The role of the 18-kDa isoform of fibroblast growth factor-2 (FGF2) in the maintenance of bone mass was examined in Col3.6–18-kDa FGF2-IREs-GFPsaph transgenic (18-kDa TgFGF2) mice in which a 3.6-kb fragment of the type I collagen 5′-regulatory region (Col3.6) drives the expression of only the 18-kDa isoform of FGF2 with green fluorescent protein-sapphire (GFPsaph). Vector only transgenic mice (Col3.6-IRES-18-kDa isoform of FGF2 with green fluorescent protein-sapphire (GFPsaph)). Vector only transgenic mice (Col3.6-IRES-GFPsaph, VTg) were also developed as a control, and mice specifically deficient in 18-kDa FGF2 (FGF2<sup>18-kDa−</sup>) were also examined. Bone mineral density, femoral bone volume, trabecular thickness, and cortical bone area and thickness were significantly increased in 18-kDa TgFGF2 mice compared with VTg. Bone marrow cultures (BMSC) from 18-kDa TgFGF2 mice produced more mineralized nodules than VTg. Increased bone formation was associated with reduced expression of the Wnt antagonist secreted frizzled receptor 1 (sFRP-1). In contrast to 18-kDa TgFGF2 mice, FGF2<sup>18-kDa−</sup> mice have significantly reduced bone mineral density and fewer mineralized nodules, coincident with increased expression of sFRP-1 in bones and BMSC. Moreover, silencing of sFRP-1 in BMSC from FGF2<sup>18-kDa−</sup> mice reversed the decrease in β-catenin and Runx2 mRNA. Assay of Wnt/β-catenin-mediated transcription showed increased and decreased TCF-luciferase activity in BMSC from 18-kDa TgFGF2 and FGF2<sup>18-kDa−</sup> mice, respectively. Collectively, these results demonstrate that the 18-kDa FGF2 isoform is a critical determinant of bone mass in mice by modulation of the Wnt signaling pathway.

A variety of tissues, including bone, produce FGF2<sup>2</sup> where osteoblasts deposit it in newly forming bone matrix (1). A single

**This work was supported, in whole or in part, by National Institutes of Health Grant AG021189-05A1 (to M. M. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.**

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2 The abbreviations used are: FGF2, fibroblast growth factor-2; BMD, bone mineral density; BMC, bone mineral content; BV/TV, bone volume/tissue volume; sFRP-1, secreted frizzled receptor 1; β-catenin, TOP-flash; GFP, green fluorescent protein; GFPsaph, with green fluorescent protein-sapphire; BMSC, bone marrow stromal cell; PBS, phosphate-buffered saline; KO, knock-out; VTg, vector only transgenic mice; FZD, frizzled; sFRP, secreted FZD-related protein; FGFR, FGF receptor; WT, wild type; SMI, structure model index; RANKL, receptor activator of the NF-κB ligand; Tb.Th, trabecular thickness; HO, homozygote; ALP, alkaline phosphatase; DXA, dual beam x-ray absorptiometry; siRNA, short interfering RNA.
ized the bone phenotype of mice with selective deletion of the 18-kDa isoform of FGF2 (10).

Recent studies reported a role for modulation of Wnt signaling by fibroblast growth factor receptor-2 (FGFR2) and the ligand fibroblast growth factor-1 (FGF-1) in intramembranous bone formation (11). However, possible cross-talk between FGF2 and Wnt signaling during postnatal bone formation has not been investigated, so we examined a putative role of the Wnt pathway in the bone anabolic effect mediated by 18-kDa FGF2. Wnt proteins are a family of secreted glycoproteins that play important roles in many biological processes, including skeletal development (12) as well as postnatal bone formation (13–15). Wnt proteins initiate a signaling cascade by binding to a membrane receptor complex composed of the frizzled (FZD) G protein-coupled receptor that is combined with a low density lipoprotein receptor-related protein to activate downstream signaling pathways (15). Wnt signaling is controlled by both extracellular and intracellular proteins (15). Secreted FZD-related proteins (sFRPs) are extracellular proteins that are able to bind Wnts or FZD receptors thereby attenuating all Wnt-activated pathways. Published results show that sFRP1 is a negative regulator of trabecular bone mass as demonstrated by increased trabecular bone mineral density in sfrp1−/− mice (16). Interestingly, FGF2 and FGFR signaling were shown to modulate heparin-induced sFRP1 accumulation in the HEK293 cell line (17). Because of the similarities between the Sfrp1 and the 18-kDa TgFGF2 mice bone phenotypes, we examined sFRP1 mRNA and protein expression in 18-kDa TgFGF2 and VTg mice as well as mice with selective deletion of the 18-kDa FGF2 isoform. We also examined whether differential expression of sFRP-1 altered β-catenin that is important in osteoblast differentiation. These studies demonstrate that transgenic overexpression of the 18-kDa FGF2 protein isoform increased bone mass and that the increase in bone mass is regulated through modulation of the Wnt pathway.

