IGF1 Signaling Play a Critical Role in Fracture Repair and Bone Homeostasis by Regulating Osteogenic Differentiation of Bone Lining Cells through CXCL12 Expression

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ABSTRACT

The type 1 insulin-like growth factor ligand and receptor (IGF1/IGF1r) both have key anabolic roles in postnatal skeletal development. Because IGF1/IGF1r knockouts (KOs) mice die either early postnatally or prenatally, studying their downstream mediators has been difficult. Although periosteal/endosteal cells play critical roles in bone development and regeneration their nature still remain elusive and whether IGF1r signaling is involved in their function has never been investigated. We have previously reported that in bone regeneration, the regulation of the expression of C-X-C-motif-chemokine-ligand-12 (CXCL12) determines the fate of a population of CXCL12+,NG2+,αSMA+ endosteal/perivascular cells either to osteogenic differentiation or to a supportive role in angiogenesis (1). To test the hypothesis that IGF1r signaling regulates CXCL12's expression to maintain bone homeostasis and promote fracture healing, we performed in-vitro and in-vivo studies of IGF1rKO. Our results reiterate that IGF1r is essential to maintain bone homeostasis and uncover a new function of IGF1 signaling in regulating the expression of CXCL12 in bone lining cells, critical to promote fracture repair. µCT and histological analyses are in progress.

INTRODUCTION

Insulin-like growth factor 1 (IGF1) is an important and unique peptide that functions via endocrine/paracrine and autocrine mechanisms across a wide variety of tissues. Bone and all cell lineages in the skeleton have been shown that require IGF1 for normal development and function and, also, to respond to IGF1 via the IGF-1 receptor (IGF1r). During cartilage and bone development, blunting the expression of IGF1, IGF2, or IGF1r, globally or specifically in osteoblasts, chondrocytes, and osteoclasts, results in abnormalities in mineralization, ossification, bone resorption, and/or precursor cell proliferation and growth. The novel role of IGF1 in regulating metabolic demands of the bone remodeling unit is currently under investigation. Notably, there is an increasing volume of *in vivo* and *in vitro* data suggesting that periosteal and endosteal cells have a critical role in skeletal development. Although periosteal/endosteal cells play critical roles in bone development and regeneration their nature still remain elusive and whether IGF1r signaling is involved in their function has never been investigated. We have previously reported that in bone regeneration, the regulation of the expression of C-X-C-motif-chemokine-ligand-12 (CXCL12) determines the fate of a population of CXCL12+, NG2+, αSMA+ endosteal/perivascular cells either to osteogenic differentiation or to a supportive role in angiogenesis; we demonstrated that the endosteal cells derived from Prx1-positive OPCs are essential for proper skeletal development and uncover a new function of IGF1 signaling in regulating the expression of CXCL12 in bone lining cells.

METHODS

Mice : Prx1-Cre:IGF1rKO (IGF1rCKO) mutant mice were generated by crossing transgenic Prx1-Cre+ mice with IGF1r lox/lox mice. Cre-negative littermates were used as controls. Prx1-GFP-CreER:IGF1rKO (IGF1rICKO) mutant mice were generated by crossing transgenic Prx1-CreER+ mice with IGF1r lox/lox mice. CreER-negative littermates were used as controls.

Fracture : We performed 4OHTAM administration 3 days before and 2 days after fracture in 10-12 week-old IGF1rICKO mutant mice. Calluses from IGF1rICKO and their control littermates were collected at different post-fracture days (PFDs) for mRNA analyses. Analysis of relative gene expression was made using mouse GAPDH as housekeeping genes and data are reported as mean ± SE of triplicate repeats from 3 separate samples normalized to control. *p<0.05 compared to control by unpaired two-tailed t-test. Stabilized fracture of right tibias was done as we previously reported(2).

Isolation of Endosteal cells : Endosteal cells were isolated according to previously published protocols (3-4). Briefly, femurs and tibias from IGF1rCKO mice were cleaned of muscle and connective tissue then digested 5 consecutive times in 0.125% trypsin and 0.1% collagenase for 30minutes each. Bone marrow was then flushed and the bones fragmented before another digest in 0.1% collagenase. This fraction of endosteal cells (EC) was plated and expanded in culture for three passages and then used in the following experiments. A second digest with 0.1% collagenase was then used to isolate endosteal osteoblasts (Obs) which were likewise grown for three passages and used for profiling comparisons with endosteal cells. Osteoblastic differentiation was carried out using StemXVivo osteogenic base media and supplement.

