Hylonome

Original Article

Skeletal effects of fibroblast growth factor mimetic (F2A) in ovariectomized rats

J.I. Aguirre[1], S.E. Franz[1], M.K. Altman[1], J.N. Stabley[1], X. Lin[2], P.O. Zamora[2], T.J. Wronski[1]

[1]Department of Physiological Sciences, University of Florida, Gainesville, FL; [2]Biosurface Engineering Technologies, Inc., Rockville, MD

Abstract

The current study was designed to determine whether short-term, systemic treatment with F2A, a mimetic for FGF-2, has skeletal effects in ovariectomized (OVX) rats and adverse side effects in non-skeletal tissues. Groups of sham-operated and OVX rats were maintained untreated for 6 weeks postOVX. These groups (N=6) were then treated IP with vehicle or F2A (0.3, 1.0, or 3.0 mg/kg) daily for 14 days. Histomorphometric analyses were performed in the proximal tibial metaphyses. Hematocrit was normal in F2A-treated OVX rats. Although organ function was not evaluated, histological examination of several organs did not detect any abnormalities. F2A treatment did not increase cancellous bone mass regardless of dose, but OVX rats treated with 1 mg/kg did exhibit increased osteoclast surface. All 3 doses of F2A induced a modest increase in cancellous bone formation. Therefore, F2A appears to increase both cancellous bone resorption and formation, but these skeletal processes are in balance so that, unlike FGF-2, cancellous bone mass is not augmented. However, F2A did not induce the anemia and impaired bone mineralization associated with FGF-2. Therefore, local application of F2A for orthopedic procedures would presumably have minimal side effects, even if the peptide is released to the systemic circulation.

Keywords: Fibroblast Growth Factor, Ovariectomy, Bone Histomorphometry, Osteopenia, Bone Formation

Introduction

Most osteoporosis therapies are anti-resorptive agents with only a modest ability to increase bone mass. Bone anabolic agents are more effective in this regard, but to date, parathyroid hormone (PTH) is the only such agent approved by the FDA for the treatment of osteoporosis. Development of additional options for bone anabolic therapy is highly desirable. Preclinical studies have shown basic fibroblast growth factor (bFGF or FGF-2) to have a strong stimulatory effect on bone formation in intact and ovariectomized (OVX) rats[1-4], but its development as a potential osteoporosis therapy is slowed by adverse side effects, most notably

anemia and impaired bone mineralization[5,6]. There are 4 cell surface receptors for bFGF (FGFRs) with splice variants[7], and it is possible that an agonist that binds preferentially to one of these receptors may have a bone selective effect without adverse side effects. With this in mind, solid-phase peptide chemistry has been used to develop a synthetic peptide (F2A4-K-NS or F2A) that binds with high affinity to FGFR1c as well as heparin and mimics certain aspects of fibroblast growth factor biology[8]. For example, F2A, much like FGF-2, activates the MAP kinase pathway in cultured human endothelial cells with consequent phosphorylation of ERK1/2. Also much like FGF-2, F2A increases the proliferation and migration of endothelial cells *in vitro* and is angiogenic in subcutaneous implants in mice[8]. Furthermore, when F2A was mixed with human demineralized bone matrix (DBM) and implanted in athymic rats, it increased the mineral deposition associated with DBM[9], which is commonly used as a bone-void filler in orthopedic procedures. In a study in rabbits with a tibial mid-shaft fracture, incorporation of F2A in a bone-void filler administered locally as a single percutaneous injection at the site of the fracture enhanced callus production and biomechanical strength[10].

These encouraging results with local application of F2A provided a rationale for studies of systemic treatment with the

peptide. In a murine model for ulcerative colitis, intraperitoneal injection of F2A ameliorated the signs of this gastrointestinal disorder in a manner similar to that of FGF-2[11]. The objective of the current study is to determine whether systemic treatment with F2A has a bone anabolic effect with minimal side effects in ovariectomized rats, a well-established animal model for postmenopausal bone loss[12].

Materials and methods

The experimental animals were 30 virgin female Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) that were 90 days of age and weighed an average of 225g at the beginning of the study. All procedures involving use of rats were approved by the Institutional Animal Care and Use Committee at the University of Florida (Gainesville, FL).

