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Inhibition of the Prostaglandin EP-1 Receptor in Periosteum Progenitor Cells Enhances Osteoblast Differentiation and Fracture Repair

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Abstract

Fracture healing is a complex and integrated process that involves mesenchymal progenitor cell (MPC) recruitment, proliferation and differentiation that eventually results in bone regeneration. Prostaglandin E2 (PGE2) is an important regulator of bone metabolism and has an anabolic effect on fracture healing. Prior work from our laboratory showed $EP1^{-/-}$ mice have enhanced fracture healing, stronger cortical bones, higher trabecular bone volume and increased *in vivo* bone formation. We also showed that bone marrow MSCs from $EP1^{-/-}$ mice exhibit increased osteoblastic differentiation *in vitro*. In this study we investigate the changes in the periosteal derived mesenchymal progenitor cells (PDMPCs), which are crucial for fracture repair, upon EP1 deletion. $EP1^{-/-}$ PDMPCs exhibit increased numbers of total (CFU-F) and osteoblastic colonies (CFU-O) as well as enhanced osteoblastic and chondrogenic differentiation. Moreover, we tested the possible therapeutic application of a specific EP1 receptor antagonist to accelerate fracture repair. Our findings showed that EP1 antagonist administration to wild type mice in the early stages of repair similarly resulted in enhanced CFU-F, CFUO, and osteoblast differentiation in PDMPCs and resulted in enhanced fracture callus formation at 10 days post fracture and increased bone volume and improved biomechanical healing of femur fractures at 21 days post fracture.

Keywords

EP1; periosteum progenitor cells; osteogenic differentiation and bone fracture

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Introduction

In the United States approximately eight million bone fractures occur annually[51, 66]. Ten to twenty percent of the fractures fail to heal resulting in delayed repair or non-union causing severe disabilities. Non unions often require surgical intervention and results in increased morbidity and health care costs. Currently, non-union treatment involves bone implants with decalcified grafts or autografts[5, 20, 57]. However, high failure rates with these approaches indicate the pressing need for the development of novel therapies that will increase bone formation, enhance fracture repair rates, and treat non-unions.

Fracture healing is a complex physiological process that includes several defined stages. Healing starts with hematoma formation and an inflammatory response. Additionally, this stage is characterized by activation and proliferation of the periosteal stromal cells also known as periosteal derived mesenchymal progenitor cells (PDMPCs), resulting in a thickened periosteum. PDMPCs further differentiate into osteoblasts and chondrocytes, which eventually form bone via both endochondral and intramembranous ossification. Finally the healing process results in restoration of normal bone architecture and strength through an on-going remodeling process[18, 19].

Mesenchymal progenitor cells (MPCs) were first isolated from bone marrow and thought to be connective tissue supportive cells[49]. Later, these cells were shown to have the potential to differentiate into osteoblastic, chondrogenic and adipogenic lineages[9]. MPCs are characterized by their ability to form colonies and by expression of specific markers. They are negative for hematopoietic and endothelial markers, CD45 and CD31 respectively, and are positive for markers including CD105, Sca1, CD29, CD90, CD44, and CD73. MPCs from both the periosteum and bone marrow have been shown to participate in the repair process[16, 26, 63] and are considered the source of cells responsible for the repair process. Thus, limitation in numbers and functions of MPCs results in impaired bone healing[69]. Given the importance of MPCs on bone regeneration[7, 8], it is important to understand the mechanisms that regulate their cell fate.

The periosteum is a thin layer of osteogenic and fibroblastic cells that surrounds the outside of the bones[2]. The “cambium layer” of the periosteum is highly cellular and contains PDMPCs, osteoprogenitors, osteoblasts, and fibroblasts as well as micro vessels and sympathetic nerves. Periosteal cells have been shown to be the main cell population to participate in fracture healing, while removal of the periosteum significantly delays the healing process[16, 69]. Furthermore, lineage-tracing studies have identified periosteal cells as the main source of cells in the callus during healing[44].

The inflammatory stage of the repair process is essential for the fracture healing cascade[24]. Cyclooxogenase 2 (COX2) and its main metabolite, Prostaglandin E2 (PGE2), are major inflammatory mediators required for the healing process to take place[52, 53, 56]. PGE2 has been shown to play an important role in bone biology; systemic PGE2 administration in mice and rats resulted in increased bone formation and bone resorption[55, 64]. PGE2 exerts its effect by binding to four receptors: EP1–4[15, 46, 60]. EP2 and EP4 receptors have both bone anabolic and catabolic effects[17, 39, 48, 61, 62]. Little is known about the role of EP1 receptor

in bone biology. In bone cells, EP1 increases osteoblastic cell-line proliferation[59]. In addition, EP1 is expressed in proliferating chondrocytes, with increased expression during proliferation[11]. EP1 has been suggested to play a role in osteoclast maturation[22]. We have previously demonstrated that EP1 knock-out ($EP1^{-/-}$) mice exhibit increased bone formation *in vivo*, resulting in stronger bones and reduced bone loss with aging[67]. Moreover, $EP1^{-/-}$ mice exhibit accelerated fracture healing resulting in faster bone formation and enhanced biomechanics[68]. These findings suggest that EP1 is a negative regulator of bone formation.

Recently we demonstrated that bone marrow mesenchymal progenitor cells (BM-MPCs) harvested from $EP1^{-/-}$ mice had enhanced osteogenic potential[21]. In light of the particular importance of PDMSDs in fracture healing and our observations regarding the enhanced repair in the $EP1^{-/-}$ mice, we decided to test whether $EP1^{-/-}$ mice exhibit differences in the functions of PDMPCs. Additionally, as a potential therapeutic target, we tested whether administration of EP1 antagonist following fracture will enhance the healing process by increasing the PDMPC differentiation potential. We found that periosteum of $EP1^{-/-}$ mice consist of more progenitors with increased osteoblastic potential. We also observed that treatment with an EP1 antagonist increases the number of osteoblastic progenitors in the periosteum, and improves the biomechanical properties during fracture healing.

Materials and Methods

Mouse strains:

C57BL/6J mice were purchased from Jackson Laboratory. $EP1^{-/-}$ mice were generously provided by Matthew Breyer[27]. All animal procedures were approved by the University Committee of Animal Resources (UCAR) at the University of Rochester Medical Center. The $EP1^{-/-}$ mice used in the experiments have global gene deletion of the $EP1^{-/-}$ receptor in all cells and tissues, including bone marrow progenitor cells[68].

