YAP and TAZ Promote Periosteal Osteoblast Precursor Expansion and Differentiation for Fracture Repair

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ABSTRACT
In response to bone fracture, periosteal progenitor cells proliferate, expand, and differentiate to form cartilage and bone in the fracture callus. These cellular functions require the coordinated activation of multiple transcriptional programs, and the transcriptional regulators Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) regulate osteochondroprogenitor activation during endochondral bone development. However, recent observations raise important distinctions between the signaling mechanisms used to control bone morphogenesis and repair. Here, we tested the hypothesis that YAP and TAZ regulate osteochondroprogenitor activation during endochondral bone fracture healing in mice. Constitutive YAP and/or TAZ deletion from Osterix-expressing cells impaired both cartilage callus formation and subsequent mineralization. However, this could be explained either by direct defects in osteochondroprogenitor differentiation after fracture or by developmental deficiencies in the progenitor cell pool before fracture. Consistent with the second possibility, we found that developmental YAP/TAZ deletion produced long bones with impaired periosteal thickness and cellularity. Therefore, to remove the contributions of developmental history, we next generated adult onset-inducible knockout mice (using Osx-CreERT2) in which YAP and TAZ were deleted before fracture but after normal development. Adult onset-induced YAP/TAZ deletion had no effect on cartilaginous callus formation but impaired bone formation at 14 days post-fracture (dpf). Earlier, at 4 dpf, adult onset-induced YAP/TAZ deletion impaired the proliferation and expansion of osteoblast precursor cells located in the shoulder of the callus. Further, activated periosteal cells isolated from this region at 4 dpf exhibited impaired osteogenic differentiation in vitro upon YAP/TAZ deletion. Finally, confirming the effects on osteoblast function in vivo, adult onset-induced YAP/TAZ deletion impaired bone formation in the callus shoulder at 7 dpf before the initiation of endochondral ossification. Together, these data show that YAP and TAZ promote the expansion and differentiation of periosteal osteoblast precursors to accelerate bone fracture healing. © 2020 American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: FRACTURE HEALING; GENETIC ANIMAL MODELS; OSTEOBLASTS; TRANSCRIPTION FACTORS

Introduction
Bone is a remarkable tissue in its capacity to heal without forming a scar, and most bone injuries heal readily, with bone fractures healing at success rates of 90% to 95%. This is due, in part, to the maintenance of a skeletal stem cell population capable of recapitulating many aspects of embryological programs for adult tissue regeneration. As in development, bone formation during fracture repair can occur through both direct intramembranous ossification and endochondral ossification via a cartilage intermediate. However, the source, niche, and molecular regulation of the progenitor cells responsible for bone fracture repair are distinct from those that produce the skeleton during development. In the embryo, mesodermal mesenchymal progenitors in the limb bud form a template of the skeletal elements, while bone fracture healing initiates by expansion and differentiation of osteochondroprogenitor cells resident in the bone-lining periosteum. Understanding the mechanisms that regulate the proliferation and differentiation of these cells will be critical to develop new therapeutic strategies for accelerating fracture repair and regenerating challenging bone injuries that cannot heal on their own.

To define the roles of the molecular mediators that regulate adult periosteal progenitor cell function, we must decouple the developmental history of the osteochondroprogenitor cells that come to reside in the periosteum from the regenerative function of these cells after fracture to accurately evaluate their...
contribution to responding to injury in the adult. For example, a recent study in which PDGFRβ was deleted from Osterix-expressing cells found no notable defects in skeletal development but severe impairment of fracture healing, demonstrating contextual distinctions between development and fracture repair. Further, skeletal cell-specific gene deletion during development may alter the number, location, or niche of progenitor cells that, during injury, are activated for skeletal regeneration. In this study, we assessed the effects of conditional gene deletion from osteochondroprogenitors on endochondral bone fracture repair, with deletion performed either constitutively during development or inducibly after normal development at skeletal maturity before fracture. Several types of inducible Cre-loxP systems exist to temporally regulate Cre-mediated gene recombination, including the interferon-responsive Mx1 promoter, tamoxifen-inducible mutated estrogen and progesterone receptors, and tetracycline-controlled systems. Here, we used the Osterix-Cre (Osx-Cre) mouse in which Cre recombinase is driven by the Osterix promoter and temporally controlled by tetracycline (or its more stable derivative, doxycycline). Osteoprogenitors, including those of the primary ossification center in the embryo as well as the periosteum in the adult,

Periosteal cell expansion and differentiation require the coordinated activation of multiple transcriptional programs, and the transcriptional regulators, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), have dned multiple transcriptional programs, and the transcriptional regulators, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), have recently emerged as critical mediators of osteoblast progenitor proliferation and differentiation during bone development. Previously, we found that constitutive homozygous deletion of both YAP and TAZ from Osterix-expressing cells caused severe skeletal fragility and neonatal lethality. Mice with a single allele of either YAP or TAZ survived but sustained spontaneous long bone fractures due to both reduced bone mass and defects in bone matrix collagen that caused weakened bone mechanical properties. Despite fracture, the neonatal limbs exhibited natural reduction and eventually healed through callus formation. However, the roles of YAP and TAZ in periosteal progenitor cell function and their contributions to bone fracture healing are unknown.

Here, we conditionally deleted YAP and/or TAZ from Osterix-expressing cells using either constitutive or tetOff-inducible deletion and evaluated adult endochondral bone fracture healing. We found that constitutive YAP and/or TAZ deletion impaired both callus formation and subsequent mineralization, due to developmental deficiencies in the progenitor cell pool before fracture. In contrast, adult onset-induced YAP/TAZ deletion had no effect on cartilaginous callus formation, but impaired both the proliferation of osteoblast precursor cells located in the shoulder of the callus and their osteogenic differentiation, both in vitro and in vivo. Together, these data show that YAP and TAZ promote the expansion and differentiation of periosteal osteoblast precursors to accelerate bone fracture healing.

Materials and Methods

Animals

Mice harboring loxP-flanked exon 3 alleles in both YAP and TAZ on a mixed C57BL/6J genetic background were kindly provided by Dr Eric Olson (University of Texas Southwestern Medical Center). Tetracycline-responsive B6.Cg-Tg(Sp7-TA,tetO-EGFP;Cre) 1AMcJ (Osx-Cre tetOff) mice from The Jackson Laboratory (Bar Harbor, ME, USA) were used to generate two mouse models in which we conditionally deleted YAP and/or TAZ from Osterix-expressing cells (Table 1). In both mouse models, tetracycline (or its more stable derivative, doxycycline) administration prevents tetracycline-controlled transactivator protein (tTA) binding to the tetracycline-responsive promoter element (TRE) in the promoter of the Cre transgene, allowing Cre expression only in the absence of doxycycline for temporal control of Oss-Cre-mediated gene deletion.