On the day of surgery, all rats were anesthetized with an intraperitoneal (IP) injection of ketamine hydrochloride and xylazine at doses of 75 and 5 mg/kg body weight, respectively. Six rats were subjected to sham surgery during which the ovaries were exteriorized, but replaced intact. Bilateral ovariectomies were performed in the remaining 24 rats from a dorsal approach. The rats were housed in pairs at 25°C with a light/dark cycle of 13 h/11h. Sham-operated rats were allowed unlimited access to food (Teklad LM-485 Rat Diet, Madison, WI). The food consumption of ovariectomized (OVX) rats was restricted to that of sham-operated rats to minimize the increase in body weight associated with ovariectomy[13]. All rats were maintained in this manner for 6 weeks after surgery to allow for the development of tibial cancellous osteopenia in the OVX animals. At this time, a group of sham-operated rats and a group of OVX rats (N=6/group) were injected IP with vehicle (an aqueous solution consisting of 4% mannitol and 10 mM glycine). Three additional groups of OVX rats (N=6/group) were injected IP with F2A (Biosurface Engineering Technologies, Inc., Rockville, MD) at doses of 0.3, 1.0, or 3.0 mg/kg body weight, respectively. All vehicle and F2A treatments were performed daily for 2 weeks, which is the same treatment period for earlier studies with FGF-2[1,3]. Each rat was injected subcutaneously with demeclocycline (Sigma Chemical Co., St. Louis, MO) at a dose of 15 mg/kg BW on the 10th day before sacrifice and with the same dose of calcein (Sigma) on the 3rd day before sacrifice to label actively forming bone surfaces.

At 8 weeks after surgery (2 weeks of F2A treatment), all rats were euthanized by exsanguination from the abdominal aorta under ketamine/xylazine anesthesia. Successful ovariectomy was confirmed by observation of lack of ovarian tissue and atrophied uterine horns. Hematocrit was measured at the time of necropsy with a micro-hematocrit reader (Clay Adams, Parsippany, NJ). Portions of the lung, liver, kidney, and heart were placed in 10% phosphate-buffered formalin for 24h for tissue fixation. The right tibia from each animal was stripped of musculature, cut in half cross-sectionally, and placed in 10% phosphate-buffered formalin for 24h. The right femur was also stripped of musculature, but

placed intact in 70% ethanol for *ex vivo* measurements of bone mineral density. Serum samples were stored at -80°C for future analyses.

The soft tissues were processed by standard methods involving paraffin embedding and subsequent staining with hematoxylin and eosin (Histoserv, Germantown, MD). These tissues were examined qualitatively for abnormalities by a veterinary pathologist (Taconic, Inc., Rockville, MD).

The proximal tibiae were dehydrated in ethanol, embedded undecalcified in modified methyl methacrylate[14], and sectioned longitudinally with Jung 2065 and 2165 microtomes (Leica Corp., Rockleigh, NJ) at thicknesses of 4 and 8 μm. The 4 μm-thick sections were stained according to the von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA) whereas the 8 μm-thick sections remained unstained for collection of fluorochrome-based data. Bone measurements were performed in cancellous bone tissue of the proximal tibial metaphysis beginning at a distance of 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. This region of interest extended between 1 and 3.5 mm distal to the growth plate and excluded cancellous bone tissue within 0.25 mm of both endocortical surfaces. All measurements were performed with the Bioquant Bone Morphometry System (R&M Biometrics Corp., Nashville, TN) and the Osteomeasure System (Osteometrics, Inc., Atlanta, GA). Cancellous bone volume (as a percentage of bone tissue area) and osteoblast and osteoclast surfaces (as percentages of total cancellous perimeter) were measured in 4-μm thick, stained sections. In addition, osteoid volume (as a percentage of bone tissue area) and osteoid surface (as a percentage of total cancellous perimeter) were measured in the same manner.

Fluorochrome-based indices of bone formation were measured in unstained, 8-μm-thick sections of the proximal tibial metaphysis. The percentage of cancellous bone surfaces with a double fluorochrome label (mineralizing surface, MS/BS) and mineral apposition rate (MAR) were measured with the Osteomeasure System. In addition, bone formation rate (tissue level, total surface referent, BFR/BS) was calculated by multiplying MS/BS by MAR.