Bone grafting and periosteal cell isolation:

Periosteal cells were isolated using a protocol described by Zhang *et al*[70]. Briefly, wild type C57BL/6J or $EP1^{-/-}$ mice aged 10–14 weeks were anesthetized with Isofluorane and a 4mm femur autograft surgery performed. Briefly a 4mm segment of bone was removed from the femur, the bone marrow was flushed from 4mm autograft segment, and the graft was placed back into the femur and fixed in place with a pin. After five days of in vivo expansion of the periosteal cell population, the autografts are harvested and freed from all muscle attachments. The harvested autografts again have repeated flushing of the marrow cavity and marrow cells are discarded. The periosteal tissue attached to the bone surface is removed and digested with Collagenase D (3mg/ml) for 45 minutes with vigorous shaking at 37°C. The periosteal cells are then strained through a 70mm mesh and plated in 15mm culture dishes. Periosteal progenitor cells were allowed to adhere to the culture dish before media change and grown to 80% confluence. The periosteal progenitor cell isolation typically pools cells from 4–5 mice.

Osteogenic differentiation:

First passage periosteal progenitor cells were plated at 10,000 cells per well of a 12-well plate and grown to confluence. The media then was replaced with alpha MEM with 10% FBS, 1% penicillin streptomycin and supplemented with 100mM beta glycerol-phosphate and 50 μg/ml ascorbic acid. The media was changed every two days and cells were harvested at indicated time points for alkaline phosphatase staining and gene expression analysis.

Adipogenic differentiation:

First passage periosteal progenitor cells were plated at 10,000 cells per well of a 12-well plate and grown to confluence. Adipogenic differentiation was induced with media containing 1μM Dexamethasone, 0.5mM IBMX, 10μg/ml Insulin and 10nM Rosiglitazone for two days. The media then was then replaced with maturation media containing 1μg/ml Insulin and 10nM Rosiglitazone for an additional 6 days. Differentiation was assessed by the appearance of lipid droplets as visualized by staining with Oil Red O and measurement of absorbance at 500nm of eluted Oil red O with 100% isopropanol, as well as adipogenic gene expression by qPCR.

Chondrogenic differentiation:

First passage periosteal progenitor cells were pellet at 2.5×10^5 cells per 15ml conical tube and supplemented with 0.5ml chondrogenic media (Lonza). The media was changed every two days for 28 days. The cell pellets were either fixed with 10% Neutral Buffer Formalin, embedded in paraffin, cut to 3μm sections, and stained with Alcian Blue, or underwent mRNA expression analysis by qPCR.

Flow cytometry:

Cells were suspended in PBS containing 3% FBS and stained with the following antibodies: CD45-PerCP, CD31-PE-Cy7, Sca1-APC, CD105-PE (BD Pharmingen). Compensation controls were performed using anti-mouse Compensation Standard beads. Gates were determined using Flow Minus One (FMOs) controls.

Colony forming assay:

In low-density cultures, single colonies of fibroblast-like cells are formed, each colony arising from a single precursor cell called a colony forming unit fibroblast (CFU-F)[37]. Freshly isolated periosteal progenitor cells were plated at 2000 cells per well in a six-well plate and cultured for 10 days. The cells were then fixed and stained with 0.5% Crystal violet in methanol for CFU-F and with ALP substrate NBT/BCIP reagent (Thermo Scientific Pierce) for CFU-O. A colony was considered a cluster of more than 50 cells[36, 37].

Gene expression analysis>:

mRNA was extracted at indicated time points using RNA Easy extraction kit (Qiagen). Exactly 0.5 g of RNA was reverse transcribed into cDNA using iScript reagent (Biorad). Quantitative RT-PCR was performed using a RotorGene real-time PCR machine.

Femur open osteotomy model:

12 week-old C57BL/6J mice (males and female) were anesthetized and a 7–8mm-long incision was made in the skin, and the midshaft of the femur was exposed by blunt dissection of the muscle without disturbing the periosteum. The mid-diaphysis of the femur was cut with a dremel saw with diamond blade. The bone was stabilized using 22 gauge metal pin placed through the intramedullary canal. Starting day 1 after surgery the mice were injected i.p. with either vehicle (DMSO) or 10mg/kg EP1 antagonist (SC51089, Cayman chemical) for 5 consecutive days. The 10mg/kg dose of SC51089 used in the experiments was based on prior studies showing that the *in vivo* effects of SC51089 on analgesia in rats have a K_d of approximately 6mg/kg[2941]. In a rat model, 10mg/kg dosing of SC51089 was shown to prevent seizure activity[50].

Micro Computed Tomography:

Femurs were harvested at the indicated time points and scanned using VIVA microCT system at a voxel size of 10.5μm. From the 2D slice images generated, an appropriate threshold was chosen for the bone voxels by visually matching thresholds areas to grayscale images. The threshold and the volume of interest were kept constant throughout the analysis for each femur. To measure the new total callus volume and bone volume, contour lines were manually drawn on the key 2D slice images to exclude the cortical bone, with morphing interpolating contours between key slices performed semi-automatically and adjusted manually by the expert operator to ensure the fidelity of callus measurements and the exclusion of cortical bone.

Biomechanical testing:

Femurs were dissected 21 days after fracture from vehicle and EP1 antagonist treated mice. Torsion testing was performed in a Torsional Test Bench (Endura Tech) according to established protocols[54]. Briefly, muscle and soft tissue were completely removed from the femurs to be tested, and the pins were carefully removed without disrupting the callus. Both ends of the femur were cemented into 6.35mm^2 aluminum tube holders using polymethyl-methacrylate (PMMA) in a custom jig to insure axial alignment and to maintain a gage length of ~6.5mm, allowing at least 3mm to be potted to each end. Specimens were then mounted on the EnduraTec TestBench™ system (Bose Corporation, Minnetonka, MN) and tested in torsion at a rate of 10°/s until failure. Ultimate torque, torsional rigidity, ultimate rotation, and energy to failure were determined.

Results

$EP1^{-/-}$ mice periosteum have more osteoblastic progenitors

Based on our *in vivo* observation that $EP1^{-/-}$ mice exhibit accelerated repair, we undertook further analysis of the progenitor population in WT and $EP1^{-/-}$ periosteum. To determine whether there was a difference in the numbers of PDMPCs in $EP1^{-/-}$ mice we analyzed both the colony forming potential by CFU assay and flow cytometric analysis for cell surface markers. We observed that $EP1^{-/-}$ periosteal cells form three times more CFU-Fs than WT (WT: 13 3.5, $EP1^{-/-}$: 36±4.8, $p<0.001$). Moreover, $EP1^{-/-}$ periosteal cells formed 7-fold

more CFU-Os (WT: 0.5±0.33, $EP1^{-/-}$: 7±2.27 $p<0.05$) (Figure 1A–B). To determine if there were differences in periosteal cell proliferation we analyzed the BrdU incorporation rate in the periosteum. No change in BrdU incorporation were observed between WT and $EP1^{-/-}$ cells (WT: 38.8%±19.75, $EP1^{-/-}$ 43.64%±16, p=0.65) (Figure 1C).

