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Post Natal Expression of Prx1 Labels Appendicular Restricted Progenitor Cell Populations of Multiple Tissues

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Abstract

Currently, there is no consensus whether there is a single or multiple postnatal stem cell population(s) that contribute to skeletal homeostasis and post-natal bone formation. A known population of cells that express Prx1 contributes to post-natal bone formation. Prx1 expression also connotes calvaria and appendicular tissues during embryonic development. A transgenic tamoxifen inducible Prx1 reporter mouse was used for lineage tracking, to characterize the post-natal contribution of Prx1 expressing cells in skeletal homeostasis and bone formation. Under homeostatic conditions Prx1 labeling gave rise to a transient yet rapid turnover cell population at the periosteal and endosteal surfaces, along muscle fibers, and within the medial layers of vessels both within the muscle and marrow compartments of the appendicular skeleton. Fracture and ectopic bone formation of both fore and hind limbs showed recruitment and expansion of Prx1-derived cells in newly formed bone tissues. Prx1 labeled cells were limited or absent at axial skeletal sites during both homeostasis and after induction of bone formation. Lastly, Prx1-derived cells differentiated into multiple cell lineages including vascular smooth muscle, adipose, cartilage, and bone cells. These results show that Prx1 expression retained its embryonic tissue specification and connotes a stem/progenitor cell populations of mesenchymal tissue progenitors.

Keywords

Prx1; MSC; skeletal stem cell; Fracture; Bone Formation

Introduction

The postnatal animal has a sizable capacity to form bone tissue. This is observed with fracture repair and heterotopic ossification (ectopic bone). Both processes depend on the recruitment of skeletogenic stem and progenitor cells (SSPCs). The periosteum, endosteum, marrow, nerves, muscle, and blood vessels are all reservoirs of adult SSPCs known to contribute to fracture repair following injury, however, less is known if the cells within these differing sites also contribute to ectopic bone formation or their function during homeostatic

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bone turnover (Bragdon, Beth C. & Bahney, 2018). The cell populations of SSPCs that have been identified to contribute to fracture repair and injury induced ectopic bone formation are however marked by the expression of paired related homeobox, Prx1 (*Prrx1*) (De Lageneste et al., 2018; Esposito, Wang, Li, Miranda, & Spagnoli, 2020; Kawanami, Matsushita, Chan, & Murakami, 2009; Wang, Zhang, & Bikle, 2017). This population of Prx1 expressing cells are located within the calvaria sutures, periodontal ligament, the periosteal and bone marrow of long bones, and adipose tissue in the postnatal animal (Bassir et al., 2019; De Lageneste et al., 2018; Kawanami et al., 2009; Sanchez-Gurmaches, Hsiao, & Guertin, 2015; Wilk et al., 2017). During embryogenesis Prx1 marks the limb bud mesenchyme of the developing limbs and calvaria (Logan et al., 2002). Multiple studies have used the Prx1 Cre transgenic mouse model to lineage trace this population during fracture repair or to analyze the functional roles of proteins in a mesenchymal stem cell population (De Lageneste et al., 2018; Esposito et al., 2020; Kawanami et al., 2009; Wang et al., 2017). Although the Prx1 population is well characterized during embryogenesis and postnatal adipose tissue distribution, it is unclear as to its distribution and contribution to postnatal skeletal tissue formation during homeostasis and injury response.

In this study the location and terminal differentiation of the Prx1 population of cells was fully characterized in vivo using the tamoxifen inducible Prx1 Cre ERT transgenic reporter mouse during bone homeostasis and three models of postnatal bone formation, a closed stabilized femoral fracture and the implantation of demineralized bone matrix (DBM) to induce ectopic bone in the muscle or orthotopic bone at the femoral periosteum. The use of both surgical models allows for the postnatal bone formation in context to injury (fracture) and without injury (orthotopic and ectopic bone). The DBM implantation model was further exploited to determine whether the Prx1 cell population remains restricted to the limbs as seen during embryogenesis, by specifically placing the DBM used to induce ectopic bone in different anatomical sites at the appendicular and axial skeleton.

Materials and Methods

Animal Studies

All animal studies were approved by the Institutional Animal Care and Use Committee at Boston University. Mice were housed under standard conditions and randomly assigned to time point and implant group.

Gene expression studies for ectopic and orthotopic bone utilized the adaptive immune-deficient male mice, B6,129S7-Rag1$^{tm1/MOM}$/J ((Rag1) Jackson Laboratory, Bar Harbor, ME). All animals were 10–13 weeks old with three to five animals enrolled for each time point, post-operative day (POD) 8 and 16. The C57BL/6J (B6) male mice (Jackson Laboratory, Bar Harbor, ME) were enrolled for the gene expression study with fracture repair. One time point was used at POD 5 with three animals enrolled.

All lineage tracing studies used the Prx1 transgenic reporter mouse which was bred in house using the following mouse crosses. The Prx1CreER-GFP mouse (Kawanami et al., 2009) was crossed with the reporter mouse strain B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J (Ai14 from Jackson Laboratories to produce Prx1CreER-GFP/Ai14 offspring. These mice

were then crossed with the Rag1 mice resulting in Prx1CreER-GFP/Ai14/Rag1. These transgenic Ai14 reporter animals express CreER under control of the Prx1 promoter and are Rag1 null allowing for the use of human DBM implantation. Both male and female mice were enrolled for the implant and fracture studies. Animals were eight to nine weeks old at time of first tamoxifen injection. For the orthotopic and ectopic studies, animals were enrolled for time points, POD 8 and 16 while the fracture study used time points POD 5 and 23. Three animals were enrolled for each time point.

Surgical Models

The implantation of demineralized bone matrix (DBM) induces bone tissue at the periosteal surface of the femur (orthotopic bone) and within the muscle to induce ectopic bone as previously described (Bragdon, Beth et al., 2017). Additional details are in supplemental. For the chest implantation, mice were prepared for surgery as described, (Bragdon, Beth et al., 2017). Approximately 0.75cm incision was created at the sternum moving laterally toward the ribs. The fascial plane was cut, careful not to puncture the chest cavity, and a pouch was created using forceps. Fifty milligrams of human DBM was implanted and the incisions were closed. For the back/spine implants, mice were placed in the prone position. Ribs were identified and used to mark the location to start the incision on the back. The fascia was cut and fifty mg of human DBM was implanted near the thoracic vertebrae. The incisions were closed. Closed stabilized transverse fractures were generated at the mid-shaft of the right femur as described (Lybrand, Bragdon, & Gerstenfeld, 2015).

Histology

Tissues were harvested with surrounding soft tissue being minimally trimmed and samples were kept at 4°C while being processing for histology. 4% Paraformaldehyde (Sigma-Aldrich, St. Louis, MO) was used to fix samples for three to four days followed by decalcification using 14% Ethylenediaminetetraacetic Acid (EDTA) (Acros Organics, NJ) at approximately one week. Samples were incubated with 7.5% sucrose/PBS solution followed by 30% sucrose/PBS, then incubated in a 1:1 mixture of 30% sucrose/PBS and Tissue Plus Optimum Cutting Temperature compound. Samples were embedded using OCT and frozen blocks were stored at −80°C. Tissues were sectioned using a Reichert Jung Cryocut 1800 (Wetzlar, Germany) with sections being 8–10 μm thick.

