Bone 103 (2017) 12–19

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Sox9 positive periosteal cells in fracture repair of the adult mammalian long bone

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ARTICLE INFO

Article history:
Received 4 March 2017
Revised 2 June 2017
Accepted 11 June 2017
Available online 13 June 2017

Keywords:
Fracture healing
Skeletal progenitors
Periosteal cells
Sox9

ABSTRACT

Introduction: The phases of fracture healing have been well characterized. However, the exact source and genetic profile of the skeletal progenitors that participate in bone repair is somewhat unclear. Sox9 expression in skeletal elements precedes bone and cartilage formation and a Sox9$^+$ cell type is retained in the adult periosteum. We hypothesized that Sox9$^+$ periosteal cells are multipotent skeletal progenitors normally participating in fracture repair.

Methods: To test this hypothesis we used tamoxifen (TM)-mediated lineage tracing of Sox9$^+$ cells in *Sox9CreErt2:Td-Tomato* mice. Intact femora were analyzed with immunostaining and RNA sequencing to evaluate the skeletal distribution and gene expression profile of Td-Tomato positive, Sox9-descendent cells in the adult femur. To assess the role of Td-tomato + cells in the fracture healing process, mice underwent a closed mid-diaphyseal femoral fracture. Fractured hind limbs were analyzed by X-ray, histology and immuno-staining at 3, 9 or 56 days post-fracture.

Results: In the intact adult mouse femur, Td-Tomato-labeled cells were observed in the primary spongiosa, periosteum and endosteum. RNA sequencing showed that Td-Tomato positive periosteal cells were co-enriched for *Sox9* transcripts, and mRNAs for osteoblast and chondrocyte specific genes. In a femoral fracture model, we showed that pre-labeled Td-Tomato positive descendent cells were mobilized during the early stages of bone repair (day 3 post-op) contributing to the fracture repair process by differentiating into chondrocytes, osteoblasts and osteocytes.

Conclusion: A Sox9$^+$ skeletal progenitor population resides in the adult periosteum. Fate tracing studies show that descendants of the Sox9$^+$ periosteal progenitors give rise to chondrocytes, osteoblasts and mature cortical osteocytes in repair of the fractured femur. To our knowledge this is the first report of a reparative Sox9$^+$ progenitor population in the periosteum of the adult long bone. Taken together with developmental studies, our data suggest a broad role for Sox9$^+$ osteochondroprogenitors in development and repair of the mammalian skeleton.

1. Introduction

Fracture healing is a complex process that involves the well-orchestrated participation of growth factors, cytokines and several cell types [1]. Most fractures are treated with a form of fixation that provides stability, but allows for some degree of motion (sling or cast immobilization, external fixation, intramedullary fixation). Thus the majority of fractures heal by secondary or indirect bone healing, a process that involves both intramembranous and endochondral ossification [2]. Secondary bone healing involves three major phases: the reactive phase (hematoma and inflammatory response), the reparative phase (soft and hard callus formation) and the remodeling phase [3]. Briefly, a fracture leads to surrounding soft tissue trauma, damage to local blood vessels and disruption of the bone marrow structure. Wound healing pathways are activated and a hematoma is formed at the area of the injury. Inflammatory cells and activated platelets soon infiltrate the hematoma and start secreting cytokines that can stimulate angiogenesis and initiate cellular events associated with the later stages of fracture healing [4]. Before the inflammation stage subsides, the repair process is initiated. An early indication of skeletal repair is the appearance of a chondrocyte-derived cartilage template that bridges and temporarily stabilizes the fractured bone fragments (soft callus). The cartilaginous callus serves as a template for formation of the hard

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http://dx.doi.org/10.1016/j.bone.2017.06.008
8756-3282/

bony callus by osteoblasts. Eventually the cartilage is eliminated from the callus that is composed of woven bone. Finally a remodeling process, dominated by osteoblasts, osteocytes and osteoclasts, returns the newly formed bone to its original bone configuration.

Despite the fact that the phases of bone healing have been well characterized, the cellular origins and molecular pathways underlying bone healing are somewhat unclear. Several possible sources of skeletal progenitor cells for bone healing have been reported [5] including endosteum [6,7], periosteum [7–10], bone marrow [8,11,12], vascular walls [13] and adjacent soft tissue [14]. Of these, a pivotal role for periosteum-derived progenitor cells in bone healing has been confirmed in several *in vitro* and *in vivo* studies, though it is not clear if this is a general property of periosteal cells, or a property restricted to distinct osteochondroprogenitors within this tissue [15].

In contrast to bone repair, the cellular mechanisms underlying bone development during embryogenesis have been well documented. Here, the *SRY* (*Sex Determining Region Y*)-*Box9* (*Sox9*) transcription factor plays an essential role in determining skeletal progenitor cells' fate prior to overt chondrocyte and osteoblast development [16]. Thereafter, this osteochondroprogenitor cell population segregates into $Sox9^{+}$ chondrocyte progenitors and $Sox9^{-}$, $Runx2/Sp7^{+}$ osteoblast progenitors that deposit cartilage and bone, respectively [16,17]. Sox9 is necessary for establishing skeletal elements in the cranial, axial and appendicular systems [18–20]. In addition, Sox9 is sufficient to initiate chondrogenic programs when activated in mesenchymal stem cells, embryonic stem cells and even fibroblasts [21–24].

