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Mesenchymal Progenitors in the Epidural Fat and Dura Mater Participate in Tissue Homeostasis and Wound Healing

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Mesenchymal Progenitors in the Epidural Fat and Dura Mater Participate in Tissue Homeostasis and Wound Healing

by

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Abstract

Mesenchymal progenitor cells (MPCs) are adult cells capable of self-renewal and differentiation into cells that make up mesodermal tissues such as bone, cartilage, and fat. MPCs are believed to play a significant role in tissue maintenance and repair. MPCs are present in many adult connective tissues but are typically found in higher quantities in adipose tissues for yet unknown reasons. Recently, our research group identified MPC populations within epidural fat and the adjacent dura mater. Clinically, epidural fat is frequently considered a space-filling, biologically inert tissue; therefore, it is common practice for spine surgeons to discard it during surgical procedures. As the development and cellular origins of both epidural fat and the dura mater remain unclear, I hypothesized that epidural fat MPCs contribute to the maintenance of dural integrity throughout growth and post-injury.

Using Paired related homeobox 1 (*Prx1*) and Hypermethylated in cancer 1 (*Hic1*) transgenic lineage tracing mice, the localization of epidural fat MPCs were identified during normal maturation and at skeletal maturity. This lineage tracing revealed an overlap between $Prx1^{+}$ and $Hic1^{+}$ populations, indicating a potential hierarchical relationship between the two MPC populations. When $Prx1^{+}$/ $Hic1^{+}$ MPCs were ablated, the expression of the dural marker α-SMA was lost in adjacent dura mater suggesting these cells are required for tissue homeostasis. Both MPC populations were observed to respond to dural injuries by homing to the lesion site. The process by which epidural fat MPCs maintain the dura mater through growth and after injury was accelerated in $p21^{-/-}$ mice (known for increased tissue regeneration/ cell proliferation).

While MPCs have been identified and characterized in other adipose tissues, the role in epidural fat remained elusive. This study contributed to our knowledge of the role of epidural fat MPCs *in vivo* in aspects of growth, homeostasis, and repair of dural tissue. This thesis emphasizes the importance of revisiting the prevalent notion of epidural fat as biologically insignificant and the process of discarding it during surgery.

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Thesis Organization

This thesis is presented in the paper chapter format and consists of five chapters.

Chapter 1 is a manuscript that was published in *Bioessays*; 43(2). DOI: 10.1002/bies.202000215 under their Hypothesis section. In this chapter, the hypothesis that epidural fat mesenchymal stem/progenitor cells act as regulators of dural homeostasis and regeneration was discussed. This chapter serves as an overarching introduction, providing some background for the thesis project. Roman Krawetz and I conceptualized the hypothesis and aims within the manuscript. Sathvika Mudigonda aided in drafting the manuscript. Alim Mitha, Paul Salo and Roman Krawetz provided critical review, commentary and revisions to the manuscript.

Chapter 2 is a comprehensive literature review of topics related to the thesis that were not discussed in sufficient detail in Chapter 1. This includes adipose biology, stem cells, mouse models, and the dura mater. At the end of this chapter, the rationale, hypotheses and study aims are specifically outlined.

Chapter 3 is a manuscript entitled "*Prx1^{+}and Hic1^{+} mesenchymal progenitors are present within the epidural fat and dura and participate in dural injury repair".* This chapter outlines the methods and results of the study and addresses aims 1-5. This chapter has been submitted for publication. I contributed to the conceptualization of the hypothesis and aims, undertook the experiments, collected and analyzed the data, and drafted the manuscript.

Chapter 4 is a manuscript entitled "*Prx1^{+} mesenchymal progenitor cells display increased proliferation in vivo within the dura mater of p21$^{-/-}$ mice".* This chapter examined the role of MPCs in *p21$^{-/-}$* mice regarding the repair and/or regeneration of the dura mater during growth and in response to injury. This study also addresses aims 1 and 5 of the thesis. This chapter will be submitted for publication. I contributed to the conceptualization of the hypothesis and aims, undertook the experiments, collected and analyzed the data, and drafted the manuscript.

Chapter 5 consists of the discussion and conclusions of this thesis. An overview of the study, along with significance, future directions, and the limitations encountered are presented.

Appendix A presents data from the pilot project wherein xeno-transplantation of human epidural fat MPCs was undertaken to determine if human MPCs can also respond to dural injury.

Appendix B consists of detailed protocols used in this study.

Appendix C contains the permission form from all co-authors of the published manuscript in this thesis, as well as the license granted from Bioessays to use the published manuscript.

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CHAPER 1: Epidural fat mesenchymal stem cells: Important microenvironmental regulators in health, disease, and regeneration: Do EF-MSCs play a role in dural homeostasis/maintenance?

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ABSTRACT

Mesenchymal stem cells (MSCs) are present in fat tissues throughout the body, yet little is known regarding their biological role within epidural fat. We hypothesize that debridement of epidural fat and/or subsequent loss of MSCs within this tissue, disrupts homeostasis in the vertebral environment resulting in increased inflammation, fibrosis and decreased neovascularization leading to poorer functional outcomes post-injury/operatively. Clinically, epidural fat is commonly considered a space-filling tissue with limited functionality and therefore typically discarded during surgery. However, the presence of MSCs within epidural fat suggests that it is more biologically active than historically believed and may contribute to the regulation of homeostasis and regeneration in the dural environment. While the current literature supports our hypothesis, it will require additional experimentation to determine if epidural fat is an endogenous driver of repair and regeneration and if so, this tissue should be minimally perturbed from its original location in the spinal canal.

Graphical abstract

1.) INTRODUCTION

Mesenchymal stem cells: identity, location and function

Mesenchymal stem cells (MSCs) were first discovered by Friedenstein *et al.* who observed spindle-shaped, plastic-adherent, non-hematopoietic cells in bone marrow (1,2). Along with the ability to undergo self-renewal, MSCs exhibit multipotent differentiation ability (3–5). MSCs are heterogeneous in nature (6), and display distinct morphologies, self-renewal and differentiation potentials based on numerous factors (e.g., donor age, tissue source) (7–9). It has been hypothesized that MSCs follow a hierarchy in which a heterogeneous group of mesenchymal progenitors are derived from a common 'apex' stem cell (6). However, this hypothesis and the existence of an apex adult stem cell population is controversial (5,10), as our understanding of the biological functions and properties of MSC populations *in vivo* remains incomplete (6).

MSCs are believed to play several physiological roles *in vivo*, including the maintenance of the haematopoietic stem cell (HSC) niche, essential for sustaining haematopoiesis throughout adulthood (1,11). In this niche, stromal cells protect HSCs from pro-apoptotic stimuli and regulate their proliferation and differentiation (1,12). MSCs also play a regulatory role in the innate and adaptive immune response (1,6). MSCs inhibit the maturation of monocytes and HSCs into dendritic cells (DCs) *in vitro* (13–15) and decrease DC production of tumor-necrosis factors, reducing their ability to generate a pro-inflammatory response (16). MSCs can also inhibit the cytotoxicity of natural killer cells, as well as decrease the activity of neutrophils and macrophages in response to infection (17). MSCs exert suppressive effects on adaptive immune cells, by inhibiting antigen-specific T-cell proliferation and cytotoxicity (16,18,19), yet can also promote $CD4^+$ and $CD8^+$ memory T cell activity, as well as the production of alloantibodies (17). As such, MSCs regulate a bimodal immunomodulatory response (anti-inflammatory and immuno-stimulatory) through modulation of the cellular immune response (20,21).

MSCs have been identified in most mesodermally derived tissues in the body (3,22,23), with phenotypic differences dependent on their tissue of origin (24). Bone marrow is frequently used as a source for MSC isolation (25,26), however, harvesting marrow is invasive, accompanied by pain and the risk of infection (26). Therefore, alternative sources have been investigated and adipose tissue has been found to contain a greater number of MSCs (26) and it is relatively non-invasive to obtain in comparison to bone marrow (27).

Adipose/fat is present throughout the body at varying quantities (28), including within synovial joints (e.g., infrapatellar fat pad (29–31) and is typically harvested/discarded during cosmetic procedures and/or surgical interventions (liposuction, resection) (26). As fat is one of the largest organ systems in the body, more MSCs (in total) can be obtained from fat vs. other tissues on a per patient basis, keeping in mind the number in bone marrow declines with age (32), while fat deposition typically increases (33). Furthermore, *in vivo* lineage tracing studies in mice have demonstrated that MSCs are present within white (34) and brown fat throughout the body (35). Due to these reasons, adipose tissue is regularly used as a viable source of MSCs (25,28).

Are adipose-derived MSCs a better option for regenerative medicine applications?

Adipose tissue contains a heterogenous population of stromal cells (including MSCs) referred to as the stromal vascular fraction (SVF) (36). Adipose-derived MSCs (ADSCs) were first described by Zuk *et al.* in 2001 (37) and demonstrate equal or greater proliferative capacity and multipotency *in vitro* compared to their bone marrow cousins (22,37). Cultured ADSCs retain the ability to differentiate into adipose (38), bone (39–41) and cartilage (42–44) *in vivo*. ADSCs have also been employed in peripheral nerve repair strategies, with some studies suggesting that ADSCs can respond to injury within neighbouring tissues *in vivo* via neuronal differentiation (45), or enhance endogenous nerve repair (46) through the release of trophic factors (47,48). The interest in developing ADSCs for regenerative medicine applications has led to ADSCs being identified in most sites of fat in the adult, however, little is known about the ADSC/MSC populations in epidural fat.

Epidural fat is commonly considered as a biologically inert tissue

Epidural fat is found within the spinal canal (49), between the dura mater and bony confines of the spinal canal (**Fig. 1)**. It is unevenly distributed along the spinal canal, increasing craniocaudally and posteriorly (vs. anteriorly) (50). During development, epidural fat is more abundant adjacent to the dural sac, whereas in adulthood, its localization becomes discontinuous (51). Epidural fat is held together by vascularized pedicles, and displays a lower density vs. subcutaneous fat, which is believed to allow it to conform better to the epidural space (49). The amount of epidural fat varies in proportion with body size, yet its volume is not related to sex (52–55). The commonly accepted paradigm is that epidural fat functions as a shock absorber for contents of the canal and dural sac (55), yet, little direct evidence supports this belief. It is also thought to protect the spinal cord/nerves by cushioning and buffering the pulsatile movements of the dural sac along with

facilitating sliding of the dural sac over periosteum in flexion and extension (55). Clinically, however, it is commonly considered a space-filling tissue and is typically discarded during surgery to increase the operational field of view.

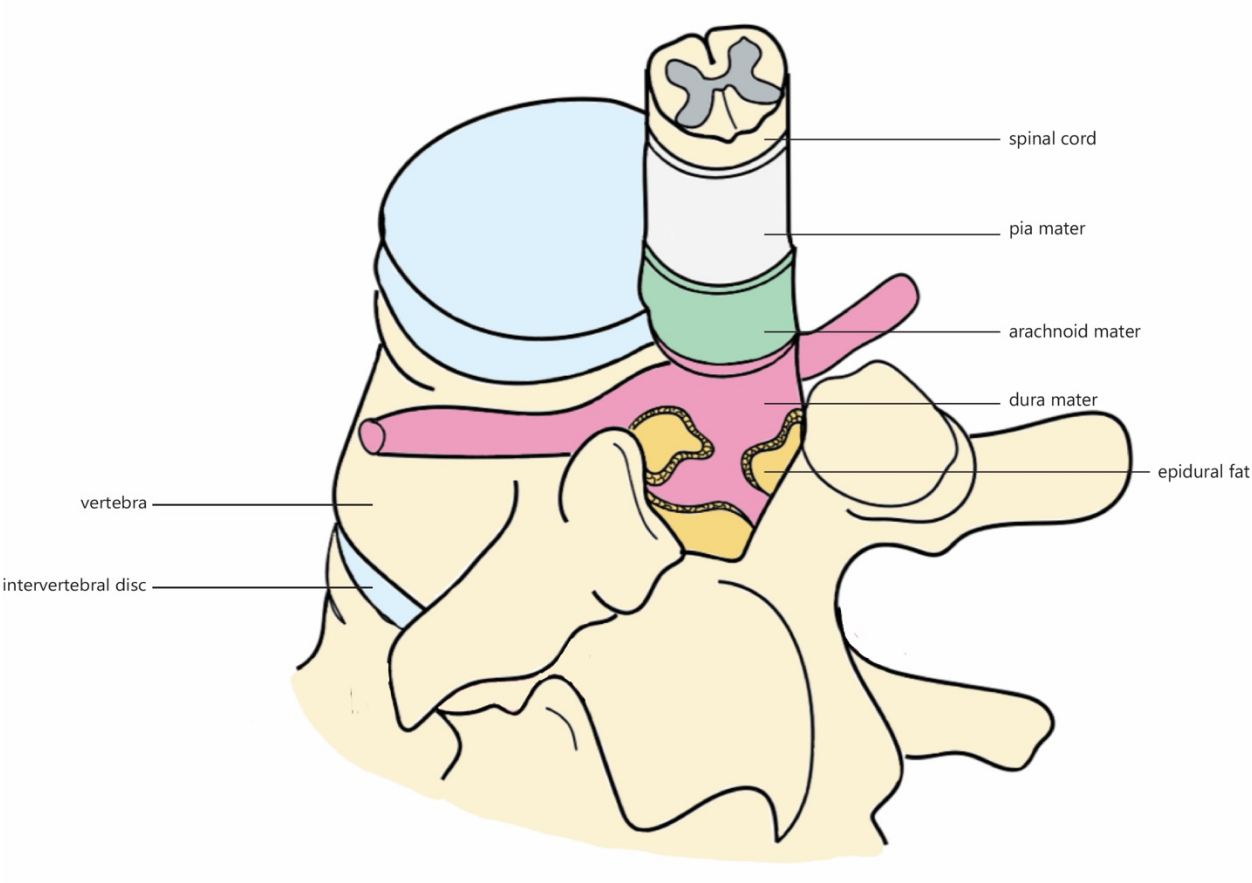

Figure 1. Three protective membranes- pia, arachnoid, and dura mater- surround the spinal cord. Epidural fat is found discontinuously within the spina canal, between the dura mater and vertebral wall.