Sections were washed with PBS and mounted using Molecular Probes Prolong Gold Antifade with DAPI. Samples of the calvaria, rib/sternum, spleen, kidney, and liver were washed with PBS and incubated with CellMask Green Plasma Membrane Stain (1:1000) at room temperature for 15 minutes. Sections were washed with PBS and mounted with Molecular Probes Prolong Gold Antifade with DAPI. Images were collected within 24 hours using an Olympus BX51 (Olympus America, Inc., Center Valley PA) with the 10x and/or 20x objective and the cellSens Dimension software (version 4.1.0.0, Olympus America).

Immunofluorescence

Sections were washed with 1X PBS and between each step of labeling. Samples were blocked (5% FBS in 1X PBS) for 60 minutes and incubated with primary antibody at 4°C overnight followed by incubated with secondary antibody for one to two hours at room

temperature protected from light. Prolong Gold Antifade Reagent with Dapi was used for mounting. Antibodies used: SMA goat (1:400 (Sigma 2500963)), Perilipin D1D8 rabbit (1:200 (Cell Signaling Technology 9349)), Osterix rabbit (1:100 (Abcam 22552)), and ColX rabbit (1:1000 (Molecular Probes A21206)). Labeling of SMA and Perilipin, Triton X-100 (0.1%) was added to blocking buffer (5% FBS in 1X PBS) and antibody dilution buffer (1% BSA in 1X PBS). Secondary antibody used with SMA was Alexa Fluor 488 anti goat (1:1000 (Molecular Probes A21467)). For ColX, secondary antibody was Alexa Fluor 488 anti rabbit (1:1000 (Molecular Probes A21206)). The Alexa Fluor 488 anti rabbit (1:2000) was used with Perilipin and Osterix primary antibodies. Primary and secondary antibodies were diluted using 1% BSA in PBS. Labeling with secondary antibodies were used as negative controls.

Statistics

The gene expression data is plotted for each individual sample which are represented by a dot on the graph while the mean for each time point is represented by a black bar. Sample size is based on previously published results (Bragdon, Beth et al., 2017). Standard error is represented by error bars. One way ANOVA was performed followed by Tukey test to identify significant differences (JMP Pro version 14.1.0 (SAS Institute Inc.; Cary, NC)).

Results

The localization of the Prx1-derived cell population during homeostasis

Lineage tracking was used to determine the localization and distribution of the Prx1 cell population under homeostatic conditions. We used the transgenic reporter mouse strain, Prx1CreER-GFP/Ai14/Rag1, which allows for tamoxifen induction and Prx1 conditional expression of dTomato. While exposed to tamoxifen, the Prx1 expressing cells and hence all progeny will express dTomato. Following tamoxifen exposure, only the cells derived from these originally exposed cells will express dTomato allowing for temporal lineage tracking of a specific population of cells. Cells actively expressing Prx1 should also express GFP independent of tamoxifen, however, we found the GFP to be not detectable (Supplemental 1). Samples were collected from multiple skeletal sites at three days post injection to identify the population of cells actively expressing Prx1. The expression of *Prrx1* was also surveyed across multiple tissues to examine its body wide distribution (Supplemental 2). The dTomato positive cells were not present in the liver and spleen, and only a few dTomato cells were identified within the kidney. There were multiple cells found within the rib/sternum, calvaria, femur, and appendicular musculature. Within the appendiculum, the majority of dTomato cells were localized to the endosteal bone surfaces with a small number of cells located within the bone marrow and central blood vessel within the bone marrow; not the vessels within the surrounding muscle. A small number of dTomato cells were also located at the growth plate near the secondary ossification center (Figure 1). While a limited number of dTomato cells were identified at the periosteal surface, these cells did not appear to be as bright as the endosteal cells. The muscle contained many smaller dTomato cells on the periphery of the muscle fibers. Immunofluorescence for Osterix was used to determine cells that have committed to the osteogenic lineage. At the growth plate and periosteum, the Osterix positive osteoblast population was distinct from the Prx1

dTomato cell population. At the endosteal surface, some of the Prx1 dTomato cells were also Osterix positive suggesting that Prx1 expression is being co-expressed within a committed osteogenic cell population (Supplemental 3).

Femur/muscle samples were also collected at 17, 31 days and 5 months post tamoxifen injection (Figure 1). At day 17, there were many more dTomato cells lining the periosteal surface and localized to the growth plate and vessels. The muscle tissue continued to show a greater number of bright and larger dTomato cells with many cells now found in the fascial planes between the muscle bundles. At this time dTomato cells were also observed in the vascular smooth muscle (tunica media) layer of intermediate and larger vessels in the muscle compartment. By day 31, the dTomato cells returned to their three day post labeling localization with now a few cells incorporated into the cortical bone. Majority of cells once again, were small and dim within the periosteum and muscle. At five months, a portion of the stem cell population has again expanded into the same heterogenous populations seen at day 17 post labeling, suggesting a continuing process of transient stem cells turning over with a fraction of cells continuing to expand into more progenitors and terminal progeny.

Prx1-derived cells contribute to post-natal bone formation

The expression and contribution of the Prx1 population of cells to post-natal bone generation was investigated by inducing ectopic and orthotopic bone (figure 2–4) and fracture repair (figure 5). Previous studies showed DBM induces endochondral ossification with peak chondrogenesis and peak osteogenesis at POD 8 and POD 16, respectively (Bragdon, Beth et al., 2017). There was an upregulation of *Prrx1* mRNA expression at both ectopic bone tissue sites and fracture repair (Figures 2 and 5). Although there was an increased expression of Prx1, it was not clear if the Prx1 stem and progenitor cells expand to contribute to bone formation or if a population of cells are induced to express Prx1 in response to the DBM implantation and fracture. Therefore, lineage tracking was performed.

Based on our initial studies in figure 1, animals were dosed with tamoxifen followed by a month washout period to allow for tamoxifen to be fully eliminated from the animal. This labeling protocol produces a more definitive lineage tracking of the stem cell by creating a bias towards the tracking of the stem cells at the time of new bone is induced and not the cell population(s) that express Prx1 following stimulation or injury. This is also based on the turnover of the transient progenitors seen at between day 17 and one month. Samples were collected at POD day 8 and 16 for ectopic and orthotopic bone formation (Figures 3 and 4) and POD 5 and 23 for fracture repair (Figure 5). Each time point represents early and late phases of endochondral ossification for each bone model respectively.

At POD 8, Prx1-derived cells were recruited to the sites of DBM implantation for ectopic and orthotopic bone development (Figure 3). Hematoxylin and eosin was used to identify areas of the DBM implant and tissue response. Serial sections were used to identify the fluorescently labeled dTomato cells that corresponded to the regions of interests. Nuclear staining with dapi was used to identify cells. The Prx1-derived cells were primarily located to the periphery of the implant of the ectopic bone. While the orthotopic samples showed Prx1-derived cells contributing to all regions of the developing bone tissue, including a hypertrophic chondrocyte region and an osteogenic region. By POD 16, bone tissue was

observed with Prx1-derived cells contributing to both the ectopic and orthotopic bone tissue (Figure 4). Within the ectopic bone, the Prx1-derived cells were more disbursed throughout the tissue compared to POD 8. The Prx1-derived cells were also found throughout the orthotopic bone tissue, but at a higher density along the surface of the orthotopic bone.