Treatment with inhibitor (AMD3100) : For in vivo treatment, mice were i.p.-injected with 2.5 mg/g AMD3100-PBS solution twice per day 2 days once prior to fracture and then six times beginning 2 days after fracture (day 2 through day 7) followed by harvest at 14 days postfracture. For in vitro treatment of confluent endosteal cells, beginning on day 7, 400 mM of AMD3100 dissolved in water was added to the cells every 3 days until day 14 when cells were harvested.

Alizarin-Red staining : Mineralization was determined by staining with a 2% solution of Alizarin red for 15 min following fixation in formalin.

Staining and Immunofluorescence : For post-mortem studies, tibiae from at 8 weeks old mice were harvested and fixed in 4% PFA. For histology, samples were decalcified, embedded in paraffin, sectioned, and stained for H&E. Frozen sections of a right tibias of adults female IGF1rCKO mice 8 weeks-old, were subjected at immunofluorescence for CXCL12, CXCR4 and PECAM.

All procedures were approved by the institutional IACUC of Rush University Medical Center (Chicago, IL, USA).

RESULTS

In IGF1rCKO mice, we found impaired bone formation and abnormal cortical bone neo-angiogenesis, associated with an increase of bone lining CXCL12+/CXCR4+ cells surrounding endothelial PECAM+ cells (Fig. 1-2). To evaluate the role of IGF1/CXCL12 interplay in fracture repair, we generated inducible IGF1rICKO mice. To this purpose, adult mice received 4OHTam 3 days before and 2 days post fracture (PFD). Calluses from IGF1rICKO and controls were collected and analyzed at different PFDs. µCT analyses at PDF14 showed that IGF1rICKO mice have reduced total callus, soft tissue and new bone volumes compared to controls (Fig.3). mRNA expression analyses of IGF1rICKO PDF14 calluses showed an increase of CXCL12, CXCR4, PECAM and pericytes markers, as well as a decrease of osteo-chondrogenic markers, compared to controls (Fig.3). To further evaluate the IGF1r-CXCL12 axis, we isolated endosteal cells from the hind-limb long bones of IGF1rCKO mice and controls. IGF1rCKO endosteal cells showed an increase in pericyte markers, and a decrease in osteogenic markers and Alizarin-Red (AR) staining. Furthermore, in IGF1rCKO endosteal cells CXCL12, CXCR4 and PECAM mRNA expressions were respectively 5-, 2- and 4-fold higher than controls (Fig.4). Next, we treated IGF1rCKO endosteal cells with AMD3100, an inhibitor of CXCL12 signaling. AMD3100 rescued the osteogenic differentiation defect of IGF1rCKO endosteal cells, as shown by an increase in osteogenic marker and AR-staining (Fig. 4). We then evaluated the effect of AMD3100 *in vivo*. IGF1rICKO mice and controls were treated with AMD3100 (2.5 µg/g by intraperitoneal injection given twice a day from PFD2 through PFD7). Analyses of calluses obtained respectively at PDF14, showed that in IGF1rICKO, AMD3100 treatment normalized mRNA levels of CXCL12, CXCR4, PECAM, pericyte and osteochondrogenic markers (Fig.5).

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Figure 1 : Phenotype of IGF1rCKO mice

