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Disruption of the *Fgf2* Gene Activates the Adipogenic and Suppresses the Osteogenic Program in Mesenchymal Marrow Stromal Stem Cells

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Abstract

Here we determine the Fibroblast Growth Factor-2 (FGF2) dependency of the time course of changes in bone mass in female mice. This study extends our earlier reports that knockout of the FGF2 gene (*Fgf2*) caused low turnover bone loss in *Fgf2*^{-/-} male mice by examining bone loss with age in *Fgf2*^{-/-} female mice, and by assessing whether reduced bone formation is associated with differentiation of bone marrow stromal cells (BMSCs) towards the adipocyte lineage. Bone mineral density (BMD) was similar in 3 month old female *Fgf2*^{+/+} and *Fgf2*^{-/-} mice but was significantly reduced as early as 5 months of age in *Fgf2*^{-/-} mice. In vivo studies showed that there was a greater accumulation of marrow fat in long bones of 14 and 20 month old *Fgf2*^{-/-} mice compared with *Fgf2*^{+/+} littermates. To study the effect of disruption of FGF2 on osteoblastogenesis and adipogenesis, BMSCs from both genotypes were cultured in osteogenic or adipogenic media. Reduced alkaline phosphatase positive (ALP), mineralized colonies and a marked increase in adipocytes were observed in *Fgf2*^{-/-} BMSC cultures. These cultures also showed an increase in the mRNA of the adipogenic transcription factor PPAR γ 2 as well as the downstream target genes aP2 and adiponectin. Treatment with exogenous FGF2 blocked adipocyte formation and increased ALP colony formation and ALP activity in BMSC cultures of both genotypes. These results support an important role for endogenous FGF2 in osteoblast (OB) lineage determination. Alteration in FGF2 signaling may contribute to impaired OB bone formation capacity and to increased bone marrow fat accumulation both of which are characteristics of aged bone.

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Keywords

FGF2 NULL MICE; OSTEOPOROSIS; AGING; ADIPOGENESIS

Introduction

Age-related bone loss occurs in animals and humans regardless of sex steroid status [1–3] and is associated with reduced osteoblasts (OB) production and an increased number of adipocytes in the bone marrow resulting in fatty marrow [4]. OBs and adipocytes are both derived from mesenchymal marrow progenitors (BMSCs) [1]. Lineage specific differentiation of BMSC is controlled by extra-cellular factors and intracellular signals [5–9]. However, there is little and conflicting data on the role of FGFs, in particular FGF2, in adipocyte differentiation.

FGF2 binds to high affinity tyrosine kinase FGF receptors to activate multiple downstream signaling pathways important in the proliferation and differentiation of a variety of cell types including OB [10]. FGF2 is stored in the extracellular matrix (ECM), is expressed by OBs [10] and is an important modulator of cartilage and bone growth and differentiation [10]. Sustained FGF2 treatment inhibits bone formation [11–14], while intermittent FGF2 treatment stimulates bone formation in vitro [13] and in vivo [15]. Similar to studies in rodents [16–17], FGF2 treatment has both inhibitory [18–19] and stimulatory effects [20–22] on human bone marrow stromal cells (hBMSC). However, the preponderance of studies show that FGF2 enhanced the osteogenic phenotype of hBMSCs [20–22] resulting in the deposition of greater amounts of mineralized matrix than in control cultures. The importance of FGF2 in bone is supported by our studies showing that *Fgf2*^{-/-} mice [23] developed decreased bone mass and bone formation, suggesting a role for endogenous FGF2 in maintaining bone mass [24]. In addition, FGF2 can stimulate osteoclast (OCL) formation, as well as increase bone resorption in rodent models [25–26]. *Fgf2*^{-/-} mice also formed fewer OCLs in culture [27], thus exhibiting a low turnover osteopenia that is similar to that observed in age-related osteoporosis [28]. Because *Fgf2*^{-/-} mice have several characteristics of senile osteoporosis, we assessed whether loss of FGF2 modulated mesenchymal cell lineage determination. The current study demonstrates for the first time the occurrence of increased adipogenesis in the bone marrow of *Fgf2*^{-/-} mice, along with progressive osteopenia, and upregulation of key adipogenic signaling molecules. These data suggest that the *Fgf2*^{-/-} mice represent a worthwhile model to study the mechanism of age-related bone loss.