During fracture healing, the Prx1-derived cells were recruited to the site of the fracture as early as POD 5 and contributed to callus formation at POD 23 (Figure 5). Similar to the development of the orthotopic bone, the Prx1-derived cells were located in regions of developing cartilage and at a high density at the surface of the fracture callus at the later time point. Besides the surface, the Prx1-derived cells were also localized to the fracture callus gap.

In Vivo Multipotency of Prx1 derived cells

During post-natal bone formation, the Prx1-derived cells are localized to the regions of cartilage and bone. In addition, Prx1-derived cells were also localized within vasculature tissues. Sanchez-Gurmaches et al also showed that Prx1 cells can differentiate to adipocytes in vivo (Sanchez-Gurmaches et al., 2015). These results suggest that the Prx1 cell population is multipotent. In order to confirm the different lineages of the Prx1 cells, histological sections were used from the different bone forming experiments and immunofluorescence was performed (Figure 6). Smooth muscle actin (SMA) was used to label smooth muscle of blood vessels, perilipin labeled adipocytes, collagen X (ColX) labeled hypertrophic chondrocytes, and osterix marked osteoblasts. The Prx1-derived cells were found to co-localize with the four different lineages: smooth muscle, adipocytes, hypertrophic chondrocytes, and osteoblasts. These data suggest that the Prx1 population of cells are multipotent.

Anatomic restriction of Prx1 recruitment during post-natal bone formation

During embryogenesis, the Prx1 cell population is restricted to the developing appendicular skeleton and calvaria. In the post-natal animal, Prx1 expression is detected in the femur as well as the calvaria and the Prx1-derived cells contribute to post-natal bone formation at both sites. Surprisingly, the ribs also showed high levels of Prx1 expression (Supplemental 2). During development, the Prx1 cells have not been shown to contribute to the ribs or sternum. It is unclear if the contribution to post-natal bone formation by the Prx1-derived cells continue to be restricted to the skeletal sites that are observed during embryogenesis and therefore are not able to be recruited to other anatomical sites (chest and spine). Lineage tracking was used with the DBM implantation model to induce post-natal bone formation at the forelimb, chest (near sternum and ribs), and back (near spine) shown in Figure 7. Samples were collected at POD 16, when mineralized bone tissue is formed. Radiological images were taken to confirm that mineralized bone tissue was induced at the various sites. Fast green and Safranin O stain was then used to identify the DBM-induced bone tissue and surrounding soft tissues. All three sites demonstrated mineralized bone tissue present at the DBM implant sites. Throughout the bone tissue that developed within the forelimb there was recruitment and contribution of the Prx1-derived cells. Similar to the orthotopic bone found at the periosteum, there was a high density at the surface. Sections of the spinal implant however did not show any Prx1-derived cells being recruited or contributing to the

ectopic bone tissue. Surprisingly, the ectopic bone at the chest site showed two patterns of contribution. The Prx1-derived cells were only contributing to small regions of the boney shell and the amount of contribution was varied between samples. However, Prx1-derived cells were mostly absent from the center of the developing bone tissue.

Discussion

Although other studies show the Prx1 cell population contributes to fracture repair and ectopic bone formation, our study focuses on characterizing and determining the function of this cell population(s) during homeostatic bone turnover and bone formation with varying degrees of injury. The mouse model used for these studies were previously reported to have GFP expression driven by the Prx1 promoter to identify the actively Prx1 expressing cell population, however, GFP was not detected with either light microscopy or flow cytometry with the studies reported here. This could be due to the use of an old IRES version or mutations introduced during the many generations of breeding this mouse in house. This should be noted for future studies. We also utilized a two-tamoxifen injection method with the CreERT to label cells with dTomato expression. It should be noted that this tamoxifen regimen is conservative and the use of five tamoxifen injections may yield additional labeling similar to other studies. Using the dTomato reporter, multiple organs and tissue regions of the postnatal animal were surveyed to fully depict homeostatic cell localization, response in a steady state and after injury as well as their contribution to bone formation using different surgical models. We determined that Prx1 expression labels multiple cell populations, a SSPC cell population that is multi-potential with limited anatomical distribution in the musculoskeletal system. These cells further have a primary role of responding to osteogenic conditions, such as DBM implantation and fracture repair. A second Prx1 positive population was identified that expressed Osterix, an osteoblast population within the bone tissue.

Under homeostatic conditions, the Prx1 postnatal cell population was identified within the kidney, sternum, calvaria, and appendicular limbs. Interestingly, during development, these same tissues have been identified to contain the Prx1 cell population (Kawanami et al., 2009; Leussink et al., 1995; Logan et al., 2002; Meijlink, Beverdam, Brouwer, Oosterveen, & Berge, 2004) suggesting that the Prx1 cell retains its embryological specification. Further detail was collected for the appendicular limbs due to its role in bone formation. The Prx1 expressing cell population reside within the bone marrow, endosteum, periosteum, and muscle. Osterix immunofluorescence was performed to differentiate between the stem/progenitor cells and committed osteoblasts at three days post tamoxifen injection. Within the growth plate, two cell populations were identified, a Prx1 dTomato cell population and an Osterix positive osteoblast population. However at the endosteal surface most Prx1 dTomato labeled cells co-labeled for Osterix. This suggests there are at least two Prx1 cell subpopulations within specific bone tissue locations, one a SSPC localized to the growth plate while a second Osterix positive committed cell population localized to the endosteal surface.

Interestingly there was a temporal cellular progression that demonstrated a transient expansion of the original Prx1 labeled cell populations that were localized to specific

regions, including the growth plate, adipose tissue, and medial layer of blood vessels muscles surrounding the long bones after 17 days. By day 31 the Prx1-derived cells showed a similar distribution and cellular appearance as at day three. Interestingly, after five months, a portion of the cell population had expanded and accumulated. This suggests that the Prx1 cell population moves through is a heterogenous transient progenitor stage and only a fraction of these cells becomes terminally differentiated into various end stage lineage cell types. This is consistent with bone marrow stromal stem cells, that have a slow regenerative potential with the maintenance of only a very small cell population (Park et al., 2012) and the αSMA cell population residing within the periosteum (Matthews et al., 2021). The failure to have an increase in dTomato labeled cells at day 31 further suggests that although the Prx1 cells expand in number, the majority of these cells do not continue to differentiate under homeostatic conditions and most likely undergo programed cell death (apoptosis). This conclusion is further consistent with osteoblasts being replenished from a larger progenitor pool (Park et al., 2012). It is unclear as to the localization of the Prx1-derived dTomato cells found within the blood vessels originate, since these cells were not identified at day three. This suggests a small/rare population of cells contribute to vessel maintenance however additional studies are needed to identify this cell population.

Previous studies mostly used strategies to label the Prx1 expressing cells at the time of fracture and follow cells and progeny during fracture repair (De Lageneste et al., 2018; Kawanami et al., 2009; Wang et al., 2017; Wilk et al., 2017). Our study, however, utilized a different approach by labeling the stem/progenitor cells a month prior to post-natal bone formation. This reduces the effect of tamoxifen has on bone formation and allows for the labeling of the stem/progenitor cell population to reach a homeostatic balance while excluding other cell populations in which, Prx1 expression is induced by injury. Based on the cell labeling during homeostasis, only a small population of cells are labeled at 31 days post tamoxifen injection. Our studies show however that there are large numbers of labeled Prx1 derived cells contributing to early callus formation and later phase of bone remodeling during fracture healing indicating an expansion of the Prx1-derived cells in response to injury. This is consistent with the emerging picture that stem cells cycle back and forth through an “alert” stage ready to respond to injury (Belenguer et al., 2021; Lee et al., 2018; Malam & Cohn, 2014).