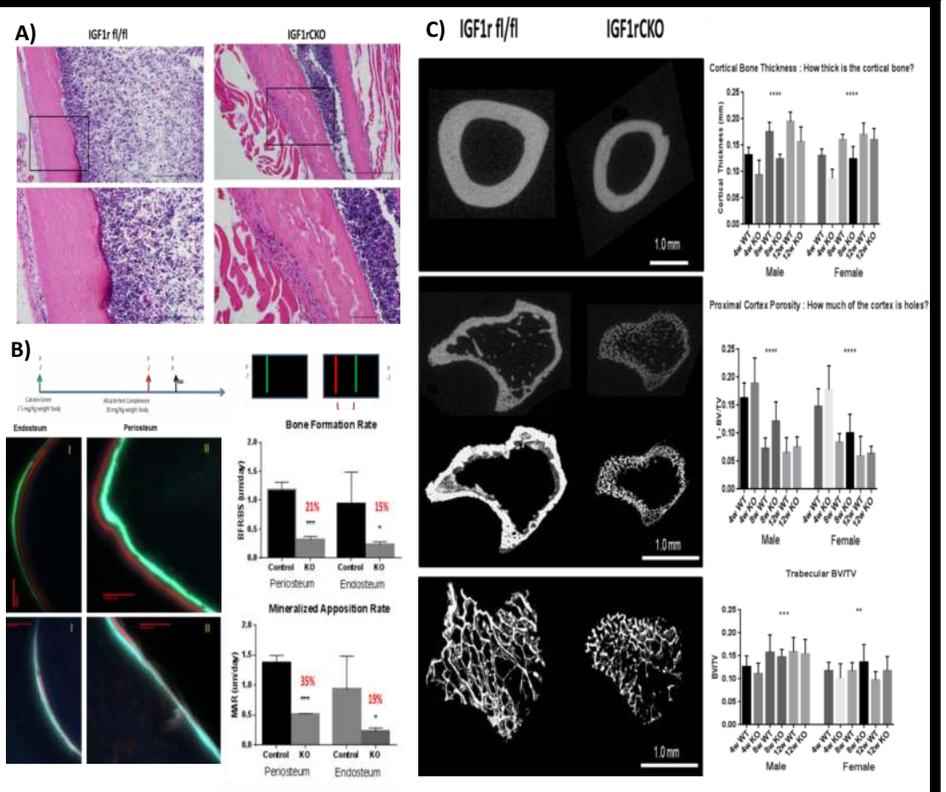

Fig 1. Phenotype of IGF1rCKO. A) Histological analysis: for post-mortem studies, tibiae from 8 weeks female mice were harvested, fixed in 4% PFA, decalcified, embedded in paraffin, sectioned, and stained for H&E. IGF1rCKO mice had a thicker, hypercellular periosteum and porous cortical bone compared to controls. B) Bone histomorphometry analysis: we evaluated skeletal growth by labeling the developing bone front with injections of calcein (day -7) and alizarin red (day -2). After 2 days from last injection, we harvested and fixed tibiae in 70% ethanol. At 8 weeks, female IGF1rKO mice had decreased BFR and MAR compared to controls. (N=3; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). C) mCT analysis: fixed tibiae were evaluated by mCT at 4W, 8W and 12W in 70% ethanol. At all timepoints, IGF1rCKO mice were skeletally deficient compared to controls: they showed a reduced tibial length, a reduced cortical area and thickness in the cortical midshaft, a reduced bone volume fraction and presence of pores in the trabecular bone region.

