2024

Role of Schwann cells in neuroregeneration and skeletal pain during femoral fracture repair

BOSTON UNIVERSITY

ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

ROLE OF SCHWANN CELLS IN NEUROREGENERATION AND SKELETAL PAIN DURING FEMORAL FRACTURE REPAIR

By

LILLIAN YANG

B.A., New York University, 2022

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2024

Approved by

First Reader ____

Louis Gerstenfeld, Ph.D.
Professor of Orthopedic Surgery

Second Reader ____

Beth Bragdon Ph.D.
Assistant Professor of Orthopedic Surgery

ACKNOWLEDGMENTS

I would like to thank Dr. Gerstenfeld for providing me this opportunity to conduct this research and Dr. Bragdon for serving as the second reader. I would like to thank both for all their guidance and mentorship throughout this year. I would also like to thank my parents and my friends at MAMS for their constant motivation and support.

ROLE OF SCHWANN CELLS IN NEUROREGENERATION AND SKELETAL PAIN DURING FEMORAL FRACTURE REPAIR

LILLIAN YANG

ABSTRACT

Intense bone pain is the primary marker for progression of healing and is associated with many skeletal pathologies including long bone fractures. New mechanism-based analgesics to relieve bone pain and augment bone recovery post-fracture is required but, further understandings of the pathophysiology of bone pain are necessary before we can develop novel mechanism-based alternative treatments. The four stages of bone healing have been established to be in the following order: hematoma formation, granulation tissue formation, bony callus formation, then bone remodeling. Studies have shown that bone fracture repair processes and the neuronal regrowth that occurs following a peripheral nerve injury share many genes and proteins that interplay with one another to aide in fracture healing and repair. However, little to no research has been done on such set of genes and proteins and their role in the sensation of pain associated with normal bone healing.

Expressions of messenger RNA for marker genes for cartilage and bone formation, along with neuronal factors involved with peripheral nerve repair, were assessed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis over a specific time course of healing, ranging from pre-fracture (day 0) to day 35 post-fracture. Structural identification and quantifications of specific fracture callus contents were assessed using staining and immunohistochemistry techniques, using day

10, day 14, and day 21 tissue samples. Potential pain-associated mechanisms and related genes to neuronal regrowth including SCPs markers were analyzed in a temporal manner.

The biological assessments of fracture healing demonstrated temporal differences in their relative peak expressions and quantity throughout the 35 day period studied. Genes associated with initial hematoma formation and neuroinflammation, including IL-6, NGF, and SC markers including PLP1, SOX2, and SOX10, reached a peak in expression early in the time course. However, SC-related genes including MAG and MOG did not reach their highest peaks in expression until mid-course around day 14, and day 20, when the bony callus and bone remodeling processes are occurring, respectively. Neuronally, such processes are occurring simultaneously as axonal reinnervation and remyelination is occurring with new vessel formation. Osteogenesis genes like BGLAP and NCAM, showed a later burst of maximal expression at day 14 and 20, associated with the later occurrence of bony callus formation and bone remodeling (secondary bone formation). Anti-Collagen type I, CNTF and SOX10 antibody staining demonstrated its presence are restricted to the lining of blood vessels and the bone marrow.

These results suggest that further research in the pain mechanisms involved with peripheral nerve damage occurring during bone fractures are necessary in understanding and developing further treatments related to bone pain. With each stage of the bone repair model, pain associated with specific genes and proteins are expected to be peak and wane off with completion of each stage. Future studies need to be carried out to determine the length of pain and localization of pain-related factors are associated with a healing fracture callus.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACAN ………………………………………………………………………..… Aggrecan

BGLAP …………………………………………Bone Gamma-Carboxyglutamate Protein

cDNA ……………………………………………Complementary Deoxyribonucleic Acid

CNS …………………………………………………….……..… Central Nervous System

CNTF…………………………………………………….……Ciliary Neurotrophic Factor

COX ……………………………………………………………………Cyclooxygenase

CRYAB ………………………………………….……………… Crystallin Alpha B

DAB………………………………………………………...….3,3' - Diaminobenzidine

DNA ………………………………………………………………Deoxyribonucleic Acid

DRG ……………………………………….……………….…Dorsal Root Ganglion

ECM ……………………………………….……………..…..Extracellular Matrix

EDTA …………………………………………………Ethylenediamine Tetra acetic Acid

IL-6 ……………………………………………………………………… Interleukin -6

MAG ……………………….…………………………Myelin Associated Glycoprotein

MOG……………………….…………………... Myelin Oligodendrocyte Glycoprotein

MSC ……………………….…………………………………Mesenchymal Stem Cells

NCAM ………………….……………..……………… Neural Cell Adhesion Molecule

Ng ……………………………………………………………………………. nanogram

NGF …………………….………………….……………………… Neuronal Growth Factor

NSAID ………………………………………….. Nonsteroidal Anti-Inflammatory Drugs

PBS ………………….…………………………………….. Phosphate-Buffered Saline

PCR ………………………………..……………………..… Polymerase Chain Reaction

PLP……………………………..……………………………… Phospholipid Protein

PNI…………………..………………..…………………………… Peripheral Nerve Injury

PNS …………………..………..……………………………… Peripheral Nervous System

qPCR ………………….………………………...Quantitative Polymerase Chain Reaction

RANK …………………..…………………………… Receptor Activator of Nuclear Factor kB

RANKL …………………….……………..Receptor Activator of Nuclear Factor kB Ligand

RNA …………………..…………………………………………….……Ribonucleic Acid

rRNA …………………..…………………………………………… Ribosomal Ribonucleic Acid

RNase …………………..…………………………………………………….Ribonuclease

SC …………………..………..…………………………………………… Schwann Cell

SCPs ……………..…….…………………………………………Schwann Cell Precursor

SOX2…………………..…………………………………………….……… SRY-box 2

SOX10…………………..…………………………………………………….SRY- box 10

STAT3…………………..………………Signal transducer and activator of transcription 3

TrkA…………………..……………………………………Tropomyosin Receptor Kinase A

µg ……………………..………..…..….………………………………… microgram

µL ……………………..…………………………………………….………microliter

INTRODUCTION

The prevalence of bone fractures has increased globally from the 1990's with approximately a 33.4% increase in absolute incident cases or about 178 million new fractures in 2019; corresponding with the rapidly aging society (Cauley, 2021). While the majority of fractures heal properly, approximately 10% are problematic. Fracture healing is an intricate ordeal involving several biological systems and pathways on the cellular and molecular levels. It comprises an anabolic process characterized by the augmentation of tissue volume, leading to the formation of new skeletal tissue, followed by an extended phase of tissue remodeling, restoring the original structure and function through catabolic mechanisms (Einhorn and Gerstenfeld, 2014).

Skeletal pathologies are most often associated with intense pain, causing a major burden on individuals' quality of life and health care systems worldwide. Schwann cell precursors (SCPs) and their plasticity within the peripheral nervous system (PNS) is a critical attribute during bone repair as they endure complex cell reprogramming designed to enhance nerve regeneration and functional recovery (Nocera and Jacob, 2020). Within minutes of a bone fracture, nociceptors innervating the bone become activated or sensitized by a variety of mediators and signaling molecules. The attenuation of bone pain is associated with normal bone healing, whereas the continuation of pain is associated with delayed or incomplete healing (Mitchell et al., 2018). In recognizing the distinct characteristics of repair Schwann cells and their cell specific control mechanisms during fracture injury response, it suggests that manipulating such cells and mechanisms may be a means to new developments of effective targeted therapies for bone pain.

Normal Fracture Healing Processes and Bone Remodeling

The normal fracture healing process differs in temporality depending on fracture types and severity of trauma. Many aspects of the bone repair process relate to certain features of embryonic skeletal development (Gerstenfeld et al., 2003). The four interrelated stages of bone fracture repair consist of hematoma formation, fibrocartilaginous callus formation, hard callus formation, and bone remodeling (Schindeler, et al., 2008). As pictured in figure 1, a complex interplay of growth factors, signaling molecules, cells and the ECM is involved in normal fracture healing processes. While there is significant overlap in processes within the regenerating tissue, there is a continuous series of changes in cell populations and signaling processes with progression (Einhorn and Gerstenfeld, 2014).

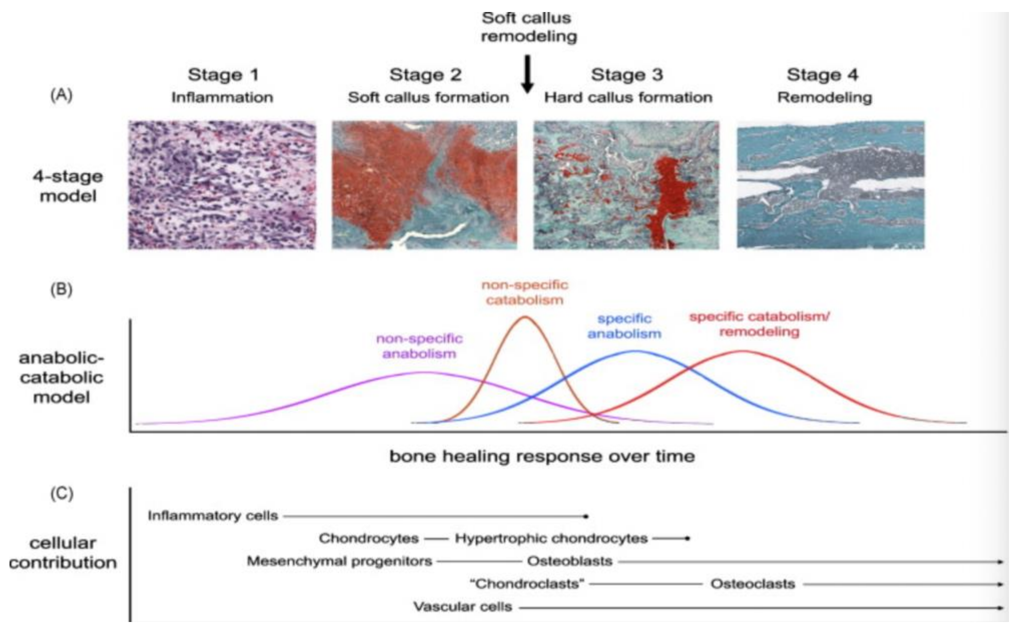

Figure 1. The Four Stage Model of Fracture Repair. Model of fracture repair and its cellular participants are demonstrated in this figure. Representative images of the four sequential stages of fracture healing (A) are mapped with the anabolic-catabolic models (B), and further mapped with the cellular components (C) to fracture repair processes. Figure taken from Schindeler et al., 2008.

Immediately following bone fracture, local soft tissue and vascular functions of surrounding soft tissues, bone marrow and endosteal/periosteal surfaces become disrupted, leading to a hematoma formation with the activation of the plasma coagulation cascade. Inflammatory cells, specifically complement proteins and neutrophils, arrive at the fracture site and danger signals from necrotic cells are released within the hours (Loi et al., 2016). As these inflammatory cells release specific inflammatory and chemotactic mediators such as interleukin 6 (IL-6), it initiates the recruitment of additional inflammatory cells including fibroblasts, mesenchymal stem cells (MSCs), and osteogenic cells from circulation, bone marrow and periosteum (Schindeler et al., 2008). IL-6 is involved in the stimulation of osteoblasts to help in the production of the fibrocartilaginous callus and angiogenesis processes that follows. Without controlled levels of IL-6, delays in mineralization and MSC differentiation processes occur (Einhorn and Gerstenfeld, 2014). In addition, IL-6 also alters the responses to mechanical stimuli and pain, as it is heavily involved in the differentiation and survival of neurons and nerve regeneration (De Jongh et al., 2003).

Continuing fracture healing, a fibrocartilaginous callus template promptly forms adjacent to the fracture line with the onboarding and differentiation of stem cells forming skeletal tissue (Einhorn and Gerstenfeld, 2014). This stage at the cellular level is dominated by chondrocytes and fibroblasts, driving the soft callus production as chondrocytes replace the fibrinous tissue with cartilage and fibroblasts continue to regenerate the extracellular matrix, laying down collagen fibers adjacent to the site of fracture (Schindeler et al., 2008). Within the extracellular matrix, aggrecan, a major structural proteoglycan specific to cartilage, is secreted and expressed by chondrocytes, providing some stability and scaffolding for endochondral bone formation. Disruption in aggrecan levels will disrupt chondrocyte differentiation and the following endochondral ossification processes at the fracture site (Zhai, 2021). Aggrecan expression is relatively highest expressed from day 5-7 and studies have shown in aggrecan knockout mice, levels were low from day 5 to 14, indicating that it is essential for chondrogenic processes (Kawakatsu, et al., 2011). Towards the end of this stage, chondrocytes hypertrophy and leaves behind a partially mineralized cartilaginous matrix while releasing various mediators before they undergo programmed cell death; this process is analogous to the function of a growth plate (Loi et al., 2016). One of the signals that demonstrates this chondrocyte to osteoblast transformation is SOX2, a vital transcription factor expressed within the nuclei of hypertrophic chondrocytes surrounding the transition zone. SOX2 plays a vital functional role in bone repair due to its pluripotency and self-renewal capacity, able to reprogram mature somatic cells into a stem-cell like state, and converting chondrogenic to osteogenic genotypes. In previous research, elimination of SOX2 resulted in smaller total callus size, bone density, and volume (Hu et al., 2017). Neural cell adhesion molecule (NCAM) is a glycoprotein that also plays a critical role in cell multidirectional differentiation and provides a functional role in chondrogenesis. NCAM is expressed in both MSCs and prechondrogenic cells but not in hypertrophic chondrocytes, suggesting that high NCAM levels could inhibit chondrocyte hypertrophic differentiation. Therefore, NCAM deficiency would enhance chondrocyte hypertrophy and therefore enhance rates of mineralization (Cheng et al., 2020).