Materials and methods

Animals

All animal protocols were approved by the University of Connecticut Health Center's Animal Care Committee. Development of *Fgf2* null mice was previously described [23]. Heterozygote *Fgf2*^{+/-} mice that are maintained on a Black/Swiss/129 Sv background were bred and housed in the transgenic facility in the Center for Laboratory Animal Care at the University of Connecticut Health Center. Genotyping of mice was performed using primers as previously described [23–24].

Bone Mineral Density

Total BMD and bone mineral content (BMC) excluding the head, was determined in 3–20 month old *Fgf2*^{+/+} and *Fgf2*^{-/-} female mice. BMD and BMC were measured by dual energy X-ray absorptiometry (DXA; PIXImus Mouse 11 densitometer (GE Medical System, Madison WI)).

Tissue preparation for histology

Preparation for Oil Red O staining—For histological analysis, *Fgf2^{+/+}* and *Fgf2^{-/-}* mice were sacrificed by CO₂ narcosis and cervical dislocation. Following euthanasia, the tibiae were removed and immediately fixed in 4% PFA at 4 °C. After samples were decalcified in 20% EDTA in PBS for seven days, each sample was embedded in Shandon Cryomatrix (Thermo Electron Corporation, Pittsburgh, PA) and completely frozen. Frozen samples were cut into 10 µm sections. The sections were stained with Oil Red O. Briefly, sections were stained with Oil Red O working solution for 10 minutes, washed in tap water, stained for 1 minute in Meyer's Hematoxylin, washed again and placed in bluing solutions for 30 seconds. Sections were next mounted in 50% glycerol in PBS.

Osmium Tetroxide Staining—Equal parts of 2% osmium tetroxide and 5% potassium dichromate were mixed and decalcified tibiae were placed in containers with this solution at room temperature under the hood for 24 hours. Tibiae were placed in cassettes, and all cassettes were placed in a strainer, and run under tap water for 2 hours. Bones were then placed overnight in 30% sucrose dissolved in PBS over night and embedded in Cryomatrix. Cryosectioning was performed on a Leica CM1900 Cryostat (D-69226; Leica, Inc., Nussloch, Germany). The Cryomatrix block containing each decalcified tibia was oriented in the block holder to obtain a 10 µm longitudinal central section that includes the central vein. Sections were collected on a special cold adhesive tape Cryofilm type IIC (10) (FINETEC Co LTD, Japan). The tapes with samples were soaked in PBS for half hour, mounted in 50% glycerol in PBS and scanned using a Zeiss Axioplan 200 inverted microscope connected with a Zeiss AxioCam color digital camera. An associated Zeiss-Improvision microscope workstation yielded 50× composite images of whole tibiae. NIH imaging was used to quantitative the Osmium positive area/Total tissue area.

Mouse bone marrow cultures

Mouse bone marrow cells (BMSC) were isolated from *Fgf2^{+/+}* and *Fgf2^{-/-}* mice as previously described [24]. The majority of the studies were conducted using female mice, however to confirm the adipogenic phenotype limited studies were conducted using aged male mice as shown in results. Briefly, tibiae, and femur were dissected free of adhering tissue. The bone ends were removed and the marrow cavity flushed with alpha minimal essential medium (αMEM, Invitrogen, Grand Island, NY). To perform an ex vivo analysis of osteogenesis and adipogenesis, BMSC from both genotypes were plated at 3×10⁶/well in 6-well dishes and cultured in basal medium (alpha-MEM+10% heat inactivated fetal calf serum (HFCS) + penicillin (100 U/ml) and streptomycin (50 µg/ml) for 10 days, then switched to either adipogenic [basal medium + rosiglitazone (0.5 µM)] or osteogenic [basal medium + phospho-ascorbate (50 µg/ml) + β-glycerophosphate (8 mM)] for an additional 7 days. Cells cultured in osteogenic media were stained for alkaline phosphatase (ALP), scanned and counter-stained for mineral by von-Kossa. Some dishes were stained for calcium using Alizarin Red S (Sigma Chemical Co, St Louis, MO). ALP staining was performed with a commercial kit (Sigma). ALP activity was determined using a kit obtained from Sigma according to the manufacturer's instructions. Cells cultured under adipogenic condition were examined under phase contrast microscopy, photographed and then stained with Oil Red O.

Since bone marrow also contains red blood cells, some experiments were conducted using nucleated cells plated at 1 × 10⁶/well in 6-well dishes under osteogenic or adipogenic conditions as described above and shown in results.

