2015

Visualisation of osteoprogenitor cells in a Prx1 murine fracture model

https://hdl.handle.net/2144/16282

Boston University
BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

VISUALISATION OF OSTEOPROGENITOR CELLS IN A PRX1 MURINE FRACTURE MODEL

by

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B.S., University of California at Los Angeles, 2013

Submitted in partial fulfillment of the requirements for the degree of

Master of Science

2015
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BLAIRE M. BEERS-MULROY

ABSTRACT

Understanding the recruitment of multipotent skeletal progenitor cells and the factors that influence their differentiation would be helpful in providing a means for harnessing the regenerative capacity of skeletal progenitor cells in bone tissue engineering. In order to track the recruitment of skeletal stem cells in fracture healing, transgenic mice containing a Tamoxifen-inducible Cre recombinase that had been placed under the control of a 2.4 kb Prx1 promotor were used to induce conditional expression in periosteal skeletal stem cells that express the Prx1 gene. In order to initially see the cells expressing Prx1, a green fluorescent protein gene (GFP) had also been put downstream to the Prx1 promoter. We then crossed these Prx1CreER-GFP transgenic mice with a second strain containing the β-galactosidase gene that becomes constitutively expressed after recombination by the Cre recombinase. The enzymatic activity of β-galactosidase was then used to generate a colorimetric staining reaction that was used to visualize the cells in which recombination had occurred based on a blue staining product. The recombination activity should only be present in Prx1 expressing cells and their progeny.

The goal of the present study was to assess several different approaches to optimize the β-galactosidase enzymatic staining protocol and to visualize the Prx1 expressing cells during fracture healing. These studies further examined those populations of cells in the fracture calluses that became labeled and arose from the stem
cell populations that had expressed Prx1 at post-operative day 7 and 14. The optimization of a staining method for histology will allow this study to track Prx1 cell fates in a fracture model both in response to specific drug treatments, mechanical loading of the fracture during healing and under pathological conditions that effect healing.
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<table>
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<tr>
<td>BUSM</td>
<td>Boston University School of Medicine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Micro-CT</td>
<td>Micro-computed tomography</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature compound</td>
</tr>
<tr>
<td>PBS</td>
<td>Sodium perborate</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>POD</td>
<td>Post-operative day</td>
</tr>
<tr>
<td>PRX1</td>
<td>Paired related homeobox 1</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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INTRODUCTION

Models of fracture healing are primarily used to evaluate bone repair and regeneration after injury. Bone repair requires the recruitment of skeletal progenitor cells as a means to lay down cartilage and eventually new bone at the injury sight. These skeletal progenitor cells are believed to come from multiple sources including, but not limited to, the bone marrow and periosteum (Ozaki 2000). An understanding of the recruitment of osteoprogenitor and chondroprogenitor cells and the factors that influence their differentiation would be beneficial to the development of skeletal regenerative interventions. A significant obstacle has been the unavailability of methods to identify these progenitor cells and to track their fate during fracture healing (Grcevic, 2012). In this study we generated a transgenic mouse in which we were able to track the fate of Prx1-expressing progenitor cells during bone repair.

Fracture Healing Cascade

Fracture healing is a dynamic process that is characterized by four overlapping steps: inflammatory response, formation of soft callus, formation of hard callus, and boney union and remodeling. The generation of a fracture disrupts the integrity of the soft tissue and this damage initiates the inflammatory response. Bleeding at the fracture site into the surrounding tissue leads to the formation of a hematoma and the release of cytokines, growth factors, and inflammatory cells. Specifically, interleukins 1 and 6, and tumor necrosis factor-α are believed to initiate the repair cascade. The increased expression of transforming growth factor β, platelet derived growth factor, fibroblast growth factors, insulin-like growth factors, and bone morphogenetic proteins are all
thought to initiate to one degree or another the recruitment and or progression of
differentiation of skeletal stem cells and skeletal progenitor cells to promote callus
formation (Ai-Aql et al., 2008). Formation of the soft callus is characterized by the
proliferation of fibroblasts and chondrocytes at and near the fracture line. Discrete
cartilaginous regions grow and converge to form a central fibro-cartilaginous plug that
binds the two sides of the fracture together. Towards the final stages of formation of the
soft callus, the chondrocytes undergo hypertrophy and mineralize the cartilage matrix
before undergoing apoptosis (Schindeler et al., 2008). The formation of the hard callus is
characterized by the high level of osteoblast activity and the development of mineralized
bone. The replacement of the cartilaginous soft callus by endochondral ossification is
initially seen at the proximal and distal margins progressing towards the fracture midline.
The presence of osteogenic factors differentiates multipotent skeletal stem cells into
osteo/chondro and osteoprogenitor cells. Osteoprogenitor cells further differentiate into
mature osteoblasts and synthesize the initial woven bone of the hard callus. Bone
morphogenic proteins are believed to be the key regulators of this process (Schindeler et
al., 2008). The final stage of fracture repair is the remodeling of the woven bone into
lamellar bone, typified by the presence of osteoclasts resorbing woven bone and
osteoblasts laying down new lamellar bone. This classic four-stage model of fracture
healing may be altered by mechanical factors and is a fundamental aspect of this study.
Figure 1 shows histological examples at each stage of the 4-stage model while identifying
cellular contributors.
The fracture model that we employed involved the generation of a closed, simple, transverse fracture by an externally applied blunt trauma. The insertion of an intramedullary fixation pin prior to the generation of the fracture provided moderate stabilization and allowed micromotion at the fracture site. This micromotion initiated repair predominantly through endochondral ossification (Lee et al., 1998). Bone repair via endochondral ossification is characterized by the formation of a cartilage intermediate and presents as a large callus at the fracture site. The stability of the fracture site determines the size of the callus. The less mechanically stable, the larger the callus. The more rigid fixation methods that prevent micromotion between cortical bone fragments lead to smaller amounts of callus formation (Morgan et al. 2010, Thompson et al., 2002).
Prx1CreER-GFP-LacZRosa transgenic mouse model