$EP1^{-/-}$ Periosteal cells have increased osteoblastic differentiation

To determine whether EP1 plays a role in PDMPC differentiation, we performed *in vitro* differentiation assays in the osteoblastic, chondrogenic and adipogenic lineages. We observed that $EP1^{-/-}$ PDMPCs have increased alizarin red staining compared to WT cells (Figure 2A). Gene expression analysis revealed a 1.5 fold increase in *Col1a1*expression (p=0.019) and 3-fold increase in *Sp7* (p=0.007) after 7 days of osteogenic differentiation (Figure 2B). No changes in chondrogenic differentiation were observed by Alcian blue staining between WT and $EP1^{-/-}$ cells (Figure 2C). However, Col2a1 expression was increased 2.5-fold in $EP1^{-/-}$ cells, along with a 1.5 -fold increase in *Acan* expression, relative to WT (Figure 2D). We observed a slight decrease in adipogenic differentiation potential in $EP1^{-/-}$ cells as indicated by an 8% decrease in Oil Red O staining (p=0.026) and a 35% decrease in *Cebpa* gene expression (p=0.025), relative to WT cells (Figure 2 E–F).

Based on the increased differentiation and increased number of osteoblastic colonies we analyzed the periosteal cells based on mesenchymal cell surface markers. Several previous studies suggested that reduced CD105 expression is associated an increase in osteoblastic differentiation potential[3, 4, 6, 38]. We sorted and removed $CD45^{-}CD31^{-}$ cells (non-hematopoietic, non-endothelial cells, respectively) and examined expression of the mesenchymal markers: Sca1 and CD105. We did not observe any significant change in the percentage of $Sca1^{+}CD105^{+}$ cells (WT 0.036%±0.02%, $EP1^{-/-}$ 0.05±0.02, p=0.25). However there was a significant increase in the percentage of the $Sca1^{+}CD105^{-}$ cells in periosteal cells obtained from $EP1^{-/-}$ mice (WT: 0.39%±0.2, $EP1^{-/-}$: 0.75%±0.26, p=0.04) (Figure 3 A–B). To confirm that these cells have different differentiation potential we sorted $Sca1^{+}CD105^{+}$ and $Sca1^{+}CD105^{-}$ cells and induced osteoblastic, adipogenic or chondrogenic differentiation. Sorted $CD105^{-}$ cells from WT or $EP1^{-/-}$ mice exhibited increased mineralization relative to matched genotype $CD105^{+}$ cells after five days of culture in osteogenic medium (Figure 3C). No differences were observed in the differentiation potential of either the $CD105^{+}$ cells or $CD105^{-}$ from the WT and $EP1^{-/-}$ mice respectively.

EP1 antagonist treatment during fracture healing changes the periosteal mesenchymal population

Since $EP1^{-/-}$ mice exhibit accelerated fracture healing[68] we tested whether systemic administration of an EP1 specific antagonist following fracture will be sufficient to enhance the repair process by inducing PDMPC differentiation.

First, we tested whether administration of EP1 antagonist induces changes to PDMPCs (Figure 4A). We observed that following EP1 antagonist treatment PDMSCs form more CFU-Fs (vehicle: 11±3.43, EP1 antagonist: 27±5.8, p=0.039) and CFU-Os (vehicle: 3 ±1.4, EP1 antagonist 12.92±4, p=0.04), resulting in a 2 fold increase in the ratio of CFUO/CFU-F

in EP1 antagonist treated PDMPCs (vehicle: 0.2±0.04, EP1 antagonist 0.43±0.05, p=0.0078, Figure 4B–D). Additionally, we observed that EP1 antagonist treatment increased the population of $Sca1^{+}CD105^{-}$ cells (2% versus 6% after treatment, N=3, $p<0.05$), resulting in a higher proportion of $Sca^{+}CD105^{-}$ than observed in $EP1^{-/-}$ PDMPCs (Figure 4E–F). The increase in the $Sca1^{+}CD105^{+}$ cells was not significant (0.05% versus 0.08% after treatment, p=0.95).

We then analyzed the fractured femurs at day 10 and 21 after fracture. μCT images showed that by day 10 after fracture, the EP1 antagonist treated group had an increase in callus volume (vehicle: 30.14±3.68mm^3, EP1 antagonist: 55±9.15, $p<0.01$) (Figure 5A, 5D). No change in bone volume was observed at this time (vehicle: 5.56±0.96 mm^3, EP1 antagonist 4.4±1.27 mm^3, p=0.88,). By day 21 the bone volume fraction (BV/TV) was significantly increased in EP1 antagonist treated fractures (vehicle: 0.22±0.007, EP1 antagonist: 0.29±0.036, p= 0.017) (Figure 5B, 5D). Additionally, mineralization density of EP1 antagonist treated fractured bone was higher compared to vehicle (vehicle-161.4±8.4 mgHA/cc, EP1 antagonist-202.4±9.10.0 mgHA/cc) (Figure 5B–D).

To test the quality of the healed fractured bones torsion testing was conducted 21 days after fracture. A 3-fold increase in torsional rigidity was observed in EP1 antagonist treated fractures, relative to vehicle treated (p=0.04). In addition, EP1 antagonist treatment resulted in increased maximum torque (2-fold increase, p=0.03), energy to maximum (2- fold increase, p=0.04), and higher yield to torque (3- fold increase, p=0.03), relative to vehicle treated fractures (Figure 6).

Discussion

We have previously demonstrated that $EP1^{-/-}$ mice have increased bone formation and accelerated fracture repair *in vivo*, as well as increased osteogenic differentiation of bone marrow cells *in vitro*[21, 68]. In the present study we sought to determine the relationship between the EP1 receptor and periosteal stem cell population and to explore the possible therapeutic potential of an EP1 antagonist to enhance bone regeneration. PGE2 has been shown to induce both anabolic and catabolic effects with overall enhanced bone formation through the activation of the EP2 and EP4 receptors[65]. While the role of EP2 and EP4 are well described, very little is known about the role of EP1 in bone homeostasis. In the present study, the data shows that EP1 receptor down regulation induces PDMPC differentiation. Furthermore, systemic administration of an EP1 specific antagonist during fracture repair accelerates healing, likely by enhancing differentiation of PDMPCs toward the osteoblastic lineage. Thus, our findings identify EP1 antagonism as a novel therapeutic approach to improve bone regeneration by modulating periosteal progenitor cell function. Using a combination of cellular-based, biomechanical and molecular assays, we provide evidence that EP1 pharmacologic inhibition alters the periosteal derived progenitor cell differentiation potential towards osteogenic lineage.