To determine whether exogenous FGF2 could rescue defective bone nodule formation in *Fgf2^{-/-}* BMSC cultures, nucleated BMSCs from both genotype were plated at 1×10⁶ cell/well in 6 well dishes. Cells were treated with Vehicle or FGF2 (0.1 nM) for the first 3 days of culture,

then cultured in the absence of FGF2 in osteogenic media for an additional 15 days. Cultures were harvested at 18 days, stained for Alizarin Red S, scanned by HP Precision Scan Pro, followed by solubilization of Alizarin Red S and spectroscopic quantitation.

In order to determine whether exogenous FGF2 could rescue the adipogenic phenotype, BMSCs were plated at 10×10^6 /well in 6-well dishes and cultured in adipogenic media in the absence and presence of FGF2 (1 nM). Cultures were harvested at 21 days, stained for Oil Red O, scanned by HP Precision Scan Pro. In order to quantify adipogenesis, Oil Red O staining was eluted by 500 μ l of 100% isopropanol per well overnight. The spectrophotometric absorbance of elution of Oil Red O was quantified at 510 nm.

Assessment of growth rate of bone marrow stromal cells from young, adult and aged Fgf2^{+/+} and Fgf2^{-/-} mice

To assess the effect of knock-out of FGF2 on viable cell number, BMSCs harvested from 5, 16 and 20 month old Fgf2^{+/+} and Fgf2^{-/-} male mice were plated in 100 mm dish. At confluence, cells were re-plated in 96 well plates at 5000 cell/cm² in α MEM+10%FBS+p/s. After 24, 48, 72, or 96 h, cells were harvested. For the last 1 h of culture, 20 μ l per well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Promega, Madison, WI) was added, and viable cell number was measured by the MTT assay. Absorbance is directly proportional to the number of proliferating and living cells.

Colony forming efficiency (CFU-F) of bone marrow stromal cells from young, adult and aged Fgf2^{+/+} and Fgf2^{-/-} mice

In order to assess colony forming efficiency (CFU-F) BMSCs were flushed from 5, 16, 20 month old Fgf2^{+/+} and Fgf2^{-/-} mice. The cell suspensions were filtered using a 70 μ m strainer and passed through 16- and 20- gauge needles to break down cell aggregates. Then the cell suspensions were filtered through a 2350 nylon cell strainer (Becton-Dickinson, Franklin Lakes, NJ) in order to obtain to singly cells. The red blood cells were lysed using 0.1% Acetic Acid. Nucleated cells were plated in 6 well dishes at 1×10^5 cells/well in α MEM+10%FBS+p/s. On day 14 the cultures were fixed and stained with Crystal Violet. BMSC colonies containing 50 or more cells were counted using a dissecting microscope. CFU (number of colonies per 1×10^5 nucleated marrow cells) was calculated.

mRNA isolation and gene expression

Total RNA was extracted from cells by using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. For real-time, quantitative RT-PCR analysis, RNA was reverse-transcribed by the Super-ScriptTM First-Strand Synthesis System for reverse transcription PCR (Invitrogen Life Technologies, Carlsbad, CA). Quantitative PCR was carried out using the QuantiTectTM SYBR Green PCR kit (Qiagen) on a MyiQTM instrument (BIO-RAD Laboratories Inc Hercules, CA). The primer sequences for the genes of interest are shown in Table 1. β -actin was used as an internal reference for each sample. Using a formula described previously [29], the relative change in mRNA was normalized against the β -actin mRNA level.

Western blot analysis

Briefly, protein was extracted from cultured BMSCs using 1 $\times$ cell lysis buffer (Cell Signaling Technology, Inc., Beverly, MA), and total protein concentration was assayed with BCA protein assay reagent (Pierce, Rockford, IL). After SDS-polyacrylamide gel electrophoresis on 10–20% gels, proteins were transferred to Immobilon-P membranes (BIO-RAD). Membranes were blocked overnight in TBS-T containing 5% nonfat dry milk (BIO-RAD). Membranes were then incubated with an anti-mouse fatty acid binding protein 4 (FABP4, AP2) antibody

(R&D Systems, Minneapolis, MN) for 1 h, washed 1 h with TBS-T, and then incubated with a rabbit anti-mouse secondary antibody (Amersham Biosciences Piscataway, NJ) in TBS-T/1% nonfat milk for 1 h. After incubation with antibodies, membranes were washed 1 h with TBS-T. Western Lighting TM chemiluminescence reagent (PerkinElmer Life Sciences, GE Health Care UK Limited, Buckinghamshire, UK) was used for detection.

