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IL-17A and the Receptor are Regulated by Runx3 during Fracture Healing

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This thesis is dedicated to my parents, who gifted me with life and love, and to my treasured companion in the USA, Inu, who steadfastly supported me throughout my time in Connecticut.

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ABSTRACT

Enhancing bone repair is the key process which facilitates the improvement of fracture healing outcomes. Despite of many studies regarding osteogenesis induction, Bone morphogenetic proteins-2 (BMP-2) and Platelet-derived growth factor-BB (PDGF-BB) are still the only two the Food and Drug Administration (FDA)-approved treatment[1, 2]. To develop potential treatment modalities, it is essential to understand the role of transcription factors in fracture healing and how they may be specifically targeted in preclinical models to enhance bone regeneration. While the Runt-related transcription factor 2 (Runx2) is known to play a crucial role in osteoblastogenesis, the role of Runx3, a homologue of Runx2, in osteoblastogenesis remains unclear. Our laboratory has previously reported that targeted deletion of the Runx3, in the periosteum resulted in enhanced fracture healing by increased bone formation and biomechanical properties of the fracture callus in mutant mice[3]. Other factors participate in fracture healing including inflammatory cytokines. They are known to be involved in fracture healing by initiating the repair process following injury. Recent studies demonstrated that one of the most important inflammatory cytokines controlling fracture healing was Interleukin 17 (IL-17)[4]. It was previously published that IL-17 was expressed by mesenchyme cells, periosteal cells, and chondrocytes within the fracture calluses of wild type mice[5]. The goal of this study was to investigate the mechanism of how Runx3 regulated fracture healing and the role of IL-17 in this process for future translation into a preclinical model of fracture healing. The hypothesis was that conditional knockout of Runx3 in the periosteum would enhance osteogenesis through upregulation of IL-17 signaling. To investigate this hypothesis, Runx3 expression was conditionally abrogated in limb mesenchymal

progenitors. Paired related homeobox 1 (Prx1)-Cre Runx3 $^{F/F}$ mice, which lacked Runx3 activity were compared to control mice (Runx3 $^{F/+}$) with 100% Runx3 activity. Mid-diaphyseal fracture surgery was performed on 12 week-old female mice, and the mice were then euthanized at 7, 14, and 21 days post-fracture followed by harvesting femurs for molecular and histology analysis. The absence of Runx3, IL-17A and IL-17RA were confirmed by RT-PCR. The localized expression of IL-17A and the receptor IL-17RA was assessed by immunohistochemistry to identify which cells were responsible for the changes in expression.

INTRODUCTION

Fracture healing is a complex process which involves many types of cells to regenerate new bone. To improve the fracture healing, osteogenesis is the key process and seminal papers have been published regarding this process over the years[6]. Osteogenesis occurs through two types of ossification: intramembranous ossification and endochondral ossification. Intramembranous ossification occurs mostly in the flat bones of the skull forming mesenchymal stem cell differentiation directly to osteoblasts, while endochondral ossification process occurs when mesenchymal stem cells differentiate first into cartilage following by a subsequent process of bone replacement[6, 7]. To explore capable modalities in order to accelerate bone regeneration is essentially critical for fracture healing. There are only two the Food and Drug Administration (FDA)-approved orthobiologic treatment modalities to induce osteogenesis: Bone morphogenetic proteins (BMPs) and Platelet-derived growth factor-BB (PDGF-BB).

BMPs induce mesenchymal stem cells to differentiate into osteoblasts to form bone in targeted tissues[8]. In 2002, the BMP-2 Evaluation in Surgery for Tibial Trauma Study Group (BESTT) published the results from a 450 patient global clinical study showing that rhBMP-2 loaded collagen sponges greatly benefited patients undergoing severe, open tibial fracture repair surgeries with intramedullary nail fixation[9]. This result led to the FDA's approval of Medtronic's INFUSE®. However, the efficacy remains unclear[1, 10].

PDGF-BB is also known to stimulate osteoblast or osteoprogenitor cell activity in favor of fracture healing[11]. However, many groups have raised concerns regarding the efficacy of PDGF-BB resulting in conflicting reports[2].

Treatments to accelerate bone regeneration are critical not only in long bone fracture healing, but also in craniofacial skeleton such as congenital and acquired facial bone abnormality and traumatic bone fracture. Currently BMP-2 has been used for sinus lift and alveolar ridge augmentation and PDGF-BB has been used for periodontal disease and bone graft for the implant treatment.

While establishing the molecular cues that govern fracture callus formation and remodeling in our lab, our laboratory previously reported that targeted deletion of the Runx3 (Runt Related Transcription Factor 3), a homologue of the osteogenic master regulator Runx2, in the periosteum resulted in enhanced fracture healing[3]. Runx3 is a transcription factor, known to be related to cancer suppression due to reduction of the angiogenesis and also found in growth plate during endochondral bone formation[12]. Runx3 is a well-established key regulator of chondrogenesis[13, 14]. While Runx2 plays a crucial role in osteoblastogenesis[15, 16], the mechanism of Runx3 in osteoblastogenesis remains unclear. Our laboratory's study indicated that the enhanced bone healing due to Runx3 deletion was due to increasing bone formation and biomechanical properties of the fracture callus in mutant mice.

Moreover, our laboratory found that the Runx3 deletion in the periosteal cell populations induced angiogenic as well as osteogenic differentiation of progenitor cells within the healing calluses. This was evidenced by increasing vessel number as well as increased angiogenic tube formation using cells harvested from fractured limbs of Runx3 conditional knockout mice compared to control littermates. Angiogenesis is essential for fracture healing to make blood supply from existing blood vessels. Additionally, colony forming unit formation was also increased when Runx3 conditional

knockout-derived periosteal cells were cultured in osteogenic media, comparing to cells harvested from control mice.

Inflammatory cytokines are known to be involved in fracture healing by initiating the repair process following injury. They are immediately secreted after injury at the injury site by macrophages, inflammatory cells, and mesenchymal origin cells[5]. Interleukin 17 (IL-17) is a family of cytokines composed of six members[17, 18]. IL-17A was cloned in a T cell hybridoma as the first member of this new class of cytokines. Other isoforms homologous to IL-17A include IL-17B, C, D, E and F[5].

IL-17C, D, and E were mainly expressed by lymphocytes such as T cells and Th17 cells. However, IL-17A, B and IL-17F are expressed by progenitor cells in fracture callus. Recent studies demonstrated that IL-17A was highly induced immediately after bone injury and promoted bone regeneration by accelerating osteogenesis via its effects on injury-associated mesenchymal cells[4]. Kokubu and his colleague reported that IL-17B was localized in chondrocytes of both the growth plate and the fracture callus in rat with closed fracture[19]. Additionally, IL-17B was expressed exclusively in the inflammatory cartilage and neutralization of IL-17B significantly suppressed the progression of arthritis and bone destruction in arthritis mice[20]. Despite the fact that both IL-17A and F bind to a receptor subunit IL-17RA[17], the IL-17F binds with relatively low affinity[21] suggesting that IL-17A is the more important interaction.