The Cre-loxP system has been used for gene manipulation based on site-specific recombination catalyzed by the Cre integrase enzyme between two 34-basepair consensus recognition sequences. The advantage to this system is that mutation can be triggered conditionally instead of leading to ubiquitous expression of the enzyme. In our Prx1CreER-GFP-LacZRosa transgenic mouse, the expression of Cre recombinase is under the control of a 2.4 kb Prx1 promoter, meaning that the recombination activity should only be present in cells that express Prx1 (Kawanami et al., 2009). Recombination is achieved when Cre becomes covalently attached to a modified estrogen receptor ligand-binding domain creating a fusion protein Cre-ERt. Tamoxifen binds this ERt and mediates nuclear translocation and recombination. The recombination allows for the transcription of the LacZ gene. The activity of the LacZ on X-gal can be utilized to visualize the recombined cells based on the production of a blue product (Maes et al., 2007). This inducible system allows the Cre-ERt-expressing cells to be tagged upon administration of Tamoxifen in a conditional manner in Prx1-expressing cells at a given point in time, and to track the subsequent life cycle of the cells that express the tag following their migration over time within the callus tissue as it forms.

Specific Aims/Objectives

The goal of the present study is to visualize Prx1-expressing cells during fracture healing. Specifically,

(1) To identify the best staining method for LacZ Rosa to visualize the Prx1-expressing cells at post-operative day 7.
(2) To identify the localization of Prx1-expressing cells at post-operative days 7 and 14 using the staining method identified to be optimal in aim 1.

(3) To analyze the mRNA expression of Prx1 compared to other marker genes for cartilage and bone formation within the fracture model.

It is the hope that this study will elucidate the source and direction of movement of Prx1-expressing cells. These results would further explicate the underlying mechanism for osteoprogenitor and chondroprogenitor recruitment during fracture healing. Understanding the location and signaling of these progenitor cells may help identify cell sources and provide a means for harnessing the regenerative capacity of skeletal stem cells in bone tissue engineering.
METHODS

Animals & Surgery

Institutional Animal Care and Use Committee at Boston University approved all animal studies. Prx1CreER-GFP transgenic mice were obtained from Case Western Reserve University Orthopaedic department, and were bred in house with 129S4–Gt(Rosa)26 B6 mice (Jackson Laboratories) to produce a Prx1CreER-GFP-LacZ Rosa lineage. The Prx1CreER-GFP-LacZ Rosa transgenic mutant mice were housed at the BUSM animal facility under standard conditions. Prior to aseptic surgery, the experimental animals were anesthetized in an anesthesia chamber with a mixture of oxygen and isoflurane. Once the mouse was unconscious, it was removed from the chamber and a nose cone administering anesthesia was placed over the head of the animal. The right knee was shaved with an electric shaver and the animal received subcutaneous injections of 0.1 mL Buprenex® to relieve pain and 0.01 mL Baytril® to prevent infection immediately prior to surgery. The surgical site was washed with Hibiclens® followed by a wash of 70% alcohol starting from the middle working outwards. The surgical field was wiped with Betadine. The right leg was bent and with a No. 15 scalpel blade, an anterior longitudinal incision was made over the patella in a superior to inferior direction. Once the distal femur was exposed, a hole was made in the femoral condyle by the insertion of a U-100 27 gauge insulin syringe and was inserted the length of the femur to the proximal end. The syringe was removed and the stylette of a 25 gauge spinal needle (fixation pin) was inserted the length of the femur. The spinal needle stylette was cut at the distal end of the femur with wire cutters and was then
buried under the condylar surface with closed forceps. The leg was straightened and the incision was closed with two sutures using 5-0 USP Perma Sharp suture material. The mouse was placed in the fracture device, the right leg was straightened, and a spring-loaded blunt blade was released over the mid-diaphyseal shaft to produce a single transverse closed fracture. An initial fracture assessment was made by palpation. The nose cone was then removed and the animal was positioned for x-ray by a Dental Unit at 70 kV for 0.10 sec, which utilizes Kodak Ultra Speed DF-50, Size 4 x-rays. The animal was transferred to a weighing box, the weight was recorded, and the animal was placed on a heating pad to recover. The x-rays were developed in a hand developing box within the procedure room to confirm the presence of a fracture. If the x-ray was negative for a fracture, the animal was re-anesthetized and another fracture attempt was made at a higher setting. This process was continued until a positive fracture x-ray was obtained. If a fracture was not obtained after five attempts, the animal did not undergo further fracture attempts and was euthanized and excluded from the study. The animals were monitored during recovery and were then placed back in their cages after an unrestricted gait was observed.