The distal half of the right tibia was dehydrated and defatted in 100% ethanol and acetone, then embedded undecalcified in a styrene monomer that polymerizes into a polyester resin (Tap Plastics, San Jose, CA). The tibial diaphysis 1-2 mm proximal to the tibiofibular junction was sawed into cross sections of ~150 μm thickness with an Isomet low-speed saw (Buehler, Lake Bluff, IL). These cross sections were then ground to a thickness of 50 μm for histomorphometric measurements with the Osteomeasure System, including cortical bone area and width, and periosteal MS/BS, MAR, and BFR/BS, as previously described[15]. These cortical bone analyses were performed in the vehicle-treated control and OVX rats, and the OVX rats treated with the high dose (3 mg/kg) of F2A.

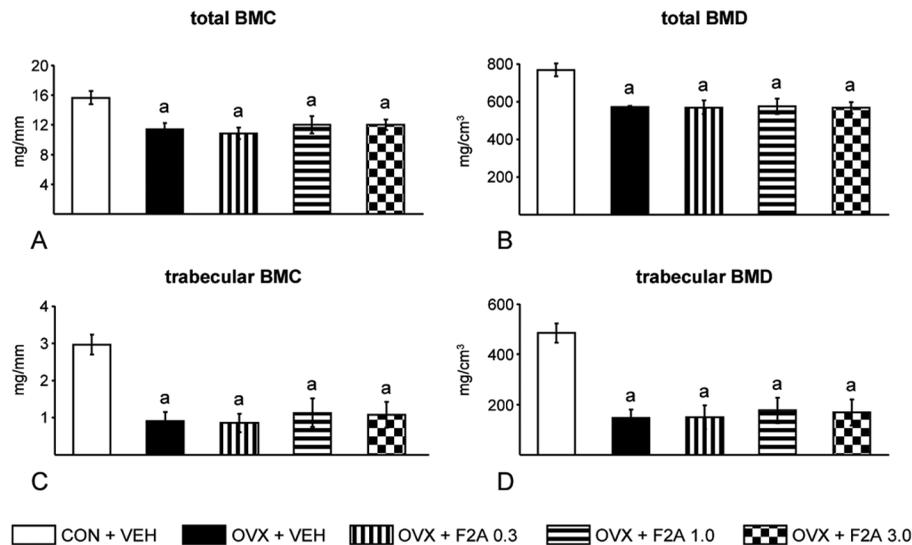The right femur was scanned by peripheral quantitative

Figure 1. Results of pQCT analyses of bone structure in the distal femoral metaphysis, including total mineral content (**A**), total density (**B**), trabecular mineral content (**C**), and trabecular density (**D**). Groups of sham-operated control (CON) and ovariectomized (OVX) rats were treated IP with vehicle (VEH) daily for 14 days (CON+VEH and OVX+VEH). Other groups of OVX rats were treated in the same manner with F2A at doses of 0.3 mg/kg (OVX+F2A 0.3), 1.0 mg/kg (OVX+F2A 1.0), or 3.0 mg/kg (OVX+F2A 3.0). Each value is the mean ± SD of 6 rats. [a]Significantly different from CON+VEH group.

computed tomography (pQCT) with a Stratec XCT Research M instrument (Norland Medical Systems, Fort Atkinson, WI). Scans were performed at a distance of 5 mm proximal to the distal end of the femur for measurements of cancellous and cortical bone structure. This site is at the level of the secondary spongiosa of the distal femoral metaphysis, where major bone structural changes after ovariectomy are known to occur in rats. The structural variables that were measured include: total area, total bone mineral content (BMC), total bone mineral density (BMD), trabecular BMC, and trabecular BMD.

Data are presented as the mean ± SD for each group. The statistical analysis consisted of ANOVA followed by the Fisher protected least significant difference (PLSD) test for multiple comparisons among groups. P values less than 0.05 were considered to be significant.

Results

General Observations

All rats gained weight during the course of the study and remained outwardly healthy. Despite pair-feeding, vehicle-treated OVX rats weighed significantly more than vehicle-treated control rats (320.8±15.7g vs. 283.7±18.7 g, P<0.05). In comparison to the former animals, treatment of OVX rats with the 3 doses of F2A did not affect body weight as the mean values for these groups ranged from 321-323 g. Mean values for hematocrit ranged from 37-42% for the 5 groups

of rats with no significant differences among them. Therefore, F2A did not induce anemia in OVX rats, as has been commonly observed in intact and OVX rats treated with FGF-2[3-6]. Histologic evaluation of the heart, kidney, liver, and lung did not reveal any pathologic changes in these organs induced by ovariectomy or F2A treatment.

