Descriptive Analysis of Possible Dural Stem/Progenitor Cells Activated in a Rat Critical-Sized Calvarial Defect Bone Regeneration Model

By

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Thesis submitted to the Faculty of the
Oral Biology Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Master of Science 2019

DISSERTATION APPROVAL SHEET

Descriptive Analysis of Possible Dural Stem/Progenitor Cells Activated in a Rat Critical-Sized Calvarial Defect Bone Regeneration Model

This thesis is submitted by Ryan T. McGary and has been examined and approved by an appointed committee of the faculty of the Uniformed Services University of the Health Sciences.

The signatures that appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore in partial fulfillment of the requirements for the degree of Master of Science.

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ACKNOWLEDGEMENTS

I would like to thank the members of the committee (Drs. Erley, Herold, Johnson, O'Bryhim) for their instruction, aid, and guidance in support of accomplishing this goal. Additionally, I could not have done this without the tireless work of lab partner and co-resident, Dr. Richard Hill. Additionally Dr. Dickinson, who aided in the concept and facilitated the instruction in immunohistochemistry and helped section slide after slide of samples.

DEDICATION

To my wife Jen who is my rock and motivator. To my children Paxton and Reagan who are the light in my life.

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[Month Day, Year of BOR meeting, check thesis manual for dates]

ABSTRACT

"Descriptive Analysis of Possible Dural Stem/Progenitor Cells Activated in a Rat Critical-Sized Calvarial Defect Bone Regeneration Model"

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Bone regeneration requires local availability of stem/osteoprogenitor cells that can differentiate into osteoblasts and eventually mature bone. Current limitations in the clinical induction of bone regeneration instead of repair, are due in part, to an incomplete understanding of stem/progenitor cells, and signaling systems, such as BMP's, that regulate their activities.

The purpose of this study was to utilize immunohistochemistry to investigate iPSC transcription factors effect on stem/osteoprogenitor cells located in the dural region, of a critical-sized rat calvarial defect model, and their healing response in the presence of varying concentrations of recombinant human bone morphogenic protein 2(rhBMP-2).

Our results demonstrated KLF-4 and Sox-2 serve as indicators of the Yamanaka transcription factors, are present in similar time points, and respond to rhBMP-2 playing a role in the healing of a critical sized defect. Better understanding of the cell populations and their potential lend to improvement in the possibilities of regeneration procedures.

TABLE OF CONTENTS

Descriptive Analysis of Possible Dural Stem/Progenitor Cells Activated in a Rat Critical-Sized Calvarial Defect Bone Regeneration Model i
DISSERTATION APPROVAL SHEET ii
ACKNOWLEDGEMENTS iii
DEDICATION iv
COPYRIGHT STATEMENT v
ABSTRACT vi
LIST OF TABLES ix
LIST OF FIGURES ix
LIST OF ABBREVIATIONS x
INTRODUCTION 1
STATEMENT OF THE PROBLEM 1
SIGNIFICANCE 2
REVIEW OF THE LITERATURE 2
Introduction 2
US Army Battlefield Injuries 2
Regeneration versus Repair 3
Bone Cell Biology 4
Osteoprogenitor cells 5
Oseteogenesis 6
Bone Remodeling and Regeneration 6
RANKL/OPG 7
Healing of Bone 7
Signaling and Early Bone Healing 8
Critical size defect 8
Early wound healing 8
Transcription Factors and Stem Cell Markers 9
Yamanaka Factors 9
Sox-2 10
KLF-4 10
OCT-3/4 11
c-MYC 11
MUSASHI 11
SOX-9 11
SDF-1 11
Growth Factors 12
BMP-2 12
PDGF and VEGF 12
Summary 13
PURPOSE 13
HYPOTHESES 13
HYPOTHESIS #1 13
Table 1 14

HYPOTHESIS #2 14
Table 2 14
MATERIALS AND METHODS 14
OVERVIEW 14
Table #3 16
Detailed Methodology 16
Immunohistochemistry 16
Data Description 18
Independent Variables 18
Dependent Variable 18
RESULTS 19
Defect Description 19
Proliferating Cell Nuclear Antigen (PCNA) 19
Krüpple-like factor 4 (KLF-4) 23
Sex Determining Region Y-Box 2 (SOX-2) 33
Sex Determining Region Y-Box 9 (SOX9) 40
Scleraxis Homolog A (SCXA) 41
RNA-binding Protein Musashi Homolog 1 (Msi1) 43
DISCUSSION 50
FUTURE STUDIES 53
References 54

LIST OF TABLES

TABLE #	DESCRIPTION	PAGE
1	Various Stem/Progenitor Markers	14
2	iPSC Transcription Factors	14
3	Time samples and BMP-2 dosages	16

LIST OF FIGURES

FIGURE		PAGE
1.	Somatic Cell to IPSCs	1
2.	Full Critical Size Defect Sample Example	19
3.	PCNA Positive Control Day1-20ug 1.25-11 Condyle	20
4.	PCNA Positive Control Day1-0ug 1.10-14 Skin 20x	21
5.	PCNA Day5: (a) 5ug 5.95-9- 20x; (b) 20ug 5.90-4 Dura - 20x	21
6.	PCNA Day7-20ug 7.127-8 Dura - 20x	22
7.	PCNA Day14-5ug 14.71-5 Dura - 20x	22
8.	KLF-4 Day1-0ug 1.10-6, 20X Dura	23
9.	KLF-4 Day1-5ug 1.8-15, 20x Dura	24
10.	KLF-4 Day1-20ug 1.25-14, 20x Dura	24
11.	KLF-4 Day3-0ug 3.37-14, 20x Dura	25
12.	KLF-4 Day3-5ug 3.32-9, 20x	26
13.	KLF-4 Day3-20ug 3.39-9, 20x	26
14.	KLF-4 Day5-0ug 5.103-2, 20x	27
15.	KLF-4 Day5-5ug 5.95-4, 20x	28
16.	KLF-4 Day5-20ug 5.90-15, 20x	28
17.	KLF-4 Day7-0ug 7.131-7, 20x	29
18.	KLF-4 Day7-5ug 7.126-15, 20x	30
19.	KLF-4 Day7-20ug 7.127-3 20x	30
20.	KLF-4 Day14-0ug 14.70-4, 20x	31
21.	KLF-4 Day14-5ug 14.71-13, 40x	32
22.	KLF-4 Day14-20ug 14.63-5 20x	32
23.	SOX2 Day1-0ug 1.1-15, 20x Dura	34
24.	SOX2 Day1-5ug 1.5-9, 20x Dura	35
25.	SOX2 Day1-20ug 1.3-13, 20x Dura	35
26.	SOX2 Day5-0ug 5.94-6, 20x Dura	36
27.	SOX2 Day5-20ug 5.99-11, 20x Dura	36
28.	SOX2 Day7-0ug 7.131-3, 20x Dura	37
29.	SOX2 Day7-5ug 7.129-5, 20x Dura	37
30.	SOX2 Day7-20ug 7.130-12, 20x Dura	38
31.	SOX2 Day14-0ug 14.64-9, 20x Dura	38
32.	SOX2 Day14-5ug 14.59-2, 20x	39
33.	SOX2 Day14-20ug 14.63-13	39
34.	SOX9 Day7-5ug 7,127-7	40
35.	SOX9 Day14-20ug 14,63-11	40
36.	SCXA Day7-0ug 7.131-6, 20x	41
37.	SCXA Day7-20ug 7.127-4, 20x	42
38.	SCXA Day14-0ug 14.70-5, 20x	42
39.	Musashi Day3-0ug 3.28-11, 20x	43
40.	Musashi Day3-5ug 3.29-11, 20x	44
41.	Musashi Day3-20ug 3.33-9, 20x	44
42.	Musashi Day5-0ug 5.82-13, 20x	45
43.	Musashi Day5-5ug 5.104-7, 20x	45

44.	Musashi Day5-20ug 5.84-13, 40x	46
45.	Musashi Day7-0ug 7.110-8, 20x	46
46.	Musashi Day7-5ug 7.111-6, 20x	47
47.	Musashi Day7-20ug 7.133-14, 20x	47
48.	Musashi Day14-0ug 14.61-12, 20x	48
49.	Musashi Day14-5ug 14.65-9, 20x	48
50.	Musashi Day14-20ug 14.62-5, 20x	49
51.	Musashi Day14-20ug 14.62-5, 20x	49

LIST OF ABBREVIATIONS

ESC:	Embryonic Stem Cell
IF:	Immunofluorescence
IHC:	Immunohistochemistry
IPSCs:	Induced Pluripotent Stem Cells
Klf4:	Krüppel-like factor 4
Msi1:	RNA-binding protein Musashi homolog 1
MSC:	Mesenchymal stem cells
PCNA:	Proliferating cell nuclear antigen
rhBMP-2:	Recombinant human bone morphogenetic protein-2
SOX9:	Sex determining region Y-Box 9
SOX2:	Sex determining region Y-Box 2
SCXA:	Scleraxis homolog A

INTRODUCTION

STATEMENT OF THE PROBLEM

The continued military conflicts in the Middle East have some service members returning with complicated injuries that require extensive surgeries and critical care. Many of the injuries sustained can cause disfigurement and deformities with loss of hard and soft tissue. This is especially evident in the craniomaxillofacial region. Ideally, treatment and healing would lead to a replacement of lost tissue with structurally and functionally equivalent tissue; that is, regeneration would occur. However, although bone does have some innate capacity for regeneration, this capacity has limitations. This is especially evident when the injury is large enough (a critical-sized defect) that the missing tissue cannot be regenerated fully. Instead, the injury is repaired, at least in part, by way of scar tissue formation. This scar tissue is notably weaker and compromised when compared to the original structures. Thus, while regeneration of bone (and associated tissues) is the preferred response, and is sought after by clinicians to improve the clinical outcomes of their patients, the default healing pathway is typically repair. The identification of bone morphogenetic proteins, such as BMP-2, that can stimulate bone formation has provided a powerful tool for treating bone injuries, but our present inability to exploit agents such as BMP-2 to fully regenerate bone consistently limits the forms of clinical treatments available.

Bone regeneration at a site requires the local availability of stem/osteoprogenitor cells that can subsequently differentiate into osteoblasts that produce osteoid matrix and woven bone, as the first step in to formation of mature bone. BMP-2 is involved in osteoblastic differentiation and bone formation. Our current limitations in the clinical induction of predictable bone regeneration

are due in part to an incomplete understanding of these stem/osteoprogenitor cells, and the signaling systems, that regulate their activities.

A great amount of research has been done on the various factors that have been used to induce the pluripotency of native somatic cells. While much of this research has been focused on a group of transcription markers allowing for the creation of induced pluripotent stem cells (iPSCs)[1,2], little information has been documented on the effects on bone and bone healing in the presence of a critical-sized defect. The hope is that iPSCs may allow for minimal modifications to resident somatic cells, which may subsequently induce regeneration in large wounds or defects[1,2]. Some of these same transcription factors are present in the maxillofacial region and may add to the regenerative potential of stem cells if induced into pluripotency[3].

SIGNIFICANCE

If the signaling pathways activating stem/osteoprogenitor cells and leading to bone regeneration were understood in more detail, this could lead to developing improvements in current clinical methods and materials, which in turn would provide better clinical outcomes in bone formation for our patients. This would lead to a decrease in bone loss and help to improve the military readiness for the Army.

REVIEW OF THE LITERATURE

Introduction

US Army Battlefield Injuries

Changes in the form of warfare in Iraq and Afghanistan has made explosives (landmines, rocket propelled grenades, improvised explosives, and mortars) the most common cause of craniomaxillofacial injury[5]. Craniomaxillofacial battlefield injuries (CBI) involved some form of

explosive 84% of the time, compared to the 2% of the CBI that involved motor vehicle accidents[6]. The extent of these forms of injuries often goes beyond the capacity of predictable reconstruction when utilizing current treatment methodologies[7].

Regeneration versus Repair

Injury to tissues and cells initiates a program of events that encompasses the initial response to injury and begins the healing process. The act of healing, by way of either regeneration or repair, is a process conducted by the organism to combat insults that causes damage[8]. Ideally, healing would be by regeneration, a process that results in the complete restoration of damaged or lost tissue. However, even with clinical intervention, the default healing pathway is typically repair, which correctly restores some of the original tissues, but is dominated by collagen deposition and scar formation.

Regeneration is the desired route for trauma-induced fractures and surgical sites, as well as congenital defects, but much of the process of regeneration, including various critical events of early wound healing and regeneration, remains largely uncharted. A more detailed understanding of bone cell biology, and the processes of osteogenesis, wound healing, and regeneration would allow the development of rational strategies that support regeneration by exploiting and enhancing underlying regulatory mechanisms. A key target for such strategies will be stem/osteoprogenitor cells associated with bone tissues, and their differentiation into chondrocytes and osteoblasts for bone formation. Adequate bone regeneration will require a sustained production of these differentiated cells in the appropriate spatial configuration.

Bone Cell Biology

Bone cells consist collectively of primary bone cells and less differentiated stem/osteoprogenitor cells. The primary bone cells are terminally differentiated cells to include osteoblasts, osteocytes, and osteoclasts.

Secretory osteoblasts are large cuboidal or box-shaped cells responsible for the synthesis, assembly, and mineralization of the bone matrix. In this form, they express genes that are markers of terminal differentiation, such as osteocalcin (OCN), which are involved in matrix formation and mineralization. Non-dividing osteoblasts can, however, further differentiate into either endosteal bone lining cells or osteocytes[9]. Bone-lining cells are elongated structures that cover a surface of bone tissue and in uninjured bone exhibit no synthetic activity[10]. . They compromise a major cell population of the endosteum (lining the inner surfaces of trabeculae), and are also present in the cambial layer of the periosteum (lining the outer surfaces of bones). It has been demonstrated more recently that endosteal bone lining cells, thought to be terminally differentiated, can reverse the differentiation process to return to an osteoblastic phenotype[9,11].

Osteocytes are terminally differentiated cells that cannot migrate or proliferate. They are trapped within the bone matrix but remain in contact with other bone cells by thin cellular processes extending through the canaliculae of the bone. They also have the ability to both synthesize or resorb matrix substance, and provide a maintenance function by regulation of blood-calcium homeostasis. Importantly, they serve as sensors for mechanical loading, and then signal to other cells in the bone to activate bone remodeling[9].

Osteoclasts are large, multinucleated cells of the monocyte-macrophage hematopoietic cell lineage; their primary function is to degrade bone matrix in various physiologic and pathologic

contexts. Osteoclasts are stimulated by osteoblasts and play a critical role in the process of bone remodeling and healing.

Osteoprogenitor cells

Terminally differentiated cells are typically non-proliferative, or can at most only undergo a few cell divisions. Therefore, less-differentiated proliferative cell populations are required to provide adequate numbers of differentiated cells such as secretory osteoblasts for bone formation. Osteoprogenitor cell populations are lineage-committed cells that have an intrinsic ability to proliferate and differentiate into osteoblasts. Less differentiated proliferative osteochondroprogenitor cells can potentially differentiate into osteoblasts, or other cell types, such as chondrocytes, depending on the local signaling environment. In turn, these progenitors arise by lineage commitment of multipotent stem cells capable of forming different lineages of cells[12].

This process of commitment and differentiation is governed in part by programs of transcription factor expression and activation, which are in turn regulated by numerous signaling pathways connected to the external environment. Although some critical transcription factors have been identified, such as Sox-9 for chondrogenesis and Runx-2 for osteogenesis, our understanding of the process is still limited. Thus, there appears to be a near continuum in the progression from multipotent stem cells to lineage-committed progenitor cells, due to our current incomplete identification of the requisite factors mediating each stage of lineage commitment. The term stem/osteoprogenitor cell reflects this uncertainty in identifying the exact position along the pathway a particular cell might occupy. Cells at various stages along this extended pathway are present in bone marrow, endosteum, and the cambium layer of the periosteum. For bone formation following injury to occur, undifferentiated mesenchymal stem/progenitor cells (MSC's) and/or osteo(chondro)-progenitor cells must migrate to the site, proliferate, and subsequently differentiate into osteoblasts[12].

Oseteogenesis

All of the cell populations described previously play a role in normal bone development, known as osteogenesis. Osteogenesis starts during embryogenesis and can occur by two distinct pathways: endochondral or intramembranous ossification. Endochondral ossification involves continued ossification of a cartilage template formed by mesenchymal cells, and is typically seen in long bones and most of the body. Chondrocytes play an important role during endochondral ossification as they undergo rapid mitosis, stacking on one another, releasing alkaline phosphatase, and eventually dying, leaving behind concavities that osteoblastic progenitors can utilize for bone formation[13]. Intramembranous ossification is a process that proceeds without cartilage and is seen in the flat bones such as the calvarium. It is mediated by the direct mineralization of osteoid. Intramembranous ossification is of considerable importance for the healing of surgical sites, fractures due to trauma, and congenital malformations[14,15].

Bone Remodeling and Regeneration

Bone modeling and remodeling are processes of resorption and appositional bone formation allowing bone tissue to adapt to loads. When mechanical stresses are placed, the bone is able to adapt by way of osteoblastic and osteoclastic activity. Osteoclastic resorption and osteoblastic deposition gives way to bone formation. In bone modeling, the unbalanced processes of resorption and deposition result in a change in the size or shape of the bone, changing the initial bone architecture[16]. In contrast, in bone remodeling these two processes are tightly coupled, affecting the mineralized bone with no net change to the overall architecture. Hormones are key regulators of the bone remodeling cycle. These include parathyroid hormone, growth hormone, leptin, and calcitonin. The process of remodeling allows for sufficient supply and homeostasis of calcium and relies on complex signaling pathways to achieve proper rates of growth and differentiation[16].

RANKL/OPG

Osteoblasts and osteoclasts work in coordination within the remodeling process. Osteoblasts secrete receptor activator for nuclear factor-κβ ligand (RANKL), which binds to the receptor activator of nuclear factor-κβ (RANK) on osteoclasts progenitors. When bound in the presence of macrophage colony stimulating factor, differentiation of monocytes into osteoclasts and maturation of immature osteoclasts occurs[17]. Osteoblasts also secrete osteoprotegrin (OPG), which acts as a false ligand competing with RANKL. It acts to inhibit osteoclastic activity and bone resorption by occupying the RANK receptor on osteoclasts without inducing signaling. By this mechanism, osteoblasts can regulate both bone formation and resorption[18,19].

Healing of Bone

MSCs and their lineage are involved in osteogenesis, but they also function in bone healing and remodeling. During the healing process some injured tissues may fail to heal, or heal by a process of scar tissue formation. Scar tissue is a reparative process in which exhibits inferior mechanical properties. It can demonstrate less than 80% of the original strength, and acellular content is the dominant tissue type[9,20].

During the intermediate phase (repair stage) of wound healing, mesenchymal cell proliferation and angiogenesis continue as fibroblasts begin to lay down a matrix, which further aids vascular ingrowth. Angiogenesis forms the vasculature that aids in new tissue formation in bone healing and as the vascular network expands and remodels. These expanded and remodeled networks of vessels come from existing vasculature of the damaged bone trabeculae, and periosteum[9,20]. In the processes of repair and remodeling in bone, osteoblasts release an osteoid matrix. This osteoid matrix forms a soft callus that begins to increase in mineralization and eventually ossifies by intramembranous ossification. This newly ossified callus helps to form a bridge of woven bone between two segments as long as the separation is not too great. As angiogenesis progresses, endothelial cells and their precursors help assemble to form new blood vessels and

secrete key factors. The important angiogenic factors include the fibroblast growth factors (FGFs), platelet-derived growth factors (PDGF), and VEGF[21]. This then leads to the continued remodeling of the wound, which can take weeks to years, depending on the type and volume of injury.

Signaling and Early Bone Healing

Critical size defect

In research models, a "critical sized defect" is a defect that will not heal (in theory during the lifetime of the animal) without some form of intervention, because the size of the defect exceeds any innate capacity for regenerative healing[22,23]. In smaller animal models such as rats, this defect or deficit in bone is typically 5 mm or greater[24,25].

Early wound healing

Bone development, healing, and homeostasis are tied to angiogenesis. Angiogenesis is the utilization of pre-existing blood vessels to form new capillaries. This is a crucial role of the inflammation phase of wound healing. There are also a variety of transcription factors that play a role in the stimulation of cells to differentiate, but also result in the de-differentiation of mature cells, resulting in a pool of cells that are pluripotent. Local production or release of bone morphogenetic protein 2 (BMP-2) is associated with osteoblastic differentiation and bone formation[26]. Other local factors such as platelet-derived growth factor (PDGF), and vascular endothelial growth factors (VEGF) serve as additional differentiating factors. PDGF is also a potent mitogenic factor, inducing cell proliferation.

Transcription Factors and Stem Cell Markers

Yamanaka Factors

This desire to induce regeneration or at least create an environment of regeneration brings in the need for stem cells. The most commonly discussed sources of stem cells are the embryonic and adult stem cells. Whether from embryos or adult tissues, stem cells are few. But many are needed for cell therapies.

One other consideration are the ethical and political problems with using embryonic stem cells -- so if there were a way to get more stem cells from adults, it might be less controversial, also making it easier to research and study. Therefore, recent studies illuminating the possibilities of induced pluripotent stem cells (iPSCs), has caused great excitement surrounding their potential for regeneration and healing. Yamanaka et al developed the method for inducing skin cells from mice into becoming pluripotent stem cell-like and called them iPSC cells[4]. This was done with only four transcription factors. He then repeated the research in humans with a similar result. The 2012 Nobel Prize in Physiology and Medicine was awarded to Shinya Yamanaka for his discoveries on reprogramming somatic cells to pluripotency.

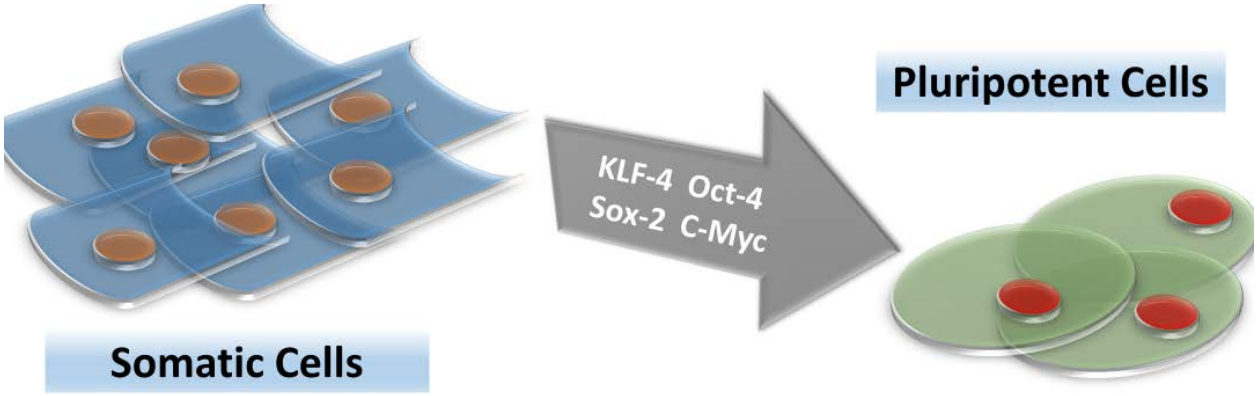

Figure #1: IPSC inducing somatic cells to de-differentiate into pluripotent stem cells.

