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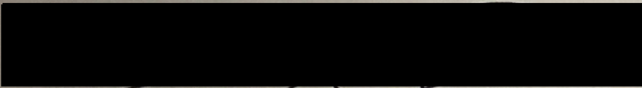

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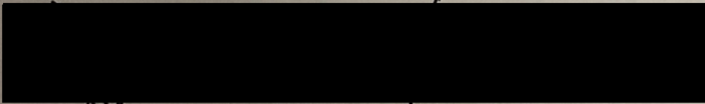

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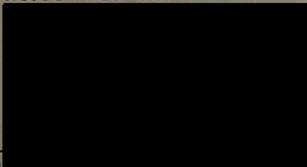

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Learning to Care for Those in Harms' Way

Ex Vivo Characterization of αSMA+ Periodontal Stem Cells and the Role of Bmp2 in *Mus Musculus*

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Short Running Title: αSMA+ Periodontal Stem Cell Characterization & Role of Bmp2

Summary (1 sentence): αSMA+ periodontal stem cells can differentiate ex vivo to produce bone, PDL fibroblasts, and possibly acellular cementum.

Abstract (250 words)

Background: Many approaches to regenerate lost periodontal tissues are studied with varying degrees of success, to include multiple sources of stem cells. The aim of this study was to examine the ex vivo differentiation of alpha smooth muscle actin positive periodontal stem cells (αSMA+ PSCs) and the effect of bone morphogenetic protein 2 (Bmp2) on differentiation capability.

Methods: αSMA+ PSCs were isolated from Bmp2-floxed mice mandibles and implanted subcutaneously in mice using absorbable gelatin sponge and denuded molar after: no additional treatment (n=4), green fluorescent protein (GFP) labeling (n=6), or Bmp2 knockout (n=3). Mice were sacrificed at 4-8 weeks, and retrieved samples underwent selected staining and immunocytochemistry (H&E, Trichrome, Bsp-Ab, Postn-Ab, αSMA-Ab, Bmp2-Ab, EdU Click-iT).

Results: 8 of 13 transplants were successfully retrieved – 5 were not present upon re-entry. Several teeth were lost from migration during implantation/healing. αSMA-Ab and GFP labeling confirmed implanted αSMA+ PSCs presence in retrieved samples. Strong bone sialoprotein signaling was observed in acellular cementum regions of several samples. Mineralized tissue consistent with bone was present and oriented adjacent to PDL-like fibroblasts in 6-week GFP sample. Periostin was seen in fibroblast cells adjacent to the root surface and throughout samples when a tooth was not present. Bmp2 was present in untreated and GFP samples. Initial observations showed Bmp2 knockout samples formed less bone and PDL fibroblasts.

Conclusions: αSMA+ PSCs can differentiate ex vivo to produce bone, PDL fibroblasts, and possibly acellular cementum. The tooth acts as a matrix for differentiation. Knockout of Bmp2 disrupts this differentiation capacity.

Key Words

Periodontium, stem cells, guided tissue regeneration, bone morphogenetic protein 2

Introduction

The most recent NHANES 2009-2014 data estimates 42% of the adult population in the United States has periodontitis.[1] Periodontal disease is a chronic health problem that can lead to tooth loss, has been linked with many systemic disorders, and significantly impacts quality of life.[2-4]

While the goal of periodontal therapy is to halt the disease, the ultimate goal would be to re-establish the lost tissues and function of the diseased periodontium, which includes alveolar bone, cementum, and the periodontal ligament. Regeneration of these tissues is a challenge, due to the complex relationship between promoting new cell development without compromising existing cell functions.[5] Many different approaches have been studied and implemented clinically with results varying widely due to this complexity.[6]

The periodontal stem cells (PSCs) for alveolar bone, cellular cementum, and periodontal ligament have been identified as alpha smooth muscle actin positive (αSMA+) progenitor cell populations within the periodontium.[7-10] A major source of these cells is associated with tiny capillaries of the periodontium.[11] Bone morphogenetic protein-2 (Bmp2) has a complex role in tooth development and periodontium formation, and it is vital in controlling the αSMA+ periodontal stem cells and the vascularization of the periodontium and bone.[8]

The purpose of this study was to examine ex vivo differentiation and characterization of αSMA+ PSCs and the effect of Bmp2 on differentiation capability.