Figure 2 : IGF1rCKO endosteal cells are CXCL12+/CXCR4+ surrounding PECAM+ cells

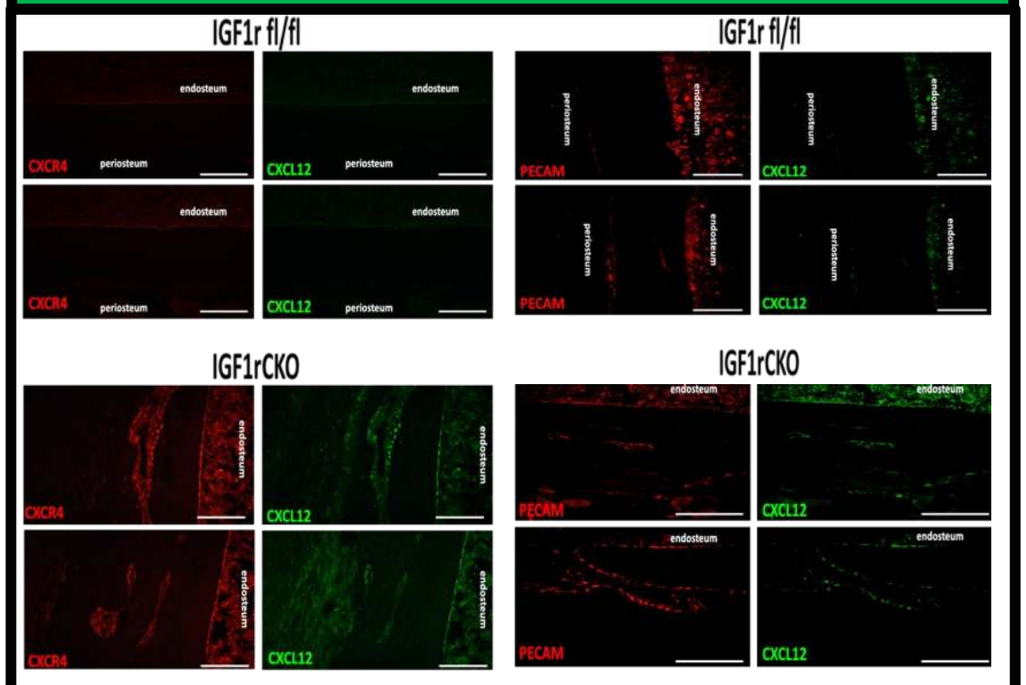

Fig 2. IGF1rCKO endosteal cells are CXCL12+/CXCR4+ surrounding PECAM+ cells. Co-immunostaining for CXCL12 and CXCR4 of adjacent frozen sections of a right tibias of an adult female Prx1-Cre:IGF1rKO mice 8 weeks-old, as well as CXCL12 and PECAM. Scale bars = 100 mm, 500mm.