The four transcription factors are Sox2 in conjunction with Oct4, c-Myc and Klf4. They were combined or co-bound to induce somatic stem cells to de-differentiate back to pluripotent stem cells. As the name implies, these iPSCs give them the ability to differentiate into other adult cells. The discovery that only four transcription factors were necessary to induce pluripotency simplified future regenerative medicine research. These iPSC cells have potential to be used in future models of regeneration including bone regeneration[1,2,4] . In addition to the capabilities of these transcription factors, they are also present in the oral maxillofacial region and have potential for induction or utilization[3].

Sox-2

The Sox family of genes has been shown to play key roles during virtually all stages of mammalian development[27] SOX2, more specifically, as a transcription factor has demonstrated that it is essential for maintaining self-renewal, and potential for pluripotency especially of the undifferentiated embryonic stem cells. Sox2 has a vital role in conservation of embryonic and neural stem cells[28]. It is also a main component of the four Yamanaka factors. Induced pluripotency is possible using adult neural stem cells, which express higher levels of Sox2.

KLF-4

Kruppel- like Factor 4 (KLF4) is plays a role in proliferation, differentiation, apoptosis and potentially somatic cell reprogramming. KLF4 has demonstrated stem-like capacity of embryonic stem cells. Additionally KLF-4 attenuates osteoblast formation, function and signaling with osteoclasts[29]. Again, it is one of 4 transcription factors that Yamanaka and his group were able to use to induce pluripotency in fibroblasts[1,2,4].

OCT-3/4

In regards to iPSCs Oct3/4 as been considered one that is seen most frequently in combination with with Sox-2[28]. Oct-3/4 has been demonstrated in a variety of oral maxillofacial areas to include: bone marrow aspirates, adult dental pulp, exfoliated deciduous teeth, periodontal ligament, and gingival tissue[3].

c-MYC

c-MYC is from the Myc family and is generally known as a regulator that codes for transcription factors. In humans it regulates expression of up to 15% of all genes and is located on chromosome 8[30]. It has been shown that in combination with the other iPSCs it is a necessary component in the pluripotency of fibroblasts[4].

MUSASHI

RNA-binding protein Musashi homolog 1 is also known as Musashi-1. As a stem cell marker, it helps to control self-renewal and terminal differentiation especially in neural stem cells[31]. Due to focus of this study on the dural region, this neural marker aids in the differentiation of which stem cells may be present.

SOX-9

Prior research suggested a chondroblast-like scaffold was deposited along the dural layer in this critical size defect model. SOX-9 is a transcription factor that has been shown to be an important component to cartilage formation[32].

SDF-1

SDF-1 is a chemokine protein with a short half-life of 2-3 hours that is induced in injured bones, specifically in the periosteum[33]. SDF-1 and its receptor CXCR-4 are upregulated by HIF-1 and help to promote endochondral bone repair by recruiting MSC's and progenitor cells. Functionally, SDF-1 regulates some reparative mechanisms, but can

also be involved in cellular inflammation and bone remodeling[33,34]. Controlled and localized release of SDF-1 augments angiogenesis and accelerates wound healing[35]. Also, experimentally it has been demonstrated that the use of heterozygous SDF-1 in mice is crucially important for recruitment of MSCs during early phases of bone fracture healing[33,35,36].

Growth Factors

BMP-2

Bone morphogenetic proteins are growth factors that induce formation of bone and cartilage. There are multiple related factors such as BMP-2, BMP-4, and BMP-7. These proteins stimulate osteoblast proliferation, differentiation, and induce production of chemotactic agents. Currently, recombinant human BMPs (rhBMPs) are used in medical and dental surgical procedures; rhBMP-2 appears to be more effective at inducing bone formation[37,38]. BMP-2 in the presence of hypoxia has an effect on osteoblasts through an HIF-1α-dependent mechanism[39]. Also noteworthy is that BMP-2 and SDF-1 may be required for differentiation of MSCs into osteoblastic cells[40,41].

PDGF and VEGF

Other locally acting growth and differentiation factors that potentially influence bone regeneration include PDGF and VEGF. VEGFs are involved in the stimulation of angiogenesis, may help in the vascularity of the bone growth[42]. PDGFs appear to promote the proliferation of undifferentiated MSC's and some progenitor cell populations. There is also an indication that PDGF's aid in tissue remodeling and cellular differentiation during later healing stages[43]. The interaction of these factors in conjunction with BMP creates a potential positive feedback loop to drive bone formation[44].

Summary

Much of the current literature contains segmented information in regards to osteogenesis, normal tissue and bone repair, and some information in regards to what regenerative mechanisms can look like. Various angiogenic, osteogenic, and neurogenic markers and factors have been described individually in one form or fashion, yet we still do not fully understand bone growth and its response to injury. We know by default that there are many cells, markers, and expression factors that must be involved, yet their correlations and interactions have not been defined in full.

PURPOSE

The purpose of this study was to investigate the potential connection of transcription factors of iPSCs to stem/progenitor cells and BMP-2 in the healing of a calvarial critical-sized defect in a rat model. An increase in the understanding of these associations would allow for the development of strategies that could make regeneration a dominant and potentially predictable treatment modality. Through the utilization of various antibody markers in conjunction with immunohistochemistry conformation of relationships and associations that could be indicative of regeneration and/or repair will be examined.

HYPOTHESES

HYPOTHESIS #1

There is a population of cells in the dura that express markers characteristic of neural crest or mesenchymal stem cells and that proliferate following injury, and respond to local BMP-2 levels by differentiating into either chondrocytes or osteoblasts *via* an osteochondroprogenitor intermediate.

Table 1

Various Stem/Progenitor Markers	
Mesenchymal stem cell	CD23, CD 73, CD90, CD105, CD166
Embryonic stem cell	Sox-2, Nestin, PCNA, KLF-4
Other	Musashi, Runx-2, Sox-9, Scx-A

Table 1: Stem/progenitor cell markers. Utilization of the various stem cell/progenitor cell markers will increase the understanding of which stem or progenitor cells are present in which location8s and at which time points. MSC stem and progenitor cell markers along with other markers were chosen based on lit review and previous rat cavarial studies done at FT. Gordon[1,2,45,46]

HYPOTHESIS #2

Co-incident with or as pre-cursors to stem/progenitor cell proliferation, iPSC transcription factors will be present and respond in a dose dependent manner to the absence or presence of BMP-2 in the critical sized defect model

Table 2

IPSCs	Transcription Factors
	Sox-2
	KLF-4
	c-Myc
	Oct-3/4

Table 2: IPSC Transcription factors. KLF-4 and Sox-2 will be used to look IPSC transcription factors, as well as stem cells (which also express this marker) at the various time points and concentrations of BMP-2[1,2,4]

MATERIALS AND METHODS

OVERVIEW

This study aims to characterize the phenotype of various cell types, such as progenitor cells, present at sites of BMP-2-stimulated bone regeneration in the rat critical-sized

calvarial defect model. The samples to be used were generated in a set of previous studies that examined the effect of recombinant human BMP-2 (rhBMP-2) on bone regeneration in this model (O'Bryhim, DeCardona, and Jusino). Animal selection, management, and the surgery protocol followed routines approved by the Institutional Animal Care and Use Committee, Dwight David Eisenhower Army Medical Center, Fort Gordon, GA. Briefly, one hundred and fifty Sprague-Dawley rats (age 11-13 weeks, weight 250-300g) were divided into three treatment groups: 20 µg/site (rhBMP-2/ACS), 5 µg/site (rhBMP-2/ACS), and absorbable collagen sponge (ACS) alone (carrier control = 0 µg/ml dilution buffer) (see Table 1). Surgical procedures were performed at the Dwight David Eisenhower Army Medical Center, Clinical Investigations, Fort Gordon, GA. The animals received a through-and-through critical-size, 8 mm-diameter, calvarial osteotomy. Once the critical osteotomy defect was created, ACS containing either rhBMP-2 or buffer alone was placed into the calvarial osteotomy defect. A 10 mm-diameter titanium mesh was placed over the graft material to provide space and wound stability, and then primary closure was obtained. Animals representing each dose were euthanized on either Day 1, 3, 5, 7 or 15, the heads collected, and fixed in 10% buffered formalin solution. Tissue blocks containing the defect site were cut coronally with a Buhler saw and divided into posterior and anterior halves. The anterior half was processed for paraffin embedding and sectioning.

These section samples will be further examined here. Immunohistochemical techniques will be used to determine the distribution of relevant markers for proliferation, stem cells, osteochondroprogenitor cells, and terminally differentiated osteoblasts in regenerative

dural cell populations in the critical-sized rat calvarial defect model. In conjunction, iPSCs transcription will be evaluated

Table #3

	Day 0	Day 1	Day3	Day 5	Day 7	Day 14
BMP-2	0 µm	0 µm	0 µm	0 µm	0 µm	0 µm
	5 µm	5 µm	5 µm	5 µm	5 µm	5 µm
	20 µm	20 µm	20 µm	20 µm	20 µm	20 µm

Table 3: Time samples and BMP-2 dosages from which the slides will be obtained and processed. Following the staining process the slides will be reviewed, looking for those markers and characteristics that would demonstrate differentiation, or specific proliferation.

Detailed Methodology

Immunohistochemistry:

Immunohistochemistry using selected antibodies and 5µm sections processed from available paraffin blocks will be performed to explore qualitatively the absolute number, proportion, and physical distribution of cells. Antigen detection in sections will use primary antibodies, and appropriate species- and immunoglobulin-matched biotinylated or ALP-conjugated secondary antibodies from various commercial sources.

Slides will be subjected to 60°C for two hours to promote tissue adherence, and then placed in a xylene series for deparaffinization, followed by rehydration in an ethanol series, and finally in deionized water. For treatment for antigen retrieval, a commercial antigen retrieval solution at 1x (Diva Decloaker, Biocare Medical, Concord, CA, USA) will be used in a carefully controlled stepwise heat treatment, with a timed step at 121°C (30 sec), in a programmable pressure chamber (Decloaking Chamber™, DC2002, Biocare Medical, Concord, CA, USA). After washing in Tris-HCl buffer with 0.9% sodium chloride (TBS), non-specific antibody binding will be blocked by pre-incubating sections

with 2.5% normal horse serum (NHS) or 2.5% normal goat serum (NGS) for 20 min at room temperature in a humidity chamber.

Appropriate dilutions of primary antibodies will be established empirically, starting with dilutions of 1.5, 1.0 and 0.5 µg/ml prepared in 1.25% horse serum or 1.25% goat serum. Sections will be incubated with the primary polyclonal or monoclonal antibodies overnight at 4°C in a humidified chamber. Sections will then be washed in TBS with 0.025% Tween 20. (TBST) and then incubated with biotinylated or ALP-conjugated secondary antibodies (diluted 1:500 in TBST) for 1 h at room temperature in a humidified chamber. After washing, sections will treated with hydrogen peroxide to block endogenous peroxidase activity, and then a commercial detection system will be employed to localize antigens; utilizing peroxidase and a diaminobenzidine (DAB) chromogen to give a brown stain. Sections will then be counterstained using hematoxylin. Slides will have cover-slips placed over them using appropriate mounting medium.

Data Description

Independent Variables

The independent variables for this study are time (continuous, but for statistical analyses will be treated as categorical ordinal), and rhBMP-2 dose (categorical ordinal).

Dependent Variable

This is primarily a descriptive study. The dependent variables are the expression of various markers (dichotomous categorical nominal) detectable by immunochemistry, These markers and antibodies provide indication of the specific cells and transcription factors that are involved in the regeneration and repair of the critical defect site at the dura and how that correlates to BMP-2 levels and time points.

RESULTS

Defect Description

Prior to a description of the individual markers and their results, it is paramount that a description of the defect is demonstrated. The samples are coronal sections and will vary in magnifications. Most of the samples will be seen from a 4x or 20x view point. Below is an example of the entire critical size defect.

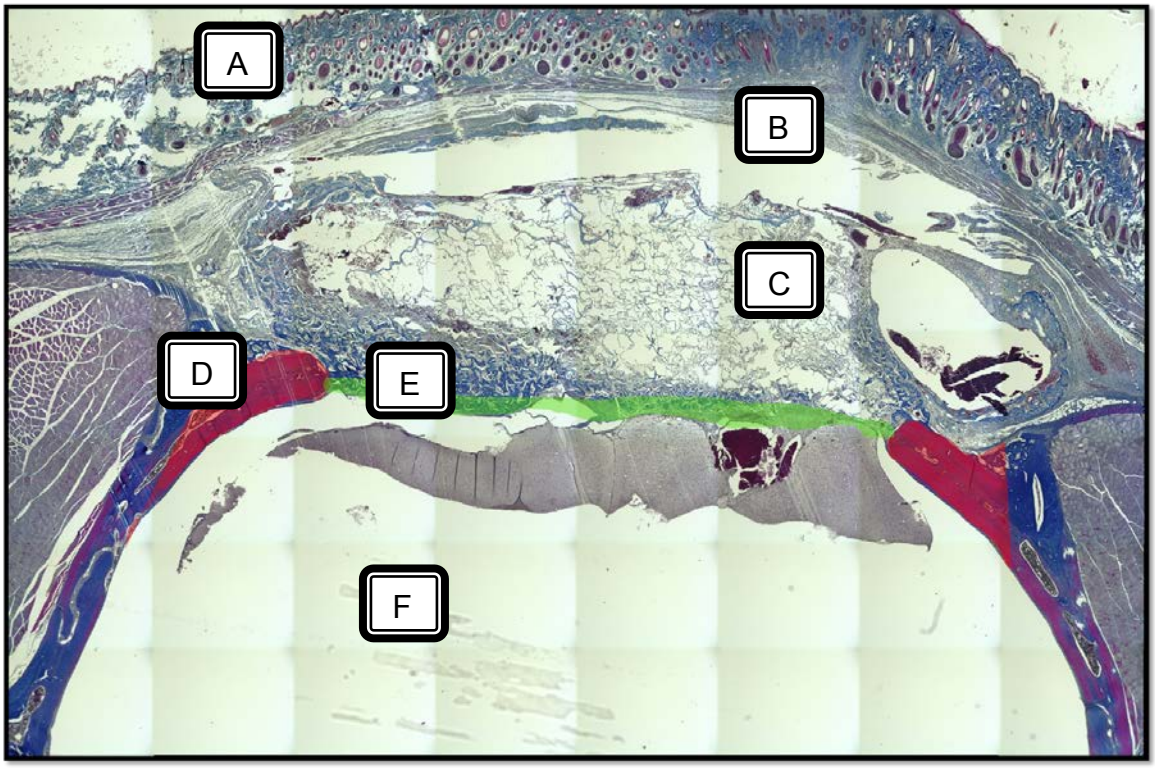

Figure 2: (Courtesy of O'Bryhim, J) This is a large scale view of the critical sized defect. **A**- Epidermis **B**-Space from titanium mesh. **C**- Collagen Sponge **D**-Parietal ridge region. **E**-Dural region. **F**-Brain/Cranial Space. Red demonstrates the most lateral aspect of the critical sized defect with green highlight is the represented dural region. These two colored areas are the areas of greatest interest and will be the focus for all of the subsequent slides.

Proliferating Cell Nuclear Antigen (PCNA)

Day #1-Day #14

PCNA demonstrates mitotic activity along the dural region to include the residual native bone on the most lateral components of the defect. The epidermal regions, condyles and hair follicles were utilized as positive controls. Osteocytes in the native bone have

stained and show a positive response contrasting the negative pattern observed in the calvarial bone. PCNA activity was most robust along new bone lining cells. PCNA activity demonstrated a slight dose-response relationship to rhBMP-2 concentrations. This was more readily seen in the osteoid substance, osteoblast-like cells and the chondroblast-like cells. There was a distinct peak of activity at the day 5 point (See Figure 5). Then moving forward to the day 7 and day 14 time points further development of chondroblast-like cells along with osteoblast-like cells and osteoid formation (see figure 6,7).

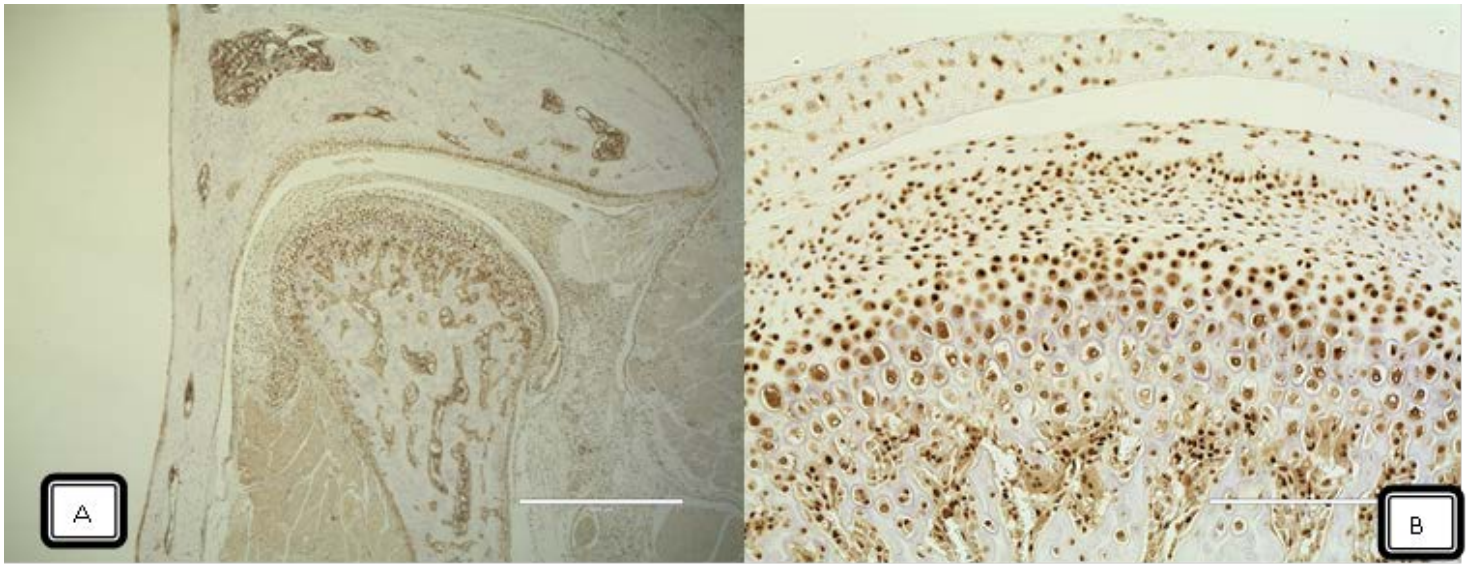

Figure #3: PCNA **Positive Controls** Day1-20ug 1.25-11 Condyle **– (a)** 4x **and (b) 20x**

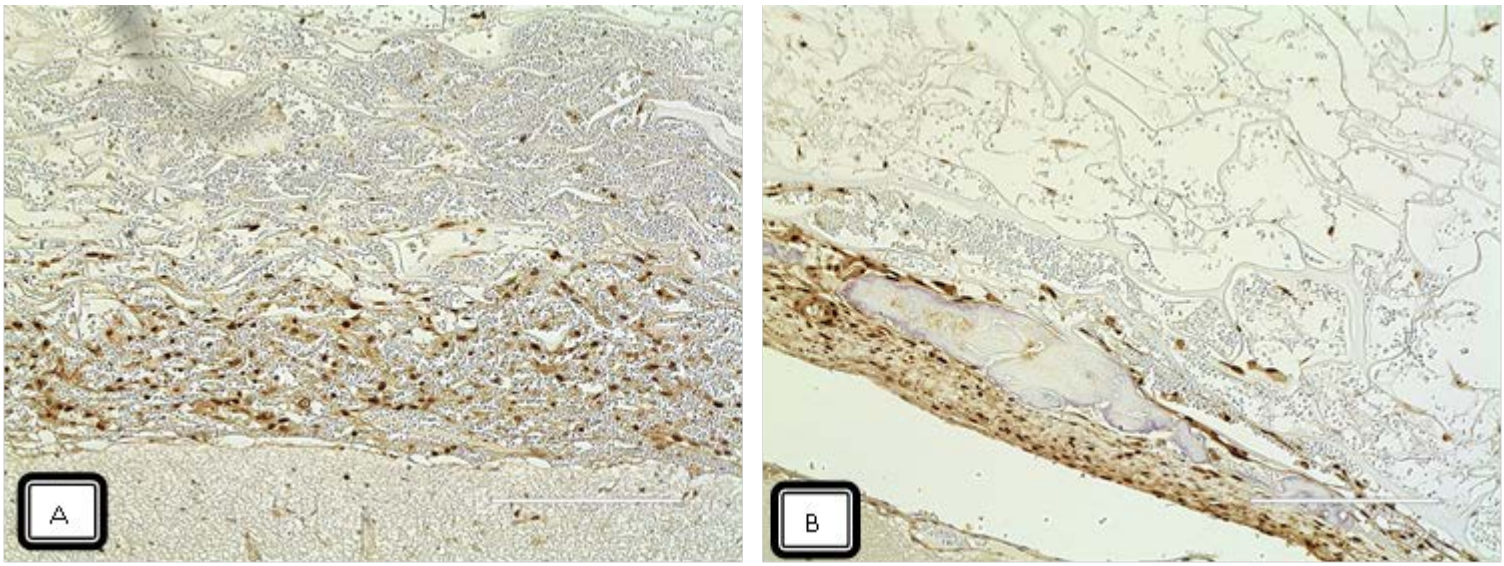

Figure #4: PCNA Positive Control Day1-0ug 1.10-14 Epidermis 20x

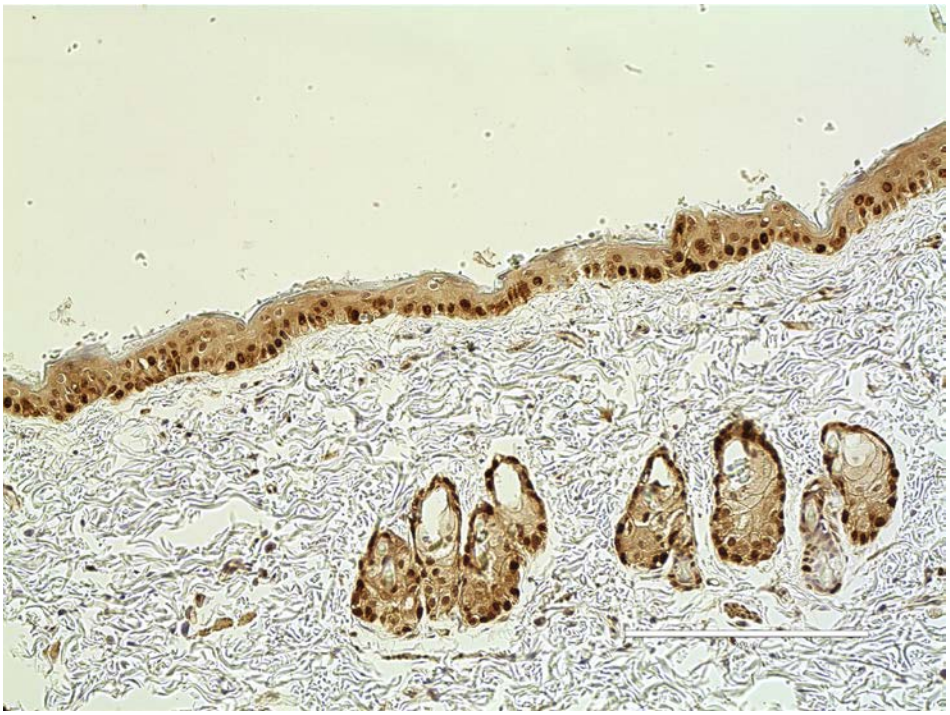

Figure #5: PCNA Day5: (a) 5ug 5.95-9- 20x; (b) 20ug 5.90-4 Dura - 20x. Increasing cellular activity along the dural border.