Materials & Methods

Mice

All mice studies were conducted under the proper guidelines for use of experimental animals as defined in the protocol and IACUC-UTHSCSA polices on the use of experimental animals. C57BL/6 Bmp2-fx/fx mice were used for cell isolation, tooth samples, and transplantation surgeries, and this mouse model has been previously established.[12] Mice used for cell isolation were three months old, while mice undergoing transplantation were four months old.

αSMA+ Periodontal Stem Cell Isolation/Preparation

αSMA+ cell populations were isolated from 3-month old Bmp2-fx/fx wild-type mice by splitting five mandibles and digesting for one hour in 10mL 0.25% collagenase 1 0.05% tripsin. Four pre-determined cuts were made through cortical plate. Cut mandibles were digested for 1.5 hours in fresh solution, rocking at 80rpm. Cells were collected and suspended in alpha minimum essential medium (αMEM) and 20% special serum. Digestion/collection/suspension was repeated twice with 10mL of fresh solution. Cells were plated on T150 presoaked in phosphate-buffered saline (PBS) with regular media changes for 10-12 days. Cells were harvested by washing plates with PBS and 2.0mmol ethylene diaminetetraacetic acid (EDTA), adding 12mL 0.2% collagenase-2 and 1.2mmol calcium chloride in PBS, and digesting for 30 minutes. Plates were scraped, cells combined, and suspended at 1200 x g for five minutes. Cells were resuspended in freeze medium and frozen with liquid nitrogen for storage.

Frozen cells were expanded in αMEM plus acetic acid and 15% fetal calf serum on eight 10cm plates coated in rat tail collagen. They were then split into three groups prepare for implantation:

no additional treatment (n=4), green fluorescent protein (GFP) labeled (n=6), and Bmp2 knockout (n=3). The first group of cells were implanted without any additional manipulation to ensure viability and growth capability. GFP labeling and Bmp2 knockout (Bmp2-cKO) was accomplished by infecting cells with adenovirus containing cytomegalovirus (CMV)-GFP or CMV-Cre+CMV-GFP at a multiplicity of infection of 3,000. Over 90% of cells were GFP+ after two days, and both groups were expanded for four days before collection.

Tooth Preparation

Molars were gently removed from the mandibles after digestion and cell wash cycles. The attached periodontal ligament cells/fibers were removed by digestion procedures, and teeth were fixed in 70% ethanol. Prior to transplantation, teeth were washed in sterile water and allowed to air-dry in a sterile hood.

Gelatin Sponge Preparation

A previously described protocol for stem cell ossicle formation was modified to incorporate mouse molars with an absorbable gelatin sponge to form the transplant.[13] αSMA+ PSCs (1-2x10^6 cells/1mL of serum-containing medium (SM)) were transferred into 1mL Eppendorf tubes, pelleted at 135 x g for 10 minutes, supernate removed, and resuspended with 75µL SM. Sterile gelatin sponges were cut into 5mm x 5mm squares. Two molars were inserted into each sponge using fine tip tweezers. Sponges were placed into SM and squeezed to remove air. They were blotted between sheets of sterile filter paper and immediately placed into the resuspended cells.

Transplantation Surgery

Mice were anesthetized using an intramuscular injection of ketamine (75 mg/kg) and dexmedetomidine (1 mg/kg). The dorsal surface was shaved, cleaned with betadine and 70%

ethanol, and a 1cm longitudinal incision was made with sterile scissors. A pocket was created by inserting sterile curved hemostats subcutaneously and opening 1cm to the anterior and posterior. Transplants were inserted into the subcutaneous pocket, 1 anterior/1 posterior (1-2 transplants/mouse). Incisions were closed with sutures, and atipamezole (0.5 mg/kg) was administered for reversal through intramuscular injection. Mice were monitored during recovery.