The aim of this study is to investigate the underlying mechanisms of Runx3 control of fracture healing, and to see whether IL-17 signaling takes in part in the fracture healing and which cells are participating the IL-17 signaling pathway. To test this, I examined expression of IL-17A and the receptor IL-17RA expression in the

periosteal cells from conditional knockout of Runx3 mouse to demonstrate whether specific cells express high levels of IL-17A to recruit progenitor cells into osteogenic lineage resulting in enhancing osteogenesis.

OBJECTIVES

The objective of this study was to test whether specific cells express high levels of IL-17A and IL-17RA as part of the underlying mechanisms of Runx3 regulation of fracture healing.

SPECIFIC AIMS

The specific aims of this study were:

1. To examine the expression of IL-17A and the receptor IL-17RA via quantitative RT-PCR (qRT-PCR) from fractured limbs of Runx3 conditional knockout and control littermates at various stages of the healing process.

2. To identify which cells from fractured limbs of Runx3 conditional knockout and control littermates at various stages of the healing process express IL-17A and the receptor IL-17RA using immunohistochemistry (IHC).

HYPOTHESIS

The hypothesis is that conditional knockout of Runx3 in the periosteum enhanced osteogenesis through upregulation of IL-17 signaling.

MATERIALS AND METHODS

1. Materials

1.1 Animals

Runx3 floxed mice (Runx3 $^{F/F}$) were crossed with Prx1-Cre transgenic mice to generate mice in which Runx3 expression is conditionally abrogated in limb mesenchymal progenitors (Prx1-Cre Runx3 $^{F/F}$). These were compared with control mice (Runx3 $^{F/+}$) with 100% Runx3 activity. Twelve-week old female mice were divided into groups and a standard mid-diaphyseal femoral fracture surgery was performed on mutant and control mice as described previously[22]. Following the surgery, the mice were euthanized at 7, 14, and 21 days post-fracture and femurs were harvested for molecular and histology analysis.

1.2 Cells

Six week-old female mice were sacrificed to harvest fractured and intact femurs 3 days after the fracture surgery (N=3 per group). Soft tissue and muscle were cleaned out and ablate to flush out the bone marrow cells. The periosteal cells were scraped from the bone under sterile conditions and cultured in hypoxia for 1 week. Cells were re-plated into 12-well plates and osteogenic media was added after 24 hours at 37^{o}C, 5% CO_2 until day 7, 14 and 21 for molecular analysis.

1.3 Antibodies

Rabbit anti-mouse polyclonal IL-17 (ab91649) and rabbit anti-mouse polyclonal IL-17A receptor (ab180904) were purchased from Abcam.

2. Methods

2-1 Molecular analysis of the callus

Total RNA was isolated from whole calluses *(in vivo)* and periosteal cells *(in vitro)* from conditional knockout mice and control littermates (N=3 per group for whole calluses, N=5 per group for periosteal cells) at day 7, 14, and 21 with TRIzol following the manufacturer's protocol (Invitrogen). Briefly, for whole calluses, the soft tissue was removed from femurs and bone marrow was ablated. Surgical tools were used to isolate the callus from the rest of the femur. Only the callus was flash frozen in liquid nitrogen, and subsequently homogenized in TRizol. For the intact control, the whole femur was used. After DNase I (Invitrogen) treatment, cDNAs were synthesized by iScript select cDNA kit following the manufacturer's protocol (Bio-Rad). The qRT-PCR was performed using SYBR Green Master mix (Applied Biosciences) and StepOnePlus real time PCR system following the manufacturer's protocol (Applied Biosciences). The specific primers used were: mouse IL-17A (forward: 5'- GCT CCA GAA GGC CCT CAG ACT -3', reverse: 5'- CCA GCT TTC CCT CCG CAT TGA -3')[23] and IL-17 RA (forward: 5'- AGT GTT TCC TCT ACC CAG CAC -3', reverse: 5'- GAA AAC CGC CAC CGC TTA C -3')[24]. The estimated amount of the gene of interest was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2-2 Immunohistochemistry

The femurs were harvested from conditional knockout mice and control littermates (N=5 per group) at day 7, 14, and 21 after the fracture surgery, fixed with 4% paraformaldehyde and embedded in paraffin. The sections were deparaffinized and serially rehydrated by incubation 3 times in Xylene for 5 minutes, 2 times in 100%

Ethanol (EtOH) for 10 minutes, 2 times in 95% EtOH for 10 minutes, 2 times in H_2O for 5 minutes followed PBS for 5 minutes. The sections were then next antigen retrieved by incubation in sodium citrate buffer, pH6.0 (SIGMA) for 15 minutes at a sub-boiling temperature, and subsequently removed to cool on the bench top for 30 minutes. After rinsing in H_2O, the sections were incubated with 3% hydrogen peroxide (Thermo Fisher Scientific) for 10 minutes to quench endogenous peroxidase activity followed by immunohistochemical staining. The slides were then incubated with 5% normal goat serum blocking buffer for one hour at room temperature. Subsequently, the slides were incubated with rabbit polyclonal IL-17A (Abcam) in 1:720 dilution or rabbit polyclonal IL-17RA (Abcam) in 1:200 dilution at 4°C overnight. Then peroxidase-conjugated rabbit SignalStain Boost IHC Detection Reagent (Cell Signaling) was used as secondary antibody using the manufacturer's recommended dilutions and incubating for 30 minutes at room temperature. Visualization was done using the 3,3-diaminobenzidine (DAB) substrate kit (Cell Signaling) following the manufacturer's protocol followed by coverslipping with VectaMount AQ (Vecta). All images were taken at 4x, 10x, 20x and 40X magnifications using an Olympus microscope.

3. Statistical analysis

All data were reported with means ± standard deviation of the mean. Parametric data were tested using a student T-test followed by an F-test to determine normality and differences between groups. Statistical significance was established at $p<0.05$.

RESULTS

Expressions of IL-17A and the receptor RA were increased in Prx1-Cre Runx3 $^{F/F}$ mice and the induction was prominent in fractured mice.

I first investigated whether IL-17A and the receptor RA were expressed in whole callus *(in vivo)* and periosteal cells *(in vitro)*. For whole callus, qPCR revealed that the expression of IL-17A was increased in Prx1-Cre Runx3 $^{F/F}$ groups, whereas IL-17RA did not show differences in the intact bone. In the fractured mice, both IL-17A and IL-17RA expressions were significantly increased in Prx1-Cre Runx3 $^{F/F}$ mice at day 14 (Figure 1).