**EXPERIMENTAL PROCEDURES**

*Generation and Identification of Mice Expressing 18-kDa Isoform of Human FGF2*—To elucidate the role of the endogenous 18-kDa isoform of FGF2 in a bone-specific manner, we generated a construct, called Col3.6–18-kDa FGF2 isoform IRES-GFPsaph. Col3.6–18-kDa FGF2 isoform IRES-GFPsaph was built by replacing a chloramphenicol acetyltransferase fragment in a previously made Col3.6-CAT-IRES-GFPsaph (18) by digestion with BMD and bone mineral content (BMC) were measured using a Dual Beam X-ray Absorptiometry (DXA).
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Piximus Mouse 11 densitometer (GE Medical Systems, Madison, WI).

Micro-CT Scanning of Femurs—After measuring the BMD and BMC, the femurs were analyzed by micro-CT system (μCT-20, Scanco Medical, Zurich), as reported previously (20). Using two-dimensional data from scanned slices, three-dimensional analysis was performed to calculate morphometric indices, including bone volume density (bone volume (BV)/tissue volume (TV)), trabecular thickness (Tb.Th = 2 × BV/bone surface). These parameters were calculated by the parallel plate model of Parfitt et al. (21). The structure model index (SMI), which reflects the degree of normal plate-like trabeculae versus abnormal rod-like trabeculae, was also determined as described previously (22).

Tissue Preparation for Histology—For histological analysis, mice expressing VTg and 18-kDa transgenes were sacrificed by CO₂ narcosis and cervical dislocation. Following euthanasia, the femurs and other tissue samples were removed and immediately fixed in 4% paraformaldehyde at 4 °C. After processing each sample was embedded in Shandon Cryomatrix (Thermo Electron Corp., Pittsburgh, PA) and completely frozen. In selected cases, femurs were decalcified in 20% EDTA in PBS for 2 days, and paraffin blocks were prepared by standard procedures. Paraffin or frozen samples were cut into 5-μm sections. The sections were stained with von Kossa. For immunohistochemistry staining, sFRP1 (Santa Cruz Biotechnology, Inc.) antibody was used at 1:200 dilution following product protocol.

Mouse Bone Marrow Cultures—Bone marrow stromal cells (BMSCs) were isolated using a modification of previously published methods (8). Tibiae and femurs from VTg and 18-kDa mice or from WT and FGF2<sup>−/−</sup> mice were dissected free of adhering tissue. Bone ends were removed, and the marrow cavity was flushed with α-minimum Eagle’s medium by slowly injecting medium into one end of the bone using a sterile 25-gauge needle. Marrow cells were collected into tubes washed twice with serum-free α-minimum Eagle’s medium. BMSCs were plated in 6-multiwell plates (2 × 10<sup>6</sup> cells/well) in αMEM containing 10% heat-inactivated FCS and on day 3 changed to differentiation medium, and cultures were fed every 3 days by replacing 80% of the medium with fresh medium. BMSCs for ALP or xylene orange staining were harvested on days 7, 14, and 21 of culture. ALP staining was performed with a commercial kit (Sigma). Dishes were scanned and then counterstained for mineral by the von Kossa method as described previously (8).

mRNA Isolation and Gene Expression—Total RNA was extracted from BMSCs or shaft of flushed long bone by using TRIzol reagent (Invitrogen) according to the manufacturer’s instruction. For real time quantitative reverse transcription-PCR analysis, RNA was reverse-transcribed by the SuperScript<sup>™</sup> first-strand synthesis system for reverse transcription-PCR (Invitrogen). Quantitative PCR was carried out using the QuantiTect<sup>™</sup> SYBR Green PCR kit (Qiagen) on a MyiQ<sup>™</sup> instrument (Bio-Rad). β-Actin was used as an internal reference for each sample. Using a formula described previously (23), the relative change in mRNA was normalized against the β-actin mRNA level.

RNA Interference—BMSCs were transfected with sFRP-1 siRNA that is able to down-regulate the sFrp1 gene in living cells by introducing a homologous double-stranded RNA. A control siRNA (scrambled sequence) was used as a negative control. The siRNA transfection was performed according to the protocol provided by the manufacturer (Santa Cruz Biotechnology, Inc.). Cells were harvested at 48 h post-transfection and were used for ALP activity assay and/or RNA extraction. The specificity of the silencing was confirmed in three different experiments by real time PCR.

Transient Transfections—To determine changes in β-catenin trans-activating activity, bone marrow stromal cells were cultured to 70% confluence in 24-well dishes and transiently transfected with the TOP-flash reporter construct using Lipofectamine reagent and PLUS reagent (25 μl Lipofectamine reagent/160 ng of TOP-flash DNA/80 ng of cytomegalovirus 5’-regulatory region DNA) according to manufacturer’s instructions (Invitrogen). Cotransfection with a construct containing the cytomegalovirus 5’-regulatory region driving the β-galactosidase gene (a gift from Dr. Anne M. Delany, University of Connecticut) was used to control for transfection efficiency. Cells were exposed to the Lipofectamine Reagent/PLUS Reagent/DNA mix for 3 h, transferred to regular medium for 48 h, washed twice with PBS, and harvested in a reporter lysis buffer (Promega). Luciferase and β-galactosidase activities were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT). Luciferase activity was corrected for β-galactosidase activity to control for transfection efficiency.