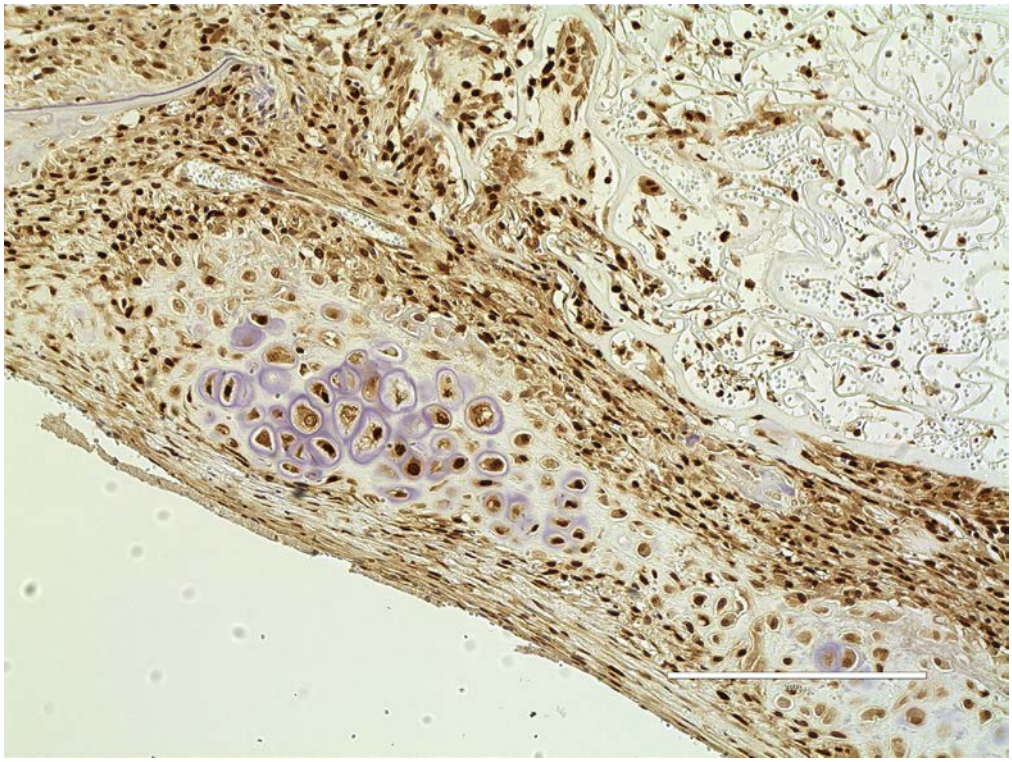

Figure #6: PCNA Day7-20ug 7.127-8 Dura - 20x Osteoblast-like cells seen in purple. Osteoid deposition in the surrounding area.

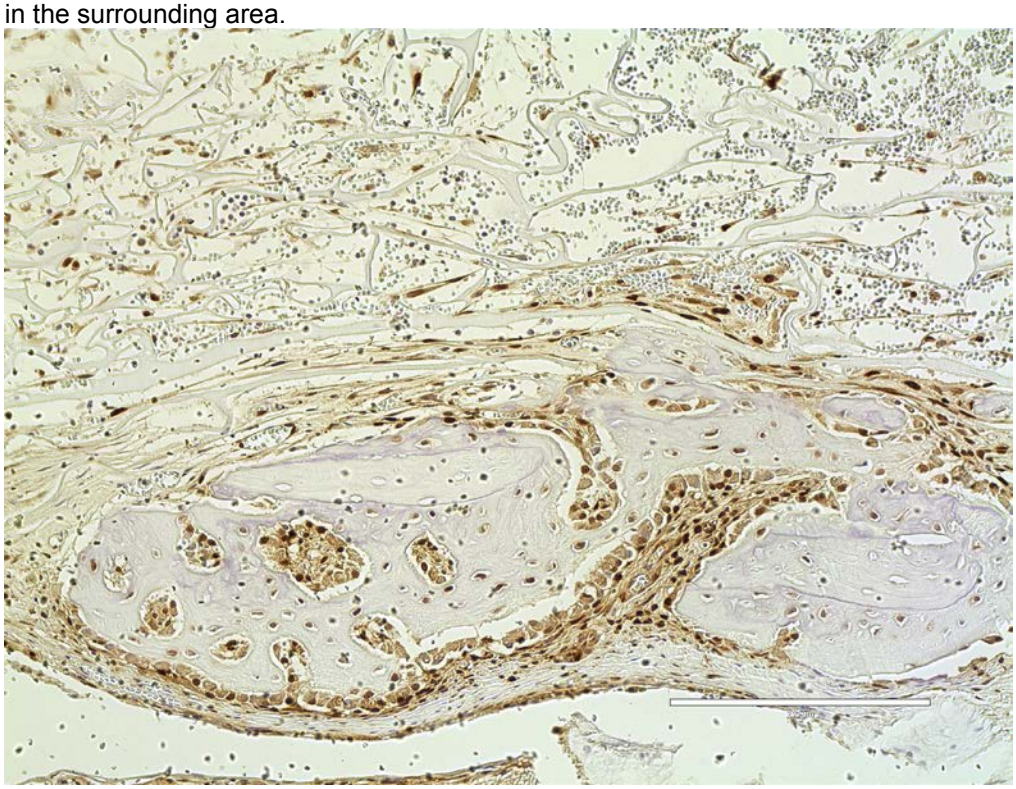

Figure #7: PCNA Day14-5ug 14.71-5 Dura - 20x Formation of islands of bone with cellular activity continuing coronally in the ACS.

Krüpple-like factor 4 (KLF-4)

Day #1

At day 1, minimal positive KLF-4 signal was observed in any sections regardless of rhBMP-2 concentration. At the initial onset there was no significant presence of KLF-4, but some positive inflammatory cells was seen throughout the lining the defect. Slight increase in the mitotic difference between the rhBMP-2 0 ug and 5ug concentrations, but potential for an increasing response in the 20ug were noted (see figures 8-10)

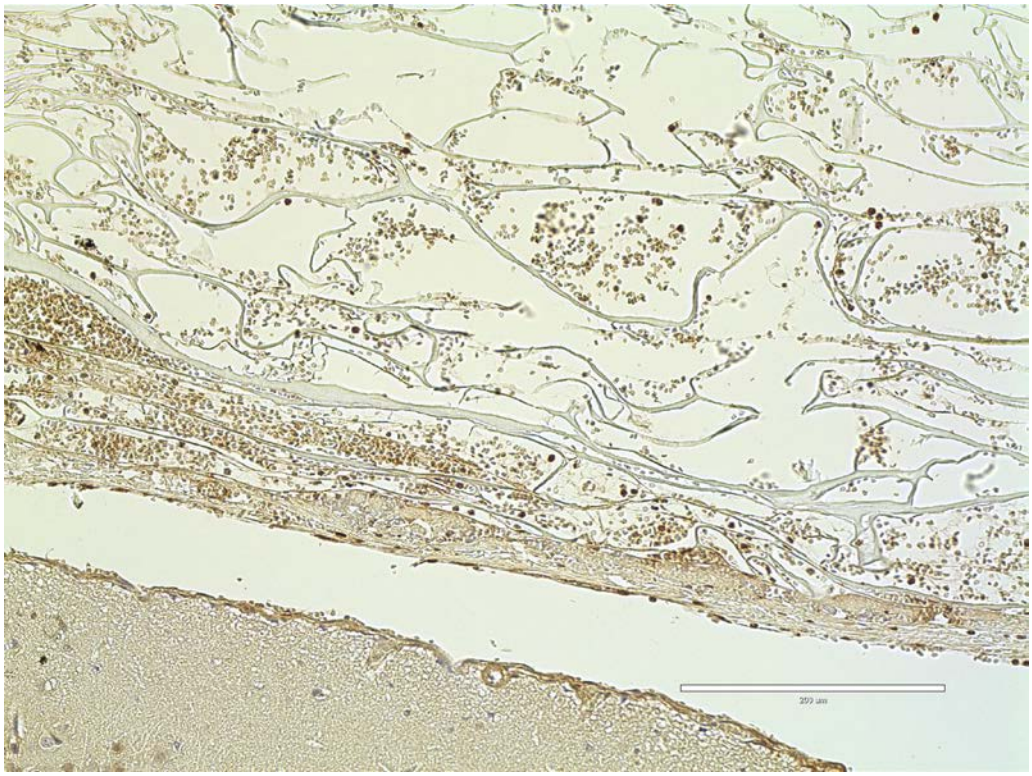

Figure #8: KLF-4 Day1-0ug 1.10-6, 20X Dura. Minimal mitotic acivity

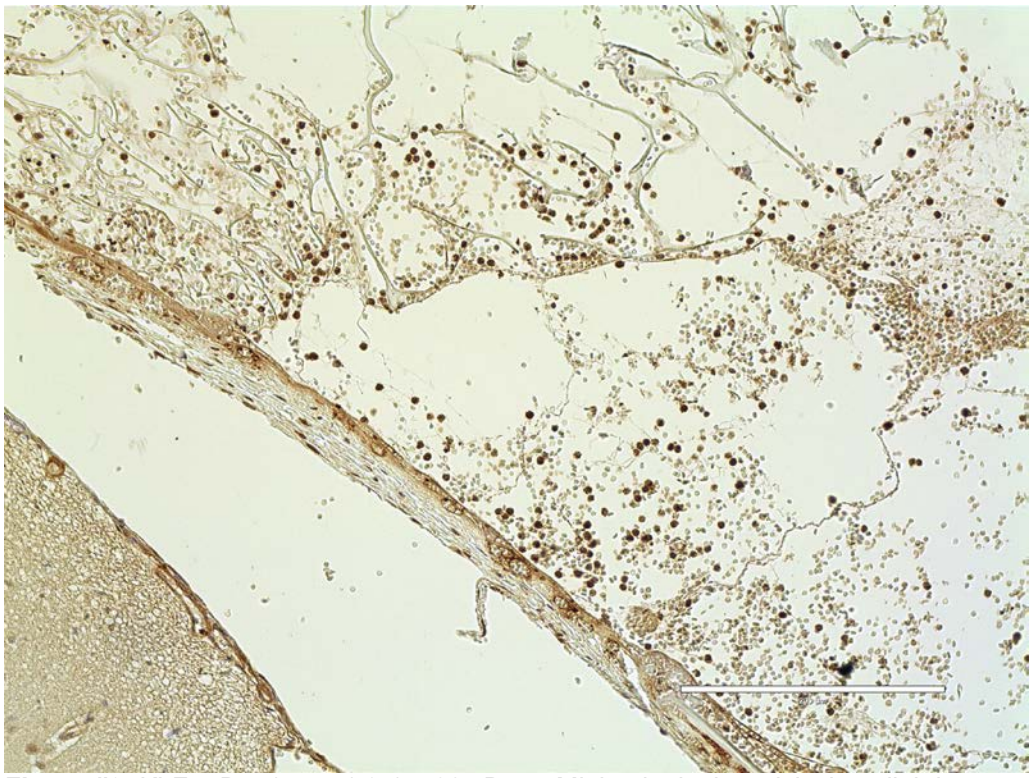

Figure #9: KLF-4 Day1-5ug 1.8-15, 20x Dura. Minimal mitotic activity but slightly more than 0 ug samples.

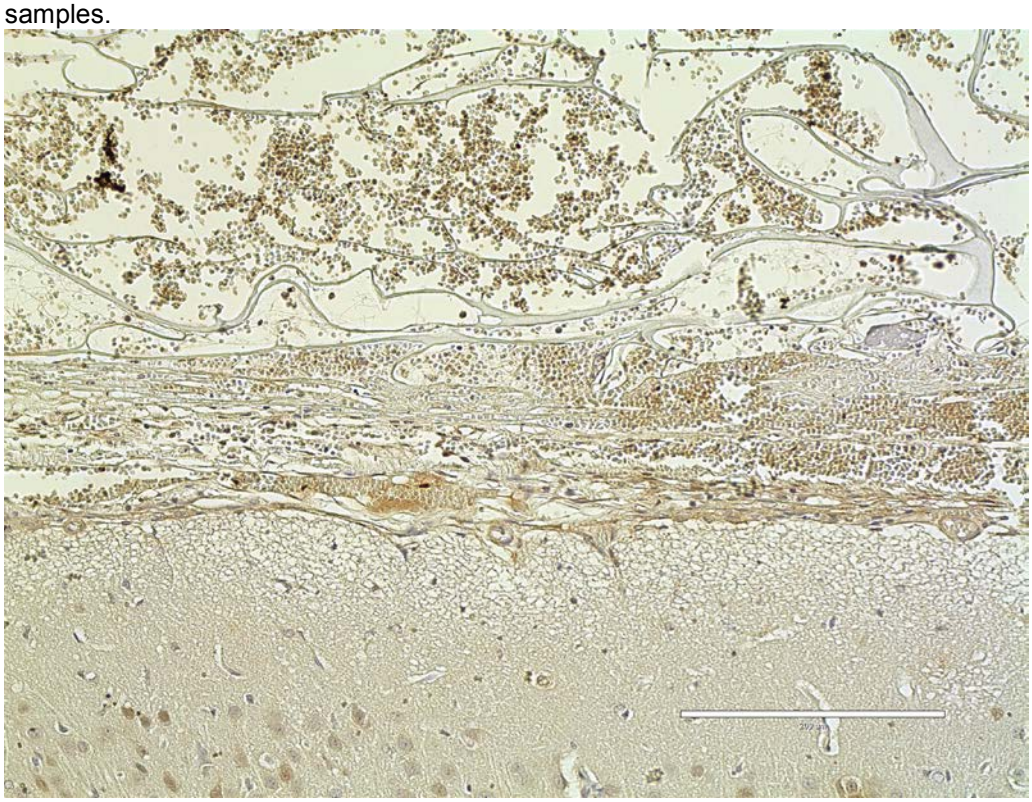

Figure #10: KLF-4 Day1-20ug 1.25-14, 20x Dura. Slightly more mitotic activity seen increasing into the ACS than with the 0ug and 5ug samples.

Day #3

The amount of cellular expression appears to be increasing. The signal is more prominent than day 1 and the activity seems to express from the lateral aspects of the defect and proliferate towards the center of the defect. The cellular response seems to be consistent in all concentrations of rhBMP-2 minimal increase to be expressly dependent on rh-BMP-2. Some minimal signs of osteoid is beginning to appear (see figure 11).

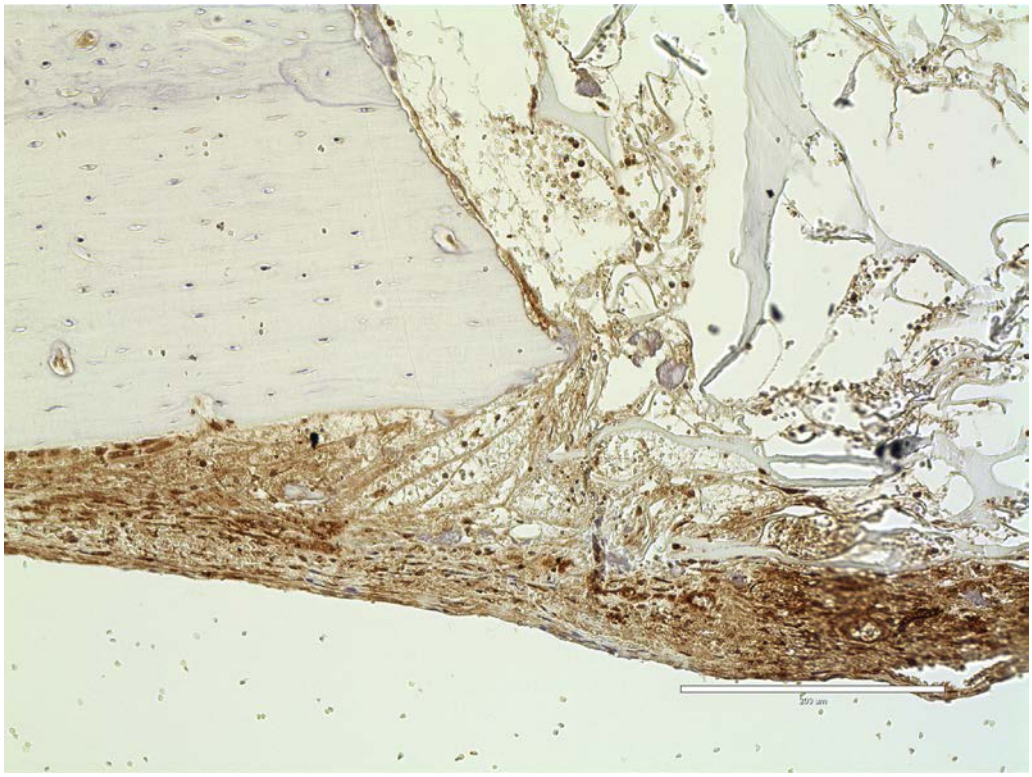

Figure #11: KLF-4 Day3-0ug 3.37-14, 20x Dura

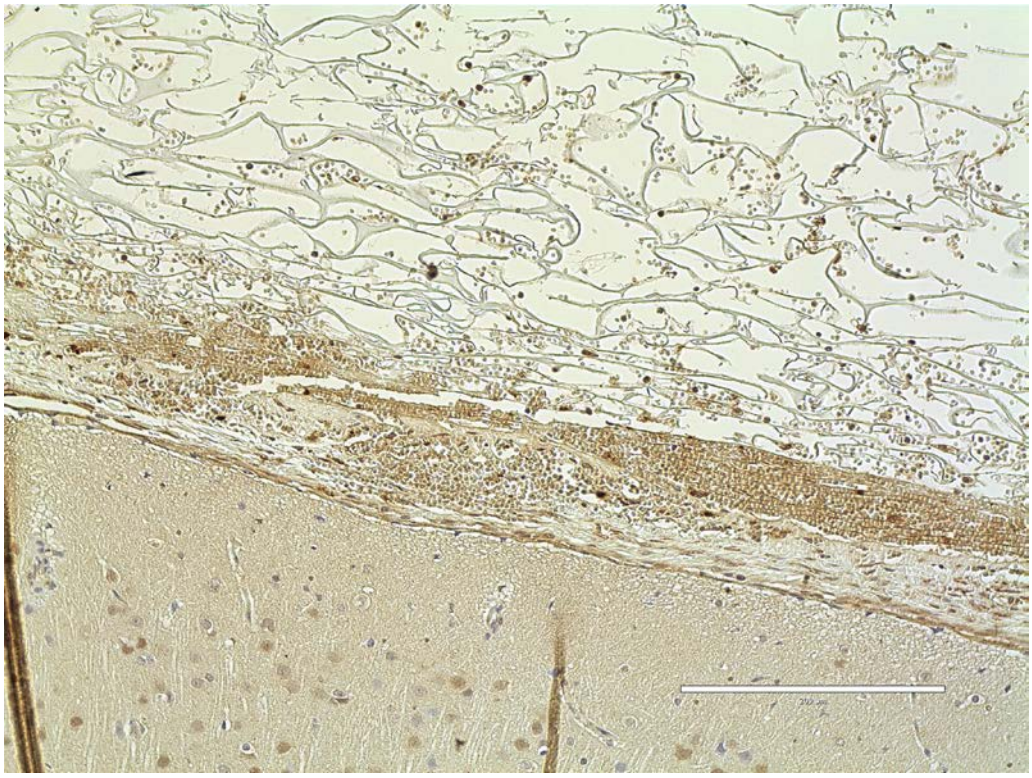

Figure #12: KLF-4 Day3-5ug 3.32-9, 20x

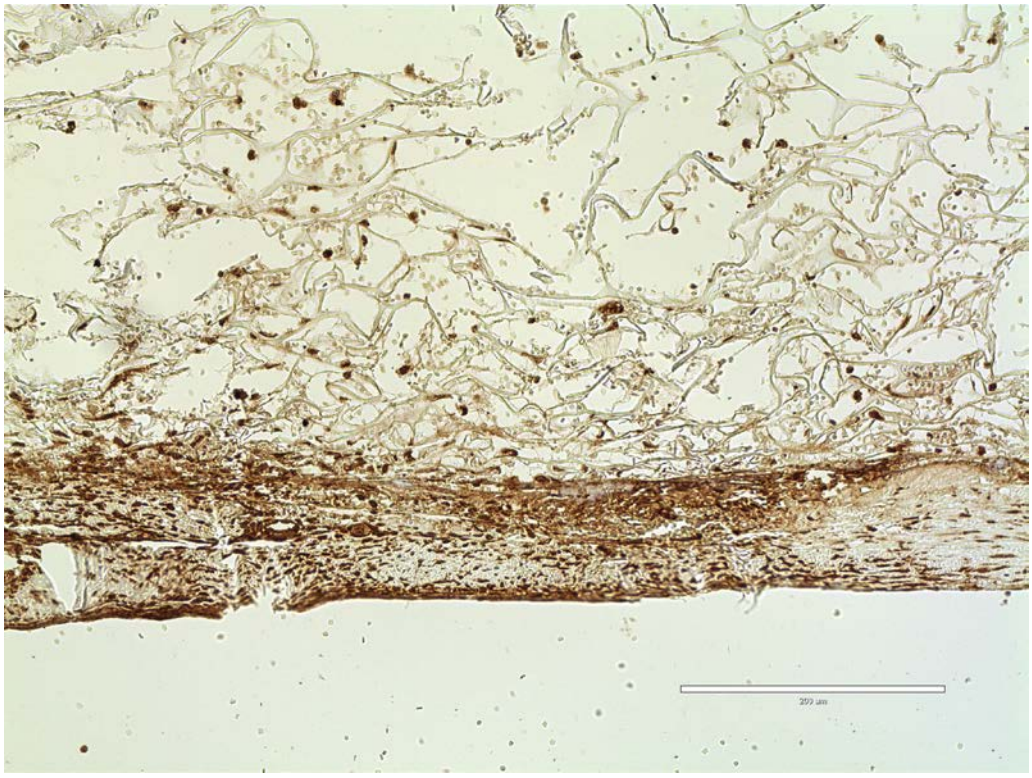

Figure #13: KLF-4 Day3-20ug 3.39-9, 20x

Day #5

At day 5, activity of regenerative cells was abundant with speckled locations of osteoblast-like cells and areas of osteoid were observed signifying potential of new proliferation of cells (see figure 16). Osteoid formation was observed and did not seem to correlate with the level of micrograms present at each concentration of rhBMP-2.