Epidural fat: more than a space-filling tissue

Only recently have human epidural fat MSCs (EF-MSCs) been isolated and found to retain self-renewal and trilineage differentiation capacity *in vitro* (56,57). Through the use of lineage-tracing mouse models, EF-MSCs were identified *in vivo*, with their progeny appearing to contribute to the dura mater (57). The existence of EF-MSCs suggests that epidural fat is not solely a space filling tissue and therefore we **hypothesize** that: the loss of epidural fat and/or EF-MSCs will result in increased inflammation, fibrosis, and degeneration of adjacent tissues (such as dura) within the

vertebral environment. Furthermore, these outcomes could be rescued with the delivery of epidural fat, EF-MSCs or epidural fat engineered from EF-MSCs. If this was demonstrated, epidural fat and by extension EF-MSCs, would be endogenous drivers of repair/regeneration that should be minimally perturbed from their location in the spinal canal.

2.) IS EPIDURAL FAT IMPORTANT?

If epidural fat and/or EF-MSCs are required to maintain the dural micro-environment, then epidural fat should be negatively impacted post-injury and with the onset of disease. Moreover, removal or damage to the epidural fat should have a negative impact on the local micro-environment (**Fig. 2**). We present evidence here that support both **sub-hypotheses**.

Under certain pathological conditions, the distribution of epidural fat within the spinal column is altered; for example: *Spinal epidural lipomatosis* presents with an accumulation of epidural fat and can occur idiopathically, or due to prolonged exogenous steroid use (49,52,58). *Spinal stenosis* is narrowing of the spinal canal, wherein pressure can be placed on the spinal cord and/or nerve roots (59). Although epidural fat is often decreased in the stenotic area (49), posterior decompression for treatment of lumbar spinal stenosis includes further removal of posterior epidural fat (60). *Kyphoscoliosis* is a combination of both kyphosis and scoliosis (abnormal outward and lateral curvature of the spine, respectively). This condition leads to asymmetrical distribution of epidural fat within the concave portion of the spinal curvature, ultimately displacing the spinal canal and its contents in the opposite direction (49).

In the treatment of these diseases as well as other spine conditions that are treated surgically, the removal of epidural fat is commonplace to increase the operational field of view and is not looked upon negatively since epidural fat is typically viewed as inert or as having limited biological activity. Recently, this process has been questioned as the removal of epidural fat has been shown to alter post-operative results, such as pain and function post-surgery (61). Among older adults with chronic axial low-back pain, better performance-based and self-reported physical function were positively correlated to increased epidural fat (61). In another study, following lumbar decompression surgery for lumbar spinal stenosis, postoperative outcomes worsened (pain intensity and a deteriorated score on the Oswestry (low back) Disability Index) amongst the group whose posterior epidural fat was removed (60).

While these studies suggest that epidural fat plays some role in the normal function of the epidural micro-environment, many of the studies are clinical in nature and therefore direct mechanisms cannot be ascertained. Hence, to demonstrate if epidural fat is important for the homoeostasis of this micro-environment, animal models in where epidural fat is removed/debrided (in the absence of any other type of insult) followed by outcome measures focused on pain and function should be employed. In these models, it would also be of interest to determine if the level of inflammation and/or fibrosis increased after epidural fat removal since previous studies have suggested a link between these events. Moreover, after injury, neovascularization of damaged tissue plays a critical part in repair and regeneration. It has therefore been postulated that MSCs not only need to promote tissue repair, but also aid (or at least not inhibit) vascularization (62). A number of requirements have been developed to define the role of MSCs in regenerative medicine strategies: 1) post-transplantation, MSCs should support regeneration of the damaged tissue *and* its vasculature (62,63), 2) MSCs are expandable *in vitro* and their differentiation should be reproducible (62), and 3) MSCs should be obtainable without donor site morbidity (62,64). Keeping such conditions in mind, transplantation studies employing EF-MSCs would be needed to determine their response to injury and if their isolation from epidural fat results in donor site morbidity. Specifically, murine epidural fat and/or EF-MSCs could be transplanted in a dura injury mouse model, and if regeneration of the damaged tissue and its vasculature was observed, this would suggest a potential reparative/regenerative role of EF-MSCs. To determine if human EF-MSCs retain this ability, xeno-transplantation of epidural fat/EF-MSCs into mouse dural injury model could be employed. These experiments would help identify the role of these EF-MSCs *in vivo* and if mouse vs. human EF-MSCs share a similar functional phenotype. If human EF-MSCs were found to contribute to the restoration of epidural fat and/or dural tissue in mice, this would refute the common paradigm of epidural fat being a biologically irrelevant tissue.

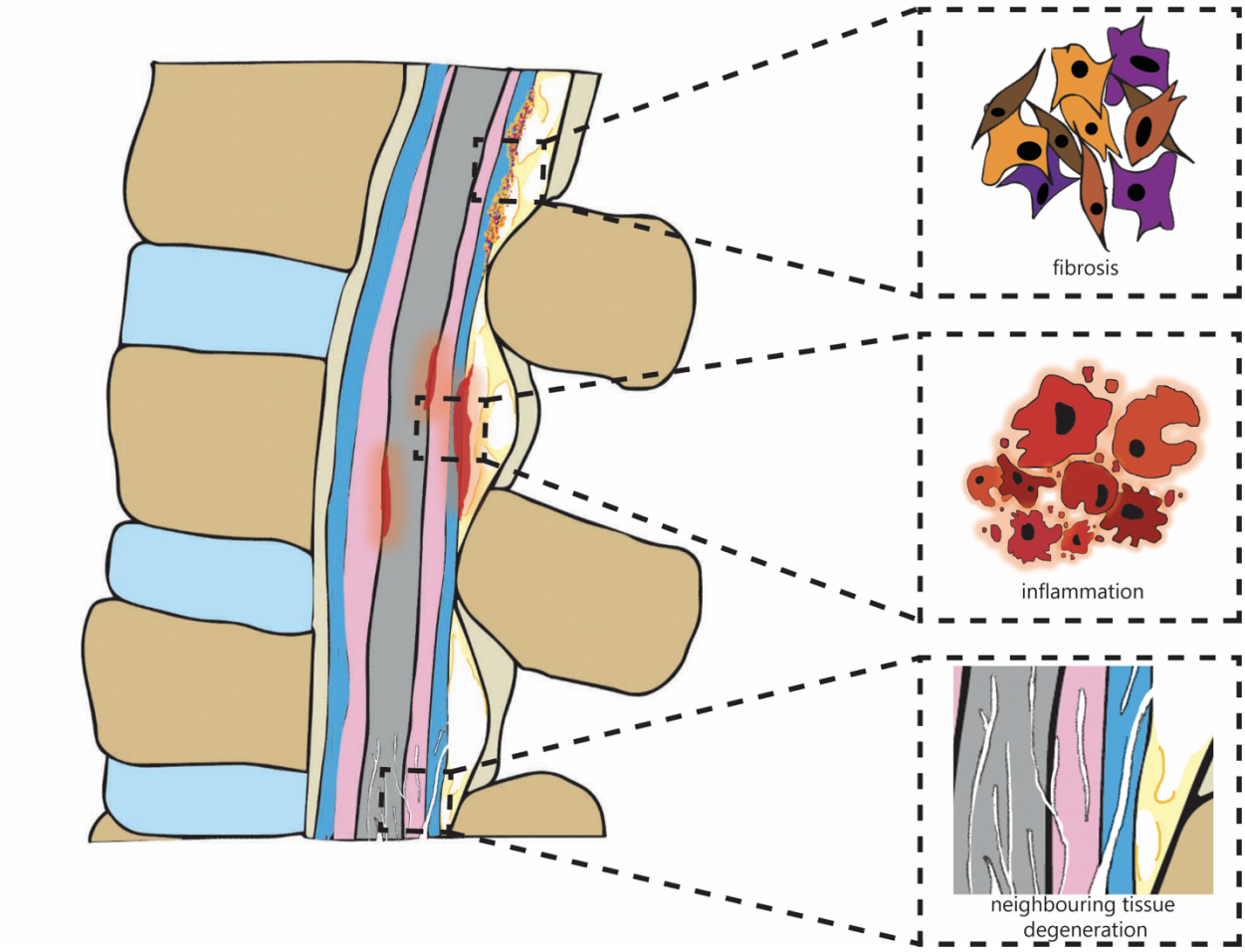

Figure 2. Potential outcomes of epidural fat loss/damage include fibrosis, inflammation, and neighbouring tissue degeneration.

3.) DOES EPIDURAL FAT REGULATE FIBROSIS?

Epidural fibrosis may be one of the main variables related to failed back surgery syndrome - a common complication in spine surgery. It is believed that this occurs due to epidural fat destruction and subsequent invasion of fibroblasts originating from disrupted paravertebral muscles in the surgical wound (65). This fibrotic tissue then adheres to the dura, compressing nerve roots (65). Since this complication is believed to be a direct consequence of the loss of epidural fat leading to increased fibrosis and inflammation, biomaterial (66) and anti-inflammatory (67) therapeutics have been explored, but have yet to be proven effective. Free-fat grafting is the most common approach to prevent fibrosis; however, the efficacy and safety of this technique is still questioned. Free-fat grafts can decrease in size over time (68), migrate (69) or herniate into the spinal column resulting in symptomatic space-occupying lesions (70), or dural compression causing cauda equina syndrome (71,72). These studies among others demonstrate that epidural fat appears to negatively

regulate fibrotic processes (**Fig. 2)** in the micro-environment and that simply 'filling' the empty cavity made by the loss of epidural fat is not an effective means of replacing the tissue. Furthermore, it also suggests that the epidural fat is biologically active and that a heterotopic tissue transplant cannot replicate these lost functions. Pursuing this line of questioning to further determine exactly what cell(s) types and/or secreted factors within the epidural fat allow it to mitigate fibrotic and inflammatory responses would not only go a long way in understanding how epidural fat works at a mechanistic level, but this knowledge could also be exploited to control fibrosis and inflammation in other organ/tissue systems. To address this, single cell sequencing approaches could be undertaken on normal vs. diseased/injured epidural fat to determine what cell types are present and what gene products are being differentially regulated in homeostatic vs. non-homeostatic conditions. An investigation of secreted factors would likely be a good starting point, as recombinant protein(s) could be transplanted post-epidural fat debridement (in sponges or other biomaterials) and the levels of fibrosis and/or inflammation could be determined. Furthermore, as fibroblasts/myo-fibroblasts are implicated in the fibrotic response in many tissues, this cell population and its interaction with epidural fat could be studied specifically using *in vitro* (co-culture, scratch assay) and *in vivo* (targeted ablation of fibroblast populations with/without addition of epidural fat specific growth factors) to elucidate if epidural fat and/or it constituents have the ability to inhibit myo-fibroblast differentiation and these cells' ability to induce a pro-fibrotic response.

4.) CAN THE EPIDURAL FAT BE RE-ENGINEERED?

Since epidural fat appears to be an important component of the micro-environment and its absence may promote inflammation and fibrosis, the reconstruction of epidural fat in animal models has been attempted through transplantation of engineered adipose tissue (65), or injectable extracellular matrices (73). When ADSCs were seeded on to a poly(lactic-co-glycolic acid) scaffold, exposed to adipogenic agents and implanted in a dorsal laminectomy rabbit model between the dura and paraspinal muscles, epidural fat like-tissue was regenerated. The fat tissue overlying the dura mater displayed similar cell morphology and arrangement as native epidural fat (65). In a separate study, decellularized adipose matrix supplemented with hyaluronic acid hydrogel was loaded with ADSCs and injected in a laminectomized rat model (73). This approach inhibited scar tissue invasion of the wound site and reduced adhesion to the dura post-surgery.