Statistical Analysis

All results were expressed as means $\pm$ 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 (ANOVA) was used, and the significant difference was determined by the Bonferroni test (StatView 4.1J Abacus Concepts, Inc, Berkeley, CA).

Results

Effect of aging on bone density in female *Fgf2*^{+/+} and *Fgf2*^{-/-} mice

In order to assess the time course of changes in bone mass in female mice with disruption of the *Fgf2* gene, BMD and BMC was measured in 3–20 month-old female *Fgf2*^{+/+} and *Fgf2*^{-/-} mice. As shown in Fig. 1, DXA analysis revealed that at 3 months of age there were no significant differences in BMD or BMC in *Fgf2*^{+/+} and *Fgf2*^{-/-} mice. In contrast, there was an age-related reduction in BMD in *Fgf2*^{-/-} female mice at 5, 14 and 20 months, *p*<0.05, compared with their *Fgf2*^{+/+} littermates. BMC was decreased by 8, 15 and 21% in 5, 14 and 20 month-old *Fgf2*^{-/-} mice respectively, compared with *Fgf2*^{+/+} mice.

In vivo analysis of bone marrow fat in female *Fgf2*^{+/+} and *Fgf2*^{-/-} mice

To assess whether there was increased fat in bone marrow of *Fgf2*^{-/-} mice, we conducted limited studies utilizing osmium tetroxide labeling as an (*in vivo*) method to assess bone marrow fat accumulation. As shown in figure 2A, at the proximal end and diaphysis of the tibiae, osmium positive cells were localized at the periphery. At the distal end, there was a greater increase in osmium labeling in tibiae from 5 and 14 and 20 month-old *Fgf2*^{-/-} mice. Quantitation of osmium area/total tissue area showed a significant increase in osmium labeling in 20 month old *Fgf2*^{-/-} marrow (Fig. 2B).

To further demonstrate marrow fat accumulation, histological examination was performed on frozen sections of the proximal end of tibiae from 5, 14 and 24 month-old female mice of both genotypes that were stained with Oil Red O to detect fat. There was a slight increase in Oil Red O staining in tibiae of 5 month old *Fgf2*^{-/-} mice compared with *Fgf2*^{+/+} mice. However, as the mice aged Oil Red O positive fat accumulation was increased in both *Fgf2*^{+/+} and *Fgf2*^{-/-} tibiae, but there was more fat accumulation in tibiae of 14 and 20 month-old *Fgf2*^{-/-} mice compared with WT littermates (Fig. 3).

In vitro analysis of osteogenesis and adipogenesis in bone marrow stromal cultures from female and male *Fgf2*^{+/+} and *Fgf2*^{-/-} mice

In order to extend the *in vivo* observations of decreased BMD and increased bone marrow fat, *ex vivo* analysis of osteogenesis and adipogenesis was performed using BMSCs harvested from 5, 14 and 20 month old *Fgf2*^{+/+} and *Fgf2*^{-/-} mice. Under osteogenic conditions there were significantly reduced numbers of ALP+ mineralized colonies in BMSC from 5 month, (4A), 14 month (Fig. 4B) and 20 month old (Fig. 4C) *Fgf2*^{-/-} mice. However, the greatest reduction in mineralized colonies was observed in BMSC from 20 month old *Fgf2*^{-/-} mice (Fig. 4D). Quantitation of the total area of mineralized bone nodule showed a significant decrease in *Fgf2*^{-/-} BMSC cultures at 5 and 14 months of age (Fig. 4E).

As shown in Figure 4F and 4G by Alizarin Red S staining, decreased mineralized bone nodule formation in cultures from *Fgf2*^{-/-} mice was rescued by adding exogenous FGF2.