Structural Analyses

pQCT analyses of the distal femoral metaphysis indicated that vehicle-treated OVX rats had normal total area (data not shown), but decreased total BMC and BMD compared to vehicle-treated control rats, which was due primarily to markedly decreased trabecular BMC and BMD in the former animals (Figure 1). Similarly, histomorphometric analyses of the proximal tibial metaphysis indicated that vehicle-treated OVX rats had markedly decreased cancellous bone volume compared to vehicle-treated control rats (Figure 2). The observed cancellous osteopenia in OVX rats was due to a reduction in trabecular number rather than a decrease in trabecular thickness. Treatment of OVX rats with F2A did not affect trabecular BMC and BMD, cancellous bone volume, and trabecular number regardless of dose. All groups of rats had a minimal osteoid volume of <0.1% (data not shown).

Histomorphometric analyses of the tibial diaphysis indicated that there were no significant differences in cortical bone structure (area and width) among the vehicle-treated control, vehicle-treated OVX, and high dose F2A-treated OVX groups (data not shown).
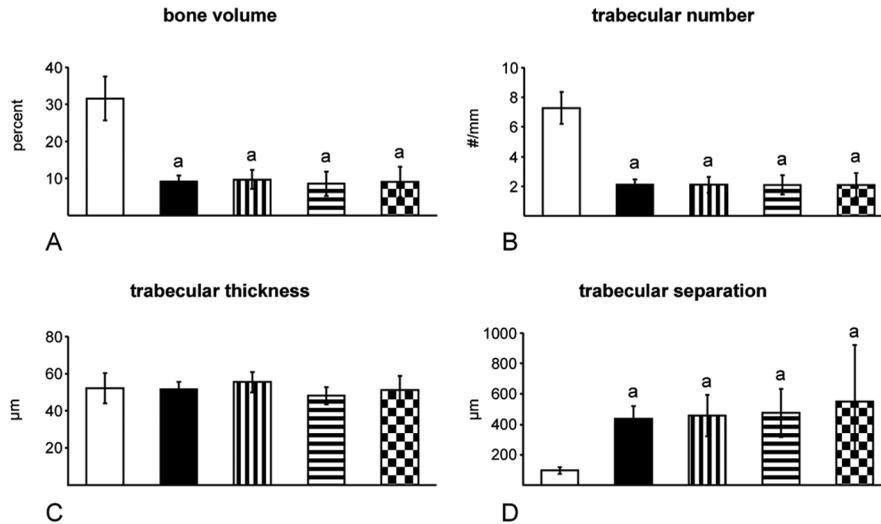
Figure 2. Results of histomorphometric analyses of cancellous bone structure in the proximal tibial metaphysis, including cancellous bone volume (**A**), trabecular number (**B**), trabecular thickness (**C**), and trabecular separation (**D**). See legend for Figure 1 for details of treatment groups. Each value is the mean ± SD of 6 rats. [a]Significantly different from CON+VEH group.

Static Histomorphometric Analyses

As expected, vehicle-treated OVX rats exhibited increased cancellous bone turnover compared with vehicle-treated control rats, as indicated by highly significant increases ($p<0.01$) in osteoblast, osteoid, and osteoclast surfaces (Figure 3). Treatment of OVX rats with F2A did not significantly affect osteoblast and osteoid surfaces compared with vehicle treatment of OVX rats, although the OVX rats treated with 1.0 mg/kg of F2A exhibited a strong trend ($p<0.07$) for an increase in these indices of bone formation. However, this dose of F2A, but not the other doses, did significantly increase osteoclast surface, an index of bone resorption.

Dynamic Histomorphometric Analyses

All fluorochrome-based indices of cancellous bone formation, including MS/BS, MAR, and BFR/BS were significantly increased in vehicle-treated OVX rats compared to vehicle-treated control rats (Figure 4). Treatment of OVX rats with all 3 doses of F2A induced a significant increase in MS/BS and BFR/BS, and the 2 higher doses significantly increased MAR as well, when compared to vehicle-treated OVX rats.