While these results seem to imply that ADSCs can compensate for the loss of epidural fat and/or EF-MSCs in terms of regulating the fibrotic response post-injury, it remains unknown if EF-MSCs have any additional properties that are required in the epidural environment. As a specific example, in our teams' previous study on EF-MSCs, we were able to demonstrate that the EF-MSCs also contribute to the dura mater (57). Based on this result, it would be important to determine if ADSCs derived from non-epidural fat sources can also migrate and populate the dura mater. While we demonstrated EF-MSCs are present in the dura mater (57), the role of these cells in the dura remain unknown. It will be essential to determine if EF-MSCs contribute to the maintenance and/or repair of the dura mater and if other ADSCs populations (from other fat sources) could also replicate this EF-MSC function. At the level of tissue functionality, since epidural-like fat can be derived from non-epidural fat ADSCs, it would be interesting to undertake a more detailed molecular study of native epidural fat vs. epidural-like fat regenerated from ADSCs. This could be accomplished through various OMICS techniques and would give a clearer picture of the tissues within the microenvironment. In regard to the accumulation of EF-MSCs in the dura, if these cells from epidural-like fat (and/or other ADSCs?) are incapable of contributing to the dura and/or responding to injury, this would call attention to the existence of additional properties specific to native epidural fat. Additionally, this novel property of EF-MSCs may suggest that trilineage differentiation potential may not be the outcome measure and characteristic required of fat within the epidural space. If there is a functional difference in EF-MSCs vs. ADSCs in terms of dural maintenance/repair, it would then be interesting to compare the transcriptomic and proteomic signature of these stem cell populations to determine what similarities and differences exist between these cell types based on the type of fat the stem cells were derived from and how these distinctions might regulate and explain phenotypic and functional differences.

5.) WHY IS IT IMPORTANT TO MAINTAIN THE INTEGRITY OF THE DURA MATER?

Both the brain and spinal cord are covered in the same three membranes, known as the meninges. The meninges (pia, arachnoid and dura mater from inner to outermost layers) appear during the early embryonic stages of cortical development (74), and lie adjacent to epidural fat within the spinal canal (**Fig. 1**). These layers along with the cerebrospinal fluid, protect the central nervous system (CNS). Meninges anchor the CNS to surrounding bones, such that the dura mater is

attached to the skull and spine (74). In early embryogenesis, mesenchymal and neural crest-derived cells give rise to the primary meninx, the latter of which forms the dura mater (74). Along with mechanical protection (anchoring spinal cord within the spinal canal and casing fluid), the dura mater plays a vital role in providing immunological protection against infections, such that, together with the other two meninges, the dura mater forms a uniform organ of its own (75). Defects in the dura can be the result of congenital abnormalities, inflammation, surgical procedures or trauma and are of concern as the dura mater is essential for cerebrospinal fluid turnover and maintaining intracranial pressure (76). Dural ruptures are commonly detected intraoperatively and consequently repaired, however if gone unnoticed, they can cause severe complications. Cerebrospinal fluid leakage can lead to fistula, with a risk of subsequent meningitis and epidural abscess that can be fatal if left untreated (77).

While we currently know that EF-MSCs are found within the dura mater from lineage tracing experiments, it remains unknown if these EF-MSCs play a functional role in this tissue or act as a reservoir of cells for repair and/or regeneration in this microenvironment. By identifying dura markers, an *in vitro* dural differentiation protocol could be developed. To a great extent, meninges are composed of meningeal fibroblasts, which were recently characterized at the molecular level (78). Transcriptional signatures of embryonic dural fibroblasts defined via single-cell RNA sequencing revealed that *Slc5a6, Slc16a9* and *Slc38a2* (all transporters) were uniquely enriched in the dural cluster. Other genes included *Rpl* & *Rps* (constituents of large and small ribosomal subunits) and *Foxp1* & *Six1* (transcription factors). Finally, genes such as *Fxyd5* (ion transport regulator) and *Nov* & *Smoc2* (encode matricellular proteins) were also selectively expressed in dural fibroblasts. In-situ hybridization labeling of these genes confirmed expression within the dura only (78). In another study, a transgenic mouse with an alpha-smooth muscle actin (α-SMA) promoter driving a GFP transgene was used to test α-SMA as a marker of skeletal precursor cells. Along with periosteum and capillary-associated cells, $\alpha\text{-SMA}^{GFP}$ was also detected in the dura (79). Furthermore, neural precursor markers, such as *Nestin, Vimentin and Sox2* have been identified in rat meninges both during development and in adulthood (80). Using such markers as evidence, MSCs from various sources (adipose vs. bone-marrow vs. epidural fat) could be subjected to a dural differentiation protocol. If only EF-MSCs were capable of giving rise to dura, this would further elucidate the importance of epidural fat in maintaining the dura. This type of experimental design could also be used *in vivo* wherein undifferentiated vs. dural differentiated

MSCs could be transplanted into normal vs. dural injury mouse models. Moreover, expression of surrogate markers in the dura, such as α-SMA, could allow for *in vivo* analysis of growth and aging patterns in homeostasis and post-injury.

6.) EF-MSCs AS AN ENDOGENOUS DRIVER OF REPAIR/REGENERATION

Similar to how dural fibrosis caused by epidural fat destruction led to the study of novel therapeutic approaches to reconstitute it as previously discussed; the clinical requirement to repair dural defects has led to numerous biological (pericardium, fascia, decellularized matrices) and synthetic (polymers, gels) graft materials (76). As grafts are exogenously delivered, measures need to be taken to avoid neurotoxic or inflammatory responses (76). Given that EF-MSCs were only recently discovered, and their role *in vivo* remains elusive, it is not surprising that little investigation has been undertaken on endogenous dural repair mechanisms. To our knowledge, there are currently no methods to induce endogenous dural repair, and therefore, if EF-MSCs play a role in dural maintenance (57), then it is possible that cells within the epidural fat could be mobilized in dural repair strategies. While no direct evidence exists to support this hypothesis, given that the debridement/destruction of epidural fat results in a pro-inflammatory and pro-fibrotic response, EF-MSCs can be tracked to the dura mater *in vivo*, and damage to the dura mater can result in a myriad of complications including death; we believe that some type of endogenous repair mechanism exists in regard to the dura otherwise clinical interventions required for dural repair would be much more common.

To begin addressing these questions, it is essential to have a more complete understanding of EF-MSCs and how they function in the epidural/dural environment. One such inquiry could make use of transgenic reporter mice in which a dural injury is induced and the reparative response of EF-MSCs observed. If the dura was directly repaired by EF-MSCs, labelled cells would be found filing the site of injury. If the injury was repaired but only non-labelled cells filled the defect, then it might be possible that EF-MSCs are not essential for dural repair or that EF-MSCs are playing an immunomodulatory role by recruiting other cells to complete the repair.

7.) EXPERIMENTAL APPROACHES TO HELP UNDERSTAND EF-MSC BIOLOGY AND FUNCTION

Although epidural fat has not been rigorously studied, and its significance at a tissue level has yet to be fully elucidated, it does share phenotypic characteristics with white fat: cells are unilocular with a large lipid vacuole, appear white/yellow and can be marked by *Prx1* expression in mouse (49,81). Epidural fat specific properties, such as its semifluid nature, rarefaction, scarcity of connective tissue, and slits/sliding spaces suggest that this tissue is not a standard white fat deposit (51). These findings may support the previously mentioned mechanical role of epidural fat, however, the distinct localization of epidural fat in the spinal cavity suggests a functional and metabolic role, rather than being an incidental tissue solely responsible for shock absorption. Studies of stem cell populations have revealed anatomic site-specific differences in functional properties (24). These molecular and functional differences specific to EF-MSCs (vs. ADSCs/MSCs) require further investigation. The contribution of EF-MSCs throughout growth of the spinal cavity can be investigated using animal models. Using the *Prx1*$^{\text{CreERT2-GFP}}$R26R$^{\text{TdTomato}}$ mouse, where employing a common MSC lineage marker gene- Paired related homeobox-1 (*Prx1/Prrx1)*- EF-MSCs could be labelled and their localization, migration and fate tracked over time. *Prx1* has been previously shown as a robust adipose MSCs marker in white adipose tissue (82), and *Prx1* positive cells have been identified in mouse epidural fat (57). Whereas EF-MSCs have been seen contributing to the dura mater (57), this study would be conducted over the course of normal skeletal and sexual maturation, therefore bringing to light the possibilities of EF-MSCs actually modulating neighbouring tissue growth over normal aging. Further building on this, EF-MSC specific ablation could be attempted and the subsequent effects on the epidural fat, the dura mater, and paraspinal muscles observed. For example, by crossing the *Prx1*$^{\text{CreERT2-GFP}}$R26R$^{\text{TdTomato}}$ mouse with a R26R$^{\text{DTA}}$ mouse (DTA: diphtheria toxin subunit A; inhibits protein synthesis), highly- specific ablation would be achieved in the *Prx1* positive cells synthesizing DTA. If surrounding tissues disintegrated and/or responded with a pro-inflammatory, fibrotic response, this would demonstrate the role of EF-MSCs in tissue maintenance. Additionally, this ablation concept could be extended to dural injuries to determine if EF-MSCs are required for the repair of this tissue. These types of experimental approaches would shed light on the role of epidural fat and the putative MSC population within this tissue.

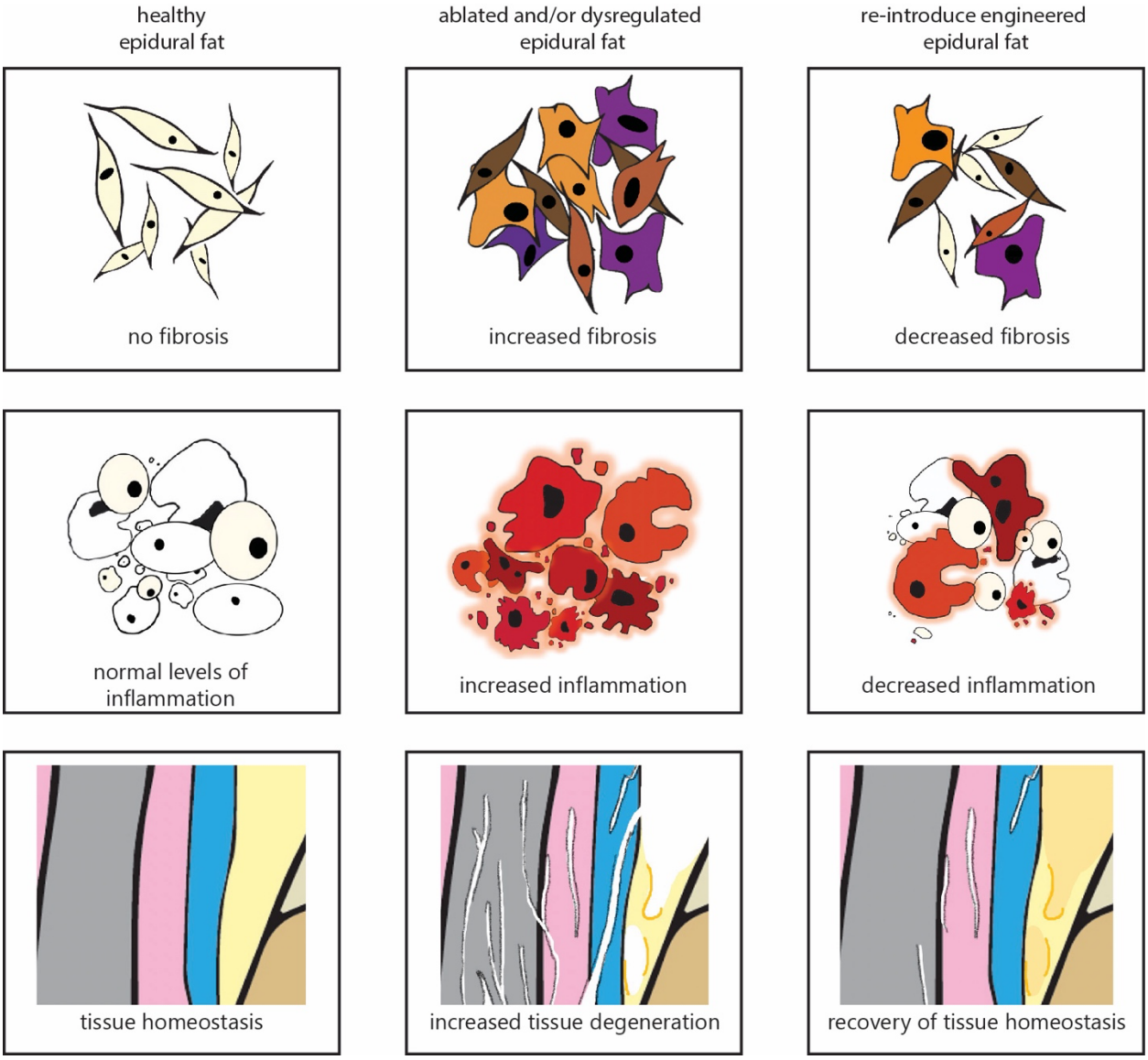

Figure 3. Dysregulation or removal of epidural fat causes increased fibrosis, inflammation, and degeneration of neighboring tissues. These processes might be partially rescued through the introduction of engineered epidural fat and EF MSCs.