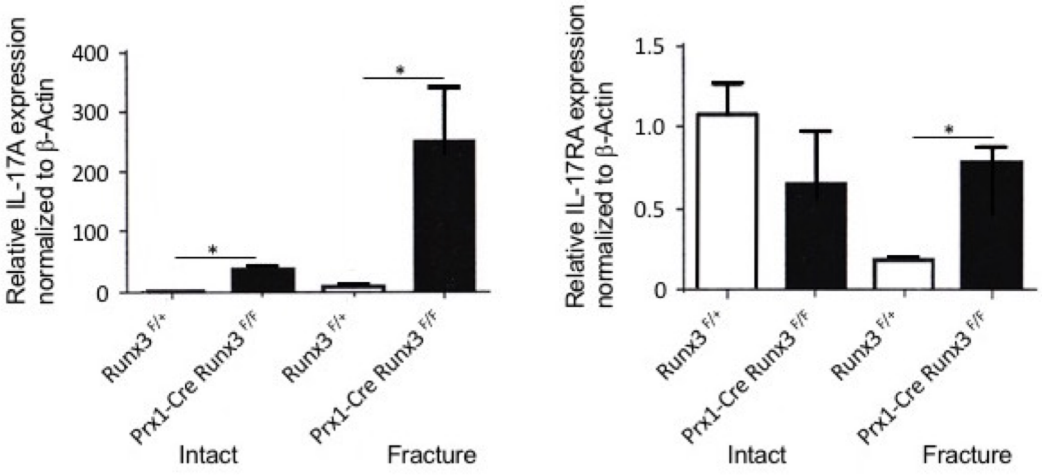

Figure 1. IL-17A and IL-17RA were increased in whole callus in Prx1-Cre Runx3 $^{F/F}$ mice at day 14 post fracture.

For periosteal cell culture, qPCR revealed that the expressions of IL-17A and IL-17RA were increased in Prx1-Cre Runx3 $^{F/F}$ mice, except at day 21 in IL-17RA (Figure 2).

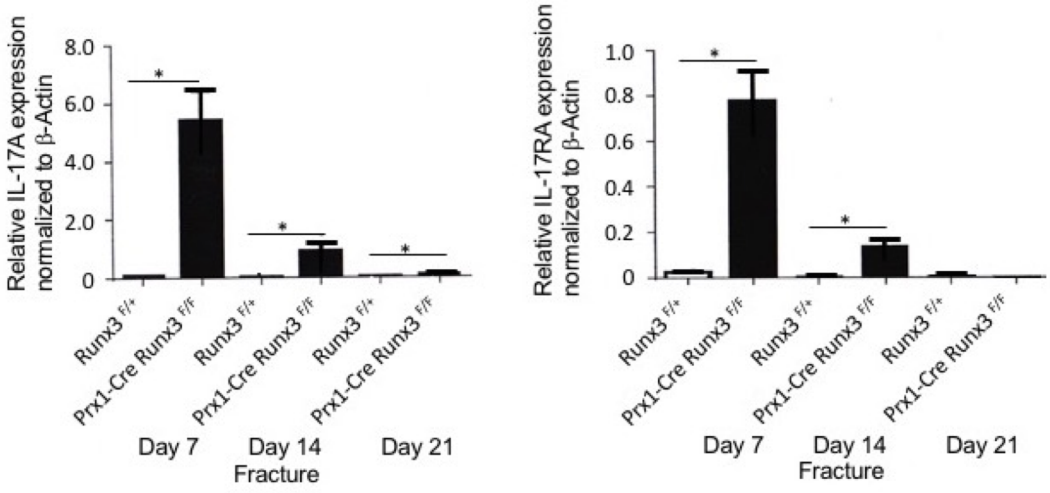

Figure 2. IL-17A and IL-17RA were increased in periosteal cells in Prx1-Cre Runx3 $^{F/F}$ mice at day 7, 14 and 21 in culture.

These results suggested that whole callus expressed IL-17A and IL-17RA and that the induction was significantly pronounced in Prx1-Cre Runx3 $^{F/F}$ mice.

Hypertrophic chondrocytes and periosteal cells were responsible for Runx3 conditional knockout-induced expressions of IL-17A and IL-17RA

qPCR results revealed that the expression of IL-17A and IL-17RA was induced in the fractured mice with targeted deletion of Runx3 in the periosteum. For these experiments, I used whole calluses, which were heterogeneous subsets involving

chondrocytes, osteoblasts, osteoclasts, mesenchymal precursor cells, and hematopoietic precursor cells.

To determine which cells were responsible for the induction of IL-17A and IL-17RA in the fracture, I employed IHC on femur sections for IL-17A and IL-17RA at day 7 and 14 after fracture surgery. Growth plates were used for positive control against the antibodies. Figure 3 and 4 showed that IL-17A and IL-17RA were expressed in hypertrophic chondrocytes and periosteal cells in Prx1-Cre Runx3 $^{F/+}$ mice.