**Tamoxifen**

Tamoxifen 10 mg/mL solution in corn oil was prepared by combining 11 mL corn oil and 110 mg Tamoxifen and sonicating the tube for 3 minutes total with 30 seconds on and 5 seconds off. Sonication was repeated until the powder was completely dissolved in solution. The solution was then sterilized by filtration through a 0.22 micron sterile syringe filter. The Tamoxifen 10 mg/mL solution in corn oil was aliquoted into 2 mL
sterile freezing vials and frozen at -20°C. When ready to use, the freezing vials were thawed and the experimental mice were injected 10 µL/g body weight.

The day 7 experimental animal group received intraperitoneal Tamoxifen injections 4 days pre surgery, day 1 and day 3 post surgery. The day 14 experimental group received intraperitoneal Tamoxifen injections 4 days pre surgery, day 1, day 3, day 6, day 8 and day 10 post surgery. Control animals received intraperitoneal injections of sterile corn oil according to the same injection schedule as the experimental animals in the specified time point. Mice were euthanized at two time points, day 7 and 14 post-surgery. A flow chart of the study design is shown in figure 2.

![Figure 2: Tamoxifen Injection Schedule](image)

Harvest

The mice were euthanized by carbon dioxide inhalation in a chamber appropriate for species, followed by cervical dislocation. A post-mortem x-ray was taken using the
Faxitron MX-20 Specimen Radiography System, at 30 kV for 40 seconds, with Kodak BioMax XAR Scientific Imaging Film. After euthanasia and post-mortem x-ray, four different methods of staining were attempted in order to recover and visualize the highest number of Prx1-expressing cells.

1. **Bulk X-gal stain and paraffin embedding**

   The right femur was dissected from the experimental mouse and the surrounding soft tissue was removed. The fixation pin was carefully removed from the distal end of the femur without altering the integrity of the fracture callus. The femur was placed in 4% PFA at 4˚C for approximately 1 hour. After 1 hour, the specimen was removed and washed for 3 x 30 minutes in X-Gal wash buffer. X-Gal wash buffer was made with 2.0 mL 1M MgCl₂, 10 mL 1% Deoxycholic Acid, 2 mL 10% Nodidet-P40, 23 mL 1M Monobasic Sodium Phosphate pH 7.3, 77 mL 1 M Dibasic Sodium Phosphate pH 7.3, and 886 mL dH₂O. After the third 30 minute wash, the wash buffer was removed and enough X-Gal stain was added to cover specimen. X-Gal stain must be made fresh in the dark with 0.6 mL 50 mg/mL X-Gal in DMF, 1.0 mL 200 mM K-ferrocyanide, 1.0 mL 200 mM K-ferricyanide, 1.0 mL 1 M Tris pH 7.5, and 46.4 mL wash buffer. The sample was developed in the dark covered with foil for 2 days at 4˚C. After 2 days, the stain was removed and the sample was washed 3 x 1 hour in 1x PBS. The sample was post-fixed in 4% PFA at 4˚C overnight. The sample was washed with 1x PBS and kept in 1x PBS until further processing.

   For decalcification, samples were placed in Tissue-Tek® Mega-cassette® and submerged in EDTA at 4˚C for 5 days with shaking. After decalcification, the samples
were rinsed with distilled water, processed into paraffin using the LEICA ASP300 and embedded into Paraplast Extra. Samples were stored at 4°C. Samples were sectioned at 5 microns and dried overnight at 44°C in the oven. To stain and cover-slip, samples were first deparaffinized 3 x 5 minutes in xylene and rehydrated in 100% EtOH for 5 minutes, 100% EtOH for 2 minutes, 95% EtOH for 2 minutes, 75% EtOH for 2 minutes, and deionized water for 5 minutes. The sample slides were washed in 1x PBS 3 x 5 minutes each at room temperature. The slides were stained for 5 minutes in 1% aqueous eosin at room temperature and rinsed in distilled water until clear. Slides were allowed to air dry and then cover-slipped using Molecular Probes ProLong© Gold Antifade reagent with DAPI.

2. **Bulk X-Gal Stain and frozen sectioning**

   This staining method was identical to the previous method up until post decalcification processing. After the sample was removed from the cold EDTA, it was incubated through a series of sucrose gradients starting with 30% sucrose on a rotor at 4°C overnight. The sample was then placed in a 1:1 mixture of OCT and 30% sucrose under the same conditions for another night. The sample was frozen in cooled liquid nitrogen with tetra fluorethane for a rapid freeze and stored at -80°C.