8.) CONCLUSIONS AND OUTLOOK

Investigation of the *in vivo* role of MSCs derived from epidural fat will provide new insights regarding the development, growth and maintenance of the dura mater. This line of research into a recently discovered MSC population within the spinal microenvironment will further develop new avenues of research in how to overcome dural (and maybe even spinal) injuries including post-operative complications such as dural fibrosis and ruptures. Additionally, a shift in focus from exogenous repair mechanisms to one that is endogenous in nature may directly impact clinical

care. Our hypothesis that epidural fat and EF-MSCs play a role in the regulation of health, disease and regeneration in the dural microenvironment will need to be tested using both *in vitro* and *in vivo* methodologies to gain a more complete understanding of the importance of this tissue. However, we suggest prioritizing experiments that focus on the ablation of epidural fat/EF-MSCs and experiments that attempt to restore tissue function once the epidural fat has been lost/damaged (**Fig 3**). These types of experiments will explain the necessity (or lack thereof) of epidural fat in the homeostatic regulation of the dural microenvironment as well as elucidate if exogenously delivered and/or engineering replacements have promise as a therapeutic for injuries and/or diseases that impact this microenvironment. In the meantime, while this research in underway, we propose that epidural fat not be (or at least minimally) disturbed from its original location when undertaking spine surgery as its loss may negatively impact endogenous repair post-surgery. While epidural fat has been long ignored, research into this novel source of MSCs will facilitate our knowledge of this unique adipose tissue with the real potential to generate clinically impactful outcomes.

CHAPER 2: Literature Review

This literature review will explore in greater detail concepts that were not already discussed in Chapter 1. A general overview of adipose biology, focusing on white and brown adipose tissue is presented. This is followed by an overview of stem cell biology, omitting specific details on adipose derived stem cells (which can be found in Chapter 1). This is followed by background on the animal models used in this thesis, highlighting the use of transgenics, finishing with a rationale for our dural injury model.

Adipose Biology

Adipose tissue (better known as fat) arises from the mesoderm (83) and consists of lipid-rich cells called adipocytes. It is found at various sites in the mammalian body, specifically in areas of loose connective tissue. The primary function of fat is surplus energy storage in the form of lipids, however, it also plays a role in body insulation and mechanical support (84). Moreover, fat is the body's largest endocrine organ (85) due to its ability to secrete a vast array of adipokines, from hormones like leptin (86) and adiponectin to inflammatory cytokines like tumor necrosis factor-alpha (TNFα) (87). Although depot-specific differences exist in fat (88,89), its main classification is by colour: white adipose tissue (WAT) and brown adipose tissue (BAT). A general overview of the development and characteristics of brown and white adipose can be seen in **Figure 1**.

WAT is the predominant form of fat in the body (subcutaneous to visceral) and contains a multitude of cells known as the stromal vascular fraction. This fraction contains pre-adipocytes (which give rise to adipocytes), fibroblasts, and immune cells like macrophages and leukocytes (90). Due in part to this heterogenous pool of cells, WAT plays a significant role in mediating metabolism and inflammation (87); the former including functions like cell differentiation and energy homeostasis and the latter including inflammatory control and neo-angiogenesis (90). White adipocytes are variably sized spheres containing a unilocular lipid droplet (91,92), which store and release energy as triglycerides and free fatty acids, respectively (84,91).

BAT emerges earlier than WAT during development (85), is abundant in neonates, and is found mainly around the neck and larger blood vessels of the thorax (84,91). BAT is specialized to quickly use its triglyceride storage to generate heat in a process known as adaptive thermogenesis

(93) or “nonshivering” thermogenesis (94). This specialization helps neonates avoid temperature loss. Although BAT is still present in adults (to a lesser degree) (95), this specialization appears to be lost as additional strategies to keep warm are established during maturation (84,91). Moreover, the distribution of BAT seen in adults (cervical, paravertebral, supraclavicular, axillary, upper abdomen) indicates a potential role in warming blood supply to vital organs (96). BAT has a greater oxygen demand (91), therefore it is found to be highly vascularized and innervated (97). In contrast to white adipocytes, brown adipocytes have multilocular lipid droplets and a significantly greater number of large mitochondria, allowing for an increased oxidation rate (97). The high vascularization and mitochondria count taken together can be attributed for the brown colour of this tissue (98).

In the inner mitochondrial membrane, a proton gradient exists wherein a flow of these protons generates adenosine triphosphate (ATP). The dissipation of heat seen in brown adipocytes is due to uncoupling protein 1 (UCP1) in the inner membrane of the mitochondria providing protons in these cells with a “short-circuit” route where the proton flow does not synthesise ATP (94). UCP1 is a defining brown adipocyte marker.

Studies looking at the possibility of WAT gaining BAT features have been conducted (99,100), terming this process as browning. Brown adipocytes found in WAT are called beige or brite. Subcutaneous WAT is the most prone to adopting a beige phenotype (92). Beige adipocytes are distinct from both white and brown adipocytes (101) and have the ability to strongly upregulate UCP1 expression and increase oxygen consumption upon adrenergic innervation or cold (102). Interestingly, brown and beige adipocytes do not share a common precursor; brown adipocytes are derived from *Myf5* expressing precursor cells (shared with muscle) (103) whereas progenitor cells in WAT give rise to beige adipocytes (104). This idea implies that these cells may behave differently in response to normal physiological cues, or insult.

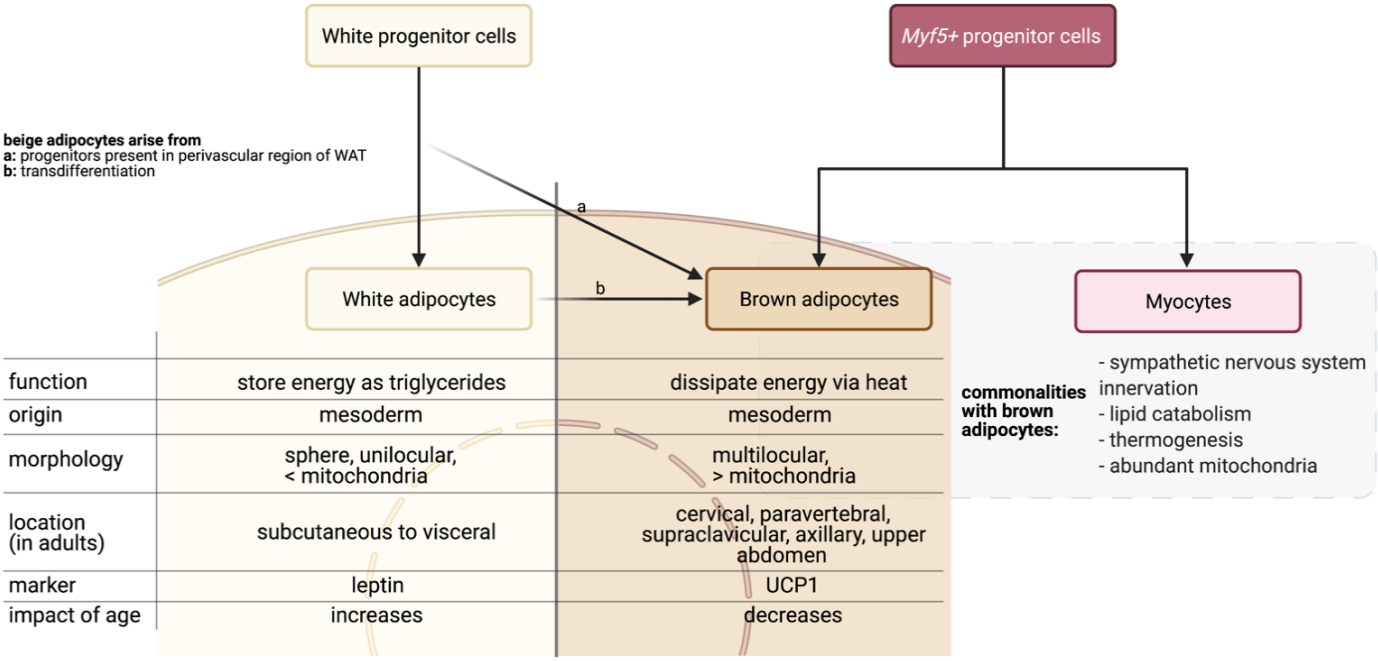

Figure 1. The development of white and brown/beige adipocytes and their characteristics. Although beige adipocytes have been seen to arise from adipocyte progenitors in the perivascular region of WAT, it remains unknown if white adipocytes can also transdifferentiate in to beige adipocytes (83,91,92,94,98).

Stem Cells

There are two defining properties of a stem cell: (1) it must replicate in order to replenish its pool (**self-renewal**), and (2) it must commit and give rise to other cell types/lineages (**differentiation**). Although self-renewal is not distinct from the regular process of cellular division in which a parental cell becomes two daughter cells, stem cells can give rise to cells that are not always identical. Symmetrical division of a stem cell gives rise to two more stem cells (stem cell expansion), or to two progenitor cells (stem cell depletion). Asymmetrical division will give rise to one of each. Generally, stem cells during early development will display symmetrical self-renewal whereas adult tissue stem cells will divide asymmetrically (105).

A stem cell's differentiation potential is known as its potency. The different types of potency from highest to lowest are

- Totipotent: can differentiate into any cell type of the embryo or adult, as well as extraembryonic tissue
- Pluripotent: can differentiate into cell types of all three germ layers
- Multipotent: differentiation becomes limited to a single germ layer or distinct tissue
- Mono- or uni-potent: can give rise to a single cell type

The zygote and the cells produced from cell division immediately after fertilization are the only true totipotent cells that occur naturally in mammals. However, because these cells do not self-renew, they are not stem cells by definition (106). By the time the cells reach the blastocyst stage, they are pluripotent. As cells continue to divide and specialize, they further lose their potency and become multipotent. By this stage, cells are limited in both self-renewal and differentiation (in comparison to the stem cells with higher potency) (105,107). Stem cells are typically split in two broad classes: embryonic and adult (or somatic) (106).

When stem cells commit to differentiation, they give rise to progenitors. Some in the field use the term progenitor interchangeably with stem cells; however, this is technically incorrect. Progenitors are an important link between a stem cell and its fully differentiated fate. As stem cells represent a negligible population of cells in adult tissues and it is extremely difficult to identify cells that meet the formal definition of stem cells *in vivo*, the term progenitor is likely to be correct when

examining a group of stem/progenitor cells *in vivo*. Progenitors retain similarities with the parental stem cell, however, are more specified (or fate determined) and thus typically demonstrate a reduced differentiation capacity. They are early derivatives of stem cells, predestined for differentiation, and essentially expand cell number (108). Hence, progenitors have come to be known as transit amplifying cells (TACs). As TACs differentiate, they also progressively lose proliferative ability (105). A general overview of stem cell characteristics and potency can be seen in **Figure 2**.

Embryonic Stem Cells (ESCs)

ESCs are pluripotent and isolated from the inner cell mass of the blastocyst (pre-implantation embryos). They can perpetually divide symmetrically (proliferation) even though the cells from which they originate are transient. It is important to note that no totipotent or pluripotent cells exist within the adult body under normal circumstances (105–107). While differentiation is often considered a unidirectional process *in vitro* and in *vivo* where differentiated cells cannot convert back to their stem cell origin, somatic cells can be converted back to a pluripotent state *in vitro* under artificial conditions in a process known as reprogramming. This process was discovered in mice in 2006 by researchers Takahashi and Yamanaka (109,110). Principally, gene expression in adult cells is altered as to recapitulate an early developmental state (111), however this process is slow and not as efficient (109). There are two main approaches to reprogramming:

1. Therapeutic cloning: an adult cell nucleus is injected into an enucleated fertilized egg. Proteins and other soluble factors in the cytoplasm of the oocyte are believed to affect the gene expression profile of the injected nucleus thus reprogramming/reverting it back to that of an embryonic stem cell. These cells are called somatic cell nuclear transfer embryonic stem cells (105).
2. Introduction of Yamanaka factors: Three (Oct3/4, Sox2, Klf4c) (112) or four transcription factors (above three and also c-Myc) known to maintain pluripotency in embryonic stem cells are introduced in an adult cell to alter gene expression/regulate transcription. These cells are called induced pluripotent stem cells.

Because of their ability to differentiate in to any cell type of the adult body, embryonic stem cells have great therapeutic potential for disease modeling and drug development (113), as well as

regenerative medicine applications such as restoration of damaged tissue and cells (114). However, there is a stigma associated with the derivation of embryonic stem cells (110), particularly revolving around the "destruction" of human embryos (115). This concern has largely been overcome with the discovery/use of induced pluripotent stem cells which have now been generated from mice, humans, cows, cats and birds to name a few (110). Regardless, tumorigenicity, immune response and undesired differentiation remain safety concerns in the clinical translation of these cells (107,115).

Adult Stem Cells

Adult stem cells are multipotent (or unipotent) and found in specific postnatal tissues, and are believed to retain some level of 'epigenetic memory' of their tissue or origin (116). This can be observed in what is known as differentiation bias, an example being that stem cells derived from fat tissues show a greater propensity to differentiate into adipocytes (117,118). Adult stem cells typically divide asymmetrically and infrequently (105). The two main types of adult stem cells in the mesodermal lineage are hematopoietic and mesenchymal (107). Previously, it was believed that adult stem cells serve as a replenishment pool only for the specific cells of the tissue wherein they reside, however greater plasticity in these cells has been observed, such that tissue-specific adult stem cells can give rise to cells from other lineages; for example, bone marrow or hematopoietic stem cells can give rise to cells of muscle, endothelium, liver, and brain (119–125) *in vitro*. Moreover, a "pluripotent" stem cell was isolated from rat bone marrow which was capable of differentiating into cells of the three germ layers (5). While numerous studies have witnessed trans-differentiation across germ layers *in vitro*, little corroborating *in vivo* evidence exists, potentially suggesting that some of these observations may result from signal transduction aberrations due to non-physiological culture environments.