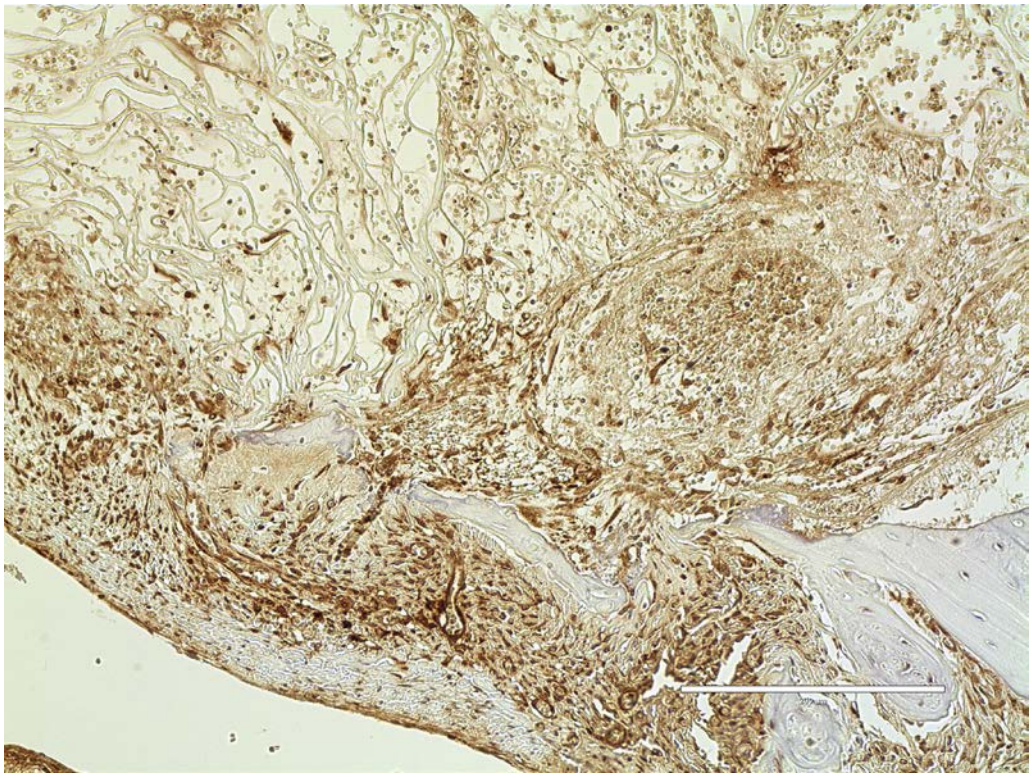

Figure #14: KLF-4 Day5-0ug 5.103-2, 20x Cuboidal cells at the dura and osteoid further in the ACS with continued mitotic activity

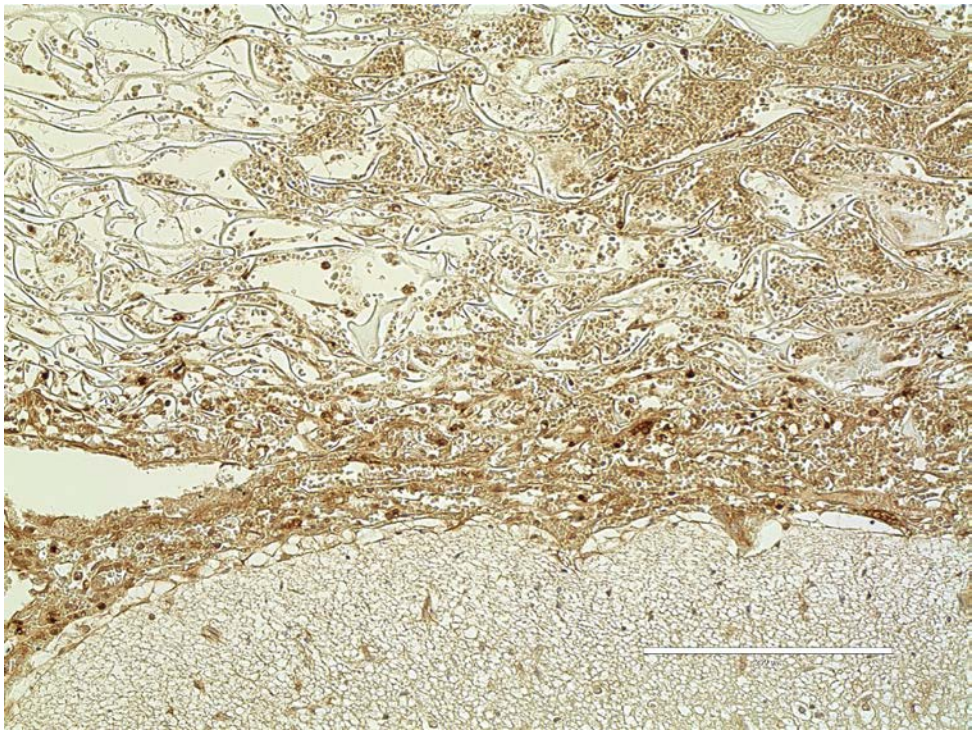

Figure #15: KLF-4 Day5-5ug 5.95-4, 20x. Further increase in the mitotic activicty and progression through the defect.

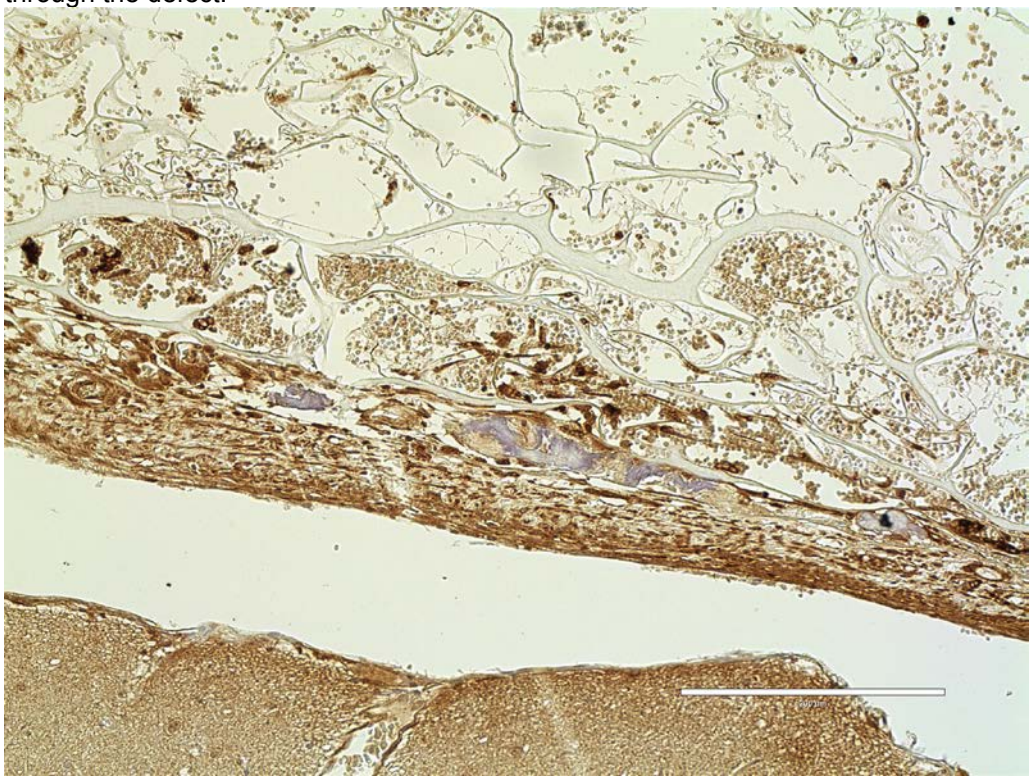

Figure #16: KLF-4 Day5-20ug 5.90-15, 20x. Dense areas of mitotic activity with the dura and proliferating into the ACS. Purple areas positive for osteoid being secreted.

Day #7

Continued areas or islands of osteoid and osteoblast like cells are present. High cellular activity is seen continuing coronally through the ACS (see figures 17-19). It seems to act as the front to the cellular progression. These cuboidal cells present along the periosteal layer appear to be the birthplace of much of the secretory activity (see figure19).

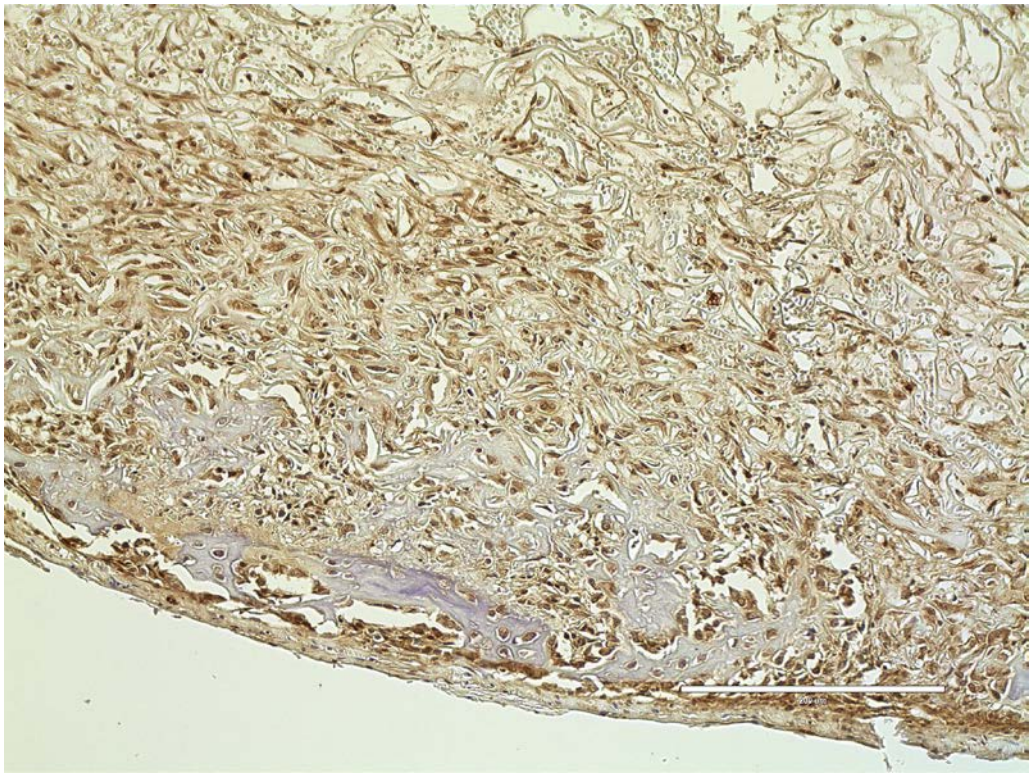

Figure #17: KLF-4 Day7-0ug 7.131-7, 20x

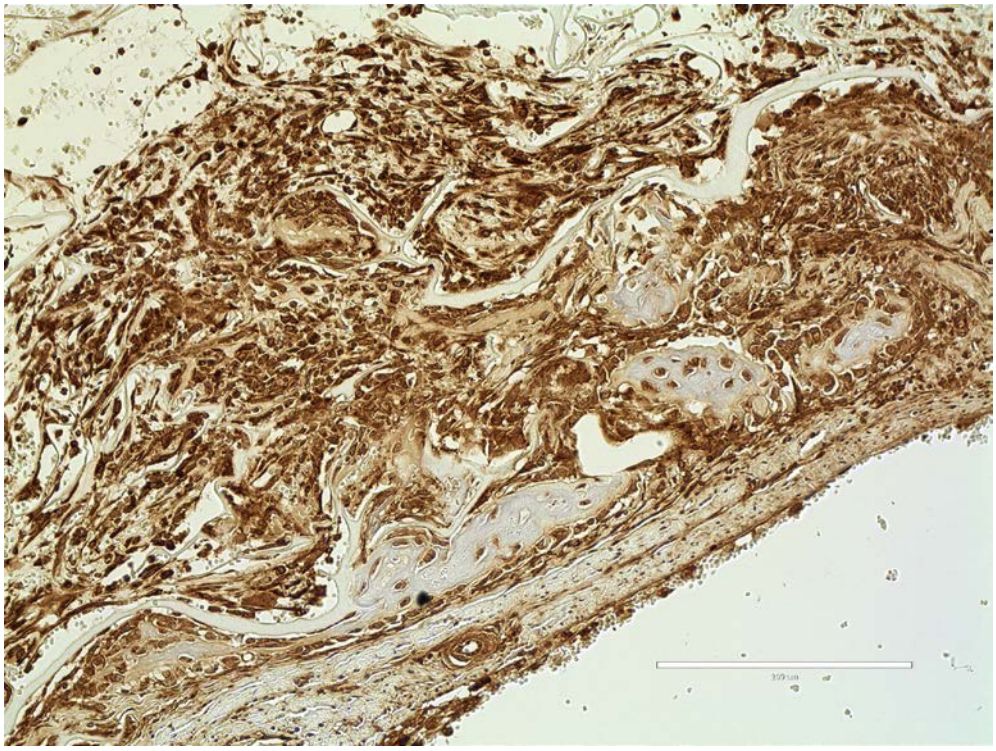

Figure #18: KLF-4 Day7-5ug 7.126-15, 20x

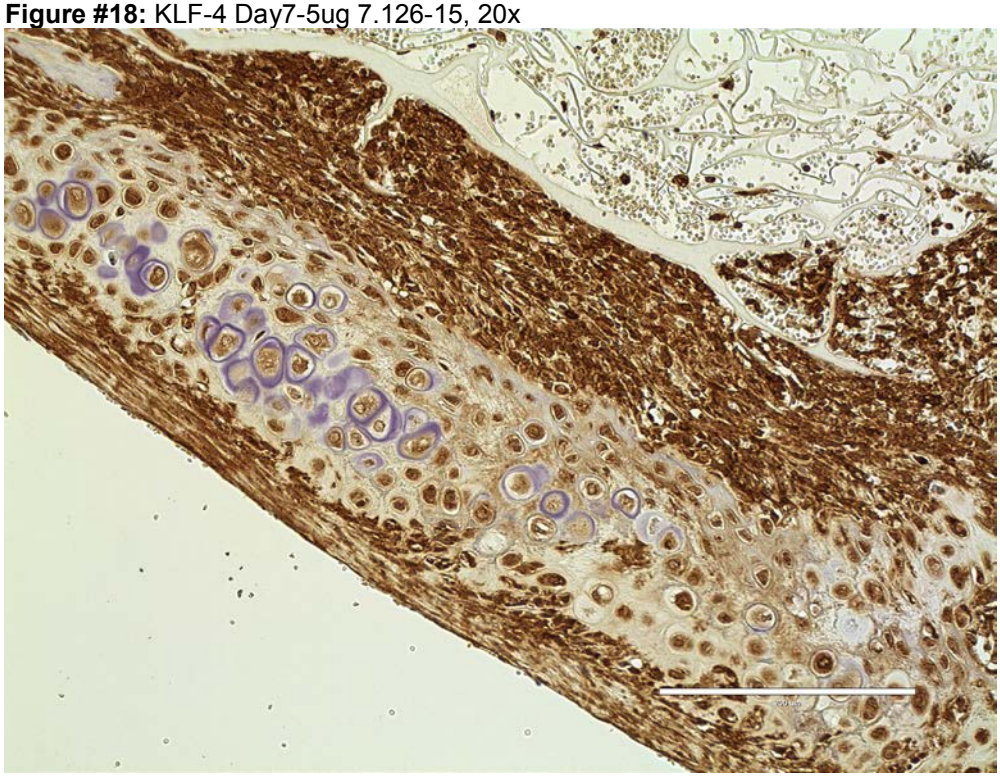

Figure #19: KLF-4 Day7-20ug 7.127-3 20x. Expansive fill of the defect by way of osteoblast-like cells with progression of the mitotic activity. Also see in this sample is a good example of the cuboidal shaped cells and maturation of the osteoid.

Day #14

By day 14, new bone formation is occurring and development of other boney healing processes are becoming evident. Reversal lines (see figure 21) and proliferation of trabeculae all began appearing on the most lateral aspects of the critical sized defect and then progressed towards the more medial aspect of the defect. Cuboidal osteoblast-like cells were present in areas of new bone formation signifying healing was still active (see figure 22). Large boney islands are present throughout the defect and are demonstrative of the fairly rapid healing of the critical sized defect (see figure 20-22).

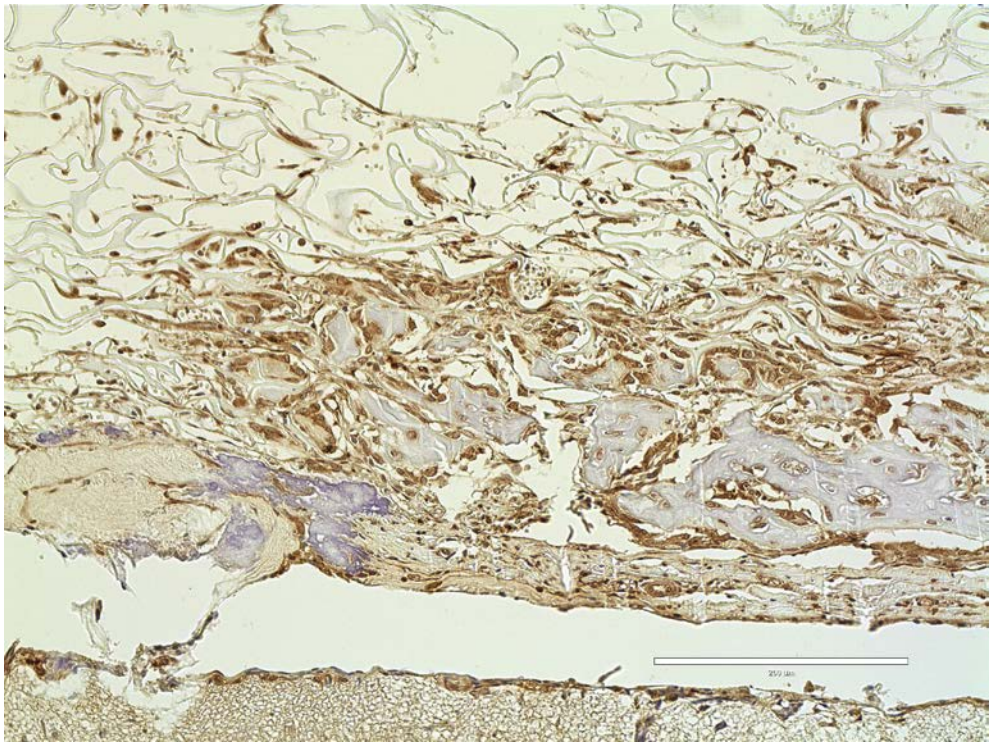

Figure #20: KLF-4 Day14-0ug 14.70-4, 20x continued progression of proliferation and maturation of the osteoid.

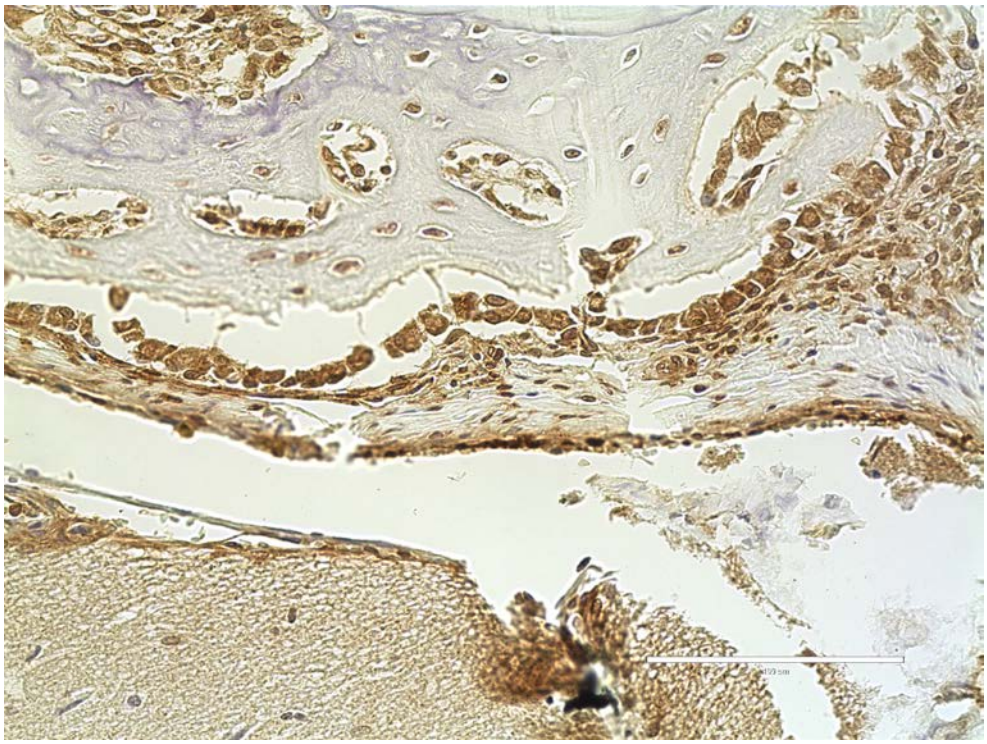

Figure #21: KLF-4 Day14-5ug 14.71-13, 40x Reversal line noted at the most superior portion of the boney complex. Continued maturation present. Cuboidal cells noted at the dural lining.

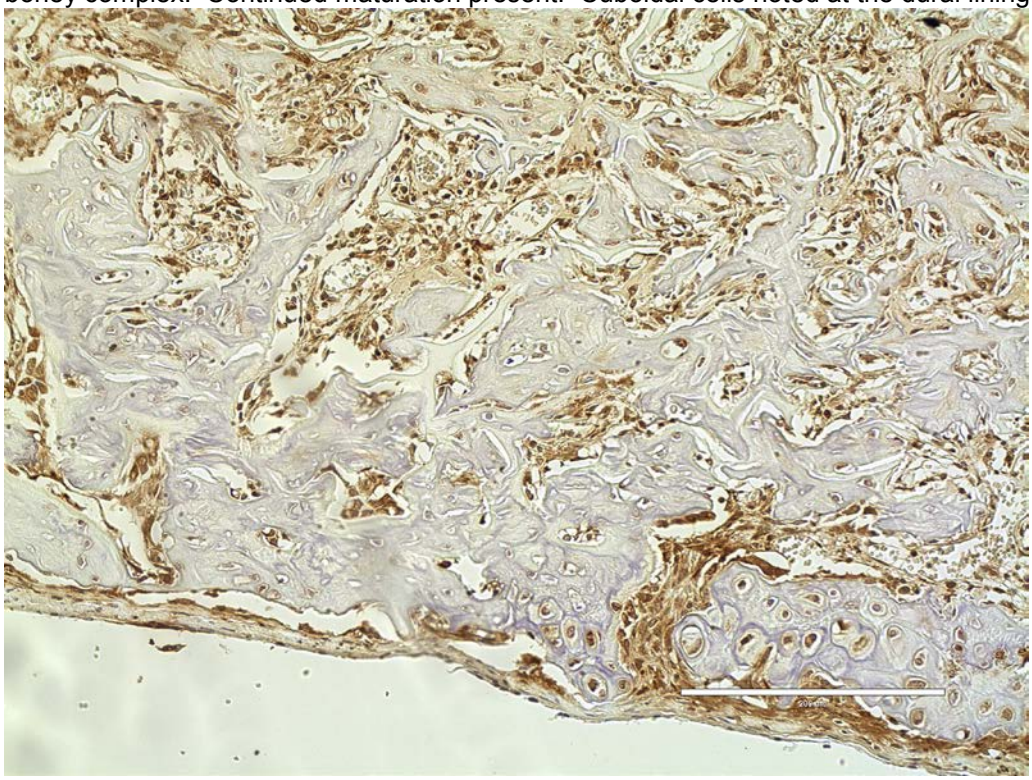

Figure #22: KLF-4 Day14-20ug 14.63-5 20x. Extensive amount of maturing bone. With progression of the proliferation at the most superior component.