For bone repair to progress, the primary soft callus must be resorbed and replaced by a usually irregular bony callus, increasing total bone volume and matrix deposition. Osteoprogenitor cells migrate and proliferate into mature osteoblasts, representing the most dynamic phase of osteogenesis (Schindeler et al., 2018). As the cartilaginous callus undergoes endochondral ossification, signaling mechanisms involving RANK and RANKL stimulate the further differentiation of osteoblasts, osteoclasts and chondrocytes, causing the calcification of the callus. Below the periosteum, woven bone begins to be laid down, angiogenesis progresses, allowing for the re-establishment of vasculature and for the migration of MSCs (Sheen et al., 2023). A cytokine present in the circulation and nervous system involved in the osteoblast differentiation process is ciliary neurotrophic factor (CNTF). It is detected locally on bone forming surfaces as well as in hypertrophic chondrocytes and believed to inhibit osteoblastic activity and remineralization (McGregor et al., 2010). Osteocalcin, also known as bone gamma-carboxyglutamic acid (BGLAP), is also involved during remineralization as the most abundant non-cartilaginous protein of bone extracellular matrix. BGLAP is first synthesized as pre-pro-osteocalcin before cleavage and post-translational modifications occur. Post-carboxylation, osteoblasts release BGLAP and is deposited into bone matrix. This causes the recruitment, maturation and relocation of pre-osteoclasts (Li et al., 2016). Osteocalcin has been shown to be involved with the early stages of bone repair, crucial in regulating osteoblastic activity as it is distributed in the ECM of both cortical and trabecular bone (Rammelt et al., 2005). During this period, cytokine levels including IL-6, are downregulated and its expressions are absent (Gerstenfeld et al., 2003).

Secondary bone formation represents the final stage of the four-stage model of bone repair, the remodeling of woven bone into cortical and/or trabecular bone (Schindeler et al., 2008). The bone resorption occurring in this phase differs from primary bone formation as IL-6 levels and other cytokines are expected to show increased expressions again (Gerstenfeld et al., 2003). Osteoclasts, as the major cell type responsible for bone resorption, and osteoblasts, the main cell type involved in bone deposition, must provide coordinated and regulated balance between the catabolic and anabolic states during the months to years long bone remodeling process (Schindeler et al., 2008). The coupled osteoblastic-osteoclastic activity eventually causes the borders of the hard callus to become restored by lamellar bone while the center is restored with cortical bone, resulting in the subsequent regeneration of normal pre-injury bone structures (Sheen at al., 2023).

Bone Pain and Bone Repair Mechanisms are Controlled by the Nervous System

The peripheral nervous system (PNS) and bone interact via innervated axons, neurotrophins, other signaling molecules, and resident bone cells. As pictured in figure 2, the PNS serves as a linkage between the central nervous system, the periosteum, cortical bone, and the bone marrow space of the bone. Nerves are often divided into two types: sensory and sympathetic nerves and they translate peripheral information to the CNS (Liu et al., 2023).

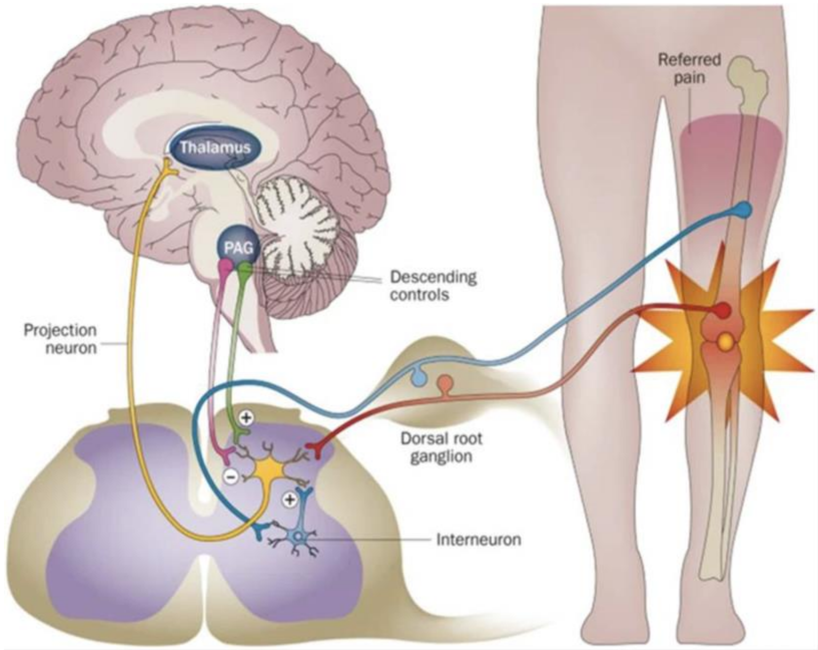

Figure 2. Relationship between PNS, CNS and Bone. The schematic relationship between bone and the nervous systems is demonstrated in this figure. The PNS serves as a relay station, transmitting information such as pain, between the CNS and bone. Figure taken from Liu et al., 2023, reprinted from Springer Nature.

The main function of the nerves within bone itself is largely related to perceiving pain in which the type of pain depends on the type, intensity, and location of the bone injury (Liu et al., 2023). Alongside the spinal cord, pseudo-unipolar primary sensory neurons in bone houses their cell bodies in the dorsal root ganglia (DRG) in which the central projection bifurcates into the brain and peripheral projection into the bone, connecting the peripheral receptive fields to the dorsal horn for processing of pain (Brazill et al., 2019). Small, myelinated or unmyelinated neurons with free nerve endings are the major type of primary afferent neurons of the DRG innervating the bone marrow and periosteum, and a few larger neurons with confined nerve endings are also found in the periosteum (Nencini et al., 2016). The periosteum contains the highest density of nerve fibers with the same abundance of Aδ- fibers (myelinated, small diameter) and C-fibers (unmyelinated) organized in bundles or branches of single nerve fibers. The stimulation of Aδ- fibers often presents a well-localized, sharp pain sensation while the stimulation of C-fibers often results in a dull, well-dispersed pain (Steverink et al., 2021). The varying perceptions of pain support the hypothesized correlation between innervation density and pain intensity encountered upon different types of bone traumas (Steverink et al., 2021).

Upon immediate bone fracture, mechanosensitive Aδ and C-fibers innervating bone are mechanically distorted, resulting in swift communications and signaling of the initial sharp pain to the spinal cord and brain (Mitchell et al., 2019). Under normal conditions, both Aδ and C-fibers are silent; they are only activated when exposed to a noxious stimulus which then becomes sensitized upon inflammation, the first stage of bone fracture repair (Kendroud et al., 2022). Not only do afferent sensory neurons become sensitized but the brain also undergoes sensitization via a phenomenon known as central sensitization (Woolf, 2010). Upon peripheral nerve injuries, spinal cord neurons amplify its neural signaling eliciting pain hypersensitivity, such that allodynia, a reduction in threshold of previously innocuous stimuli and/or hyperalgesia, an increased sensitivity to noxious stimuli, may take place (Woolf, 2010). Nerve growth factor (NGF) is fundamental for the differentiation and survival of sensory and sympathetic neurons innervating bone. In response to injury, NGF will activate TrkA (tropomyosin receptor kinase A) in which an assortment of downstream channels, receptors and neurotransmitters expressed become sensitized or upregulated (Mitchell et al., 2019). In bone tissue, high densities of TrkA-positive fibers are located within the periosteum, bone marrow and mineralized bone, exhibiting a longitudinal or branching pattern enwrapping blood vessels, driving skeletal pain. If signaling through the NGF/TrkA pathway is disrupted, skeletal pain may be attenuated and impaired fracture healing and improper regeneration of sensory innervation results (Li et al., 2019). Because NGF is secreted by several types of cells including Schwann cells, fibroblasts, and immune cells, they all can factor in to hyperreactive pain in damaged peripheral nerves (Lim et al., 2021).

Ectopic nerve sprouting is another mechanism possibly involved with bone pain. Mechanosensory fibers innervating bone become mechanically injured and stromal and/or inflammatory cells release neurotrophic factors, including NGF (Mitchell et al., 2019). This results in excessive innervation of the bone marrow, periosteum, and mineralized bone, impelling the sensation of pain, as pictured in figure 3 (Mitchell et al., 2019). These neurotrophic factors can induce ectopic nerve sprouting in poorly innervated bone regions (mineralized bone), as well as sensitize newly sprouting nerve fibers such that normal movements of the fractured bone are hypersensitized to be a highly distressing event (Mitchell et al., 2019). Because ectopic sprouting generally occurs in the callus of fracture sites with normal bone healing, NGF levels are expected to taper off and sprouted fibers are to be “pruned” back to its normal, non-sensitized state (Mitchell et al., 2019). In fractures that do not completely heal, ectopic nerve sprouting is detected in the normally homogeneous marrow space and will induce significant pain (Chartier et al., 2014).

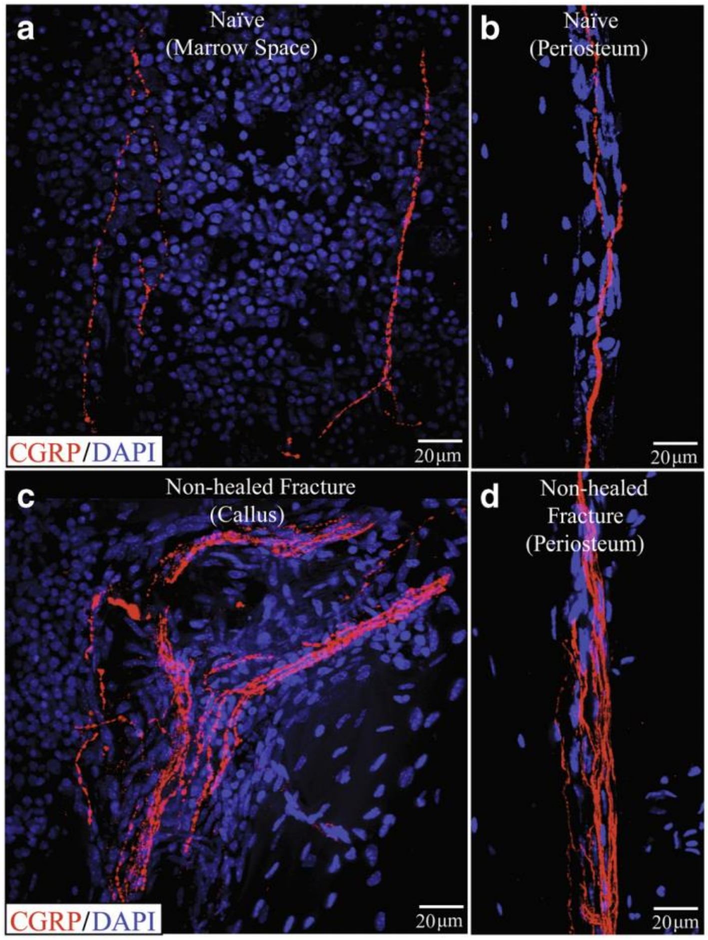

Figure 3. Ectopic sprouting of sensory nerves in a non-healed fracture callus and periosteal compared to a normal femur. Calcitonin gene-related peptide (CGRP) is labeled red demarcating sensory nerve fibers. **(a/b)** Newly formed sensory fibers located in the marrow and periosteum of non-injured femur. **(c/d)** Fracture callus and periosteum show the expansion and hyperinnervation during fracture repair. Figure obtained from Mitchell et al., 2019

Schwann Cell Precursors and its Multipotent Capacities

Unlike central nerve injuries, peripheral nerve injuries (PNIs) are capable of effectively regenerating their functions. The major glial cell type in the PNS, Schwann cells (SCs), are extremely important in its interactions at the site of injury with other cell types during neuronal regeneration and repair (Tian, et al., 2021). Derived from neural-crest cells, Schwann cell precursors (SCPs) are multipotent and able to differentiate into both myelinating and non-myelinating (Remak) SCs but also have the capacity to differentiate into all lineages of neural crest cells (Solovieva and Bronner, 2021). Remak SCs are typically surrounding smaller axons as they do not form myelin sheaths, and myelinating SCs create myelin sheaths around single, larger axons. Disruption of normal myelination processes could potentially result to disproportionately higher fiber densities of axons associated with Remak bundles, leading to nontypical hypersensitivity to pain. (Fornaro, et al., 2021). A common regulator of peripheral glial cell differentiation is transcription factor SOX10 as its expression is initiated in neural crest cells and remains throughout its migration. However, only in glial and melanocyte lineages does its expression remain present in the later stages. In SOX10 mutants, a lack of peripheral glial cells led to the degeneration of sensory and sympathetic neurons and SCPs are not generated (Britsch et al., 2001). Proteolipid protein (PLP) on the other hand, is a marker that shows some expression in neural crest cells, but its expressions are highest in mature myelinating SCs (Huang et al., 2020). Due to its hydrophobic nature, it helps insulate the axons longitudinally and contributes to myelin stability (Kister and Kister, 2023). Because SCs are in essentially all tissues due to their associations with tissue innervation, they also express an assortment of CNS myelin proteins, including myelin-oligodendrocyte glycoprotein (MOG). MOG has been primarily constrained to the external surfaces of myelin sheaths and oligodendrocytes however, studies have suggested there is a widespread distribution of low levels of MOG, suggesting that it may be used as a marker for Schwann cells (Pagany et al., 2003).