In our first study, we evaluated constitutive allele dose-dependent deletion of YAP and/or TAZ in skeletal mature mice 16 to 21 weeks of age (Table 1). Mice with homozygous floxed alleles for both YAP and TAZ (YAPfl/fl;TAZfl/fl) were mated with double heterozygous conditional knockout mice (YAPfl/w;TAZfl/w;Osx-Cre) to produce eight possible genotypes in each litter, but only Cre-positive and YAPfl/w;TAZfl/w;Osx-Cre animals were compared (Table 1). Because constitutive, dual homozygous YAP/TAZ deletion using Osx-Cre is perinatal lethal, we could not evaluate this genotype in the constitutive deletion fracture healing model. Here, the littermate YAPfl/fl;TAZfl/fl mice were considered the wild-type control genotype. All of these mice were bred, raised, and evaluated without tetracycline administration to induce gene recombination in Oss-Cre-expressing cells during embryonic development, for the duration of the analyses in the constitutive deletion model. In the constitutive deletion fracture healing model, 5 to 8 mice per genotype were evaluated at 14- and 42-day post fracture (dpf) using micro-computed tomography (microCT) and mechanical testing.

In our second study, we allowed mice to develop to skeletal maturity and induced homozygous YAP/TAZ deletion 2 weeks before fracture at 16 to 18 weeks of age (Table 1). In the inducible deletion model, both littermate YAPfl/fl;TAZfl/fl (YAPfl/w;TAZwt/w) mice and separately bred Osx-Cre tetOff mice were evaluated as wild-type control genotypes (Table 1). All mice were bred and raised until skeletal maturity with doxycycline in their drinking water to prevent Cre-mediated gene recombination. For all in vivo fracture healing assessments, doxycycline was removed 2 weeks before fracture surgery and normal drinking water was provided for the remainder of the study. For periosteal progenitor cell isolations from fractured limbs, doxycycline was provided for both YAPfl/w;TAZwt/w and YAPfl/w;TAZkO/kO;TAZkO/kO mice throughout skeletal development and the duration of the fracture healing experiment. In the inducible deletion fracture healing model, 3 to 9 mice per genotype were evaluated at 4, 7, and 14 dpf using qPCR, histology, and microCT.

In both studies, mice were tail or ear clipped after weaning or before euthanasia and genotyped by an external service (Transnetyx, Inc., Cordova, TN, USA). All mice were fed regular

Table 1. Experimental Fracture Healing Models, Genotypes, and Abbreviations

| Deletion approach | Genotype | Abbreviation |
|-------------------|----------|--------------|
| Constitutive      | Yapfl/fl;Tazfl/fl | YAPWT;TAZWT |
| Yapfl/fl;Tazfl/fl;Osx-Cre | YAPHE;TAZHE |
| Yapfl/fl;Tazfl/fl;Osx-Cre | YAPKO;TAZKO |
| Yapfl/fl;Tazfl/fl;Osx-Cre | YAPKO;TAZKO |
| Osx-Cre tetOff | Oss-Cre |
| Yapfl/fl;Tazfl/fl | YAPWT;TAZWT |
| Yapfl/fl;Tazfl/fl;Osx-Cre tetOff | YAPKO;TAZKO |

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In both studies, mice were tail or ear clipped after weaning or before euthanasia and genotyped by an external service (Transnetyx, Inc., Cordova, TN, USA). All mice were fed regular
chow (PicoLab Rodent Diet, cat# 0007688, Purina LabDiet, St. Louis, MO, USA) ad libitum and housed in cages containing 2 to 5 animals each. Mice were maintained at constant 25°C on a 12-hour light/dark cycle. Both male and female mice were evaluated with the same fracture healing procedure for both the constitutive and inducible deletion models of fracture healing (Table 1). All protocols were approved by the Institutional Animal Care and Use Committees at the University of Notre Dame and the University of Pennsylvania. All animal procedures were performed in adherence to federal guidelines for animal care and conform to the Animal Research: Reporting of in vivo Experiments (ARRIVE) guidelines.

Open femoral fracture model and timepoints

An open, unilateral, and intramedullary pin-stabilized femoral fracture model was used to study bone repair in both the inducible and constitutive deletion studies. Femora were surgically exposed and manually fractured by applying a bending moment with a scalpel but stabilized with an intramedullary pin. For the surgical procedure, animals were anesthetized using isoflurane (1% to 5%), all hair was removed from the surgical site, and the area was cleansed with sterile water followed by betadine. A (1% to 5%), all hair was removed from the surgical site, and the area was cleansed with sterile water followed by betadine. A 25-gauge needle was inserted in a retrograde manner into the intramedullary canal of the right femur. Subsequently, the muscle surrounding the same femur was blunt dissected to expose the femoral midshaft and a reproducible fracture was created by applying a three-point bending moment using a scalpel in the femur containing the intramedullary pin. The contralateral leg was left intact. Any animals that displayed intramedullary pin displacement or fractures that were comminuted or too oblique were removed. The mice were allowed to recover under a heating lamp and after awakening returned to their cages and allowed to ambulate freely. In the constitutive deletion model, mice were euthanized at 14 dpf and 42 dpf. In the inducible deletion model, mice were euthanized at 4 dpf, 7 dpf, and 14 dpf. At 4 dpf, mice were injected intraperitoneally with 5-ethynyl-2'-deoxyuridine (EdU; E10187; Invitrogen, Carlsbad, CA, USA) at 10 mg/kg 3 hours before euthanasia to assay cellular proliferation.

