ORIGINIAL ARTICLE

Opposing effects of Sca-1+ cell-based systemic FGF2 gene transfer strategy on lumbar versus caudal vertebrae in the mouse

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Our previous work showed that a Sca-1+ cell-based FGF2 therapy was capable of promoting robust increases in trabecular bone formation and connectivity on the endosteum of long bones. Past work reported that administration of FGF2 protein promoted bone formation in red marrow but not in yellow marrow. The issue as to whether the Sca-1+ cell-based FGF2 therapy is effective in yellow marrow is highly relevant to its clinical potential for osteoporosis, as most red marrows in a person of an advanced age are converted to yellow marrows. Accordingly, this study sought to compare the osteogenic effects of this stem cell-based FGF2 therapy on red marrow-filled lumbar vertebrae with those on yellow marrow-filled caudal vertebrae of young adult W41/W41 mice. The Sca-1+ cell-based FGF2 therapy drastically increased trabecular bone formation in lumbar vertebrae, but the therapy not only did not promote bone formation but instead caused substantial loss of trabecular bone in caudal vertebrae. The lack of an osteogenic response was not due to insufficient engraftment of FGF2-expressing Sca-1+ cells or inadequate FGF2 expression in caudal vertebrae. Previous studies have demonstrated that recipient mice of this stem cell-based FGF2 therapy developed secondary hyperparathyroidism and increased bone resorption. Thus, the loss of bone mass in caudal vertebrae might in part be due to an increase in resorption without a corresponding increase in bone formation. In conclusion, the Sca-1+ cell-based FGF2 therapy is osteogenic in red marrow but not in yellow marrow.

INTRODUCTION

Osteoporosis is a systemic bone disease of low bone mass associated with impaired bone architecture, characterized by disruption of trabecular continuity through trabecular perforation that results in reduced connectivity of the bone structure and conversion of the normal plate-like trabeculae into thinner rod-like structures. It is resulted from insufficient bone formation to compensate for increases in bone resorption. Most current FDA-approved therapies for osteoporosis, such as bisphosphonates, selective estrogen receptor modulators and RANKL-inhibitory monoclonal antibody (Denosumab), aim to suppress bone resorption and have demonstrated clinical efficacy. Owing to the relatively low bone regenerative capacity of these anti-resorptive drugs (as reflected by only very modest increases in bone density), they are generally inadequate for patients with severe osteoporosis, who have large bone deficits. The acquisition rate of new bone formation achieved with the only FDA-approved anabolic therapy (teriparatide (parathyroid hormone, PTH) injection therapy) may also be insufficient for patients with the highest fracture risk. Patients with severe bone deficits require a potent osteogenic therapy capable of generating large amounts of structurally and mechanically sound new bone within a realistic timeframe. Accordingly, there is still unmet demand for a potent osteogenic therapy, especially for patients with severe bone deficits.

Our laboratory has been interested in developing a systemic osteogenic growth factor gene transfer-based therapy for osteoporotic patients with large bone deficits. Our approach was to take advantage of the propensity of hematopoietic stem cells (HSCs) to home to and engraft in the HSC niches within bone marrow cavities, and to develop a HSC-enriched stem cell antigen-1 positive (Sca-1+) cell-based ex vivo gene transfer strategy. This strategy used genetically engineered Sca-1+ cells to deliver a bone growth factor to the endosteum to promote endosteal bone formation. Our decision of utilizing the fibroblast growth factor (FGF2) transgene was based on findings that disruption of the Fgf2 gene in mice reduces bone formation and impairs fracture repair and that FGF2 is a potent osteogenic factor capable of stimulating endochondral bone formation in both young and aged ovariectomized rats. Accordingly, systemic administration of recombinant FGF2 protein promotes rapid bone formation and increases trabecular bone mass on the endosteal surface without significant effects on periosteal bone formation in ovariectomized rats. FGF2 also appears to be required for the anabolic action of PTH in bone.

Our recent studies have provided compelling proof-of-concept evidence for the feasibility of this systemic stem cell-based FGF2 gene transfer strategy. Specifically, a single transplantation of genetically modified Sca-1+ cells expressing a human FGF2 gene into sub-lethally irradiated recipient mice yielded robust de novo trabecular bone formation and increased trabecular...
connectivity on the endosteum of long bones. Although this systemic gene transfer strategy delivering a FGF2 gene showed huge bone formation-promoting capacity using the same strategy but delivering the bone morphogenetic protein-4 (BMP4) gene only resulted in modest gains in bone mass, and the use of the human growth hormone (hGH) gene even led to significant bone loss. This stem cell-based FGF2 strategy also increased bone resorption and caused osteomalacia, which presumably was due to secondary hyperparathyroidism developed in response to hypocalcemia that was a consequence of a high circulating level of FGF2 and too rapid of bone formation. However, these adverse effects can be avoided with the use of β-globin promoter to drive FGF2 to confine FGF2 expression to the bone marrow cavity.

