Activation of the Gs G protein–coupled receptor Rs1 in osteoblasts increases bone mineral density by 5- to 15-fold in mice and recapitulates histologic aspects of fibrous dysplasia of the bone. However, the effects of constitutive Gs signaling on bone tissue quality are not known. The goal of this study was to determine bone tissue quality in mice resulting from osteoblast-specific constitutive Gs activation, by the complementary techniques of FTIR spectroscopy and synchrotron radiation micro-computed tomography (SRμCT). Col1(2.3)-tTA/TetO-Rs1 double transgenic (DT) mice, which showed osteoblast-specific constitutive Gs signaling activity by the Rs1 receptor, were created. Femora and calvariae of DT and wild-type (WT) mice (6 and 15 weeks old) were analyzed by FTIR spectroscopy. WT and DT femora (3 and 9 weeks old) were imaged by SRμCT. Mineral-to-matrix ratio was 25% lower (P = 0.010), carbonate-to-phosphate ratio was 20% higher (P = 0.025), crystallinity was 4% lower (P = 0.004), and cross-link ratio was 11% lower (P = 0.025) in 6-week DT bone. Differences persisted in 15-week animals. Quantitative SRμCT analysis revealed substantial differences in mean values and heterogeneity of tissue mineral density (TMD). TMD values were 1,156 ± 100 and 711 ± 251 mg/cm³ (mean ± SD) in WT and DT femoral diaphyses, respectively, at 3 weeks. Similar differences were found in 9-week animals. These results demonstrate that continuous Gs activation in murine osteoblasts leads to deposition of immature bone tissue with reduced mineralization. Our findings suggest that bone tissue quality may be an important contributor to increased fracture risk in fibrous dysplasia patients.

Keywords Fourier transform infrared spectroscopy · Synchrotron micro-computed tomography · Bone formation · Bone quality · Mineralization · Gs signaling · Rs1 transgenic mice · Receptors activated solely by synthetic ligands (RASSLs) · G protein–coupled receptors · Fibrous dysplasia of bone

Osteoblast G protein–coupled receptors (GPCRs) are fundamental regulators of skeletal maintenance and repair. Activation of osteoblast Gs-GPCR signaling increases bone mass, as in pathological conditions such as fibrous dysplasia of the bone and McCune-Albright syndrome [1, 2]. However, the affected bone is associated with increased fragility. In contrast, intermittent activation of the parathyroid hormone receptor (PTH1R) by recombinant parathyroid hormone (teriparatide) [3] increases bone
formation and is an important treatment for osteoporosis. Manipulation of skeletal GPCR signaling, therefore, is a potentially potent technique for regulating disease processes and enhancing repair of bone tissue [4].

The skeletal effects of Gs-GPCR signaling on bone formation were investigated with the ColII(2.3)-tTA/TetO-Rs1 mouse model of fibrous dysplasia of the bone. This model uses a synthetic biology approach [4] to activate the Gs signaling pathway in maturing mouse osteoblasts by the constitutive Gs activity of the Rs1 engineered receptor activated solely by a synthetic ligand (RASSL) [5, 6]. RASSLs are engineered receptors that no longer respond to endogenous hormones but are activated by synthetic small-molecule drugs. They have been useful for studying the roles of G-protein signaling in a variety of in vivo systems [7–10]. In the ColII(2.3)-tTA/TetO-Rs1 mouse model, Rs1 expression was sufficient to induce a dramatic increase in skeletal bone mass and trabecular bone formation [6]. In addition, cortical bone was lost, the normal bone marrow canal was obliterated, and the normal bone marrow cellular components were replaced by a large number of small cells that histologically resemble young osteoblasts. These features show strong similarity to the bony lesions in patients with fibrous dysplasia of the bone, a disease that also results from activation of the Gs signaling pathway [1, 2].

While elevated osteoblast Gs signaling has significant effects on bone mass and structure, the quality of the resulting bone tissue has not been thoroughly investigated. Characteristics of bone tissue quality, including mineral content and composition, crystal size and perfection, and collagen matrix stability, are critical to the mechanical competence [11–14] and remodeling characteristics [15, 16] of bone. Therefore, evaluation of tissue quality metrics is crucial for understanding the anabolic effect of constitutive Gs signaling on bone and for developing effective treatments for skeletal pathology.