Micro-computed tomography

Micro-computed tomography was performed according to published guidelines on two separate systems correlating with the constitutive and inducible model. In accordance to published microCT guidelines, the same threshold was applied across all genotypes for a given system. In the constitutive deletion study, 14- and 42-dpf fractured limbs were dissected free from surrounding musculature and the intramedullary pins were removed. Samples from 14- and 42-dpf limbs were wrapped in PBS-soaked gauze and frozen at −20°C. When removed from the freezer, bones were allowed to thaw while being imaged using a μCT 35 system (Scanco Medical, Bruttisellen, Switzerland). Samples from 14- and 42-dp bone were imaged with an X-ray intensity of 114 μA, energy of 70 kVp, integration time of 200 ms, and resolution of 15 μm. We again defined the fracture callus mineralization threshold as 50% of the mineral density that we used to segment intact cortical bone under these conditions on this system. 2D tomograms of the entire fracture callus, both excluding and including the intact cortical bone were manually contoured, stacked, and binarized by applying a Gaussian filter (σ = 0.8, support = 1) at a threshold of 345 mg HA/cm³. 3D quantification was conducted in two ways. First, to determine the callus volume and mineralization percentage of the callus, microCT slices were contoured pre-segmentation to produce a region of interest that excluded the intact cortical bone but included the nonmineralized callus. Total callus volume, percent callus mineralization, and volumetric bone mineral density of the mineralized tissue were evaluated using the segmentation and thresholding above. Second, to evaluate the contours of the outer boundary of the callus, including both nonmineralized and mineralized tissues, microCT slices were contoured pre-segmentation and the polar moment of inertia (pMOI) was computed on a transverse slice-by-slice basis as described in detail below. Five to 8 mice were analyzed per group. Investigators were blinded to animal genotype during scan quantification.

In the inducible deletion study, 7- and 14-dpf limbs were dissected free from surrounding musculature and the intramedullary pins were removed. Bones from 7 and 14 dpf were then snap-frozen in liquid nitrogen-cooled isopentane for 1 minute, wrapped in gauze, and imaged on a vivaCT 80 system (Scanco Medical). Samples from 7 and 14 dpf were imaged with an X-ray intensity of 114 μA, energy of 70 kVp, integration time of 200 ms, and resolution of 15 μm. We again defined the fracture callus mineralization threshold as 50% of the mineral density that we used to segment intact cortical bone under these conditions on this system. 2D tomograms of the entire fracture callus, both excluding and including the intact cortical bone were manually contoured, stacked, and binarized by applying a Gaussian filter (σ = 0.8, support = 1) at a threshold of 254 mg HA/cm³. 3D quantification was applied as described above. Six to 8 mice were analyzed per group. Investigators were blinded to animal genotype during scan quantification.

To quantify the contours of the outer boundary of the callus along the length of the bone axis, the “Bone Midshaft” evaluation script (Scanco Medical) was used to measure the pMOI, also known as the second polar moment of area, a measure of radial distribution. Limbs from 14 dpf in the constitutive deletion model and 14 dpf from the inducible deletion model were analyzed using a negative, nonphysiological threshold within the contours of the callus to include both mineralized and nonmineralized tissues in the Bone Midshaft evaluation. Limbs from 42 dpf were scanned on the μCT 35 system and were analyzed using the same threshold for mineralized tissue above (345 mg HA/cm³) to include only the mineralized tissue in the Bone Midshaft evaluation. In both cases, pMOI values for all groups were binned into 25 equal-distance bins from the center of the fracture using a custom MATLAB script. Data presented as the mean polar moment of inertia at each axial position, with error bars corresponding to the standard deviation (SD).

Mechanical testing

After microCT scanning, 14- and 42-dpf limbs from the constitutive deletion model were tested in torsion to failure. For torsional testing, we used fixtures and a custom potting apparatus that allowed us to reproducibly align and pot the fractured limbs in polymethylmethacrylate bone cement. After the fractured limbs were potted, they were loaded in torsion at a rate of 1°/s until failure using a custom-designed micro-torsional testing system. Recorded torque-rotation data were normalized by gauge length on a per-sample basis. Torsional rigidity, maximum torque to failure, work to maximum torque, and work to failure were quantified from the normalized torque-rotation data using a custom MATLAB script. Five to 8 mice were analyzed per group per
time point. Investigators were blinded to animal genotype during data quantification.

Histology, immunohistochemistry, and immunofluorescence

Limbs from 7 and 14 dpf were fixed with 10% neutral buffered formalin for 48 hours and decalcified for 4 weeks with 0.25 M EDTA (pH 7.4) at 4°C. Paraffin sections (5 μm thickness) were processed for either immunohistochemistry or histology. Primary antibodies were compared with normal rabbit sera IgG control sections. For immunostaining, anti-OSX (1:500, ab22552; Abcam, Cambridge, MA, USA), anti-YAP (1:500, 14074; Cell Signaling, Danvers, MA, USA), and anti- TAZ (1:250 NB110-58359; Novus Biologicals, Littleton, CO, USA) primary antibodies were applied overnight. Next,

Fig. 1. Constitutive, combinatorial YAP/TAZ deletion from Osterix-expressing cells impaired fracture healing. (A) MicroCT reconstructions at 14 days post-fracture (dpf). Quantification of 14 dpf callus architecture: (B) total callus volume and (C) mineralized callus percentage. Quantification of 14 dpf callus mechanical testing in torsion to failure: (D) maximum torque and (E) torsional rigidity. (F) MicroCT reconstructions at 42 dpf. Quantification of 42 dpf callus architecture: (G) total callus volume and (H) mineralized callus percentage. Quantification of 42 dpf callus mechanical testing in torsion to failure: (I) maximum torque and (J) torsional rigidity. Data are presented as individual samples in scatterplots and boxplots corresponding to the median and interquartile range. Data were evaluated by one-way ANOVA with post hoc Tukey's multiple comparisons tests. Groups with significant pairwise comparisons are indicated by bracketed lines and p values adjusted for multiple comparisons. Sample sizes, n = 5–8. Scale bars = 1 mm for microCT reconstructions.
Fig. 2. Constitutive, combinatorial YAP/TAZ deletion from Osterix-expressing cells reduced callus size. (A) MicroCT reconstructions at 14 dpf showing longitudinal cut-planes within the callus. Dotted lines indicate the callus boundary, the region within which the total callus moment of inertia was quantified. (B) Polar moment of inertia distributions of the entire callus for each of the YAP/TAZ allele dose-dependent knockout genotypes. Data were binned into 25 equal distance bins from the center of the callus and presented as dots representing the mean and error bars corresponding to the standard deviation (SD). Sample sizes, n = 5–8. Scale bar = 1 mm for microCT reconstructions.

Fig. 3. Constitutive, combinatorial YAP/TAZ deletion from Osterix-expressing cells impaired periosteal development in intact bone. (A) Representative micrographs of 18- to 21-week-old distal femur cortical bone stained by aqueous H&E. Dotted lines indicate the periosteum. Quantification of (B) periosteal thickness, (C) periosteal area, and (D) periosteal cell number per bone surface (N.Pc /PS). Data are presented as individual samples in scatterplots and boxplots corresponding to the median and interquartile range. Data were evaluated by one-way ANOVA with post hoc Tukey's multiple comparisons tests. Groups with significant pairwise comparisons are indicated by bracketed lines and p values adjusted for multiple comparisons. Sample sizes, n = 6–7. Scale bars = 50 μm for all images.
sections were incubated with corresponding biotinylated secondary antibody, avidin-conjugated peroxidase, and diaminobenzidine substrate chromogen system (329ANK-60; Innovex Biosciences, Lincoln, RI, USA), which allowed for immunohistochemical detection of positively stained cells. Hematoxylin and eosin stains (H&E), Safranin-O, and Picrosirius Red stains were used to stain for bone, cartilage, and collagen.