Other cell populations have been shown to contribute to fracture repair, including αSMA, Mx1, Periostin, and Cathepsin K, all of which have been found within the periosteum and responds to injury (De Lageneste et al., 2018; Matthews et al., 2021; Ortinau et al., 2019). It is not clear if these cell populations are distinct or the degree of overlap between these cell populations. However, the Prx1 expressing cell population was shown to overlap greatly with the αSMA+/Mx1+ periosteal cell population under homeostatic conditions. And recently, the αSMA periosteal cell population was shown to consist of a long-term progenitor cell and an injury activated cell population, with both contributing to 60% of the fracture callus while the long-term progenitor contributes about 20% of the fracture callus. Although our results suggest the long-term Prx1 progenitor cell is a major contributor to the formation of the callus, the period between tamoxifen dosing and fracture was only 28 days in this study while it was 45 days in the αSMA study. Further, the contribution of the endosteal Prx/Osterix cell population to fracture repair cannot ruled out.

This study also utilized a non-injury model to induce post-natal bone formation on the periosteal surface (orthotopic) and within the muscle of the upper hind limb (ectopic) by utilizing DBM implantation. Both sites of post-natal bone showed recruitment, expansion, and contribution of the Prx1-derived cells. In all models the expression of Prx1 was upregulating indicating either expansion of the Prx1 stem/progenitor cell population or induction of the local cell populations to express Prx1. It is less likely that the Prx1/Osterix cell population found within the bone contributed to the ectopic bone formation. Our histological analysis suggests that there is expansion of the Prx1 cell population and that they directly contribute to post-natal bone formation. An injury model of heterotopic ossification also demonstrated Prx1 cells contributing to bone formation through endochondral ossification (Agarwal et al., 2016). Although a large number of cells are Prx1 derived from the early labeling scheme, there are cells that are not labeled, indicating other population(s) contribute to post-natal bone formation.

Our results illustrated that the Prx1 labeled cells localized to the sternum. Using the method of labeling the Prx1 stem cells a month prior to surgery, ectopic bone was induced at the chest and spine. It was determined that the Prx1 cell population continued to retain its embryonic specification. The chest ectopic bone had varying levels of recruitment and/or proliferation, with some regions of ectopic bone being absent of labeled Prx1-derived cells with other regions showing recruited labeled Prx1-derived cells. It appeared that the implants that were closest to the sternum showed increased numbers of Prx1 labeled cells. No labeled Prx1-dervied cells were found within the ectopic bone located at the spine. These results indicate that other stem cell populations are recruited for bone development for the axial skeleton and that the Prx1 stem cells do not migrate under homeostatic conditions. This is similar to Hox11 paralogous genes that specify the patterning of the developing tibia/fibia and ulna/radius. In the postnatal animal, Hox11 genes keep their distinct location and contribute to only the ulnar fracture, not the fracture within the femur (Rux et al., 2016).

Immunofluorescence confirmed that the Prx1 derived cells are multipotent in vivo with differentiation towards chondrocytes, osteoblasts, adipocytes (perilipin) and localized to vessel walls (SMA alpha). Although expression of *Prrx1* is high in the vasculature during development, it was shown that Prx1 is not involved with the vessel wall patterning, but it was suggested to be involved with the matrix synthesis within the vessel wall (Bergwerff et al., 1998; Leussink et al., 1995; Meijlink et al., 2004). It is also possible that the Prx1 cells localized to vessels are pericytes since they co-localize to SMA alpha and previously been shown to express NG2, a pericyte marker (De Lageneste et al., 2018).

Our results agree with previous reports that Prx1 expressing cells are a stem/progenitor cell population that predominantly contributes to injury or stimulating environment but not to the growth or maintenance of bone tissue (Tsuji et al., 2006). The progenitor cell population has a quick turnover rate. These results also suggest that there are at least two Prx1 cell populations within the bone tissue, but it remains unclear if the Prx1 cell populations within the different compartments are regulated by its niche or rather each stem cell population is unique and express Prx1 separately. However, with the correct osteo-inductive conditions the Prx1 stem/progenitor cell population is multipotent, greatly expands, and contributes throughout the endochondral ossification process for post-natal bone formation.

This responsive cell population is restricted to its embryological sites indicating that there are different skeletal stem cells that respond to injury and bone homeostasis depending upon the skeletal site.

Data Accessibility Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Agarwal S, Loder S, Brownley C, Cholok D, Mangiavini L, Li J, … Levi B (2016). Inhibition of Hif1alpha prevents both trauma-induced and genetic heterotopic ossification. Proceedings of the National Academy of Sciences of the United States of America, 113(3), 338. doi:10.1073/pnas.1515397113 [doi] [PubMed: 26719419]

Bassir SH, Garakani S, Wilk K, Aldawood ZA, Hou J, Yeh SA, … Intini G (2019). Prx1 expressing cells are required for periodontal regeneration of the mouse incisor. Frontiers in Physiology, 10, 591. [PubMed: 31231227]

Belenguer G, Duart-Abadia P, Jordán-Pla A, Domingo-Muelas A, Blasco-Chamarro L, Ferrón SR, … Fariñas I (2021). Adult neural stem cells are alerted by systemic inflammation through TNF-α receptor signaling. Cell Stem Cell, 28(2), 285–299. e9. [PubMed: 33207218]

Bergwerff M, Gittenberger-De Groot AC, Deruiter MC, Iperen LV, Meijlink F, & Poelmann RE (1998). Patterns of paired-related homeobox genes PRX1 and PRX2 suggest involvement in matrix modulation in the developing chick vascular system. Developmental Dynamics: An Official Publication of the American Association of Anatomists, 213(1), 59–70. [PubMed: 9733101]

Bragdon BC, & Bahney CS (2018). Origin of reparative stem cells in fracture healing. Current Osteoporosis Reports, 16(4), 490–503. [PubMed: 29959723]

Bragdon B, Lam S, Aly S, Femia A, Clark A, Hussein A, … Gerstenfeld LC (2017). Earliest phases of chondrogenesis are dependent upon angiogenesis during ectopic bone formation in mice. Bone, 101, 49–61. [PubMed: 28412469]

De Lageneste OD, Julien A, Abou-Khalil R, Frangi G, Carvalho C, Cagnard N, … Colnot C (2018). Periosteum contains skeletal stem cells with high bone regenerative potential controlled by periostin. Nature Communications, 9(1), 773.

Esposito A, Wang L, Li T, Miranda M, & Spagnoli A (2020). Role of Prx1-expressing skeletal cells and Prx1-expression in fracture repair. Bone, 139, 115521. [PubMed: 32629173]

Kawanami A, Matsushita T, Chan YY, & Murakami S (2009). Mice expressing GFP and CreER in osteochondro progenitor cells in the periosteum. Biochemical and Biophysical Research Communications, 386(3), 477–482. [PubMed: 19538944]

Lee G, Santo AIE, Zwingenberger S, Cai L, Vogl T, Feldmann M, … Nanchahal J (2018). Fully reduced HMGB1 accelerates the regeneration of multiple tissues by transitioning stem cells to GAlert. Proceedings of the National Academy of Sciences, 115(19), E4463–E4472.