Sample Harvest/Fixation

At 4- to 8-weeks post-transplantation, mice were sacrificed using carbon dioxide inhalation and cervical dislocation prior to retrieving samples. 5-ethynyl-2'-deoxyuridine (EdU) was injected for evaluation of proliferation one day prior to sacrifice. All samples were fixed with 4% paraformaldehyde overnight and decalcified with 15% EDTA for two weeks, changing solution twice per week, then split between paraffin and cryostat embedding protocols. 8µm sections were prepared for staining and immunocytochemistry: H&E, Masson's Trichrome, EdU-Ab-clickiT kit, αSMA-Ab, Bsp-Ab, Postn-Ab, Bmp2-Ab, and Col12-Ab.

Analysis

Light microscope and confocal microscope were used for histological evaluation of cell proliferation, arrangement, and mineralization. Qualitative data are presented.

Results

Eight of 13 transplants were successfully retrieved. Two Bmp2-cKO and three GFP-labeled samples were not present upon re-entry. Teeth were lost in several samples due to migration from the sponge during implantation/healing, and some samples had more teeth upon retrieval than during transplantation due to this migration (Figure 1).

Characterization of αSMA+ Periodontal Stem Cells

αSMA-Ab (untreated samples) and GFP labeling confirmed the presence of implanted αSMA+ PSCs in the retrieved samples (Figure 2). Strong bone sialoprotein (Bsp) signaling was observed in the acellular cementum region adjacent to the implanted tooth (Figure 3). Areas of bone structure with osteocytes and osteoblasts and mineralization were present and oriented adjacent to PDL-like fibroblasts in the 6-week GFP sample (Figure 4). Periostin (Postn), a marker for mature PDL fibroblast cells were adjacent to the root surface (Figure 5).

Comparison of Proliferation in Bmp2-cKO Model

Bmp2 was present in untreated and GFP samples (Figure 6). Initial observations showed the αSMA+ PSCs in which the endogenous Bmp2 is removed formed less bone structure and PDL fibroblasts, as assayed with H&E, Trichrome staining, and immunocytochemistry for Postn and Bsp.

Discussion

Non-embryonic stem cell sources, such as adult stem cells, offer several advantages for regenerative treatment. Isolation and use of adult stem cells encounter fewer legal and ethical issues, form fewer tumors when implanted due to a more restricted differentiation capacity, and provide a potential autologous source for implantation.[5] Identification of the correct cell type, optimal harvest, differentiation, and delivery mechanisms are essential for effective use in regenerative procedures. The current study demonstrated αSMA+ PSCs could be harvested, expanded, and implanted as an allogenic source with potential to differentiate into the components of the periodontium ex vivo. These findings support previous studies showing PDL progenitor cells as the only cell source to produce alveolar bone in addition to the other required

periodontium components.[14] The other sources studied – dental follicle, dental papilla, and mesenchymal stem cells – were also unable to form organized elongated cell shapes required as a prerequisite for functional PDL fiber formation.

Bone sialoprotein (Bsp) is an early marker of osteoblast and cementoblast differentiation, thought to nucleate hydroxyapatite crystal formation, and vital for acellular cementum formation.[15] Bsp is often absent in exposed cementum of diseased teeth, which is thought to be due to structural and compositional changes in the matrix. These changes may influence the capacity for new connective tissue attachment and periodontal regeneration, since functional cementum is necessary for proper and functional PDL attachment and arrangement.[16] The presence of Bsp in this study's samples show promise for acellular cementum formation, osteogenesis, and a functionally arranged periodontium.

Periostin (Postn) is a critical extracellular matrix protein involved in PDL space maintenance and periodontal homeostasis. Previous studies evaluated a Postn knockout model which replicated the appearance of periodontitis, producing bone loss, PDL inflammation, and pocket formation. These studies also found faster bone formation from PDL progenitor cells than other bone-forming progenitor cells.[9] The presence of Postn in the retrieved samples of the present study indicates initial stages of periodontium formation and organization.