Although other investigators have previously used FGF2 protein therapy to promote endosteal bone formation and had produced very encouraging results, our HSC-based FGF2 gene transfer strategy is novel with respect to the innovative use of HSCs as the cell vehicle for FGF2, which allows targeted FGF2 expression specifically at the stem cell niches along the endosteum. Although direct comparison of the efficacy of the FGF2 protein therapy with that of our stem cell-based strategy is difficult owing to differences in animal models, experimental designs and treatment duration, our strategy appeared to be ~10 times more potent than the protein-based strategy in increasing the percentage of trabecular bone area on the endosteum and in increasing trabecular bone parameters. Furthermore, because the administered FGF2 protein is readily metabolized and cleared from the circulation, multiple (daily) injections of the FGF2 protein are required for the protein therapy to produce a detectable biological effect. This requires large supplies of the FGF2 protein, which would be a significant limiting factor. In contrast, the engrafted genetically modified stem cells in our strategy continuously produce FGF2 at the endosteal niches to promote robust endosteal/trabecular bone formation, making multiple administrations of the therapy unnecessary. The combination of these unique and potent regenerative properties renders this systemic stem cell-based FGF2 gene transfer strategy potentially a more attractive regenerative therapeutic option than the FGF2 protein therapy for treatment of osteoporotic patients with severe bone deficits.

Previous work on the FGF2 protein therapy of Wronski and coworkers, however, has also disclosed an intriguing observation in that the osteogenic effect of the FGF2 protein therapy seen in red marrow skeletal sites (proximal femurs and lumbar vertebrae) was greatly attenuated in skeletal sites containing largely yellow marrows (distal tibia and caudal vertebrae). As almost all of the bone marrows in a person of an advanced age is converted to yellow marrows, and because osteoporosis is largely an aging disorder and patients with severe osteoporosis are usually the elderly, our stem cell-based FGF2 gene transfer strategy must also be able to promote bone formation in yellow marrow cavities for it to be useful in treating elderly osteoporotic patients. Accordingly, the issue as to whether our strategy is also effective in yellow marrow sites is highly relevant to its clinical potential. Consequently, our primary goal for the present study was to determine whether our Sca-1+ cell-based FGF2 gene transfer strategy is as efficacious in the yellow marrow-containing caudal vertebrae as in the red marrow-containing lumbar vertebrae.

RESULTS

Marrow transplantation of Sca-1+ cells expressing FGF2 increased circulating and local FGF2 levels in recipient mice

To assess whether our systemic Sca-1+ cell-based FGF2 gene transfer strategy would promote trabecular bone formation in lumbar vertebrae, we transplanted W41/W41 recipient mice with MLV-gfp-transduced or MLV-FGF2-transduced Sca-1+ cells (n = 7 per group). Engraftment of donor cells, which was assessed by measuring the relative percentage of peripheral gfp-expressing blood cells at 6 weeks post transplantation, was ~70% (data not shown). To confirm an effective engraftment of MLV-FGF2-transduced Sca-1+ cells in recipient mice, we measured the relative levels of FGF2 genomic DNA in extracts of peripheral blood cells of recipient mice by real-time PCR, using a primer set that is specific for the human FGF2 transgene and that does not recognize the endogenous murine Fgf2 gene of the host. The human FGF2 DNA content in recipient mice of MLV-FGF2-transduced Sca-1+ cells at both 6 and 14 weeks post transplantation was each ~600-fold greater than that in recipient mice of MLV-gfp-transduced Sca-1+ cells recipient control mice (Figure 1a). The serum level of FGF2 protein in recipient mice of MLV-FGF2-transduced cells was also 15-fold greater than that in the control group at 14 weeks post transplantation (Figure 1b). These findings confirm an effective and relatively stable engraftment of the donor FGF2-expressing Sca-1+ cells in recipient mice.

Our previous studies showed that marrow transplantation of FGF2-expressing Sca-1+ cells not only promoted robust endosteal/trabecular bone formation, but also caused secondary hyperparathyroidism due to hypocalcemia developed in response to the rapid increase in bone formation. Consistent with the

Figure 1. Effects of marrow transplantation of MLV-FGF2- or MLV-gfp-transduced Sca-1+ cells on relative levels of engraftment (a), as well as serum levels of FGF2 (b) and serum PTH (c) in recipient mice. In (a), engraftment of FGF2-expressing Sca-1+ cells was assessed by measuring the relative level of human FGF2 genomic DNA content in peripheral blood cells of recipient mice of MLV-FGF2-transduced cells (FGF2) or control MLV-gfp-transduced cells (GFP) at 6 or 14 weeks post transplantation, respectively. N = 7 per group. In (b), serum FGF2 levels of recipient mice of MLV-FGF2-transduced cells (FGF2 mice) or the control MLV-gfp-transduced cells (GFP mice) at 14 weeks post transplantation were assayed with a commercial ELISA kit. N = 7 per group. In (c), serum PTH levels of recipient mice of MLV-FGF2- or MLV-gfp-transduced Sca-1+ cells at 14 weeks post transplantation were measured with a commercial ELISA kit. N = 7 per group.
previous findings. Figure 1c shows that the serum PTH level of recipient mice of MLV-FGF2-transduced Sca-1\(^+\) cells at 14 weeks post transplantation was more than 10-fold that in control mice receiving MLV-gfp-transduced Sca-1\(^+\) cells (> 100 pg ml\(^{-1}\) in FGF2 mice vs 9.8 ± 0.8 pg ml\(^{-1}\) in gfp control mice). Serum PTH level in the gfp-transduced control mice was not significantly different from that of untreated control mice (10.7 ± 0.6 pg ml\(^{-1}\)), indicating that the increase in serum PTH was in response to FGF2 transgene expression and not to Sca-1\(^+\) cell transplantation per se.