Assessment of the proliferative capacity of bone marrow stromal cultures from young, adult and old *Fgf2*^{+/+} and *Fgf2*^{-/-} mice

To test whether aging and FGF2 modulate cell proliferation, the proliferative capacity of BMSC from young, adult and old *Fgf2*^{+/+} and *Fgf2*^{-/-} mice was determined. Confluent BMSCs were re-plated in 96-well plates at a density of 5000 cells/well in α MEM containing 10% FBS. After 24, 48, 72, and 96 h cells were harvested, and viable cell number was measured by the MTT assay. Absorbance is directly proportional to the number of live cells. As shown in figure 5, viable cell number was significantly decreased with age in *Fgf2*^{+/+} mice. At 72 h after plating, viable cell number was decreased by 15%, 16% and 11% from 5, 16, and 20 month old *Fgf2*^{-/-} mice respectively compared with their *Fgf2*^{+/+} counterpart (Fig. 5). At 96 h after plating, viable cell number was significantly decreased by 30% and 19% in 5 and 16 month old *Fgf2*^{-/-} mice respectively in compared with *Fgf2*^{+/+} of the same age. But there was no significant difference in viable cell number between *Fgf2*^{+/+} and *Fgf2*^{-/-} at 20 month of age at this time point of culture. These data suggested that *Fgf2*^{-/-} impaired the BMSC proliferation at both young and old age.

Comparison of the effects of aging on Colony forming Efficiency (CFU-F) of bone marrow stromal cells from *Fgf2*^{+/+} and *Fgf2*^{-/-} mice

To test the status of the bone marrow stromal cell population as a function of aging, CFU-F assay was performed using BMSC suspension prepared from 5, 16 and 20 month old male mice of both genotype (Fig. 6). As mice aged CFU-F was significantly decreased in both genotype. FGF2 knockout significantly decreased CFU-F in 16 and 20 month old mice but not in 5 month old mice. These data suggest that since CFU-F was not decreased in the *Fgf2*^{-/-} mice at a young age, the increase in adiposity is not due to an increased senescence of the cells in the *Fgf2*^{-/-} mice.

Effect of exogenous FGF2 on bone nodule formation in BMSCs cultures from *Fgf2*^{-/-} mice in vitro

Since we observed that knockout of endogenous *Fgf2* gene resulted in decreased bone formation both in vivo and in vitro, we next explored the ability of exogenous FGF2 to rescue the in vitro phenotype. BMSCs from 24 (Fig. 7A) or 19 month (Fig. 7B) old male mice were plated at 2 million cells per well in 6 well dishes. Cells were treated with vehicle or 1 nM FGF2 for the first three days then cultured in osteogenic media. On day 11 ALP staining and ALP activity were performed. Both ALP staining and activity was significantly decreased in *Fgf2*^{-/-} vehicle treated cultures compared with *Fgf2*^{+/+} vehicle treated cultures. In cultures treated with exogenous FGF2, ALP staining and activity was significantly increased in both *Fgf2*^{+/+} and *Fgf2*^{-/-} cultures compared with cultures treated with vehicle. But there was a greater increase in ALP stained colonies in *Fgf2*^{-/-} compared with *Fgf2*^{+/+} (199% vs 46% increase). ALP activity in *Fgf2*^{-/-} cultures treated with FGF2 reached the level of the *Fgf2*^{+/+} cultures treated with vehicle. These data suggest that adding exogenous FGF2 can rescue the reduced bone formation observed in *Fgf2*^{-/-} BMSC cultures in vitro.

Effect of knockout of the *Fgf2* gene on adipocyte formation in BMSCs cultures from *Fgf2*^{-/-} mice in vitro

Adipocyte formation was determined in parallel BMSC cultures from 5, 14 and 20 month old *Fgf2*^{+/+} and *Fgf2*^{-/-} mice cultured for 7 days in adipogenic media. Both phase contrast (Fig. 8A) and Oil Red O staining (Fig. 8B) revealed increased numbers of fat cells in BMSC cultures from 20 month-old female *Fgf2*^{+/+} mice compared with 5 and 14 month-old *Fgf2*^{+/+} mice,

however there were more adipocytes in 5, 14 and 20 month old *Fgf2*^{-/-} compared with *Fgf2*^{+/+} mice. The greatest increase was observed in BMSC cultures from 20 month old *Fgf2*^{-/-} mice.

In vitro analysis of adipogenic related genes in bone marrow stromal cultures from aged *Fgf2*^{+/+} and *Fgf2*^{-/-} female mice

Since the greatest increase in fat cells was observed in aged mice, total RNA was extracted from BMSCs of 20 month old *Fgf2*^{+/+} and *Fgf2*^{-/-} mice that were cultured in the presence and absence of an adipogenic stimulus (roziglitazone) in order to examine the expression of genes that have been shown to be important in adipogenesis. As shown in Fig. 9A and Fig. 9B, basal expression of the mRNA for the pro-adipogenic transcription factors C/EBP α and PPAR γ -2 was increased, with a greater induction by roziglitazone in *Fgf2*^{-/-} BMSC cultures. In addition, *Fgf2*^{-/-} cultures showed a greater increase in adiponectin mRNA (Fig. 9C), Fabp4/aP2 mRNA (Fig. 9D) and FABP4/AP2 protein (Fig. 9E), all markers of differentiated adipocytes.