Bmp2 has been shown to play a role in vivo in tooth development, cytodifferentiation, cementogenesis, and organization of the PDL. The PDLs in Bmp2-cKO mice were dysmorphic, and decreased vascularization led to a reduction in candidate periodontal stem cells.[8] The initial

findings of the current study also showed decreased cell differentiation, bone formation, and PDL fibroblast formation in Bmp2-cKO αSMA+ PSC samples compared to untreated and GFP-labeled samples. Decreased Postn expression is found in Bmp2-cKO mice models, as was also seen with decreased Postn expression in Bmp2-cKO αSMA+ PSC samples.

A major limitation of this study was the loss of several samples and migration of teeth out of the sponges, which limited the findings to qualitative analysis. Five samples in three mice were not present upon re-entry, but all mice were from the same litter as previous subject animals. The failed samples were from the same cell line; however, one successful sample was produced in the mouse whose second sample failed during the same implantation cycle. Unknown orientation of the tooth within the sample during sectioning was another drawback for obtaining clear sections through the long axis of the tooth. The malleable nature of the absorbable gelatin sponge did not allow for consistent orientation once implanted. Natural movements of the mouse during healing may have displaced the teeth from the original orientation within the sponges.

Further research is needed to confirm these findings and expand the knowledge of the differentiation capacity and quality of tissues produced by αSMA+ PSCs. While the current study proved the capacity of these cells to produce components of the periodontium ex vivo, the orientation and timing of the production of each tissue type greatly impacts the functionality of the periodontium and qualification as true periodontal regeneration.

In conclusion, the results of this study showed αSMA+ PSCs can differentiate ex vivo to produce bone, PDL fibroblasts, and possibly acellular cementum. In this application, the tooth acted as a matrix for differentiation, and knockout of Bmp2 disrupted the differentiation capacity.

Acknowledgements

The authors declare no conflicts of interest with this study. This study was supported by the NIH Grant 5R01DE024797-04: Bmp2 and Sost Genes and Their Interactions in Stem Cells of the Periodontium.

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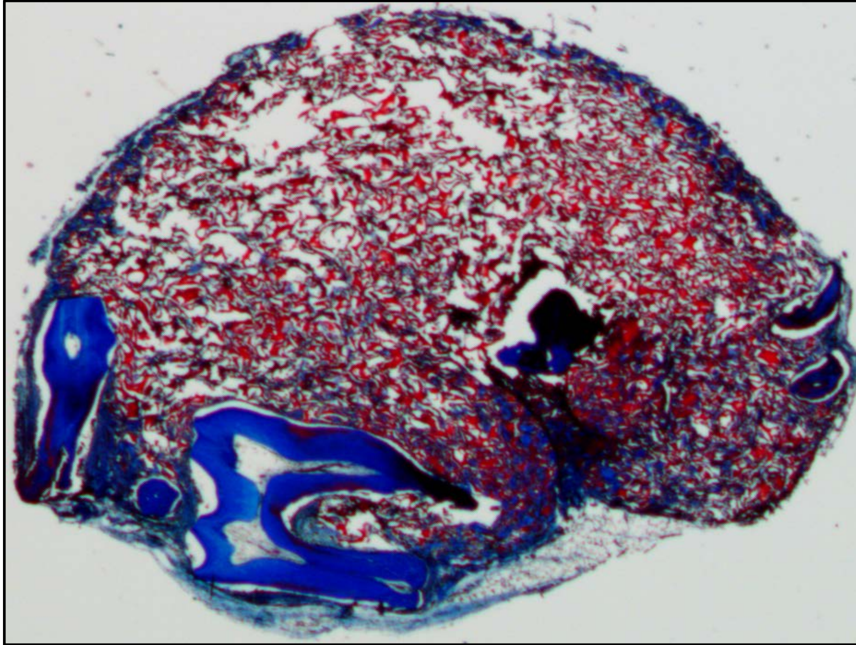
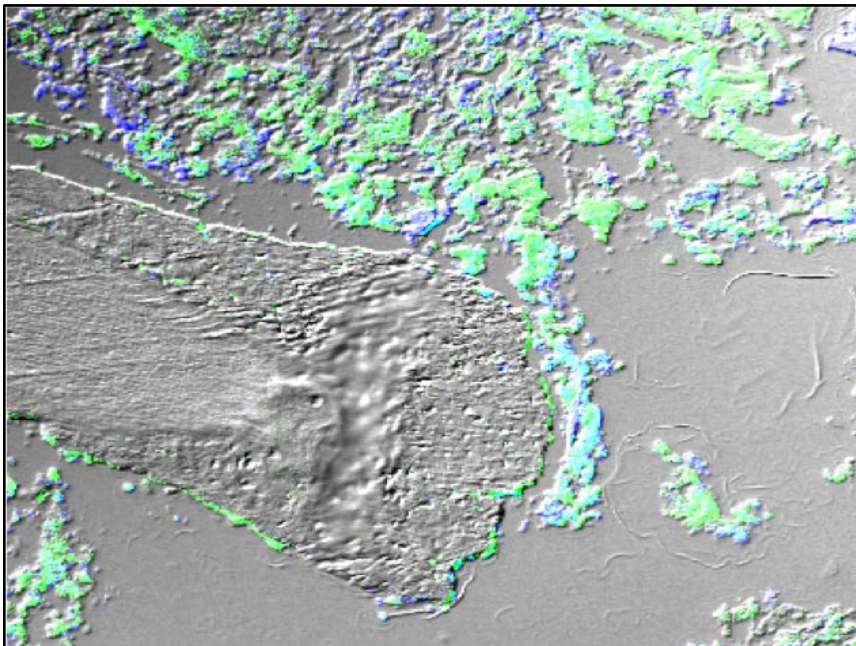
Figures