Sca-1\(^-\) cell-based FGF2 gene therapy promoted robust bone formation but caused osteomalacia in lumbar vertebrae

The robust enhancing effects of the Sca-1\(^+\) cell-based FGF2 gene transfer strategy on endosteal/trabecular bone formation in femurs of recipient mice have been reported previously.\(^{17,18}\) To determine whether this FGF2 gene therapy would also similarly promote de novo trabecular bone formation in lumbar vertebrae, cross-sections of L3 vertebrae of recipient mice of Sca-1\(^+\) cells transduced with MLV-FGF2 and those of recipient mice of MLV-gfp-transduced Sca-1\(^+\) cells were stained with Goldner's trichrome dye for mineralized bone matrices. Figure 2 shows dramatic histologic changes in vertebrae of mice with high expression levels of FGF2 (right panel) compared with gfp control mice (left panel). There were large increases in newly formed bone matrix areas that nearly filled the entire marrow cavity of recipient mice of the FGF2 group. However, a large majority of these newly formed matrix areas were un- or under-mineralized (stained red). Nevertheless, the osteoid layers exhibited a matrix pattern similar to newly laid lamellar bone and not woven bone. The pattern of wide layers of un- or under-mineralized lamellar bone in the FGF2 group suggests that the gene transfer strategy caused similar robust increases in trabecular bone formation in lumbar vertebrae as seen in long bones.\(^{17,18}\) In addition, similar to those in the FGF2 gene therapy-treated femurs, the large amounts of largely un- or under-mineralized newly formed bone in lumbar vertebrae are presumably also the result of the hypocalcemia and secondary hyperparathyroidism.

We next measured quantitative static bone histomorphometry parameters on the L3 vertebra. The results were averaged over six consecutive microscopic fields, at ×10 magnification, beginning from a point 350 μm distal from the inferior edge of the bone growth plate. As shown in Figure 3, the % osteoid area (%O.Ar) (increased 4.6-fold), % forming surface (%L.Pm) (increased 1.9-fold) and trabecular width (Tb.Wi) (increased 1.3-fold) in L3 vertebrae of recipient mice transplanted with MLV-FGF2-transduced Sca-1\(^+\) cells were each significantly increased compared with those in the gfp control recipient mice. Conversely, the % mineralized bone area (%Md.Ar) was significantly decreased (by 27%) in the FGF2-transplanted group compared with the gfp control group. The trabecular number (Tb.N) was not different between the two groups. These quantitative histomorphometry measurements of L3 vertebra were compatible with the microscopic bone changes that show a pattern of large increases in new bone formation but with significant reduction in the amounts of mineralized bone (Figure 2). These findings indicate that the Sca-1\(^-\) cell-based FGF2 gene therapy led to similar robust trabecular bone formation in lumbar vertebrae as seen in long bones.\(^{17,18}\) Similar to those in the FGF2 gene therapy-treated femurs, the large amounts of largely un- or under-mineralized newly formed bone in lumbar vertebrae are presumably also the result of the hypocalcemia and secondary hyperparathyroidism.

![Figure 2](Image 2)

**Figure 2.** Sca-1\(^+\) cell-based systemic FGF2 gene therapy not only promoted massive trabecular bone formation but also caused osteomalacia in recipient mice. In (a), a cross-sectional slice (5 μm in thickness) of L3 lumbar vertebrae of a representative control mice receiving the marrow transplantation of MLV-gfp-transduced Sca-1\(^+\) cells at 14 weeks post transplantation stained with the Goldner’s trichrome dye for mineralized bone. In (b), a cross-sectional slice (5 μm in thickness) of L3 lumbar vertebrae of a representative mice receiving the marrow transplantation of MLV-FGF2-transduced Sca-1\(^+\) cells at 14 weeks post transplantation stained with the Goldner’s trichrome dye for mineralized bone. Mineralized bone is stained dark blue in color, whereas un-mineralized bone matrix is stained in red.

![Figure 3](Image 3)

**Figure 3.** Static bone histomorphometric parameters of L3 vertebra of recipient mice of MLV-gfp-transduced Sca-1\(^+\) cells (green bars) or MLV-FGF2-transduced Sca-1\(^+\) cells (yellow bars) at 14 weeks post transplantation. %Md.Ar, % mineralized bone area; %O.Ar, % osteoid area; %L.Pm, % bone forming surface; Tb.Wi, trabecular width; and Tb.N, trabecular number. N = 7 per group. N.S. = not significant.
developed in response to excessively high levels of FGF2 expression and too rapid of an increase in bone formation.\textsuperscript{17}

Transplantation of MLV-FGF2-transduced Sca-1\textsuperscript{+} cells in mice led to substantial loss of trabecular bone mass in caudal vertebrae. To examine the possibility that donor FGF2-expressing Sca-1\textsuperscript{+} cells might target the highly vascularized red marrow skeletal sites rather than the poorly vascularized yellow marrow sites for engraftment, we performed a second transplantation experiment to compare osteogenic effects of the Sca-1\textsuperscript{+}-based FGF2 gene therapy on the proximal femur metaphysis (a red marrow skeletal site) with that on the caudal vertebra (a yellow marrow skeletal site). In this experiment, W\textsuperscript{51}/W\textsuperscript{51} recipient mice were transplanted with 500 000 MLV-gfp-transduced or MLV-FGF2-transduced Sca-1\textsuperscript{+} cells. We first measured levels of serum FGF2, serum and tibial extract alkaline phosphatase (ALP) activity, and serum PTH of each recipient mouse at 14 weeks post transplantation to ensure successful transplantation. Consistent with our previous findings, recipient mice transplanted with MLV-FGF2-transduced Sca-1\textsuperscript{+} cells showed ~4000-fold increase in serum FGF2 (Figure 4a) and ~8-fold increase in serum PTH (Figure 4b) compared with control recipient mice transplanted with MLV-gfp-transduced Sca-1\textsuperscript{+} cells. The FGF2 group of mice also exhibited elevated serum (Figure 4c) and bone (Figure 4d) ALP levels (a biomarker of bone formation).