Upon a peripheral nerve injury, the injured neurons and SCs distal to the injury undergo substantial changes in its differentiation states, allowing for the shift in functions from cell-cell signaling to axonal regeneration and myelin regeneration, as pictured in figure 4. Both non myelinating and myelinating SCs change phenotypes, adopting a new function as repair SCs (Fornaro et al., 2021). Numerous transcriptional regulators, signaling pathways, and epigenetic mechanisms govern these processes and SCs remain in the spotlight continuously throughout the entire duration of healing (Nocera and Jacob, 2020). Through the upregulation of neurotrophic factors and downregulation of myelin-related genes throughout different stages of the PNI repair process attributes to the idea that SCs can both induce pain and relieve pain, depending on the progression of nerve repair and the microenvironment created surrounding the fracture site (Zhang et al., 2023).

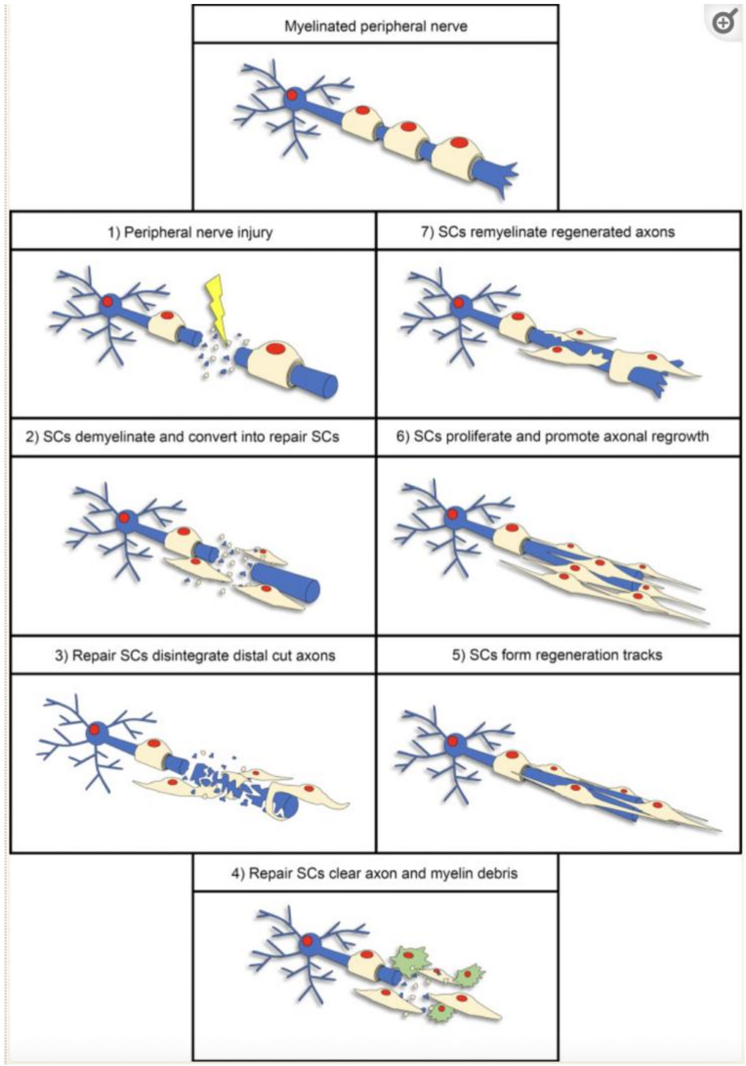

Figure 4. Major events of the PNI repair program coordinated by Schwann Cells. This figure illustrates the principal steps of repair in the PNS, orchestrated by SCs following a PNI. A single neuron (blue) interacts with SCs (yellow) within a peripheral nerve undergoing a traumatic lesion. In step 4, it is the role of macrophages (green) that help SCs clear axon and myelin debris. Figure taken from Nocera and Jacob, 2020.

Normal Nerve Regeneration and Repair Post-Fracture

In a process known as Wallerian degeneration, a cascade of molecular and cellular events distal to injured nerve fibers occur, including the disintegration of the blood-nerve barrier, expansion of SCs, recruitment of macrophages, and the reorganization of the endoneurial space takes place (Dubovy, 2011). Upon a traumatic injury to the peripheral nerve, axons lose functional connections to peripheral tissue. Molecular changes related to inflammation occur leading to modulations of the productions and secretions of neurotrophins and cytokines. Axonal cytoskeletal elements such as the axoplasm and axolemma actively degrade by means of an intrinsic self-destructive process activated by the calcium influx and activation of calpains (proteases) in SCs, which also causes an upregulation of cytokine release and stimulation of early SC proliferation. This is important for the accumulation of SC pool due to gradual apoptosis in the long term during the regeneration of nerves (Dubovy et al., 2013). CNTF is also released by SCs to help alert nerve damage and enhance the pain sensation via the initiation of the immune cascade, also inducing IL-6 production in sensory neurons via STAT3, an activating signal transducer (Hu et al., 2020). Proinflammatory cytokines including IL-6 released by SCs and macrophages promote neuritic outgrowth and NGF is also upregulated contributing to the preparations of axonal regeneration at the distal stump (Svennigsen and Dahlin, 2013). NGF provides guidance for axons, promoting axonal sprouting and stimulating the myelination processes by SCs (Shakhbazau et al., 2012) Following pro-inflammatory cytokine and chemokine secretions, monocytes are recruited from circulation into the distal branch of damaged axons and differentiate into macrophages. Matured macrophages and resident bone macrophages along with de-differentiated SCs, play a crucial role in phagocytosing myelin sheaths (ovoids) and axonal debris (Lim et al., 2021). A mediator that promotes the clearance of myelin debris is SOX2, due to its ability to recruit macrophages (Yuan et al., 2022). Because myelin debris contains molecules that inhibit axonal growth including myelin associated glycoprotein (MAG), causing the increase in local pressure at the fracture site impeding nerve repair, improper clearance may lead to delayed regeneration (Yuan et al., 2022). MAG has been shown to be a potent inhibitor of axonal regeneration from DRG neurons, specifically in adults, binding to neurons and inhibiting neuritic outgrowth (DeBellard, et al., 1996). AlphaB-crystallin (CRYAB), a heat shock protein provides some protective functions in preventing harmful effects of prolonged neuroinflammation. CRYAB suppresses the inflammatory response and helps manage the degree of macrophage recruitment to fracture site and therefore dampens hypersensitivity to painful stimuli in a controlled and timely manner (Lim et al., 2021). Due to the temporal regulation and coordination of demyelination and remyelination processes, the clearance of myelin debris is positively impacted by the Wallerian degeneration and inhibition of myelin regeneration by MAG (Yuan et al., 2022).

As axons begin to regenerate, SCs continue proliferating and migrating in alignment within the nerves, creating bands of Bungner, providing directional cues for the regrowth of axons (Svenningsen and Dahlin, 2013). In the bands of Bungner, SCs produce more growth promoting factors, including CNTF and NCAM. Endogenous CNTF released from SCs now directly provides axonal guidance and directional cues for proper reinnervation, especially when working in synergy with NGF. It has been shown through in vivo morphological studies that CNTF drastically potentiates axonal rejuvenation by promoting the elongation of axon tips into the distal stump (Sahenk et al., 1994). NCAM influences the assortment of axons and non-myelinating sensory

SCs, preferentially promoting the regeneration of sensory axons at the proximal and distal stumps (Thornton et al., 2008). CRYAB is crucial in the remyelination process as well, regulating the communication between regenerated axons and SCs. CRYAB is highly expressed in mature nerves, and it's suggested that it plays a role in regulating the conversion of dedifferentiated SCs back into a myelinating phenotype (Lim et al., 2016). The renewal of the axon-Schwann cell interaction then triggers the restoration of myelin and the physiological functions of fibers, also causing macrophages to exit the basal lamina once remyelination occurs (Gaudet et al., 2011). Interestingly, though Plp is specifically expressed in SCs, Plp1 does not seem to be involved with the myelin itself, as it is preferentially excluded from the SC membrane (Puckett et al., 1987). MAG, expressed on the periaxonal space on the innermost myelin membrane, is directly adjacent to the axon surface and therefore plays a role in stabilizing axon-myelin interactions. MAG is not necessarily required for remyelination, but it has been shown to regulate the axon cytoskeleton and helps establish nodes of Ranvier (Schnaar and Lopez, 2009). NCAM expression remains during the remyelination process, specifically at the periaxonal space, proposing that NCAM plays a role in the axon-SCs interactions as well. (Dolapchieva, et al., 2002).

The repair and regeneration of axons involved with a peripheral nerve injury is extremely complex and involves a large variety of cells, signaling molecules and targets. Throughout all stages of peripheral nerve repair, orchestrated events must occur under spatially and temporally precise circumstances for proper nerve regeneration and gain of function to occur. Pictured in figure 5, an overview of all steps of nerve repair is presented. Mutations in specific genes involved with nerve regeneration or improper progressions during any of the processes may lead to disease or delayed healing.

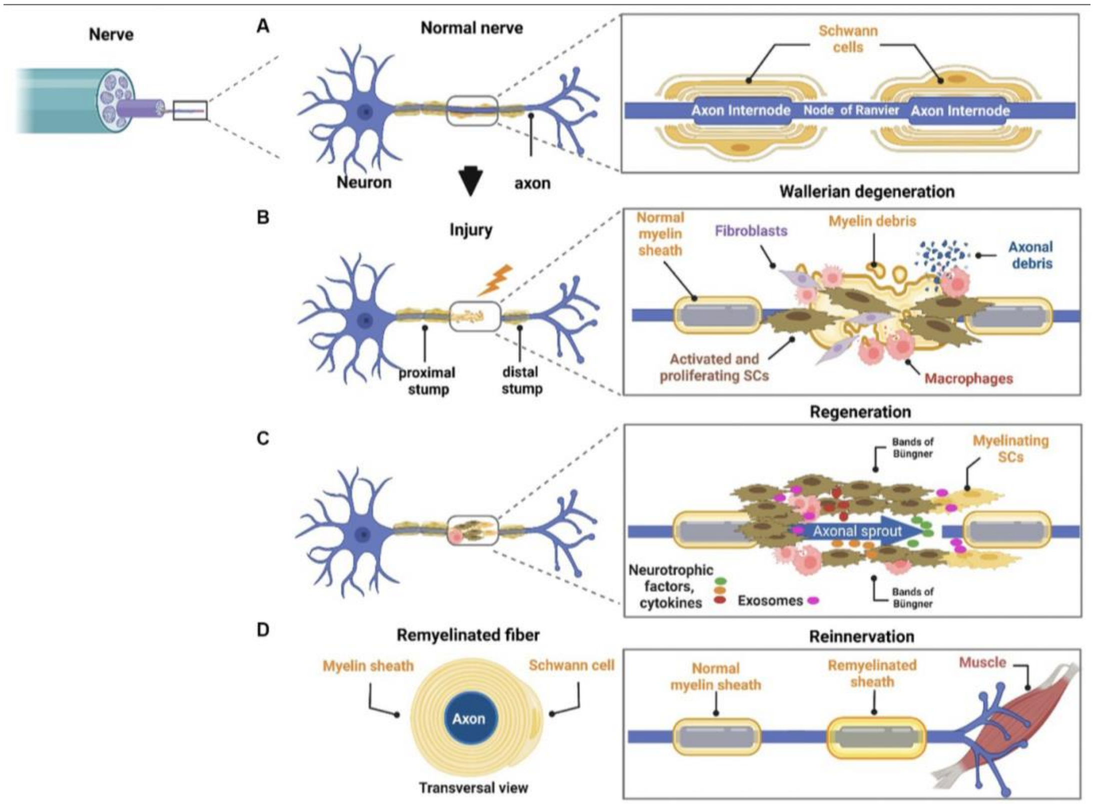

Figure 5. Role of Nerves and Schwann Cells in Peripheral Nerve Regeneration. Axons and SCs, responsible for the formation of peripheral nerves, are necessary for neurogenic homeostasis and regeneration. **(A)** Myelinating SCs support and engulf large axons. **(B)** The dedifferentiation and proliferation of SCs marks the location for axonal degradation related debris clearance by macrophages. **(C)** Reinnervation of the target organ occurs in response to neurotrophic factors, cytokines, and exosomes secreted by activated SCs associated into bands of Bunger during the remyelination process. **(D)** Reinnervation of the target organ. Figure taken from Oliveira et al., 2023.

Current Treatments for Bone Pain

Although skeletal injuries are one of the leading causes of chronic pain globally, we currently have few therapeutics that can diminish this pain without significant side effects that potentially could negatively affect individuals' lifestyles. For example, the common use of nonsteroidal anti-inflammatory drugs (NSAIDs) and opiates have proven to be useful in attenuating acute pain however, it becomes problematic with long term use (Chartier et al., 2014). The use of NSAIDs have proven to inhibit or decrease cyclooxygenase (COX) activity, important in the initial steps of the production of prostaglandins, leading to delayed bone healing (Gerstenfeld, et al., 2003). Similarly, opioid analgesics seems to be affiliated with an increased risk of repeating fractures and impaired bone healing, specifically affecting the differentiation and maturation of osteoclasts, reducing bone resorption (Coluzzi et al., 2020). To establish treatment regimens without off-target interference into the bone repair process, deeper insights into the pain mechanisms underlying fracture repair and neuroregeneration are essential for the establishment of treatment regimens without off-target interference into the bone repair process.