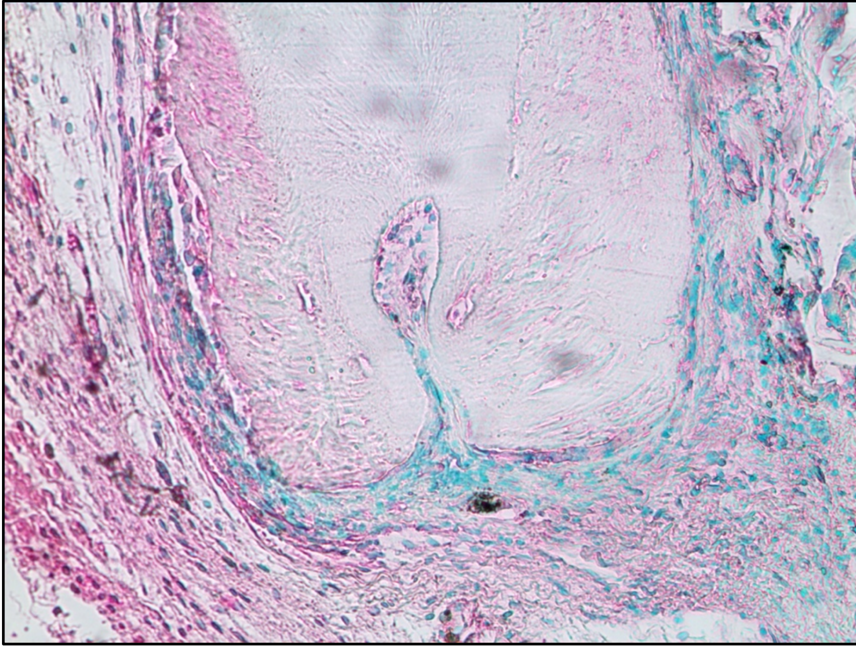
1. Migration of teeth within transplant and from adjacent transplant (4 weeks, Masson's Trichrome, 0.5x)