Lims from 4-dpf and intact femora were fixed with 10% neutral buffered formalin for 48 hours at 4°C, transferred to 30% sucrose in PBS overnight at 4°C, and then embedded in OCT compound (Tissue-Tek, Sakura Finetek USA, Torrance, CA, USA). Thin sections (7 μm thickness) were made from undecalciﬁed fractured femurs using cryofilm IIC tape (Section Lab Co. Ltd., Hiroshima, Japan) as previously described and processed for immunofluorescence and/or aqueous H&E staining. Taped sections were glued to microscope slides using a UV-adhesive glue, rehydrated and then decalcified with 0.25 M EDTA (pH 7.4) for 3 minutes before staining. 5-ethyl-2′-deoxyuridine (EdU) staining was performed using the Click-IT Plus EdU Assay kit (C10339; Invitrogen) according to the manufacturer’s instructions.

Imaging and histomorphometric analysis

Histological and immunohistochemical sections were imaged on either an Axio Observer Z1 (Zeiss, Thornwood, NY, USA) at the 10x and 25x objectives or using an Axioscan microscope (Zeiss) at the 10x and 20x objective. Histomorphometry quantification at 4, 7, and 14 dpf was performed using ImageJ (NIH). To evaluate bone and cartilage area parameters at 7 and 14 dpf, the entire callus area was evaluated using ImageJ on at least three sections per sample with 3 to 4 mice per group per time point. To evaluate the number of positively immunostained cells at 7 and 14 dpf, individual cells were manually scored as either positive or negative, using ImageJ, for each given antibody. Data are reported as the percentage of positively stained osteoblasts, osteocytes, and chondrocytes per total number of each cell type on each sample. For all samples, approximately 500 cells were evaluated where osteoblasts and osteocytes were primarily evaluated in the “shoulder” region of the callus, while chondrocytes were evaluated in the cartilage near the fracture line. To evaluate periosteal parameters at 4 dpf, four regions of interest were outlined per sample 1 to 3 mm from the fracture on the periosteal cortical bone, which corresponded in this femoral fracture model to areas of the callus where intramembranous ossification occurred.

Samples from 7 and 14 dpf were stained with Picrosirius Red and imaged under polarized light using an Axioscan microscope (Zeiss) at the 20x objective and using second harmonic generated (SHG) microscopy. SHG images were taken on a TCS SP8 Multiphoton Confocal microscope (Leica, Buffalo Grove, IL, USA) at a fundamental wavelength of 880 nm with the 10x and 40x objective on sections oriented in the same direction for all groups. All SHG images were quantiﬁed using ImageJ and reported as mean pixel intensity within the cortical and cal-

Fig. 4. Periosteal thickness and cellularity developed normally in adult onset-induced, Osterix-conditional, homozygous YAP/TAZ knockout mice. (A) Representative micrographs of 16- to 18-week-old cortical bone stained by aqueous H&E. Dotted lines indicate the periosteum. Quanti-

manually scored as either positive or negative, averaged together from each of the four regions, and reported as percent positively stained per total number of periosteal cells in ImageJ. Periosteal area and average thickness from these same four regions of interest were outlined in ImageJ using both immunofluorescence and aqueous H&E sections and averaged together with 6 to 9 mice per group.

Periosteal cell isolation and osteogenic differentiation

Mouse periosteal cells were isolated from either WT or Osterix-conditional YAP/TAZ-deficient (YAPcKO;TAZcKO) femurs on 4 dpf and cultured at 37°C and 5% O2, as described previously. Briefly, mice were anesthetized by carbon dioxide inhalation and euthanized via cervical dislocation. Fractured limbs were carefully dissected of all non-osseous tissue, the epiphyses were then removed, and marrow cavities were ﬂushed. The periosteum was scraped and enzymatically digested for 1 hour at 37°C on an orbital shaker (0.5 mg/mL collagenase P, 2 mg/mL hyaluronidase in PBS). After washing, 2 × 10^5 cells/cm^2 were seeded in growth medium (α-MEM, 10% FBS, 1% penicillin–streptomycin, and 1 μg/mL doxycycline) and cultured in 5% oxygen for the first 4 days. Half of the media was changed on day 4 and cultures were then incubated in 21% O2. Primary cells reached
confluence by day 7 and were passaged once into osteogenic differentiation experiments.

Passage 1 periosteal cells were then seeded at 21% O₂ into 24-well plates (15 x 10³ cells/cm²) and cultured in growth medium. After reaching confluence, primary periosteal cell cultures were induced toward osteogenic differentiation (50 μg/mL ascorbic acid and 4 mM β-glycerophosphate) for 21 days. Osteogenic media was changed every 2 days before RNA isolation and Alizarin Red staining for mineral deposition at 21 days.

RNA isolation and qPCR

Limbs from 7 and 14 dpf were carefully dissected and removed of all non-osseous tissue. The intramedullary pin was removed, the ends of the femur were cut, and marrow flushed before snap-freezing the entire callus in liquid nitrogen-cooled isopentane for 1 minute before microCT imaging and storage at −80°C until processing. Tissues were then homogenized via mortar and pestle and RNA from the sample was collected using Trizol Reagent (15596026; Life Technologies, Carlsbad, CA, USA) followed by centrifugation in chloroform. RNA from fractured limb tissue and cell culture experiments were purified using the RNA Easy Kit (74106; Qiagen, Valencia, CA, USA) and quantified by spectrophotometry using a NanoDrop 2000 (ThermoFisher Scientific). Quantitative polymerase chain reaction (qPCR) assessed RNA amount using a StepOnePlus Real-Time PCR System (Thermo-Fisher Scientific) relative to the internal control of 18S ribosomal RNA (18S rRNA). Data were presented using the ΔΔCt method. Six mice per group were used. Specific mouse primer sequences are listed (Supplemental Table S1).