Effect of exogenous FGF2 on adipocyte formation in bone marrow stromal cultures from *Fgf2*^{-/-} mice in vitro

In order to test whether add back of FGF2 would inhibit adipocyte formation, BMSCs from 19 month old male mice were plated at 10 million per well in 6 well dishes. The cells were treated with FGF2 or vehicle and cultured in proliferation medium for 11 day then switched to adipogenic media for another 10 days. Oil Red O staining (Fig. 10A) and quantitation of solubilized Oil Red O (Fig. 10B) was performed. Roziglitazone induced more adipogenesis in *Fgf2*^{-/-} culture compared with *Fgf2*^{+/+} culture. Exogenous FGF2 blocked adipogenesis in both *Fgf2*^{+/+} and *Fgf2*^{-/-} cultures as demonstrated by reduced Oil Red O staining (Fig. 10A) and reduced Oil Red O concentration (Fig. 10B).

Discussion

We previously reported that FGF2 modulates mesenchymal stromal stem cell differentiation and that knockout of the *Fgf2* gene caused low turnover osteopenia in *Fgf2*^{-/-} male mice [24]. The principal observation of this report is that endogenous FGF2 is also necessary for maximal bone formation and the maintenance of bone mass in female mice. Furthermore, our in vitro and in vivo studies show that endogenous FGF2 also modulates bone marrow adipogenesis which has been shown to be important in osteopenic states in both humans and rodents [1–3].

As previously noted, there are limited and conflicting data on the effects of FGFs on adipocyte differentiation. Acidic FGF (FGF1) promoted adipogenesis of primary human preadipocytes and murine adipogenic 3T3-L1 cells [30]. Although FGF1 has some similarity in action to FGF2 in bone, we do not believe that increased bone marrow adipogenesis in *Fgf2*^{-/-} mice is due to a compensatory increase in FGF1, since *Fgf1* was not increased in *Fgf2*^{-/-} mice [23].

Previous studies suggested both pro and anti-adipogenic effects of FGF2. In support of a pro-adipogenic role, FGF2 treatment was reported to enhance adipogenesis in response to an adipogenic hormone cocktail or troglitazone in rat mesenchymal stem cell cultures [31]. Other investigators reported that dexamethasone-induced adipocyte formation from human marrow stromal cells was accelerated but not increased by FGF2 [32]. FGF2 was also reported to induce adipose tissue development when implanted in Matrigel in mice [33]. However, although the data was not shown, the authors noted that in the absence of Matrigel, FGF2 did not induce adipogenesis in these mice. In support of an anti-adipogenic role for FGF2, other investigators, using the 3T3L1 cell line and TA1 cell lines, (the latter derived from C3H-10T1/2 mouse embryo fibroblast-derived cell line) [34], reported that FGF2 inhibited adipoblast

differentiation [35]. In addition, treatment of differentiated TA1 adipocytes with FGF2 reversed the adipocyte phenotype [36]. Prusty et al showed that while short term FGF2 treatment (6h) enhanced adipogenesis, prolonged exposure, (12–24h) inhibited adipogenesis in the 3T3L1 cells [36]. The observation of increased bone marrow fat in *Fgf2* null mice would support an anti-adipogenic effect of FGF2 in bone.

Increased adipogenesis in bone involves modulation of the transcription factors RUNX2 and PPAR γ 2, which are key determinants of MSC commitment to the OB and adipocyte lineage, respectively [3]. Therefore we hypothesized that loss of FGF2 modulates these key molecules that are important in osteogenesis/adipogenesis. In addition to promoting OB differentiation, RUNX2 also promotes OCL differentiation and it is interesting that selective deficiency of RUNX2 is associated with both impaired bone formation, OB maturation and OCL formation resulting in low turnover osteopenia [37], reminiscent of the phenotypes of the *Fgf2*^{-/-} mice. We previously reported that there was reduced accumulation of *Runx2* mRNA in bone marrow stromal cells of adult *Fgf2*^{-/-} mice [38], therefore in the present report we examined the expression of pro-adipogenic genes in the absence of endogenous FGF2.