Western Blot Analysis—The expression of FGF2 isoform protein was determined by Western blot. Briefly, protein was extracted using 1 × cell lysis buffer (Cell Signaling Technology, Inc., Beverly, MA), and total protein concentration was assayed with BCA protein assay reagent (Pierce). After SDS-PAGE on 10–20% gels, proteins were transferred to Immobilon<sup>™</sup> transfer membranes (Millipore). Membranes were blocked overnight in TBS-T containing 5% nonfat dry milk (Bio-Rad). Membranes were incubated with a mouse monoclonal anti-FGF2 antibody that recognizes all isoforms of FGF2 (BD Biosciences) for 1 h, washed 1 h with TBS-T, and then incubated with a rabbit anti-mouse secondary antibody (Amersham Biosciences) in TBS-T, 1% nonfat milk for 1 h. After incubation with antibodies, membranes were washed 1 h with TBS-T. Western Lightning<sup>™</sup> chemiluminescence reagent (PerkinElmer Life Sciences and GE Healthcare) was used for detection. Band density was quantified by densitometry. The membranes were stripped and re-probed for sFPR1 (Santa Cruz Biotechnology, Inc.) or β-catenin (BD Biosciences).

Biochemistry—Blood was collected from euthanized animals by cardiac puncture. After clotting, the blood was spun, and serum was collected for analysis. Serum phosphate and calcium were measured using the FAST 340 phosphorous reagent SET/calcium reagent SET (Eagle Diagnostics, Desoto, TX). Creatinine was measured using QuantiChrom<sup>™</sup> creatinine assay kit (DICT-500) (Sigma). Serum FGF2 level was measured using QuantiKine<sup>™</sup>HS human FGF basic immunoassay kit (R & D Systems) according to the manufacturer’s instructions.
Indirect Immunofluorescence Staining for sFRP1 in BMSCs—
BMSC cells from 2-month-old FGF2lmw/H11002/WT littermate mice were plated at 2 \times 10^6 cells/well in 6-well dishes containing coverslips and cultured for 2 weeks on coverslips fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After rinsing twice with PBS, a 1:50 dilution of the rabbit anti-human sFRP1 antibody was added to the cells and then incubated for 1 h at 37 °C. For control studies, primary antibody was replaced with PBS alone. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) was then applied to the cells at a dilution of 1:60 for 1 h at 37 °C. After washing, cells were mounted in 50% glycerol in PBS. The cells were examined and photographed with a Zeiss Axioshot fluorescence microscope.

**Statistical Analysis**—All results were expressed as means ± S.E. Differences between groups were analyzed using the Student's t test, and differences were considered significant at p values of less than 0.05. For the comparison among multiple groups, analysis of variance was used, and the significant difference was determined by the Bonferroni test (StatView 4.1J Abacus Concepts, Inc., Berkeley, CA).

**RESULTS**

Characterization of 18-kDa TgFGF2 Mice—Fig. 1A shows the maps for the vector only and 18-kDa Fgf2 transgenes that were utilized to develop the vector control (VTg) and 18-kDa TgFGF2 mice. Several founder mice were identified and two viable 18-kDa TgFGF2 lines (18-kDa(305) and 18-kDa(308)) carrying the human 18-kDa FGF2 isoform transgene were established. Analysis was performed on HO 18-kDa TgFGF2 mice compared with HO VTg mice as controls unless noted. Since these mice are on an FVB/N background, HO-breeding pairs were maintained to generate sufficient VTg, 18-kDa(305)TgFGF2, and 18-kDa(308)TgFGF2 mice necessary for experimentation. PCR analysis of genomic DNA sample collected from tail samples demonstrated that both lines of 18-kDa TgFGF2 mice carried the transgene, whereas their non-transgenic littermates lacked the transgene (Fig. 1B). Western blot analysis of protein from tissues of 7-day-old (P7) 18-kDa TgFGF2 mice demonstrated

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**FIGURE 1. Establishment of VTg and 18-kDa TgFGF2 transgenic mice.** Targeting strategy for generation of the 18-kDa TgFGF2 transgenic mice is shown. A, schematic of 18 kDa-GFP expression vector. PA, polyadenylation. B, example of PCR genotyping results with DNA from heterozygote 18-kDa TgFGF2 mice and their non-transgenic littermates. A 525-bp fragment from the 18-kDa FGF2-GFP was generated by PCR; note the absence of expression in non-transgenic (NTg) littermate. C, Western blot analysis. Protein isolated from various mouse tissues (B = long bone; C = calvariae; L = liver; K = kidney; S = skin) at 2 weeks of age showed high level expression of the 18-kDa FGF2 isoform in long bone and calvariae from 18-kDa TgFGF2 mice. D, frozen section of femur from 24-day-old mice showed targeted expression of GFP driven by a 3.6-kb fragment of the COL1al 5′-regulatory region in osteoblast (arrow) and osteocytes (arrowhead) (magnification x200). E, serum FGF2 level. There was no difference in serum FGF2 level between VTg and 18-kDa TgFGF2 mice.