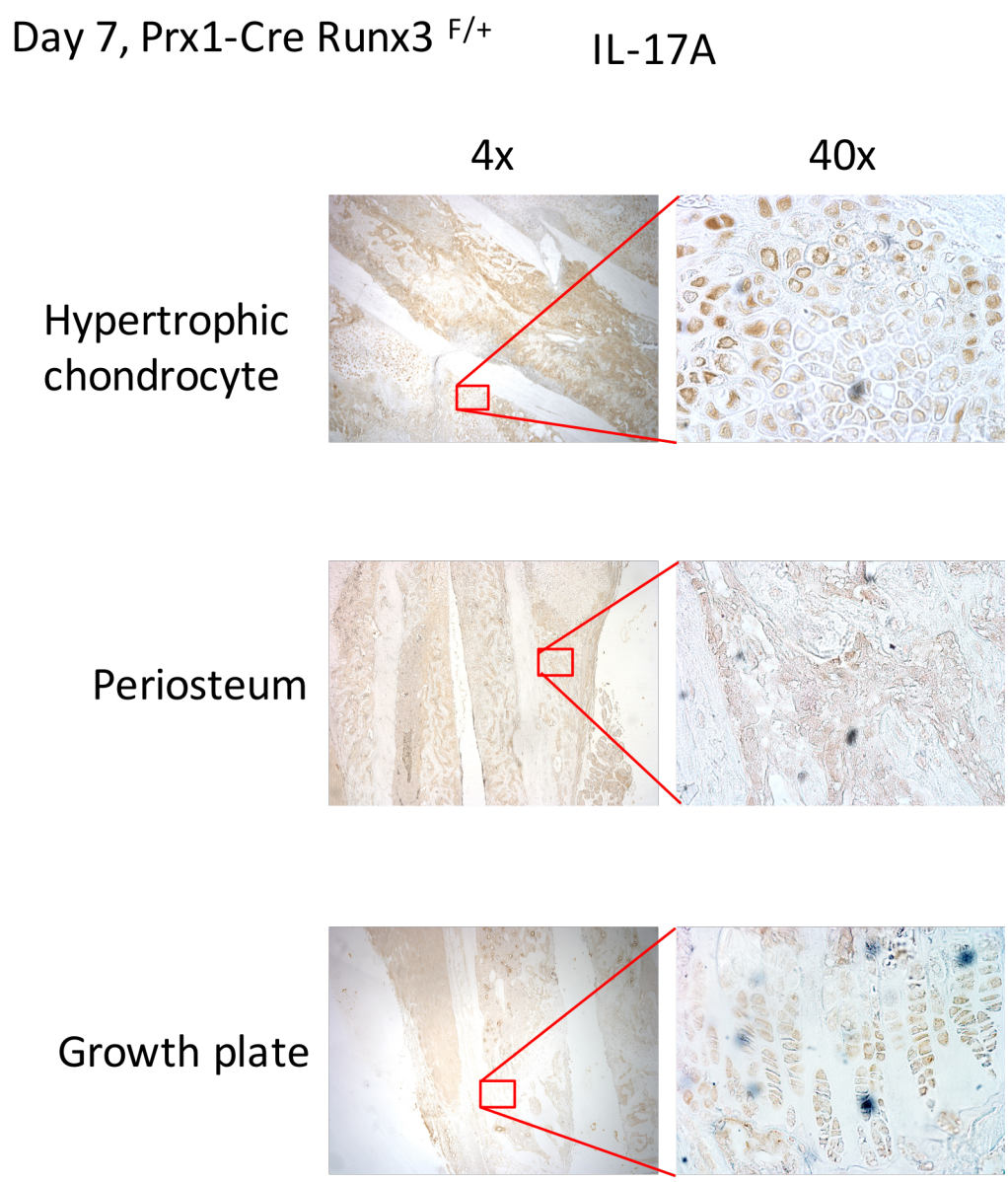

Figure 3. IL-17A was expressed in hypertrophic chondrocytes and periosteal cells in Prx1-Cre Runx3 $^{F/+}$ mice at day 7. The left images were 4x and the right images were 40x magnification.

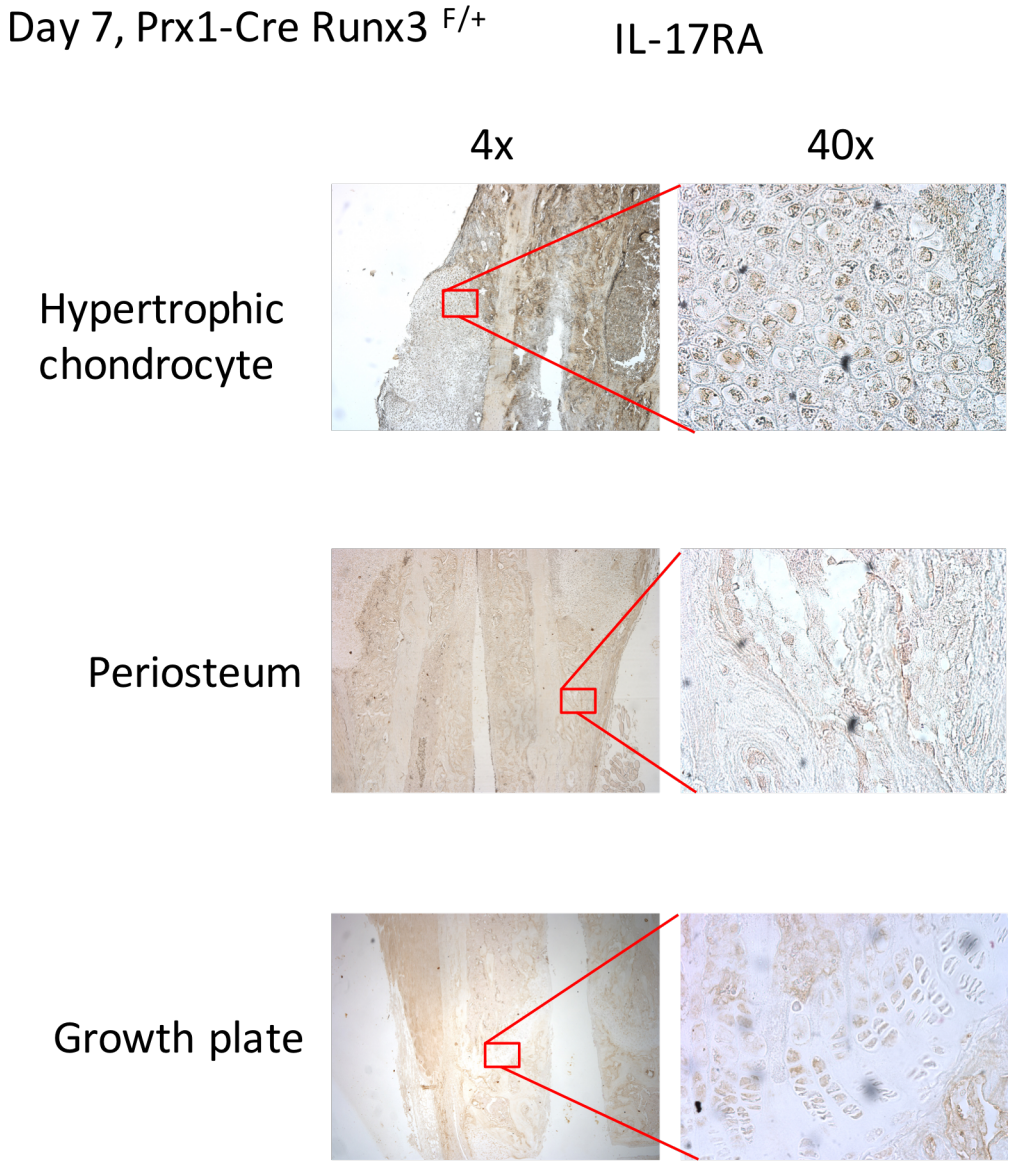

Figure 4. IL-17RA was expressed in hypertrophic chondrocytes and periosteal cells in Prx1-Cre Runx3 $^{F/+}$ mice at day 7. The left images were 4x and the right images were 40x magnification.

Figure 5 and 6 showed that IL-17A and IL-17RA were expressed in hypertrophic chondrocytes and periosteal cells in Prx1-Cre Runx3 $^{F/F}$ mice.