Among the pro-adipogenic genes are several families of transcription factors that directly modulate fat cell development [39,6]. These include the CCAAT/enhancer binding proteins (C/EBP α) and PPAR γ 2 which modulate the expression of several genes characteristic of the adipocyte phenotype such as the adipocyte selective fatty acid binding protein FABP4(aP2) [6]. There are two protein isoforms of PPAR γ (PPAR γ 1 and PPAR γ 2) [6] and studies have shown that PPAR γ 2 negatively regulated OB development and bone formation from bone marrow stromal cells, while positively regulating marrow adipocyte differentiation [40]. In addition, PPAR γ haplo-insufficiency resulted in increased bone mass and increased osteoblastogenesis in mice [41]. It is noteworthy that in humans a PPAR γ polymorphism due to a C to T transition is associated with reduced BMD [42]. Consistent with previous reports on the importance of PPAR γ in adipogenesis, we observed increased expression of PPAR γ 2 as well as downstream genes associated with differentiated adipocytes in cells from *Fgf2*^{-/-} mice.

Our data clearly shows that knockout of the endogenous *Fgf2* gene promoted adipogenesis and impaired bone formation. We do not believe that a decrease in colony forming efficiency caused the increased adipocyte formation in *Fgf2*^{-/-} mice, since colony forming efficiency was similar in BMSCs from *Fgf2*^{+/+} and *Fgf2*^{-/-} at 5 months of age. These studies also demonstrate that exogenous FGF2 was sufficient to normalize the increased adipocyte formation of *Fgf2*^{-/-} cells further indicating that FGF2 plays a critical role in the blockade of adipocyte formation in BMSCs.

In summary, these results support an important role for endogenous FGF2 in OB lineage determination. Alteration in FGF2 signaling may contribute to impaired bone formation and to increased adiposity, both characteristics of aged bone.

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Fig. 1.

DXA Analysis of age-related changes in bone mineral density (BMD) and bone mineral content (BMC) in 3–20 month-old *Fgf2*^{+/+} (WT) and *Fgf2*^{-/-} (KO) female mice. Whole body BMD, excluding the head was determined by Piximus -DXA as described under methods. Values are the mean $\pm$ SEM for 6 bones/group *Significantly different from WT mice. $p < 0.05$.

Fig. 2.

Osmium tetroxide labeling and quantification for bone marrow fat accumulation in tibiae from *Fgf2*^{+/+} (WT) and *Fgf2*^{-/-} (KO) mice. (A) Histologic examination of sections of tibiae from 5, 14 and 20-month-old female WT and KO mice for accumulation of fat by osmium tetroxide staining was performed as described under methods (Composite image, magnification $\times 50$). There were increased osmium tetroxide positive cells in the distal end of tibiae from KO mice compared with WT. (B) Osmium positive area/total tissue area was measured by NIH Image 1.63 of tibiae from 20 month old mice. Osmium positive area/total tissue area was significantly increased by 15% in KO mice of both genotypes. Values are the mean $\pm$ SEM for 3 bones/group *Significantly different from WT mice. $p < 0.05$.

Fig. 3. Oil Red O labeling for age-related changes in bone marrow fat accumulation in tibiae from *Fgf2*^{+/+} (WT) and *Fgf2*^{-/-} (KO) mice. Histologic examination of proximal ends of cryo-sectioned tibiae from 5, 14 and 20 month-old female WT and KO mice for accumulation of fat by Oil Red O staining was performed as described under methods (magnification $\times 100$). There was increased Oil Red O positive cells in tibiae from KO mice compared with WT.

Fig. 4.

In vitro analysis of bone nodule formation in bone marrow stromal cells (BMSC) from (A) 5 month, (B) 14 month and (C) 20 month old female *Fgf2*^{+/+} (WT) and *Fgf2*^{-/-} (KO) mice. BMSCs were cultured as described in methods. Reduced ALP positive mineralized colonies were observed in KO cultures in 5, 14 and 20 month old mice. (D) Quantitative analysis revealed significantly reduced mineralized colony number in 5, 14 and 20 month old KO BMSC cultures. (E) Quantitative analysis revealed a significant decrease in total mineralized colony area in 5 and 14 month old KO BMSC cultures compared with WT. At 20 months of age, total area of mineralized bone nodule was decreased by 30% in KO culture but was not significant. Values are the mean ± SEM for 6 determinations/group *Significantly different from WT mice. $p < 0.05$. (F) Alizarin Red S staining and (G) Quantification showed that exogenous FGF2 rescued the defective bone nodule formation in BMSC cultures from KO mice. Values are the mean ± SEM for 3 determinations/group Significantly different from *corresponding WT group, # KO-Vehicle group. $p < 0.05$.