Indirect Immunofluorescence Staining for sFRP1 in BMSCs—
BMSC cells from 2-month-old FGF2<sup>tmw-/-</sup> and WT littermate mice were plated at 2 \times 10^6 cells/well in 6-well dishes containing coverslips and cultured for 2 weeks on coverslips fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After rinsing twice with PBS, a 1:50 dilution of the rabbit anti-human sFRP1 antibody was added to the cells and then incubated for 1 h at 37 °C. For control studies, primary antibody was replaced with PBS alone. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) was then applied to the cells at a dilution of 1:60 for 1 h at 37 °C. After washing, cells were mounted in 50% glycerol in PBS. The cells were examined and photographed with a Zeiss Axioshot fluorescence microscope.

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high levels of 18-kDa FGF2 in femurs and calvariae compared with VTg controls. Low levels of FGF2 were observed in liver, but other tissues such as kidney and skin did not overexpress 18-kDa FGF2 (Fig. 1C). There was no difference in serum FGF-2 levels between VTg and 18-kDa TgFGF2 mice (Fig. 1E), confirming bone specificity of FGF-2 overexpression.

Expression of the 18-kDa FGF2-GFP transgene in vivo was analyzed in frozen undecalcified sections of femurs from 24-day-old male mice (Fig. 1D). GFP expression was seen in bone-forming osteoblasts and osteocytes, which is consistent with observations in other transgenic animals that have been generated using the col3.6 5′- regulatory region construct (18). Osteoblasts expressing the transgene were present on the surface of trabecular bone and on the endosteal surface and periosteal surface of the metaphyseal cortical bone. GFP expression was stronger in osteoblasts found on trabecular surfaces, osteal surface of the metaphyseal cortical bone. GFP expression was seen in epithelium of the papilla and collecting ducts (24).

To determine whether there were extra-skeletal side effects from targeted overexpression of 18-kDa FGF2 in bone, hematocrit, serum creatinine, serum calcium, and phosphorus were measured. There were no differences in any of these parameters between VTg and 18-kDa TgFGF2 mice (data not shown). Histologic examination of hematoxylin and eosin-stained sections of the kidneys showed that targeted overexpression of 18-kDa FGF2 in bone did not cause enlargement, vacuolation and karyomegaly of podocytes in glomeruli, dilatation and cast formation in tubules, thickening of the media in lobular arteries, or hyperplasia of the epithelium of the papilla and collecting ducts (24).

We assessed body weight and femoral bone lengths in mice in VTg and 18-kDa TgFGF2 mice. Mean body weights were 27.6 ± 0.6 for VTg mice versus 29.4 ± 0.3 and 29.7 ± 0.3 g for 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice, respectively (p < 0.05). Femoral bone lengths were 14.37 ± 0.60 for VTg mice versus 14.87 ± 0.26 and 14.68 ± 0.13 mm for 18-kDa(305)/TgFGF2 and 18-kDa(308)/TgFGF2, respectively. There were no significant differences in bone lengths; therefore, the overexpression of the 18-kDa FGF2 isoform did not cause dwarfism or shortened femurs.

**DXA Analysis of BMD and BMC in VTg and 18-kDa TgFGF2 Mice**—BMD and BMC were determined by DXA analysis of femurs and tibiae from two independent lines of 18-kDa TgFGF2 mice compared with VTg. Tibial BMD (Fig. 2A) was increased by 16 and 12% in 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice, respectively (p < 0.05). Tibial BMC (Fig. 2B) was significantly increased by 29 and 18% in 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice, respectively (p < 0.05). Femoral BMD (Fig. 2C) was significantly increased by 8 and 6% in 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice, respectively (p < 0.05).

**Micro-CT Analysis of Femurs from VTg and 18-kDa TgFGF2 Mice**—To investigate the structural effects of 18-kDa FGF2 isoform overexpression on different bone compartments, bone micro-architecture was examined in adult mice by micro-CT. Three-dimensional images of the femurs from VTg, 18-kDa(305) TgFGF2, and 18-kDa(308) TgFGF2 mice are shown in Fig. 3A. Structural analysis showed that BV/TV was significantly increased by 19% in 18-kDa(305) TgFGF2 and increased by 24% in 18-kDa(308) TgFGF2 mice (Fig. 3B) (p < 0.05). The SMI describes the normal plate-like structure of bone, the closer to 0 the more plate-like the trabecular structure, and the closer to 3 the more abnormal rod-like the trabeculae. As shown in (Fig. 3C), SMI was significantly decreased by 10 and 12% in 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice, respectively (p < 0.05). Trabecular thickness (Tb.Th) (Fig. 3D) was significantly increased by 8 and 13% in 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice, respectively, compared with VTg mice (p < 0.05).