We applied two complementary strategies for assessing bone quality in mice with osteoblast-specific constitutive Gs signaling activity: Fourier transform infrared (FTIR) spectroscopy, to investigate tissue quality at the molecular level, and synchrotron radiation micro-computed tomography (SRμCT), to provide accurate quantification of bone tissue mineral density (TMD) at high spatial resolution.

FTIR spectroscopy provides a method for examining tissue quality and composition by measuring molecular bond vibration frequencies [17, 18]. FTIR spectra of calcified tissues typically provide information on the structure and environment of the carbonate and phosphate groups of the mineral phase and the amide groups of the organic matrix. Metrics of bone quality can be calculated from these data. Specifically, these parameters include mineral-to-matrix ratio (correlated to ash density [19, 20]), carbonate-to-phosphate ratio (reflecting the level of carbonate substitution into the apatite lattice [15, 21]), crystallinity (related to crystal size and perfection as determined by X-ray diffraction [22, 23]), and collagen cross-link ratio (the ratio of nonreducible to reducible cross-links, indicating collagen maturity and stability [24]). These FTIR parameters provide information on the structure and mineralization of the inorganic and organic components of bone.

SRμCT enables accurate TMD quantification at high spatial resolution. In this technique, a high photon flux monochromatic X-ray beam extracted from a synchrotron beam replaces the standard polychromatic X-ray beam used in conventional μCT devices. The use of a single X-ray beam energy eliminates beam-hardening artifacts, the high photon flux produces a high signal-to-noise ratio and high spatial resolution, and the use of a nearly parallel beam enables exact 3D tomographic reconstruction. Due to these factors, TMD quantification by SRμCT is more accurate than that by conventional μCT. SRμCT TMD assessment has been verified against gravimetric methods (ashing) [25] and 2D microradiography [26] and is currently considered the gold standard for high-resolution 3D TMD evaluation.

In this study, we used FTIR spectroscopy and SRμCT to evaluate measures of bone tissue mineralization, composition, and maturity in mice with osteoblast-specific constitutive Gs signaling activity. Our results show that the bone formed by activated Gs signaling is more immature and has lower mineralization than that of wild-type (WT) controls. These findings suggest that reduced bone quality contributes to increased bone fragility in fibrous dysplasia patients. In addition, our results illustrate that metrics of bone tissue quality are important in investigating signaling mechanisms.

Materials and Methods

Mouse Model

All transgenic mouse studies were approved by and performed in accordance with the Institutional Animal Care and Use Committee and the Laboratory Animal Research Center at the University of California, San Francisco. ColII(2.3)-tTA/TetO-Rs1 double transgenic (DT) mice were generated by heterozygote crosses of mice carrying the TetO-Rs1 transgene [6] with mice carrying the ColII(2.3)-tTA transgene [27], as described [6]. Transgene expression was activated by maintaining the mice on regular mouse chow without doxycycline (LabDiet 5053; PMI Nutrition, St. Louis, MO). These transgenic mice display osteoblast-specific constitutive Gs signaling activity through the Rs1 receptor. Previous qPCR assays on whole femora of 6-week-old adult mice showed that Rs1 expression was
selectively and highly induced in the bones of DT mice [6]. All studies compared DT mice to WT littermate controls.

**FTIR Spectroscopy**

Femora and calvariae of DT and WT mice were analyzed by FTIR spectroscopy. Mice of two ages were evaluated: 6 weeks ($n = 6$ WT, 6 DT) and 15 weeks ($n = 6$ WT, 6 DT). Bones were excised, stripped of soft tissue, and fixed in 70% ethanol. The desired region of each specimen was isolated and desiccated through an ethanol series, followed by exposure in a desiccant chamber. The sampled regions were the mid-diaphysis of the left femur and the left posterior quadrant (parietal bone) of the calvaria (Fig. 1). For each sample, a 2-mg (approximately 2 mm long) bone segment was isolated and a homogenized powder mixture was created of 1% bone by weight in potassium bromide (KBr; Thermo Electron, Waltham, MA). The powder mixture was compressed using a manual die to create a pellet for FTIR spectroscopy.