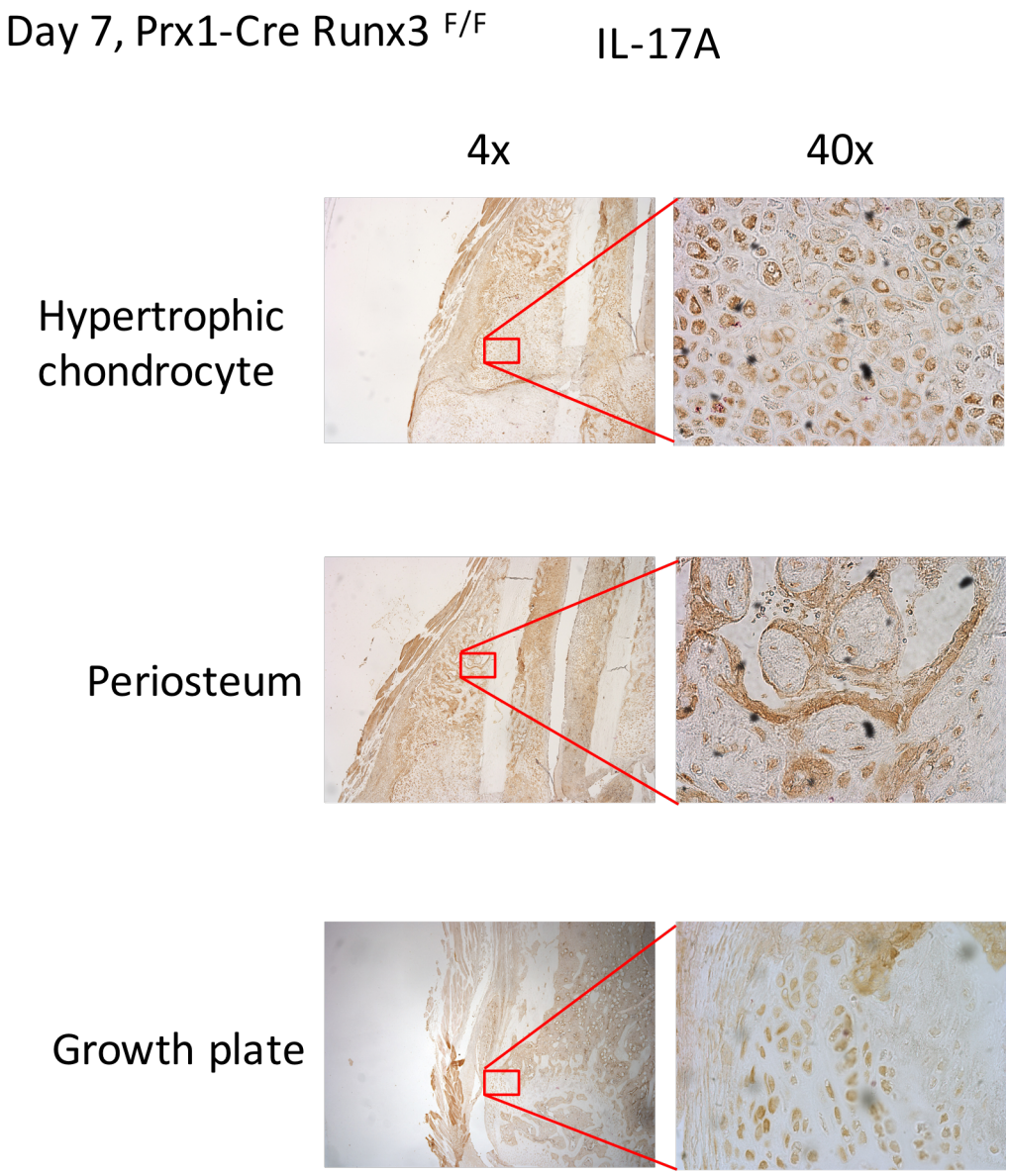

Figure 5. IL-17A was expressed in hypertrophic chondrocytes and periosteal cells in Prx1-Cre Runx3 $^{F/F}$ mice at day 7. The left images were 4x and the right images were 40x magnification.

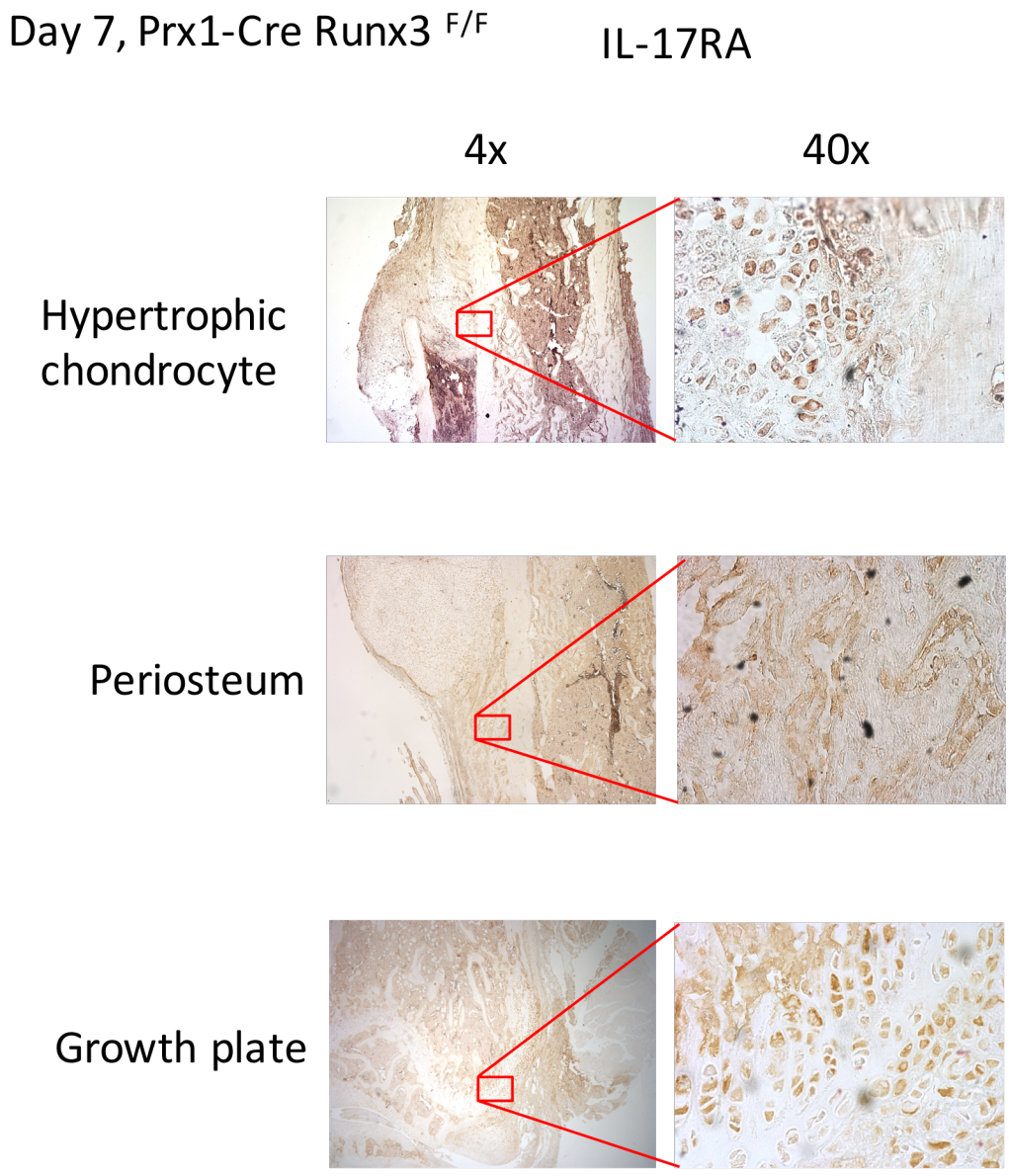

Figure 6. IL-17RA was expressed in hypertrophic chondrocytes and periosteal cells in Prx1-Cre Runx3 $^{F/F}$ mice at day 7. The left images were 4x and the right images were 40x magnification.