Figure 3 : Fractured IGF1rICKO mice

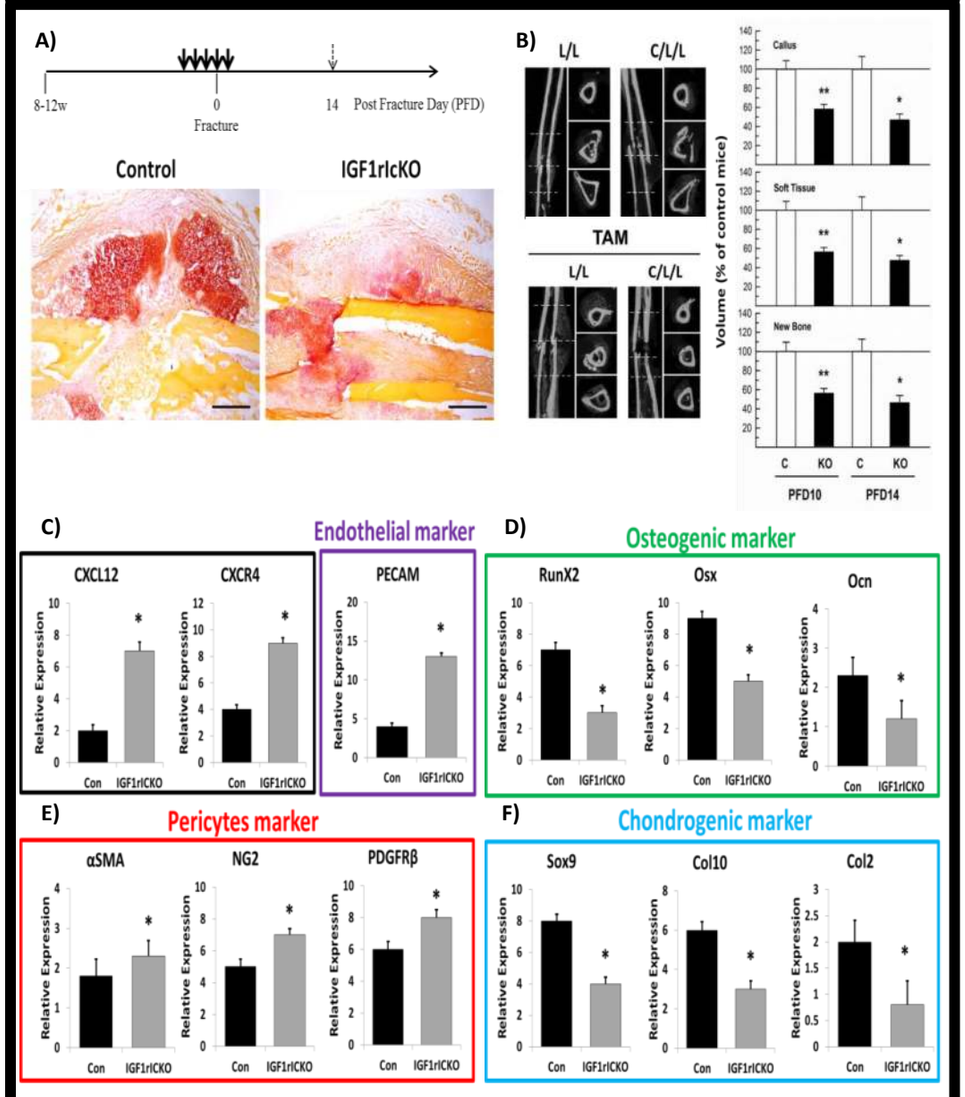

Fig 3. Tibia callus in an IGF1rICKO mouse and its littermate controls at PFD10 and PFD14. A) Saf.O/Orange G staining of right tibias of TAM-injected IGF1rICKO mice and controls at PFD14. B) At PFD10 and PFD14, right tibias of TAM-injected IGF1rICKO double mutant (C/L/L) mice and controls (L/L), as well as their non-injected counterparts, were subjected to µCT analysis. **Note** that the mice receiving TAM treatment exhibited a larger callus, compared to their non-treated counterpart, and that the IGF1rICKO mouse, i.e., C/L/L mutant mouse receiving TAM, had much smaller callus. Thus, only TAM-treated control mice are suitable to serve as experimental control for IGF1rICKO mice. A line is drawn at the control level to facilitate comparison. Data represent mean ± SD of 3 - 6 samples. *, $p < 0.05$; **, $p < 0.01$; compared to their respective controls.
C) mRNA expression analysis from fractured IGF1rICKO mice and their control littermates showed increased CXCL12 and CXCR4 expression, as well as CD31 and D) pericytes marker, and decreased E) osteo- and F) chondrogenic marker expression. Data represent mean ± SD of 4 samples. *, $p < 0.05$; t-test; compared to their respective controls.

Figure 4 : In vitro treatment of isolated endosteal cells from IGF1rCKO mice with AMD3100

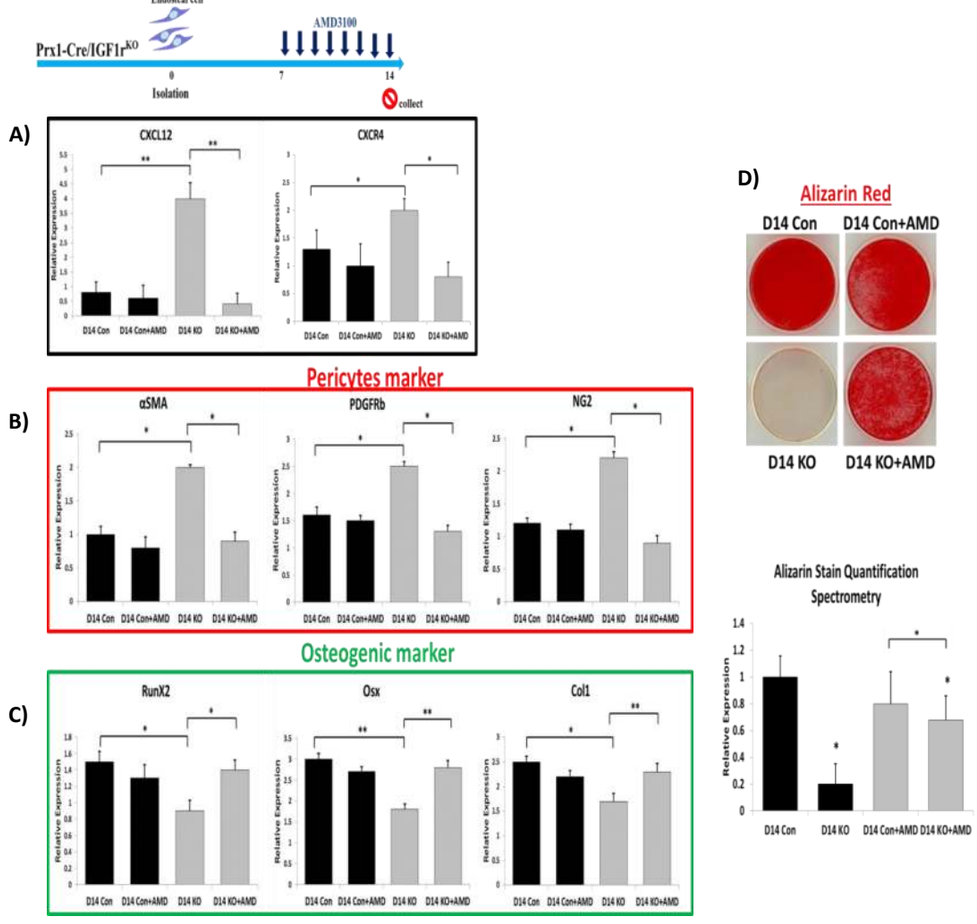

Fig 4. Gene expression analysis of isolated endosteal cells from IGF1rCKO mouse before and after AMD3100 treatment. A) mRNA expression analysis of isolated endosteal cells from IGF1rCKO mice and their control showed increased CXCL12 and CXCR4 expression, as well as B) pericytes marker, and decreased C) osteogenic marker expression. AMD3100 rescued the osteogenic differentiation defect of IGF1rCKO endosteal cells, as shown by an increase in osteogenic marker and D) AR-staining. Data represent mean ± SD of 3 - 6 samples. *, $p < 0.05$; **, $p < 0.01$; compared to their respective controls (t-test).