Spectroscopy was performed on a benchtop interferometer system (Nexus 870, Thermo Electron). Spectra were acquired using 256 scans at a spectral resolution of 4 cm$^{-1}$. A background scan was recorded immediately after each sample scan to facilitate background correction. After acquisition, the spectra were transferred to chemical imaging software (Isys; Spectral Dimensions, Olney, MD) for analysis. Spectra were baseline-adjusted and the integrated areas of the amide I (1,595–1,720 cm$^{-1}$), $\nu_3$ phosphate (PO$_4^{3-}$, 895–1,215 cm$^{-1}$), and $\nu_2$ carbonate (CO$_3^{2-}$, 840–890 cm$^{-1}$) bands were calculated. Mineral-to-matrix (PO$_4^{3-}$/amide I), carbonate-to-phosphate (CO$_3^{2-}$/PO$_4^{3-}$), and carbonate-to-matrix (CO$_3^{2-}$/amide I) ratios were calculated from integrated areas of the respective peaks. Additionally, peak heights were measured at specific wave numbers: 1,020, 1,030, 1,660, and 1,690 cm$^{-1}$. From these, a series of absorbance ratios were calculated to determine additional spectroscopic parameters. The ratio of 1,030 to 1,020 cm$^{-1}$ represents the ratio of stoichiometric apatite to nonstoichiometric apatite, a measure of crystallinity. Finally, the ratio of 1,660 to 1,690 cm$^{-1}$ represents the proportion of nonreducible to reducible cross-links in the collagen, indicative of collagen maturity.

**FTIR Spectroscopy—Repeatability**

Left femora from each of three 15-week-old WT mice were used to assess repeatability of FTIR measures. From each femur, a mid-diaphyseal section was isolated and processed into a homogeneous powder mixture of 1% bone by weight in KBr as described above. Three pellets were created from each batch of the homogenized mixture, and the remaining powder was stored in the desiccator to prevent hydration...
between each pellet preparation. Spectroscopy and analysis of the three pellets from each bone were performed consecutively on a single day, following the methods detailed in the previous section.

Synchrotron μCT Imaging

One representative femur from 3- and 9-week-old WT and DT mice was imaged using SRμCT. Femora were dissected, stripped of soft tissue, fixed in 10% neutral buffered formalin for 24 h, and stored in 70% ethanol before imaging. The distal surface of the femoral condyles was mounted onto a block for imaging with a wax-based adhesive. Synchrotron imaging was performed at the European Synchrotron Radiation Facility (Grenoble, France). Beamline ID19 of this facility is equipped with a μCT acquisition system capable of producing high-resolution 3D images with high contrast and signal-to-noise ratio [28]. Images were acquired using a single energy (21 keV) chosen to provide suitable contrast. The transmitted monochromatic X-ray beam was recorded using a scintillator coupled to a 2D 1,024 × 1,024 charge coupled device (CCD) camera. The optical system was arranged to produce a pixel size of 5 μm. Dark current and reference images without the specimen were taken during acquisition for flat field correction. Three-dimensional images were reconstructed by means of a 3D filtered back-projection algorithm from 900 projections. A local thresholding scheme [29] was employed to segment the bone from background (Fig. 2). This local thresholding scheme was necessitated by the variations in voxel intensity observed in the synchrotron images; global thresholds failed to capture the entire mineralized region, particularly in DT bone, and therefore underestimated TMD variation and miscalculated mean TMD. After segmentation, average linear attenuation was calculated and directly converted to TMD with a theoretical relationship modeling linear attenuation coefficient as a function of hydroxyapatite concentration.

DXA Analysis

Whole-body DXA scans of WT and DT mice were performed for animals aged 3 weeks (n = 10 WT, 14 DT), 6 weeks (n = 10 WT, 10 DT), 9 weeks (n = 8 WT, 14 DT), and 15 weeks (n = 7 WT, 7 DT) to assess phenotype progression. Whole-body DXA scans were performed under anesthesia with 1% isoflurane in a Lunar PIXImus2 (GE Lunar, Waukesha, WI), as described previously [6].

Histology

Femora of 6-week-old DT and WT mice collected for histology were fixed in 10% neutral buffered formalin for 24 h. Undecalciﬁed femora were embedded in polymethyl methacrylate [6], and 4-μm sections were cut on a hard-tissue microtome. Decalciﬁed bones were processed in 10% EDTA, embedded in parafﬁn, sectioned, and stained with hematoxylin and eosin (H&E) by the Gladstone Institutes Histology Core (San Francisco, CA). Mid-diaphyseal regions of interest were visualized to grossly assess phenotypic changes.