EXPERIMENTAL AIMS AND GOALS

The focus of this study was on the molecular mechanisms of skeletal cell development along with the role of Schwann cell precursor cells in the bone fracture repair processes following injury using specific neuronal biomarkers. Under normal conditions, fractures are expected to fully regenerate itself to its original structure and function. Nociceptive pain should attenuate with successful completions of specific stages of healing as the cortical bone, bone marrow or the periosteum undergo cellular changes. In previous research studying fracture callus transcriptomes, they have shown that genes associated with neurogenesis were the most prevalently expressed gene ontologies in callus tissues and were expressed earlier than those associated with skeletogenesis. The pain intensity during the initial periods of fracture healing is most closely linked with endochondral bone formation and subsequent pain resolution demonstrates signs of clinical healing. If pain remains unsolved, it is a sign of delayed healing.

For this study, we hypothesize that the expansion of Schwann Cell precursors is related to injury-induced nerve sprouting associated with the hypersensitivity of pain but that it is transient and regresses as bone heals and repairs. To test this hypothesis, mRNA expression, fracture callus structures, and cartilage and cellular contents were measured and observed. We then assessed and monitored SCPs and nerve sprouting by tracking the lineages of SCPs along with the osteogenic and chondrogenic processes during bone repair using COL-1, CNTF and SOX10 immunohistochemical analyses. The high plasticity demonstrated in SCPs and extensive research on their functions in neuroregeneration through their mediations of inflammatory, neurogenic, and neuropathic signaling processes, leads to the expectations to find that skeletal tissue injury will stimulate SCPs expansion, support injury-induced sprouting of peripheral nerves, initiating and sustaining dorsal root ganglion activation necessary for the development of chronic neuropathic pain. SCPs expansion will transiently occur during the early periods when pain is maximal and during new bone formation. We expect these cells to disappear with regression of the sprouted nerves when bone healing is completed. If validated, it will further show that SCPs are a major cell type in mediating skeletal pain, which can then further research can be exploited in the development of new therapies for pain management. New mechanism-based analgesics to relieve bone pain are necessary but, further understandings of the pathophysiology of bone pain are needed.

METHODS

cDNA Generation and qPCR

A collection of RNA samples from murine mice were obtained, ranging from day 0 (control) to day 35. A total of 22 samples were used, ranging from day 0, 3, 5, 7, 10, 14, 18, 20, and 35. To first quantify the concentrations of each sample, a Thermo Fisher NanoDrop Spectrophotometer was used, assessing the OD 260 of each oligonucleotide. An absorbance reading of 1.0 at 260 nm in 1.0 nL of solution using a 1 cm light path was used to quantify each oligonucleotide to obtain values with a concentration unit of ng/µl. Taking the inverse of each RNA concentration given in ng/µl provided us with volume of RNA to be used in µl for each sample. All RNA samples were then diluted in a 0.2 mL Eppendorf tube to a total of 10.4 µl using specific volumes of RNAse-free water, to give a total RNA concentration of 96 ng/µl. A reverse transcriptase reaction was performed using Taqman Reverse Transcription Kit (Applied Biosystems Cat# N8080234) which consisted of the following reagents: MgCl2, dNTP Mix, Random Hexamers and 10XRT Buffer. All reagents were pipetted in specific ratios and vortexed. RNase inhibitor and Taqman Reverse Transcriptase enzymes were then added to the reagents mix. 19.6 µl of the RT-PCR reagents and enzymes mix was added to each of the 10.4 µl of RNA for a final volume of 30 µl. Each sample was carefully mixed and vortexed before being placed into a thermal cycler to run the PCR cycle. The following set up was used for the PCR cycling: 25° C for 10 minutes, 37° C for 60 minutes, 95° C for 5 minutes, and held at 4° C. A normalized cDNA concentration of 33.28 ng/µl was obtained for each sample. Each cDNA sample was then diluted in a 1:50 or 1:5 ratio depending on specific gene expression levels, using RNase free water and stored at -20° C. Samples diluted 1:50 gave a cDNA concentration of about 0.66 ng/µl and samples diluted 1:5 gave a cDNA concentration of 6.66 ng/µl.

Quantitative real time polymerase chain reaction (qPCR) was performed using Taqman Universal Master Mix (Applied Biosystems Cat# 4304437). Each primer was carefully added in a 1:10 ratio of primer of interest:TaqMan. Loading a 96-well qPCR plate on ice, 9µl of the diluted cDNA and 11µl of the primer/master mix was added to each well for a total of 20µl per well. The loaded 96-well plate was covered using a clear film (Applied Biosystems Cat# 4306311) then centrifuged at 1500 rpm for 2 minutes. The qPCR reaction was set to: 50°C/2 minutes, 95°C/ 10 minutes, 95°C/15 seconds, 60°C/1 minute, repeated 40x. Each sample was performed in duplicates, then normalized to 18S rRNA (housekeeping gene) of the same samples. Each Ct value of a sample set was averaged then normalized to the 18S by subtracting the averaged normalization value from the sample value, providing the relative expression (ΔCt). To calculate ΔΔCt, the fold expression, the normalized control (day 0 samples) was subtracted from the samples and unlogged in Excel using (=2^(-ddCT). Two samples of RNA per time point was averaged and used to obtain the ΔCt and ΔΔCt values with an exception to day 10 and day 20 samples where one sample of RNA was used.

Safranin-O Slide Staining

A collection of femoral fracture calluses was obtained ranging from day 10 to 21. To prepare for staining, slides were placed on a warmer with a cover for at least 1 hour and at most 24 hours before staining. They were then pressed with a wet bibulous paper and roller, allowing it to dry for another half hour without the warmer cover. All slides were placed on a universal slide rack for the dipping procedures. To first deparaffinize the slides, slides were immersed into a solution of xylene three times, for ten minutes each. Then, rehydrating the slides, slides were dipped into wash baths in a descending series of ethanol: 100% ethanol for 5 minutes, 100% ethanol for 2 minutes, then into a solution of 95% ethanol for 2 minutes, then 75% ethanol for another 2 minutes, and then double distilled water for 5 minutes before blotting onto a paper towel. To stain the slides, the following solutions were used in the order of: Gill No. 2 Hematoxylin for 1 minute, dipped about four times into double distilled water or until the water cleared, then blotted on a paper towel, Fast green (0.25g/500mL ddH_2O) for 8 minutes, 1% acetic acid for about 3-4 dips, then Safranin-O (0.1% in dH_2O) for 10 minutes. To dehydrate the slides, an ascending series of ethanol was used: 95% ethanol two times for about 4-5 dips each time. Then dipped 4-5 times into 100% ethanol before being submerged into 100% ethanol for 5 minutes. Next, slides were dipped into xylene for two rounds, for two minutes each. Finally, to mount the slides, they were individually air-dried then a glass coverslip was applied using Fisher Chemical ™ Permount ™ Mounting Medium.

Picro-Sirius Red Staining

Similarly prepped as the Safranin-O staining protocol, a collection of femoral fracture calluses was obtained ranging from day 10 to 21. Slides were placed on a warmer with a cover for at least 1 hour and at most 24 hours before staining. They were then pressed with a wet bibulous paper and roller, allowing it to dry for another half hour without the warmer cover. All slides were placed on a universal slide rack for the dipping procedures. Similar to the Safranin-O procedures, to first deparaffinize the slides, they were immersed into a solution of xylene three times, for ten minutes each. Then, rehydrating the slides, slides were dipped into wash baths in a descending series of ethanol: 100% ethanol for 5 minutes, 100% ethanol for 2 minutes, then into a solution of 95% ethanol for 2 minutes, then 75% ethanol for another 2 minutes, and then double distilled water for 5 minutes before blotting onto a paper towel. A Picro-Sirius Red Stain Kit (Connective Tissue Stain) ab150681 was used to effectively stain the slides. An adequate amount of Picro-Sirius red solution to completely cover the tissue sections was applied and incubated for 60 minutes. Next, slides were rinsed in two changes of Acetic Acid solution (0.5%) before being rinsed in absolute alcohol for 5 minutes. Following, all slides were dehydrated and incubated in two changes of absolute ethanol for 5 minutes each. Lastly, slides were individually air-dried and then mounted using a glass coverslip sealed using Fisher Chemical ™ Permount ™ Mounting Medium.

DAB Staining Immunohistochemistry

For the following immunohistochemistry protocols, a Vectastain® Elite® ABC-HRP Kit was obtained and utilized. All Reagents were ready-to-use and no dilutions were necessary. The kit consisted of Normal Horse Serum, 2.5%, Biotinylated Universal Antibody, R.T.U (Horse Anti-Mouse/Rabbit IgG), and Vectastain Elite ABC Reagent, R.T.U. All were stored at 2-8 ℃.

Immunohistochemistry for Identification of Collagen Type 1, SOX10, and CNTF

Paraffin sections of femoral fracture calluses were obtained, ranging from day 10, 14 and 21. All slides were first deparaffinized and hydrated using a series of xylenes and a graded series of ethanol solutions before being washed in a solution of PBS. Antigen unmasking was performed using a Tris-EDTA buffer (10mM Tris base, 1mM EDTA solution, 0.05%, 0.05% Tween 20, pH 9.0) for both Collagen Type 1 and SOX10. Antigens were retrieved using a sodium citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for CNTF. The appropriate buffer solution was heated to 95℃ before slides were incubated until it reached room temperature. Next, slides were incubated in BLOXALL ® Blocking Solution (SP-6000) for 10 minutes before washing in PBS for 5 minutes. Next, slides were incubated with Normal Horse Serum, 2.5% for 20 minutes. The serum was then tipped off from sections before being incubated with a primary antibody diluted in PBS containing 1.5% blocking serum for 30 minutes. All slides were incubated in a 1:50 or 1:100 dilution of antibody to PBS containing 1.5% blocking serum. Slides were then washed for 5 minutes in PBS. Following that, slides were incubated for 30 minutes with Biotinylated Universal Antibody, then washed again in PBS for 5 minutes. The slides were next incubated with R.T.U. VECTASTAIN Elite ABC Reagent for 30 minutes and washed with PBS for 5 minutes. Finally, they were incubated in a peroxidase substrate solution using a ratio of 1 mL of ImmPACT® DAB diluent to 1 drop (~30 μL) of ImmPACT® DAB Reagent 1 until the desired stain intensity developed. Both the diluent and reagent were obtained from the ImmPACT ® DAB Substrate Kit, Peroxidase from Vector Laboratories. After a rinse in tap water, a counterstain was performed using hematoxylin. Lastly, slides were dried and cleared, then mounted using a glass coverslip sealed using Fisher Chemical ™ Permount ™ Mounting Medium.

Imaging

All sections stained with hematoxylin, Safranin-O, and DAB were imaged with an Olympus BX51 light microscope, attached to an Olympus DP70 digital CCD camera at either 1.25x, 4x, 10x, and/or 20x. Safranin-O slides were imaged using the brightfield microscopy filter attachment. The software cellSens Dimension, Olympus Corp. was used to download all images.

RESULTS

qPCR Analysis: Chondrogenesis and Osteogenesis

Specific genes were chosen for analysis, based on their role in chondrogenesis and/or osteogenesis during the fracture repair process. Genes under investigation involved in chondrogenesis were Aggrecan (ACAN) and bone gamma-carboxyglutamate protein (BGLAP) and neuronal cell adhesion molecule (NCAM). Figure 6 illustrates the relative fold change in expression for a group of mRNAs expressed during the chondrocyte and osteoblast differentiation processes. Results were normalized to unfractured B6 control fracture calluses (day 0) and gene expression profiles are presented in temporal order of the progression of chondrocyte differentiation and osteogenic processes.

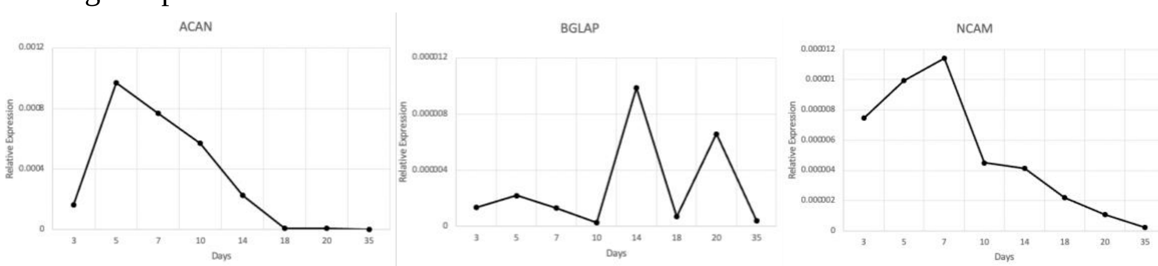

Figure 6. qPCR Analysis: Chondrogenesis and Osteogenesis. These graphs show the temporal gene expression profiles of three genes (ACAN, BGLAP & NCAM), associated with chondrogenesis processes (ACAN/NCAM) and osteogenesis processes (BGLAP) occurring during femoral fracture repair.