The effect of 18-kDa FGF2 isoform overexpression on femoral cortical bone was also determined by micro-CT. Segmented bone area (Fig. 3E) was significantly increased by 17 and 15% in 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice, respectively, compared with VTg mice (p < 0.05). Circumferential cortical thickness (Fig. 3F) was increased by 7% in both 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice (p < 0.05). Circumferential periosteal perimeter (Fig. 3G) was increased by 9 and 7% in 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice, respectively, compared with VTg mice (p < 0.05). Circumferential endosteal perimeter (Fig. 3H) was significantly increased by 9 and 8% in 18-kDa(305) TgFGF2 and 18-kDa(308) TgFGF2 mice, respectively (p < 0.05). By histo-
morphometry, von Kossa staining of non-decalcified sections of femurs showed increased mineralized trabeculae in 18-kDa TgFGF2 mice when compared with VTg mice (Fig. 3).

Analysis of BMSC Cultures from VTg and 18-kDa TgFGF2 Mice—Western blot (Fig. 4A) of cell matrix showed overexpression of 18-kDa FGF2 protein isoform in BMSC cultures from both lines of 18-kDa TgFGF2 mice. We also measured FGF2 in conditioned media of BMSC cultures of VTg and 18-kDa mice. As shown in supplemental Fig. 2, there was no difference in FGF2 protein between the genotypes. Furthermore, as shown by Western blot in supplemental Fig. 3A, overexpression of 18-kDa FGF2 did not increase the production of other FGF2 isoforms in bones. In addition, as shown by Western blot in supplemental Fig. 3B, overexposure of a membrane preparation from BMSCs cultured for 3 weeks revealed similar levels of endogenous high molecular weight FGF2 isoforms between both genotypes. We observed that GFP expression (Fig. 4B) was greater in 18-kDa TgFGF2 cultures compared with VTg. The time course of formation of ALP-positive colonies and mineralized nodules is shown in Fig. 4C, and quantization of mineralized colony number and colony area are shown in Fig. 4, D and E, respectively. At each time point, the number of ALP-positive colonies and ALP-positive colony area was significantly increased in 18-kDa(305)TgFGF2 and 18-kDa(308)TgFGF2 mice compared with VTg. As shown in Fig. 4F, ALP activity was significantly increased by 55% in BMSC cultures from 18-kDa TgFGF2 mice. In order to determine the relative prevalence of ALP-positive colonies per total cell count, the dishes that were initially stained for alkaline phosphatase after 1 week of culture were counterstained with crystal violet to stain all colonies. Total crystal violet-stained colonies as well as ALP-positive colonies in both lines of 18-kDa transgenic mice were compared with VTg controls. The stained dishes and graphic representations of total colonies, ALP-positive and calculated ALP-negative colonies are shown in supplemental Fig. 4. There is a significant reduction in ALP-negative colonies in both lines of 18-kDa compared with VTg.

Since FGF2 signals via multiple FGFRs, we determined whether there was modulation in the pattern of FGFR gene expression in 18-kDa TgFGF2 versus VTg BMSCs that were cultured for up to 3 weeks. At 1 week of culture FGFr2 mRNA (Fig. 4G) was significantly increased (p < 0.05) by 50% in BMSC from both lines of 18-kDa TgFGF2 mice relative to VTg mice. There was a progressive increase in FGFr1 mRNA expression over 3 weeks of culture that was similar in both VTg and 18-kDa
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TgFGF2 mice. FGF3 mRNA expression was increased at 3 weeks of culture but was similar in BMSC from VTg and 18-kDa TgFGF2 mice (supplemental Fig. 5).

We also assessed the mRNA and protein expression for Runx2/cbfα1, a transcription factor important in osteoblast differentiation whose expression is regulated by FGF2. We observed that Runx2 mRNA was increased by 38 and 54% in 1-week-old BMSC cultures from 18-kDa(305)TgFGF2 and 18-kDa(308)TgFGF2 mice, respectively, compared with VTg (Fig. 4H). As shown in Fig. 4I, at 1 week, Runx2 protein was increased by 48% in BMSC from 18-kDa(305)TgFGF2 mice compared with VTg. As shown in Fig. 4J, the mRNA for osteocalcin, a target gene for Runx2 (25), was significantly increased in BMSC from both lines; that was evident as early as 1 week of culture. Since the transcription factor osterix can also modulate osteoblast differentiation (26), and it can be regulated by Runx2 (27), we also measured Osterix protein levels. As shown in supplemental Fig. 6, there was no difference in Osterix protein expression in 18-kDa BMSC cultures compared with VTg.