By analyzing the colony forming ability, we observed a higher number of both CFU-Fs and CFU-Os of $EP1^{-/-}$ mice and a higher ratio of CFU-O/CFU-Fs in periosteal cells from $EP1^{-/-}$ mice compared to WT mice. Considering that the cultures were not stimulated with

osteogenic medium, the increased osteogenic differentiation of EP1$^{-/-}$ cells suggests that these cells were already committed to the osteogenic lineage at the time of isolation. We recently had similar findings in wild type and EP1$^{-/-}$ bone marrow progenitor cell populations, suggesting that progenitor cells in bone marrow and in the periosteum are both regulated by EP1 gene deletion[21]. We characterized the cells by analyzing the cell surface markers Sca1 and CD105 in the PDMPCs isolated from WT and EP1$^{-/-}$ mice. We observed that a higher percentage of cells from the EP1$^{-/-}$ mice are Sca1+/CD105 negative. Moreover, we showed that treatment of WT mice with an EP-1 antagonist similarly resulted in an increase in the percentage of Sca1+/CD105- cells. PDMPCs isolated from EP1 antagonist treated mice also had enhanced *in vitro* osteoblast differentiation.

While markers specific for different stages of mesenchymal progenitor cell differentiation are not well defined[10, 12, 40], there are multiple studies demonstrating that progenitor cells that lose the expression of CD105 (also known as Endoglin) represents a subpopulation of multipotent stromal cells with increased osteogenic and/or chondrogenic differentiation capacities. Differentiation of umbilical cord MSCs into osteogenic and chondrogenic lineage was accompanied by reduction in CD105 expression[3, 6]. In adipose tissue, two separate studies showed that CD105^{-} cells have increased osteogenic potential. Moreover, down regulation of CD105 expression using shRNA against CD105 resulted in enhanced osteoblastic differentiation of adipose derived MSCs[32, 38]. A recent study showed that the loss of CD105 in tendon isolated stromal cells was associated with enhanced differentiation into the chondrogenic lineage[4]. Most importantly, CD105^{-} cells had increased *in vivo* repair capacity as demonstrated using the calvarial critical size model[38]. These collective studies suggest that CD105^{-} cells represent a subpopulation of progenitors with increased differentiation potential. In line with this, we observed that in *in vitro* assays periosteal CD105^{-} cells exhibit increased potential to form mineralized nodules and increased expression of osteoblastic markers compared to CD105^{+} cells. Considering that we did not observe differences in the nodule potential formation between WT CD105^{+} and EP1$^{-/-}$ CD105^{+} cells or between WT CD105^{-} cells and EP1$^{-/-}$ CD105^{-} cells, we can speculate that the enhanced differentiation of EP1$^{-/-}$ periosteal cells is due to the higher number of the CD105^{-} cells compared to WT periosteal cells.

Interestingly, when testing for the differentiation potential, we observed that EP1$^{-/-}$ PDMPCs had increased osteoblastic differentiation with slight but significant decrease in adipogenic differentiation potential. As the osteogenic and adipogenic differentiations have been shown to have reciprocal potential[13, 33], it is possible that in EP1$^{-/-}$ mice the enhanced differentiation into the osteoblastic lineage results concomitantly in decrease in the adipogenic differentiation. This potentially can be an important research area as part of the age-related changes in bone is increased differentiation into adipocytes instead of into osteoblasts, resulting in bone loss[30]. More studies need to be done to test the role of EP1 receptor in adipogenic differentiation.

Based on this observation that down regulation of EP1 increases the number of committed progenitors we tested whether blocking the EP1 receptor pharmacologically can be used as a drug target to enhance repair. The use of a specific EP1 antagonist in animal studies was previously described in other systems[1, 23, 50]. We used the established dosage in these

studies to treat wild type animals and analyze the repair process.[29, 41, 50] To direct the effect of the antagonist to the inflammatory stage, we inhibited EP1 receptor activity by EP1 specific antagonist on days 1–5 after fracture. This treatment strategy provides clues for understanding the role of EP1signaling during the early MSC proliferation and differentiation phases of fracture repair.

Our first observation was that following treatment with EP1 antagonist the PDMPCs change their phenotype, as indicated by an increase in the population of $CD105^{-}$ cells. We observed that after 5 days of treatment the percentage of the $CD105^{-}$ cells increased as well as the CFU-O numbers, suggesting that EP1 antagonist treatment promotes the number of committed progenitors at the periosteum, reaching similar percentage as in $EP1^{-/-}$ mice. It is worth noting that we did not observe any changes in flow cytometry analysis of $EP1^{-/-}$ mice following EP1 antagonist administration, suggesting that there was not an off-target effect. This increase in percentage of committed progenitors resulted in increased callus at day 10 after fracture and resulted in bigger bone area at day 21after fracture. The bone volume and the mineralization of the callus was significantly higher at the EP1 antagonist treated group. This supports our *in vitro* data that EP1receptor down regulation specifically promotes PDMPC differentiation into the osteoblastic lineage. Remarkably, the biomechanical properties of EP1 antagonist treated fractures were significantly increased compared to the vehicle treated animals.

The significance of the findings is enhanced by the fact that EP1 antagonist treatments are in Phase II clinical trials and show limited side effects[14, 35]. Moreover, efficacy has been demonstrated in the treatment of esophageal pain hypersensitivity in patients with gastric reflux disease, indicating that an EP1 receptor inhibition approach can be translated to clinical trials to improve fracture healing.[7,8] An added advantage of EP1 antagonist treatment is that inhibition of EP1 has been shown to reduce pain perception[42, 43, 58]. Currently, the anti-inflammatory drugs that are used as analgesics are COX2 inhibitors (NSAIDS), which block PGE2 production. However, since PGE2 has an bone anabolic effect mediated by the EP2 and EP4 receptors, NSAIDs used for analgesia can impair fracture healing[25, 34, 47]. Interestingly, studies suggest the reduction in pain following NSAID mediated by COX-2/PGE2 inhibition may in fact be secondary to decreased EP1 receptor activation[28, 31, 45]. Thus, pharmacologic agents that selectively inhibit EP1 may have the dual effect to of directly relieving pain and stimulating fracture healing.