Fracture healing has been characterized by many as the postnatal analogue of embryonic skeletal development, since many of the molecular mechanisms that control differentiation and growth during embryogenesis recur during fracture repair [25]. Since Sox9 defines osteochondroprogenitor cells during skeletogenesis and a similar differentiation program is likely shared between skeletal development and adult long bone repair, we hypothesized that Sox9 might play a major role in adult long bone repair. In this study, we demonstrate that an osteochondroprogenitor cell population positive for Sox9 resides in the periosteum of adult long bones and that upon activation by fracture stimulation, these osteochondroprogenitor cells direct fracture repair, differentiating into chondrocytes, osteoblasts and osteocytes.

2. Material and methods

2.1. Mouse lines and lineage tracing

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Southern California (IACUC # 11892) and carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. A double heterozygous *Sox9CreErt2:Td-Tomato* mouse line was used for the lineage tracing experiments. These double heterozygous mice, carrying one allele of *Sox9CreErt2* driver and one allele of *Td-Tomato* reporter, were generated by crossing heterozygous *Sox9CreErt2* (*$Sox9^{tm1(cre/ERT2)Haak}$*) [26] with homozygous *Td-Tomato* (*$Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze}$*) [27] mice purchased from the Jackson Laboratory (https://www.jax.org. Bar Harbor, ME). The mice received 3 intraperitoneal injections of 100 μL tamoxifen (TM) solution (20 mg/mL diluted in corn oil, Cat# T5648-1g, Sigma-Aldrich, St. Louis, MO), at 48-hour intervals, 2 weeks pre-euthanasia or pre-operatively depending upon the group, to induce Td-Tomato reporter expression in Sox9-expressing cells and their descendants. Thirty-six, 12–16-week old, *Sox9CreErt2:Td-Tomato* mice, were used in the study; 24 mice for analysis of intact adult femora and 12 for the femoral fracture assay. Intact mouse femora were harvested 2 weeks after the last tamoxifen injection and analyzed with a) frozen histology and immunostaining, to evaluate the distribution of Sox9CreErt2 + descendent cells in adult long bones and b) RNA sequencing, to determine the gene expression profile of periosteal cells of the femur. The remaining mice were used to assess the contribution of Sox9CreErt2 + cells at different stages of the fracture healing process.

2.2. Femoral fracture

Twelve animals received 3 consecutive doses of TM, 2 weeks before a closed mid-diaphyseal femoral fracture was created unilaterally using an established fracture model [28–30]. Briefly, the mice were anesthetized with inhalational anesthesia (2% isoflurane) and their left hind limbs were shaved and prepared with three alternating scrubs of betadine and 70% isopropyl alcohol. Using aseptic surgical technique, a 3 mm incision was made medial to the patellar tendon. The patella was dislocated laterally to expose the femoral condyles. A small hole was then drilled into the trochlear groove and a 26-gauge needle was inserted in retrograde fashion into the femoral intramedullary canal, not exiting through the greater trochanter. The dislocated patella was reduced and a careful closure with absorbable sutures was performed. A closed, mid-diaphyseal femoral fracture was then created using a modified Bonnarens & Einhorn's fracture apparatus described by Marturano et al. [28,29]. Radiographic images of the fractured femora were obtained right after intramedullary fixation/fracture creation to verify production of a transverse, mid-diaphyseal fracture. Post-operatively, mice received buprenorphine subcutaneously for 2 days and antibiotics through the drinking water for 5 days. The animals were allowed to bear weight immediately and to eat and drink *ad libitum*.

The mice were euthanized at different time points post-operatively (1, 3, 9, and 56 days) and fractured and contralateral normal limbs harvested for further analysis (radiographic evaluation, H&E and safranin O/Fast green histology and frozen histology and subsequent immunostaining).

2.3. Radiographic evaluation

Radiographs of the fractured femora were obtained using a Faxitron X-ray device (Faxitron Bioptics, Tucson, AZ) immediately post-fracture, to verify the type of fracture and initial pin fixation, and at the time of euthanasia, to monitor callus formation at different time points (Postoperative days 1, 3, 9, 28 and 56).

2.4. Histologic analysis

After limb harvesting, dissection of the adjacent soft tissue, and removal of the intramedullary fixator for the fractured femora, limb specimens were processed for histology. Fractured femora were analyzed with both standard and frozen decalcified histology, whereas intact femora were processed for frozen histology only.

For standard histology, fractured femora were fixed in 10% formalin for 24 h, decalcified in 10% EDTA for 14 days at room temperature, then embedded in paraffin and cut longitudinally. Sections were stained with H&E or Safranin O/Fast green and imaged using a Zeiss Axio Imager 2 microscope (Carl Zeiss Microscopy, Thornwood, NY).

For decalcified frozen histology specimens were fixed in 4% paraformaldehyde/PBS (PFA) for 4 h and decalcified in 14% EDTA/PBS for 14 days. Next, samples were soaked in 30% sucrose/PBS overnight at 4 °C. After embedding in Tissue Tek OCT compound (Cat#25608-930; VWR, Radnor, PA), samples were cut longitudinally with a cryostat (Leica, Nussloch, Germany) to generate 8–10 μm sections which were mounted onto glass slides. Sections were stored at −20 °C until use.

2.5. Immunostaining

Immunostaining was carried out following a previously established protocol [22]. Briefly, frozen sections were washed with PBS for 3 × 5 min, then fixed again in 4% PFA for 15 min. Fixed sections were treated with 0.1 M Glycine/PBS for 25 min and washed