At day 14, hypertrophic chondrocytes were not observed in callus. Thus, I examined sections of periosteal cells and growth plate. Figure 7 and 8 showed that IL-17A and IL-17RA were expressed in periosteal cells in Prx1-Cre Runx3 $^{F/+}$ mice.

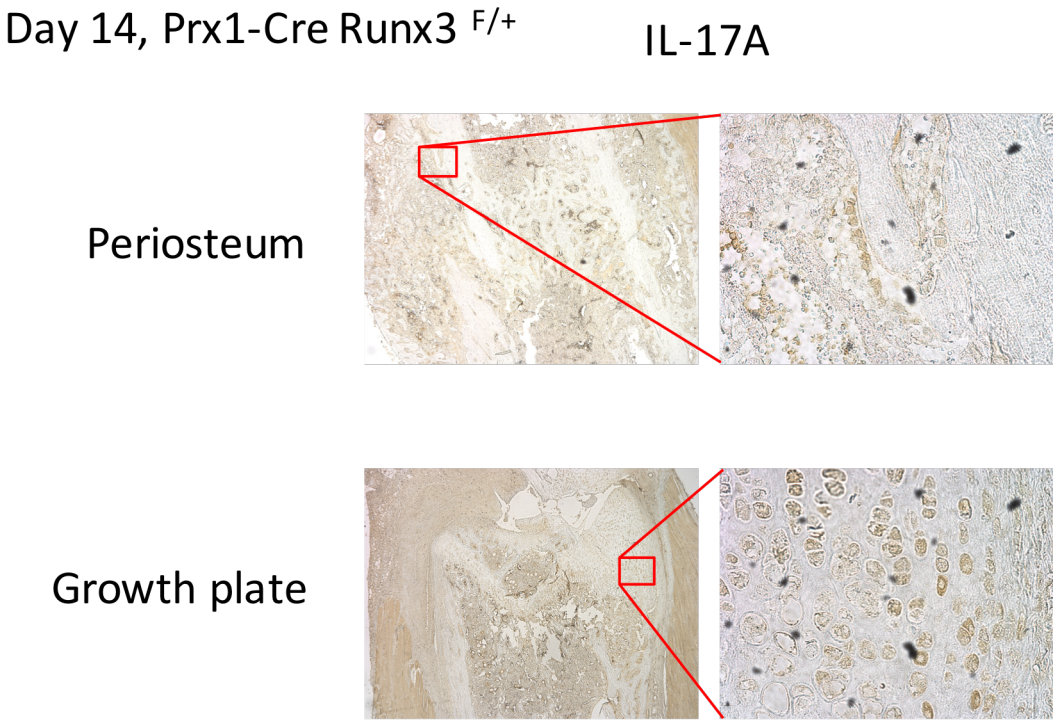

Figure 7. IL-17A was expressed in periosteal cells in Prx1-Cre Runx3 $^{F/+}$ mice at day 14. The left images were 4x and the right images were 40x magnification.

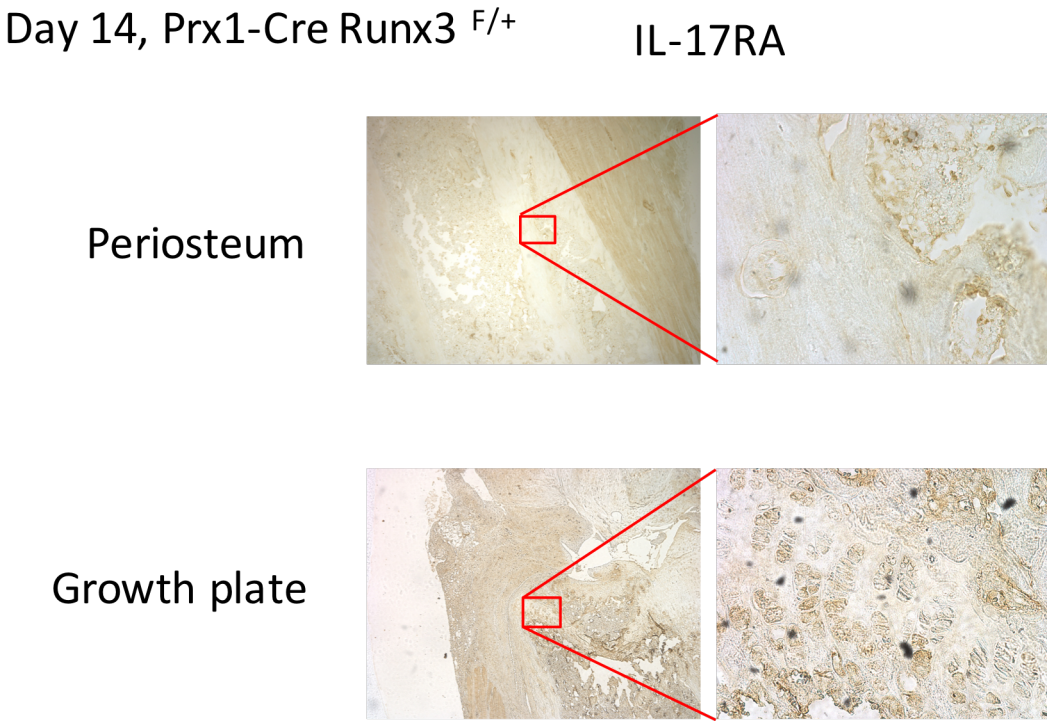

Figure 8. IL-17RA was expressed in periosteal cells in Prx1-Cre Runx3 $^{F/+}$ mice at day 14. The left images were 4x and the right images were 40x magnification.