We next performed micro-computed tomography (\(\mu\)-CT) analysis on the metaphysis of femur and on the S3 caudal vertebra of each recipient mouse. Top panels of Figure 5a shows the \(\mu\)-CT three-dimensional reconstruction of the femur metaphysis of representative recipient mice, each transplanted either with MLV-gfp-transduced Sca-1\textsuperscript{+} cells or with MLV-FGF2-transduced cells. As expected, FGF2 gene therapy yielded large increases in trabecular bone volume (Tb.BV/TV) and thickness (Tb.Th), without altering trabecular number (Tb.N) or separation (Tb.Sp) at the femur metaphysis (Figure 5a, bottom panels). Conversely, transplantation of MLV-FGF2-transduced Sca-1\textsuperscript{+} cells not only did not yield robust increases in trabecular bone mass in the S3 caudal vertebra, but in fact led to substantial reduction in trabecular bone mass compared with control mice (Figure 5b). The findings of the ~40\% reduction in Tb.N, the approximately twofold increase in Tb.Sp, along with no significant changes in Tb.Th, are consistent with an increase in bone resorption in caudal vertebra (Figure 5b, bottom panel). Consequently, it appears that the absence of an increase in bone formation, along with the apparent increase in bone resorption, resulted in substantial loss of trabecular bone mass in the caudal vertebra.

Sca-1\textsuperscript{+} cell-based FGF2 gene therapy-associated trabecular bone loss in caudal vertebrae was not due to insufficient local FGF2 production

To determine whether the lack of an anabolic response in caudal vertebrae of recipient mice was caused in part by inefficient engraftment of donor Sca-1\textsuperscript{+} cells at yellow marrow skeletal sites, we performed a third marrow transplantation experiment, in which we compared the relative engraftment efficiency of 500 000 donor Sca-1\textsuperscript{+} cells isolated from TgN-GFP-transgenic mice, transduced with MLV-FGF2 (test group) or MLV-\(\beta\)-gal (control group), in the marrow cavity of femurs at 6 weeks post transplantation with that in the marrow cavity of S3 caudal vertebrae of recipient mice. To assess engraftment efficiency, bone marrow cells were flushed out of femurs of each recipient mouse. Total RNA of marrow cells was isolated and reverse-transcribed to cDNA and the relative gfp mRNA level (as an index of engraftment of donor cells) was determined by real-time PCR. Because marrow cells of caudal vertebrae could not be flushed out easily, we opted to isolate total RNA from the entire caudal vertebra. Thus, the caudal vertebrae RNA samples differed from

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Serum FGF2 (a), serum PTH (b), serum ALP activity (c) and bone ALP activity (d) of recipient mice receiving either MLV-FGF2-transduced Sca-1\textsuperscript{+} cells (FGF2) or MLV-gfp-transduced Sca-1\textsuperscript{+} cells (GFP) after 14 weeks post transplantation in the second marrow transplantation experiment. FGF2 and PTH were measured with respective commercial ELISA kits. ALP activity was assayed as described in Materials and methods. \(N=7\) for the GFP group and \(N=4\) for the FGF2 group.
the femur marrow cell RNA samples, in that the caudal vertebra samples contained RNAs derived from both bone marrow cells and bone cells. Figure 6a shows that the gfp mRNA levels between recipient mice of MLV-β-gal-transduced gfp transgenic Sca-1+ cells (FGF2) or MLV-gfp-transduced Sca-1+ cells (GFP) after 14 weeks post transplantation did not differ statistically from each other at the S3 caudal vertebra or at the femur. On the other hand, comparison of the relative gfp mRNA expression level between the two bone sites of the FGF2 group of mice alone (without normalizing against each respective MLV-β-gal control) showed that gfp mRNA expression was significantly higher in bone marrow cells of femurs than in bone extracts of S3 caudal vertebra (P < 0.0002) (Figure 6b). However, because the caudal vertebra samples contained RNA derived not only from marrow cells but also from vertebral bone, the possibility that the different amounts of gfp mRNA detected in femur bone marrow cells (as opposed to that in caudal vertebra) may be due to the different cellular sources of RNA from the two skeletal sites cannot be ruled out.

To further evaluate the possibility that the lack of an osteogenic response in caudal vertebrae of recipient mice transplanted with FGF2-expressing Sca-1+ cells was caused by an inefficient expression of FGF2 at yellow marrow sites, we compared the relative fold increases in the FGF2 mRNA level in bone marrow cells of the femur with that in S3 caudal vertebra by real-time reverse transcriptase polymerase chain reaction (RT-PCR). As expected, FGF2 expression at the femur and caudal vertebra was significantly higher in the FGF2 group of mice compared with β-gal control group of mice at both skeletal sites.

Figure 5. Micro-CT analyses at the secondary spongiosa of the distal femur (a) or at S3 caudal vertebra (b) of recipient mice receiving either MLV-FGF2-transduced Sca-1+ cells (FGF2) or MLV-gfp-transduced Sca-1+ cells (GFP) after 14 weeks post transplantation. Top panels show a representative three-dimensional reconstruction of the trabecular structure each at the femur metaphysis (a) or at S3 caudal vertebrae (b) for each treatment group. Bottom bar graphs show the quantitative analyses of the three-dimensional bone parameters at each site. N=7 for the GFP group and N=4 for the FGF2 group.
measuring the relative GFP compared with respective GFP control group. N.S.

Relative expression levels of Ppia mRNA.