Sex Determining Region Y-Box 2 (SOX-2)

Day #1-Day #14

SOX-2 expression was weak initially, up through day 3. It was on day 3 at the 20ug concentration that a beginning sign of cellular activity was present. Then on days 5-14 we see an increase in cellular activity with an apparent increase in the osteoblast-like cells, along with increasing osteoid deposition and seem to show some increase with increasing rhBMP-2 concentrations, although this marker is hard to differentiate this specifically. On day 5 the osteoblast-like and chondroblast-like cells present that are contributing to the maturation of the defect. This also seems to be more efficient with an increase in the concentrations of rhBMP-2 (see figure 27). By day 14 islands of bone noted with continued progression of the defect maturation that seems to move from lateral to medial and inferior to superior (see figure 31-33).

This pattern of activity seems to follow the pattern that was seen with the KLF-4 marker. In that the most abundant increase in activity seemed to happen on around day 5 and that the subsequent time points of day 7 and day 14 seemed to show maturation of the cellular processes (see figures 30 and 33). Being that both of these markers are transcription factors that were described by Dr. Yamanaka and that concurrently have the potential to cause de-differentiation of somatic cells, it is promising that the markers are showing similar staining patterns and activity.

While both KLF-4 and Sox-2 were tested separately, these findings seem to be consistent with the potential that they may be co-binding and contributing to the healing

of the defect. Immunofluorescence and confocal microscope would be need for confirmation.

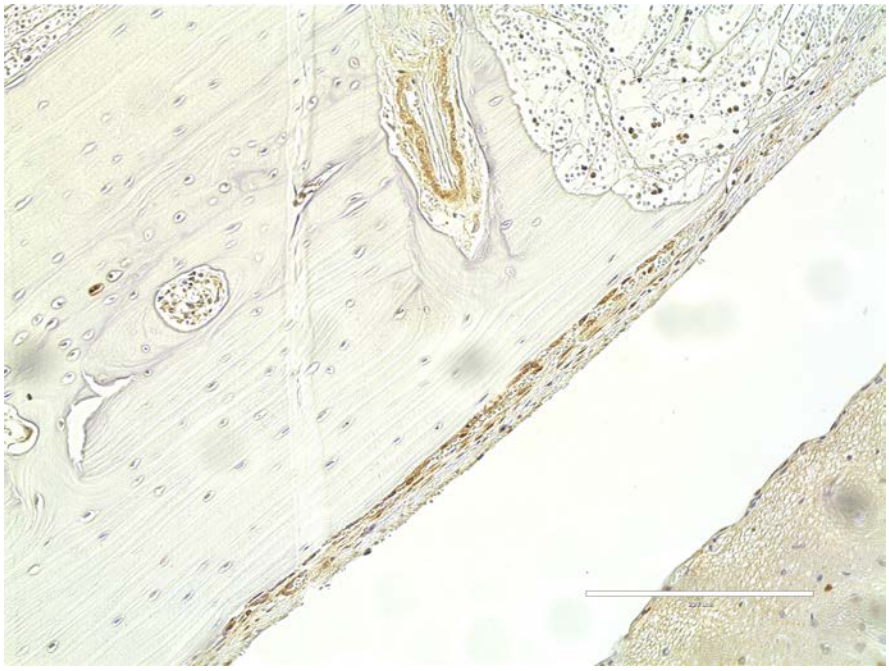

Figure #23: SOX2 Day1-0ug 1.1-15, 20x Dura

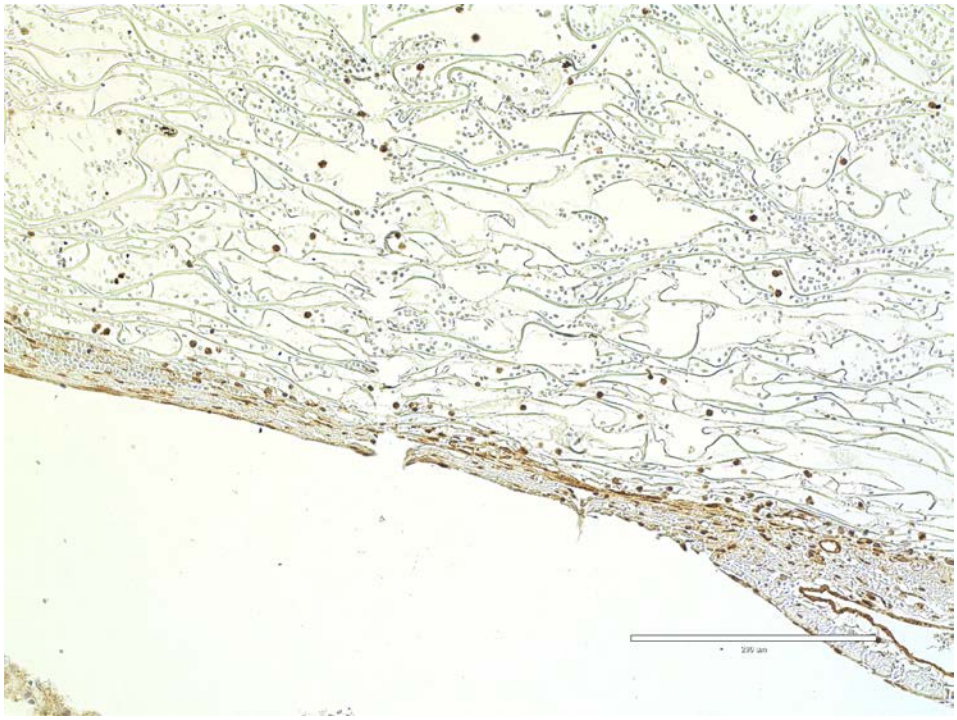

Figure #24: SOX2 Day1-5ug 1.5-9, 20x Dura. Minimal mitotic activty

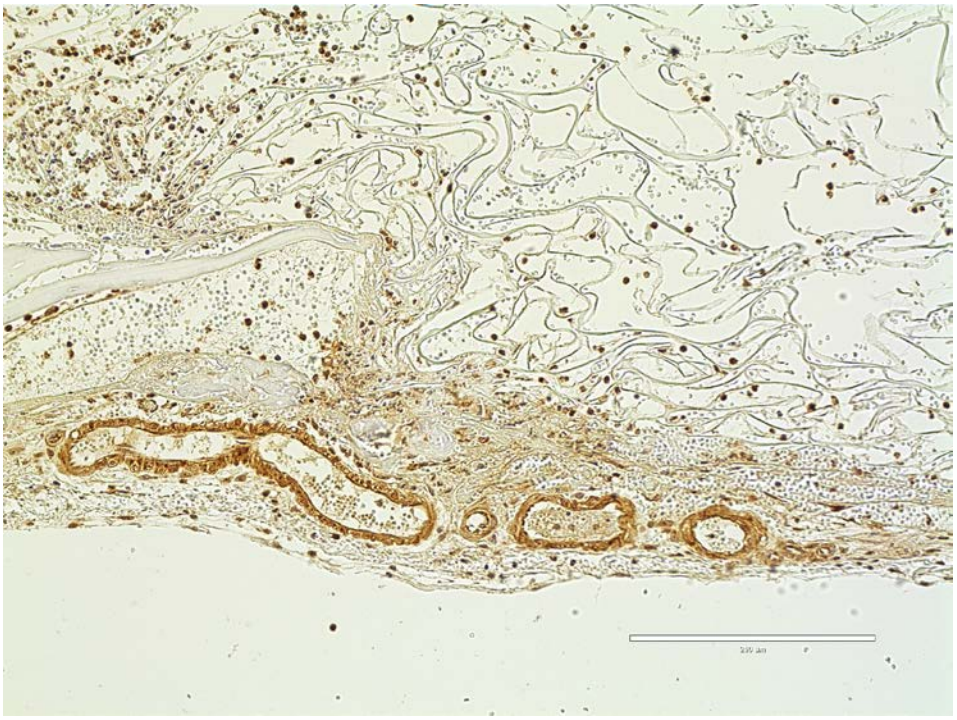

Figure #25: SOX2 Day1-20ug 1.3-13, 20x Dura. Blood vessels present in the dura. Mitotic activity is present but minimal

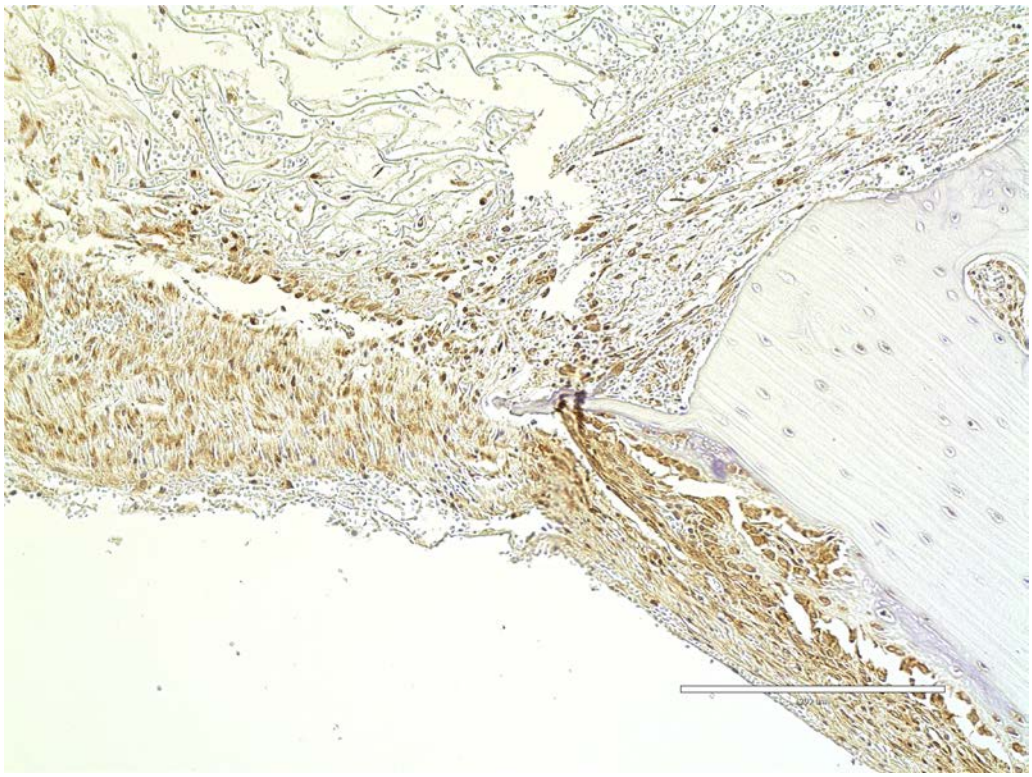

Figure #26: SOX2 Day5-0ug 5.94-6, 20x Dura. Dramatic increase in the mitotic activity that seems to express from the dura move superior in direction. Some beginning signs of osteoid.

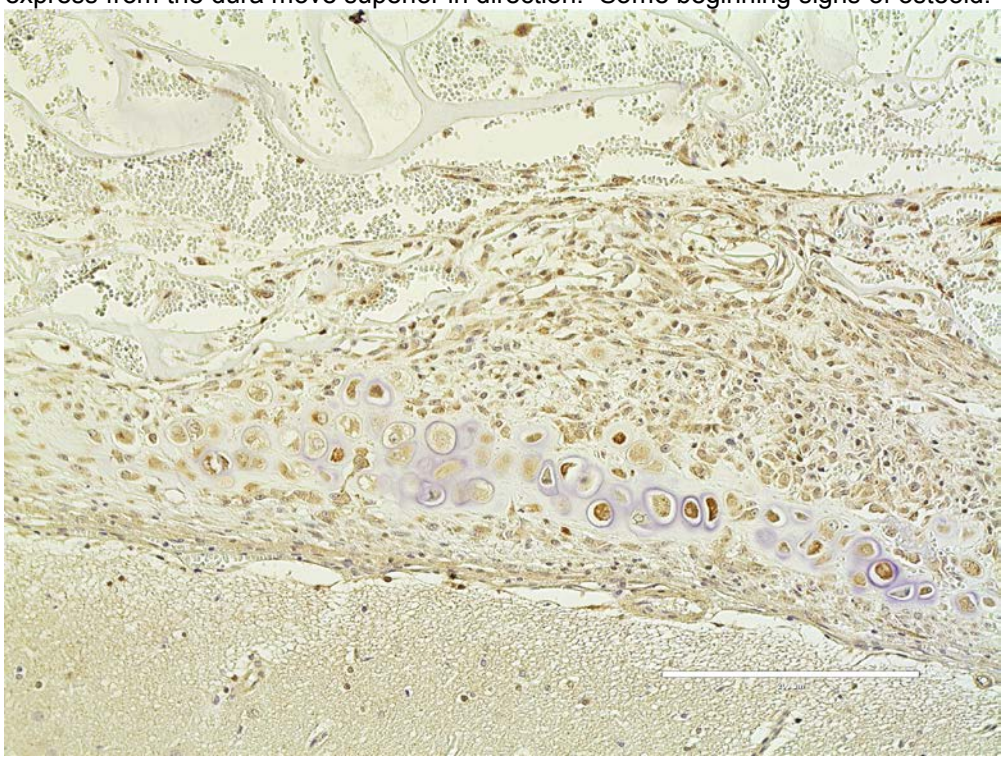

Figure #27: SOX2 Day5-20ug 5.99-11, 20x Dura. Osteoblast-like and chondroblast-like cells present that are contributing to the maturation of the defect.

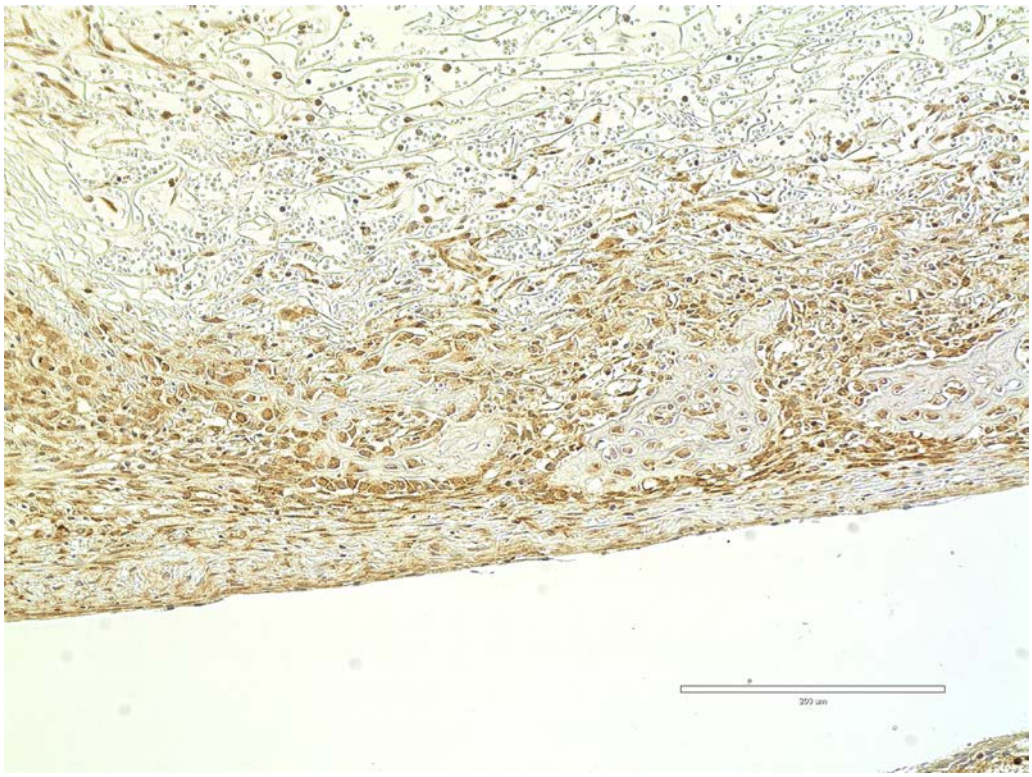

Figure #28: SOX2 Day7-0ug 7.131-3, 20x Dura

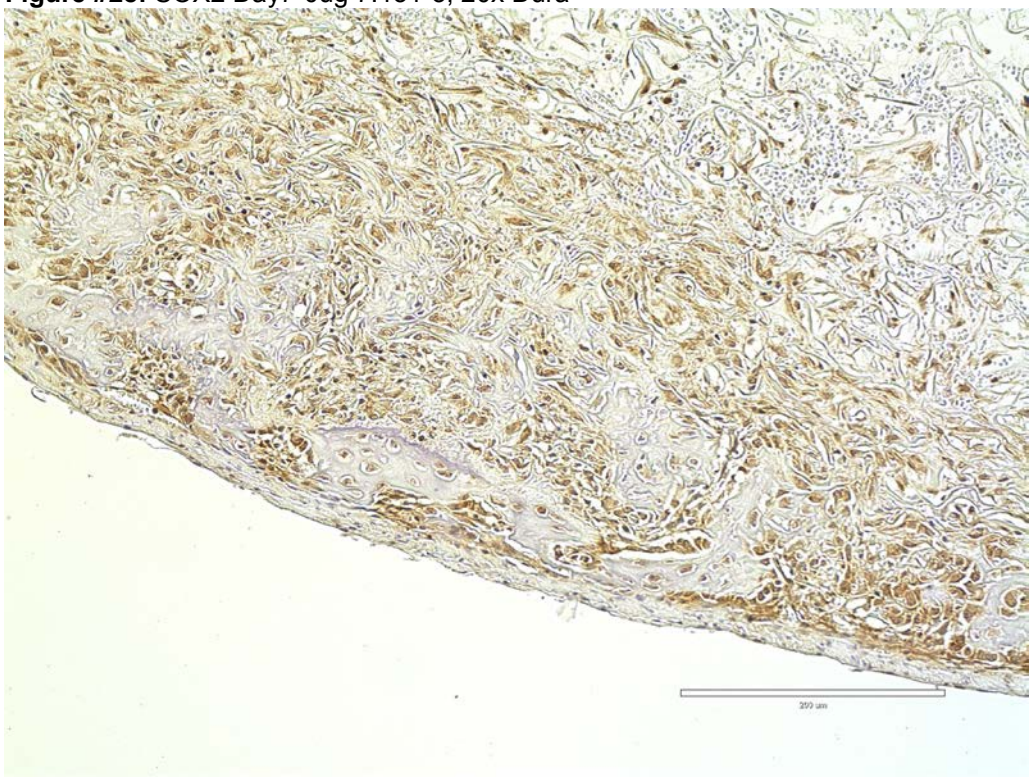

Figure #29: SOX2 Day7-5ug 7.129-5, 20x Dura. Extensive progression of osteoid, and cuboidal osteoblast-like cells.

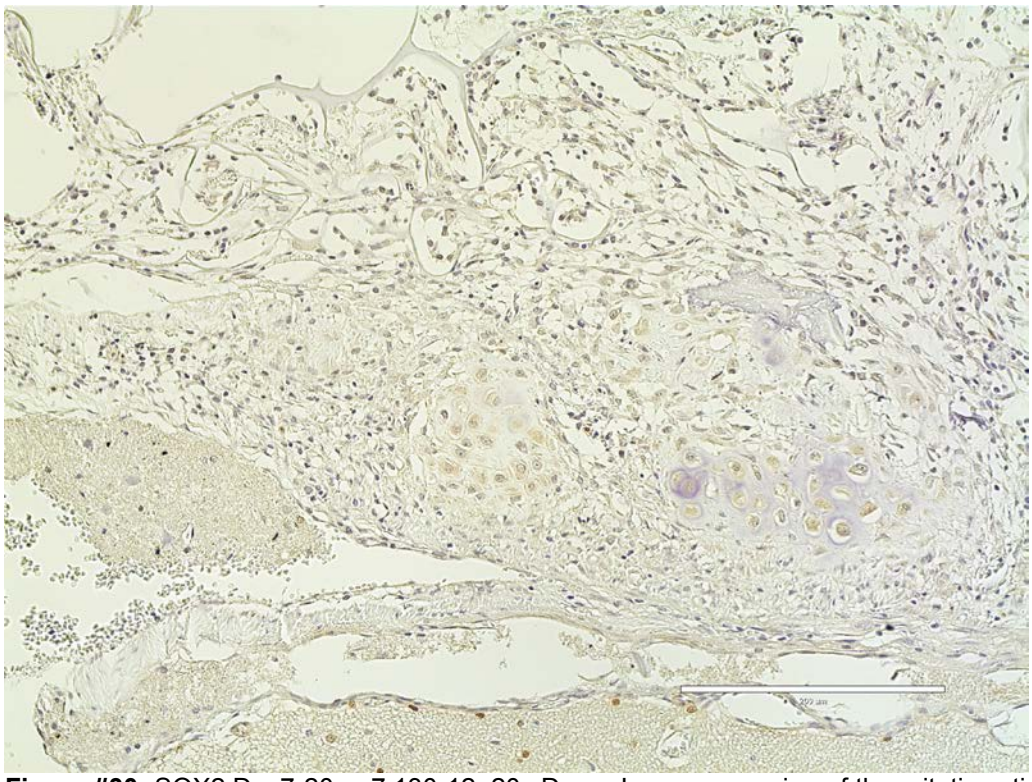

Figure #30: SOX2 Day7-20ug 7.130-12, 20x Dura. Large expansion of the mitotic activity and maturation of the osteoid that is present.

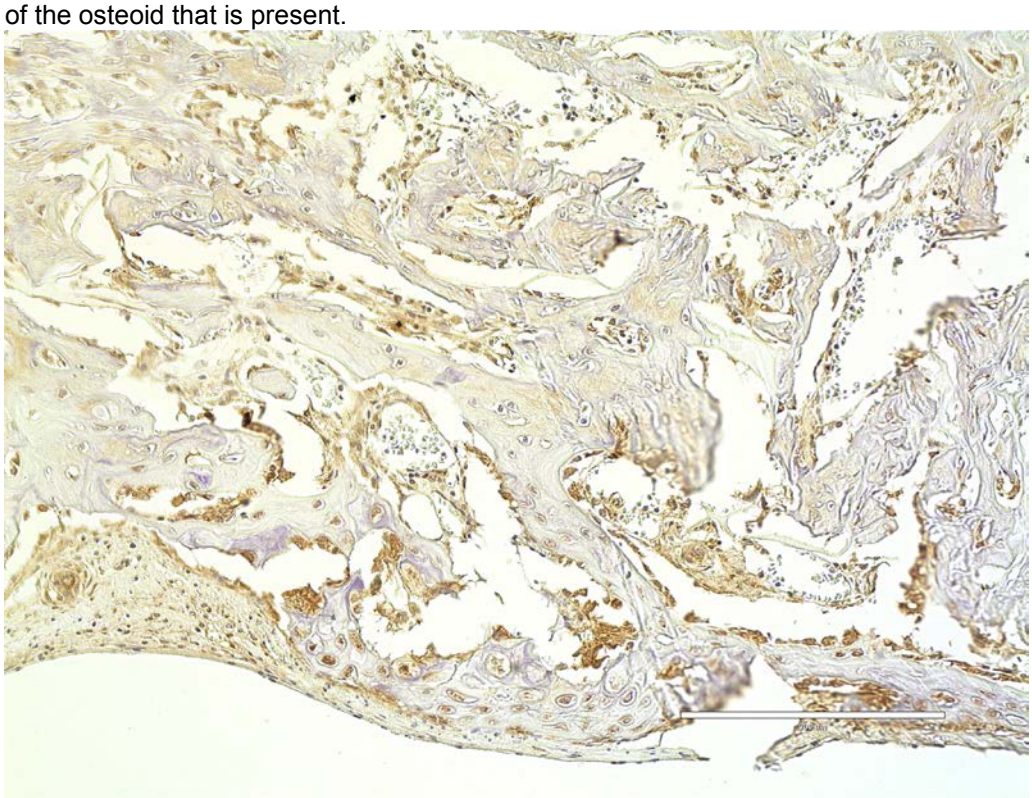

Figure #31: SOX2 Day14-0ug 14.64-9, 20x Dura. By day 14 islands of bone noted with continued progression of the defect maturation that seems to move from lateral to medial and inferior to superior.