The specific expressions of marker genes associated with the chondrogenesis, and osteogenesis process were used in following the progression of fracture healing. The ACAN gene is responsible for the production of aggrecan, a structural proteoglycan found specifically in cartilage (Doege, et al., 1991). As expected, the expression of ACAN peaked at day 5, early during the fracture repair processes as chondrogenesis and the cartilage formation occurs before new bone formation and lay down occurs. Aggrecan is expressed by chondrocytes and plays an important mediating role in chondrocyte-chondrocyte and chondrocyte-matrix interactions due to its ability to bind hyaluronan, forming the soft callus (Kiani et al., 2002). Its expression decreases temporally whereby day 18, its expression was about 100-fold less than day 5 during its peak, by which then increasing amounts of collagen has already been remodified into bone. BGLAP, a protein secreted by mature osteoblasts and an indicator for bone function, is highly expressed during the bone mineralization process. Due to its role in regulating new bone formation and resorption via interactions with Vitamin D and influencing the differentiation of osteoblasts, it is expected that it peaks later at around day 14 and day 20 of the fracture repair process. Increased levels of BGLAP in circulation has been thought to be associated with increased states of bone turnover, and thus BGLAP may represent a marker for fracture bone repair (Kerner et al., 1989). It is likely that the first peak and highest expression at day 14 is correlated with the bony callus formation and the smaller peak at day 20, to be associated with the bone remodeling phase. Similarly, to ACAN, its expression was strongly downregulated by day 35. Neural cell adhesion molecule (NCAM) has been shown to be strongly expressed in most osteoblasts in bone, which coincides with the presence of collagen I activity. Its expression has been shown to be transient, such that it is not expressed in MSCs before osteogenic condensation and is lost in late osteocytes (Lee and Chuong, 1992). Presuming its activity is closely related to ACAN and the presence of collagen, its peak expression fittingly occurs at around day 5-7 following fracture repair, analogous to the ACAN peak, and similarly to the BGLAP peak, it decreases at day 10. Its expression decreases temporally, however, it does not reach its minimum until day 35, unlike ACAN.

qpCR analysis: Pain-Associated and Neuronal Growth Markers

To examine the temporal effects of pain during fracture repair processes, expressions of specific genes including interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF), nerve growth factor (NGF), and Crystallin-alpha B (CRYAB) were assessed. Figure 7 shows the relative fold change in expression of genes involved with pain. All results were normalized to unfractured B6 control bones and profiles of gene expression are presented in a temporal order of the progression of the inflammatory processes during initial bone and neuronal repair.

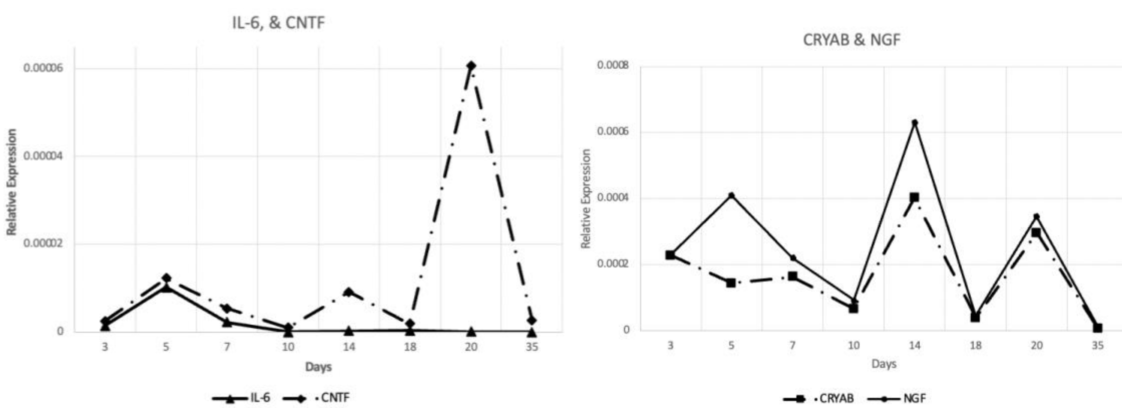

Figure 7. qPCR Analysis: Inflammatory and Neuronal Growth Processes. These graphs show the temporal gene expression of four genes (IL-6, CNTF, CRYAB & NGF) associated with the inflammatory and neuronal repair processes post fracture.

Bone fracture healing processes are postulated to be closely linked to the phases of neuroinflammation and nociceptive pain. Specific expression of marker genes such as IL-6, CNTF, NGF, and CRYAB are thought to be involved during the regeneration of axons and reinnervation of bone that occurs simultaneously to the bone repair processes. Previous studies suggest that fracture-induced pain-related behaviors will peak about 1-4 days post-fracture and return to baseline by day 21 (Mitchell et al., 2019). From previous studies, it has been suggested that IL-6 and its receptor are upregulated post nerve-injury, linked to microglia activation and directly influences the establishment of neuropathic pain (Sebba, 2021). The level of IL-6 is limited to the inflammatory phase, and therefore should remain at baseline in the remodeling phases of fracture healing, which is appropriately shown in figure 7 (Mountziaris and Mikos, 2008). Our data supports these findings such that its greatest expression occurred at day 5 post fracture, and returns to baseline quickly after, remaining low throughout the 35 days. Ciliary neurotrophic factor (CNTF), part of the IL-6 cytokine family, is expressed in osteoblast-like cells including proliferating chondrocytes, osteoblasts, osteocytes and osteoclasts (McGregor et al., 2010). Its expression in chondrocytes may explain for the small peak early on at day 5, during the soft callus formation and, its peaks at day 14 and 20 may be due to the upregulation of osteoblastic activity during bony callus formation and bone remodeling. Because the neuronal regeneration is closely associated to the bone repair and regeneration processes, CNTF is also expressed in the peripheral nervous system, released after nerve injury to promote neuronal regeneration. Perhaps an explanation for this phenomenon lies in the Schwann cells propensity for trans-differentiation into cell types that assist in the bone repair process. Unlike IL-6, CNTF does not have a signal peptide and is thought to be released from disrupted SCs such that damaged and dead neurons release CNTF, acting on viable neurons to promote its survival (Sango et al., 2007). Our peak present early in the neuronal repair system at day 5, may explain for such processes taking place. It has also been established that CNTF plays a crucial role in supplementing axonal regrowth and maturation post-PNI (Ma et al., 2021). This suggests in our studies, that the large peak present at day 20 represents the communications between axons and SCs during the remyelination processes. NGF, promotes bone healing by inducing nerve growth within the fracture callus and therefore is a mediator of inflammatory pain. In previous studies, NGF was shown to be upregulated between 3-7 days post-fracture, with increases in axonal ingrowth and sprouting, followed by vascularization and ossification (Qin et al., 2022). It also plays a role in the cartilage-to-bone transition during the healing process, as shown with the peak at day 5 correlating to the chondrogenesis processes and a larger peak at day 14, corresponding to its ability to promote osteoblast growth and differentiation and osteoclast formation (Sun et al., 2021). NGF and TrkA, its receptor that it activates on periosteal sensory nerves, has been shown to be found in fracture callus marrow stromal cells, osteoblasts, young osteocytes, new capillary endothelial cells, and periosteal osteoprogenitor cells, therefore capable of sensitizing nociceptive neurons and inducing pain. Its peak at day 20, during bone remodeling phase explains for the reinnervation of vascularized bone tissue. CRYAB, a heat shock protein, has several protective functions including anti-inflammatory, pro-survival, and remyelinating abilities. From previous research, CRYAB has been shown to be present in accompanying pro-inflammatory macrophages following nerve injury, reducing the production of cytokines, and decreasing inflammation after PNS injury (Lim et al., 2021). Because CRYAB helps lessen the hypersensitivity to painful stimuli rather than generating pain, it should and it does, peak after pro-inflammatory cytokine IL-6 peaks. It interestingly but not surprisingly decreases when IL-6 reaches its peak at day 5 as well. It's been suggested that CRYAB is expressed by both peripheral axons and SCs (Lim et al., 2017), and our data supports such findings such that when CRYAB peaks at day 14 and 20, during bony callus formation and bone remodeling respectively, its concurrently playing a role in the remyelination and pro-survival functions of the newly regenerated sensory neurons forming.

qPCR analysis: Schwann Cell Markers

The remaining genes studied are neuronal or Schwann cell related markers present during the bone fracture repair and remodeling processes. Figure 8 presents the assessments of the mRNA expressions for SRY-box 2 (SOX2), SRY-box 10 (SOX10), myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG), and proteolipid protein 1 (PLP1). The figure shows the relative fold change in expression of the genes related to Schwann cells. All fold changes were normalized to unfractured B6 control bones.

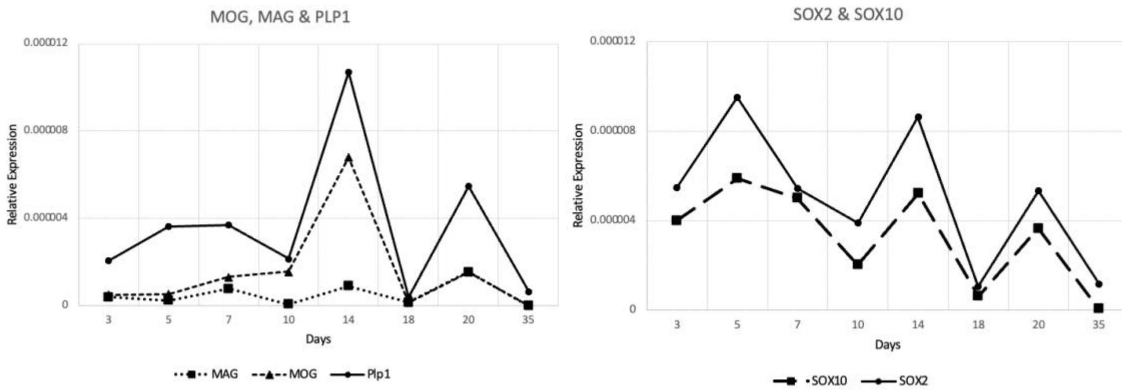

Figure 8. qPCR Analysis: Schwann Cell Markers. This graph shows the gene expressions of five Schwann cell related markers (MOG, MAG, PLP1, SOX2 & SOX10) associated with the neurogenetic processes during femoral fracture repair.

MOG, MAG and PLP1 have been established as Schwann cell biomarkers during tissue, specifically neuronal repair and regeneration. MAG is secreted by SCs and has been postulated to play an important role during remyelination processes. Its mRNA remained relatively low from the onset of the fracture until about day 7, most likely due to the early Wallerian degeneration processes of myelin debris clearance by macrophages. Because MAG acts as an inhibitor of axonal regeneration during this phenomenon, damaged tissue requires time to be cleared. Its expression was highest at day 14, when BGLAP, the bone matrix gene reached its peak as well, possibly due to the revascularization processes occurring, providing oxygen, nutrients and stems cells to the fracture site allowing for the differentiation of osteoblasts. It’s expression also peaked at day 20, similarly to BGLAP again, possibly due to the influx of ions necessary for remineralization during the remodeling phase, carried out by the new blood vessel formation (Stegen at al., 2015). MOG on the other hand, is expressed on the surface of myelin and studies has shown that its expression is typically 10-fold lower in the PNS than in the brain. However, in our study, we found higher levels of mRNA during the bone repair and remodeling processes, peaking at day 14 and day 20, respectively (Pagany et al., 2003). This may possibly contribute to the reinnervation and myelination processes occurring as neuronal regeneration occurs with the upregulation of Schwann cells at and surrounding the fracture site. Proteolipid protein-1 (PLP1), a major myelinating protein expressed primarily in SCPs also contributes to the preservation of peripheral axons. Its peaks are similar to both MAG and MOG as illustrated in figure 8, reaching its highest expression at day 14, and smaller peaks at day 7 and day 20. Its peak at around 5-7 can be speculated to be due to its involvement with the early stages of Wallerian degeneration, as SCs proliferate and expand. Its peak at day 20, is likely due to its role in the remyelination of axons, occurring later in the neuronal repair stage. From previous studies, SOX10 has been shown to play a critical role in the regulation of SC proliferation and myelination (Fujiwara et al., 2014). SOX10 is expressed from the neural crest cells to all phenotypes of SCs, and its early peak in our study may represent its role in regulating SC de-differentiation following peripheral nerve damage, transforming from SCPs to repair SCs to myelinating SCs. SOX10 has also been shown to be actively inducing the expressions of myelin genes, including MAG during myelination processes (Bremer et al., 2011). This may explain why its expressions in comparison to MAG, reached higher levels both before and at day 5, when MAG peaked as well. Its later peaks are likely to be due to its role in maintaining the functions of the myelin sheaths, at day 21, as SCs take on its myelinating phenotype. Reasons as to why its expression dropped at day 10, 18 and 35, may require further research or trials. Similarly, transcription factor SOX2, in response to initial nerve injury, it is involved with regulating Schwann cells undergoing phenotypic changes. Its expression is a marker of immature SCs and downregulated as they differentiate into mature phenotypes, promoting proliferation, suppressing de-differentiation (Balakrishnan et al., 2021), perhaps explaining for the lower expressions relative to SOX10 present at day 3. High expression and peak at day 5 may explain for the promotion of macrophages into the nerve, for myelin and axonal debris clearance during Wallerian degeneration. Its expression drops at day 18, likely to correlate with the beginning of the remyelination processes, due to the concept that sustained expression of SOX2 blocks myelination (Balakrishnan et al., 2021). Its overall relative expression prior to day 18 is high, most likely attributable to its role in suppressing the remyelination processes, before clearance, reconstruction and axonal regeneration are properly completed.

Safranin-O Staining Analyses

Safranin-O staining of the transverse sections of the fracture callus tissues at day 10, 14, and 21 were analyzed. The safranin-O staining procedure allowed for the quantification and identification of the acidic proteoglycans and glycosaminoglycans in cartilage tissue which stained red/pink, and other connective tissues including bone, to stain blue. Representative histological sections are shown in figure 9, providing information on important stages of the healing based on cellular and tissue responses. In increasing magnifications moving from left to right, and in increasing time (days), moving from top to bottom, are shown.