Mesenchymal Stem Cells (MSCs)/ Mesenchymal Progenitor Cells (MPCs)

MPCs have been isolated from various tissues including bone marrow stroma, blood, bone, adipose tissue, synovium, muscle, and placenta (126). The International Society for Cellular Therapy proposes a minimal criteria to define human MPCs: plastic-adherent, capable of trilineage differentiation (in to bone, cartilage and fat), express surface markers CD105, CD73, CD90, and lack the expression of CD45, CD34 and CD14/19 (127) (CD44, CD29, CD105, Sca-1, and CD34,

TER-119, CD45, CD11b respectively in mice) (128). Because MPC isolation is not accompanied by the same ethical and moral concerns associated with embryonic stem cells, and because MPCs are present in many tissues throughout the body, expand *in vitro*, have a negligible risk of teratoma formation *in vivo* (129), and can differentiate in to multiple mesodermal lineages, they are seen as a powerful tool in regenerative medicine approaches for the treatment of numerous chronic and acute disorders, particularly skeletal tissue damage and inflammatory diseases (130).

Research investigating the microanatomical location of MPCs *in vivo* has shown that these cells are commonly found adjacent to blood vessels in various tissues and organs of the body (131), a trait that is not unique to MPCs but applicable to other multipotent progenitors as well (132). This association was due to the fact that many pericytes (or mural cells; cells around capillaries) display MPC features. Pericytes and MPCs are not interchangeable however, as pericyte is a vague term referring to heterogenous cells adjacent to capillaries alone, whereas MPCs are also found near other blood vessels, such as arteries and veins (132).

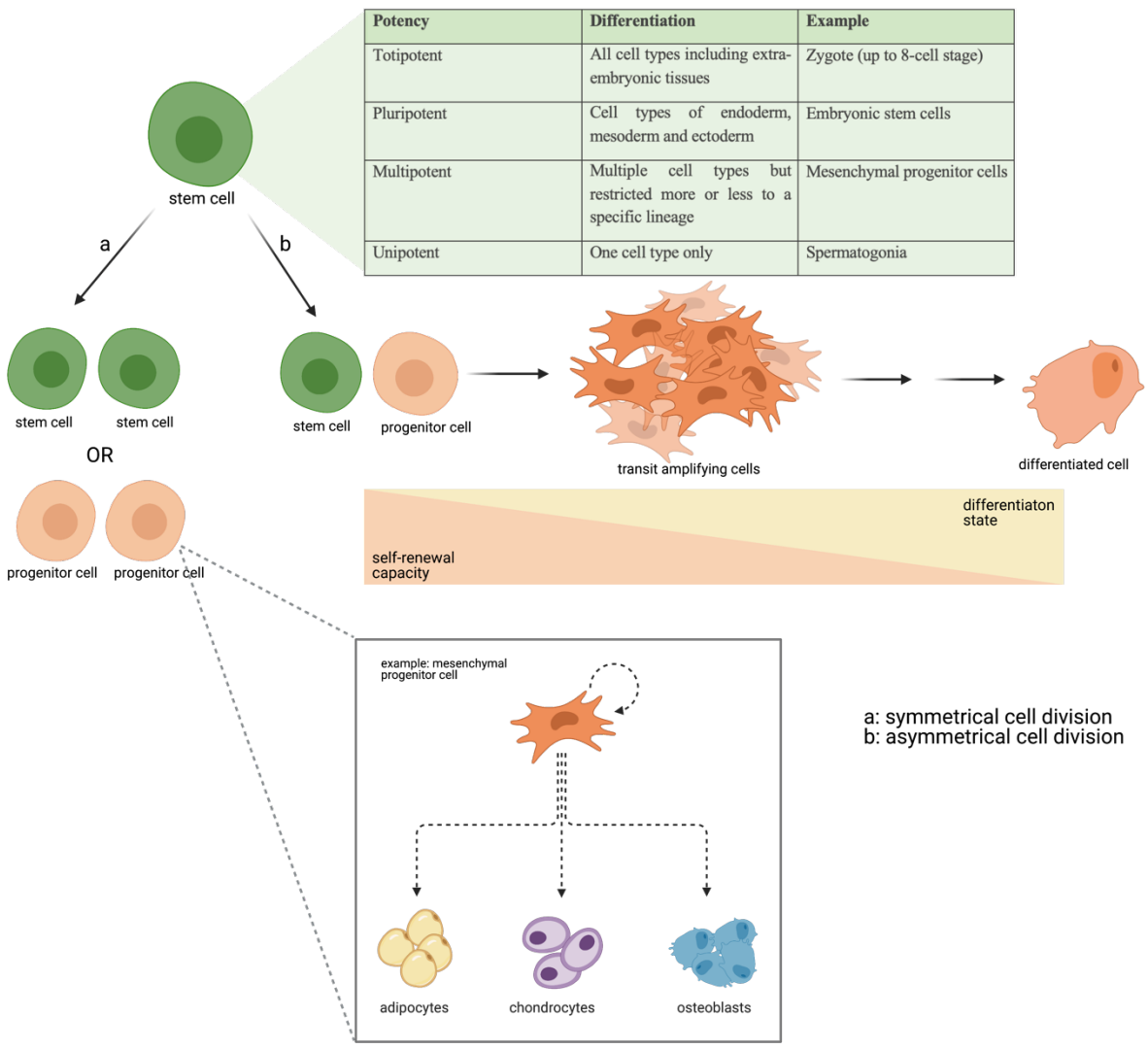

Figure 2. General overview of defining stem cell characteristics and potency. As a progenitor cell moves towards its differentiated fate via transit amplifying cell intermediates, proliferative capacity declines at every step whereas state of terminal differentiation increases.

Mouse Models

Genetic modifications in mice allow for the study of both development and disease and are generated through the deletion and/or insertion of genes in specified regions of the genome. The resultant animal of these types of genetic modifications are collectively referred to as transgenic mice (133). One method to identify and track MPC lineages *in vivo* is to employ genetically modified reporter mice. These animals carry a reporter gene inserted into their genome under the control of an endogenous gene promoter known to be specific/enriched in MPCs. Therefore, the reporter's expression also becomes regulated by these MPC specific factor(s). A commonly used class of reporters are fluorescent proteins such as the well-known green fluorescent protein (GFP), which was identified in jellyfish that encodes for a protein which emits green fluorescent light when examined via fluorescent microscopy. Therefore, one can identify MPCs *in vivo* using these types of lineage markers in mice that have been genetically modified with the insertion of reporter genes.

While there are many different MPC marker genes, controversy remains over which genes mark which types (or all) MPCs. One commonly used MPC lineage marker gene is Paired related homeobox gene-1 (*Prx1/Prrx1)*. *Prx1* is a paired-type homeobox transcription factor, a class of transcription factors that control development and differentiation (134) and are essentially master regulators of morphogenetic processes across species (135). *Prx1* is a transcription co-activator highly enriched in developing mesodermal tissues (e.g., limb buds) (136), is found in the ectomesenchyme of the face (137), and is also expressed in adult tissues like the heart (138), and regulates neural progenitors stemness(139). Targeted mutation of *Prx1* has shown the essential role this gene plays in regulating limb skeletal development such that disruption led to perinatal death with limb/craniofacial deformations (140). *Prx1* expression has also been identified in MPCs capable of differentiating into bone (141), cartilage (142) and fat *in vivo* (82). In relation to this thesis, *Prx1* has been previously shown as a robust adipose MPCs marker in white adipose tissue (82). Moreover, along with (*Prx1*$^{+}$) presumptive MPC expression in the dura mater, *Prx1*$^{+}$ cells have been identified in mouse epidural fat (143). Therefore, in this project, a *Prx1* reporter lineage tracing mouse that has GFP expression under the control of the *Prx1* reporter was employed to visualize *Prx1*$^{+}$ cell expression in epidural fat and in the dura mater.

Another lineage marker, hypermethylated in cancer 1 *(Hic1*), is a transcription factor gene ubiquitously expressed in normal tissues, however in cancer cells, it is hypermethylated and under expressed (144). *Hic1* is a tumour suppressor which has been found to suppress growth and viability in tumour cells (144). Upon deletion of the entire *Hic1* coding region, the involvement of *Hic1* in developing structures was defined; Several developmental defects (craniofacial/limb defects, underdeveloped ear) were found in these *Hic1*$^{-/-}$ mice (145). Moreover, *Hic1* is transcriptionally regulated by many cell cycle genes such as *p53* (146), *p21* (147) and *E2F1* (148), highlighting its role in cell cycle regulation. Recently, *Hic1* was identified in MPCs presented within skeletal muscle, yet was found to only be expressed in quiescent MPCs (149). *Hic1*$^{+}$ MPCs give rise to transit-amplifying cells that support regeneration post-injury (149). Moreover, it has been found that *Hic1* deletion in mice affects skeletal muscle regeneration. As MPCs prove to be vital in tissue homeostasis and regeneration (149), a *Hic1* reporter lineage tracing mouse that has tdTomato (red fluorescent protein) under the expression of *Hic1* will be used in this study, as their contribution to mature mesenchymal lineages (149,150) in the vertebral environment can be investigated *in vivo*.

Prx1$^{\text{CreERT2-GFP+/+}}$R26R$^{\text{tdTomato+/+}}$

Prx1$^{\text{CreERT2-GFP+/+}}$: in these mice, a transgene was inserted which contains 2.4 kb of the mouse *Prx1* gene promoter. Under control of the *Prx1* promoter is an IRES-GFP cassette and a CreERT2 fusion gene, which encodes Cre recombinase (Cre) fused to a mutant estrogen ligand-binding domain (ERT2). When Cre is fused to ERT2, the Cre is inactive and only in the presence of the estrogen agonist tamoxifen (and not endogenous estrogen), is the Cre released to become active and enter the nucleus (151). Mice expressing Cre can mediate Cre-*loxP* recombination, where genetic sequences between two *loxP* (DNA recognition) sites will be excised. These mice are bred to the following mouse to generate the *Prx1*$^{\text{CreERT2-GFP+/+}}$R26R$^{\text{tdTomato+/+}}$ strain.

R26R$^{\text{tdTomato+/+}}$: these mice contain the tdTomato reporter gene under the control of the strong, synthetic CAG promoter used to drive increased gene expression. This transgene is present in the Rosa26 locus of the mouse which is thought to be resistant to gene silencing (e.g., always active; constitutive). Between the CAG promoter and the tdTomato reporter gene is a STOP cassette flanked by two *loxP* sites. The STOP cassette blocks the activity of the CAG promoter so that tdTomato is not expressed. After Cre mediated recombination, the STOP cassette is excised,

allowing for the permanent expression of tdTomato. Post-tamoxifen induction, if the cells continue to express *Prx1,* GFP and tdTomato expression will be co-localized to give the impression of a yellow cell. Once the *Prx1*$^{+}$ cell has committed to a specific lineage, it will lose *Prx1* expression (GFP) and therefore these now *Prx1*$^{-}$ cells will still be permanently marked with tdTomato. See **Supplementary Figure 1** in Chapter 3.

Hic1$^{CreERT2+/+}$R26R$^{tdTomato+/+}$

The *Hic1*$^{CreERT2+/+}$ mice were generated by introducing an CreERT2 in to the 3' untranslated region of the *Hic1* gene. For lineage tracing purposes, these mice were bred to the R26R$^{tdTomato+/+}$ to generate the *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ mouse strain (149). Like the *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$ mouse, a STOP cassette flanked by two *loxP* sites restricts the expression of the tdTomato reporter gene until tamoxifen is administered. After Cre mediated recombination, the STOP cassette is excised, allowing for the permanent expression of tdTomato. In this mouse however, *Hic1* expressing cells and their differentiated progeny will both express tdTomato. See **Supplementary Figure 2** in Chapter 3.

Prx1$^{CreERT2\text{-}GFP+/-}$ R26R$^{tdTomato+/-}$R26R$^{DTA+/-}$ and *Hic1*$^{CreERT2+/-}$ R26R$^{tdTomato+/-}$R26R$^{DTA+/-}$

Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$ and *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ mice were bred to R26R$^{DTA+/+}$ to generate these strains. The R26R$^{DTA+/+}$ mouse contains a *loxP*- flanked STOP cassette linked to an attenuated DTA (diphtheria toxin subunit A), all under the control of a CAG promoter at the Rosa 26 locus. Diphtheria toxin is a cytotoxic protein which binds a particular cell-surface receptor and is subsequently endocytosed via receptor-mediated endocytosis. The enzymatically active A subunit of the protein is then translocated to the cytosol of the cell (152), where it efficiently ablates cell lineages by inactivating eukaryotic elongation factor 2 (an essential factor for protein synthesis) and therefore inhibiting protein synthesis in the cells where it is expressed. This is followed by restriction on DNA and RNA synthesis and energy metabolism, rapidly causing cell death (153). When this mouse is crossed to the *Prx1*$^{CreERT2\text{-}GFP}$R26R^{tdTomato} and *Hic1*^{CreERT2}R26R^{tdTomato} mice (Cre recombinase expressing strains), highly specific cell ablation can be achieved (154). Because mice (and not humans) lack the functional receptor for diphtheria toxin (152), diphtheria toxin cannot be administered to the mice unless the diphtheria toxin receptor is first genetically engineered into the genome. Therefore, the approach in this model is

to genetically encode DTA, which lacks the B subunit which is essential for cell membrane penetration (155). As such, ablation becomes restricted to cells that synthesize the protein only, and neighboring cells remain unaffected by the toxins created/released by the dying cells (156).

Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; *p21*$^{-/-}$

Prx*1*$^{CreERT2\text{-}GFP+/+}$ mice were crossed with *p21*$^{-/-}$ mice (*Cdkn1a*^{tm1Tyj}/J; stock no. 003263 from The Jackson Laboratory, backcrossed with C57BL/6 mice for 10 generations prior to the start of the study) and genotyped to select *Prx1*$^{CreERT2\text{-}GFP+/+}$ *p21*$^{-/-}$ mice. R26R$^{TdTomato+/+}$ mice were crossed with *p21*$^{-/-}$ mice and genotyped to select R26R$^{TdTomato+/+}$ *p21*$^{-/-}$ mice. The two lines were then bred and genotyped to generate *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; *p21*$^{-/-}$ mice. DNA damage within the cell typically induces the expression of *p53*, also known as the guardian of the genome due to its function as a tumor suppressor. *p53* activates a number of pathways to repair the genetic damage and/or trigger apoptosis if the cell is beyond saving. The first step in repairing the genetic damage is to inhibit cell proliferation/DNA replication and *p53* accomplishes this through activation of *p21*. *p53* is a transcription factor with high affinity to binding sites upstream of the *p21* promoter, and this interaction leads to the increased transcription of *p21* mRNA (157). *p21* (*p21*^{WAF1}/Cip^{1}; cdkn) is a 165 amino acid protein which binds to and inhibits the activity of cyclin- cyclin dependent kinase (1/2) complexes (Cdks). Cdks are cell cycle regulators which bind to cyclin, resulting in the phosphorylation of multiple substrates essential for cell cycle progression (158). By regulating Cdks, *p21* acts as a *p53*-dependent cell cycle inhibitor at the G_1 cell cycle checkpoint (159). Under normal conditions, checkpoints regulate cell cycle progression such that disruption to these mechanisms can lead to cell transformation and tumorigenesis. As *p21* promotes cell cycle arrest in response to DNA damage, it is also commonly labeled as a tumor suppressor. Although the canonical function of *p21* is within the *p53/p21* axis, *p21* is involved in many cellular processes, and can also promote anti-proliferative activities via *p53*-independent pathways (159). The non-canonical (i.e., independent of its interaction with Cdks) roles of *p21* include regulation of gene transcription, apoptosis, and DNA repair. *p21* can also maintain stem cell potential (160). A unique functional role of *p21* was recently identified when a *p21* knockout ($^{-/-}$) mouse model displayed increased regeneration capacity (161). Mammals tend to heal wounds through contraction and scar formation. This is contrary to amphibians like the axolotl, which can regenerate entire limbs with the formation of a blastema and inhibition of the fibrotic response

(162). The phenotype seen in the *p21*$^{-/-}$ mouse matched a previous wound healing ability observed in the Murphy Roths Large (MRL) strain which demonstrated increased tissue regeneration in the absence of fibrotic scarring (163). The MRL strain regenerative ability was first observed in 1988, when the MRL mouse was found capable of hair follicle, auricular and articular cartilage regeneration (161). Interestingly, it appeared that MRL mice regenerated tissue through blastema formation at wound sites, suggesting that this mouse may be an exception to the rule that mammals cannot regenerate appendage tissue (162). The MRL phenotype has been attributed to 20 loci on numerous chromosomes, however, it was noted that MRL fibroblasts demonstrated increased cell proliferation, an accumulation of DNA damage and an accumulation of cells at the G2/M checkpoint. All these observations are consistent with the loss of *p21* expression. Therefore, it was decided to examine if the deletion of *p21* could convert a non-regenerating mouse to one that undergoes epimorphic regeneration (formation of blastema). The Heber-Katz group performed this experiment and found that mice lacking *p21* expression developed a regeneration ability similar to MRL mice and possessed the same cellular phenotypes.

Dura Mater

As mentioned before, the dura mater (or pachymeninx) is the tough and fibrous outermost layer of the meninges. Mammalian dura mater is derived from neural crest cells, with infiltration and predomination of paraxial mesoderm cells in the tissue postnatally (164,165). The dura mater is composed of fibroblasts, mesothelial cell-basement lining, and an abundance of collage fibrils (166). It is both vascular and innervated, and although it has two layers (periosteal and meningeal) around the skull, only the meningeal layer extends past the foramen magnum to surround the spine (167), becoming the dural/thecal sac. The meninges appear to be vital for normal brain structure formation, such that deep penetration of meninges can be seen within the brain at every level of organization: from in-between major brain structures to sheaths of blood vessels (74).

Dural Injuries

The dural injury model used in this project was developed in collaboration with Dr. Paul Salo. The mouse vertebral column has 7 cervical (C), 13 thoracic (T), and 6 lumbar (L) vertebrae (168). The iliac crest is commonly seen to cross the midline at the L4-L5 vertebral level (169); using this as an identifier, our dural injuries were performed between L3-L4. An L3 laminectomy (removal of the posterior part of a vertebra) was undertaken to create space by enlarging the spinal canal and relieving pressure (170) prior to focally puncturing the dura mater, allowing easier access in to the canal. Dural injuries were performed in the lumbar region of the spinal canal, and specifically between L3-L4 to minimize any possibility of spinal cord damage and paralysis. The spinal cord does not in fact extend the entire length of the canal. At birth, the spinal cord ends at the L3 level however as bones in the canal continue to grow, the end of the spinal cord reaches L1-L2 by 12 months (171), forming a cone-shaped structure known as the conus medullaris. At this point, nerve rootlets extend out the spinal column (from L2 to the coccyx) creating a bundle of nerves known as the cauda equina (172). These nerves innervate pelvic organs such as the bladder and the lower limbs. The meninges are still present around the conus medullaris however the pia mater tapers off and forms a fibrous strand of tissue- filum terminale- that connects the conus medullaris with the coccyx (172). The thecal sac however surrounds the conus medullaris, the filum terminale and the cauda equina. Thus, an L3 laminectomy creates space for needle insertion between L3-L4, where risk of injury to the spinal cord is minimized, and care is taken to avoid injuring the rootlets of the cauda equina.

Granulation Tissue

Dural injury sites were identified histologically by the presence of granulation tissue in adjacent muscle.

In adult wound healing, there are typically four stages of tissue repair (173):

1. Hemostasis (formation of scab)
2. Inflammation
3. Proliferation
4. Remodelling (formation of scar)

In the proliferative stage, granulation tissue is formed. This is the initial scaffold of tissue that appears after injury and can fill wounds of any size. It is a contractile, connective tissue that is highly cellular and made of extracellular matrix, collagen/elastin and proteoglycans. The main cell types found in granulation tissue are:

- fibroblasts: synthesize and modify copious amounts of extracellular matrix and collagen.
- immune cells: primarily macrophages, neutrophils and leukocytes; these cells phagocytose debris and old tissue meanwhile protect from infections (173).
- endothelial cells: these grow into the site of injury and form anastomoses with other vessels. Angiogenesis is necessary to provide the new tissue with nutrients and remove any waste. Moreover, these cells also assist the fibroblasts by providing them with the oxygen they need to continue proliferating and synthesizing matrix (173).

In all, the three main functions of granulation tissue are immune protection, filling the wound with new tissue/vasculature, and serving as a temporary patch until fibrotic/scar tissue forms (173).

This project was based off the primary literature discussed in Chapter 1 and 2. Below is a summary of the thesis rationale as well as the hypothesis and aims of the project.

Thesis Rationale

In a previous study by Krawetz and Lyons (57), an adult mesenchymal progenitor cell (MPC) population was isolated from human epidural fat, and *Prx1*$^{+}$ cells were found within mouse epidural fat and adjacent dura. Yet, the role of these cells (if any) remained elusive. In this thesis, I explored the possibility that cells originating from the epidural fat are responsible for tissue homeostasis (during growth and post-injury) in the dura mater. The previous literature on adipose derived MPCs from other anatomical sources of fat would suggest MPCs within the epidural fat are a likely candidate to play a pivotal role in the health of the dura mater. Moreover, if these epidural fat MPCs are involved in the growth and/or maintenance of the dura, then these MPCs may also have the potential to respond to injury signals within the vertebral environment. Therefore, the purpose of this thesis project was to study the roles of epidural fat MPCs in regard to the maintenance of the dura mater both under homeostasis and after injury/insult, and to observe what effect the depletion of epidural fat MPCs have on the homeostasis of the dura mater *in vivo*.

Hypotheses

The hypotheses for this project are:

I. *Prx1* and/or *Hic1* expression in the epidural fat marks a population of MPCs *in vivo* which maintain and/or contribute to the growth of the dura mater in mice.
II. *Prx1*$^{+}$ and/or *Hic1*$^{+}$ MPCs (and/or their differentiated progeny) respond to dural injury.
III. *Prx1*$^{+}$ MPCs in a *p21*$^{-/-}$ mouse model display increased cell proliferation *in vivo*.

Project Aims

To test these hypotheses, this thesis is organized in to six main aims. An overview of the techniques used can be seen in **Figure 3**.

1. Characterize epidural fat MPC localization *in vivo* over time during normal growth/maturation (8-34 weeks of age) in both C57BL/6 and *p21*$^{-/-}$ mice.
2. Characterize epidural fat MPC localization *in vivo* in a skeletally mature mouse model (6 months of age).
3. Determine the relationship between cell populations expressing MPC lineage markers- *Prx1* and *Hic1*, and their respective contribution(s) to the dura mater.

4. Genetically ablate epidural fat MPCs and observe consequences on the dura mater.
5. Perform a focal dural injury (puncture) and investigate if epidural fat MPCs (endogenous and exogenous) respond to the injury in both C57BL/6 and *p21*$^{-/-}$ backgrounds.
6. **Pilot Project**: Deliver human epidural fat MPCs to an immune compromised dural injury mouse model to corroborate the role of epidural fat MPCs post-injury and also determine if MPC function is conserved between mice and human.

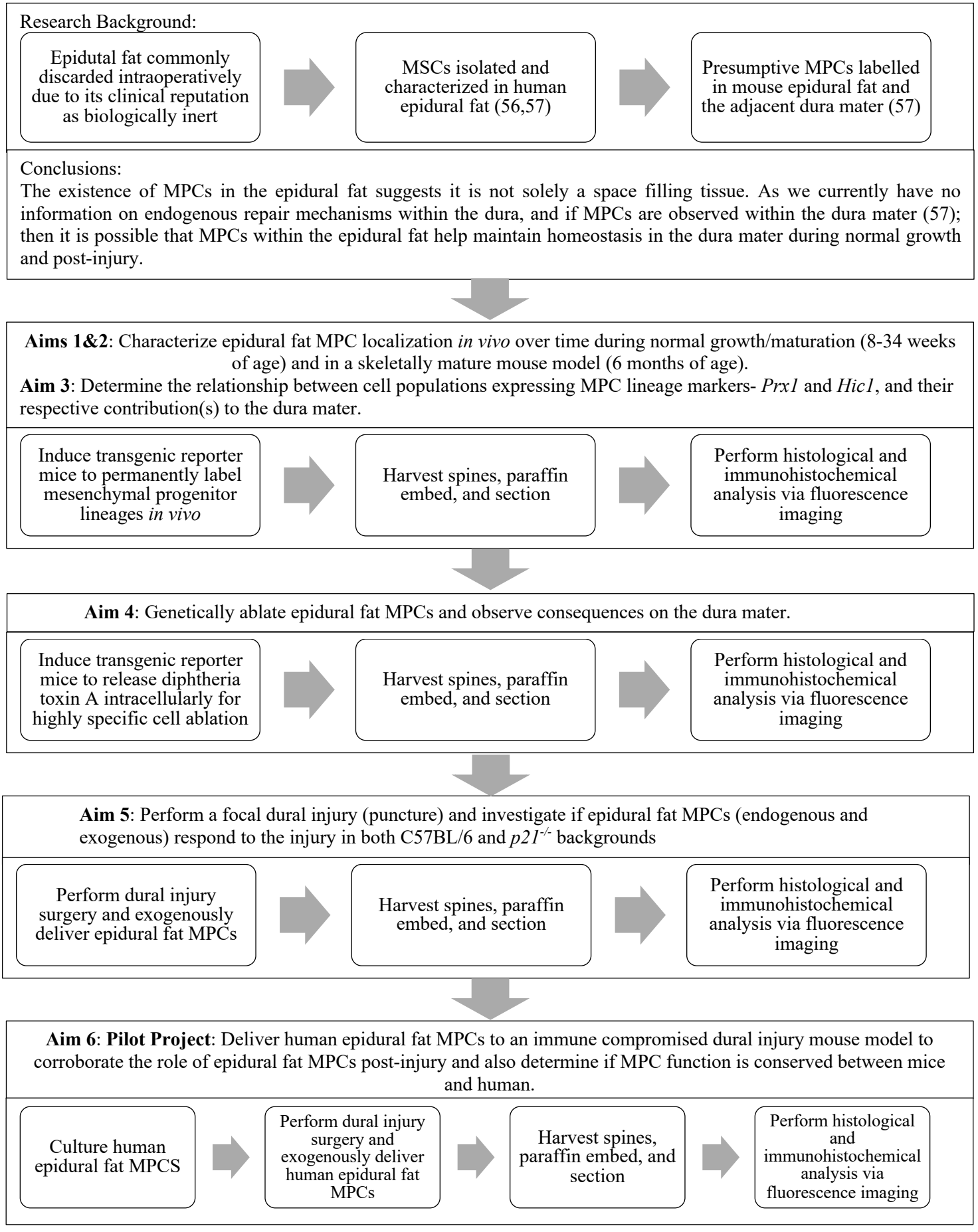

Figure 3. An overview of the techniques used in this thesis.