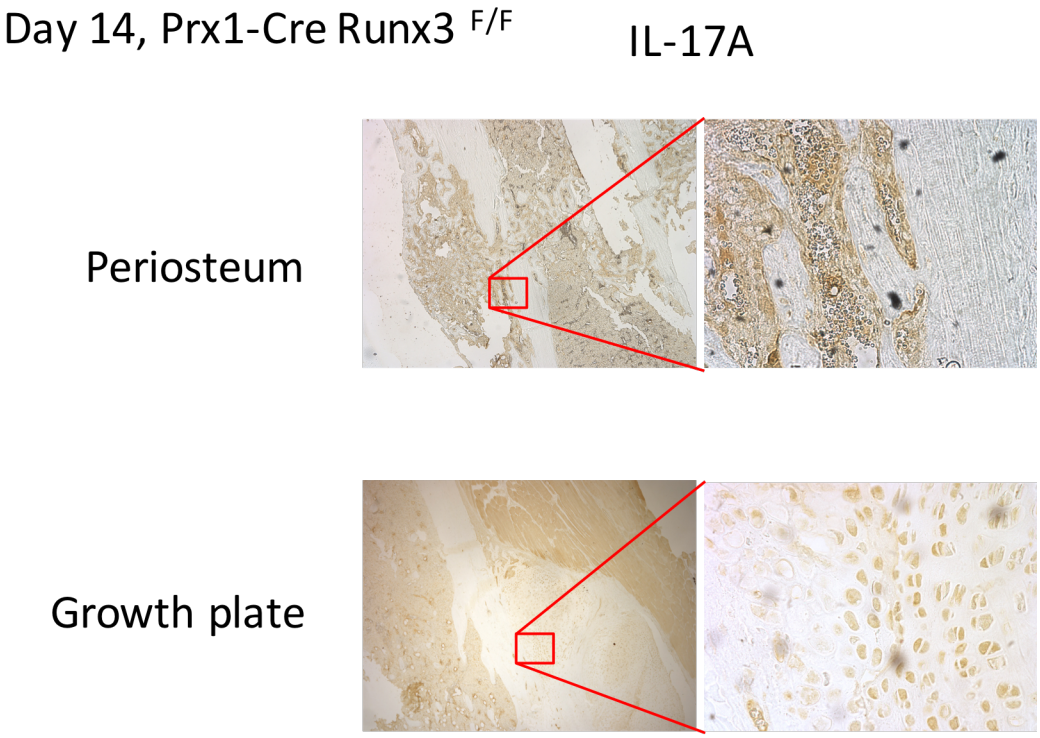

Figure 9. IL-17A was expressed in periosteal cells in Prx1-Cre Runx3 $^{F/F}$ mice at day 14. The left images were 4x and the right images were 40x magnification.

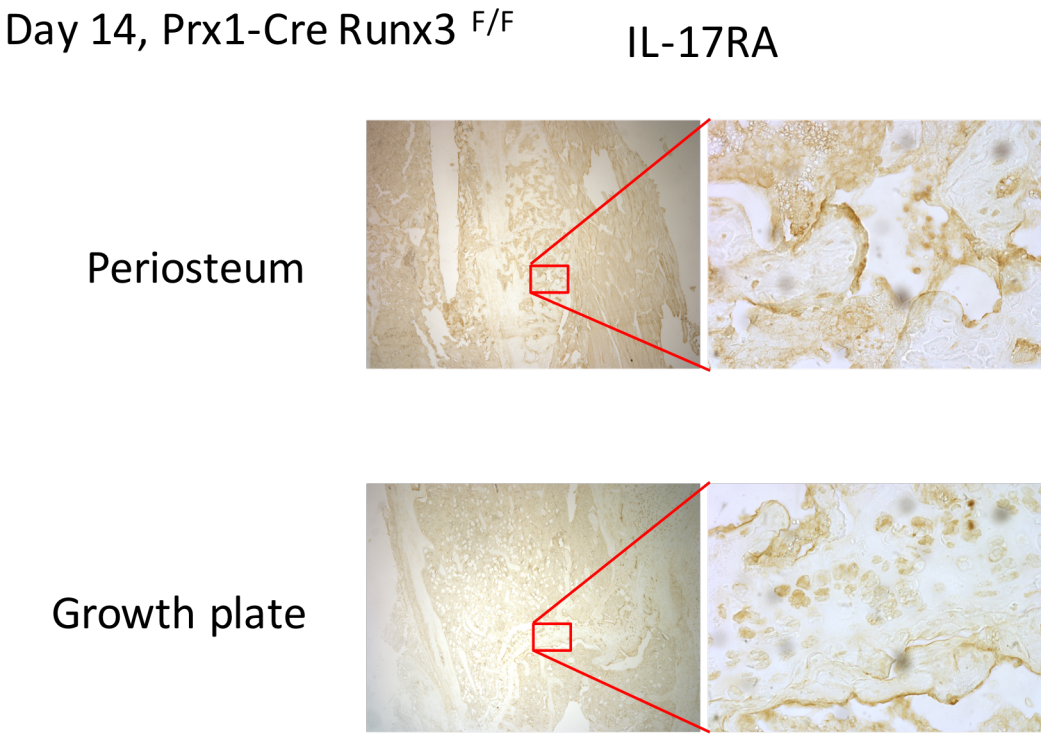

Figure 10. IL-17RA was expressed in periosteal cells in Prx1-Cre Runx3 $^{F/F}$ mice at day 14. The left images were 4x and the right images were 40x magnification.

Figure 9 and 10 showed that IL-17A and IL-17RA were expressed in periosteal cells in Prx1-Cre Runx3 $^{F/F}$ mice. Figure 11 and 12 showed the results of IHC for hypertrophic chondrocytes and periosteal cells at day 7, respectively. Figure 13 showed that the result of IHC for periosteal cells at day 14. Both IL-17A and IL-17RA expressions in hypertrophic chondrocytes were significantly enhanced in mice targeted deletion of Runx3 in periosteum compared with control mice (Figure 11). The similar trend was observed in periosteum at day 7 (Figure 12) and day 14 (Figure 13).

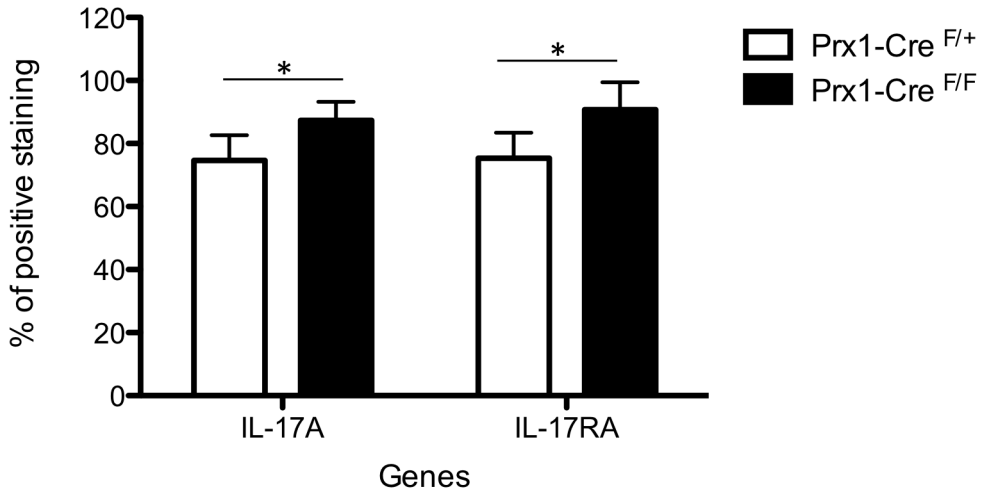

Figure 11. IL-17A and IL-17RA were significantly increased in hypertrophic chondrocytes in Prx1-Cre Runx3 $^{F/F}$ mice at day 7. *P < 0.05.