In the tibial diaphysis, periosteal MS/BS and BFR/BS (but not MAR) were significantly increased by at least of factor of 3 in both the vehicle-treated OVX rats and the OVX rats treated with the high dose of F2A compared to the vehicle-treated control rats (data not shown). However, mean values for these indices of periosteal bone formation were nearly identical in the vehicle- and F2A-treated OVX rats, with no significant differences between them.

Discussion

The current study demonstrated that F2A, a synthetic peptide designed to mimic FGF-2, has a modest stimulatory effect on cancellous bone turnover in OVX rats. This peptide was found to increase osteoclast surface, an index of bone resorption, as well as bone formation rate in the proximal tibial metaphysis of OVX rats. However, F2A treatment did not augment cancellous bone mass in these estrogen-deplete animals, which suggests that the observed increase in bone formation is in balance with the increase in bone resorption so that a net change in bone mass did not occur. Therefore, F2A has a more subtle skeletal effect than FGF-2, which markedly stimulates cancellous bone formation to a much greater extent than bone resorption and restores lost cancellous bone mass in osteopenic, OVX rats[3,4].

Despite the strong bone anabolic effects of FGF-2, its development as an osteoporosis therapy has been contraindicated by adverse side effects, such as anemia and impaired bone mineralization[5,6]. Neither of these side effects was observed in F2A-treated OVX rats. As evidence for impaired bone mineralization, OVX rats treated with FGF-2 exhibit marked accumulation of osteoid and an almost total lack of fluorochrome labeling of bone surfaces. In contrast, F2A-treated OVX rats had very low osteoid volumes and normal fluorochrome labeling of bone. Furthermore, histologic evaluation of other organs after treatment with F2A did not reveal any abnormalities known to occur in rats treated with FGF-2, such as extramedullary hematopoiesis in the liver, glomerular lesions in the kidney, and a thickening of alveolar epithelium in the lung[5,6,16]. However, the possibility that a longer treatment period with F2A may induce
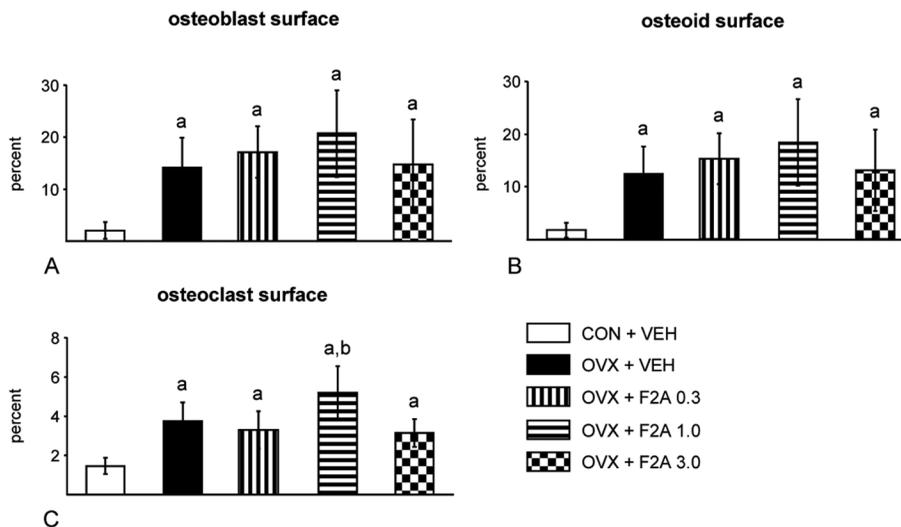
Figure 3. Mean values (±SD) for cancellous osteoblast surface (**A**), osteoid surface (**B**), and osteoclast surface (**C**) in the proximal tibial metaphysis of the 5 groups of rats. See legend for Figure 1 for details of treatment groups. [a]Significantly different from CON+VEH group; [b]Significantly different from OVX+VEH group.