Leussink B, Brouwer A, El Khattabi M, Poelmann RE, Gittenberger-de Groot AC, & Meijlink F (1995). Expression patterns of the paired-related homeobox genes MHox/Prx1 and S8/Prx2 suggest roles in development of the heart and the forebrain. Mechanisms of Development, 52(1), 51–64. [PubMed: 7577675]

Logan M, Martin JF, Nagy A, Lobe C, Olson EN, & Tabin CJ (2002). Expression of cre recombinase in the developing mouse limb bud driven by a prxl enhancer. Genesis, 33(2), 77–80. [PubMed: 12112875]

Lybrand K, Bragdon B, & Gerstenfeld L (2015). Mouse models of bone healing: Fracture, marrow ablation, and distraction osteogenesis. Current Protocols in Mouse Biology, 35–49. [PubMed: 25727199]

Malam Z, & Cohn RD (2014). Stem cells on alert: Priming quiescent stem cells after remote injury. Cell Stem Cell, 15(1), 7–8. [PubMed: 24996163]

Matthews BG, Novak S, Sbrana FV, Funnell JL, Cao Y, Buckels EJ, … Kalajzic I (2021). Heterogeneity of murine periosteum progenitors involved in fracture healing. Elife, 10, e58534. [PubMed: 33560227]

Meijlink F, Beverdam A, Brouwer A, Oosterveen TC, & Berge DT (2004). Vertebrate aristaless-related genes. International Journal of Developmental Biology, 43(7), 651–663.

Ortinau LC, Wang H, Lei K, Deveza L, Jeong Y, Hara Y, … Lee B (2019). Identification of functionally distinct Mx1 αSMA periosteal skeletal stem cells. Cell Stem Cell, 25(6), 784–796. e5. [PubMed: 31809737]

Park D, Spencer JA, Koh BI, Kobayashi T, Fujisaki J, Clemens TL, … Scadden DT (2012). Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. Cell Stem Cell, 10(3), 259–272. [PubMed: 22385654]

Rux DR, Song JY, Swinehart IT, Pineault KM, Schlientz AJ, Trulik KG, … Wellik DM (2016). Regionally restricted hox function in adult bone marrow multipotent mesenchymal stem/stromal cells. Developmental Cell, 39(6), 653–666. [PubMed: 27939685]

Sanchez-Gurmaches J, Hsiao W, & Guertin DA (2015). Highly selective in vivo labeling of subcutaneous white adipocyte precursors with Prx1-cre. Stem Cell Reports, 4(4), 541–550. [PubMed: 25801508]

Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, … Rosen V (2006). BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nature Genetics, 38(12), 1424–1429. [PubMed: 17099713]

Wang T, Zhang X, & Bikle DD (2017). Osteogenic differentiation of periosteal cells during fracture healing. Journal of Cellular Physiology, 232(5), 913–921. [PubMed: 27731505]

Wilk K, Yeh SA, Mortensen LJ, Ghaffarigarakani S, Lombardo CM, Bassir SH, … Intini G (2017). Postnatal calvarial skeletal stem cells expressing PRX1 reside exclusively in the calvarial sutures and are required for bone regeneration. Stem Cell Reports, 8(4), 933–946. [PubMed: 28366454]

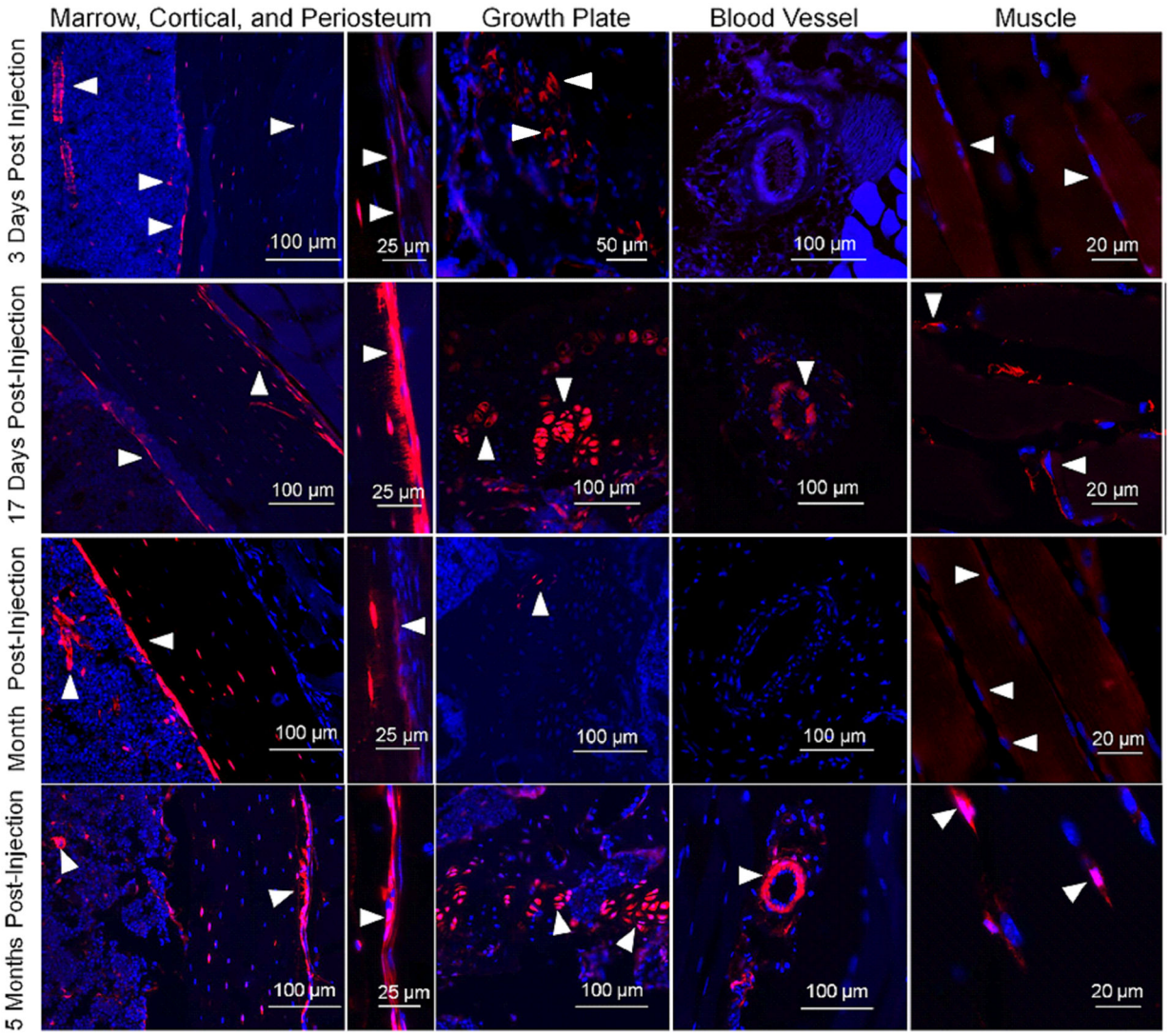

Figure 1. Characterizing *Prxx1* expression and tissue distribution of Prx1 derived cells during homeostasis.

The Prx1/Ai14/Rag mice received tamoxifen to induce dTomato expression (red) in the Prx1 cell population. Samples were harvested 3, 17, 31 days, and five months following tamoxifen (n=3). Frozen histology was performed. Dapi was used to label nuclei (blue). White arrow heads point to dTomato fluorescent positive cells.