Taken together, the studies presented here suggest that treatment with an EP1 antagonist at the first stages of the healing cascade alter the periosteal progenitor population, possibly by increasing the population of more osteoblastic progenitors. These initial changes in the cellular population result in an accelerated healing response, consistent with the healing response in $EP1^{-/-}$ mice. Moreover, the increased mechanical properties in the EP1 antagonist treated group suggest that the accelerated repair process results in functional bone, thus making the EP1 receptor a possible target to improve healing.

In summary, using a genetic model and a translational approach we demonstrated that EP1 is a negative regulator of bone formation, and therefore a novel therapeutic target for bone formation and regeneration. Future studies will determine if EP1 antagonism can stimulate

healing in animal models with compromised healing, such as aging and obesity. The ultimate goal is to establish an anabolic for EP1 antagonism in bone regeneration that can be translated to improve fracture healing in humans.

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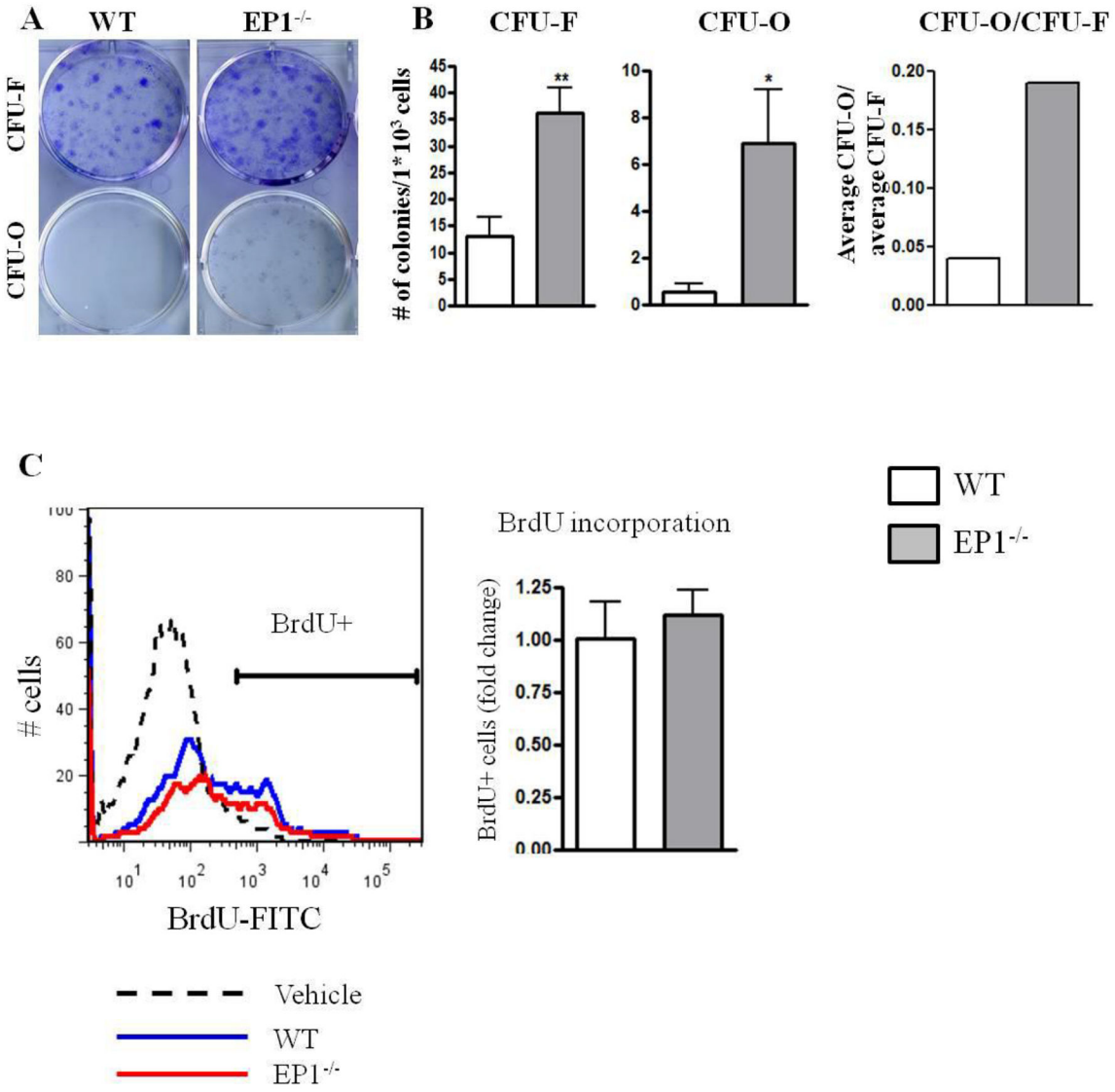

Figure 1: EP1$^{-/-}$ periosteal cells form more osteoblastic colonies.

Periosteal cells were isolated from WT or EP1$^{-/-}$ mice and plated at clonal density. Colonies were stained with Crystal Violet for CFU-F and Alkaline Phosphatase for CFU-O (A-B). Mice were injected with BrdU 2 hours before sacrificing. BrdU positive cells were measured using flow cytometry (C).

N=6 replicates per group for each assay. Error bars represent standard error of the mean. Statistical analysis was performed using paired student t-test. (*)=p<0.05 (**)=p<0.01 vs. age-matched WT.