   After it had been frozen for at least one hour, the sample was sectioned at a thickness of 8 microns and placed on a slide. The sample slides were washed in 1x PBS 3 x 5 minutes each at room temperature. The slides were stained for 5 minutes in 1% aqueous eosin at room temperature and rinsed in distilled water until clear. The slides were
allowed to air dry and then cover-slipped with Molecular Probes ProLong® Gold Antifade reagent with DAPI.

3. **X-Gal Stain of cut frozen sections** (Han and Feng 2014).

This method differs starting at dissection as it was recommended to cut both ends of the bone sample for better penetration of the marrow cavity. After dissection, the sample was fixed in ice-cold 4% PFA for 1 hour at 4°C with shaking. The sample was then washed in 1x PBS 3 x 30 minutes at 4°C with shaking. The sample was placed in a Tissue-Tek® Mega-cassette® and submerged in 0.1 M EDTA solution at 37°C overnight with shaking. After decalcification, the sample was washed with 1x PBS 3 x 30 minutes at room temperature with shaking. The sample was incubated through the same series of sucrose gradients as the previous method, embedded in OCT media, and frozen at -80°C. The sample was cut at a thickness of 10-12 microns and placed on slides. The sections were post-fixed in ice-cold 4% PFA at 4°C for 10 minutes. The sections were washed in 1x PBS 3 x 5 minutes at room temperature and incubated in X-gal solution at 37°C for 24 hours while protected from light. The slides were washed in 1x PBS 3 x 5 minutes, rinsed with distilled water, and counter-stained with 1% aqueous eosin for 5 minutes. Slides were rinsed with distilled water until clear, allowed to air dry, and cover-slipped with Molecular Probes ProLong® Gold Antifade reagent with DAPI.

4. **X-Gal Perfusion and frozen sectioning**

Immediately post-mortem, the animal was pinned supine to a Styrofoam surface. To expose the heart, the diaphragm was cut and the two sides of the ribcage were pinned open. The heart was carefully lifted and the inferior vena cava was clipped. A Vacutainer
needle was inserted into the posterior side of the left ventricle and the needle was
superglued in place. Using a 10 mL syringe, 10 mL of 4% PFA was pushed through the
Vacutainer tubing, followed by 10 mL of X-gal wash buffer, and 5 mL of X-gal stain.
The femur was then harvested and post-harvest processing proceeded according to the
Bulk X-gal stain and frozen sectioning method.

Imaging

High resolution imaging was performed using the Olympus BX51 microscope and
images were captured using the CellSens software. For each slide, stage limit, shading,
and white balance calibrations were performed. Images were captured at 10x and stitched
together in order to include the entire fracture callus at a magnification that cells can be
discriminated. A 10x stitched image of the same field for each sample was taken in bright
field and with a DAPI filter.

The fracture callus was then delineated into cartilage and ossifying bone. The
bright field image was used to manually count the number of X-gal stained cells in both
cartilage and ossifying bone. The DAPI image was used to count the total number of cells
in the specified regions of cartilage and ossifying bone. The regions of interest were
manually demarcated and the detection settings were set as such: detection area: ROI,
borders-frame: truncate, borders-ROI: include, minimum object size: 20 pixels. The
lower limit of the adaptive threshold varied among samples depending on the intensity of
staining with the upper limit always at 186. The adaptive threshold range for samples can
be seen in Table 1.
Table 1: Adaptive Threshold Range. This table depicts the adaptive threshold range in DAPI when calculating the relative object count of cells.

<table>
<thead>
<tr>
<th>Slide</th>
<th>Adaptive Threshold</th>
<th>Lower limit</th>
<th>Upper limit</th>
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<tr>
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<td>186</td>
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<td>Paraffin 2</td>
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<td>11</td>
<td>186</td>
</tr>
<tr>
<td>Paraffin 3</td>
<td></td>
<td>18</td>
<td>186</td>
</tr>
<tr>
<td>Bulk Stain Frozen 1</td>
<td></td>
<td>4</td>
<td>186</td>
</tr>
<tr>
<td>Bulk Stain Frozen 2</td>
<td></td>
<td>8</td>
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<tr>
<td>Perfusion 1</td>
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<tr>
<td>Perfusion 3</td>
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<tr>
<td>Han/Feng 5</td>
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</tr>
<tr>
<td>Han/Feng 6</td>
<td></td>
<td>15</td>
<td>186</td>
</tr>
</tbody>
</table>

A relative object count was measured and region of interest values were recorded. This quantification method was repeated on all samples with n=3.