Statistics

Wilcoxon signed-rank tests revealed no signiﬁcant differences in FTIR spectroscopic parameters between the two anatomic sites (femur, calvaria); therefore, values were averaged to produce a single result per animal. Results obtained from WT and DT mouse specimens were compared using t-tests or Wilcoxon rank sums tests as appropriate. Repeatability of FTIR measures was determined by calculating the coefﬁcient of variation (CV) for each

Fig. 2 Mid-diaphyseal femoral cross section of a 3-week-old double transgenic mouse. Gray-scale data (a) were processed using a local thresholding algorithm that incorporates edge detection (b, center panel shows result of edge detection), allowing accurate segmentation (b, right panel shows result of segmentation) despite the existence of fine structures and dramatic spatial variation in attenuation. A rendering of the resulting 3D segmented data set is shown (c)
femoral specimen, then computing mean CV for the three femora used in the repeatability study.

**Results**

**FTIR Spectroscopy**

FTIR spectroscopy was performed to assess bone quality in young (6 weeks) and mature (15 weeks) WT and DT mice. No differences in FTIR spectroscopic parameters were detected between the two anatomic sites we tested (femora and calvaria) for either WT or DT animals, despite the different bone-formation processes (endochondral vs. intramembranous) represented by the two sites.

Significant differences in FTIR spectroscopic measures of bone composition were found between 6-week-old WT and DT mice (Table 1, Fig. 3). Mineral-to-matrix ratio was 25% lower in DT bone ($P = 0.010$), carbonate-to-phosphate ratio was 20% higher ($P = 0.025$), crystallinity was 4% lower ($P = 0.004$), and cross-link ratio was 11% lower ($P = 0.025$). These differences persisted in 15-week-old animals. No differences were found between WT and DT mice in carbonate-to-matrix ratio ($P = 0.084$ at 6 weeks, $P = 0.107$ at 15 weeks). Differences associated with animal age were detected only in WT mice. WT carbonate-to-phosphate ratio increased 17% from 6 to 15 weeks ($P = 0.017$), carbonate-to-matrix ratio increased 49% ($P = 0.007$), and cross-link ratio decreased 8% ($P = 0.037$). Change in carbonate-to-matrix ratio with age approached significance in DT mice (25% increase, $P = 0.050$). These results indicate that bone tissue in DT mice is more immature than that in WT mice. In addition, while WT tissue matured as the mice aged, DT mice retained their immature tissue composition.

**FTIR Spectroscopy—Repeatability**

Mean CV values were calculated to assess repeatability of the FTIR measurements. Mean CV for FTIR measures ranged from 0.3% to 3.9%, with crystallinity displaying the lowest variation and carbonate-to-phosphate ratio displaying the highest variation for the samples tested (Table 2).

**SRµCT Imaging**

SRµCT data were evaluated to assess bone microstructure and TMD. Synchrotron imaging revealed dramatic differences in structure between WT and DT femora (Fig. 4). The 3-week-old DT femur had no distinct marrow cavity or cortical shell but, rather, a disorganized trabecular pattern throughout the cross section. This structural abnormality persisted in the 9-week-old DT femur. Quantitative analysis of the synchrotron data revealed differences in TMD mean and variance values. TMD values were $1.156 \pm 100$ and $711 \pm 251$ mg/cm$^3$ (mean ± SD) in WT and DT femur diaphyses, respectively, at 3 weeks and $1,205 \pm 72$ and $813 \pm 133$ mg/cm$^3$ in WT and DT mice, respectively, at 9 weeks. These results show that tissue composition and organization are altered in DT mice.

**DXA Analysis**

Whole-body BMD values were measured for DT and WT mice at 3, 6, 9, and 15 weeks of age to evaluate skeletal growth over the range of ages assessed via FTIR and SRµCT (Fig. 5). At 3 weeks of age DT mice had BMD values statistically indistinguishable from those of WT mice. BMD of DT mice was 103% that of their WT littermates by 6 weeks of age and 165% by 9 weeks ($P < 0.003$). BMD of DT mice remained constant through 15 weeks, with no statistically significant change detectable relative to the 9-week measurement. These results indicate that the accumulation of bone mineral in DT mice persists through sexual maturity, then plateaus in mature mice (>8 weeks).