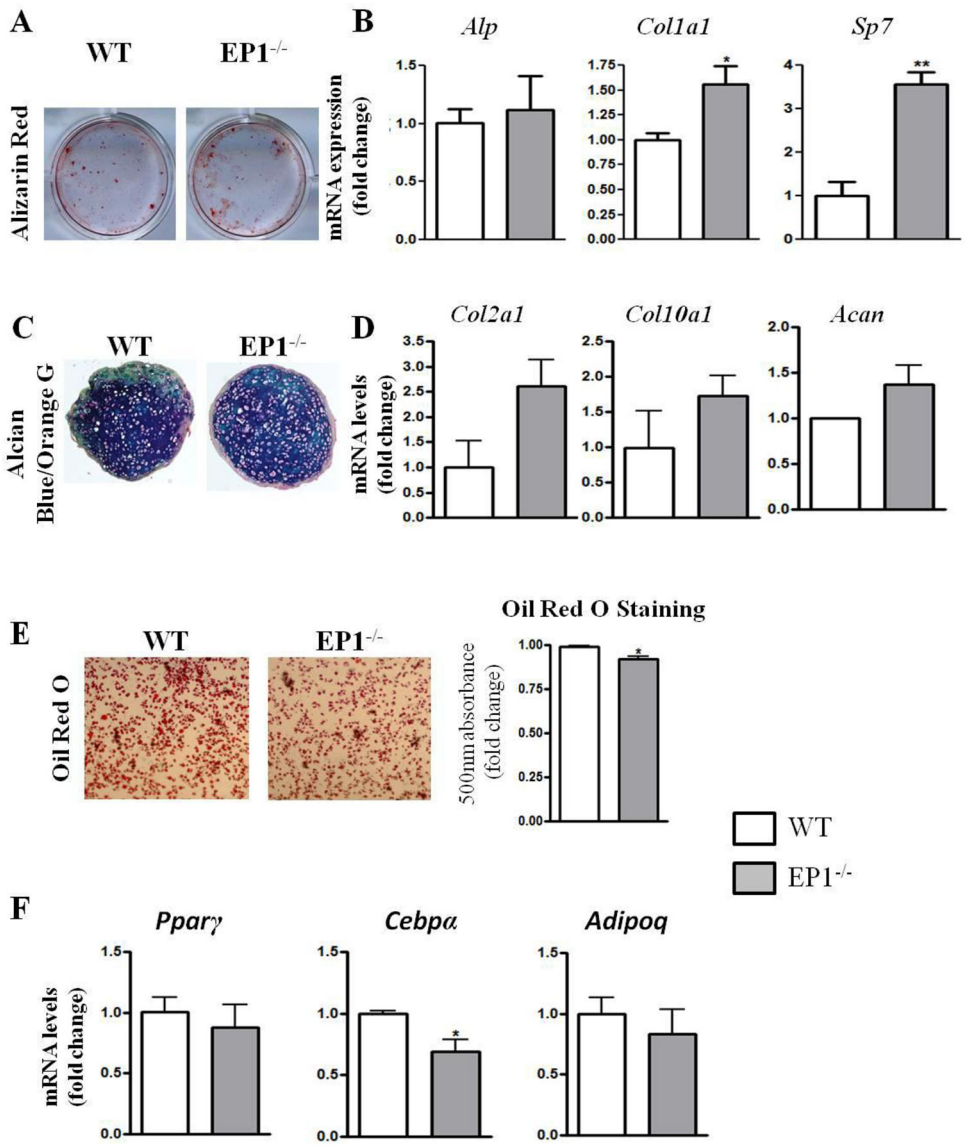

Figure 2: EP1$^{-/-}$ periosteal cells exhibit enhanced osteoblastic differentiation.

Alizarin Red (A) staining and osteoblastic gene expression (B) following 10 days of induction of osteoblastic medium. Cell pellets sections and stained with Alcian Blue (C) and gene expression analysis for chondrogenic genes (D). Oil Red O staining (E) and adipogenic gene expression (F) following 6 days of induction with adipogenic medium.

N=5 replicates per group for each assay. Error bars represent standard error of the mean. Statistical analysis was performed using paired student t-test. (*)=$p<0.05$ (**)=$p<0.01$ vs. age-matched WT.

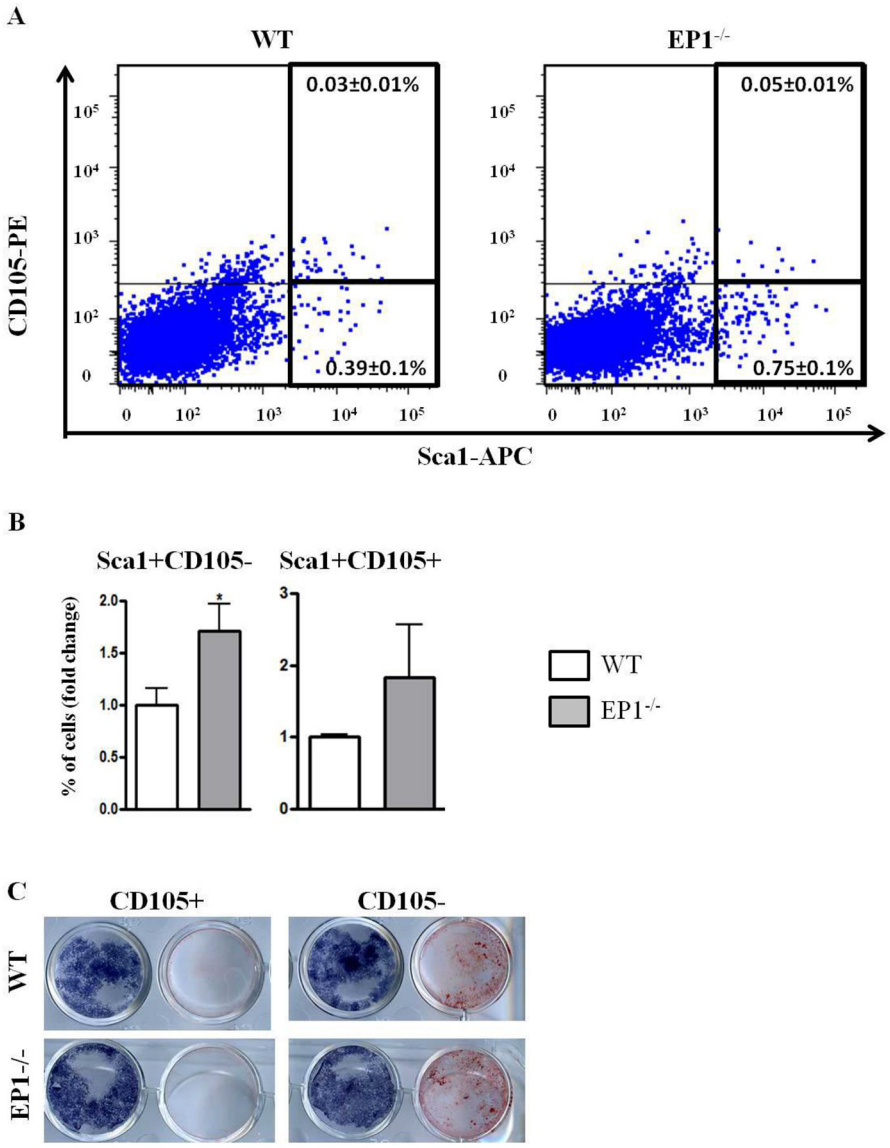

Figure 3: EP1$^{-/-}$ periosteum contains more CD105^{-} progenitors.

A-B, freshly isolated periosteal cells were stained with antibodies against: CD45-PrcP, CD31-PEcy7, CD105-PE, Sca1-APC (A-B). The cells were selected for CD45 and CD31 negative cells and the percentage of Sca1 + and CD105+ cells were analyzed. (C), Sca1^{+}CD105^{+} and Sca1^{+}CD105^{-} cells were sorted and cultured with osteogenic medium for 10 days. In the paired culture wells shown in the figure, the well on the left is stained for alkaline phosphatase and the well on the right is stained with alizarin red to detect mineralization in the cultures.