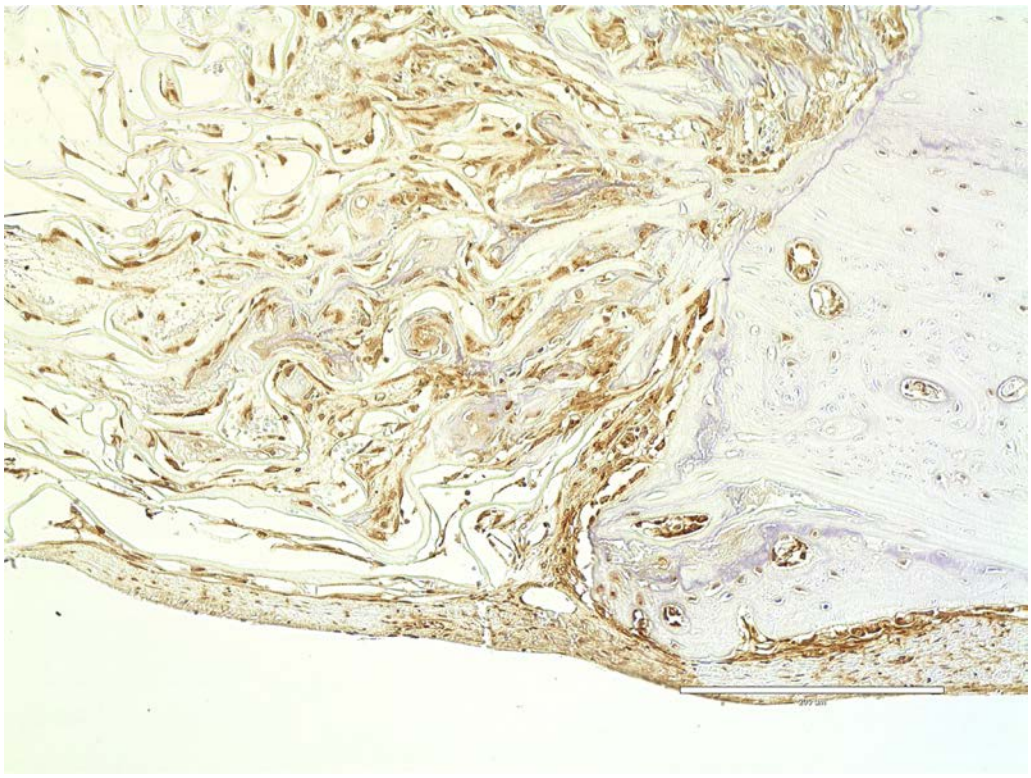

Figure #32: SOX2 Day14-5ug 14.59-2, 20x Dura. Progression of healing from lateral to medial

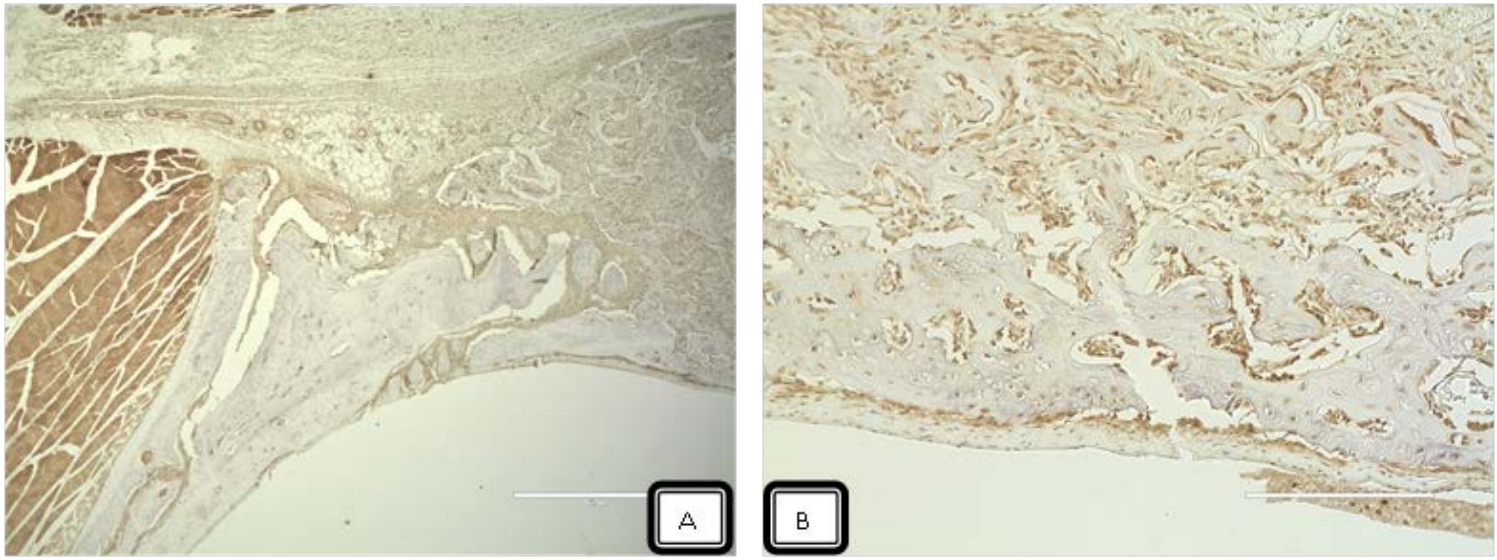

Figure #33: SOX2 Day14-20ug 14.63-13, (a) 4x lateral aspect of dura and ACS with osteoid through entire sponge on lateral side (b) 20x of same sponge in more medial aspect

Sex Determining Region Y-Box 9 (SOX9)

SOX9 expression was demonstrated at the later time points of day 5 but most prevalent at day 7. Very distinct chondroblast-like cells are seen and seem to be induced by the rhBMP-2 dosages (see figure 34). Expression present in the condyle region serves as a positive control for the staining series. The chondroblast-like cells seem to give way to more mature osteoid like activities as it approaches the day 14 time point.

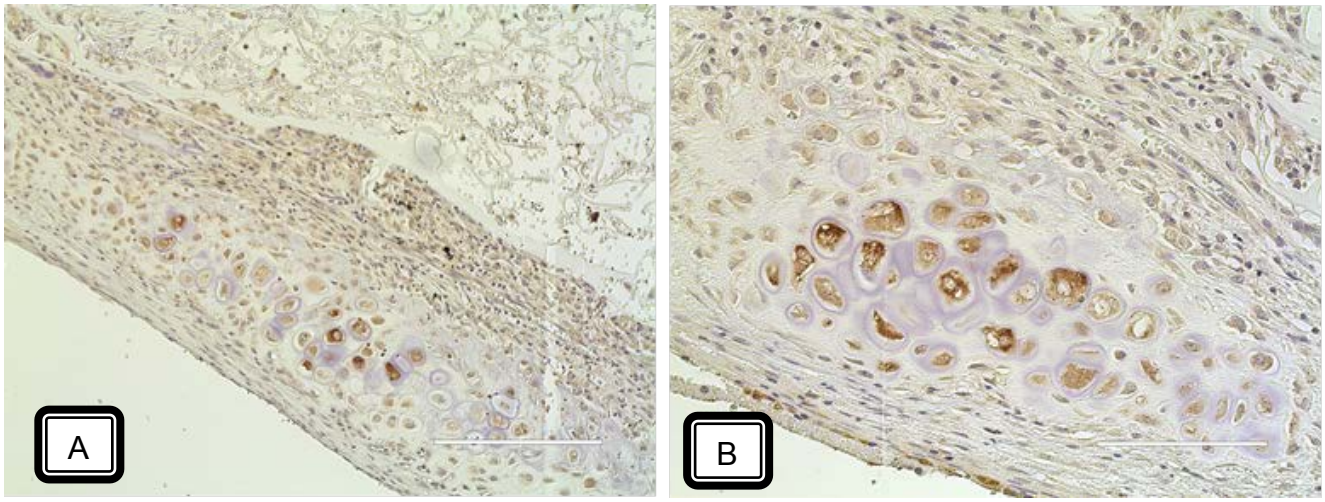

Figure #34: SOX9 Day7-5ug 7,127-7, Dura (a) 20x (b) 40x Osteoblast-like and Chondroblast-like cells. Darker staining cells are most likely the chondroblast-like cells

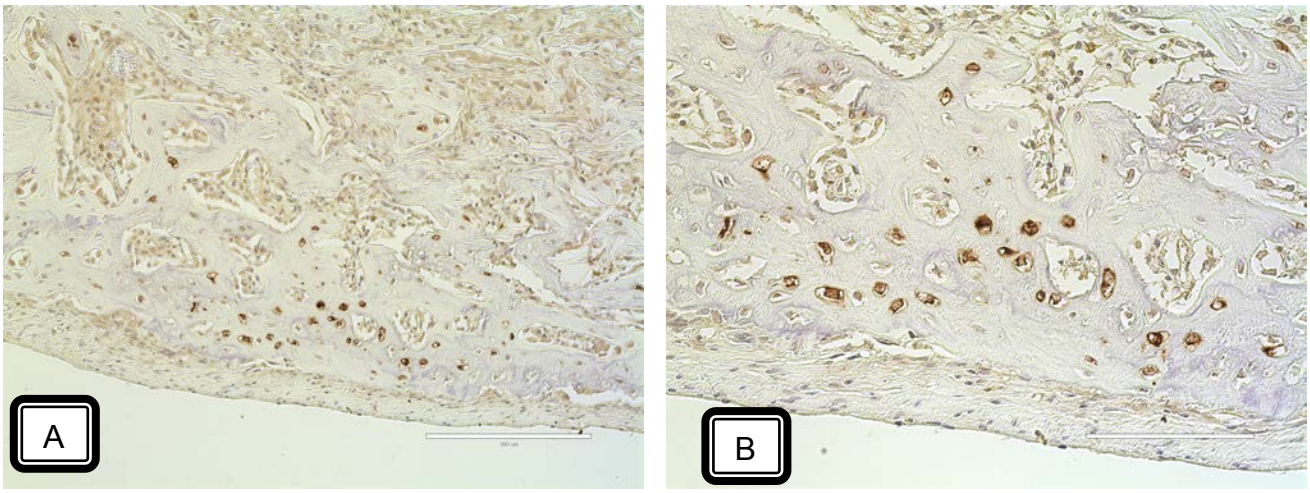

Figure #35: SOX9 Day14-20ug 14,63-11, Dura (a) 20x (b) 40x

Scleraxis Homolog A (SCXA)

Day #1-Day #14

Scleraxis was initially selected for is ability to detect high mitotic areas near muscle and tendon attachment. It was intended to show and demonstrate areas of cellular activity near the muscle attachments. The signal seemed to decrease as areas of cellular activity matured and was strongest prior to new bone formation. Surprisingly there was some activity noted in the dural region. Osteoblast-like cells seemed to be present starting on about day 7 (see figure 37). Additionally there were islands of dense bone that were forming on around day 14 (see figure 38). All of these events noted with SCXA expression were unaffected by rhBMP-2 concentrations.

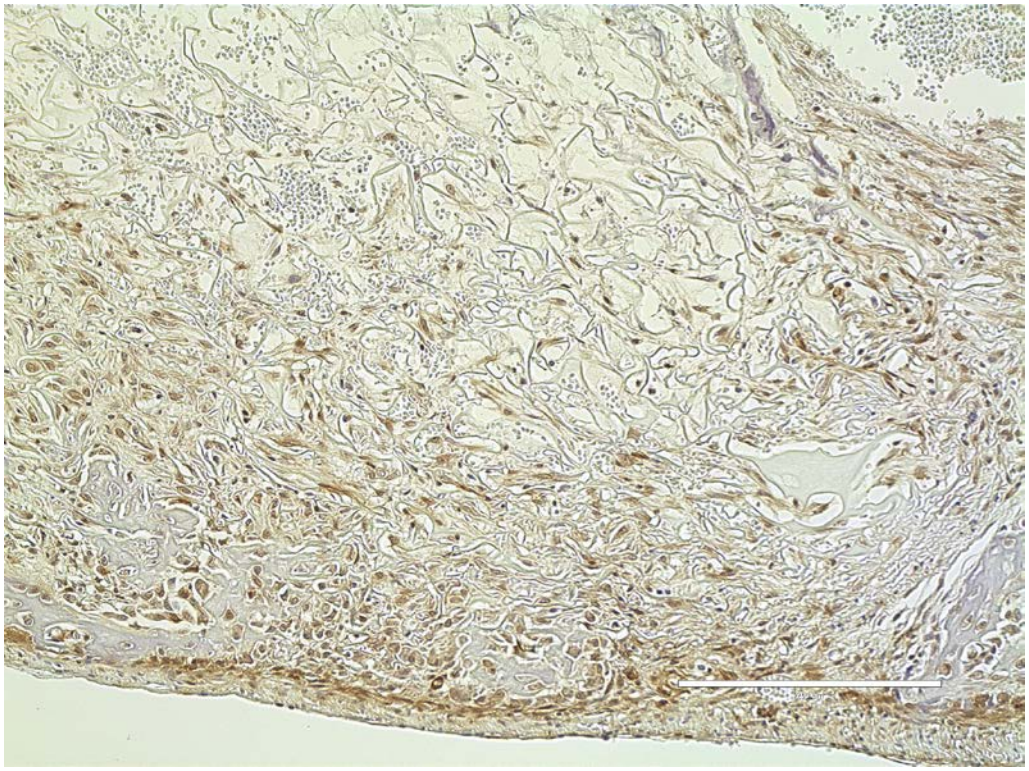

Figure #36: **SCXA Day7-0ug 7.131-6, 20x Dura. Areas of high cellular activity and osteoid formation**

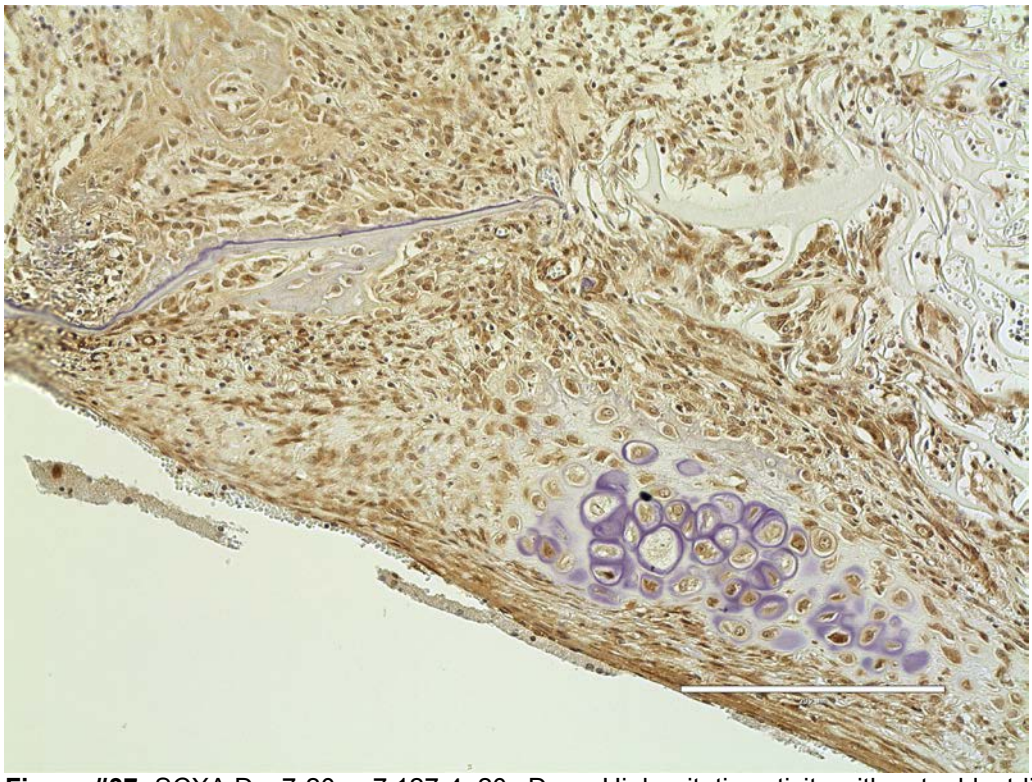

Figure #37: SCXA Day7-20ug 7.127-4, 20x Dura- High mitotic activity with osteoblast-like cells

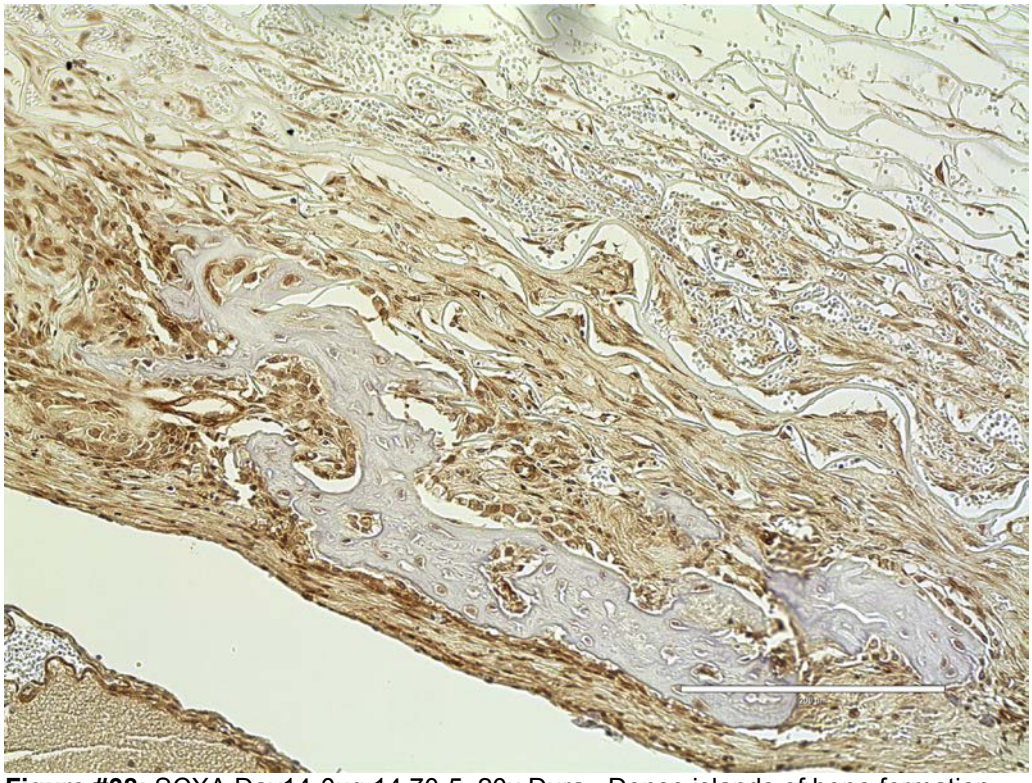

Figure #38: SCXA Day14-0ug 14.70-5, 20x Dura. Dense islands of bone formation

RNA-binding Protein Musashi Homolog 1 (Msi1)
Day #1-14

The initial time points demonstrate minimal activity. Some beginning stages of proliferation being demonstrated at the dural region. Osteoblast-like cells have begun to proliferate and lay down osteoid. Fibroblast-like cells stained positive for the presence of Msi1. This activity is best observed in Figure 44. Msi1 positive cells are present in the dura and appear to proliferate coronally and medially. During the later time points of 7 days and 14 days there are large amounts of osteoid and then later bone islands there are present. Cellular activity continues to migrate coronally through the defect (see figures 46-47,51). With an increasing rhBMP-2, there appears to have an increasing presence of osteoid, maturation of bone and osteoblast-like cells (see figures 45-47).