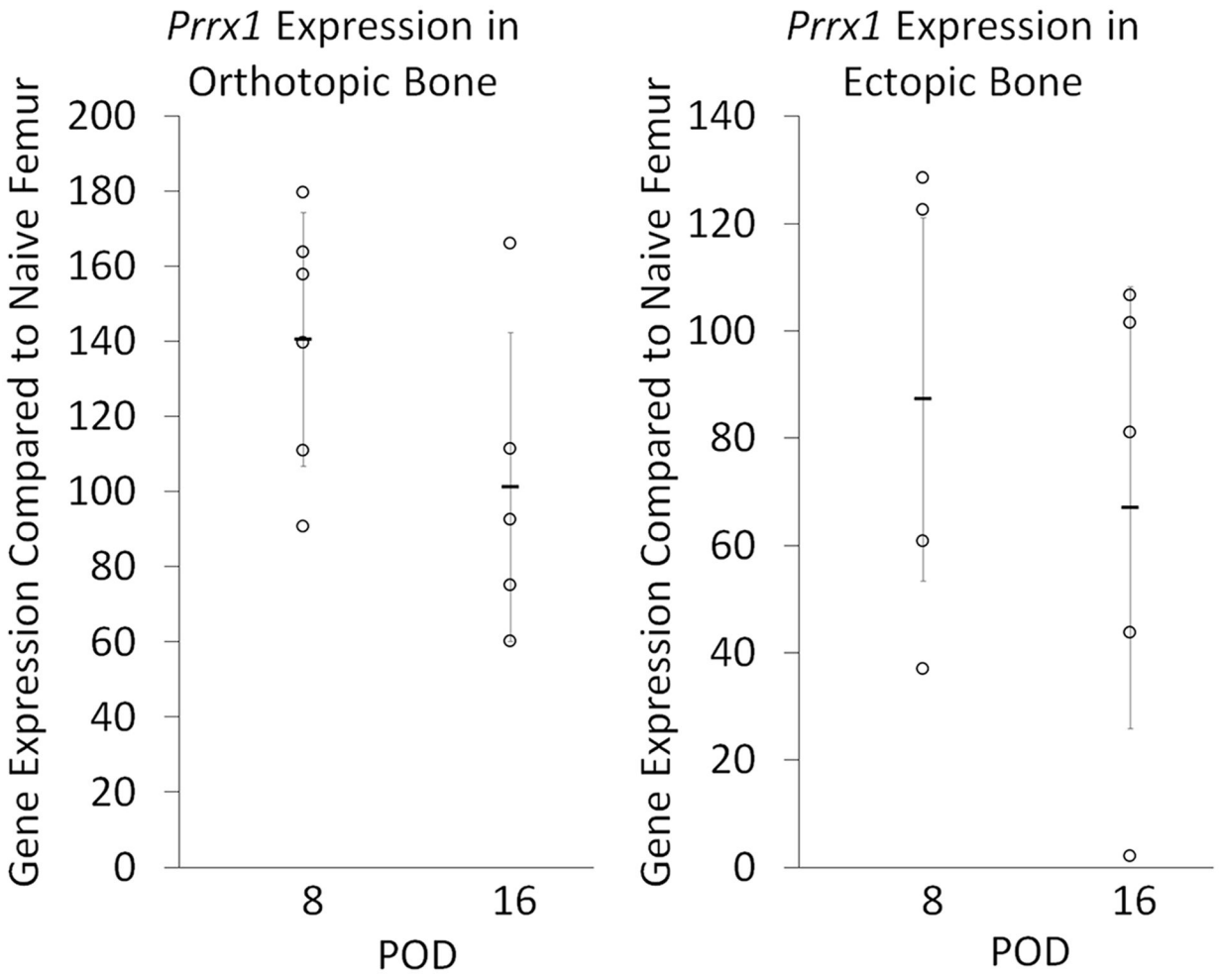

Figure 2. Expression of *Prrx1* following DBM implantation.

Orthotopic (left panel) and ectopic (right panel) bone formation was induced by DBM implantation. Samples were collected at post-operative day (POD) 8 (orthotopic n=6 and ectopic n=4) and 16 (orthotopic n=5 and ectopic n=5). Relative expression of *Prrx1* was compared to naïve bone. Individual dots represents each sample and the black bar shows the average. The error bars represent standard deviation.

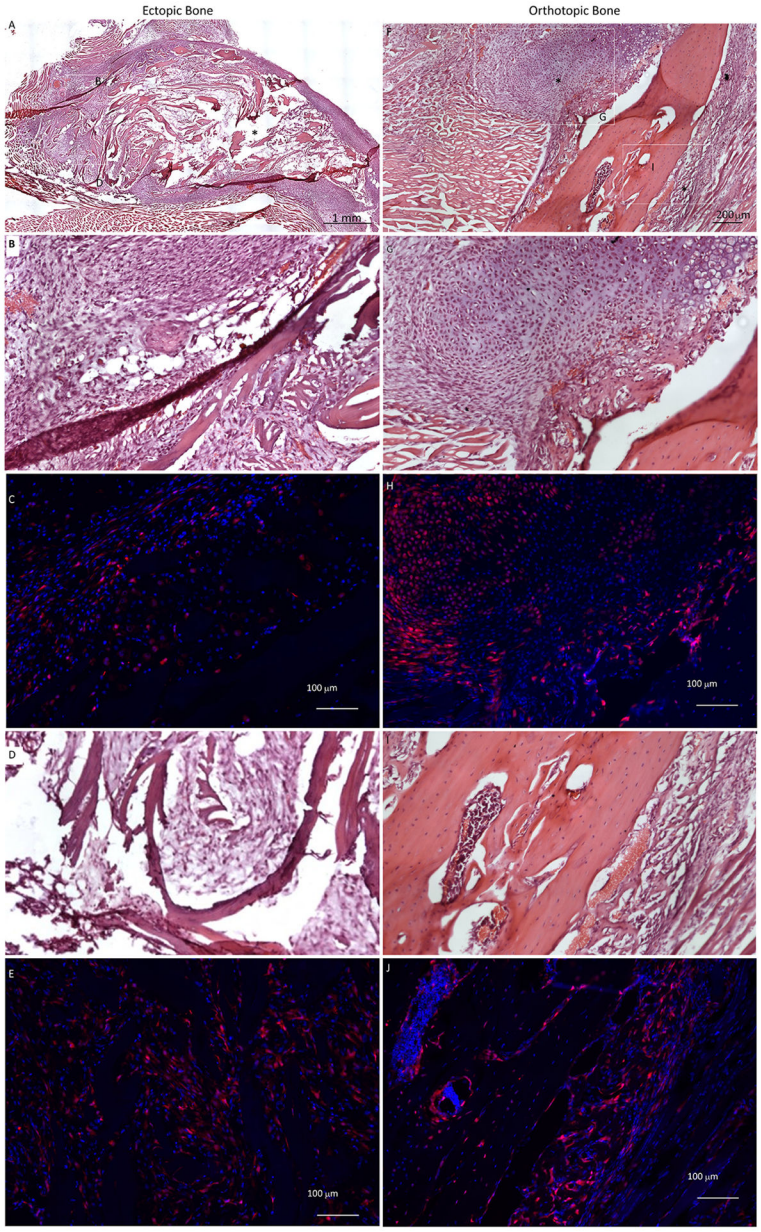

Figure 3. Prx1 derived cells are recruited to ectopic and orthotopic bone at POD 8.