Quantitative real-time polymerase chain reaction analysis

Femur samples from C57BL/6J B6 mice (Jackson Laboratories) that underwent the same previously described surgery and harvest were used in the gene expression analysis. These mice were housed under similar conditions but did not receive corn oil or Tamoxifen injections. Post-harvest, femur samples were stored at -80°C until RNA extraction was performed.
Femur samples were removed from storage and kept in liquid nitrogen. Each sample was then placed in a 2 mL tube filled with 0.75 mL Qiazol Lysis Reagent® and flash-frozen in liquid nitrogen for 15 seconds. A 2 mm stainless steel bead was added to the 2 mL tube and placed into the Qiagen Tissue Lyser II® for 2 minutes at 30 Hz for lysing. If the sample had not homogenized or thawed, the sample was placed back in liquid nitrogen and the previous steps were repeated. Once homogenized, the resulting solution was removed and added to a tube with 1 mL of Qiazol Lysis Reagent® and put on ice. After all samples were lysed, 200 µl of chloroform was added to each sample, vortexed, and placed back on ice for at least 2 minutes.

Each sample was re-vortexed and centrifuged at 14000 RPM at 4° C for 15 minutes. After centrifugation, the aqueous phase was removed and added to an RNase free 1.5 mL tube. An equal volume of isopropanol was added and the tube was inverted until the liquid appeared clear. The sample was then centrifuged at 14000 RPM at 4° C for 30 minutes and the supernatant was removed and washed with 500 µl of 70% ethanol and centrifuged again at 14000 RPM at 4° C for 5 minutes. This process was repeated, the ethanol was removed, and the tube was inverted on a sterile Kimwipe to dry. Once dry, the pellet was dissolved in 35 µl of RNase-free water and stored at -80° C.

A quality test of the RNA was performed via electrophoresis on a 2% agarose gel at 100V for approximately 40 minutes. The concentration of the RNA was determined by adding 1 µL of the diluted RNA to 99 µl of 10 mM Tris buffer and was placed into a UV/Vis Spectrophotometer (Beckman Coulter DU 350). Absorbance values of 260 nm were recorded.
Extracted RNA from each sample was thawed on ice and 2 μg of RNA was increased to a volume of 10.4 μL with RNase-free water in a 0.2 mL PCR tube. The reverse transcriptase reaction was performed using a mixture of the following reagents per sample from the Taqman Reverse Transcription Kit®: 6.61 μL MgCl₂, 6.0 μL dNTP Mix, 3.0 μL 10X RT Buffer, and 1.5 μL Random Hexamers. The reagents were vortexed and the reverse transcriptase enzymes 0.6 μL RNase Inhibitor and 1.89 μL Reverse Transcriptase were added to the reagent mixture. The 19.6 μL of the reverse transcription polymerase chain reaction reagents and enzymes were added to the 10.4 μL of RNA for a total of 30 μL and was lightly mixed. The samples were then placed in a thermal cycler (Eppendorf Mastercycler®) to run the PCR cycle as follows: 25° C for 10 minutes, 37° C for 60 minutes, 95° C for 5 minutes, and held at 4° C. The subsequent samples of complementary DNA (cDNA) were then diluted 1:50 in RNase-free water and stored at -20° C until use for qPCR.

QPCR was performed in order to amplify the DNA primers of interest. For each polymerase chain reaction, 9 μL of the diluted cDNA was added to 10 μL of Universal PCR Master Mix (Applied Biosystems®) and 1 μL of the particular primer set (Prx1, Gremlin, Sox9, and Osterix). A 96 well plate was used for each reaction and covered with a clear adhesive film. The plate was placed into the centrifuge and gently spun down at 1500 rpm for 2 minutes. The quantitative Real-Time polymerase chain reaction (qRT-PCR) was performed using an ABI 7700 Sequence Detector® (Applied Biosystems) and set up as follows: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute and run for 40 cycles. Samples were run in duplicate and normalized to 18s
rRNA from the same sample. The fold change in expression was normalized to samples from non-fractured B6 control mice.

Statistical analysis was performed using Microsoft Excel 2013 where group averages at each time point (n=4) for qPCR data and standard deviations were computed. Graphs were produced for mRNA expression of Prx1, Gremlin, Sox9, and Osterix, including standard deviation error bars in both directions.
RESULTS

Histological Evaluation of POD7 Staining Methods

Figures 3-6 depict representative 10x stitched images in bright field and DAPI from each fracture callus at POD7 among the four staining methods: Paraffin, Bulk Stain Frozen, Han/Feng, and Perfusion.

Figure 3: 10x Stitched Image of Fracture Callus at POD7 Paraffin Method. A: Bright field B: DAPI with total cells counted in red.
Figure 4: 10x Stitched Image of Fracture Callus at POD7 Bulk Stain Frozen Method. A: Bright field B: DAPI with total cells counted in red.
Figure 5: 10x Stitched Image of Fracture Callus at POD7 Han/Feng Method. A: Bright field B: DAPI with total cells counted in red.
Figure 6: 10x Stitched Image of Fracture Callus at POD7 Perfusion Method. A: Bright field B: DAPI with total cells counted in red.
A qualitative comparison of the cartilage and newly ossifying bone between the different staining methods shows the LacZ stained cells a faded blue in the Paraffin method and a more intense blue in the Bulk Stain Frozen, Han/Feng and Perfusion.