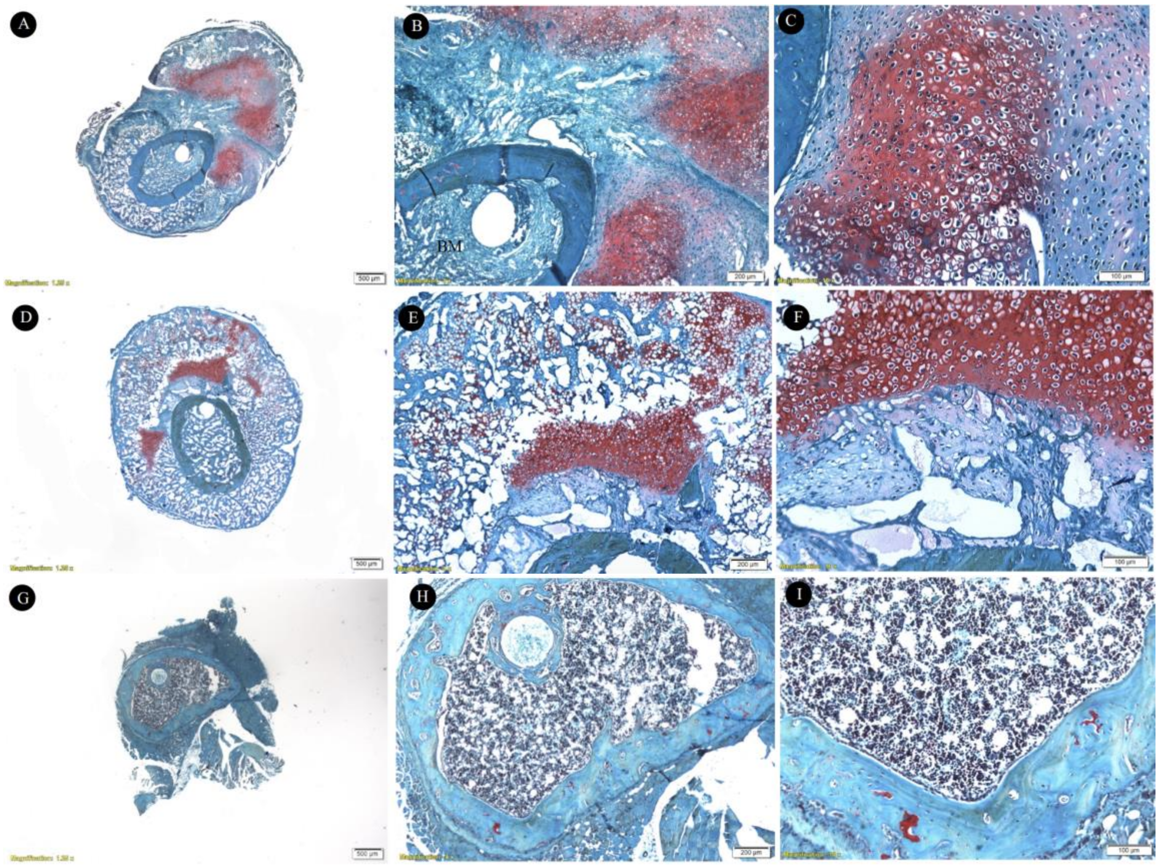

Figure 9. Safranin-O Histology Slides. A-C: Day 10: 1.25x, 4x, 10x respectively; **D-F:** Day 14: 1.25x, 4x, 10x respectively, **G-I:** Day 21: 1.25x, 4x, 10x respectively. The sizing of the scale bars in the 1.25x, 4x, and 10x imaging slides are of the following: 500µm, 200 µm, and 100 µm, respectively.

Comparing histology slides from day 10, 14 and 21, as expected, the quantity of cartilage tissue decreases, and the quantity of bone tissue increases with the progression of fracture healing. These findings are analogous to the findings from the qPCR results such that by day 20-21, we'd expect to see increasing osteoblastic activity and lay down of bone with the transition from cartilage to bone tissue. In comparison between day 14 and 21, day 10 images of the callus show the highest amount of cartilage to bone ratio with some chondrocyte hypertrophy and inflammation. Part of the reparative phase during fracture healing, a soft callus composed of fibrous and hyaline cartilage is visible, and a sizable red cartilage matrix is observed. Day 14, slides D-F, the appearance of chondrocyte hypertrophy is evident, with an increasing amount of mineralization of the cartilaginous matrix surrounding the bone. Increased amounts of cartilage have transformed into woven bone, and hypertrophied cartilage began to calcify into bone; a reduction of the cartilaginous matrix is observed. Day 21, represented by slides G-I, endochondral calcification continues to take place, and cartilage resorption endures. Almost no cartilage (red) is present as a ring of trabecular bone forms, representing the remodeling phases of bone regeneration. Cells within the bone marrow are strongly visible and an increased number of vessels are shown present in bone. Comparing the overall fracture callus size looking at the slides under 1.25 magnification, demonstrates that with increasing fracture repair progress, the callus size decreases.

Picro-Sirius Red Staining Analyses

Picro-Sirius red stained slides were viewed using standard light microscopy with a polarizing light filter, allowing for easier differentiation of collagen fibers from the background. With the use of a polarizing light filter, it is expected that type I collagen fibers display a yellow/orange birefringence, while type III collagen fibers display a green birefringence. Fracture calluses obtained from day 10, day 14, and day 21 post-fracture samples were observed.

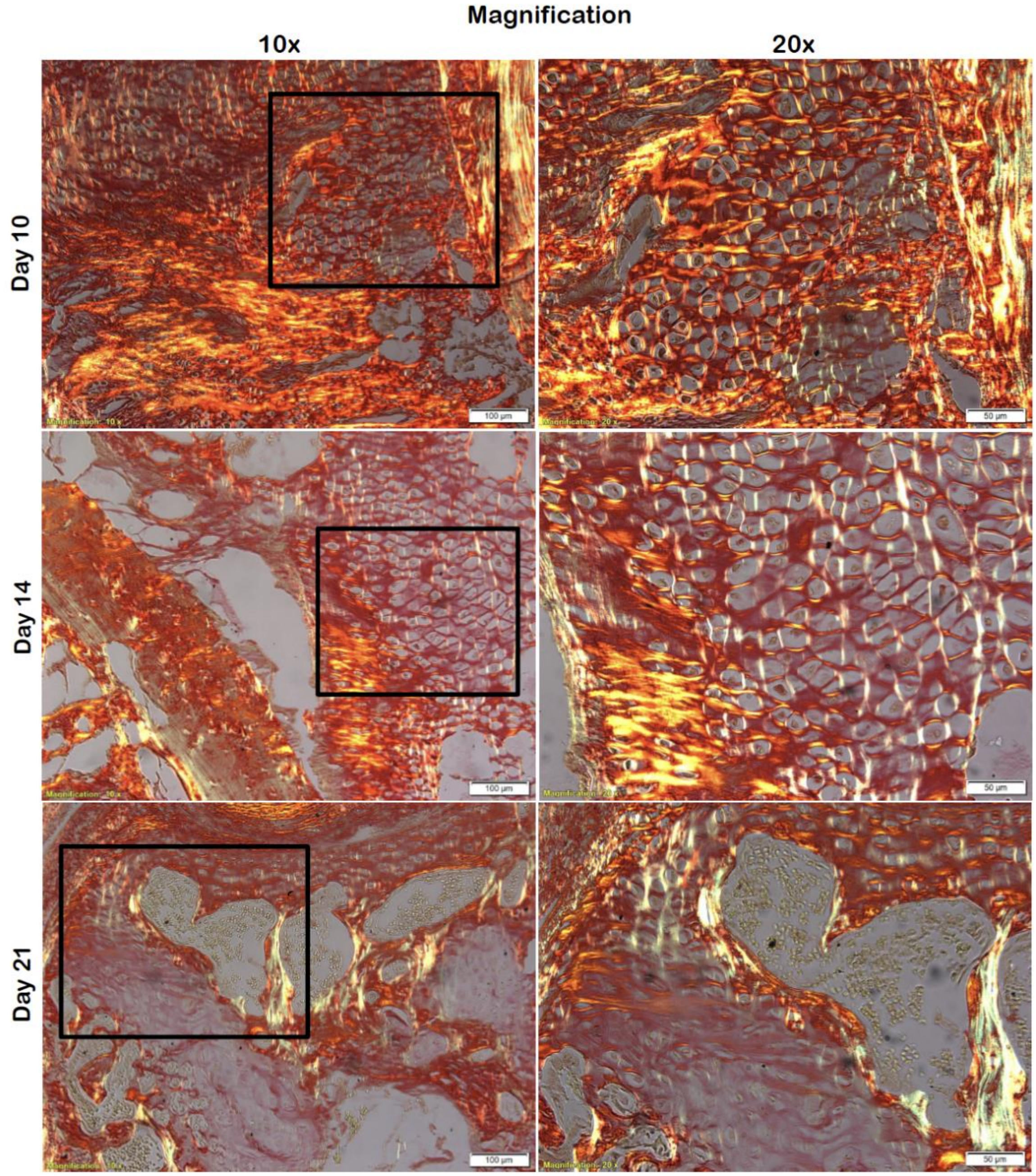

Figure 10. Picro-Sirius Red Stained Slides: top Day 10:10x, 20x respectively; **middle:** Day 14:10x, 20x respectively, **bottom:** Day 21:10x, 20x respectively. Red/orange birefringence represents type I collagen. Sizing of the scale bar of the 10x and 20x slides are: 100 μm and 50 μm, respectively.

Picrosirius red staining showed that the healing fracture callus express an abundant amount of type I and type III collagens, with decreasing amounts as time goes on. Because collagen type I is the predominant type of collagen present in bone, little to no type III collagen (green) was shown in these slides. In a temporal manner, the amount of fibrous tissue decreased, indicative of the fracture callus repairing processes occurring with the replacement of fibrous tissue to bone tissue. Shown in day 10 histology slides, copious amounts of collagen are present in a honey-comb like pattern, represented by the high intensity of birefringence present. Relative to day 14 and 21, the intensity of birefringence brightness was strongest at day 10. Relative to day 10, day 14 slides still demonstrated a strong presence of fibrous tissue at the fracture callus. However, its birefringence brightness was less intense, and its density are reduced with increasing time. The thickness of collagen fibers themselves also has decreased, as more white birefringence color is present in the day 14 samples, and a darker orange/red color is shown in the day 10 histological slides. At day 21, a substantial decrease in fibrous tissue is shown. With an increased amount of cartilaginous tissue replaced by bone, an increase in cellularity but a decrease in fibrous tissue is shown. Areas with brightest birefringence in day 21 slides were organized in layers concentric to blood vessels. These histological slides findings support the expected bone repair and remodeling framework.

Immunohistochemistry Analysis of Anti-Collagen Type 1, SOX-10, and CNTF

Deparaffinized tissue samples were stained with specific antibodies marking for proteins, collagen type I, SOX-10, and CNTF shown in figure 11. DAB staining was used, staining for the specific antibody of interest a brown coloring.

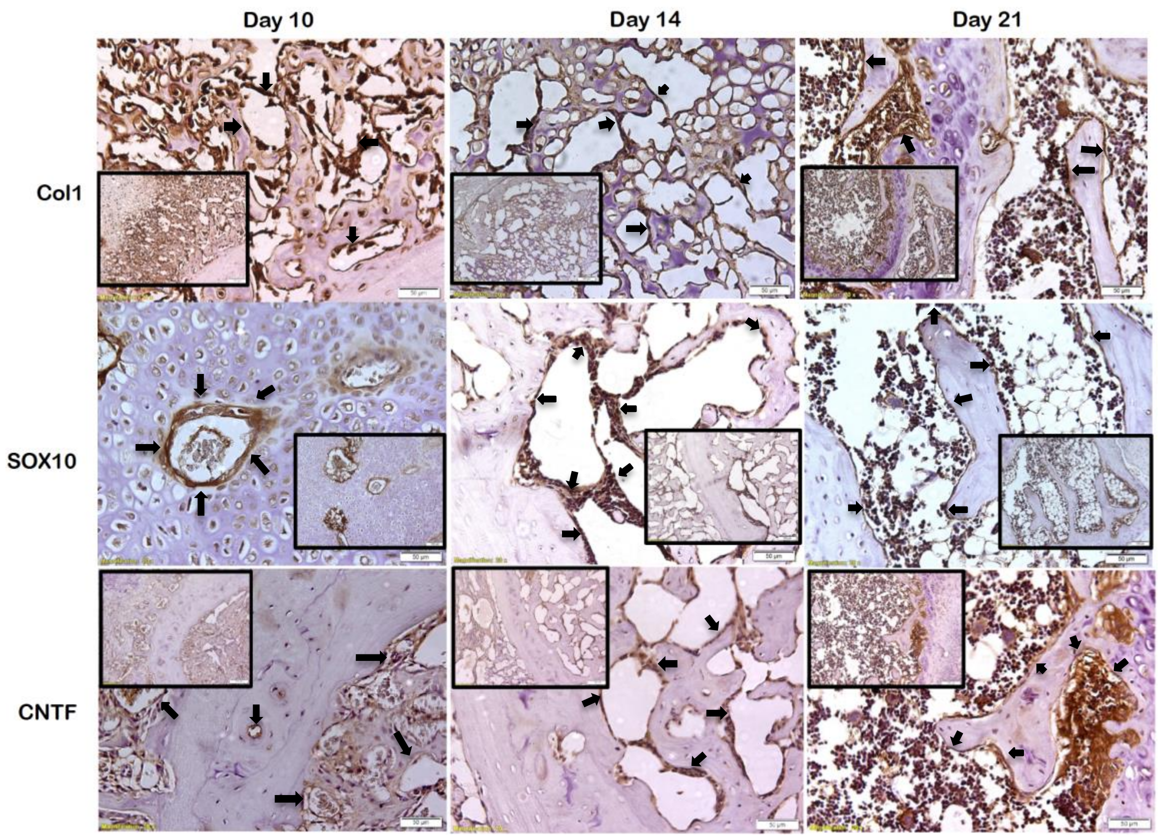

Figure 11. Immunohistochemical Slides, using primary antibodies targeting collagen type I, SOX-10, and CNTF. Slides were immunostained and counter-stained with hematoxylin. Magnification of 20x with a scale bar size μm of 50 μm is shown and a magnification of 10x with a scale bar size of 100 μm is represented by the image within the black outlined box. Black arrows indicate immune-positive staining.