**Histology**

Undecalcified sections were processed to visualize cortical and trabecular structure. Decalcified sections were stained...
with H&E to reveal cellular, matrix, and marrow components. A disordered mineralization pattern with effacement of the cortical compartment and elimination of the medullary canal was seen in these images (Fig. 6), in agreement with our previous finding that Rs1-induced basal \( G_s \) signaling in osteoblasts of DT mice increased trabecular bone and decreased cortical bone in lesions, reminiscent of fibrous dysplasia of the bone [6].

**Discussion**

Our results show that continuous \( G_s \) activation in mouse osteoblasts leads to deposition of large quantities of immature trabecular bone with reduced mineralization. FTIR spectroscopy revealed a 25–29% deficit in mineral-to-matrix ratio in the bones of mice expressing Rs1, and synchrotron imaging showed a reduced mean TMD. These findings are consistent with low levels of bone mineralization. Measures of mineral composition, mineral maturity, and collagen maturity also indicate significant abnormalities in bone formation induced by \( G_s \) activation in maturing osteoblasts. These alterations in tissue quality accompany dramatic structural changes, including greatly increased bone mass, increased heterogeneity of mineralization, disorganization of trabecular morphology, effacement of the cortical shell, and elimination of the medullary canal.

Our model is particularly relevant for GPCR diseases found in humans, including primary hyperparathyroidism and fibrous dysplasia of the bone such as that occurring in McCune-Albright syndrome. Patients with primary hyperparathyroidism present with marked cortical thinning and decreased cortical BMD [30, 31], possibly related to increased cortical porosity [32–34]. The elimination of the cortical compartment in DT mice is an extreme version of the cortical loss seen in primary hyperparathyroidism. However, the massive increase in trabecular volume observed in DT mice is not generally seen in patients with hyperparathyroidism. Bone from McCune-Albright syndrome patients shows a fibrous infiltrate, significant increases in trabecular bone formation, ablation of the marrow cavity, and an increased propensity to deformation and fracture [35]. In addition, these patients accumulate unmineralized osteoid with a nonlamellar structure as well as mineralized tissue with markedly reduced mineral content [1, 36]. The mineralization abnormalities found in our mouse model of fibrous dysplasia may reflect those in patients with fibrous dysplasia of the bone.

FTIR spectroscopic measures provide bone tissue characterization at the molecular level and, importantly, are correlated with mechanical integrity and remodeling properties of the tissue. Mineral-to-matrix ratio increases as
both primary and secondary mineralization progress and, therefore, is positively associated with tissue age [12, 37]. Studies of human tissue and animal models demonstrated positive correlations between mineral-to-matrix ratio and tissue stiffness and hardness [12, 38, 39]. Mineral-to-matrix ratio explains 50–60% of the variation in both tissue modulus and hardness [37, 40]. In our study, decreased mineral-to-matrix ratio in DT bone suggests the presence of immature bone with reduced resistance to deformation. Since FTIR crystallinity values are positively associated with tissue age, tissue yield strength, and stiffness [12, 14, 37], the decreased crystallinity we observed in DT bone substantiates the presence of immature tissue with reduced mechanical properties. We previously showed that DT animals have markedly elevated bone-turnover markers; display irregular, punctate bone formation by von Kossa staining and double fluorescent labeling; and have increased numbers of TRAP-positive osteoclasts [6]. These characteristics support the conclusion that DT bone lesions contain regions with extremely high rates of bone formation and turnover, consistent with our findings of accumulated immature tissue.

Increased carbonate substitution has been associated with increased tissue age [12, 15, 41, 42], increased tissue...
indentation modulus and hardness [40], and—by a mechanism of reduced ductility—increased incidence of fracture [43] and inferior mechanical properties at the whole-bone level [20]. Carbonate substitution in the hydroxyapatite lattice leads to a change in lattice dimensions and increased disorder of the crystalline structure [44]. Further, mineral solubility is affected by carbonate content and increased carbonate content is thought to enhance bone resorption [45]. Carbonate-to-phosphate ratio was significantly elevated in DT bone, indicating an alteration in crystal synthesis and perhaps playing a role in the high turnover observed in DT animals [6].