N=5 replicates per group for each assay. Error bars represent standard error of the mean. Statistical analysis was performed using paired student t-test. (*)=p<0.05 (**)=p<0.01 vs. age-matched WT.

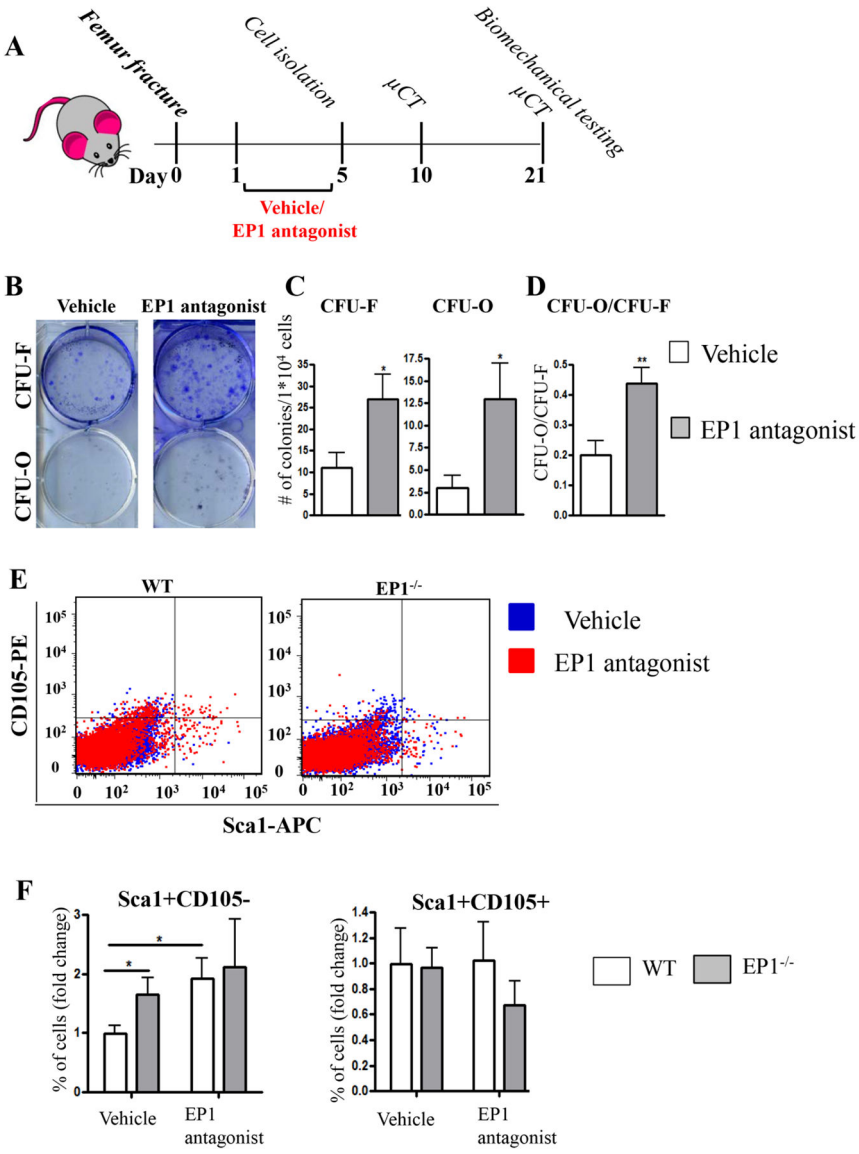

Figure 4: EP1 antagonist administration increase the number of committed progenitors at the periosteum.

(A) Schematic describing experimental design. (B-D) CFU assays on primary isolated periosteal cells. Colonies were stained with Crystal Violet for CFU-F and Alkaline Phosphatase for CFU-O. (E-F) freshly isolated periosteal cells were stained with antibodies against: CD45-PrcP, CD31-PEcy7, CD105-PE, Sca1-APC. The cells were selected for CD45 and CD31 negative cells and the percentage of Sca1 + and CD105+ cells were analyzed. N=5–6 replicates per group for each assay. Error bars represent standard error of the mean. Statistical analysis was performed using paired student t-test. (*)=p<0.05 (**)=p<0.01 vs. age-matched vehicle treated animals.