Statistics

Sample sizes were selected a priori by power analyses based on effect sizes and population standard deviations taken from published data on the developmental bone phenotype of Osterix-conditional YAP/TAZ deletion.(15) Because fracture healing outcomes tend to have more variability than developmental phenotyping outcomes, we assumed 1.5 times the standard deviation for outcomes from Osterix-conditional YAP/TAZ deletion,(15) which resulted in an effect size of 1.94, a power of 80%, and α = 0.05. From the power analysis, a sample size of six was selected. Some samples were lost during initial protocol optimization. All statistics and power analyses were performed in GraphPad Prism or R (version 3.5.1; GraphPad, La Jolla, CA, USA). Comparisons between two groups were made using the independent t test, whereas comparisons among three or more groups were made using a one-way ANOVA with post hoc Tukey’s multiple comparisons test, if the data were normally distributed according to D’Agostino-Pearson omnibus normality test and homoscedastic according to Bartlett’s test. When parametric test assumptions were not met, data were log-transformed, and residuals were evaluated. If necessary, either the nonparametric Kruskal-Wallis test with post hoc Dunn’s multiple comparisons or the nonparametric Mann–Whitney test were used. A p value <0.05 (adjusted for multiple comparisons) was considered statistically significant.

Fig. 5. Adult onset-inducible, homozygous YAP/TAZ deletion from Osterix-expressing cells impaired callus mineralization but not size. (A) MicroCT reconstructions at 14 dpf. Quantification of 14 dpf callus architecture: (B) total callus volume, (C) mineralized callus percentage, and (D) volumetric mineral density. Data are presented as individual samples in scatterplots and boxplots corresponding to the median and interquartile range. Data were evaluated by one-way ANOVA with post hoc Tukey’s multiple comparisons tests. Groups with significant pairwise comparisons are indicated by bracketed lines and p values adjusted for multiple comparisons. Sample sizes, n = 6–8. Scale bars = 1 mm for microCT reconstructions.
multiple comparisons) was considered significant. On the graphs, exact \( p \) values to three decimal places were indicated above the bracketed lines to indicate significant comparisons.

Post hoc comparisons were performed using adjusted post hoc Tukey’s multiple comparisons test for comparisons among three or more groups or two-tailed \( t \) tests when comparing.
two groups. If a p value is not indicated between two groups, the differences between those groups were not statistically significant. Data are presented as individual samples in scatterplots and boxplots corresponding to the median and interquartile range.

**Results**

Constitutive Osterix-conditional YAP and/or TAZ deletion impaired fracture healing

To evaluate the roles of YAP and TAZ in fracture repair, we used Cre-lox to delete YAP and/or TAZ from Osterix-Cre expressing cells during embryonic development.\(^{[13]}\) We selected a breeding strategy that generated YAP/TAZ allele dosage-dependent Osterix-conditional knockouts with four genotypes (Table 1). Constitutive, Osterix-conditional, dual homozygous YAP/TAZ knockouts were not evaluated because of perinatal lethality.\(^{[15]}\) We then evaluated adult bone fracture repair at 14 and 42 dpf.

All genotypes exhibited callus formation by 14 dpf (Fig. 1A). However, constitutive, Osterix-conditional YAP and/or TAZ deletion reduced total callus volume and mineralized callus percentage (ie, bone volume per tissue volume [BV/TV]) at 14 dpf in an allele dosage-dependent manner (Fig. 1B, C). Similarly, constitutive Osterix-conditional YAP and/or TAZ deletion also reduced mineralized tissue volume and volumetric mineral density at 14 dpf (Supplemental Fig. S1A, B). We then tested 14 dpf limbs in torsion to failure and observed a similar reduction in maximum torque to failure and torsional rigidity. However, work to max torque and work to failure did not reach statistically significant differences between genotypes (Supplemental Fig. S1C, D).

At 42 dpf, all genotypes underwent hard callus formation and remodeling (Fig. 1F). However, constitutive Osterix-conditional YAP and/or TAZ deletion again delayed healing with reduced mineralized callus percentage, maximum torque to failure, and torsional rigidity, but at this time point differences in total callus volume did not reach statistical significance (Fig. 1G–J). At 42 dpf, constitutive, Osterix-conditional YAP and/or TAZ deletion reduced volumetric mineral density but did not significantly reduce mineralized tissue volume, work to maximum torque, or work to failure (Supplemental Fig. S1E–H).

Constitutive Osterix-conditional YAP and/or TAZ deletion reduced callus size

To evaluate the radial distribution of the total callus at 14 dpf, we quantified the polar moment of inertia of the entire callus, including bone, cartilage, and fibrous tissue. Independent of mineralized tissue formation, constitutive, Osterix-conditional YAP/TAZ deletion reduced total callus size, particularly in the YAP\(^{cHET};TAZ\(^{cKO}\) mice, which are homozygous for TAZ deletion and heterozygous for YAP (Fig. 2A, B). At 42 dpf, similar results were observed where the polar moment of inertia distribution of mineralized tissue within the callus was reduced in the YAP\(^{cHET};TAZ\(^{cKO}\) mice (Supplemental Fig. S2A, B). At 42 dpf, we performed an analysis of covariance (ANCOVA) using linear regression to decouple the contributions of callus mineralization and geometric distribution from the mechanical behavior,\(^{[15,27]}\) since constitutive, Osterix-conditional YAP/TAZ deletion reduced both callus mineralization and geometry. We found that individual regression lines for each genotype best predicted maximum torque to failure and torsional rigidity, suggesting that differences in connectivity or composition also contribute to mechanical behavior (Supplemental Fig. S2C, D).

Constitutive Osterix-conditional YAP and/or TAZ deletion impaired periosteal development

The impairment of bone fracture healing observed in the constitutive, Osterix-conditional YAP/TAZ deletion knockout model could be a consequence of defective periosteal stem cell supply, expansion, and/or differentiation. To address this question, we generated adult-inducible, dual homozygous YAP/TAZ knockout mice in which the periosteal progenitor population was allowed to develop normally before fracture. Here, we induced homozygous Osterix-conditional YAP/TAZ deletion 2 weeks before fracture. During those 2 weeks before fracture, inducible Osterix-conditional YAP/TAZ deletion did not significantly affect periosteal cell thickness, area, or cell number (Fig. 4A–D).

To analyze the recombination efficiency of inducible Osterix-Cre-mediated YAP/TAZ deletion after fracture, we evaluated YAP/-TAZ expression in chondrocytes, osteoblasts, and osteocytes.
within the callus at 14 dpf by immunohistochemistry and qPCR (Supplemental Fig. S3A–C). The percent of YAP-positive cells were significantly reduced for chondrocytes (23% reduction), osteoblasts (23% reduction), and osteocytes (26% reduction) (Supplemental Fig. S3D–F). YAP mRNA expression was reduced by 59% in full callus lysate preparations (Supplemental Fig. S3G). The percent of TAZ-positive cells were moderately reduced for chondrocytes (11% reduction; \( p = 0.106 \)) and significantly reduced for osteoblasts (18% reduction), and osteocytes (12% reduction) (Supplemental Fig. S3H–J). TAZ mRNA expression was reduced by 49% in full callus lysate preparations (Supplemental Fig. S3K).