Collagen matrix biochemistry is also related to tissue age, mineralization, and mechanics. Intermolecular cross-linking provides the matrix tensile strength and influences whole-bone strength [46–49]. Cross-link formation also alters the rate of mineralization and microdamage accumulation [50], thereby providing a second mechanism for regulating the mechanical properties of bone [51]. The ratio of mature to immature, reducible cross-links—quantified as cross-link ratio—increases with tissue maturity [52, 53] and correlates positively with indentation modulus [12]. In DT bone, low cross-link ratio indicates immature tissue with reduced stiffness.

FTIR analyses showed that DT bone does not exhibit maturation between 6 and 15 weeks. In contrast, specimens from 6- and 15-week-old WT mice showed significant differences in carbonate-to-matrix and carbonate-to-phosphate ratios, indicative of increasingly mature tissue. Cross-link ratio decreased in WT animals, in contrast to the established association between cross-link ratio and matrix maturation.

SRµCT imaging was used to quantify mean TMD and distribution of TMD values, measures complementary to those determined through FTIR analysis. Consistent with the deficit in mineral-to-matrix ratio identified by FTIR, DT bone had mean TMD values 39% and 33% lower than WT bone at 3 and 9 weeks, respectively. SRµCT also revealed increased heterogeneity in mineral content in DT mice as reflected by the large standard deviation of TMD in DT bones at 3 and 9 weeks (151% and 88% higher, respectively). The alteration in distribution of mineralization values might significantly affect the overall material properties of DT bone; heterogeneous regions of tissue mineralization may hinder crack propagation and toughen tissue. It is intriguing to consider that the increased variation in TMD may represent a compensatory mechanism by which DT mice counteract the deficit in tissue mineralization. Increased tissue volume in DT animals may represent an additional adaptive response to the formation of hypomineralized tissue and loss of cortical structure. These changes may partially or fully compensate for any loss of whole-bone strength or stiffness. No spontaneous fractures have been identified in DT animals, perhaps supporting this hypothesis.

The presence of immature bone in our mouse model of fibrous dysplasia suggests that strategies for modulating
bone formation, such as antiresorptive medications, may have therapeutic value for these patients. Since the fibrous
dysplastic bone lesions appear to be reversible by inhibiting
Gs signaling [54], the immature bone formation seen in
our DT mice might also be reversed. This would be an
important metric as potential therapies for fibrous dysplasia
are developed.

Despite identifying significant effects of Rs1 signaling
on bone mineralization, our study has several limitations.
First, we were unable to perform direct measurement of
mechanical competence in DT mice. Mechanical testing is
challenging in DT specimens due to morphological
abnormalities and heterogeneous mineralization. However,
the compositional measures derived from FTIR and
SRµCT data are highly associated with mechanical prop-
erties of bone tissue and give us insight into the mecha-
nisms of increased fragility in fibrous dysplastic bone.
Second, the ages of animals characterized by FTIR and
SRµCT were different; this should be taken into consid-
eration when making direct comparisons between analysis
results. However, for each analysis technique, the younger
animals (3 and 6 weeks old) were in the rapid skeletal
growth phase, while the older animals (9 and 15 weeks old)
had reached a plateau of bone accumulation. This is sup-
ported by BMD measurements and consistent with the
observed sexual maturity window of 7–8 weeks in both
WT and DT mice. Finally, our findings are based on
samples from a relatively small number of animals. The
significant abnormalities in tissue quality and skeletal
structure we observed in our TD mice were surpris-
ingly conserved between the two different anatomic sites sam-
ped (femora and parietal bones of calvariae). This finding
allowed us to compile results from the two skeletal sites
and perform a conservative statistical analysis. Despite
these limitations, we believe that our results provide new
insight into the roles of Gs signaling in regulating the
matrix formation process.

In conclusion, our results illustrate that activation of the
Gs signaling pathway in maturing osteoblasts leads to a
significant degradation of bone tissue quality in a mouse
model of fibrous dysplasia. The striking influence of the
fibrous dysplasia model on tissue quality metrics reinforces
the paradigm that tissue quality, and not just quantity and
structure, must be considered in the evaluation of any
disease processes or potential therapies.

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