Figure 5.

Comparison of viable cell number in BMSCs from 5, 16, 20-month-old *Fgf2*^{+/+} (WT) and *Fgf2*^{-/-} (KO) mice. BMSCs were prepared from 5, 16, and 20-month-old male WT and KO mice as described under “methods” and cultured in 10% FBS for 24–96 h. Then an MTT assay was performed as described under “methods.” Values are the mean ± S.E. for 3 determinations/group.

Figure 6.

Colony forming efficiency (CFU-F) of bone marrow stromal cells from young, adult and aged *Fgf2*^{+/+} (WT) and *Fgf2*^{-/-} (KO) mice. (A) Crystal violet staining of CFU-F in BMSC cultures from 5, 16, and 20-month-old WT and KO male mice. (B) Quantification of CFU-F. BMSC colonies containing 50 or more cells were counted using a dissecting microscope. CFU-F (number of colonies per 1×10⁵ nucleated marrow cells) was calculated. Values are the mean ± SEM for 3 determinations/group. Significantly different from * correspondent WT group, # 5 month old WT, @ 5 month old KO. p<0.05.

Figure 7. Effect of exogenous FGF2 on defective bone nodule formation in old *Fgf2*^{-/-} (KO) mice. (A) ALP staining of BMSCs from 24 month old male WT and KO mice cultured in osteogenic media with or without FGF2 treatment. (B). ALP activity assay of BMSC culture from 19 month old male mice cultured with or without FGF2 treatment. Values are the mean $\pm$ SEM for 3 determinations/group. Significantly different from *WT-Vehicle group, # WT-FGF2 group, @ KO-Vehicle. $p < 0.05$.

Fig. 8.

In vitro analysis of adipogenesis in BMSCs from 5, 14 and 20 month old female *Fgf2*^{+/+} (WT) and *Fgf2*^{-/-} (KO) mice. (A). Phase contrast analysis shows increased adipocytes in 17 day BMSC cultures from 5, 14 and 20 month old KO mice. (B). Oil-red-O staining confirmed increased fat in BMSC cultures from *Fgf2*^{-/-} mice compared with WT cultures.

Fig. 9.

Representative analysis of adipogenic gene expression in BMSC from 20 month old female *Fgf2*^{+/+} (WT) and *Fgf2*^{-/-} (KO) mice. Total RNA was extracted from parallel dishes of cultures from experiments described in Fig. 8. BMSC cultures from KO mice show an increase in β -actin-normalized mRNA expression of (A) PPAR γ 2, (B) C/EBP α , (C) Adiponectin, and (D) Fabp4/aP2 by real time RT-PCR. (E). Western blot analysis showed a corresponding increase in FABP4/AP2 protein in KO BMSC after 14 and 20 days of culture. Values are the mean $\pm$ SEM for 3 determinations/group *Significantly different from WT mice. p<0.05. Similar results were obtained in 2 additional experiments.

Figure 10.

Effect of exogenous FGF2 on adipogenesis in BMSC cultures from *Fgf2*^{+/+} (WT) and *Fgf2*^{-/-} (KO) mice. (A) Oil Red O staining for adipocytes in BMSC cultures from 19 month old male WT and KO mice cultured in adipogenic media with or without FGF2 treatment. (B). Quantification of Oil Red O of BMSC cultures from 19 month old male mice with or without FGF2 treatment. Values are the mean $\pm$ SEM for 3 determinations/group. Significantly different from *correspondent vehicle group, # WT-ROSI group, @ KO-ROSI group, ^ KO-ROSI group. $p < 0.05$.

Table 1

Primers used in PCR

Gene	Forward	Reverse
β -actin	5'-atggctggggtgtgaaggt-3'	5'-atctggcaccacacattctacaa-3'
Adiponectin	5'-atcctggccacaatggcaca-3'	5'-caagaagacgtcatctcct-3'
aP2	5'-caccgagatttcctcaaaact-3'	5'-gccatctagggttatgatgc-3'
PPAR γ 2	5'-gctgttatgggtgaaactctg-3'	5'-ataaggtggagatgcaggttc-3'
C/EBP α	5'-cagcaacgagtaccgggta-3'	5'-tgcgtctccacgttcgctt-3'