All genotypes underwent initial callus formation by 14 dpf (Fig. 5A). Inducible Osterix-Cre-mediated YAP/TAZ deletion reduced mineralized callus percentage and volumetric bone mineral density, but differences in total callus volume between groups were not observed (Fig. 5B–D). Further, inducible Osterix-Cre-mediated YAP/TAZ deletion qualitatively increased variability in the polar moment of inertia distribution of the total

Fig. 7. Adult onset-inducible, homozygous YAP/TAZ deletion from Osterix-expressing cells impaired periosteal osteoblast precursor cell expansion and osteogenic differentiation. (A) Representation of the regions of interest in the callus “shoulder,” where bone formation initiates by intramembranous ossification and where we evaluated periosteal osteoblast precursor expansion and proliferation. (B) Representative micrographs of EDU+ periosteal cells (red) at 4 dpf, with all nuclei counterstained by DAPI (blue). White dotted lines indicate periosteal cell expansion zone; m = muscle; c = cortical bone. Quantification of the expanded (C) periosteal area, (D) average thickness, (E) number of periosteal cells per bone area (N.Pc/BA), and (F) percentage of EDU+ periosteal cells. (G) Activated periosteal cells isolated from 4 dpf limbs were cultured in osteogenic media for 21 days. (H) Representative Alizarin Red staining of mineral deposition after osteogenic induction. (I) Yap, Taz, Ctgf, and Cyr61 and (J) Runx2, Osx, Alp, and Bsp mRNA expression, relative to 18S rRNA, from periosteal progenitor cell cultures after 21 days of osteogenic induction. Data are presented as individual samples in scatterplots and boxplots corresponding to the median and interquartile range. Data were evaluated either by one-way ANOVA with post hoc Tukey’s multiple comparisons tests or by two-tailed Student’s t tests. Groups with significant pairwise comparisons are indicated by bracketed lines and \( p \) values adjusted for multiple comparisons. \( n = 6–9 \) per group for in vivo data and \( n = 3 \) for in vitro data. Scale bars = 100 μm for all high-power EDU images and 1 mm for high-power Alizarin Red images.
callus size in comparison to YAP\textsuperscript{WT}, TAZ\textsuperscript{WT} and Osx:Cre mice (Fig. 5B; Supplemental Fig. S4A, B).

Inducible Osterix-conditional YAP/TAZ deletion did not alter callus size but reduced bone formation

Because the formation of a cartilaginous callus template is a critical step during endochondral fracture healing,\textsuperscript{3–5} we histologically evaluated cartilage, bone, and fibrous tissue formation at 14 dpf (Fig. 6A, E–G; Supplemental Fig. S5). Consistent with microCT, inducible Osterix-Cre-mediated YAP/TAZ deletion did not affect total callus area at 14 dpf (Fig. 6E). Differences in total cartilage area, percent cartilage area, and the number of Osterix (OSX)-positive hypertrophic chondrocytes were not detected between groups (Fig. 6F–H). Fibrous tissue composition was evaluated by qualitative analysis of H&E- and Picrosirius Red-stained adjacent sections to differentiate between fibrotic tissue and bone, but differences in fibrous tissue were not observed between groups (Supplemental Fig. S5A; Fig. 6B). At 14 dpf, inducible Osterix-Cre-mediated YAP/TAZ deletion did not significantly alter mRNA expression of markers for chondrogenesis, including SRY-Box Transcription Factor 9 (Sox9), Aggrecan (Acan), and collagen, type II, alpha 1 (Col2a1) (Supplemental Fig. SSD).

After formation of the cartilaginous callus, bone formation occurs through both intramembranous and endochondral ossification.\textsuperscript{4} Therefore, we next evaluated endochondral ossification and bone formation at 14 dpf (Fig. 6J; Supplemental Fig. S5A). At 14 dpf, inducible Osterix-Cre-mediated YAP/TAZ deletion reduced the number of OSX-positive osteoblasts per bone surface, relative SHG measurement of collagen organization, and histomorphometric bone area and percent bone area (Fig. 6I–N). Further, inducible Osterix-Cre-mediated YAP/TAZ deletion reduced mRNA expression of markers for hypertrophic chondrocytes, including collagen, type X, alpha 1 (Col10), and vascular endothelial growth factor (Vegfa) (Fig. 6O). Similarly, inducible Osterix-Cre-mediated YAP/TAZ deletion reduced mRNA expression of markers of collagen, including collagen type I, alpha 1 (Col1α1), and collagen type I and alpha II (Col1α2) but not serpin family H member 1 (SerpinH1) (Fig. 6P). Lastly, inducible Osterix-Cre-mediated YAP/TAZ deletion reduced mRNA expression markers of osteogenesis, including osteoblast-specific transcription factor Osterix (Osx) and alkaline phosphatase (Alp), while reductions in

Fig. 8. Adult onset-inducible, homozygous YAP/TAZ deletion from Osterix-expressing cells impaired periosteal osteoblast precursor bone formation. (A) MicroCT reconstructions at 7 days post-fracture (dpf). Quantification of 7 dpf callus architecture: (B) callus volume, (C) mineralized callus percentage, and (D) volumetric bone mineral density. Quantification of total callus histomorphometry at 7 dpf of (E) total callus area, (F) cartilage area, (G) percent cartilage, (H) bone area, (I) percent bone area, and (J) Osterix-positive osteoblasts per bone surface (OSX+N.Ob/BS). (K) Representation of the callus “shoulder,” where bone formation initiates by intramembranous ossification and where we evaluated (L) anti-Osterix (αOSX) immunostaining at 7 dpf. Data are presented as individual samples in scatterplots and boxplots corresponding to the median and interquartile range. When appropriate, an independent t test was used and p values are shown with bracketed lines. If not shown, p value for comparison >0.05. Sample sizes, n = 3–8. Scale bars = 1 mm for microCT reconstructions and 50 μm for micrographs.
runt-related transcription factor 2 (Runx2) or bone sialoprotein (Bsp) did not reach statistical significance (Fig. 6Q).