CHAPTER 3: *Prx1*+ and *Hic1*+ mesenchymal progenitors are present within the epidural fat and dura and participate in dural injury repair

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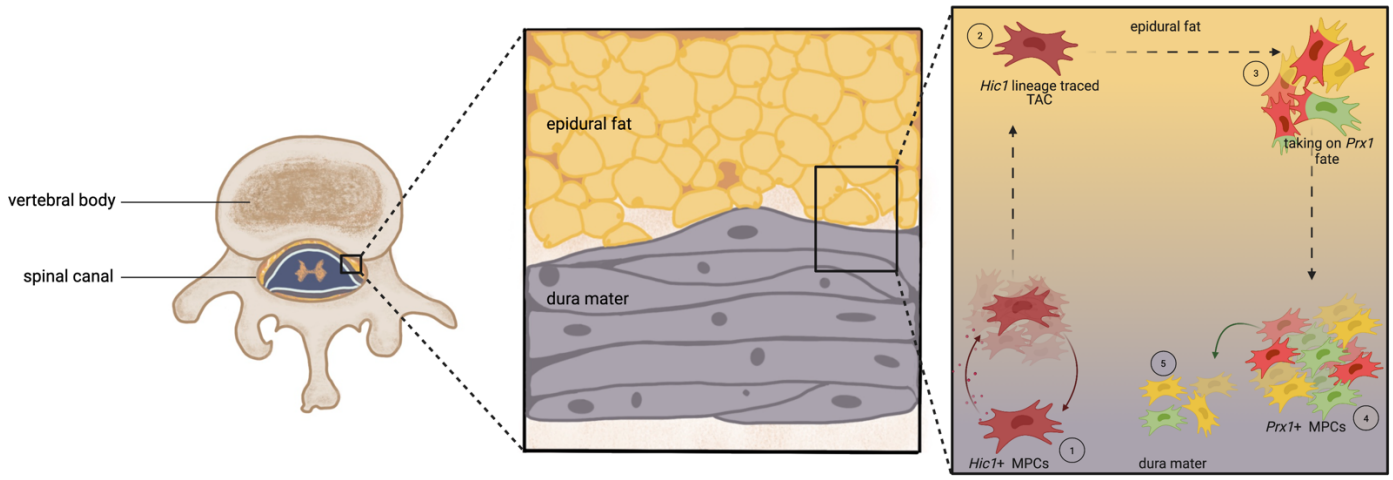
Keywords: Adipose stem cells, adult stem cells, animal models, Cre-loxP system, *in vivo* tracking.

Abstract

Epidural fat is commonly discarded during spine surgery to increase the operational field. However, mesenchymal progenitor cells (MPCs) have now been identified in human epidural fat and within murine dura mater. This led us to believe epidural fat may regulate homeostasis and regeneration in the vertebral micro-environment. Using two MPC lineage tracing reporter mice (*Prx1* and *Hic1*), not only have we found that epidural fat MPCs become incorporated in the dura mater over the course of normal skeletal maturation but have also identified these cells as an endogenous source of repair and regeneration post-dural injury. Moreover, our results reveal a partial overlap between *Prx1*$^{+}$ and *Hic1*$^{+}$ populations, indicating a potential hierarchical relationship between the two MPC populations. This study effectively challenges the notion of epidural fat as an expendable tissue and mandates further research into its biological function and relevance.

Graphical abstract



1. Introduction

Since their discovery in bone marrow by A.J. Friedenstein in 1976 (174), mesenchymal progenitor cells (MPCs) have been identified in almost all human tissues (175). Although tissue of origin confers site specific differences in MPC characteristics - such as morphology, fate commitment biases and immune-phenotype (24), overall, MPCs from all sources retain the ability to self-renew and differentiate into multiple cell types of mesodermal lineage. MPCs also possess bimodal immunomodulatory properties wherein they can enhance cells from both the innate and adaptive immune systems and inhibit the release of pro-inflammatory cytokines in damaged tissue (1,176) Using these properties and likely other mechanisms, MPCs home to, and proliferate in injured/inflamed environments where they promote repair/regeneration, angiogenesis, and cellular recruitment (20). Adipose tissue is an abundant source of MPCs, with less-invasive isolation methods and a higher MPC yield compared to bone marrow (177). As such, MPCs derived from adipose tissue have become a promising tool in regenerative medicine approaches for the treatment of numerous chronic and acute disorders (130).

Recently, a progenitor population—expressing MPC surface markers and capable of trilineage differentiation—was found in human epidural fat (56,57). Using a *Prx1* lineage tracing mouse, it was also found that these epidural fat MPCs populated the dura mater (57). The biological function for epidural fat is still debated and therefore it is commonplace for epidural fat to be discarded during surgical procedures to increase the operational field of view. We have previously questioned this practice and suggested that epidural fat may regulate homeostasis and regeneration in this environment such that perturbation from its original location could lead to degeneration or fibrosis of neighbouring tissues, such as the dura mater (178). Therefore, we undertook the current study using two MPC lineage tracing reporter mice to determine if epidural fat MPCs are an endogenous source of repair and regeneration in the spinal canal and maintain the integrity of the dura mater throughout growth and post-injury.

2. Materials and Methods

2.1 Experimental Outcome: The experimental outline of the study is presented in **Figure 1**.

2.1 Ethics Statement: Animal studies were carried out in accordance with the recommendations in the Canadian Council on Animal Care Guidelines and approved by the University of Calgary Health Sciences Animal Care Committee (AC20-0042).

2.2 Lineage tracing: *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$ (derived from stock no. 029211 and 007914 from The Jackson Laboratory; **Supplementary Figure 1**) and *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ (courtesy of Dr. T. Michael Underhill, University of British Columbia; **Supplementary Figure 2**) reporter mice were employed in this study. The active Z isomer of Tamoxifen ((Z)-4-OHT, *Sigma Aldrich*) was administered to both mice strains intraperitoneally (1mg/injection) for four consecutive days to drive Cre mediated recombination and permanently label the cells with tdTomato. *Prx1* and *Hic1* MPC lineage tracing was performed (mice aged 2 months) at 1, 2, 4 weeks and at 4 months post-tamoxifen induction. Additionally, *Prx1*$^{+}$ MPC lineage tracing was performed on aged mice (6 months) at 1-, 2-, and 4-weeks post-tamoxifen induction. Mice were sacrificed via CO_2 asphyxiation and intact spines removed and fixed for seven days in 10% neutral buffered formalin (NBF; *Fisherbrand*), then decalcified in 10% EDTA (pH=7) for 14 days. After decalcification, samples underwent tissue processing and paraffin embedding. EverBrite™ Hardset Mounting Medium with DAPI (emission wavelength 420-470nm*; Biotium*) was applied to slides and endogenous GFP and tdTomato florescence was assayed using an Axio Scan.Z1 Slide Scanner microscope (*Carl Zeiss*) outfitted with a Plan-Apochromat objective (10x/0.8 M27). The following filters were applied: DAPI (353 nm/465 nm), EGFP (493 nm/517 nm), DsRed (563 nm/581nm).

2.3 Immunohistochemistry: To prepare samples for immunostaining, serial sagittal paraffin sections (10 μm) were deparaffinized in CitriSolv (*Fisher Scientific*) and rehydrated through a series of graded ethanols to distilled water. Next, samples were subjected to antigen retrieval (10 mM sodium citrate, pH 6.0) and blocking (1:500 dilution; 100 μL goat serum:50 mL TRIS-buffered saline, 0.1% Tween 20 (TBST) for 1 hour). steps were performed prior to going through TBST wash and antibody application steps. Antibodies conjugated to fluorophores for cell

proliferation (Ki67 – AF647, Clone # SolA15, *eBioscience*), a dural marker (α-SMA – AF647, Clone # 1A4, *Biolegend*), Hic1 (Clone # H6, *Santa Cruz*), Prx1 (*Novus Biologicals*) were applied at 4°C overnight. Sections were then washed 3 times at 10 minutes/wash in TBST and mounted using EverBrite™ Mounting Medium with DAPI (*Biotium*) for nuclear counterstaining and cover slipped.

2.4 MSC ablation: *Prx1*$^{\text{CreERT2-GFP+/+}}$R26R$^{\text{tdTomato+/+}}$ and *Hic1*$^{\text{CreERT2+/+}}$R26R$^{\text{tdTomato+/+}}$ mice were crossed with R26R$^{\text{DTA+/+}}$ mice (DTA: diphtheria toxin subunit A; stock no. 010527 from The Jackson Laboratory) mice to generate the *Prx1*$^{\text{CreERT2GFP+/-}}$R26R$^{\text{tdTomato+/-}}$R26R$^{\text{DTA+/-}}$ and *Hic1*$^{\text{CreER2+/-}}$R26R$^{\text{tdTomato+/-}}$R26R$^{\text{DTA+/-}}$ strains. (Z)-4-hydroxytamoxifen (1mg/injection) was administered to mice (aged 2 months) intraperitoneally for ten days consecutively to drive Cre recombination, and subsequent release of DTA, to ablate the *Prx1* and *Hic1* expressing MPCs. Spines were harvested 1 day after the last injection of tamoxifen.

2.5 Dural injuries: The injury model was performed on both induced *Prx1* and *Hic1* reporter mice. Mice were anesthetized (isoflurane 3.0vol/vol% with 1L/min O2), the skin of their back shaved and disinfected, and the dorsal aspect of the spinal column exposed at the L3 vertebrae. Paraspinal muscles were mobilized and retracted, with hemostasis secured by bipolar cautery. An L3 laminectomy was performed, and the dura mater focally punctured with a 30-gauge needle. Evident leakage of cerebrospinal fluid was used as indication of a successful puncture. The muscle was repaired with 6-0 vicryl and the skin closed with stainless clips. Mice were sacrificed two weeks post-injury for histology (Safranin-O and Fast Green) and immunohistochemistry.

2.6 Cell enumeration and statistical analysis: Cell counting was performed using a methodology previously described (179,180). Briefly, cells positive for the reporter and/or antibodies of interest were enumerated within two regions of interest (Area = ~1.12 x 10^5 sq. μm): epidural fat and dura mater from n=3 animals at each data point. For each region of interest (per animal), a total of n=3 tissue sections were counted for each fluorescent filter (e.g., EGFP, R-PE, DsRed, APC) or in combination when applicable. GraphPad Prism software (Version 7.0) was used to summarize the variables (mean ± SD) and perform standard descriptive statistics. Significance

was reported at the level of $p \leq 0.05$. Statistical analysis was by one-way or two-way ANOVA (as appropriate) followed by Tukey's *post hoc* testing.

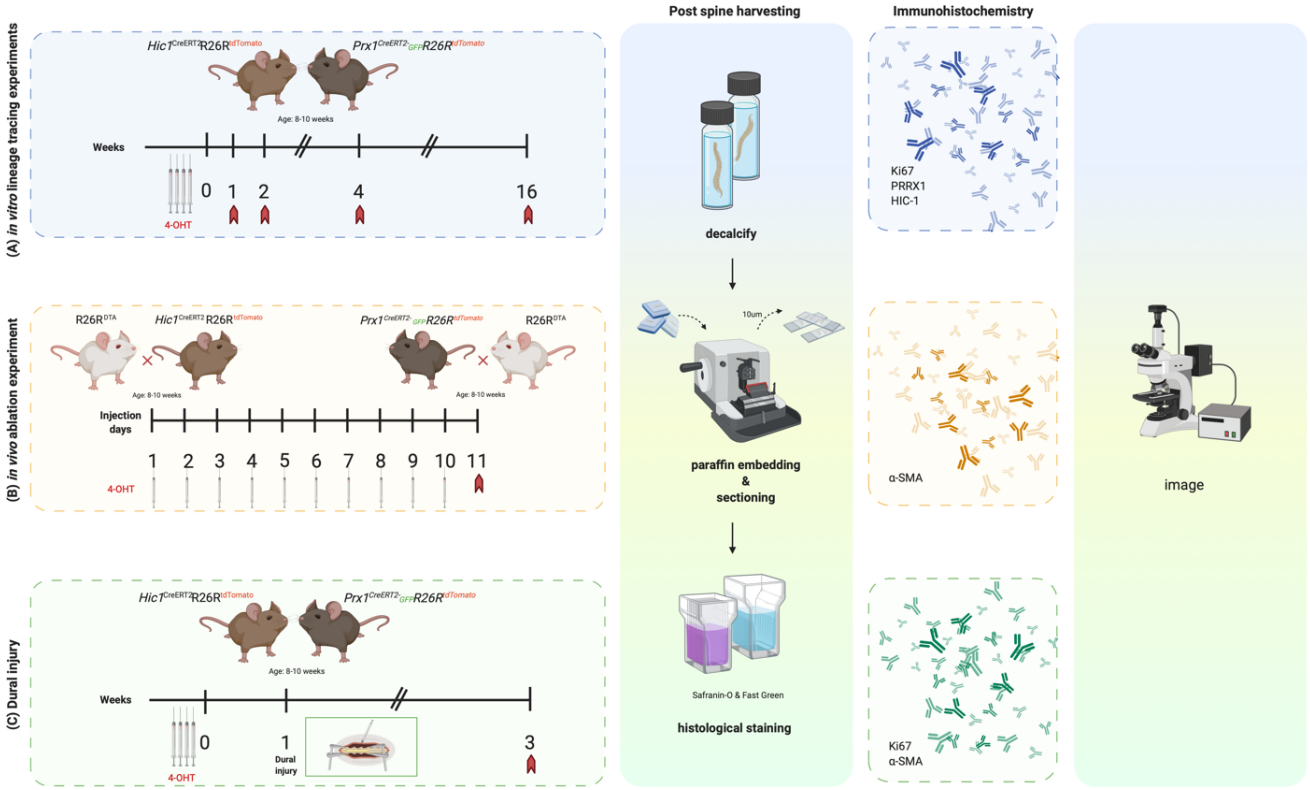


Figure 1. Schematic overview of the experimental design employed in the current study.

