD lesions upon expression of GαsR201C in SSC lineage.

Fig. 6. GαsR201C leads to increased bone resorption via the Rankl pathway. (A) Tartrate-resistant acid phosphatase (TRAP) staining of limb bone indicates the presence of numerous osteoclasts in GαsR201C mice (n = 4). (B) Serum level of tartrate-resistant acid phosphatase 5b (TRACP 5b) was elevated in GαsR201C mice (n = 4). Scatterplots are plus mean (Middle line). (C) qPCR analysis shows significantly increased Rankl and Rankl/Opg ratio (n = 6). (D) Serum level of Rankl was significantly increased in GαsR201C mice (n = 4). Scatterplots are plus mean (Middle line). (E) Immunofluorescence staining shows colocalization of Rankl with EE tag in fibroblastic-like cells expressing GαsR201C. (F) Immunofluorescence staining shows binding of Rankl with osteoclasts, which are marked by cathepsin K (Ctsk). Opg, osteoprotegerin; Rankl, receptor activator of nuclear factor kappa-B ligand. Data are presented as mean ± SD, and significance was calculated by Student’s t test (**P < 0.01 and ***P < 0.001).
FD and establish the strict dependence of FD on persistent GαsR201C activity to support the maintenance of FD lesions.

Together, the data lend support to the hypothesis that a cure for FD could be brought about by reducing the expression of GαsR201C and/or its associated downstream molecular events. Regarding the latter, we observed a remarkable activation of PKA upon mutant GαsR201C expression in BMSCs. In turn, we can postulate that PKA-regulated transcriptional events may promote the expression of the Rankl promoter, similar to that induced by PTH or 1,25 dihydroxyvitamin D3 [1,25(OH)2D3] (36). However, why PKA stimulation may halt the terminal differentiation of osteoblastic precursors is still unknown. While detailed analysis of transcriptional programs elicited in vivo by GαsR201C can now be explored in our animal model, recent findings supporting that PKA can control histone modification marks (37, 38) may provide new avenues in the search of a treatment for FD. Specifically, our recent study revealing that PKA controls stem cell fate in the skin (17) and recent evidence that PKA activation promotes epigenetic gene repression by histone H3 with acetylated lysine 9 (H3K9) demethylation, suggests that many new cancer therapies targeting epigenetic mark writers, readers, or erasers can now be deployed for FD therapy. In addition, recent high-throughput small molecule library screens have identified multiple novel drug candidates, however, their evaluation for FD/MAS treatment has been hampered by the dearth of suitable animal models of this disease (39). In this regard, several...
of the features of our mouse model, specifically rapid onset of phenotype with appropriate histological FD manifestations, and the reversion of FD lesions after blocking pathologic GnaαsR201C expression, may represent an ideal platform to discover precision treatment approaches disrupting GnaαsR201C-driven growth, and/or restoring normal skeletal stem cell differentiating circuits in FD.

Materials and Methods

Generation of GnaαsR201C Mice. The disease model mice are triple transgenic mice obtained by breeding a strain that express Cre driven by the Prx1-derived enhancer [B6.Cg-Tg(Prrx1-cre)1Cju, referred to as “Prrx1-Cre” mice], with ROSA26-LSL-RTA-IRES-GFP mouse able to express reverse tetracycline transactivator (tTA) after Cre combination [B6.Cg-Tg(Rosa26SlrtTA)2KsG(cre)1Vko, EGFP(Nagy), referred to as linker mice], with mice carrying the mutated human GNASαsR201C gene and bearing a long internal EE tag, described previously (17) (referred to as “Tet-Gαs” mice). To demonstrate sites of Cre combination, the GnaαsR201C mice were bred with double-fluorescent Cre reporter mice (Rosa26 mTmG). All mice except Tet-Gαs mice were obtained from The Jackson Laboratory.

All mice were of mixed background between C57BL/6 and FVB/N. All animal studies were carried out according to NIH Intramural Animal Care and Use Committee-approved protocols and the University of California San Diego (UCSD) Institutional Animal Care and Use Committee-approved protocols, in compliance with the Guide for the Care and Use of Laboratory Animals. Both male and female mice were used in the studies, if not specified. All experiments were conducted using littermate controls. In the tetracycline-inducible mice, doxycycline (Dox) (Sigma-Aldrich) was administered in a concentration ranging from 0.01 to 1 g/L in drinking water or 6 g/kg in the diet (Newco specialty division). Treatment was started between embryonic day 4.5 and 2 wk after birth, for up to 2 consecutive weeks, as indicated in each experimental group.

Tissue Collection and Processing. Fresh bone tissues were frozen in dry ice immediately after collection and homogenized. Bone tissue samples were collected and fixed in 10% formalin buffered fixative solution at room temperature overnight and then stored in 70% ethanol for further embedding. Fixed skeletal elements were either decalcified in 4% buffered EDTA for paraffin and optimal cutting temperature (OCT) frozen embedding or embedded in PMMA (40) without decalcification. H&E staining sections were obtained from paraffin sections for further histological assessment. Please see SI Extended Materials and Methods for additional details.

X-Ray, Microcomputed Tomography. Radiograms were taken using a Faxitron MX-20 Specimen Radiography System and microcomputed tomography (μCT) analysis was performed according to the standard guidelines (41) using a SCANCO Medical μCT 40 system (SCANCO Medical). We also assessed the bone volume fraction (BV/TV) in the regions of limb with FD lesions and the corresponding regions in controls. Please see SI Extended Materials and Methods for additional details.