Inducible Osterix-conditional YAP/TAZ deletion reduced periosteal osteoblast precursor expansion in vivo and osteogenic differentiation in vitro

Given the osteogenic defect resulting from inducible Osterix-Cre-mediated YAP/TAZ deletion, we evaluated activated periosteal progenitors at 4 dpf in four regions distal and proximal to the fracture line, in which periosteal osteoblast precursors are primarily fated to form bone through direction intramembranous ossification(6) (Fig. 7A). At 4 dpf, inducible YAP/TAZ deletion reduced periosteal osteoprogenitor cell expansion in terms of total area and average thickness (Fig. 7B–D). However, inducible YAP/TAZ deletion did not reduce the number of periosteal osteoprogenitor cells per expanded periosteal area but significantly reduced the percentage of proliferating, EdU-positive periosteal osteoprogenitor cells (Fig. 7E, F).

Though unexplored in periosteal osteoprogenitor cells, YAP and TAZ are known to mediate osteogenic differentiation in the mesenchymal stem cells (MSCs),28–30 which originate from a common mesenchymal embryonic lineage.(33) To elucidate if inducible YAP/TAZ deletion regulated periosteal osteoprogenitor differentiation, we isolated activated periosteal progenitor cells at 4 dpf (Fig. 7G–H). After culture for 21 days in osteogenic media, inducible, Osterix-conditional YAP/TAZ deletion reduced mineral deposition stained with Alizarin Red (Fig. 7G–H). As expected, Osterix-conditional inducible YAP/TAZ deletion in vitro reduced mRNA expression of Yap and Taz as well as their canonical downstream target, Ctgf and Cyr61, in periosteal progenitor cells from YAPcKO;TAZcKO mice (Fig. 7I). Lastly, Osterix-conditional inducible YAP/TAZ deletion in vitro reduced mRNA expression of osteogenic differentiation genes, including Runx2, Col1a1, Alp, and Bsp, while reductions in Osx (p = 0.052) did not reach statistical significance (Fig. 7J).

Given the defective periosteal osteoblast precursor expansion and osteogenic differentiation, we histologically evaluated the callus at 7 dpf. All genotypes underwent periosteal expansion and mineralization (Fig. 8A). Further, inducible Osterix-Cre-mediated YAP/TAZ deletion did not alter total callus volume but reduced mineralized callus percentage and volumetric mineral density at 7 dpf (Fig. 8B–D). Significant differences in cartilaginous callus formation or fibrous tissue were not detected at 7 dpf (Fig. 8E–G; Supplemental Fig. S6). Similarly, inducible Osterix-Cre-mediated YAP/TAZ deletion did not significantly alter endochondral ossification, matrix collagen, or osteogenic gene expression at 7 dpf (Supplemental Fig. S7). At 7 dpf, differences in bone area did not reach statistical significance (Fig. 8H, I). Yet inducible Osterix-Cre-mediated YAP/TAZ deletion reduced the percentage of bone within the total callus area (Fig. 8H, I). Lastly, inducible Osterix-Cre-mediated YAP/TAZ deletion reduced OSX-positive osteoblasts per bone surface within regions of the callus undergoing intramembranous bone formation (Fig. 8J–L), consistent with our observations at 4 dpf in vivo and in vitro.

Discussion

This study identifies new roles for the transcriptional regulators, YAP and TAZ, in bone fracture healing, adding to our understanding of periosteal osteoblast precursor cell regulation. Here, we show that YAP and TAZ promote expansion and osteoblastic differentiation of periosteal osteoblast precursors to promote bone fracture healing. Constitutive YAP and/or TAZ deletion from Osterix-expressing cells impaired bone fracture healing by reducing both callus size and subsequent mineralization, due in part to developmental defects in the periosteal progenitor supply. When we allowed for the development of a normal periosteal progenitor population before fracture, adult onset-induced YAP/TAZ deletion did not impair callus size but delayed mineralization as a result of impaired osteoblast precursor cell differentiation. Together, these data demonstrate that the transcriptional co-activators, YAP and TAZ, promote the expansion and differentiation of periosteal osteoblast precursors to accelerate bone fracture healing.

Fracture healing recapitulates many aspects of embryonic skeletal development but features a unique postnatal environment, resulting in contextual differences.3 We previously found that Osterix-conditional YAP/TAZ deletion in the embryo caused a severe skeletal fragility phenotype,(15) whereas Xiong and colleagues induced Osterix-conditional YAP/TAZ deletion at postnatal day 21 (P21) and performed skeletal phenotyping at P84, observing increased osteoblast numbers but no measurable effect on whole bone microarchitecture.(16) Our present data resolve the differences between these two studies, establishing a critical role for YAP and TAZ in the development of the postnatal osteoprogenitor niche and demonstrating critical roles for YAP and TAZ in osteoblast precursor proliferation and differentiation in a context of rapid bone formation, similar to that which occurs during bone development, in contrast to postnatal growth and homeostasis. The present data are further consistent with other reports. For example, deletion of the YAP/TAZ-regulated transcription factors, Snail and Slug, from Osterix-expressing cells reduced both the proliferative potential of bone surface-associated osteoprogenitors and osteogenic differentiation capacity of adult skeletal stem cells.32 Similarly, conditional deletion of YAP from Osteocalcin-expressing cells reduced osteoblast progenitor cell proliferation and differentiation, further supporting a role for YAP and TAZ in promoting osteoblast progenitor cell function.17

Endochondral bone fracture repair includes both the formation of a cartilage template as well as subsequent osteoblast-mediated mineralization.3–5 Here, we found that while constitutive deletion impaired callus size, adult onset-inducible YAP/TAZ deletion did not significantly affect cartilage formation during fracture healing. A previous study found that YAP overexpression in developing chondrocytes, using the Col2a1 promoter, as well as deletion of MST1/2 using Dermo-Cre impaired endochondral fracture healing,33 which appears to contradict the results described here. However, both models exhibit a developmental skeletal phenotype before fracture33 and, in particular, observed that YAP/TAZ negatively regulates chondrogenesis. Thus, inducible targeting models are needed to decouple the developmental history from the process of fracture repair.34,35 Here, we selected the tetOFF Osterix-Cre inducible system instead of the tamoxifen-inducible Osterix-CreERT2,(36) as tamoxifen is rapidly cleared,(37) resulting in transient Cre-activity38 in newly generated Osterix-positive cells during fracture repair. Furthermore, targeting conditional gene inactivation in chondrocytes versus osteoblast-lineage cells can result in drastically different phenotypes. For example, conditional deletion of Runx2 in chondrocytes using Col2a1-Cre phenocopied global Runx2 gene inactivation with perinatal lethality and a lack of mineralization, while conditional deletion of Runx2 in osteoblasts using 2.3 kb-Col1a1-Cre resulted in a moderate low bone mass phenotype.39,40 Nonetheless, hypertrophic chondrocytes are
known to express Osterix during endochondral ossification,\(^{(41,42)}\) and we observed moderate Osterix-Cre-mediated YAP/TAZ recombination in hypertrophic chondrocytes, suggesting that the relative contributions of YAP and TAZ during endochondral ossification are potentially stage-dependent.\(^{(15-17,33)}\) Future studies will identify the temporal and cell-specific contributions of YAP and TAZ to bone development and repair.