Figure 6. (a) Relative engraftment of transplanted β-galactosidase (β-gal)-expressing cells at the marrow cavity of femurs (left columns) or at the entire caudal vertebra (right columns) of recipient mice receiving MLV-FGF2-transduced Sca-1+ cells (FGF2, N = 20) after 14 weeks post transplantation compared with that of control recipient mice receiving MLV-gfp-transduced Sca-1+ cells (GFP, N = 4). N.S. = not significant. (b) Relative engraftment of gfp-expressing cells in the marrow cavity compared with that in the entire caudal vertebra of recipient mice receiving MLV-FGF2-transduced Sca-1+ cells, N = 20 per group. Engraftment in each panel was assessed by measuring the relative gfp mRNA levels (normalized against Ppia mRNA).

![Graph showing relative engraftment of β-galactosidase (β-gal)-expressing cells](image)

Figure 7. Relative expression levels of FGF2 mRNA in the marrow cavity or in the entire caudal vertebra of recipient mice receiving MLV-FGF2-transduced Sca-1+ cells (N = 20) compared with respective FGF2 mRNA expression levels at each bone site of control recipient mice receiving MLV-gfp-transduced cells (N = 4). ***P < 0.001 compared with respective GFP control group. N.S. = not significant.

![Graph showing relative FGF2 mRNA expression](image)

DISCUSSION

In this study, we confirmed that our stem cell-based FGF2 gene transfer strategy is highly effective in promoting trabecular bone formation at the endosteal surface throughout the marrow cavity of femurs.17,18 It also provided compelling evidence that this strategy is highly osteogenic in the lumbar vertebra. This conclusion is based on the findings of large increases in un-mineralized bone area (Figure 2), %O.Ar (Figure 3) and mineralized bone mass (as evidenced by the increase in Tb.BV/TV data not shown), as well as large increases in % forming surface (%L.Pm) and trabecular width (Tb.Wi) in lumbar vertebrae of FGF2-treated mice compared with control mice. The findings that this therapeutic approach is capable of promoting robust trabecular bone formation and regeneration of trabecular structure in both proximal femurs and lumbar vertebrae could have clinical implication, because the lumbar spine and the hip are two of the most common skeletal sites for non-traumatic osteoporotic fractures.

Previous studies reported that the FGF2 protein therapy was unable to promote bone formation in skeletal sites with yellow marrows, such as the caudal vertebrae.24,25 The FGF2 protein therapy strategy differs from our strategy in many aspects, such as differences in tissue-targeting mechanisms, delivery kinetics, achievable local FGF2 concentrations and metabolism clearance rates. Unlike red marrows, which are highly vascularized, there is very little vascular structure in yellow marrows. This reduced vascular network may limit the accessibility of the systemically administered FGF2 protein in yellow marrow cavities. In contrast, FGF2-expressing Sca-1+ cells engraft at HSC niches, presumably in both red and yellow marrows, and produce FGF2 continuously at local sites, resulting in relatively high local FGF2 levels. To our surprise, we also found that, similar to the FGF2 protein therapy,24,25 our stem cell-based therapy also did not yield significant increase in trabecular bone formation in caudal vertebra. More surprisingly and contrary to the findings of the FGF2 protein therapy that did not result in any bone loss in caudal vertebrae,24,25 our therapy in fact caused substantial trabecular bone loss in caudal vertebrae, as reflected by >40% reduction in Tb.BV/TV and Tb.N (Figure 5).

The mechanistic reason for the apparent lack of an osteogenic action of the two FGF2-based therapies in yellow marrow-filled caudal vertebrae is not well understood. Previous studies have suggested that the lack of an osteogenic response to FGF2 in caudal vertebrae was not due to an insufficient expression of the FGF2 receptors, because there was no significant difference in expression levels of the various surface FGF2 receptors between cells on the endosteal surface of red marrow sites compared with that of yellow marrow in caudal vertebrae.24,25 Similarly, the lack of an osteogenic effect of our stem cell-based systemic FGF2 gene therapy was also not due to inadequate expression of the FGF2 transgene locally, because we found no significant differences in the relative engraftment efficiency of the transplanted GFP-expressing Sca-1+ cells in femurs from that in caudal vertebrae of recipient mice (Figure 6), and because marrow transplantation of FGF2-expressing Sca-1+ cells yielded >1000-fold increase in the local FGF2 mRNA level in both femurs and in caudal vertebrae (Figure 7). A major difference between the two types of vertebrae is that lumbar vertebrae contain largely red marrow with some amounts of adipose tissues; but caudal

[Image 53x413 to 267x732]

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vertebrae is made up of predominantly yellow marrow, which is filled largely with adipocytes,\(^2\)\(^2\) thus raising the possibility that the site-specific differences in the skeletal response to the FGF2 treatment could be in part due to differences in the cellular composition between red and yellow marrows. In this regard, mesenchymal stem cells (MSCs) are more abundant in red marrow than in yellow marrow. Multipotent MSCs can be committed into progenitor cells of osteogenic, chondrogenic or adipogenic lineages, depending on the type of cellular signals released from the local milieu.\(^2\)\(^7\) FGF2 stimulates osteoblastic differentiation of MSCs and osteoblastic bone formation.\(^5\),\(^2\)\(^8\)–\(^3\)\(^1\) In red marrow sites, MSCs favor the osteogenic (or chondrogenic) lineage; whereas MSCs in yellow marrow tend to commit to the adipocytic lineage. As a result, there may be limited numbers of MSCs committed to the osteoblastic lineage in yellow marrows when compared with red marrows, thus greatly reducing the osteogenic response to FGF2. The difference in response to FGF2 therapies between lumbar and caudal vertebra could also be due, at least in part, to differences in weight bearing at the two sites. These are interesting possibilities, some of which will be evaluated in our future studies.