Figure 4. Mean values for cancellous mineralizing surface (**A**), mineral apposition rate (**B**), and surface-referent bone formation rate (**C**) in the proximal tibial metaphysis of the 5 groups of rats. See legend for Figure 1 for details of treatment groups. [a]Significantly different from CON+VEH group; [b]Significantly different from OVX+VEH group.
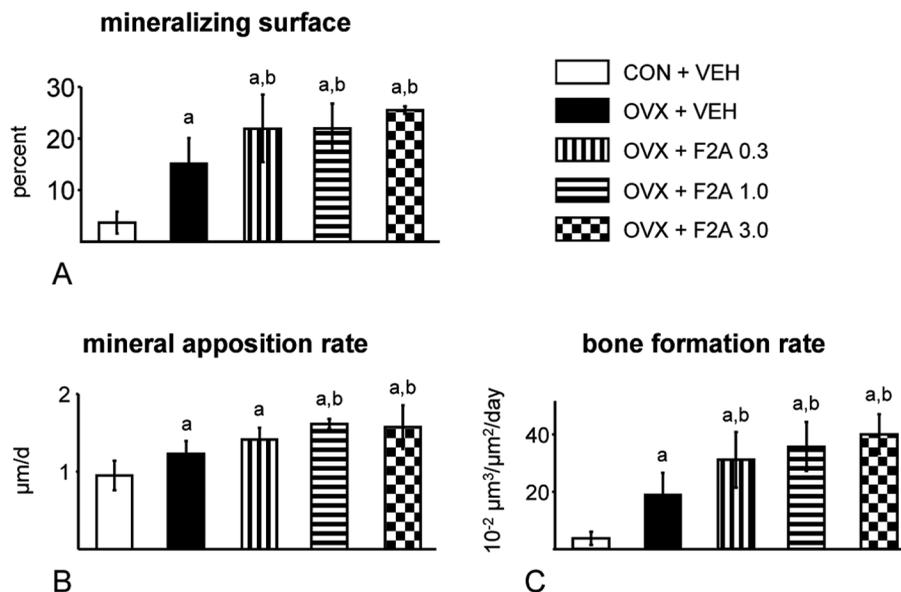
adverse side effects in both skeletal and non-skeletal tissues cannot be ruled out. Also, the current analyses consisted of histologic observations only and did not include functional assays of organs. Nevertheless, if the current short-term study is predictive of the long-term biologic effects of F2A, local application of the peptide for orthopedic procedures, even if some of the F2A circulates systemically, is unlikely to induce adverse side effects in organs that exhibit a biologic response to FGF-2.

F2A is known to bind to FGFR1c with an affinity approximately one third that of FGF-2[8]. However, once bound, the dissociation rate of F2A from this receptor is nearly 10 times slower than FGF-2[8]. The binding affinity of F2A to other FGF receptors is currently unknown. Despite the presumed activa-

tion of FGFR1c, OVX rats treated with F2A exhibited a much smaller increase in cancellous bone formation compared with FGF-2 treatment. Although dose comparisons between F2A and FGF-2 may be problematic due to differences in pharmacokinetics, F2A at a dose of 1.0 mg/kg induced a nonsignificant, 50% increase in osteoblast surface, whereas the same dose of FGF-2 induced a highly significant, 6-8 fold increase in this cellular index of bone formation[3,4]. These findings suggest that binding of FGF-2 to FGFR1c may be minimally involved in the strong bone anabolic effects of the growth factor in estrogen-deplete rats. Similarly, despite presumptive activation of FGFR1c, F2A-treated OVX rats were not found to be anemic, which is commonly observed in intact and OVX rats treated with FGF-2[3-6]. Although *in vitro* evidence has implicated this particular FGF receptor in the regulation of hematopoiesis[17], our findings in rats treated with F2A suggest that activation of FGFR1c may not play a major role in the anemia induced by systemic treatment with FGF-2.

The findings of the current study should be considered with some limitations in mind. Only one index of bone resorption (osteoclast surface) was measured, which is indicative of the effects of F2A on osteoclast numbers, but not on osteoclastic activity. Furthermore, although F2A did not affect cancellous bone mass after 2 weeks of treatment, it is possible that longer treatment with the peptide may have a positive effect on cancellous bone balance. Nevertheless, it can be concluded that, under the conditions of this study, F2A did not induce the strong bone anabolic response previously observed in intact and OVX rats treated with FGF-2[1,3]. In fact, indices of bone formation such as osteoblast and osteoid surfaces were found to be significantly increased in OVX rats after only 2 days of treatment with FGF-2[18].