Recent and ongoing studies have revealed remarkable diversity in both the cellular identity and regulatory signals that contribute to periosteal function. GH1,\(^{(43)}\) Prx1,\(^{(31)}\) αSMA,\(^{(44)}\) cathepsin K,\(^{(17)}\) and Osterix\(^{(45)}\) mark both overlapping and distinct periosteal progenitor cell populations, while markers previously thought to define bone marrow stromal cells, including CD73, CD90, CD105, PDGFR\(\alpha\), Gremlin 1, Cxcl12, and Nestin,\(^{(31,46-49)}\) also show high expression in the periosteal progenitors. Ongoing efforts continue to uncover new skeletal progenitor cell populations that contribute to fracture repair,\(^{(48-52)}\) and the intersection of this cellular diversity with YAP/TAZ signaling remains unclear. Here, we observed significant reductions in mRNA expression of endochondral-, collagen-, and osteogenesis-related gene expression signatures at 14 dpf, which can be explained either by indirect shifts in the cell populations that express these targets or by direct YAP/TAZ-mediated transcriptional regulation of those genes. Further research will be required not only to systematically identify the transcriptional co-effectors of YAP and TAZ in each cell type of interest, but also to delineate the periosteal progenitor subpopulations affected by YAP/TAZ signaling.

YAP and TAZ may regulate osteoblast precursor cell proliferation and osteogenic differentiation through a variety of mechanisms. Conditional deletion of YAP in osteoblasts using Osteocalcin-Cre reduced osteoblast progenitor proliferation as well as osteogenic differentiation and proposed YAP stabilized β-catenin to promote β-catenin-mediated osteogenesis.\(^{(17)}\) However, evidence exists for YAP and TAZ playing both a positive and negative role in WNT/β-catenin signaling,\(^{(33,34)}\) suggesting that further investigating into YAP/TAZ-dependent regulation of this pathway in periosteal progenitors is needed. A similar study demonstrated that Snail and Slug form stable protein–protein complexes with both YAP and TAZ in tandem to promote osteoprogenitor proliferation and differentiation.\(^{(32)}\) In osteoprogenitors, the Snail/Slug-YAP/TAZ axis promotes proliferation by interacting with TEAD to enhance TEAD-dependent transcriptional activity and downstream expression of YAP/TAZ-TEAD target genes, such as Ctgf and Ankrtd.\(^{(32)}\) In contrast, the Snail/Slug-YAP/TAZ axis promotes osteogenic differentiation via Snail/Slug-TAZ interactions with Runx2 to promote Runx2-dependent transcriptional activity and downstream expression of osteogenic target genes, such as Osterix and Alp.\(^{(32)}\) Accordingly, evidence for TAZ interacting with Runx2 to promote downstream osteogenic gene expression in vitro is strong,\(^{(50,55)}\) but YAP has been observed to both inhibit and promote downstream osteogenic gene expression in vitro.\(^{(56-59)}\) Thus, future studies to identify the molecular mechanisms by which YAP and TAZ control periosteal progenitor expansion and differentiation are needed.

This study has several limitations. First, small sample sizes (n = 3–9) produced underpowered analyses for some fracture-healing outcome measures, in particular histomorphometric analysis of cartilage. Nonetheless, we did observe statistically significant differences in osteogenic outcome measures of fracture healing in both the constitutive and inducible model, suggesting a larger effect size of Osterix-mediated YAP/TAZ deletion on osteogenesis compared with chondrogenesis during fracture healing. Regardless, not all the studies performed had sufficient power to compare sex as a variable, although we did not observe sexually dimorphic behavior for any outcome measure, and our prior assessment of YAP/TAZ regulation of bone development did not show an effect of sex.\(^{(15)}\) Second, we used an open fracture model, which may affect the kinetics and immunology of the fracture repair process.\(^{(50,61)}\) We initially began these experiments using a closed fracture model, following the Einhorn method,\(^{(62)}\) but this produced a high percentage of comminuted fractures in the constitutive, Osterix-conditional YAP and/or TAZ knockout genotypes. We therefore moved to an open fracture model in which the bending moment could be applied with lower kinetic energy. This observation suggests that YAP/TAZ deletion during development impaired the bone matrix fracture toughness, consistent with our prior report on the bone fragility phenotype.\(^{(15)}\) However, in the current study, we did not observe differences in post-fracture work to maximum load or work to failure, suggesting potential differences between post-yield plastic deformation and elastic deformation of the calluses. Third, adult onset-inducible Osterix-conditional knockout increased variability in response to fracture, adding an additional layer of complexity to the already challenging study of endochondral bone fracture healing biology.\(^{(5)}\) The drug used to prevent Cre-mediated recombination, doxycycline, is a tetracycline derivative. Tetracycline exhibits high affinity for exposed mineral and is therefore commonly used as a label for dynamic bone histomorphometry.\(^{(63)}\) Potential embedding of doxycycline into the bone matrix during skeletal development and subsequent release after fracture could impair robust Cre-recombination and reduce the observed effect size for adult onset-inducible YAP/TAZ knockout mice. We recommend additional study to quantify the kinetics of tetOff inducible systems and efficiency of Cre-mediated inducible recombination in bone. Fourth, although callosus size was equivalent in inducible knockout mice, we did not measure to ensure an equal length of cortical bone in samples prepared for PCR, which may contribute to the variability of observed outcomes. Lastly, the Osterix-Cre transgene is known to cause fracture callus formation\(^{(62)}\) and defects in craniofacial development\(^{(64)}\) depending on genetic background. However, on this background, we did not observe differences between Osx-Cre and YAP\(^{WT}\);TAZ\(^{WT}\) wild-type mice, demonstrating phenotypic specificity for YAP/TAZ deletion.

In conclusion, this study identifies the transcriptional co-activators, YAP and TAZ, as regulators of bone fracture healing that promote periosteal osteoblast precursor proliferation and osteogenic differentiation to accelerate bone healing. Further elucidation of the mechanisms by which YAP and TAZ control the periosteal progenitor cell response to fracture may help guide the development of future targeted therapies to enhance bone fracture healing.

**Disclosures**

All authors state that they have no conflicts of interest.

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PEER REVIEW

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