Anti-Collagen Type 1 Immunohistochemistry Analysis

Collagen type 1 constitutes about 90% of the bone matrix and plays a crucial role in remineralization and therefore bone repair processes. The differentiation of osteoblasts and mineralization of MSCs is induced by collagen adhesion, specifically, type I collagen binding to bone-inducing growth factors (Yigiter, et al., 2019). Shown in the top row of figure 11, immunostained with anti-collagen type I histological sections demonstrate the presence of collagen lining blood vessel walls and other connective tissues. Though type I collagen is the most abundant collagen in the body, it is absent from hyaline cartilage. This is shown by the absence of antibody staining and presence of hematoxylin staining purple/pink, in hyaline cartilage areas. Areas where chondrocytes have apoptosed are lined and shown in the day 14 slide. Type I collagen is also present lining the bone marrow as shown in the day 21 slide. As expected, the predominantly fibrocartilage cores positively stained throughout for type I collagen.

Anti-SOX10 Immunohistochemistry Analysis

Anti-SOX10 antibody was used to localize the presence of osteoblasts and osteogenic cells during bone regeneration along with the presence of peripheral glial cells. With the process of angiogenesis that occurs almost simultaneously with the neuronal regeneration processes post fracture, we expect to see SOX10-positive staining lining blood vessels and the bone marrow space. The day 10 slide in the middle row of figure 11 may represent the presence of a new blood vessel formed, surrounding hypertrophic chondrocytes, undergoing apoptosis as cartilage begins to be replaced by bone. The day 14 slide appropriately demonstrates the presence of SOX10 present lining blood vessels in which they carry such proteins to support the growth and regeneration of new neurons. Lastly, day 21 slide illustrates the presence of SOX10 lining the bone marrow space and cells within the bone marrow space. Positive-stained cells within the bone marrow is likely to demonstrate the role of SOX10 in regulating SC differentiation and proliferation processes following peripheral nerve damage.

Anti-CNTF Immunohistochemistry Analysis

The bottom row of figure 11 represents immunohistological slides stained with an anti-CNTF. The primary source of CNTF are Schwann cells in the PNS in which it signals to the sensory neurons upon peripheral nerve damage. Similar to the concept of SOX10 being found lining blood vessels, CNTF-positive staining is found surrounding blood vessels while cartilage and bone were negative for CNTF and shown to be pink due to the counterstain with hematoxylin. In the slide representing day 10, a vessel is observable within the ring of bone and within the marrow space where an abundance of blood vessels is found. An increase in number and size of blood vessels are shown with progression of the fracture repair. Nutrients and oxygen from such blood vessels provide the necessary substances for the growth of new axons and neuronal connections post-fracture. The day 21 slide illustrates the bone marrow with increased cellularity and is likely to also contain a numerous amount of Schwann cells.

DISCUSSION

Study Findings

The crossover between neuronal regeneration with bone repair and remodeling has been shown that there is a strong overlap in the two processes supporting one another. Many factors have been identified to be involved with the bone healing processes however, little research has been done on how the same factors can promote peripheral nerve sprouting and if these are a cause of neuropathic pain associated with bone healing. The role of the peripheral nervous system in regulating fracture healing is required to understand the connections between fracture healing and pain. The goal of the study was to focus on the mechanisms of skeletal cell development and determine if SCPs contribute to these processes by using specific neuronal biomarkers. We hypothesized that the upregulation of Schwann Cell precursor cells is related to injury-induced nerve sprouting associated with the hypersensitivity of pain and that such neuropathic pain should regress with proper completion of healing.

Looking at mRNA expression profiles, the different peaks present specific to each gene demonstrates the overlap between bone repair and neuronal repair processes occurring almost simultaneously post-fracture. Looking at genes related to the early phases of bone fracture repair, as expected, the ACAN gene peaked early, associated with the production of aggrecan and the formation of the soft callus. Following nerve injury, it was also expected to find an early peak in genes such as NGF, IL-6, and CNTF, as all are related to the neural inflammatory processes and neuronal tissue repair. Neural mechanisms occurring in the process of fracture repair serves to be a controller of pain and swelling, and a key factor in promoting bone healing. Pro-inflammatory cytokines such as IL-6 and CNTF are expected to peak during the inflammation phase, which plays a role in promoting the growth of nerve fibers. Recent studies have hypothesized that anti-NGF may be able to attenuate neuropathic pain without affecting bone healing (Parker et al., 2024). NGF/TrkA signaling has been shown to be involved with nerve sprouting that occurs following injury and such sprouting, is expected to be involved with neuropathic pain. It can be proposed that only when NGF expression levels decline, do the newly sprouted fibers prune back and nociceptors return to a non-sensitized state, does the loading or movement of the fractured bone get perceived to be a non-noxious event (Mitchell et al., 2018). CRYAB and its role in preventing prolonged inflammation, expectedly decreased in expression as pro-inflammatory factors increased in expression early during the repair process.

Only following the initial pain and inflammation, should we expect genes such as SOX10, SOX2, MAG, and MOG to reach its highest peak in expression during the axonal reinnervation and remyelination processes that occurs after inflammation is cleared. SOX2 is a key transcription factor of neurogenic lineage, and its expression would be expected to increase if SCs are present during repair since they are needed to clear myelin and axon debris. The expression of proteins like MAG are also unique to SCs and promotes the recruitment of macrophages while SOX10 regulates the differentiation of SCPs for new neuronal connections to be reformed. Both SOX10 and SOX2 reach their highest expression levels at day 14, providing evidence for the presence of the SCs and the formation of regenerated tracks and promoting axonal regrowth during this period. During this process, CNTF and NGF are released by SCs providing axonal guidance for proper innervation, and both genes also generated a peak in expression at day 21. We have speculated that the peaks found at day 21 represents the SCs remyelinating the regenerated axons for normal function.

To further corroborate the temporal changes and differences found across specific genes using qPCR, we looked at fracture callus tissue sections and stained for cartilage, collagen type I, CNTF, and SOX10. In a temporal manner, we found that cartilage decreased in density and number while an increasing amount of new bone was seen, comparing day 10, 14 and 21 histological slides stained with Safranin-O and Picro-Sirius Red. Coupling our data from qPCR and staining slides, our data complements each other, as ACAN peaked early and declined 100-fold by day 18 and in the Safranin-O stained day 21, little to no cartilage (red) was shown in the fracture callus. BGLAP also did not reach its peak until day 14, representing the new turnover from cartilage to trabecular bone. Immunohistological slides treated with anti-CNTF demonstrated its localization towards blood vessels. When blood vessels are disrupted within the periosteum and bone marrow, neurotransmitters and neuropeptides secreted by immune cells and SCs promote angiogenesis, regulating the influx of nutrients and ions to the fracture callus site, further promoting the reinnervation processes (Morris et al., 2024). CNTF-positive areas were constrained to the lining of blood vessels and along the periosteum in immunohistochemical slides using anti-CNTF. Similarly, SOX10 positive areas were limited to the lining of blood vessels and the periosteum in anti-SOX10 stained slides, as well as inside the bone marrow space, where the differentiation and trans-differentiation of SCs takes place.

Conclusions and Future Studies

To gain further understanding of the results, more investigations need to be done to clearly show the effects of the specific genes and proteins studied and their associations with neuropathic pain. First, in order to confirm the relative peaks present in the qPCR data, more RNA samples should be collected at each timepoint of fracture healing and amplified using qPCR. A limitation found in our study was that only one sample was used for day 10 and day 20 while the Ct values of the rest of the days studied was an average across at least two RNA samples taken from the same day. This may explain for the decreased in expression levels at certain timepoints in the relative expression vs. days graphs that were not explained for in our results. Increasing the timepoints studied from 35 to 60 (2 months) may also be helpful in further understanding the processes of fracture healing and neuronal regrowth during bone fracture repair. Another limitation present in this study was the lack of different timestamps used during staining and immunohistochemical analyses. Only three time stamps, day 10, 14, and 21 were studied. While this may represent the major phase changes occurring it does not entirely represent all neuronal, bone, or vascular changes that takes place following a fracture and peripheral nerve injury. Other methods of staining such as using silver staining, may help further and better understand the localization and presence of neuronal tissue within the fracture callus.

Future exploration of how the peripheral and central nervous systems regulate fracture repair and how they promote neuropathic pain is critical. In the future, researchers should study more genes related to the regeneration of bone and peripheral nerves that more clearly delineates the impact of different nerve types on fracture healing. Remodeling the microenvironment during peripheral nerve repair and studying the exogenous application of specific local and systemic modulators of nerve regeneration may allow us to further understand the interactions of different cell types and regulators of peripheral nerve repair. With a deeper understanding of the complex mechanisms taking place following a fracture, the development of novel therapies targeting specific aspects of the nervous system that can stimulate fracture healing speeds and quality, alleviating complications such as neuropathic pain, could significantly improve patient outcomes and the healthcare systems.

REFERENCES

Balakrishnan, Anjali, et al. “Insights into the role and potential of Schwann cells for peripheral nerve repair from studies of development and injury.” *Frontiers in Molecular Neuroscience*, vol. 13, 25 Jan. 2021, https://doi.org/10.3389/fnmol.2020.608442.

Brazill, Jennifer M, et al. “Nerves in bone: Evolving concepts in pain and anabolism.” *Journal of Bone and Mineral Research*, vol. 34, no. 8, 26 July 2019, pp. 1393–1406, https://doi.org/10.1002/jbmr.3822.

Britsch, Stefan, et al. “The Transcription Factor Sox10 Is a Key Regulator of Peripheral Glial Development.” *Genes & Development*, vol. 15, no. 1, 1 Jan. 2001, pp. 66–78, doi:10.1101/gad.186601.

Cauley, Jane A. “The Global Burden of Fractures.” *The Lancet Healthy Longevity*, vol. 2, no. 9, Sept. 2021, https://doi.org/10.1016/s2666-7568(21)00183-5.

Chartier, Stephane R., et al. ``Exuberant sprouting of sensory and sympathetic nerve fibers in non healed bone fractures and the generation and maintenance of chronic skeletal pain.” *Pain*, vol. 155, no. 11, 6 Sept. 2014, pp. 2323–2336, https://doi.org/10.1016/j.pain.2014.08.026.

Cheng, Bin-Feng, et al. “Neural Cell Adhesion Molecule Regulates Chondrocyte Hypertrophy in Chondrogenic Differentiation and Experimental Osteoarthritis.” *Stem Cells Translational Medicine.*, vol. 9, no. 2, Feb. 2020, pp. 273–283, doi:10.2139/ssrn.3321477.

Coluzzi, Flaminia, et al. “The effect of opiates on bone formation and bone healing.” *Current Osteoporosis Reports*, vol. 18, no. 3, 6 Apr. 2020, pp. 325–335, https://doi.org/10.1007/s11914-020-00585-4.

DeBellard, Maria-Elena, et al. “Myelin-associated glycoprotein inhibits axonal regeneration from a variety of neurons via interaction with a sialoglycoprotein.” *Molecular and Cellular Neuroscience*, vol. 7, no. 2, Feb. 1996, pp. 89–101, https://doi.org/10.1006/mcne.1996.0007.

De Jongh, Raf F., et al. “The role of interleukin-6 in nociception and pain.” *Anesthesia & Analgesia*, Apr. 2003, pp. 1096–1103, https://doi.org/10.1213/01.ane.0000055362.56604.78.

Doege, K J, et al. “Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. human-specific repeats, and additional alternatively spliced forms.” *Journal of Biological Chemistry*, vol. 266, no. 2, 15 Jan. 1991, pp. 894–902, https://doi.org/10.1016/s0021-9258(17)35257-2.

Dolapchieva, S., et al. “Expression of Neural Cell Adhesion Molecule (NCAM) in the Peripheral Nerve Fibers Demonstrated by Post-Embedding Immunogold Method on Ultrathin Sections.” *Annals of Anatomy - Anatomischer Anzeiger*, vol. 184, no. 2, Mar. 2002, pp. 137–140, doi:10.1016/s0940-9602(02)80006-1.

Dubový, Petr. “Wallerian Degeneration and Peripheral Nerve Conditions for Both Axonal Regeneration and Neuropathic Pain Induction.” *Annals of Anatomy - Anatomischer Anzeiger*, vol. 193, no. 4, 10 Mar. 2011, pp. 267–275, doi:10.1016/j.aanat.2011.02.011.

Dubový, Petr, et al. “Role of Inflammation and Cytokines in Peripheral Nerve Regeneration.” *International Review of Neurobiology*, vol. 108, 2013, pp. 173–206, doi:10.1016/b978-0-12-410499-0.00007-1.

Einhorn, Thomas A., and Louis C. Gerstenfeld. “Fracture healing: Mechanisms and interventions.” *Nature Reviews Rheumatology*, vol. 11, no. 1, 30 Sept. 2014, pp. 45–54, https://doi.org/10.1038/nrrheum.2014.164.

Fornaro, Michele, et al. “Dynamic Environmental Physical Cues Activate Mechanosensitive Responses in the Repair Schwann Cell Phenotype.” *Cells*, vol. 10, no. 2, 17 Feb. 2021, p. 425, doi:10.3390/cells10020425.

Gaudet, Andrew D, et al. “Wallerian Degeneration: Gaining Perspective on Inflammatory Events after Peripheral Nerve Injury.” *Journal of Neuroinflammation*, vol. 8, no. 1, 30 Aug. 2011, p. 110, doi:10.1186/1742-2094-8-110.