Whole-Mount Skeletal Staining (Alizarin Red and Alcian Blue Staining). Mice were killed, skinned, and eviscerated, and then fixed in 100% ethanol overnight. The specimens were then immerged in staining solution for 3 d and processed according to standard protocols for Alizarin Red S (ARS) staining. Please see SI Extended Materials and Methods for additional details.

Sirus Red Staining. Paraffin-embedded tissue sections were deparaffinized and stained in Picro-Sirius Red solution, counterstained with methyl green (Vector), dehydrated, and evaluated under both transmitted light and polarized light microscopy. Please see SI Extended Materials and Methods for additional details.

TRAP Staining. Sections were incubated at 37 °C in freshly prepared TRAP staining solution, rinsed in distilled water, counterstained with methyl green (Vector), dehydrated, and mounted. Please see SI Extended Materials and Methods for additional details.

Bone Histomorphometric Analyses. Bone histomorphometric measurements were performed on undecalcified PMMA-embedded bone sections after Toluidine Blue and von Kossa/nuclear fast red staining, using OsteoMeasure software (OsteoMetrics). The region of interest excluded the area either 0.25 mm from the growth plate. All histomorphometric parameters were calculated and expressed according to the suggestions made by the American Society for Bone and Mineral Research nomenclature committee (42).

RNA Isolation and qRT-PCR. Total RNA was isolated from both bone tissues and cells using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed followed by qRT-PCR analysis using iQ SYBR Green Supermix (Bio-Rad) on a Bio-Rad iCycler IQ multicolor real-time PCR detection system. Target gene expression levels were normalized with Gapdh. Please see SI Extended Materials and Methods for additional details and oligoribonucleotides used for amplification.

Immunoblot Analysis. Western blot assays were performed and repeated at least three times as previously described (17). Please see SI Extended Materials and Methods for additional details and all antibodies used.

Immunohistochemistry and Immunofluorescence. For immunohistochemistry, paraffin sections were deparaffinized and rehydrated, endogenous peroxide activity was blocked using 3% hydrogen peroxide, and heat-mediated antigen retrieval was performed. The sections were blocked with 10% FBS (Gibco) in PBS for 1 h then incubated with primary antibodies overnight at 4 °C followed by incubation of biotinylated secondary antibodies for 30 min at room temperature. Sections were detected using ABC and DAB systems, and then counterstained with hematoxylin. The slides were scanned using an Aperio AT2 Slide Scanner. For double-labeling immunofluorescence, the OCT-embedded frozen sections were blocked and incubated with primary antibodies overnight as were the paraffin sections. Sections were then incubated 30 min with mouse secondary antibodies (Alexa Fluor-conjugated secondary antibodies) and nuclei stained with DAPI (4’,6-diamidino-2-phenylindole; Sigma-Aldrich) for 5 min. Images were taken using a confocal microscope, as described below. Please see SI Extended Materials and Methods for additional details and the antibodies used. Each immunostaining was repeated in at least three independent mice or three independent experiments; multiple fields were reviewed.

Fluorescent Imaging. Confocal fluorescent images were used to analyze the double-labeling immunofluorescence staining in both control and FD tissues. Please see SI Extended Materials and Methods for additional details.

Edu Proliferation Assay. The proliferation assay was performed as previously described (43). Briefly, 100 μL of 10 mM Edu per 10 g of mouse body weight were administered 3 h before killing, before proceeding to decalcification and sectioning. The click staining and the following immunofluorescence staining were performed according to the manufacturer’s instructions (Invitrogen, Click-IT Edu Alexa Fluor 647 Imaging Kit).

Bone Marrow Stromal Cell Culture and Osteogenic Induction. Primary mouse BMSCs were isolated and cultured as previously described (44). The osteogenic differentiation induction was performed as previously described (45). To induce the osteoblast lineage, ARS staining was performed to assess the mineralization of extracellular matrix after 14 d of osteogenic induction. Cells were rinsed with PBS and fixed in 4% paraformaldehyde for 1 h at room temperature. After washing with PBS, cells were stained with freshly made ARS staining solution and then washed in distilled water. Please see SI Extended Materials and Methods for additional details.

ELISA. For CAMP ELISA, BMSCs were seeded in 24-well plates at a density of 1 × 10⁴ cells per well and incubated overnight in serum-free medium. Then cells were incubated in the presence of 5 μg/mL Dox for 2 h. The phosphodies- terase inhibitor, 0.2 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich) was added to the medium 30 min before harvest. Intracellular CAMP levels were measured using the Mouse/Rat cAMP Parameter Assay Kit (R&D systems). For tranylcypromine-resistant acid phosphatase Sb (TRACP Sb) and TNP-resistant activation-induced cytokine (TRANCE, Rankl) ELISA, mice were killed for serum collection. Serum samples were then analyzed by ELISA according to manufacturer’s instructions. Please see SI Extended Materials and Methods for additional details.

Statistical Analysis. Statistical analyses were performed from at least three independent experiments. Unpaired t test or ANOVA were used to evaluate significance in two or more than two sample groups, respectively. All analyses were carried out using Prism 6 statistical analysis program (GraphPad). Statistical significance was reported as follows: NS for not significant or P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001. All data are reported as mean ± SD.

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