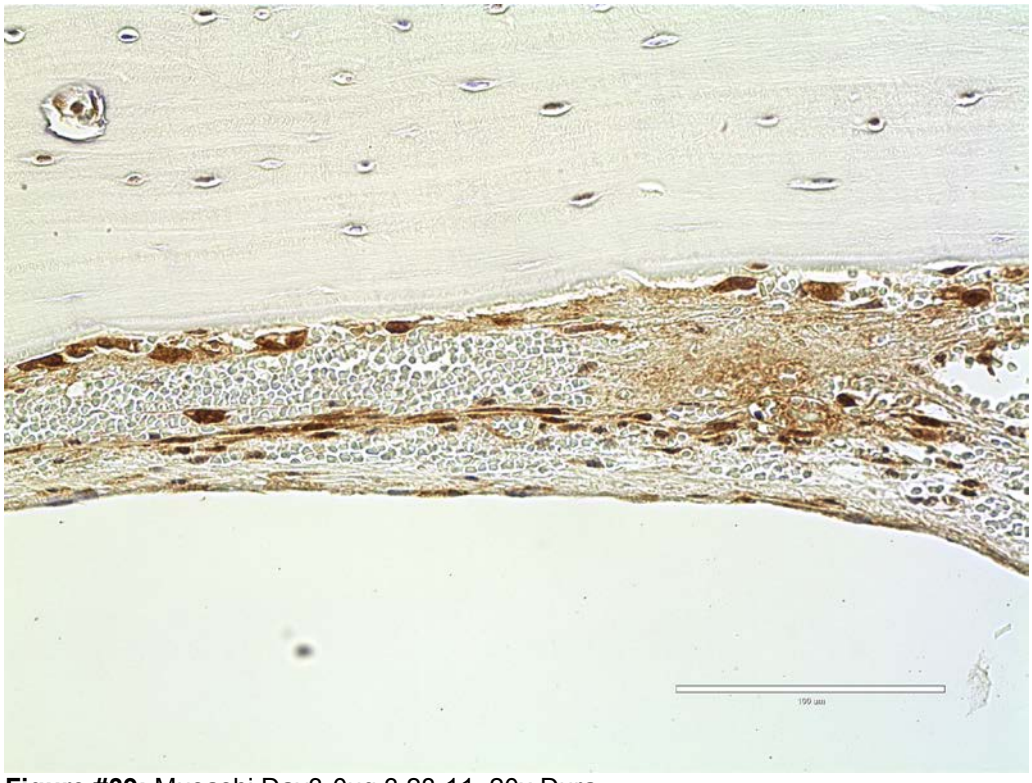

Figure #39: Musashi Day3-0ug 3.28-11, 20x Dura

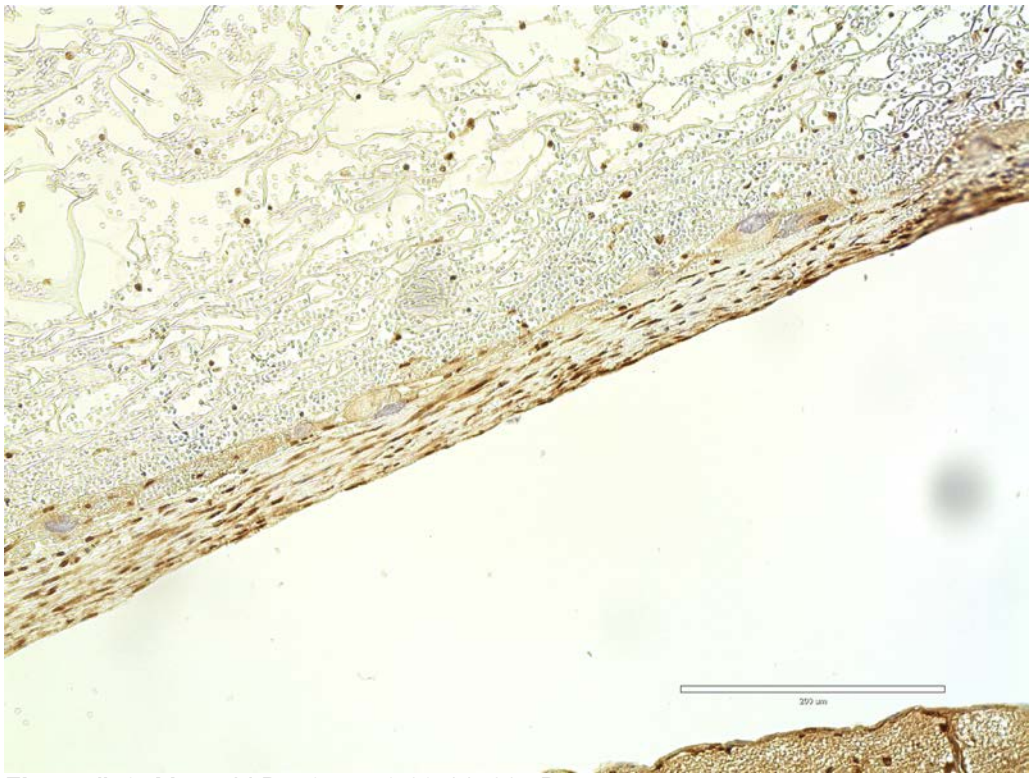

Figure #40: Musashi Day3-5ug 3.29-11, 20x Dura

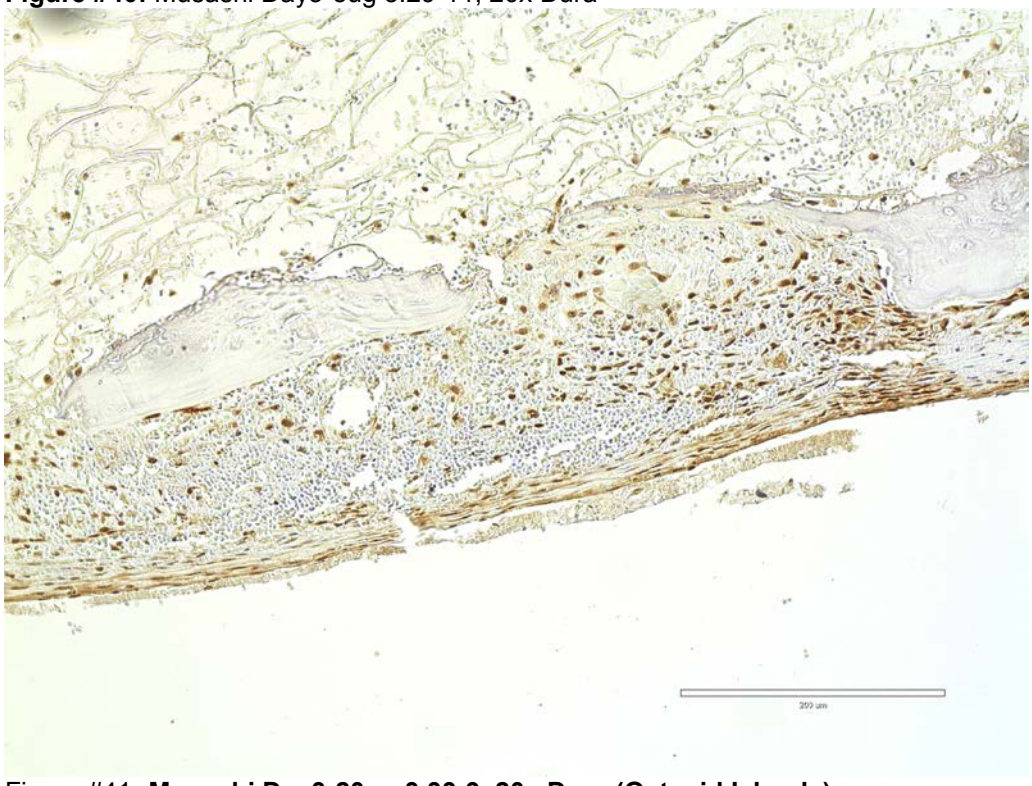

Figure #41**: Musashi Day3-20ug 3.33-9, 20x Dura (Osteoid Islands)**

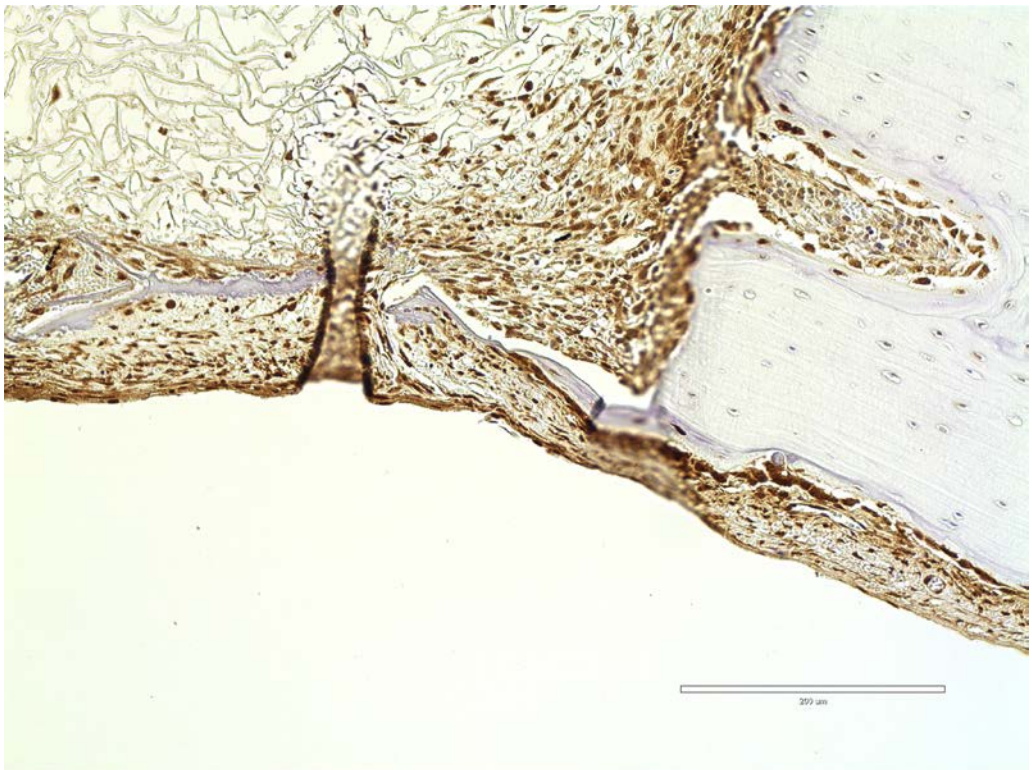

Figure #42: Musashi Day5-0ug 5.82-13, 20x Dura. High mitotic activity along the dural lining. Cells appear to migrate from the lateral borders and the dura itself

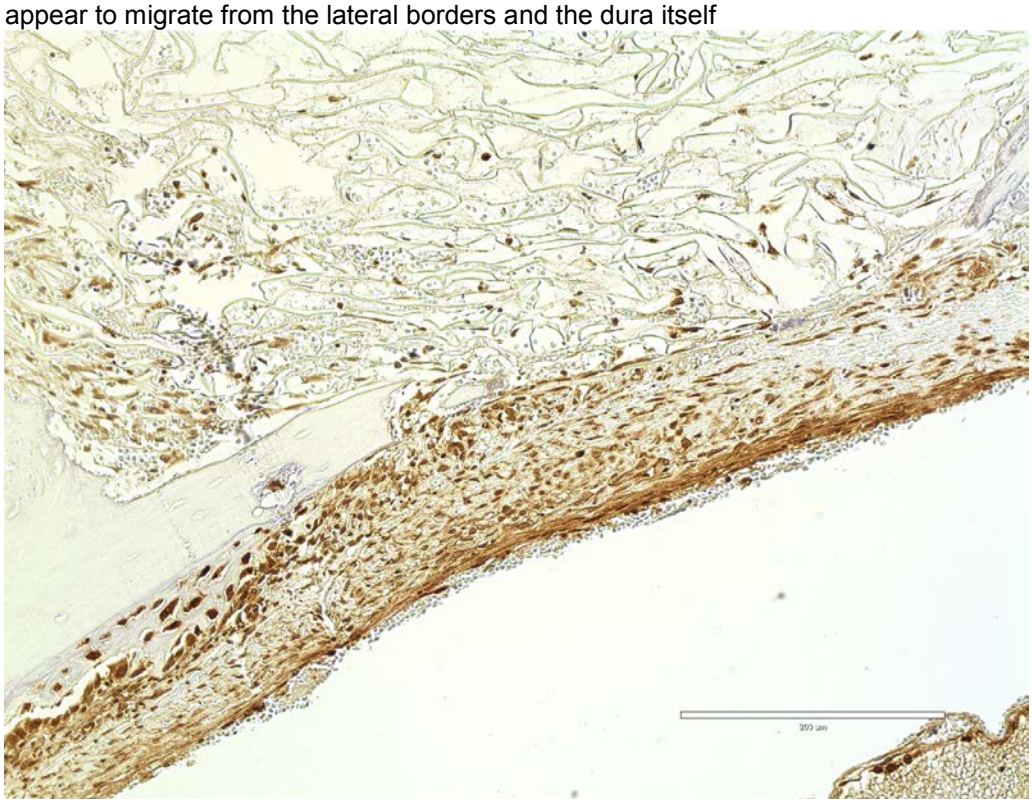

Figure #43: Musashi Day5-5ug 5.104-7, 20x Dura

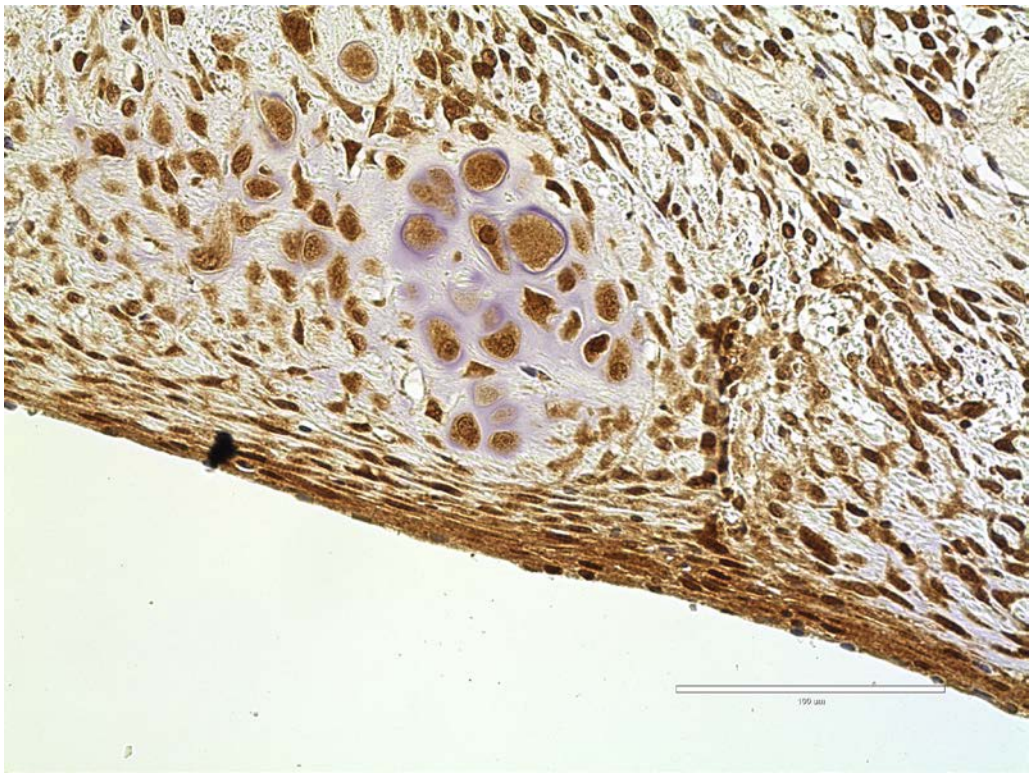

Figure #44: **Musashi Day5-20ug 5.84-13, 40x Dura. Osteoid and osteoblast-like cells in higher concentrations of rhBMP-2**

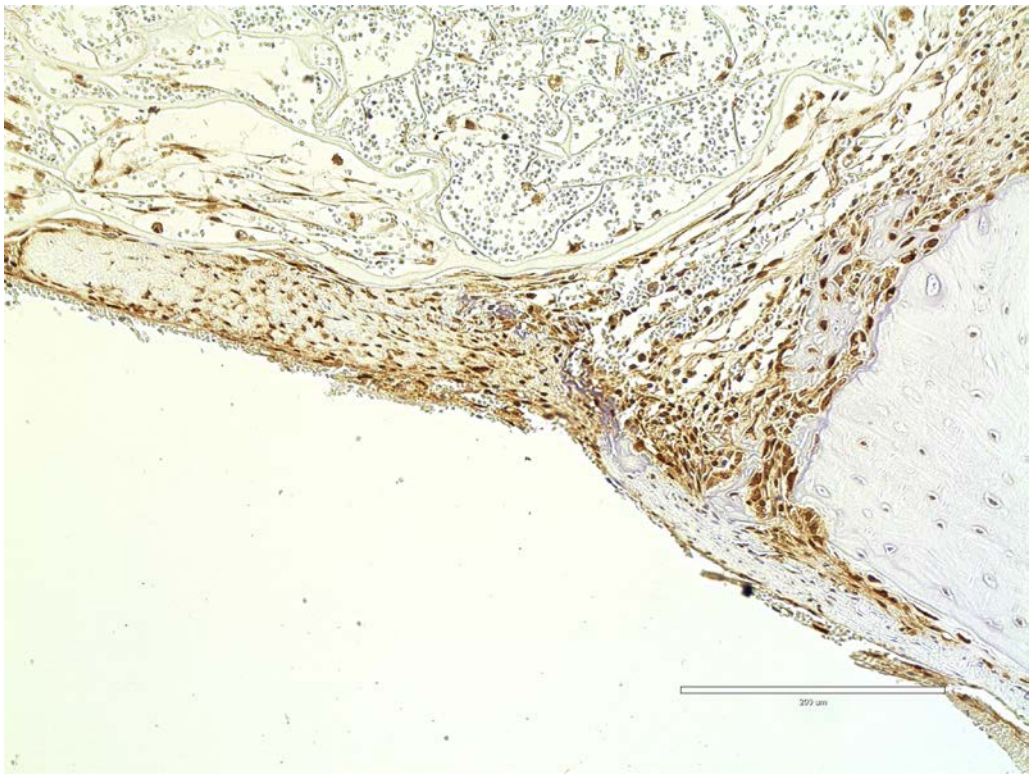

Figure #45: Musashi Day7-0ug 7.110-8, 20x Dura. Beginning stages of osteoid and cuboidal cells

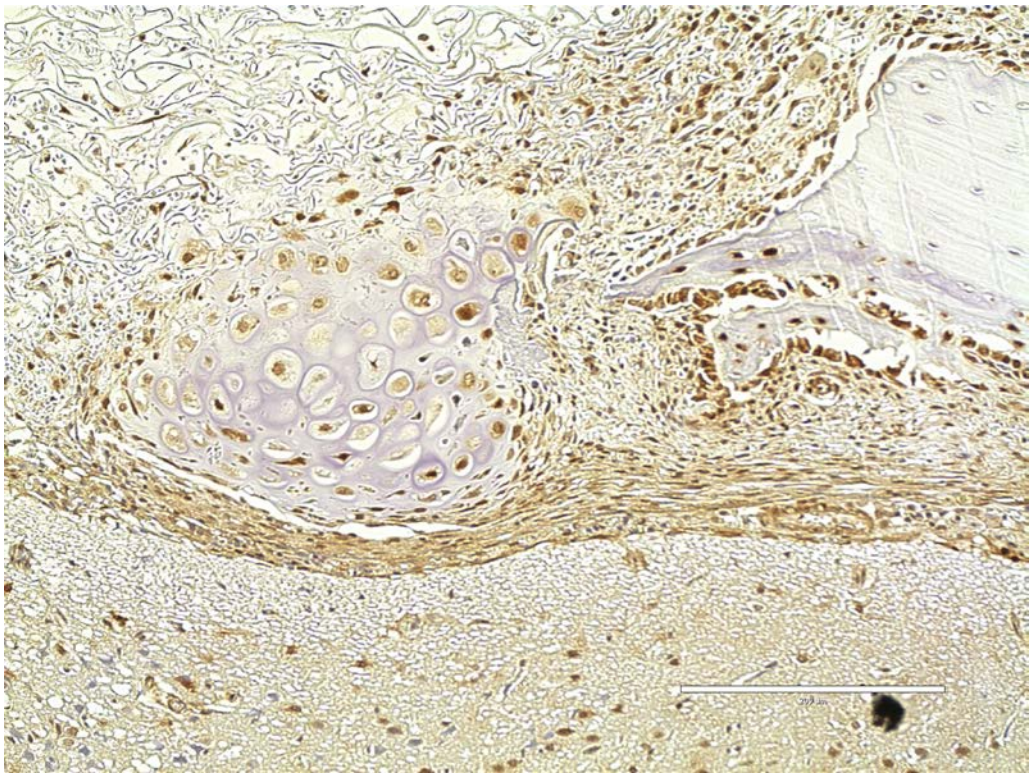

Figure #46: Musashi Day7-5ug 7.111-6, 20x Dura. With an increasing rhBMP-2, There appears to have an increasing presence of osteoid, maturation of bone and osteoblast-like cells.

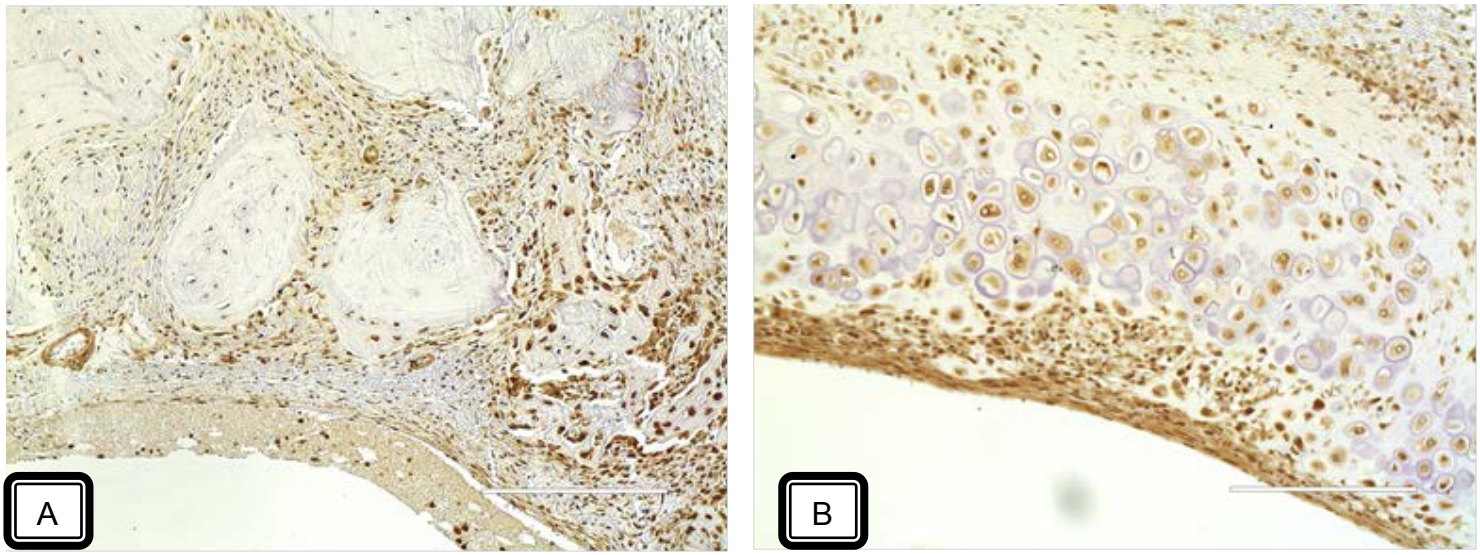

Figure #47: Musashi Day7-20ug 7.133-14, 20x Dura. (a) Dense bone islands (b) Osteoblast-like cells with osteoid present. Both show high mitotic activity.