Targeted Deletion of 18-kDa FGF2 (FGF2lmw/J/j), Effects on Bone Mineral Density, In Vitro Bone Formation, and Gene Expression—To confirm the specificity of the anabolic effect of the 18-kDa FGF2 isoform on bone, limited analysis of the bone phenotype in 18-kDa FGF2KO (FGF2lmw/J/j) mice was conducted and compared with 18-kDa TgFGF2 mice. Specifically, vertebral BMD and BMC of 2-month-old vector and 18-kDa Tg FGF2 mice was compared with 2-month-old WT and FGF2lmw/J/j littermate mice. Similar to the analysis of femoral bones, vertebral BMD (Fig. 5A) and BMC (Fig. 5B) were significantly increased in 18-kDa TgFGF2 compared with vector mice (p < 0.05). By contrast, vertebral BMD (Fig. 5C) and BMC (Fig. 5D) were reduced by 16 and 30%, respectively, in FGF2lmw/J/j mice compared with

FIGURE 4. Analysis of FGF2 protein isoform, bone nodule formation, and gene expression in BMSCs from VTg and 18-kDa TgFGF2 mice. Starting on day 3 of culture, BMSC were fed with osteogenic differentiation media, and cells were cultured for 1–3 weeks. A, Western analysis revealed increased 18-kDa FGF2 protein in cultures from 18-kDa TgFGF2 mice. Densitometry shows FGF2 protein normalized to α-tubulin standard. B, there were more GFP-positive colonies in 18-kDa BMSC at 2 and 3 weeks of culture. C, increased ALP-positive colonies and mineralized nodules (von Kossa) were observed in BMSC from both 18-kDa TgFGF2 mice. D, colony number; E, colony area were measured by NIH Image. F, ALP activity was measured after 1 week of culture as described under “Experimental Procedures.” *, significantly different from VTg, p < 0.05. G, real time PCR analysis of FGFr3 mRNA; H, Runx2 mRNA; I, Runx2 protein; J, osteocalcin mRNA expression in BMSC cultures from VTg and 18-kDa Tg mice. BMSC cultures were grown under differentiation conditions and then harvested at 1, 2, and 3 weeks. Total RNA was extracted for real time PCR analysis as described under “Experimental Procedures.” **, significantly different from VTg, p < 0.05.
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WT mice (p < 0.05). In order to assess the mechanism of the reduced BMD, BMSC from FGF2<sup>lmw</sup>-/- and WT littermate mice were cultured for up to 3 weeks. After 1–3 weeks of culture, the number of ALP-positive, mineralized, as well as xylanol orange (XO)-positive colonies were observed in BMSC from FGF2<sup>lmw</sup>-/- mice. Colony number and area (Fig. 5G) were significantly decreased in BMSC from FGF2<sup>lmw</sup>-/- mice (p < 0.05).

To determine whether FGFr and Runx2 gene expression were altered in FGF2<sup>lmw</sup>-/- mice, total RNA was extracted from BMSC of WT and FGF2<sup>lmw</sup>-/- littermates that were cultured for up to 3 weeks. As shown in Fig. 5H, there was a marked reduction in FGFr2 mRNA expression at 1 and 3 weeks in BMSC cultures from FGF2<sup>lmw</sup>-/- mice. At 1 week, Runx2 mRNA was decreased by 37% in BMSC cultures from FGF2<sup>lmw</sup>-/- compared with WT (Fig. 5I).

Secreted FZD-related Protein 1 (sFRP1) mRNA and Protein and β-Catenin mRNA Expression—To assess the role of sFRP1 in 18-kDa FGF2 regulation of osteogenesis, we first examined its expression in flushed bone using quantitative real time PCR. sFRP1 mRNA expression was significantly (p < 0.05) reduced by 27% in flushed bone from 18-kDa TgFGF2 mice compared with VTg mice (Fig. 6A). To confirm that decreased expression of sFRP1 mRNA was associated with decreased sFRP1 protein in differentiated bone, Western blot analysis was performed. As shown in Fig. 6, C and D, sFRP1 protein expression was significantly decreased by 42% in whole bone from 18-kDa TgFGF2 mice compared with VTg mice. Decreased sFRP1 protein expression was shown in immuno-stained femurs from 18-kDa TgFGF2 mice compared with VTg controls (Fig. 6G). sFRP1 protein expression was detected in bone marrow cells, osteoblasts on the trabecular surface, and in the osteocytes of the cortical bone. Overall the data show decreased expression of sFRP1 protein in femurs from 18-kDa TgFGF2 mice when compared with VTg transgenic mice.