Figure 7 shows examples of the LacZ staining in cartilage and bone at POD7 among the four different staining methods. These images are purely for a qualitative comparison and are not indicative of the quantitative results.
Quantitative analysis of the histological sections at POD7 using the Paraffin staining method measures the percentage of LacZ stained cells in cartilage and ossifying bone as 0.011% and 0.001% respectively. The Bulk Stain Frozen method produces 0.034% and 0.021%, the Han/Feng 0.062% and 0.090%, and the Perfusion 0.012% and 0.007%. Table 2 depicts the cell counts for each sample, group averages, and percentages of LacZ stained cells.
Table 2: Quantitative Comparison of Staining Methods at POD7 in Cartilage and Bone.

<table>
<thead>
<tr>
<th>Slide:</th>
<th>Cartilage</th>
<th>Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total cartilage cells</td>
<td>LacZ stained cells</td>
</tr>
<tr>
<td>Paraffin 1</td>
<td>26336</td>
<td>265</td>
</tr>
<tr>
<td>Paraffin 2</td>
<td>22097</td>
<td>236</td>
</tr>
<tr>
<td>Paraffin 3</td>
<td>13401</td>
<td>207</td>
</tr>
<tr>
<td>Paraffin Average</td>
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<td>236</td>
</tr>
<tr>
<td>Paraffin % LacZ</td>
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<td></td>
</tr>
<tr>
<td>Bulk Stain Frozen 1</td>
<td>12009</td>
<td>360</td>
</tr>
<tr>
<td>Bulk Stain Frozen 2</td>
<td>11555</td>
<td>426</td>
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<tr>
<td>Bulk Stain Frozen 3</td>
<td>12051</td>
<td>416</td>
</tr>
<tr>
<td>Bulk Stain Frozen Average</td>
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<td>401</td>
</tr>
<tr>
<td>Bulk Stain Frozen % LacZ</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td>Han/Feng 1</td>
<td>13118</td>
<td>872</td>
</tr>
<tr>
<td>Han/Feng 2</td>
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<td>483</td>
</tr>
<tr>
<td>Han/Feng 3</td>
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<td>759</td>
</tr>
<tr>
<td>Han/Feng Average</td>
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<td>705</td>
</tr>
<tr>
<td>Han/Feng % LacZ</td>
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<tr>
<td>Perfusion 2</td>
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<td>Perfusion Average</td>
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<td>Perfusion % LacZ</td>
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Figure 8: Percentage of Cells Stained with LacZ: Comparison of Staining Methods at POD7 in Cartilage and Bone. This graph depicts the average number of LacZ stained cells in each tissue divided by the total number of cells in that tissue for each staining method.

Histological Evaluation of Han/Feng POD7 and POD14 Temporal Comparison

Figure 9 illustrates a representative 10x stitched image in bright field and DAPI of the fracture callus at POD14 using the Han/Feng staining method.
A qualitative comparison of the Han/Feng method at POD7 and POD14 in Figure 10 shows an increase in the amount of ossifying bone at POD14. The LacZ stained cells also appear more spread apart at POD14 than the clustering seen at POD7. The intensity of the blue stain appears consistent between the two time points.
A quantitative analysis of the Han/Feng method at POD7 and POD14 indicates an increase in the average total number of bone cells to 47,049 and the average LacZ stained bone cells to 690 at POD14 from 5,043 and 454 respectively at POD7. There is thus a decrease in the percentage of LacZ stained cells at POD14 with 0.015% in bone from the 0.090% seen at POD7. No cells were stained with LacZ in the cartilage at POD14. Table
3 reports the cell counts in cartilage and bone of LacZ stained cells compared to the total number of cells in each tissue using the Han/Feng staining method at POD7 and POD14.

### Table 3: Quantitative Temporal Comparison of Han/Feng Method at POD7 and POD14 in Cartilage and Bone.

<table>
<thead>
<tr>
<th>Slide</th>
<th>POD7 Cartilage</th>
<th></th>
<th>POD7 Bone</th>
<th></th>
<th>POD14 Cartilage</th>
<th></th>
<th>POD14 Bone</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total cartilage cells</td>
<td>LacZ stained cells</td>
<td>total bone cells</td>
<td>LacZ stained cells</td>
<td>total cartilage cells</td>
<td>LacZ stained cells</td>
<td>total bone cells</td>
<td>LacZ stained cells</td>
</tr>
<tr>
<td>Han/Feng 1</td>
<td>13118</td>
<td>872</td>
<td>6245</td>
<td>542</td>
<td>8970</td>
<td>0</td>
<td>38930</td>
<td>448</td>
</tr>
<tr>
<td>Han/Feng 2</td>
<td>8875</td>
<td>483</td>
<td>3354</td>
<td>317</td>
<td>8648</td>
<td>0</td>
<td>51185</td>
<td>841</td>
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<tr>
<td>Han/Feng 3</td>
<td>12091</td>
<td>759</td>
<td>5529</td>
<td>504</td>
<td>10828</td>
<td>0</td>
<td>51031</td>
<td>781</td>
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<tr>
<td>Han/Feng Average</td>
<td>11361</td>
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<td>454</td>
<td>9482</td>
<td>0</td>
<td>47049</td>
<td>690</td>
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<tr>
<td>Han/Feng % LacZ</td>
<td>0.062</td>
<td></td>
<td>0.090</td>
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<td>0.015</td>
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</table>
**Figure 11: Percentage of Cells Stained with LacZ: Temporal Comparison of Han/Feng Method at POD7 and POD14 in Cartilage and Bone.** This graph depicts the average number of LacZ stained cells in each tissue divided by the total number of cells in that tissue for each time point.