Figure 5 : In vivo treatment of fractured IGF1rICKO mice with AMD3100

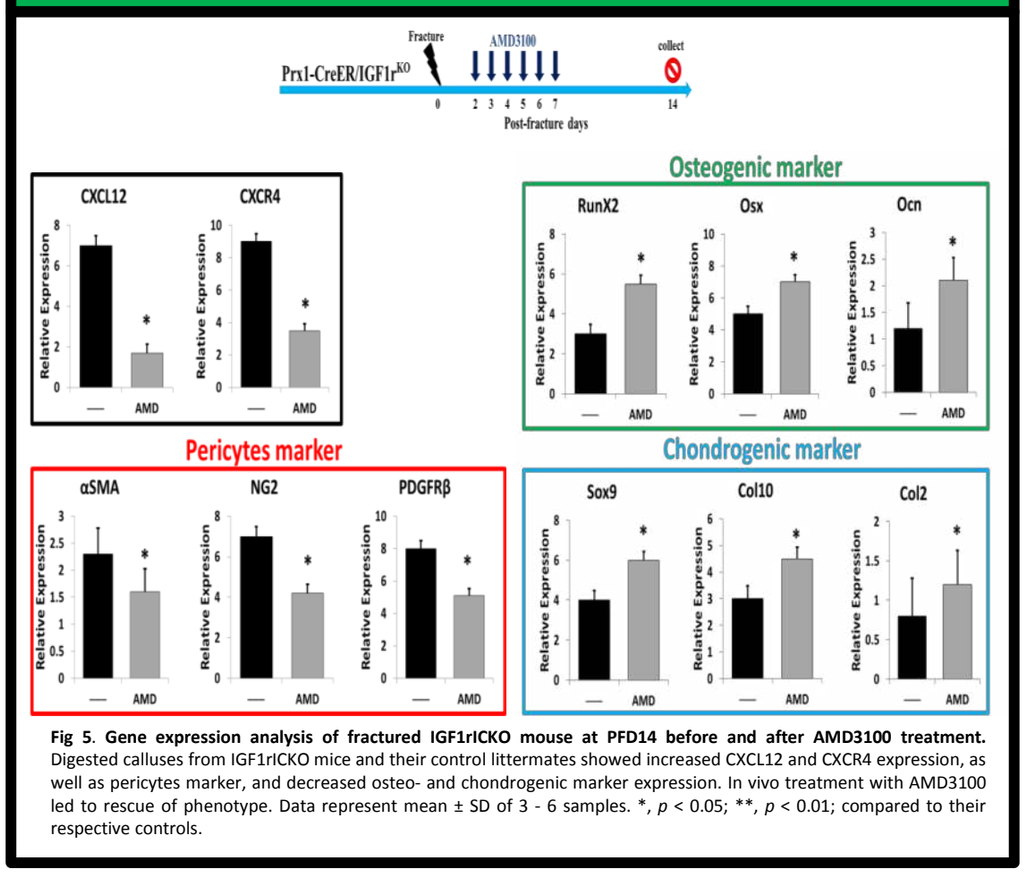

Fig 5. Gene expression analysis of fractured IGF1rICKO mouse at PFD14 before and after AMD3100 treatment. Digested calluses from IGF1rICKO mice and their control littermates showed increased CXCL12 and CXCR4 expression, as well as pericytes marker, and decreased osteo- and chondrogenic marker expression. In vivo treatment with AMD3100 led to rescue of phenotype. Data represent mean ± SD of 3 - 6 samples. *, $p < 0.05$; **, $p < 0.01$; compared to their respective controls.

CONCLUSIONS

Our findings unveil a novel role of IGF1 signaling in regulating CXCL12 expression in bone lining cells that is critical to promote fracture healing.

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