Ectopic **(A-E)** and orthotopic **(F-J)** bone was induced by DBM implantation. Samples were collected at POD 8 and processed for histology (n=3). **(A, B, D and F, G, I)** H/E stain of the ectopic and orthotopic bone. **(C, E and H and J)** Serial sections were labeled with Dapi for nuclei (blue) and fluorescent images were collected. High resolution 10x images were collected in the regions marked by the white rectangles.

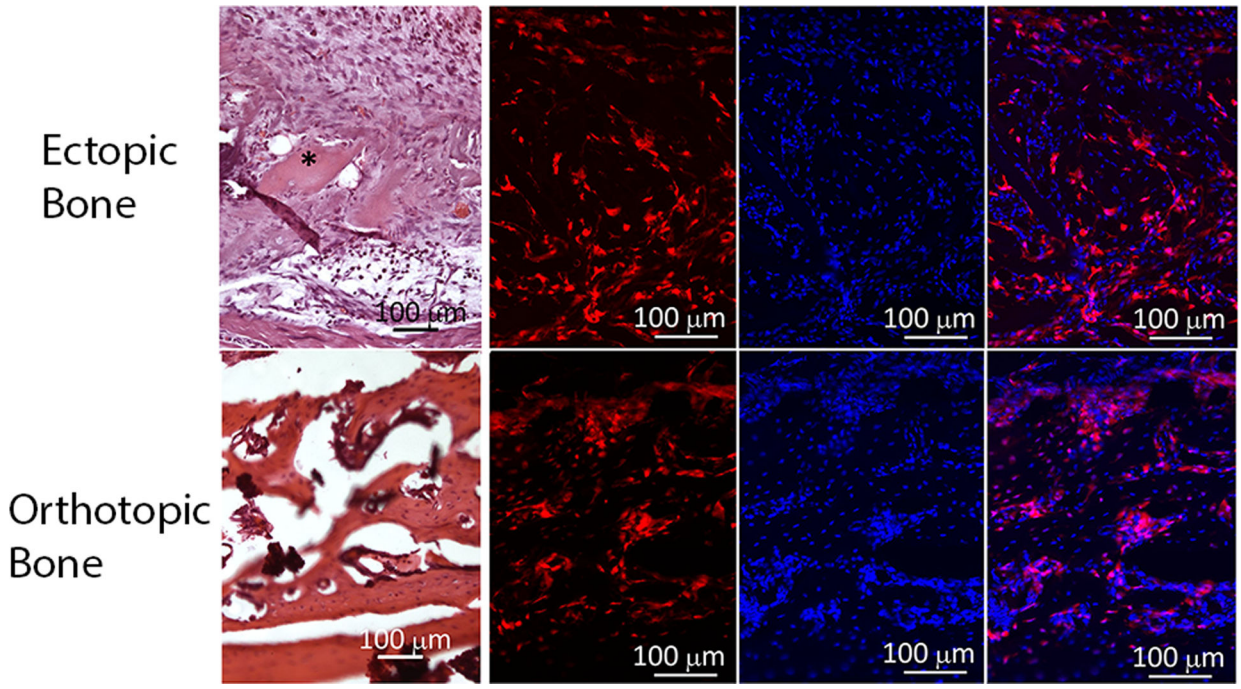

Figure 4. Prx1 derived cells are recruited to ectopic and orthotopic bone at POD 16.

Ectopic **(top)** and orthotopic **(bottom)** bone was induced by DBM implantation. Samples were collected at POD 16 and processed for histology (n=3). **(A and F)** H/E stain of the ectopic and orthotopic bone. **(B and G)** Serial sections were labeled with Dapi for nuclei (blue) and fluorescent images were collected. The Prx1 derived cells were recruited and found throughout the site of the developing ectopic and orthotopic bone. High resolution 10x images were collected in the region **(C-E and H-J)**.

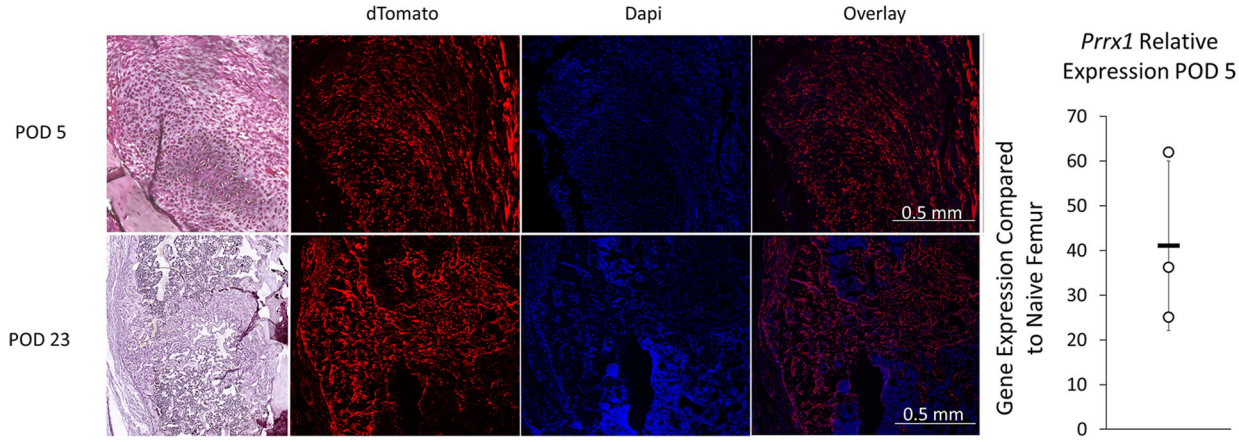

Figure 5. Prx1 derived cells are recruited to the fracture callus.

A closed transverse fracture callus was created and tissue was collected at POD 5 and 23 (n=3). High magnification (10x) images were collected in the region of the fracture gap and indicated by the white rectangle. The relative expression of *Prrx1* compared to naïve femur was determined at POD 5 (n=3). The individual dots represents each sample and the black bar shows the average. Standard deviation is shown with error bars.