The mechanistic cause for the substantial loss of trabecular bone mass in caudal vertebrae of the recipient mice is unknown at this time. FGF2 has been shown to promote osteoclastogenesis and bone resorption \textit{in vitro}.\(^3\)\(^2\)–\(^3\)\(^6\) Our findings of ~40% reduction in Tb.N and ~twofold increase in Tb.Sp., along with no significant changes in Tb.Th (Figure 5b, bottom panel) in caudal vertebra of mice treated with the FGF2 gene therapy, are consistent with an increase in bone resorption at this skeletal site. Thus, it is possible that the trabecular bone loss is due to an increase in bone resorption without a corresponding increase in bone formation. In addition, the effects of adipocytes (and their secretory factors) on osteoclastogenesis are complex,\(^1\)\(^7\) as both positive and negative effects on the survival and actions of osteoclasts and precursors have been reported. Therefore, we cannot rule out the possibility that the increased bone resorption in yellow marrows could be due to their increased lipid content. We should emphasize that recipient mice transplanted with FGF2-transduced Sca-1\(^+\) cells, especially those with high circulating levels of FGF2, developed hypocalcemia, secondary hyperparathyroidism and osteomalacia (Figures 1 and 4). Secondary hyperparathyroidism is a well-known pathologic cause of increased resorption. Therefore, inasmuch as it is possible that direct activation of osteoclastogenesis by high concentrations of local FGF2 could have a contributing role in the observed increase in bone resorption, the primary contributing factor is most likely secondary hyperparathyroidism, which presumably developed in response to the large and too rapid increase in bone formation.\(^1\)\(^7\) In support of this tentative conclusion, the FGF2 protein therapy, which did not induce hypocalcemia or secondary hyperparathyroidism, did not increase bone resorption in bone sites in yellow marrows.\(^2\)\(^4\),\(^2\)\(^5\) We have recently modified our stem cell-based FGF2 therapy to use the erythroid promoter to confine FGF2 expression to the marrow cavity, and found that this modification was effective and also avoided development of hypocalcemia, secondary hyperparathyroidism and osteomalacia.\(^1\)\(^8\) Therefore, we will in the future determine whether the prevention of secondary hyperparathyroidism with the use of the erythroid promoter could prevent the increase in bone resorption and the bone loss in caudal vertebrae.

On the basis of our findings, we have advanced a model (Figure 8) that may account for the contrasting effects of the stem cell-based FGF2 therapy on red marrows as opposed to yellow marrows. In this model, we envision that our strategy leads to sustained elevation of local levels of FGF2 in both red and yellow marrows. We postulate that there are large numbers of uncommitted MSCs and osteoprogenitors in red marrows (left side of Figure 8), where FGF2 acts on uncommitted MSCs and osteoprogenitors to promote osteoblastic differentiation. This leads to robust and rapid increases in endosteal/trabecular bone formation in red marrows. We further speculate that there is insufficient dietary calcium to meet the huge demand for calcium needed for the rapid and massive increase in bone formation. As a consequence, hypocalcemia and secondary hyperparathyroidism develop, which in turn increases bone resorption at both red and yellow marrows. Because the increase

![Figure 8](image-url)

\(\text{Figure 8. }\) A proposed model to account for the contrasting effects on the Sca-1\(^+\) cell-based systemic FGF2 gene therapy on skeletal sites with red marrows as opposed to those on skeletal sites with yellow marrows. Please see text for detailed description of the model.
in bone formation is larger than that of bone resorption in red marrows, the result is a net increase in bone mass. Conversely, there are very limited numbers of uncommitted MSCs and osteoprogenitors in yellow marrows (right side of Figure 8) for FGF2 to act on. Accordingly, the elevated FGF2 levels have little or no osteogenic effects. This lack of a significant bone formation at the yellow marrows, coupled with the increased resorption rate related to hyperparathyroidism, leads to substantial loss of trabecular bone mass in yellow marrows.

In conclusion, this study highlights an important issue that is highly relevant to the clinical potential of our stem cell-based FGF2 gene transfer therapy for severe osteoporosis. Although our strategy is capable of promoting endosteal/trabecular bone formation in proximal femurs as well as lumbar vertebrae, it lacks osteogenic effects in yellow marrows. This inability to promote regeneration of trabecular bone structure in yellow marrow skeletal sites would greatly reduce the clinical utility of this strategy, and must be overcome before it can be used to treat patients with severe bone deficits. Our future understanding of how and why FGF2 mediates trabecular bone formation in red, but not yellow marrows would provide insights as to how to improve on the efficacy of the therapy in yellow marrows. Consequently, our future work will focus on the mechanistic aspects of this therapy to improve on its clinical potential.

MATERIALS AND METHODS

Animals

C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Trans-Golgi network β-actin-enhanced green fluorescent protein (TgN-GFP) transgenic mice (in C57BL/6J genetic background) were also purchased from the Jackson Laboratories. The W41/W41 mutant strain of mice (in C57BL/6J background; originally provided by Dr. Jane Barker of the St Louis University School of Medicine (St Louis, MO, USA)) was used as transplantation recipients in all transplantation experiments. This mouse strain was used as recipient mice, because, as a result of a point mutation in the c-kit gene,38 they are deficient in the number of HSCs39 and thereby require significantly lower levels of irradiation than wild-type mice for highly efficient engraftment during the bone marrow transplantation procedure.40 The femoral bone mineral density of female (but not male) W41/M41 mice was 17% lower than that of age-matched female wild-type littermates.41 However, because total body irradiation is known to cause significant bone loss and it has been well-recognized as a major etiologic factor for osteoporosis in bone marrow transplantation,42 this mouse model may be considered as an animal model of radiation-induced osteoporosis. Animals were housed within the Veterinary Medical Unit of the Loma Linda Veterans Affairs Healthcare System. All animal procedures were reviewed and approved by two Institutional Animal Care and Use Committees (Loma Linda Veterans Affairs Healthcare System and Loma Linda University).