**qPCR Analysis**

The fold expression of genes associated with cartilage and bone formation was analyzed to track the progression of fracture healing. Prx1 is a gene expressed in both chondroprogenitor and osteoprogenitor cells, and is thus involved in both chondrogenesis and osteogenesis. Figure 12 shows the average fold expression of Prx1 with an absolute maximum at day 3 of 14.92 and a relative maximum at day 7 of 9.54.
**Figure 12: qPCR Analysis of Prx1.** This graph shows the temporal gene expression profile of Prx1 normalized to 18s rRNA from the same sample. The fold change in expression was normalized to non-fractured B6 control mice.

Gremlin is a bone morphogenic protein antagonist that has been found to define a population of osteochondroreticular stem cells in bone marrow (Worthley et al., 2015). Figure 13 shows an average fold expression for Gremlin peaking at day 28 with 1.85 fold expression. Still, there is minimal expression of Gremlin throughout this fracture model.
Figure 13: qPCR Analysis of Gremlin. This graph shows the temporal gene expression profile of Gremlin normalized to 18s rRNA from the same sample. The fold change in expression was normalized to non-fractured B6 control mice.

The gene Sox9 produces transcription factors that control the earliest commitment stage of chondrogenesis and is seen in early callus formation (Uusitalo et al., 2001). Figure 14 depicts the average fold expression of Sox9 with an absolute maximum at day 10 of 4.90. Minimal expression was detected after day 14.
Figure 14: qPCR Analysis of Sox9. This graph shows the temporal gene expression profile of Sox9 normalized to 18s rRNA from the same sample. The fold change in expression was normalized to non-fractured B6 control mice.

Osterix, produced by the gene OSX, is a transcription factor that is necessary for the differentiation of mesenchymal stem cells into osteoblasts and indicates osteoblast lineage commitment (Zhang, 2010). Figure 15 shows the average fold expression of Osterix with an absolute maximum at day 10 of 29.82.
Figure 15: qPCR Analysis of Osterix. This graph shows the temporal gene expression profile of Osterix normalized to 18s rRNA from the same sample. The fold change in expression was normalized to non-fractured B6 control mice.


DISCUSSION

Histology: Method Comparison

At POD7 the frozen histology sections appear superior to the paraffin sections when evaluating the presence and brightness of LacZ stained cells. The LacZ stained cells in the paraffin sections appear faded and do not present as the bright blue of the frozen sections. The processing of paraffin sections requires the use of xylene as well as the deparaffinization of the slides for staining. It is known that xylene has a bleaching effect on X-gal staining, thus greatly reducing the intensity of the blue staining (Han and Feng 2014). The paraffin sections preserve better than the frozen sections, but this fading of the LacZ cells diminishes the potential use of the paraffin embedding and sectioning method as the best option for the quantification and tracking of Prx1-expressing cells.

Another difference in the staining methods was the bulk stain of the X-gal versus staining once sectioned. It is difficult to determine at POD7 that this would cause any discrepancy. However, at later time points like POD14 or POD21 the X-gal stain may have trouble penetrating the callus and staining the deeper portions of the sample. Further experimentation would need to be performed to determine whether the staining of cut sections is in fact superior to the bulk stain.

The Han/Feng staining method required a hot decalcification at 37°C compared to the other methods that were decalcified at 4°C. A hot decalcification up to 42°C has been experimentally shown as a method of rapid decalcification without any loss of β-galactosidase activity (Cho et al., 2010). With the large amount of LacZ staining when employing the Han/Feng staining method, there is little reason to believe that the hot
The decalcification method poses a problem to the β-galactosidase activity without further experimentation.

The Han/Feng staining method also required a hot X-gal stain at 37˚C compared to the other methods that were stained in X-gal at 4˚C. It is possible that this method of hot staining produced exogenous staining in the bone marrow that was not seen in the other staining methods. Further, by performing the staining for 24 hours instead of 4, the weaker signals were likely intensified. Further experimentation should be done using the Han/Feng method of staining with the X-gal staining at 4˚C to determine whether this exogenous staining can be eliminated.

The Perfusion method did not seem to increase the number of LacZ cells stained and is likely unnecessary to the effectiveness of X-gal staining. The decrease in the number of stained cells from the Bulk Stain Frozen Method to the Perfusion is probably due to the differences in the callus size and variability among animals. One side of the cortical bone shell broke off during harvest and the partial sample is a likely contributor to the decreased number of LacZ stained cells. It would be expected that the Perfusion method produced similar numbers to the Bulk Stain Frozen.