Since sFRP1 has been shown to block Wnt signaling, we compared expression of β-catenin mRNA in flushed bones from VTg and 18-kDa TgFGF2 mice. As shown in Fig. 6B, β-catenin mRNA was significantly (p < 0.05) increased by 39% in flushed bone from 18-kDa TgFGF2 mice compared with VTg mice. Increased β-catenin protein in 18-kDa TgFGF2 was also detected by Western blot analysis (Fig. 6C). Analysis of Western blot data from three independent experiments showed that β-catenin protein levels increased by 36% in whole bone from 18-kDa TgFGF2 mice (Fig. 6E). Overexpression of 18-kDa isoform of FGF-2 in 18-kDa TgFGF2 was confirmed from whole bone (Fig. 6, C and F) where analysis of Western blot data from three inde-
sFRP-1 siRNA was used to deplete sFRP1 expression in BMSC to determine whether elevated sFRP1 specifically regulated WNT signaling. Whole bones showed decreased expression of sFRP1, increased expression of Wnt in 18-kDa TgFGF2 mice compared with VTg mice. 

**DISCUSSION**

The principal finding shown here is that specific transgenic overexpression of the 18-kDa FGF2 protein isoform in the osteoblast lineage results in increased BMD and bone mass in vivo, without causing dwarfism. Overexpression of 18-kDa FGF2 also increased mineralized bone nodule formation in vitro. Comparison of these results with our earlier studies, in which constitutive overexpression of all human FGF2 isoforms resulted in both dwarfism (5) and reduced bone mass (6), reveals that the pleiotropic function of the FGF2 gene lies in the protein isoforms. The fact that targeted deletion of 18-kDa FGF2 in FGF2<sup>lmw</sup>−/− mice resulted in decreased BMD as well
as decreased mineralized bone nodule formation in vitro further confirms the important role of this isoform in maintaining bone mass through the osteoblast.

Although FGF2 is a potent anabolic agent that has been shown to increase bone mass in osteoporotic animals, concerns about potential side effects would preclude its use as a therapeutic agent for disorders of low bone mass (9). Specifically, undesirable extra-skeletal effects from systemic FGF2 treatment in rats have been reported (9). The extra-skeletal side effects included transient hypotension, anemia, renal toxicity, and hypophosphatemia in rats. Since the commercially available recombinant FGF2 used in the rat studies is also the 18-kDa FGF2 isoform that was overexpressed in the 18-kDa TgFGF2 mice, we determined whether or not there is extra-skeletal side effects in those mice. We did not observe anemia or renal dysfunction in the 18-kDa FGF2. There was no difference in serum calcium or phosphorus between VTg and 18-kDa TgFGF2 mice. This is probably due to the fact that in 18-kDa TgFGF2 mice, FGF2 overexpression was targeted to the osteoblast lineage and was not increased in sera from these mice.

Previous studies showed that exogenous FGF2 modulated both cancellous as well as cortical bone (28). Consistent with this earlier study, structural analysis by micro-CT demonstrated a marked increase in bone mass in trabecular and cortical bone that suggests an anabolic effect for the 18-kDa FGF2 isoform on both compartments.

BMSCs contain pluripotent cells capable of differentiating into a variety of mesenchymal cell types, including osteoblasts, which can differentiate to express ALP, type I collagen, and osteocalcin, and they are capable of producing a mineralizable matrix (29). Previous studies showed that FGF2 modulates the growth and expression of the osteogenic phenotype of bone

FIGURE 7. sFRP1 protein expression in FGF2+/+ and FGF2lmw−/− mice. A, indirect immunofluorescence staining for sFRP1 in WT and FGF2lmw−/− BMSC cultured in differentiation medium. For negative control, primary antibody was replaced with PBS alone (magnification ×400). B, detection of sFRP1 protein expression in vertebrae from 2-month old male WT and FGF2lmw−/− mice (magnification ×100). C–G, sFRP1, β-catenin, Runx2, Col1a1, and ALP mRNA expression at 48 h post-transfection with control siRNA or sFRP1 siRNA in BMSC cultures. Total RNA samples were extracted and then assayed by real time PCR. H, sister cultures were used for measuring ALP activity assay. Data are expressed as mean ± S.E. *, p < 0.05, compared with WT-Control siRNA. #, p < 0.05, compared with FGF2lmw−/−-Control siRNA.
marrow-derived bone-like cells in vitro (30–31). Continuous treatment of BMSC with exogenous FGF2 inhibited osteoblast differentiation (30). However, there were more ALP mineralized colonies in bone marrow cultures from the 18-kDa FGF2 transgenic mice. Not only was there increased mineralized nodule number, but also increased area per nodule. These results suggest that targeted overexpression of the 18-kDa FGF2 isoform enhances osteoblast proliferation as well as differentiation. Conversely, the reduced number of ALP-positive mineralized colonies observed in the FGF218-kDa mice further supports our hypothesis that the endogenous 18-kDa FGF2 isoform is necessary for maximum bone formation.