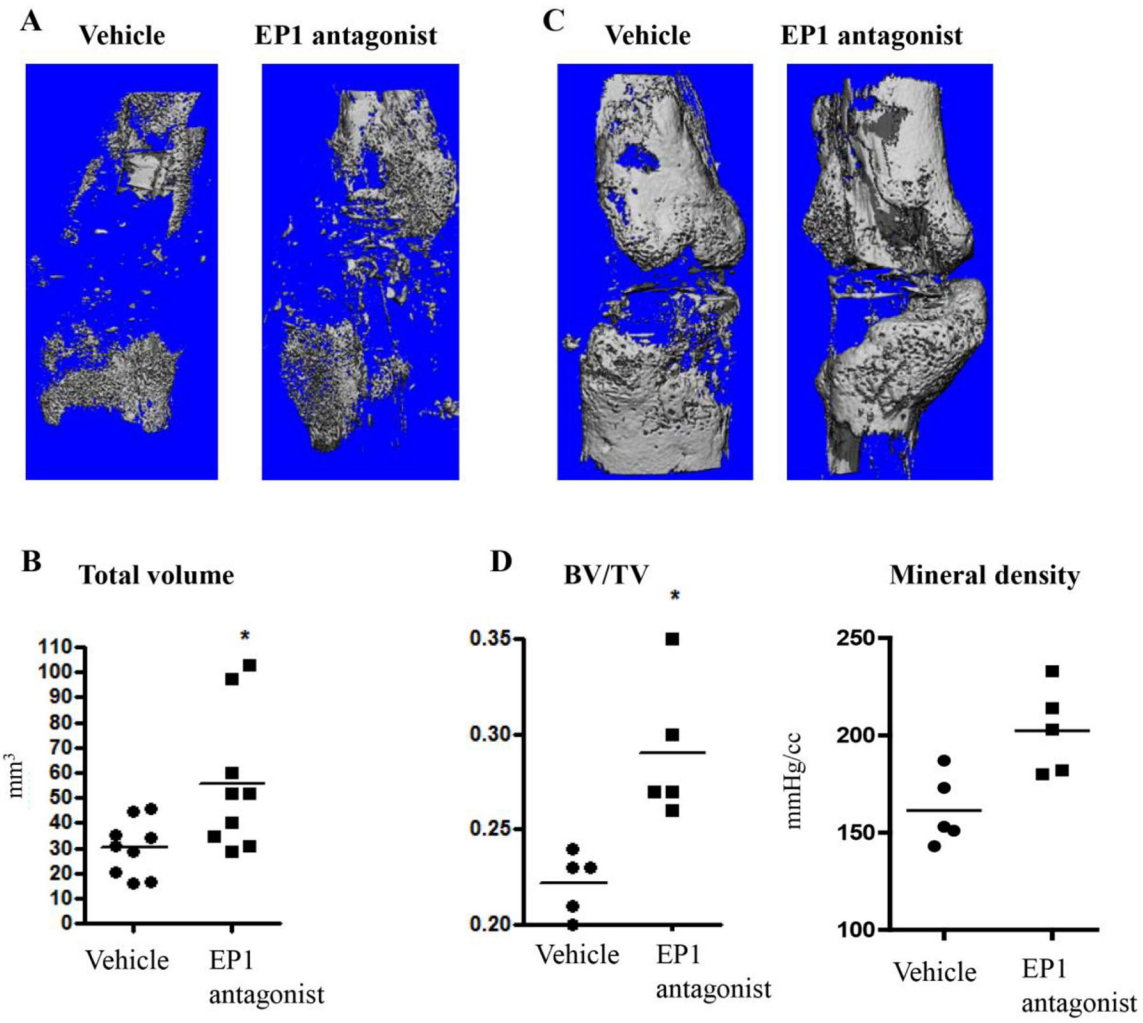

Figure 5: EP1 antagonist administration alters fractured femurs properties.

Micro-CT reconstructions of fractured femurs at day 10 (A and B) and day 21 (C and D). Representative images showing callus formation in vehicle treated mice and in EP1 antagonist treated mice at day 10 (A) and day 21 (C). (B) Day 10 BV/TV and mineralized density as determined by Micro-CT (B). Total bone volume at day 21 after fracture as measured by Micro-CT (D).

Day 21 has N=5 per group and Day 10 has 9 mice per group. Error bars represent standard error of the mean. Statistical analysis was performed using paired student t-test. (*)=$p<0.05$ vs. age-matched vehicle treated animals.

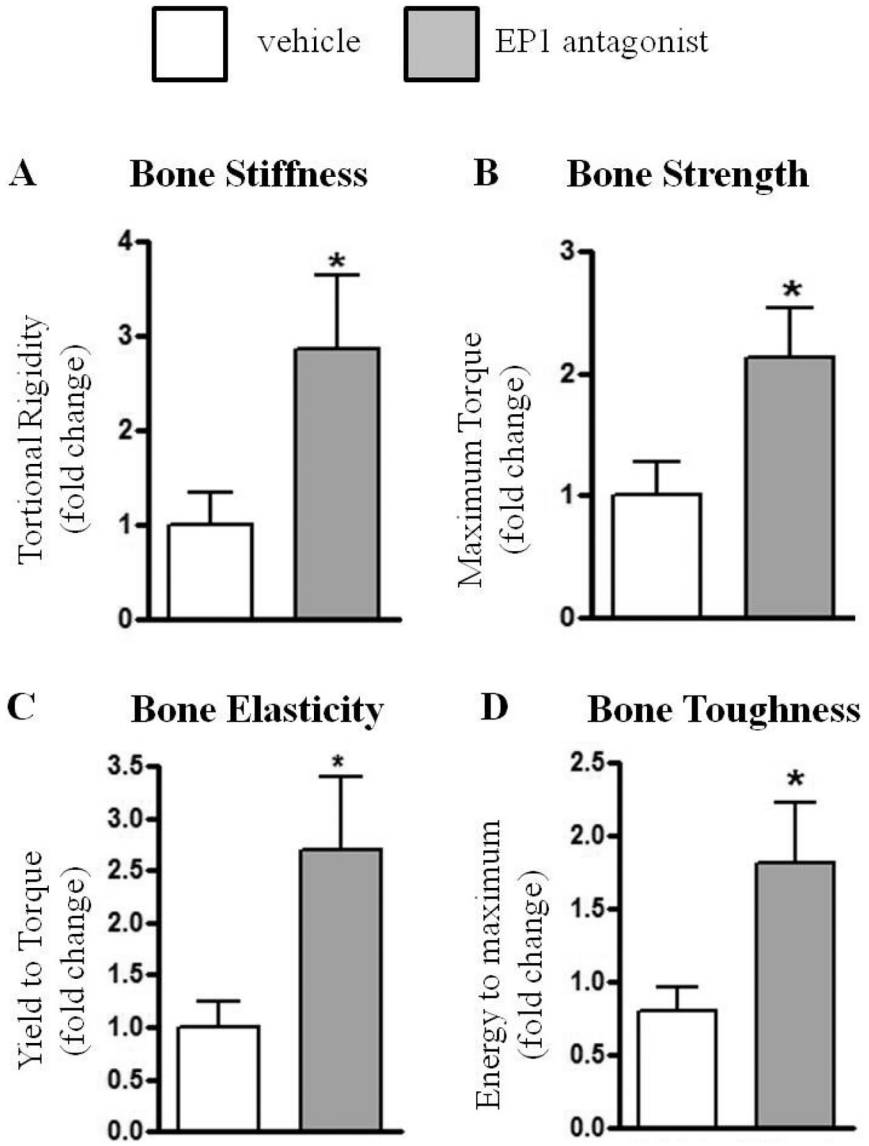

Figure 6: Femur fractures in EP1 antagonist treated mice heal with increase mechanical strength:

Biomechanical testing on day 21 after fracture. Mice were with either Vehicle or EP1 antagonist for 5 days, starting on post fracture day 1. The experiment is presented as fold differences between paired EP1 antagonist treated mice and Vehicle treated mice. Altogether an N=9 animals per group was examined in two separate experimental cohorts that were combined. Statistical analysis was performed using paired student t-test. (*)=$p<0.05$ vs. age-matched vehicle treated animals. The mean values of the biomechanical measurements for the combined groups was Torsional rigidity (Vehicle = 125.3 ± 52.92 [N.mm/(rad/mm)]; EP1 antagonist = 261.1 ± 63.7 [N.mm/(rad/mm)]); Maximum torque (Vehicle = 6.46 ± 1.98 (N.mm); EP1 antagonist = 10.62 ± 1.16 (N.mm)); Yield to torque (Vehicle = 5.17 ± 1.67 (N.mm); EP1 antagonist = 8.2 ± 0.8 (N.mm)); and Energy to maximum (Vehicle = 0.35 ± 0.068 (N.mm*(rad/mm) vehicle; EP1 Antagonist = 0.8 ± 0.19 [N.mm*(rad/mm)]).