Gerstenfeld, Louis C., et al. “Differential inhibition of fracture healing by non-selective and cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs.” *Journal of Orthopaedic Research*, vol. 21, no. 4, July 2003, pp. 670–675, https://doi.org/10.1016/s0736-0266(03)00003-2.

Gerstenfeld, Louis C., et al. “Fracture Healing as a Post-natal Developmental Process: Molecular, Spatial, and Temporal Aspects of Its Regulation.” *Journal of Cellular Biochemistry*, vol. 88, no. 5, 1 Apr. 2003, pp. 873–884, doi:10.1002/jcb.10435.

Hu, Diane P., et al. “Cartilage to bone transformation during fracture healing is coordinated by the invading vasculature and induction of the core pluripotency genes.” *Development*, vol. 144, no. 2, 15 Jan. 2017, pp. 221–234, https://doi.org/10.1242/dev.130807.

Hu, Zhongsheng, et al. “CNTF-STAT3-il-6 axis mediates neuroinflammatory cascade across Schwann cell-neuron-microglia.” *Cell Reports*, vol. 31, no. 7, 19 May 2020, p. 107657, https://doi.org/10.1016/j.celrep.2020.107657.

Huang, Zhong, et al. “Perspective on Schwann Cells Derived from Induced Pluripotent Stem Cells in Peripheral Nerve Tissue Engineering.” *Cells*, vol. 9, no. 11, 17 Nov. 2020, p. 2497, doi:10.3390/cells9112497.

Kawakatsu, Motohisa, et al. “Loss of Smad3 gives rise to poor soft callus formation and accelerates early fracture healing.” *Experimental and Molecular Pathology*, vol. 90, no. 1, Feb. 2011, pp. 107–115, https://doi.org/10.1016/j.yexmp.2010.10.011.

Kendroud, Sarah, et al. “Physiology, Nociceptive Pathways - Statpearls - NCBI Bookshelf.” *Physiology, Nociceptive Pathways*, StatPearls, 26 Sept. 2022, www.ncbi.nlm.nih.gov/books/NBK470255/. Accessed 27 Dec. 2023.

Kerner, S A, et al. “Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D3.” *Proceedings of the National Academy of Sciences*, vol. 86, no. 12, June 1989, pp. 4455–4459, https://doi.org/10.1073/pnas.86.12.4455.

Kiani, Chris, et al. “Structure and function of Aggrecan.” *Cell Research*, vol. 12, no. 1, 1 Mar. 2002, pp. 19–32, https://doi.org/10.1038/sj.cr.7290106.

Kister, Alexander, and Ilya Kister. “Overview of Myelin, Major Myelin Lipids, and Myelin-Associated Proteins.” *Frontiers in Chemistry*, vol. 10, 21 Feb. 2023, doi:10.3389/fchem.2022.1041961.

Lee, Yun-Shain, and Cheng-Ming Chuong. “Adhesion molecules in skeletogenesis: I. Transient expression of neural cell adhesion molecules (NCAM) in osteoblasts during endochondral and intramembranous ossification.” *Journal of Bone and Mineral Research*, vol. 7, no. 12, Dec. 1992, pp. 1435–1446, https://doi.org/10.1002/jbmr.5650071211.

Li, Jinqiao, et al. “An overview of Osteocalcin Progress.” *Journal of Bone and Mineral Metabolism*, vol. 34, no. 4, 8 Jan. 2016, pp. 367–379, https://doi.org/10.1007/s00774-015-0734-7.

Li, Zhu, et al. “Fracture repair requires trka signaling by skeletal sensory nerves.” *Journal of Clinical Investigation*, vol. 129, no. 12, 22 Oct. 2019, pp. 5137–5150, https://doi.org/10.1172/jci128428.

Lim, Erin-Mai F., Stan T. Nakanishi, et al. “Alphab-Crystallin Regulates Remyelination after Peripheral Nerve Injury.” *Proceedings of the National Academy of Sciences*, vol. 114, no. 9, 30 Jan. 2017, pp. 1707–17016, doi:10.1073/pnas.1612136114.

Lim, Erin-Mai F., et al. “Presence and Activation of Pro-Inflammatory Macrophages Are Associated with CRYAB Expression in Vitro and after Peripheral Nerve Injury.” *Journal of Neuroinflammation*, vol. 18, no. 1, July 2021, doi:10.1186/s12974-021-02108-z.

Liu, Songyang, et al. “Nerves within bone and their application in tissue engineering of bone regeneration.” *Frontiers in Neurology*, vol. 13, 2 Feb. 2023, https://doi.org/10.3389/fneur.2022.1085560.

Loi, Florence, et al. “Inflammation, fracture and bone repair.” *Bone*, vol. 86, May 2016, pp. 119–130, https://doi.org/10.1016/j.bone.2016.02.020.

Ma, Fukai, et al. “Controlled release of ciliary neurotrophic factor from bioactive nerve grafts promotes nerve regeneration in rats with facial nerve injuries.” *Journal of Biomedical Materials Research Part A*, vol. 110, no. 4, 18 Nov. 2021, pp. 788–796, https://doi.org/10.1002/jbm.a.37327.

McGregor, Narelle E., et al. “Ciliary Neurotrophic Factor Inhibits Bone Formation and Plays a Sex-Specific Role in Bone Growth and Remodeling.” *Calcified Tissue International*, vol. 86, no. 3, Mar. 2010, pp. 261–270, doi:10.1007/s00223-010-9337-4.

Mitchell, Stefanie A., et al. “New insights in understanding and treating bone fracture pain.” *Current Osteoporosis Reports*, vol. 16, no. 4, Aug. 2018, pp. 325–332, https://doi.org/10.1007/s11914-018-0446-8.

Morris, Ashlyn J., et al. “Cracking the code: The role of peripheral nervous system signaling in Fracture Repair.” *Current Osteoporosis Reports*, vol. 22, no. 1, 18 Jan. 2024, pp. 193–204, https://doi.org/10.1007/s11914-023-00846-y.

Mountziaris, Paschalia M., and Antonios G. Mikos. "Modulation of the inflammatory response for enhanced bone tissue regeneration." *Tissue Engineering Part B: Reviews*, vol. 14, no. 2, June 2008, pp. 179–186, https://doi.org/10.1089/teb.2008.0038.

Nencini, Sara, and Jason J. Ivanusic. "The physiology of bone pain. how much do we really know?" *Frontiers in Physiology*, vol. 7, 26 April, 2016, https://doi.org/10.3389/fphys.2016.00157.

Nocera, Gianluigi, and Claire Jacob. "Mechanisms of Schwann cell plasticity involved in peripheral nerve repair after injury." *Cellular and molecular life sciences : CMLS* vol. 77,20 (2020): 3977-3989. doi:10.1007/s00018-020-03516-9

Oliveira, Julia Teixeira, et al. "Neuron-Schwann Cell Interactions in Peripheral Nervous System Homeostasis, Disease, and Preclinical Treatment." *Frontiers in Cellular Neuroscience*, vol. 17, 12 Oct. 2023, doi:10.3389/fncel.2023.1248922.

Pagany, Maria, et al. "Myelin Oligodendrocyte Glycoprotein Is Expressed in the Peripheral Nervous System of Rodents and Primates." *Neuroscience Letters*, vol. 350, no. 3, 30 Oct. 2003, pp. 165–168, doi:10.1016/s0304-3940(03)00899-1.

Parker, Reginald S., et al. "Role of the neurologic system in fracture healing: An extensive review." *Current Osteoporosis Reports*, vol. 22, no. 1, 18 Jan. 2024, pp. 205–216, https://doi.org/10.1007/s11914-023-00844-0.

Puckett, C., et al. "Myelin-specific Proteolipid Protein Is Expressed in Myelinating Schwann Cells but Is Not Incorporated into Myelin Sheaths." *Journal of Neuroscience Research*, vol. 18, no. 4, 1987, pp. 511–518, doi:10.1002/jnr.490180402.

Qin, Qizhi, et al. "Neurovascular coupling in bone regeneration." *Experimental &Molecular Medicine*, vol. 54, no. 11, 29 Nov. 2022, pp. 1844–1849, https://doi.org/10.1038/s12276-022-00899-6.

Rammelt, Stefan, et al. "Osteocalcin enhances bone remodeling around hydroxyapatite/collagen composites." *Journal of Biomedical Materials Research Part A*, vol. 73A, no. 3, 30 Mar. 2005, pp. 284–294, https://doi.org/10.1002/jbm.a.30263.

Sahenk, Zarife, et al. "CNTF potentiates Peripheral Nerve Regeneration." *Brain Research*, vol. 655, no. 1–2, Aug. 1994, pp. 246–250, https://doi.org/10.1016/0006-8993(94)91621-7.

Schindeler, Aaron, et al. “Bone remodeling during fracture repair: The cellular picture.” *Seminars in Cell & Developmental Biology*, vol. 19, no. 5, Oct. 2008, pp. 459–466, https://doi.org/10.1016/j.semcdb.2008.07.004.

Schmelz, Martin, et al. “Nerve growth factor antibody for the treatment of osteoarthritis pain and chronic low-back pain: Mechanism of action in the context of efficacy and safety.” *Pain*, vol. 160, no. 10, Oct. 2019, pp. 2210–2220, https://doi.org/10.1097/j.pain.0000000000001625.

Schnaar, Ronald L., and Pablo H.H. Lopez. “Myelin-associated Glycoprotein and Its Axonal Receptors.” *Journal of Neuroscience Research*, vol. 87, no. 15, 15 Nov. 2009, pp. 3267–3276, doi:10.1002/jnr.21992.

Sebba, Anthony. “Pain: A review of interleukin-6 and its roles in the pain of rheumatoid arthritis.” *Open Access Rheumatology: Research and Reviews*, Volume 13, Mar. 2021, pp. 31–43, https://doi.org/10.2147/oarrr.s291388.

Stegen, Steve, et al. “Bringing new life to damaged bone: The importance of angiogenesis in Bone Repair and regeneration.” *Bone*, vol. 70, Jan. 2015, pp. 19–27, https://doi.org/10.1016/j.bone.2014.09.017.

Sheen, Jonathon R., et al. “Fracture Healing Overview - Statpearls - NCBI Bookshelf.” *Fracture Healing Overview*, StatPearls Publishing, Jan. 2023, www.ncbi.nlm.nih.gov/books/NBK551678/. Accessed 27 Dec. 2023.

Solovieva, Tatiana, and Marianne Bronner. “Schwann cell precursors: Where they come from and where they go.” *Cells & Development*, vol. 166, 1 June 2021, p. 203686, https://doi.org/10.1016/j.cdev.2021.203686.

Sun, Seungyup, et al. “No pain, no gain? the effects of pain-promoting neuropeptides and Neurotrophins on fracture healing.” *Bone*, vol. 131, Feb. 2020, p. 115109, https://doi.org/10.1016/j.bone.2019.115109.

Steverink, Jasper G., et al. “Sensory innervation of human Bone: An immunohistochemical study to further understand Bone Pain.” *The Journal of Pain*, vol. 22, no. 11, Nov. 2021, pp. 1385–1395, https://doi.org/10.1016/j.jpain.2021.04.006.

Svennigsen, Åsa, and Lars Dahlin. “Repair of the Peripheral Nerve—Remyelination That Works.” *Brain Sciences*, vol. 3, no. 4, Sept. 2013, pp. 1182–1197, doi:10.3390/brainsci3031182.

Thornton, Martin R., et al. “Neurotrophins 3 and 4 differentially regulate NCAM, L1 and N-cadherin expression during peripheral nerve regeneration.” *Biotechnology and Applied Biochemistry*, vol. 49, no. 2, Feb. 2008, pp. 165–174, https://doi.org/10.1042/ba20070040.

Tian, Heng, et al. “Interaction between Schwann cells and other cells during repair of peripheral nerve injury.” *Neural Regeneration Research*, vol. 16, no. 1, Jan. 2021, pp. 93–98, https://doi.org/10.4103/1673-5374.286956.

Woolf, Clifford J. “Central Sensitization: Implications for the Diagnosis and Treatment of Pain.” *Pain*, vol. 152, no. 3, Mar. 2011, pp. S2–S15, doi:10.1016/j.pain.2010.09.030.

Yiğiter, Özgür, et al. “The effects of type I collagen on bone defects and gene expression changes for osteogenesis: In a rat model.” *Journal of Cellular Biochemistry*, vol. 120, no. 7, 28 Feb. 2019, pp. 11525–11530, https://doi.org/10.1002/jcb.28432.

Yuan, YiMing, et al. “Review: Myelin Clearance Is Critical for Regeneration after Peripheral Nerve Injury.” *Frontiers in Neurology*, vol. 13, 16 Dec. 2022, doi:10.3389/fneur.2022.908148.

Zhai, Guangju. “Clinical Relevance of Biochemical and Metabolic Changes in Osteoarthritis.” *Advances in Clinical Chemistry*, 7 July 2020, pp. 95–120, doi:10.1016/bs.acc.2020.06.001.

Zhang, Wen-jun, et al. “Potential Role of Schwann Cells in Neuropathic Pain.” *European Journal of Pharmacology*, vol. 956, 5 Oct. 2023, p. 175955, doi:10.1016/j.ejphar.2023.175955.

Zhu, Bin, et al. “Cryab promotes osteogenic differentiation of human bone marrow stem cells via stabilizing β-catenin and promoting the Wnt signalling.” *Cell Proliferation*, vol. 53, no. 1, 22 Oct. 2019, https://doi.org/10.1111/cpr.12709.

CURRICULUM VITAE

[redacted]

[redacted]

[redacted]