In summary, short-term, systemic treatment with F2A, a mimetic for FGF-2, had a modest stimulatory effect on cancellous bone turnover in OVX rats, but failed to augment cancellous bone mass in these osteopenic animals. These findings suggest that F2A has limited potential as an osteoporosis therapy. However, F2A did not induce the adverse side effects associated with systemic FGF-2 treatment, which minimizes concerns about potential release of the synthetic peptide to the systemic circulation when F2A is delivered locally for orthopedic applications.

References

1. Mayahara H, Ito T, Nagai H, Miyajima H, Tsukuda R, Taketomi S, Mizoguchi J, Kato K. *In vivo* stimulation of endosteal bone formation by basic fibroblast growth factor in rats. Growth Factors 1993;9:73-80.
2. Nagai H, Tsukuda R, Mayahara H. Effects of basic fibroblast growth factor (bFGF) on bone formation in growing rats. Bone 1995;16:367-73.
3. Liang H, Pun S, Wronski TJ. Bone anabolic effects of basic fibroblast growth factor in ovariectomized rats. Endocrinology 1999;140:5780-8.
4. Iwaniec UT, Magee KA, Mitova-Caneva NG, Wronski TJ. Bone anabolic effects of subcutaneous treatment with basic fibroblast growth factor alone and in combination with estrogen in osteopenic ovariectomized rats. Bone 2003;33:380-6.
5. Mazue G, Bertolero F, Garofano L, Brughera M, Carminati P. Experience with the preclinical assessment of basic fibroblast growth factor. Toxicol Letters 1992;64/65:329-38.
6. Wamsley HL, Iwaniec UT, Wronski TJ. Selected extra-skeletal effects of systemic treatment with basic fibroblast growth factor in ovariectomized rats. Toxicol Path 2005;33:577-83.
7. Eswarakumar VP, Lax I, and Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev 2005;16:139-49.
8. Lin X, Takahashi K, Campion SL, Liu Y, Gustavsen GG, Pena LA, Zamora PO. Synthetic peptide F2A-K-NS mimics fibroblast growth factor-2 *in vitro* and is angiogenic *in vivo*. Int J Mol Med 2006;17:833-9.
9. Lin X., Pena LA, Zamora PO, Campion SL, Takahashi K. Augmentation of demineralized bone matrix (DBM) mineralization by a synthetic growth factor mimetic. J Orthop Res 2006;24:2051-8.
10. Fredericks DC, Bobst JA, Petersen EB, Zamora PO, Nepola JV. Enhanced repair in a rabbit tibia osteotomy model with the synthetic peptide F2A4-K-NS (F2A). Orthop Res Soc 2007; 0069: http://www.ors.org/web/transactions/6thcombined meeting/0069.pdf.
11. Lin X, Zamora PO, Takahashi K, Lui Y. Alleviation of experimental ulcerative colitis with the synthetic peptide, F2A4-K-NS (Fibratide). Dig Dis Sci 2007;52:2054-62.
12. Kalu D. The ovariectomized rat model of post-menopausal bone loss. Bone Miner 1991;15:175-92.
13. Wronski TJ, Schenck PA, Cintron M, Walsh CC. Effect of body weight on osteopenia in ovariectomized rats. Calcif Tissue Int 1987;40:155-9.
14. Baron R, Vignery A, Neff L, Silvergate A, Santa Maria A. Processing of undecalcified bone specimens for bone histomorphometry. In: Recker RR, editor. Bone Histomorphometry: Techniques and Interpretation. Boca Raton, FL: CRC Press; 1983. pp. 13-35.
15. Iwaniec UT, Moore K, Rivera MF, Myers SE, Vanegas SM, Wronski TJ. A comparative study of the bone-restorative efficacy of anabolic agents in aged ovariectomized rats. Osteoporos Int 2007;18:351-62.
16. Mayahara H, Ito T, Nagai H, Miyajima H, Tsukuda R, Taketomi S, Mizoguchi J, Kato K. *In vivo* stimulation of endosteal bone formation by basic fibroblast growth factor in rats. Growth Factors 1993;9:73-80.
17. Allouche M. Basic fibroblast growth factor and hematopoiesis. Leukemia 1995;9:937-42.
18. Power RA, Iwaniec UT, Magee KA, Mitova-Caneva NG, Wronski TJ. Basic fibroblast growth factor has rapid bone anabolic effects in ovariectomized rats. Osteoporos Int 2004;15:716-23.