Bone marrow Sca-1+ cell population isolation

Detailed description of methods for Sca-1+ cell harvest and isolation are previously described elsewhere.17 Briefly, whole bone marrow cells were harvested from donor mice by flushing tibiae and femurs with phosphate-buffered saline plus 0.5% bovine serum albumin (Sigma–Aldrich, St Louis, MO, USA) using a needle and syringe. Erythrocytes were removed by osmotic lysis and the remaining mononuclear cell preparation was enriched for Sca-1+ cells by passage twice through an automatic magnetic assisted cell separation column (AutoMacs), after incubation with magnetic microbeads conjugated with antibody specific for Sca-1 (Miltenyi Biotech, Inc, Auburn, CA, USA) according to the manufacturer’s instructions. To assess enrichment effectiveness, aliquots of each cell fraction were incubated with phycoerythrin-conjugated Sca-1-specific or phycoerythrin-conjugated rat isotype control antibody (Pharmingen, San Diego, CA, USA) and analyzed for Sca-1 and/or GFP expression by fluorescence-activated cell sorter analysis with a FACS Calibur or FACS Aria System (BD Biosciences, San Jose, CA, USA). The percentage of Sca-1+ cells was calculated by subtracting the value obtained with the phycoerythrin-conjugated isotype control antibody from that obtained with the phycoerythrin-conjugated Sca-1-specific antibody. Similar to previous work,17 approximately 70% of the cells in the isolate after the enrichment expressed the Sca-1 marker.

MLV-based human FGF2 expression vectors

The generation of MLV-based vectors expressing the enhanced GFP marker or a modified human FGF2 gene has been described in detail elsewhere.17 To increase secretion efficiency and stability of the expressed FGF2 recombinant protein, the human FGF2 gene was modified by adding the BMP2/4 hybrid secretion signaling sequence to its N terminus and mutating two key cysteines (cys-70 and cys-88) to serine and aspartic acid, respectively.43 The MLV-β-galactosidase (β-gal) was generated as described in detail elsewhere.44

Transduction of Sca-1+ cells with MLV-based vectors

Briefly, Sca-1+ cells were plated in six-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) coated with retronectin (Takara, Otsu, Shiga, Japan) at a density of 4 × 106 cells per well in Iscove’s modified Dulbecco’s medium (Invitrogen, Grand Island, NY, USA) containing 20% fetal bovine serum (BioWhittaker, Walkersville, MD, USA), 50 ng ml−1 of human R3-3, L, 50 ng ml−1 of murine stem cell factor, 50 ng ml−1 of interleukin-6, 10 ng ml−1 of murine interleukin-3, 0.1 ng ml−1 murine interleukin-1α (all from Peprotech, Rocky HIlls, NJ, USA), 100 μM l−1 of deoxyribonucleotide triphosphate (Roche Diagnostics, Indianapolis, IN, USA), as described previously.17 After overnight incubation, 40 μl of MLV-gfp, MLV-β-galactosidase (β-gal) concentrated viral stock (5 × 105 transforming units per microliter) was applied to the cells. The medium was removed 8 h later, and the transduction was repeated once. Cell yields were measured by manual count of viable cells as determined by trypan dye exclusion, and transduced cells were transplanted into recipient mice 20 h after transduction.

Marrow transplantation

The Sca-1+ transplantation procedure was performed as described previously.17 Two weeks before and 2 weeks after irradiation, recipient mice were provided sterile food and autoclaved, acidified water (pH 2.0–2.5) containing 50 mg l−1 neomycin sulfate (Sigma–Aldrich) and 13 mg l−1 polymixin B sulfate (Sigma–Aldrich). Mice were preconditioned by total body irradiation from a 60Co source delivering a single irradiation dose of 5 Gy (80 cGy min−1). Each transduced donor Sca-1+ cells were then transplanted into anesthetized recipient mice 4 h after irradiation via retro-orbital injection.

Three marrow transplantation experiments were performed. In the first experiment, 14 W41/M41 mice were transplanted with 850 000 (in 30 μl sterile saline) Sca-1+ enriched cells transduced with MLV-gfp or MLV-β-gal viral vector (N = 7 per group). Donor Sca-1+ cells were isolated from wild-type C57BL/6J mice. At 6 weeks and 14 weeks post transplantation, transduction and engraftment efficiencies were evaluated as a single measurement of FGF2 DNA levels in the cells extracts of recipient peripheral blood, by real-time PCR. Primers specific for the human FGF2 DNA sequence were used and relative fold changes were calculated by the Livak–Schmittgen Method.45 In the second transplantation experiment, 16 W41/M41 mice were transplanted with 500 000 (in 30 μl sterile saline) Sca-1+ enriched cells transduced with either MLV-gfp or MLV-β-gal viral vector (N = 8 per group). Four mice died (one from the GFP group and three from the FGF2 group) before end point. In the third transplantation experiment, we made the following three changes from the second experiment: (i) we used GFP-expressing Sca-1+ cells from TgN-GFP mice as donor cells in the transplantation, so that engraftment of GFP-transgenic Sca-1+ cells could be tracked and assessed; (ii) we used MLV-β-gal rather than the MLV-gfp as the control viral vector, because the donor cells already expressed GFP; and (iii) because we typically had seen a wide variation in FGF2 expression in FGF2-transplanted group, and in contrast we had seen consistency in our measurements with control mice, we increased the number of the test group (n = 20), that is, recipients of MLV-β-gal-transduced Sca-1+ cells, and slightly reduced the number of recipient mice in the control group (n = 4) that were transplanted with MLV-β-gal-transduced Sca-1+ cells.