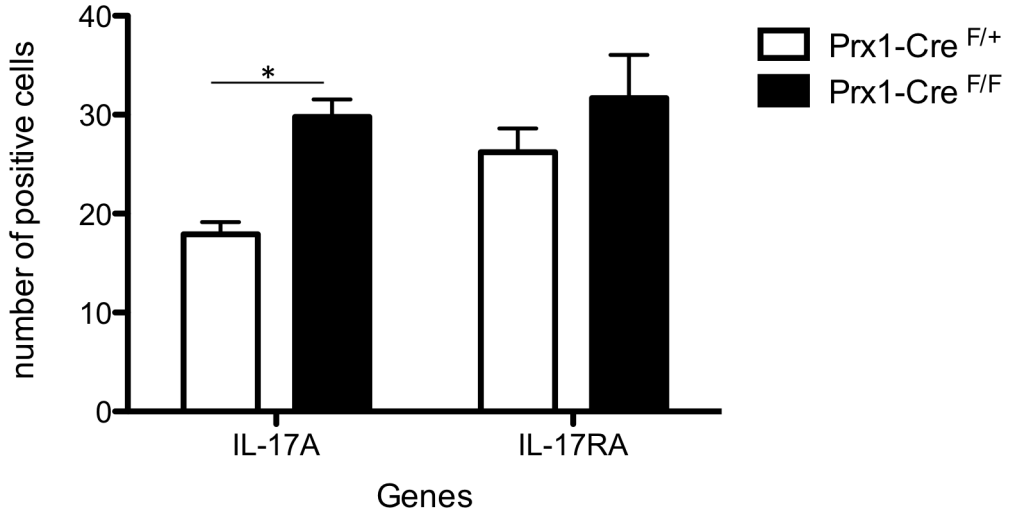

Figure 12. IL-17A and IL-17RA were increased in periosteal cells in Prx1-Cre Runx3 $^{F/F}$ mice at day 7. *P < 0.05.

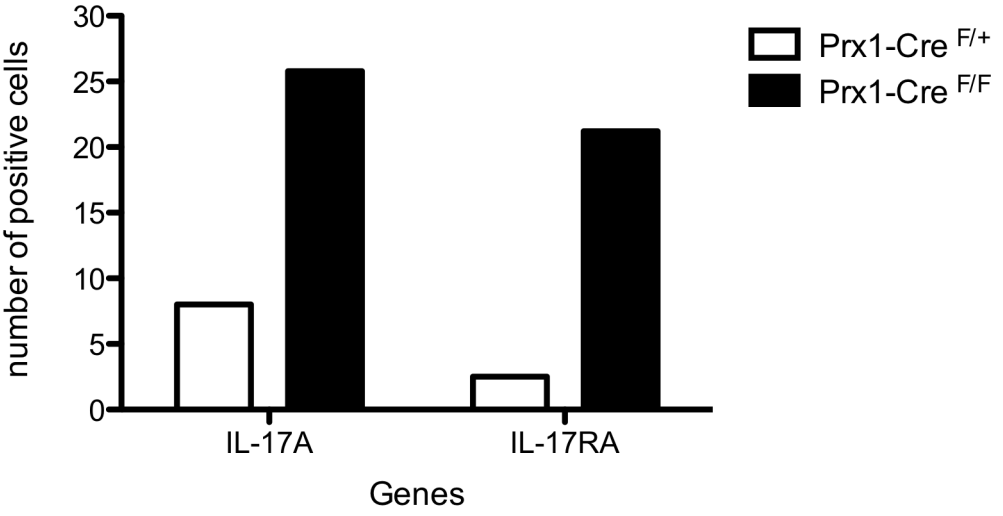

Figure 13. IL-17A and IL-17RA were increased in periosteal cells in Prx1-Cre Runx3 $^{F/F}$ mice at day 14.

DISCUSSION

The periosteum consists of multipotent progenitor cells and they play crucial roles following bone graft and in musculoskeletal pathologies. Our laboratory previously reported that targeted deletion of Runx3 in the periosteum resulted in enhanced fracture healing and that the enhanced bone healing due to Runx3 deletion was due to increasing bone formation and biomechanical properties of the fracture callus in mutant mice[3].

Inflammatory cytokines are known to be involved in fracture healing by initiating the repair process following injury. They are secreted immediately after injury at the injury site by macrophages, inflammatory cells, and mesenchymal origin cells[5]. Recent studies demonstrated that IL-17A was highly induced immediately after bone injury and promoted bone regeneration by accelerating osteogenesis via its effects on injury-associated mesenchymal cells[4]. The Runx family of transcription factors has demonstrated critical functions in the regulation of these progenitors through IL-17 signaling pathway the Runx family of transcription factors has demonstrated critical functions in the regulation of these progenitors through IL-17 signaling pathway[25, 26].

Thus, the hypothesis is that periosteal cells, following conditional knockout of Runx3, express high levels of IL-17A to recruit progenitor cells into osteogenic lineage resulting in bone healing. My study showed that the expressions of IL-17A and IL-17RA were induced periosteal cells in the mice with targeted deletion of Runx3 to the periosteum. Moreover, the induction was increased significantly in the fractured mouse group.

There is controversy regarding IL-17A and osteogenesis and bone formation. Noh reported that IL-17A increased leptin production that inhibits adipogenesis and promotes osteogenesis on human bone marrow derived mesenchymal stem cells via JAK/STAT signaling[27]. In contrast, there is a paper indicating that the combination of IL-17A and TNF-alpha activated osteoclasts leading to bone destruction due to RANKL inhibition and Schnurri-3 induction in human mesenchymal stem cells[26]. However, they discussed the possibility that this change could promote ectopic bone formation in the absence of osteoclasts. My results suggest that the induction of IL-17A and IL-17RA in callus should contribute to enhanced bone healing in Runx3 conditional knockout mice.

Many groups have reported the efficacy of IL-17A in bone formation and bone healing in mesenchymal stem cells[27]. When I investigated which cells played a key role in the enhanced bone healing, my data indicated that hypertrophic chondrocytes and periosteal cells were responsible for the Runx3 deletion-induced expressions of IL-17A and IL-17RA. Huang reported that IL-17 produced by T cell induced proliferation of human bone marrow-derived mesenchymal stem cells depending on the generation of reactive oxygen species (ROS)[25]. IL-17 signaling may promote osteogenesis and angiogenesis to lead to enhanced-bone repair in vivo.

FUTURE DIRECTIONS

Understanding the pathways underlying Runx3 deletion induced-IL-17 induction in these progenitor cells will be beneficial to the translation in clinical approaches. Currently, there are only two FDA-approved modalities for orthobiologic treatment. BMP-2 has been used for sinus lift and alveolar ridge augmentation and PDGF-BB has been used for periodontal disease and bone graft for the implant treatment. Mimicking these effects using small molecules to specifically inhibit Runx3 expression locally at the fracture site could be a new treatment modality that could be applied to oral and maxillofacial fracture.

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