There are multiple high affinity receptor tyrosine kinases through which FGF2 signals to affect downstream signaling pathways (30). Targeted deletion of FGFRs revealed their importance in osteoblast differentiation (32). Although FGF2 preferentially signals via FGFR1 and FGFR2, it is capable of signaling through other FGFRs (30). Therefore, we examined the expression of FGFR1, FGFR2, and FGFR3 at the transcriptional level. Increased expression of FGFR2 mRNA was observed at 1 week of culture of BMSCs from 18-kDa TgFGF2 mice, contrasted with reduction in FGFR2 mRNA observed in 1-week BMSC cultures from the selective FGF218-kDa KO mice. These data support a role for 18-kDa/FGFR2 signaling in osteoblast differentiation (33). FGFR3 is also important in perinatal endochondral bone development (29) and postnatal bone growth and remodeling (34). In view of these studies, we examined FGFR3 mRNA expression in both VTg and 18-kDa TgFGF2 mice. We observed that the expression of FGFR3 mRNA was similar in marrow stromal cultures from VTg and 18-kDa TgFGF2 mice after 3 weeks of culture, suggesting that signaling via FGFR3 pathway is not important in postnatal 18-kDa FGF2 regulation of bone formation.

Runx2 is a transcription factor that promotes commitment to the osteoblast lineage and osteogenic differentiation (35–37). We previously reported that intermittent FGF2 treatment increased Runx2 mRNA in osteoblast stromal cells (31). In addition, previous studies reported that Runx2 is phosphorylated and activated by FGF2 (38–39). Furthermore, partial or complete disruption of FGF2 expression reduced Runx2 mRNA expression in osteoblast/stromal cells (40). The observation that Runx2 mRNA was increased in cultured BMSC from 18-kDa TgFGF2 transgenic mice suggests that the increased bone mass and bone formation in those mice resulted in part from induction of Runx2 gene expression. Furthermore, the marked reduction in Runx2 mRNA in BMSCs from FGF218-kDa mice (relative to their WT littermates) supports a mechanism where endogenous 18-kDa FGF2 isoform modulates Runx2 to promote osteoblast differentiation. Although Runx2 is necessary for the differentiation of osteoblasts, previous studies have shown that continuous expression inhibits osteoblast maturation (41). This study is of significance to our present study, in which increased Runx2 in 18-kDa marrow cultures was only observed during the early stage of osteoblast differentiation.

The maintenance of skeletal integrity in a healthy individual requires a balanced regulation of the processes of osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Receptor activator of the NF-kB ligand (RANKL), its receptor RANK, and the endogenous soluble RANKL inhibitor osteoprotegerin play direct and essential roles in the formation, function, and survival of osteoclasts (42). Although FGF2 was previously shown to increase RANKL and decrease osteoprotegerin in osteoblast cultures (29, 43), there was no consistent change in RANKL or osteoprotegerin expression in osteoblasts from 18-kDa TgFGF2 mice (supplemental Fig. 7). Therefore, we do not believe that decreased osteoclast formation contributed to the increased bone mass observed in these mice.

Recent studies suggest that FGFs and Wnts initiate signaling cascades that can interact in a cooperative or antagonistic manner, depending on the cell and tissue type (11). Wnts are secreted glycoproteins that mediate important biological processes, including embryogenesis, organogenesis, and tumorgenesis (38–40). As stated previously, studies have shown the importance of Wnt in skeletal development and postnatal bone formation (12–14). In addition, several extracellular and intracellular proteins modulate Wnt signaling (44–46).

sFRPs are extracellular proteins that directly bind both Wnts and FZD receptors to blunt all Wnt-activated pathways (47). The studies by Bodine et al. (48) showed that sFRP1 is an important regulator of osteoblast and osteocyte survival. In addition, deletion of sFRP1 preferentially activated Wnt signaling in osteoblasts, leading to enhanced trabecular bone formation in adult mice (16). As noted previously, recent studies have demonstrated a role for modulation of Wnt signaling by FGF2 and the ligand FGF-1 in intramembranous bone formation (11). However, the role of Wnt inhibitors and FGF2 in postnatal

**FIGURE 8. Effect of 18-kDa FGF2 on β-catenin signaling.** BMSC from VTg, 18-kDa, FGF2+/+, or FGF218-kDa mice were co-transfected with a TOP-flash construct and a cytomegavirus β-galactosidase expression vector for 3 h. A, at 48 h post-transfection the basal TOP-flash reporter activity was increased in 18-kDa FGF-2-transfected BMSC compared with VTg-transfected BMSC. B, TOP-flash reporter activity was decreased in FGF218-kDa BMSC compared with WT BMSC. Data shown are for luciferase activity/β-galactosidase activity. Data are expressed as mean ± S.E. from three experiments. *, significantly different from VTg, p < 0.05. #, significantly different from FGF2+/+, p < 0.05.
18-kDa FGF2 isoform is an important determinant of bone mass in mice and strongly implicates modulation of the Wnt pathway as a mechanism for these responses.

Acknowledgments—We thank the University of Connecticut Health Center Micro-Computed Tomography Facility for performing the Micro-CT studies. We also thank Dr. T. V. Rajan from the Department of Pathology and Immunology, University of Connecticut Health Center, for analysis of the kidney histology.

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