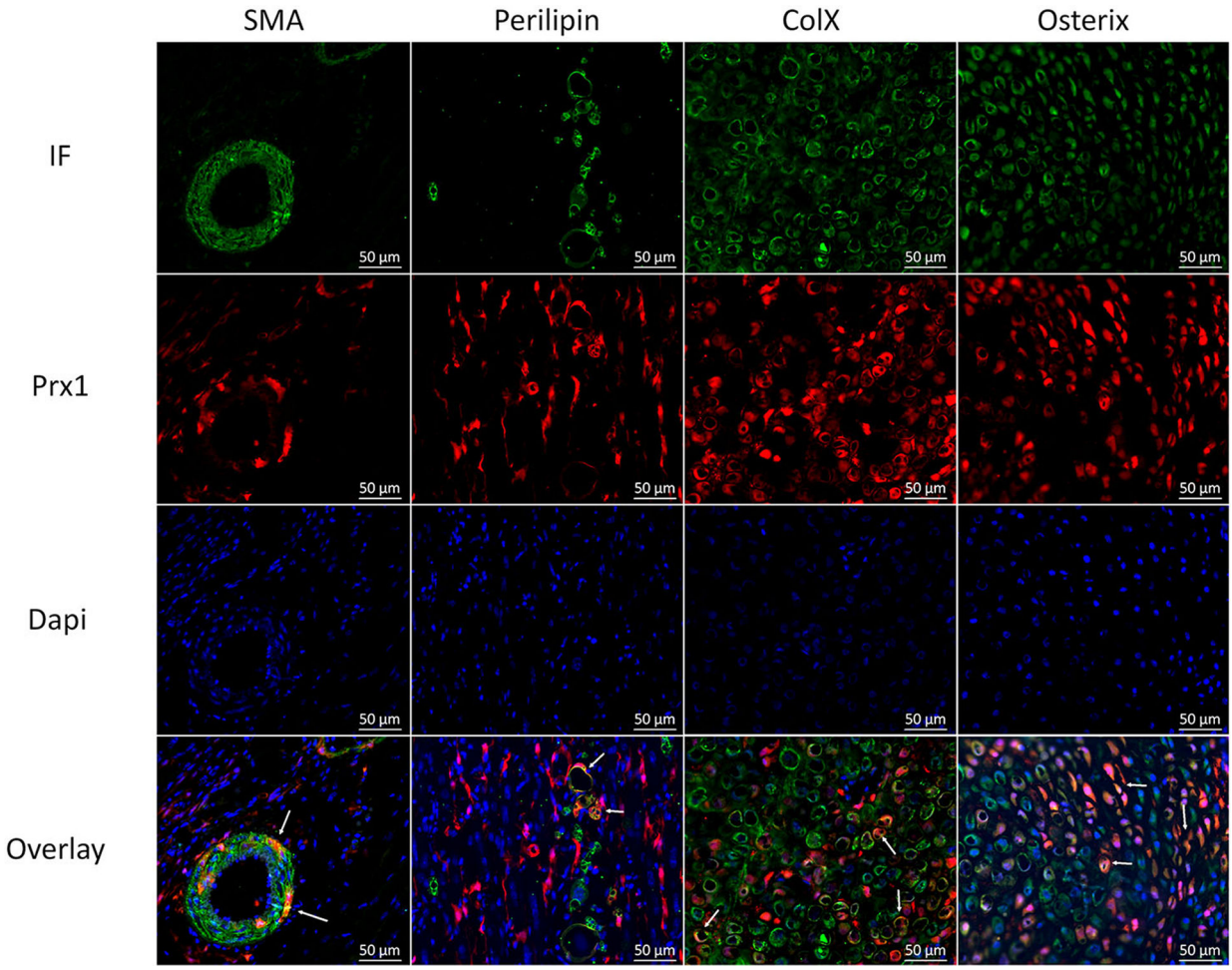

Figure 6. In Vivo Multipotency of Prx1 derived cells.

Immunofluorescence was performed on histological sections for smooth muscle actin (SMA) (n=4), Perilipin (n=3), collagen X (ColX) (n=3), or Osterix (n=3) for vessels, adipose, chondrocytes, and osteoblasts, respectively, and are shown by green label. Dapi was used to label nuclei. White arrows point to yellow cells that are labeled both red and green.

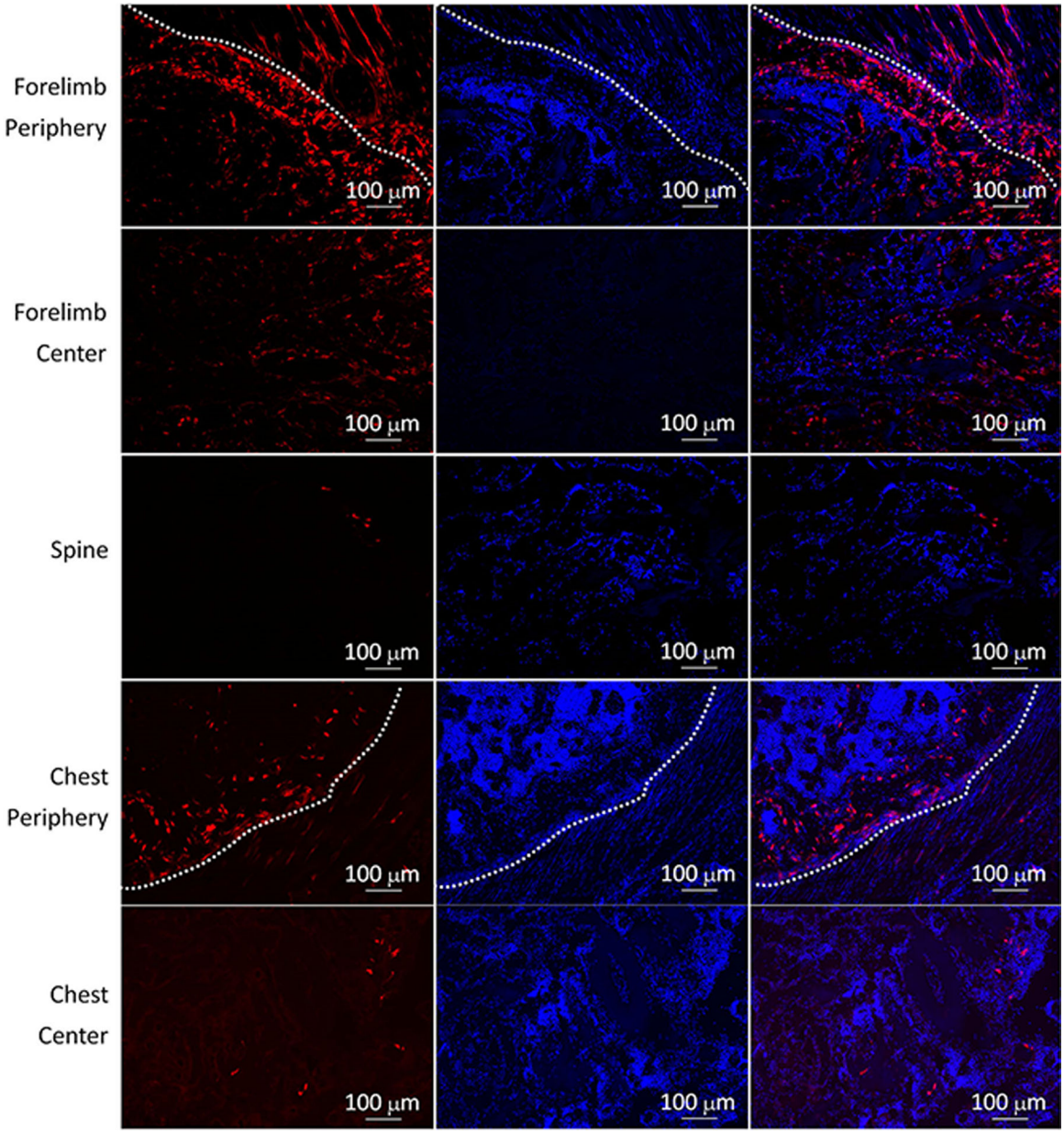

Figure 7. Anatomic restriction of Prx1 recruitment to post-natal bone formation.

The DBM material was implanted on the periosteal surface of the humerus (n=2), near the sternum/ribs (chest, n=3) and the spine (n=2) to induce orthotopic bone. Radiologic images (top panels) and histology (middle panels) show DBM-induced mineralized tissue development at each location. Serial sections were labeled with the nuclei stain, Dapi (blue). White dotted line defines the boundary between the orthotopic bone and muscle.