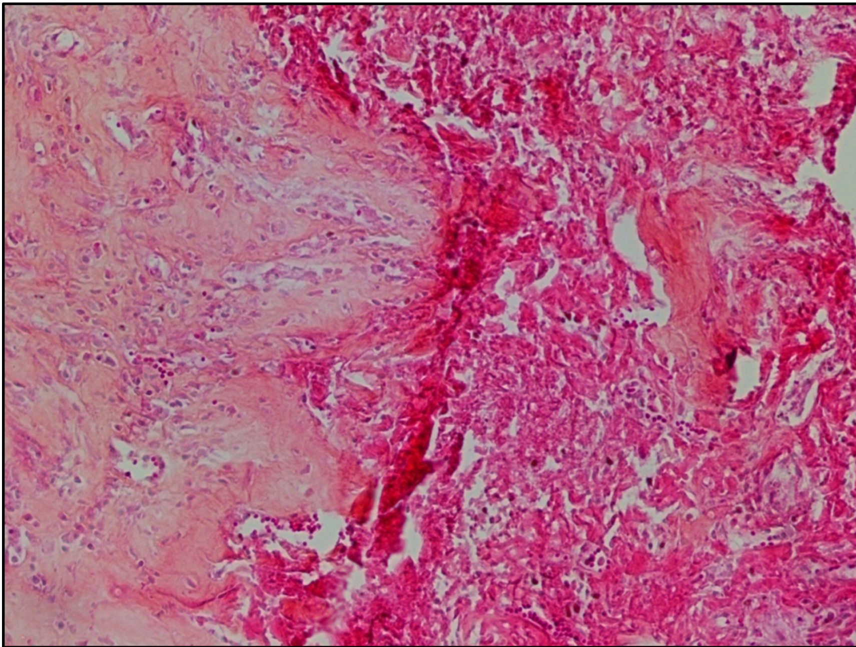
2. PSC-Ad-CMV-GFP differentiated to cementum-like cells attached to the tooth surface and adjacent matrix (4 weeks, GFP, 4x)



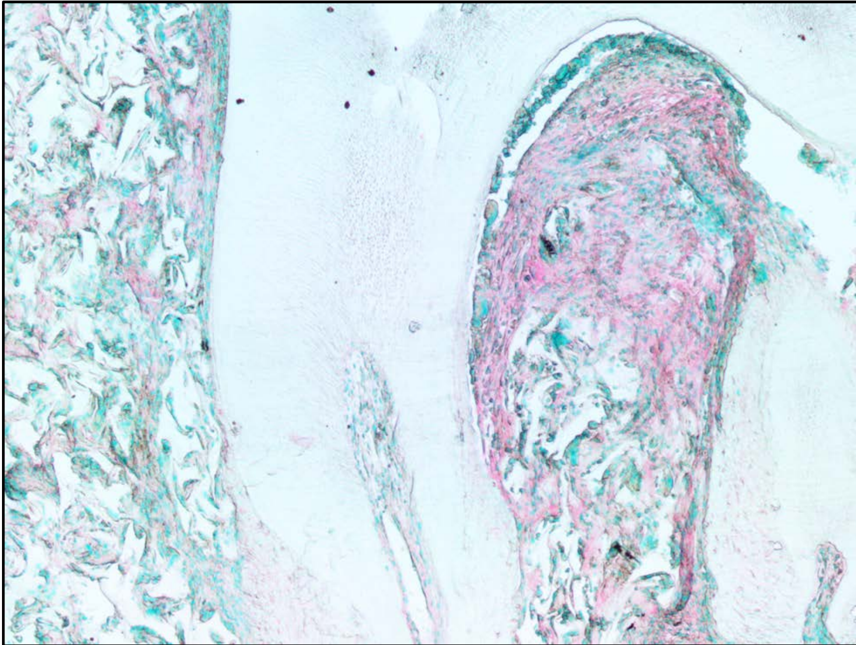
3. Bone sialoprotein+ cells adjacent to root apex (4 weeks, Bsp-Ab, 10x)



4. PSC produced bone- and cartilage-like cells with PDL-like structures (6 weeks, H&E, 10x)



5. Periostin+ PDL-like cells on root surface and in matrix (4 weeks, Postn-Ab, 4x)



6. Bone morphogenetic protein 2+ cells present in untreated PSC (4 weeks, Bmp2-Ab, 4x)