**Histology: Temporal Comparison**

When comparing the Han/Feng method at POD7 and POD14 it is evident that the number of ossifying bone cells increases at the later time point, and there are no LacZ stained cells in the cartilage at the later time point. The first observation is expected with the normal progression of fracture healing. The increase in the number of LacZ stained
cells in the bone at POD14 suggests that the Prx1 cell population in the periosteum either continues to give rise to the initial primary bone in the callus or the progeny of the original cells that developed in the callus have continued to expand in the primary bone that develops at the later time period. It is interesting to note that the former possibility is favored based on the second broad peak of Prx1 mRNA expression that was seen in the callus tissues extending from 7 days through 14 days post-operative (see discussion below). The second observation that no LacZ stained cells were seen in the cartilage at POD14 was not as expected. However, it is possible that the tagged chondrocytes that arose initially after fracture had already remodeled at POD14 and are thus not seen. The mRNA data is also suggestive of this conclusion since Prx1 showed a very strong peak of expression at POD 3 and it is the cells expressing the Prx1 at this time that most likely became tagged in the callus at day 7. The implication of this finding is that the Prx1 cells at earlier and later times are solely directed into either cartilage or bone lineages at these two different times during fracture healing. Further experimentation restricting the Prx1 induction period to either earlier or later time points needs to be done on more animals to further explore this suggestive data.

Gene Expression

Prx1 gene expression peaks at day 3 and 7 in this fracture model. This is consistent with the known expression of Prx1 in the periosteum in chondroprogenitor and osteoprogenitor cells. It is probable that the day 3 peak is indicative of the recruitment of chondroprogenitor cells to begin formation of the soft callus. The day 7 peak may
indicate the recruitment of osteoprogenitor cells to begin the formation of the hard callus. The Prx1 average fold expression remains elevated during the remainder of fracture.

Gremlin gene expression does not appear to be affected by fracture as the average fold expression never exceeds 2. It is interesting to note that it approaches the levels seen in native unfractured bone. This suggests that as the marrow is established in the callus at later times, these stem cells populate the new marrow space of the healing bone. Sox9 and Osterix average fold expression both peak at day 10 and are indicative of early callus formation.

**Future Directions**

Once the histological methods are finalized for this study, Tamoxifen will be administered in specific time windows to better track the fate of the Prx1-expressing cells. It is the ultimate goal that the Prx1CreER-GFP-LacZRosa lineage will be used for further experimentation with distraction osteogenesis.
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Education: M.S. Medical Science, Boston University School of Medicine (2015)

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Minor: Spanish Language

The Bishop’s School, La Jolla CA (2007)

Academic Achievement:
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Florence Riford Scholarship (2007)

Research Experience:
Graduate Student Researcher – Orthopaedic and Developmental Biomechanics Lab at Boston University (2014-2015)

Study: Visualization of Prx1-expressing cells in a murine fracture model
Intentions: identify the optimal staining method for LacZ Rosa to visualize Prx1-expressing cells and track cell fate
Skills: animal handling, intraperitoneal injections, aseptic surgery, animal harvest, x-ray, histology, reagent preparations, qPCR

Senior Lab Assistant - Biomaterials and Particle Analysis Lab at UCLA (2009-2013)

Study: comparing the various lab protocols used to isolate orthopedic wear particles from test lubricant against the current ASTM 561-05a and ISO 17853 standards.
Intentions: investigate the potential advantages and disadvantages of various protocols while attempting to obtain the highest recovery of particles, in order to better analyze the failure of orthopedic implants.
**Skills:** scanning electron microscopy, morphological analysis of SEM images (metamorph software), energy dispersive x-ray spectroscopy, ultracentrifugation, iridium sputter coating, reagent preparations

**Teaching Experience:**
Teaching Fellow – Fundamentals of Medical Biotechnology (2015)
Boston University School of Medicine – Graduate Medical Science

Teaching Fellow – Cellular Organization of Tissues (2014)
Boston University School of Medicine – Graduate Medical Science

**Publications:**
Reviewer – *Medical Neurobiology* (2010)

**Leadership/University Service:**
Master of Science Medical Science Subcommittee (2014-2015)
Aid the Masters of Science Medical Science Program Director in class programming, and serve as a liaison for current and prospective students.

Boston Medical Center Pediatric Unit Volunteer (2014-2015)

Student Intern – Project Brainstorm UCLA K-12 Outreach (2010)
Prepared lesson plans and taught students of varying backgrounds (ages K-12) basic aspects of neuroscience to promote their interest in studying science.


Student Group Leader – Brain Awareness Week at the Brain Research Institute UCLA (2010)
Presented preserved human brain specimens to K-12 outreach students and explained the causes and effects of brain injury through trauma and disease.

Tutored and mentored an elementary school girl each week to improve upon her weaknesses in math, reading, and writing. The Watts Tutorial Program at UCLA busses in children from the inner-city for private tutoring, and strives to deter the gang violence, poverty, and illiteracy encompassing the community.