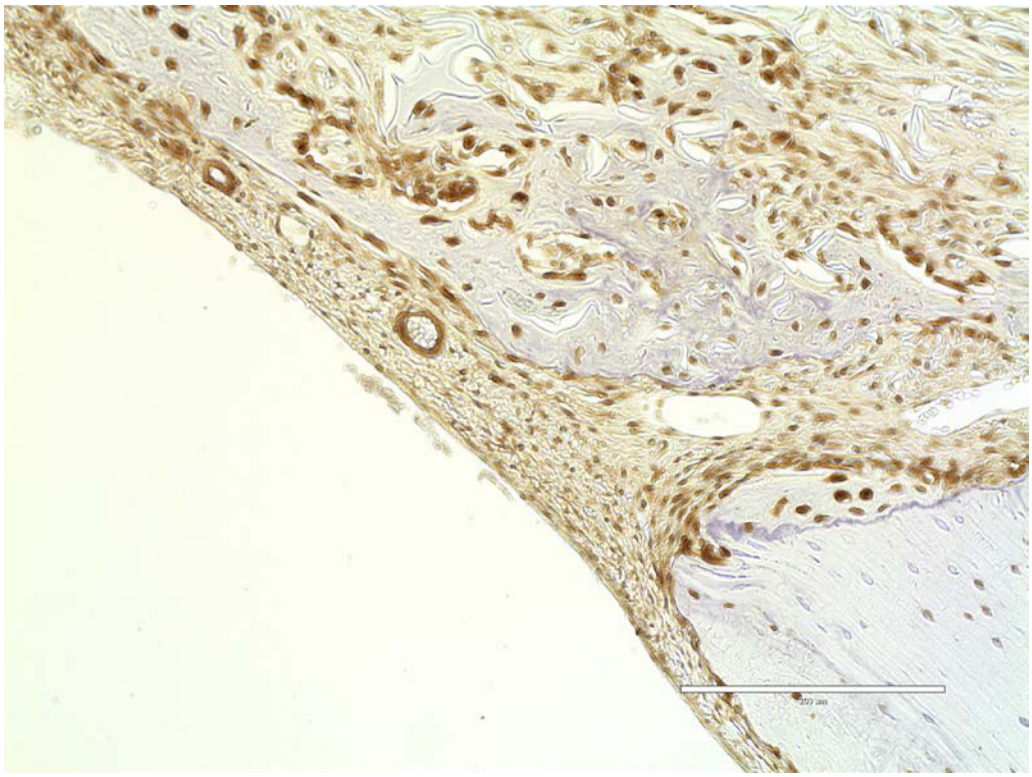

Figure #48**: Musashi Day14-0ug 14.61-12, 20x Dura**

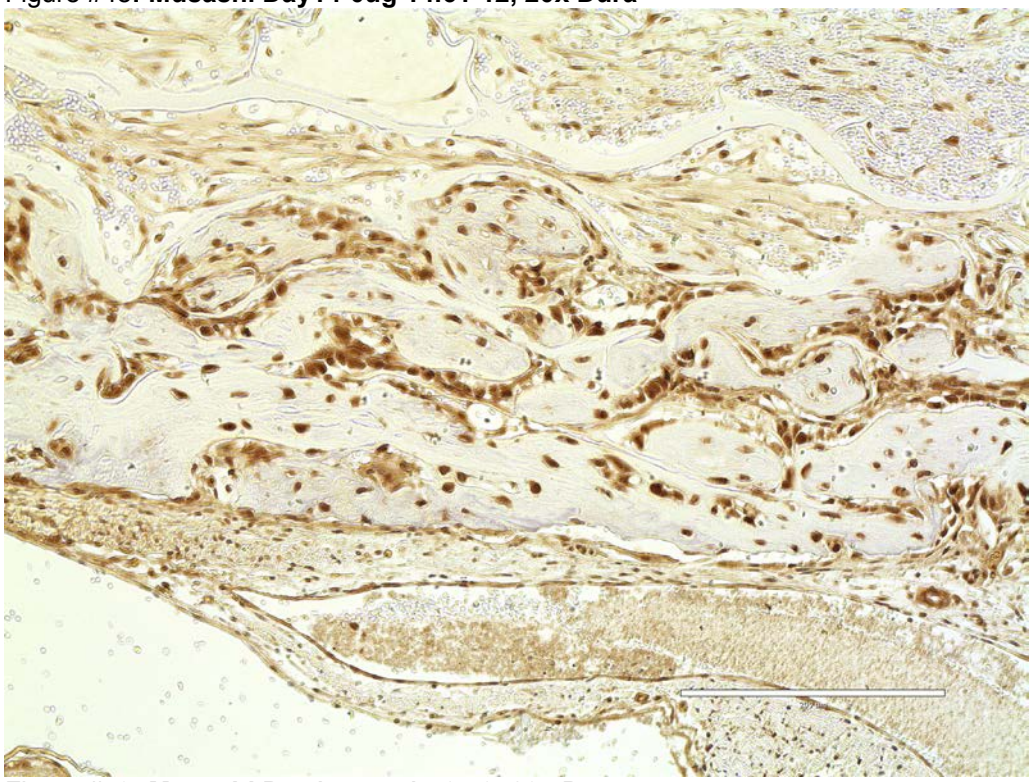

Figure #49**: Musashi Day14-5ug 14.65-9, 20x Dura**

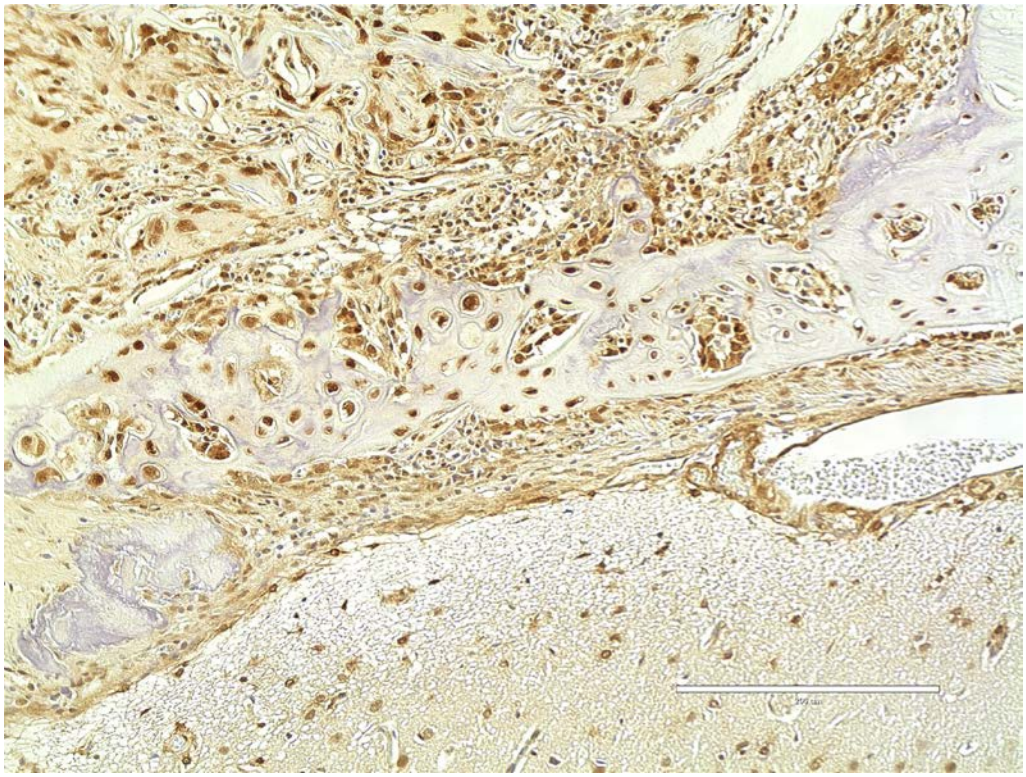

Figure #50: **Musashi Day14-20ug 14.62-5, 20x Dura 3**

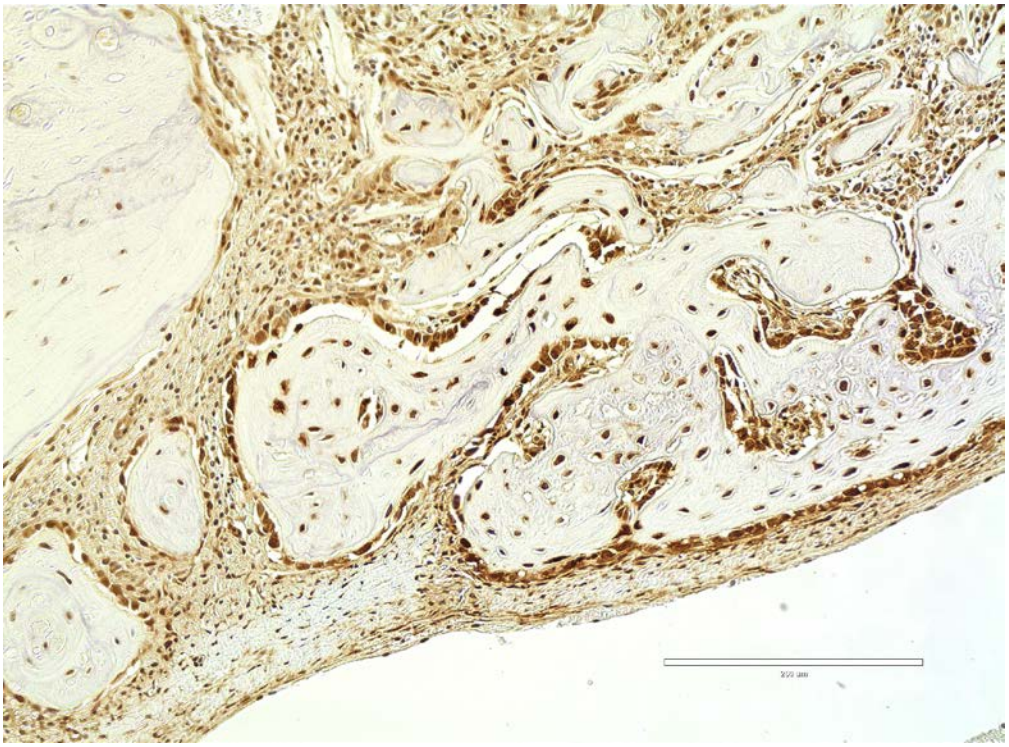

Figure #51: Musashi Day14-20ug 14.62-5, 20x Dura

DISCUSSION

The presence and quantity of iPSC cells is not quantifiable with the technique of immunohistochemistry. But what it does offer is an opportunity to see the types of signals, markers and transcription factors that are present. This also proves to be of value as certain markers, transcription factors, or signals are effected by the concentration of the rhBMP-2. Notwithstanding the potential to identify if the Yamanka transcription factors are present in a critical defect model, but also if they too, are effected by rhBMP-2.

KLF-4 and Sox-2 serve as valuable indicators for the other two Yamanaka transcription factors. The presence of both transcription factors, in similar time points and reacting similarly to rhBMP-2 gives the potential that these two transcription factors are in fact playing a role in the healing of a critical sized defect. Additionally that these transcription factors may also have a combination role, or may actually be co-binding to facilitate the regeneration process. A positive signals for KLF-4 and Sox-2 support the hypothesis that expression in regions where osteoblastic differentiation potential is suspected would be consistent with the hypothesis that pluripotent stem cells exist in the area and are capable of differentiating along the osteoblast cell lineage. And more importantly that co-incident with or as pre-cursors to stem/progenitor cell proliferation, iPSC transcription factors are present and respond in a dose dependent manner and have the highest degree of cellularity around the day 5 time point in this critical sized defect model.

In our staining series, we used PCNA results to identify high degrees of mitotic activity and confirm that the dura/ dural lining was an area of interest. The subsequent day

points demonstrated that yes there was high cellular activity and that two of the sources of activity were coming from the residual native bone on the most lateral aspect of the critical sized defect and additionally from the residual dural tissue. This information could suggest that when stem cells were activated by local signals they began to differentiate along their osteoblastic cell lineage and in some scenarios were increased in the presence of rhBMP-2.

Musashi marker, is for mesenchymal stem cells most typically of fetal and adult neural origin. Positive staining suggested that these neural stem cells are present within the dura in the healing defect. These positive cells lend to one more potential cellular process that could be a precursor to the osteoblastic lineage. Large amounts of osteoid and bone islands were seen around the 7 day time point and increased in frequency towards the 14 day time point. Scattered large heavily stained nuclei on the cells with in the dura and ACS give the appearance that the proliferation of these cells may play a role in the progression of the healing.

The general expectation was that rhBMP-2 concentrations would have an increased cellular response with increasing doses in regards to the mitotic proliferation activity. Surprisingly, some of the markers demonstrated this correlation, while others were not consistent with this hypothesis. Generally speaking the rhBMP-2 created a greater cellular response in the day 5 time point than what was seen with the marker alone. Specifically this was seen with the KLF-4 and Sox-2 markers. Our research demonstrates that in this model there is potential that these iPSC transcriptions factors are present and functioning and potentially influenced by the presence of rhBMP-2. Further research, such as immunofluorescence would confirm that the same cells are

expressing the transcription factors and then be able to quantify the number of cells and assess if the rhBMP-2 is increasing the cellular response.

The Scleraxis A (SCXA) marker was observed near the 7 day time point. This tendon transcription factor demonstrated strong signaling in the nuclear region of the cells. In addition the manifestation of SCXA was seen in the dura to a somewhat surprising degree, at least initially. This marker was selected for its ability to detect areas of tendon and tendon insertion. While focusing on the dura, one would think that such an area would not have expression of tendinous like material. However, the dura did have cellular activity that expressed the SCXA marker. Further research then lead us to the prospect that SCXA expression is also present in cells that are of the chondroblastic lineage. Along with Sox-9 this marker further clarifies that near the dural lining the healing has a few mechanisms of which chondrogenesis is one. These areas of new bone formation were not one of our expectations, but still adds to the growing data of the mechanisms, by which a large cranial defect attempts to regenerate new woven bone. There have also been other markers that were expressed in this same region and SCXA and Sox-9 may seem to serve as primers or precursors to the differentiation of osteoblastic cell line.

As has been mentioned previously, the best way to determine the exact cells and markers present would be to utilize immunofluorescence. This would allow for quantification of the data, and would be the next process to seek more definitive answers.

FUTURE STUDIES

The presence of iPSCs and their role in the healing and regenerative process poses a viable option to achieve actual regeneration. Our samples have demonstrated that these markers or transcription factors are there and in some instances are in a reasonable amount. There is potential that they are stimulate stem cells to either differentiate or de-differentiate back to pluripotent stem cell status. These stem cells seemed to respond to osteoblastic differentiation proteins. A better understanding of the cells involved and which differentiation factors are in play, could allow for regenerative abilities could be enhanced aiding in the healing process and periodontal regeneration. Future studies could test other markers specific to the iPCS and more specifically could use immunofluorescence and confocal microscopy to determine if the signals are in the same cells or different cells.

References

1. Yamanaka S. (2007) Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell;1:39–49. doi: 10.1016/j.stem.2007.05.012.
2. Yamanaka S. (2009) A fresh look at iPS cells. Cell. 137:13–17. doi: 10.1016/j.cell.2009.03.034.
3. Ebrahimi, M., & Botelho, M. (2017). Adult Stem Cells of Orofacial Origin: Current Knowledge and Limitation and Future Trend in Regenerative Medicine. *Tissue engineering and regenerative medicine*, *14*(6), 719-733. doi:10.1007/s13770-017-0078-6
4. Takahashi K, Yamanaka S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell.126(4):663–76.
5. Zachar, M. R., Labella, C., Kittle, C. P., Baer, P. B., Hale, R. G., & Chan, R. K. (2013). Characterization of mandibular fractures incurred from battle injuries in iraq and afghanistan from 2001-2010. *Journal of Oral and Maxillofacial Surgery, 71*(4), 734-742. doi:http://dx.doi.org/10.1016/j.joms.2012.10.030
6. Lew, T. A., Walker, J. A., Wenke, J. C., Blackbourne, L. H., & Hale, R. G. (2010). Characterization of craniomaxillofacial battle injuries sustained by united states service members in the current conflicts of iraq and afghanistan. *Journal of Oral and Maxillofacial Surgery, 68*(1), 3-7. doi:http://dx.doi.org/10.1016/j.joms.2009.06.006
7. Allareddy, V., & Nalliah, R. P. (2011). Epidemiology of facial fracture injuries. *Journal of Oral and Maxillofacial Surgery, 69*(10), 2613-2618. doi:http://dx.doi.org/10.1016/j.joms.2011.02.057
8. Robbins, C. (2010). Tissue Renewal, Regeneration, and Repair. In *Pathologic Basis of Disease* (8 ed., pp. 79-110). Philadelphia: Saunders Elsevier.
9. Kalfas, I. H. (2001). Principles of bone healing. *Neurosurgical Focus, 10*(4), 1-4. doi:10.3171/foc.2001.10.4.2
10. Poliard A, Nifuji A, Lamblin D, Plee E, Forest C, Kellermann O. Controlled conversion of an immortalized mesodermal progenitor cell towards osteogenic, chondrogenic, or adipogenic pathways. *J Cell Biol* 1995;130:1461-1472.
11. Kim SW, Pajevic PD, Selig M, et al. Intermittent parathyroid hormone administration converts quiescent lining cells to active osteoblasts. *J Bone Miner Res* 2012;27:2075-2084.

12. Long, M. W., Robinson, J. A., Ashcraft, E. A., & Mann, K. G. (1995). Regulation of human bone marrow-derived osteoprogenitor cells by osteogenic growth factors. The Journal of clinical investigation, 95(2), 881-887.
13. *Lee, T. J.; Jang, J.; Kang, S.; Jin, M.; Shin, H.; Kim, D. W.; Kim, B. S. (2013). "Enhancement of osteogenic and chondrogenic differentiation of human embryonic stem cells by mesodermal lineage induction with BMP-4 and FGF2 treatment". Biochemical and Biophysical Research Communications.* ***430*** *(2): 793–797.*
14. Gilbert S. Developmental Biology. 6th Sunderland (MA): Sinauer Associates; 2000. Available from: http://www.ncbi.nlm.nih.gov/books/NBK10056/.
15. Kanczler 2008
16. Frost HM. Skeletal structural adaptations to mechanical usage (SATMU): 2. Redefining Wolff's law: the remodeling problem. *Anat Rec* 1990;226:414-422.
17. Wada, T., Nakashima, T., Hiroshi, N. & Penninger, J. M. (2006) RANKL–RANK signaling in osteoclastogenesis and bone disease. *Trends in Molecular Medicine* **12,** 17-25
18. Leibbrandt A, Penninger JM. RANK/RANKL: regulators of immune responses and bone physiology. *Ann N Y Acad Sci* 2008;1143:123-150.
19. Leibbrandt A, Penninger JM. Novel functions of RANK(L) signaling in the immune system. *Adv Exp Med Biol* 2010;658:77-94.
20. Dawson, J. I., Kanczler, J., Tare, R., Kassem, M., & Oreffo, R. O. C. (2014). Concise review: Bridging the gap: Bone regeneration using skeletal stem cell-based Strategies—Where are we now? *Stem Cells, 32*(1), 35-44. doi:10.1002/stem.1559
21. Hsiong SX, Mooney DJ. Regeneration of vascularized bone. *Periodontol 2000* 2006;41:109-122.
22. Yew TL, Huang TF, Ma HL, Hsu YT, Tsai CC, Chiang CC, et al. Scale-up of MSC under hypoxic conditions for allogeneic transplantation and enhancing bony regeneration in a rabbit calvarial defect model. J Orthop Res. 2012 Aug;30(8):1213-20.
23. Alexander, K. A., Chang, M. K., Maylin, E. R., Kohler, T., Müller, R., Wu, A. C., Pettit, A. R. (2011). Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model. *Journal of Bone and Mineral Research, 26*(7), 1517-1532. doi:10.1002/jbmr.354
24. Spicer P, Kretlow J, Young S, Jansen J, Kasper F, Mikos A. Evaluation of bone regeneration using the rat critical size calvarial defect. Nature Protocols. 2012;7(September):1918-29.

25. Kunkel, N., Wagner, A., Gehwolf, R., Heimel, P., Tempfer, H., Korntner, S., . . . Traweger, A. (2015). Comparing the osteogenic potential of bone marrow and tendon-derived stromal cells to repair a critical-sized defect in the rat femur. *Journal of Tissue Engineering and Regenerative Medicine,* , n/a-n/a. doi:10.1002/term.2097
26. Johnson MR, Boerckel JD, Dupont KM, Guldberg RE. Functional restoration of critically sized segmental defects with bone morphogenetic protein-2 and heparin treatment. Clin Orthop Relat Res. 2011 Nov;469(11):3111-7
27. Kiefer JC. (2007) Back to Basics: Sox Genes. Developmental Dynamics. 236(8):2356–2366. doi: 10.1002/dvdy.21218.
28. Rizzino, A. (2009), Sox2 and Oct-3/4: a versatile pair of master regulators that orchestrate the self-renewal and pluripotency of embryonic stem cells. WIREs Syst Biol Med, 1: 228-236. doi:10.1002/wsbm.12
29. *Kim JH, Kim K, Youn BU, Lee J, Kim I, Shin HI, Akiyama H, Choi Y, Kim N (2014). "Kruppel-like factor 4 attenuates osteoblast formation, function, and cross talk with osteoclasts". The Journal of Cell Biology.* ***204*** *(6): 1063–74. doi:10.1083/jcb.201308102*
30. Gearhart J, Pashos EE, Prasad MK (2007). "Pluripotency redux--advances in stem-cell research". *The New England Journal of Medicine*. **357**(15): 1469–72. doi:10.1056/NEJMp078126. PMID 17928593.
31. Good P, Yoda A, Sakakibara S, Yamamoto A, Imai T, Sawa H, Ikeuchi T, Tsuji S, Satoh H, Okano H (1998). "The human Musashi homolog 1 (MSI1) gene encoding the homologue of Musashi/Nrp-1, a neural RNA-binding protein putatively expressed in CNS stem cells and neural progenitor cells". *Genomics*. **52** (3): 382–4. doi:10.1006/geno.1998.5456. PMID 9790759
32. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. (19999) Sox9 is required for cartilage formation. Nat Genet 22:85-9.
33. Kitaori, T., Ito, H., Schwarz, E. M., Tsutsumi, R., Yoshitomi, H., Oishi, S., Nakamura, T. (2009). Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis & Rheumatism, 60*(3), 813-823. doi:10.1002/art.24330
34. Kanki S, Segers VF, Wu W, Kakkar R, Gannon J, Sys SU, et al. Stromal cell-derived factor-1 retention and cardioprotection for ischemic myocardium. Circ Heart Fail. 2011 Jul;4(4):509-18.

35. Wise JK, Sumner DR, Virdi AS. Modulation of stromal cell-derived factor-1/CXC chemokine receptor 4 axis enhances rhBMP-2-induced ectopic bone formation. Tissue Eng Part A. 2012 Apr;18(7-8):860-9.
36. Higashino K, Viggeswarapu M, Bargouti M, Liu H, Titus L, Boden SD. Stromal cell-derived factor-1 potentiates bone morphogenetic protein-2 induced bone formation. Tissue Eng Part A. 2011 Feb;17(3-4):523-30.
37. Park J, Ries J, Gelse K, Kloss F, von der Mark K, Wiltfang J, et al. Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes. Gene Ther. 2003 Jul;10(13):1089-98.
38. Guang LG, Boskey AL, Zhu W. Regulatory role of stromal cell-derived factor-1 in bone morphogenetic protein-2-induced chondrogenic differentiation in vitro. Int J Biochem Cell Biol. 2012 Nov;44(11):1825-33.
39. Tseng WP, Yang SN, Lai CH, Tang CH. Hypoxia induces BMP-2 expression via ILK, Akt, mTOR, and HIF-1 pathways in osteoblasts. J Cell Physiol. 2010 Jun;223(3):810-8.
40. Hosogane N, Huang Z, Rawlins BA, Liu X, Boachie-Adjei O, Boskey AL, et al. Stromal derived factor-1 regulates bone morphogenetic protein 2-induced osteogenic differentiation of primary mesenchymal stem cells. Int J Biochem Cell Biol. 2010 Jul;42(7):1132-41.
41. Yamaguchi, A., Komori, T., & Suda, T. (2000). Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endocrine Reviews, 21*(4), 393-411. doi:10.1210/edrv.21.4.0403
42. Street, J. T., Wakai, A., Wang, J. H., Wu, Q. D., McGuinness, A., & Redmond, H. P. (2001). Vascular endothelial growth factor–? Osteoblast survival factor. In 47th Annual Meeting of the American Orthopaedic Research Society, San Francisco, CA (Vol. 485).
43. Su Jin Kim, Su Yung Kim, Chae Hwa Kwon & Yong Keun Kim (2007) Differential effect of FGF and PDGF on cell proliferation and migration in osteoblastic cells,Growth Factors, 25:2, 77-86, DOI: 10.1080/08977190701398977
44. Wozney, J. M. & Seeherman, H. J. (2004) Protein-based tissue engineering in bone and cartilage repair. *Curr Opin Biotechnol* **15,** 392-398. A. & Filvaroff, E. H. (2002) Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci U S A* **99,** 9656-9661.
45. Luzzani, C., Neiman, G., Garate, X., Questa, M., Solari, C., Espinosa, D.F., García, M., Errecalde, A.L., Guberman, A., Scassa, M.E. and Sevlever, G.E., 2015. A therapy-grade

protocol for differentiation of pluripotent stem cells into mesenchymal stem cells using platelet lysate as supplement. Stem cell research & therapy, 6(1), p.1.

46. Marynka-Kalmani, K., Treves, S., Yafee, M., Rachima, H., Gafni, Y., Cohen, M. A. and Pitaru, S. (2010), The Lamina Propria of Adult Human Oral Mucosa Harbors a Novel Stem Cell Population. STEM CELLS, 28: 984–995. doi:10.1002/stem.425