Serum and bone extract analyses

Serum FGF2 and PTH levels of recipient mice were determined with respective enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA and ALPCO, Salem, NH, USA, respectively). Serum
ALP activity was measured with a Hitachi 912 Clinical Chemistry analyzer (Stratec SA Plus, White Plains, NY, USA). Bone extract ALP activity was determined as previously described. Briefly, tibia of recipient mice were dissected and extracted with 0.1% Triton X-100 solution for 72 h. The extracts were then assayed in duplicate for ALP activity by colorimetric assay in 96-well plates in a total volume of 0.3 ml, containing 100 mmol l$^{-1}$ Na$_2$CO$_3$ buffer (pH 10.3), 10 mmol l$^{-1}$ p-nitrophenyl phosphate and 1 mmol l$^{-1}$ MgCl$_2$. The time-dependent increase in absorbance at 405 nm (reflecting p-nitrophenol production) was determined on a microtiter plate spectrophotometer (Model EAR 400 AT, SLT Lab Instruments, Hillsborough, NC, USA). The ALP activity was calculated as U$^{-1}$ of serum or mU per mg dry weight of bone, where one unit of activity is defined as the production of 1 μmol of p-nitrophenol per minute at 22 ± 2 °C.

Static histomorphometry of the vertebrae

At 14 weeks post transplantation, mice were killed. Vertebrae T12 to L6 were harvested from recipient mice and the bone was cleared of muscle and other soft tissues as much as possible. The vertebrae were fixed in 10% formalin for 4 h, and stored in 0.05% sodium azide/phosphate-buffered saline solution at 4 °C. L2-3 vertebrae were located by anatomical landmark (T12-rib junction). A 0.01 inch diameter hollow metal sleeve surrounding a fine copper wire was inserted into the L2-L3 intravertebral space. After confirmation of correct anatomical placement by Faxitron imaging, the metal sleeve was withdrawn, leaving the wire in place. The L2-L3 bones were then dissected and embedded into methylmethacrylate, and serial thin sections (5 μm in thickness) were then stained with Goldner’s trichrome dye for analysis of matrix mineralization.

Micro-computed tomography (μ-CT)

Three-dimensional bone parameters were assessed on distal femurs or 53 caudal vertebra by μ-CT using a Scanco vivaCT40 μ-CT scanner (Scanco Medical, Bruttisellen, Switzerland) as previously described. Trabecular measurements were performed at the secondary spongiosa of distal femur (at a site that was 10% of the full length of the femur from the distal end) or the entire 53 caudal vertebra. Accordingly, a region of 0.8 mm in thickness at 10% of the full length from the distal end of each femur was scanned. The trabecular masks were defined in a semiautomatic manner, starting from the outer mask of the femur and application of 15 erosion cycles to ensure that no cortex was included in the measurement. The slices were analyzed using the threshold setting of 230–1 000 mg cm$^{-3}$. Bone parameters were calculated using the analytical tool software of Scanco.

DNA/RNA extraction and quantitative real-time RT-PCR

Total DNA was isolated from cells using the Qiangen QIAamp DNA Blood Mini Kit (Qiangen, Valencia, CA, USA), and total RNA was isolated using the Qiangen RNeasy Mini kit. The cDNA of each RNA sample was synthesized using the Qiagen QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA), and total RNA was isolated using the Qiagen QIAamp DNA Blood Mini Kit (Qiagen). Total DNA in a 20-μl reaction was performed with 2 μl of DNA or cDNA, 10 pmol of each primer set and HotStarTaq DNA Polymerase using a QuantiTect SYBR Green PCR Kit (Qiagen) in an Opticon DNA Engine (MJ Research/Bio-Rad, Hercules, CA, USA). To normalize the data, a primer set unique to mouse peptidylprolyl isomerase A (Ppia) (accession number: NM 002006.4) was used. To distinguish transgenic human FGF2 mRNA from endogenous murine Fgf2 mRNA, the FGF2 primers used for the PCR reaction were specific to the human FGF2 gene (accession number: NM 002006.4) (forward (position 578) 5′-GGTTCTGGGTGCATTCC-3′, reverse (position 900) 5′-CAGGAACCTTGGACAGAACG-3′). As mice transplanted with cells transduced with the MLV-βgal vector do not express the human FGF2 gene, the critical cycle threshold (CT) value of 38 (the basal ‘noise’ value of our RT-PCR analysis) was assigned to the specimen of these control mice to calculate ΔCT values. Relative mRNA abundances were quantified as the critical cycle threshold (ΔCT) method: CT of the gene of interest – CT of the housekeeping gene. The results are shown as the mean ± s.e.m. of duplicates, with the difference in one cycle representing a twofold difference in relative mRNA abundance. Fold changes were calculated by the 2$^{-Δ^{Δ}CT}$ method.  

Statistical analysis

Comparisons of differences were performed using two-tailed, two-sample independent t-tests. Results were considered significant when P < 0.05. All data are reported as mean ± s.e.m.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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