3. Results

3.1 Prx1^{+} cells enrich the dura mater over time: Post-tamoxifen induction in the *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$ model, if the cells continue to express *Prx1,* GFP and tdTomato expression is co-localized (yellow cell). Once *Prx1*$^{+}$ cells commit to a fate decision, these cells lose *Prx1* (and GFP) expression, yet retain tdTomato expression. At one- and two-weeks post-tamoxifen induction, few *Prx1*$^{+}$ cells were present within the dura mater (**Figure 2 A,B**), however, by four-weeks post-induction, the dura mater appeared to be primarily comprised of *Prx1*$^{+}$ cells (**Figure 2 C**). While the abundance of *Prx1*$^{+}$ cells was altered in the dura mater over time, the presence of *Prx1*$^{+}$ cells in the epidural fat remained unchanged over the same time period (**Figure 2 A-C**). It is also important to note that the *Prx1*$^{+}$ MPCs within the dura mater remained undifferentiated as they retained expression of GFP (**Supplementary Figure 3)**. These *Prx1*$^{+}$ MPCs were non-proliferative within the dural tissue as Ki67 staining (blue) was absent over the time points examined (**Figure 2 A-C**). Interestingly, in the skeletally mature mouse (6 months old; 4 months post-tamoxifen induction), single *Prx1*$^{+}$ cells were observed interspaced every ~100μm within the dura mater (**Figure 3)**. Similar to lineage traced *Prx1*$^{+}$ cells in the younger mice, these cells also retained GFP expression suggesting they remained in an undifferentiated fate (**Figure 3)**. Yet, in contrast to the earlier timepoints, these cells expressed Ki67, suggesting they were proliferative within the dural tissue (**Figure 3**). To determine if the expansion of *Prx1*$^{+}$ MPCs in the dura mater over time was an effect of growth/maturation or a normal cyclic phenomenon, 6-month-old mice were induced with tamoxifen (**Supplementary Figure 4**). No expansion of *Prx1*$^{+}$ MPCs was observed within the dura mater over time in the aged mouse, instead lineage tracing revealed that the sparse pattern of undifferentiated (GFP^{+}) MPCs remained in the dura mater (**Supplementary Figure 4**).

3.2 Hic1+ MPCs cycle between the dura mater and epidural fat over time: In the *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ model, *Hic1* expressing MPCs, and their differentiated progeny were permanently marked by tdTomato expression post-tamoxifen injection. Unlike *Prx1*$^{+}$ MPCs, *Hic1*$^{+}$ MPCs were present within the dura mater, with little to no tdTomato expression observed in the adjacent epidural fat at one- and two-weeks post-tamoxifen induction (**Figure 2 D,E**). However, by four-weeks post-tamoxifen induction, the adjacent epidural fat was enriched with *Hic1*$^{+}$ MPCs (**Figure 2 F**). Ki67 staining revealed that nearly all the *Hic1*$^{+}$ MPCs within the dura mater were

proliferative (**Figure 2 D**) with the exception of 4 weeks, where no Ki67 staining was present within *Hic1*$^{+}$ MPCs (**Figure 2 F**). At four-months post-tamoxifen induction, *Hic1*$^{+}$ MPCs were once again observed within the dura mater, with minimal tdTomato expression observed within the epidural fat (**Figure 3 C**). Furthermore, these *Hic1*+ MPCs within the dura mater were proliferative as evidenced by Ki67 expression (**Figure 3 C**).

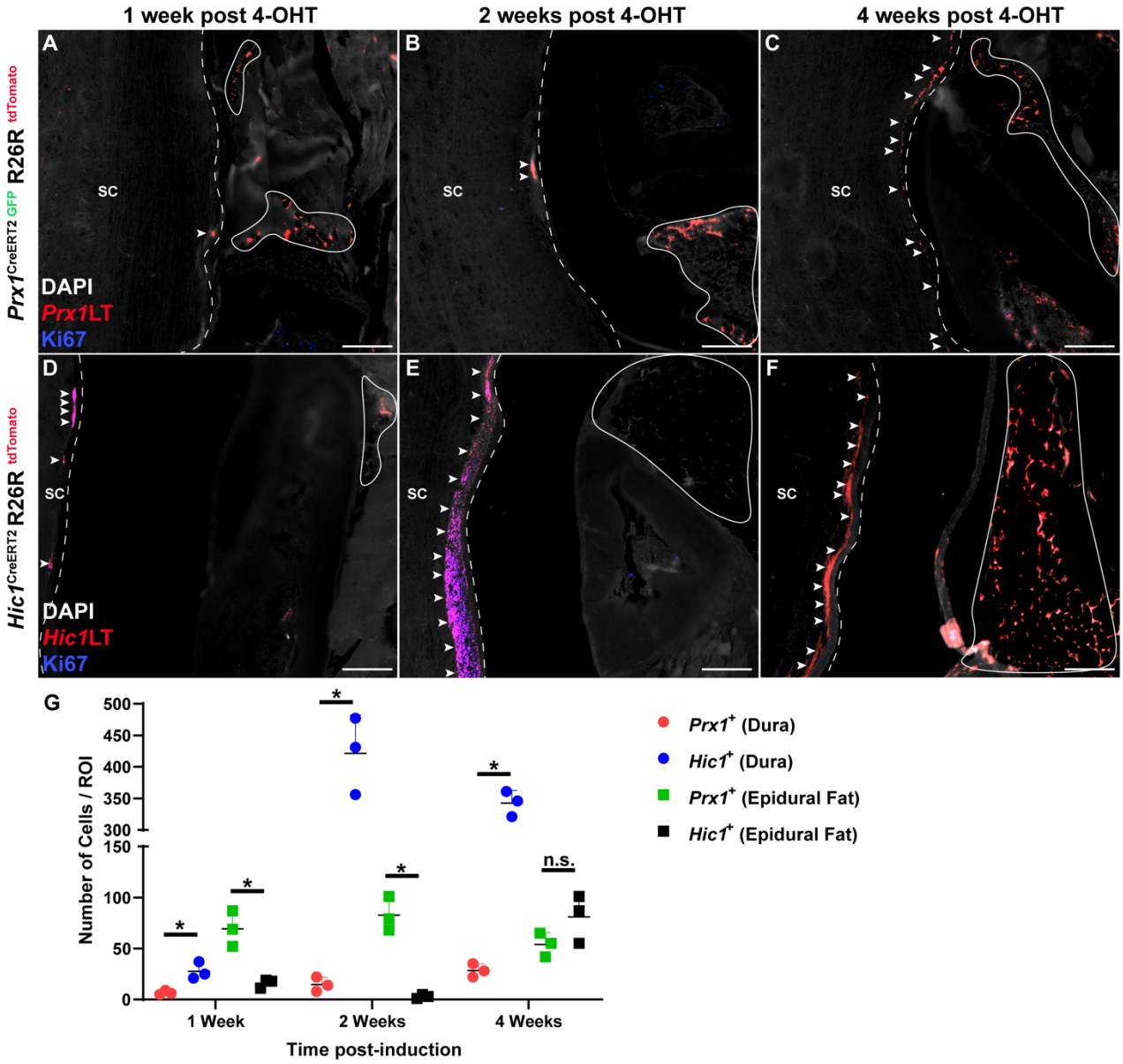


Figure 2. *Prx1*$^+$ (A-C) and *Hic1*$^+$ (D-F) MPC lineage tracing 1,2 and 4-weeks post-tamoxifen induction. Sections were also stained with Ki67 to identify proliferative cells (A-F). The dura mater is highlighted by the dashed line while the epidural fat is encircled by the solid line. Arrows indicate examples of *Prx1*$^+$ and *Hic1*$^+$ cells within the dura mater. Quantification of *Prx1*$^+$ and *Hic1*$^+$ MPCs within the dura mater and epidural fat (G). * $p<0.05$. Scale bars = 100μm; SC= spinal cord.

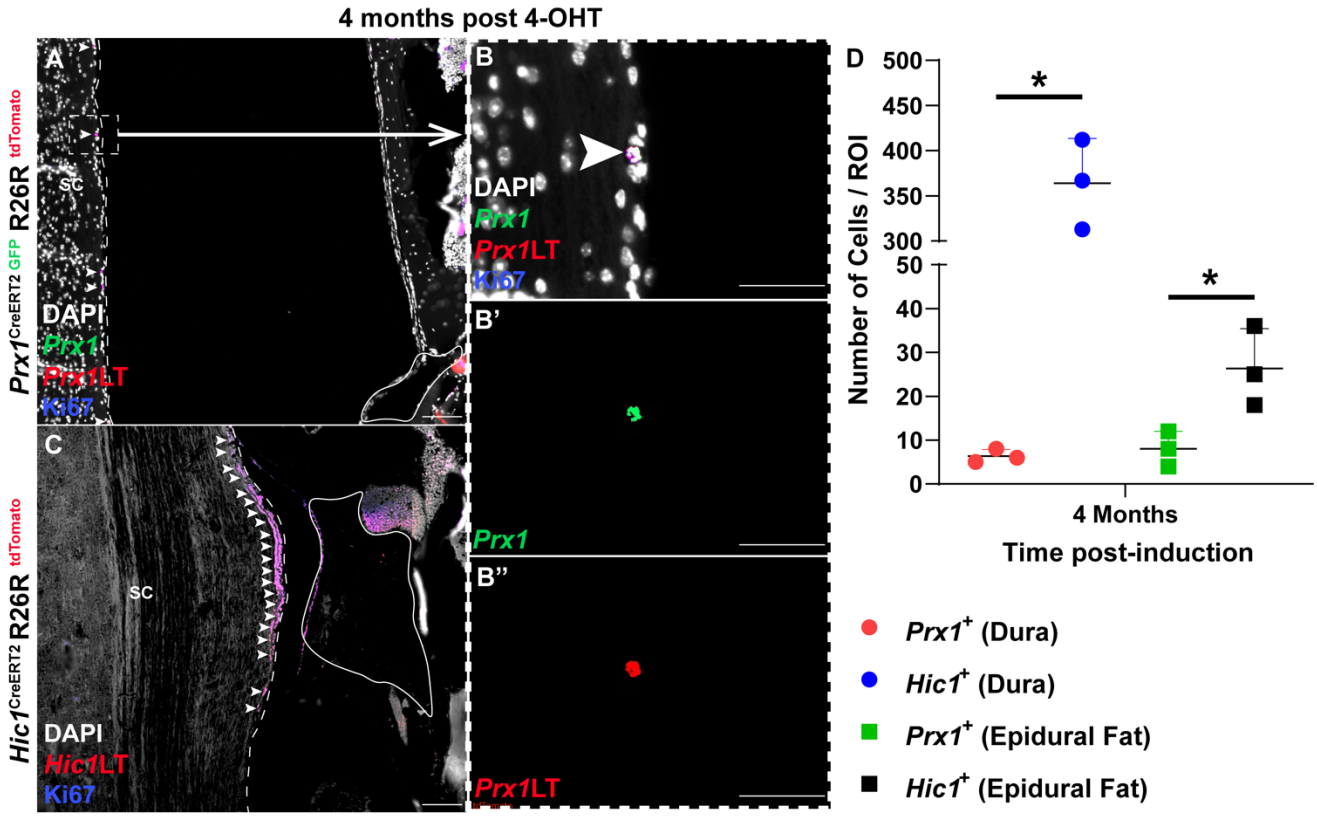


Figure 3. *Prx1*$^+$ (A-B'') and *Hic1*$^+$ (C) MPC lineage tracking (LT) in skeletally mature mice (four months post-tamoxifen induction). Individual GFP (*Prx1*) and tdTomato (*Prx1*LT) channel images (B',B'') are presented to demonstrate that the *Prx1*$^+$ LT cells remain undifferentiated. The dura mater is outlined by the dashed line while the epidural fat is encircled by the solid line. Arrows indicate examples of *Prx1*$^+$ and *Hic1*$^+$ cells in the dura mater. Quantification of *Prx1*$^+$ and *Hic1*$^+$ MPCs within the dura mater and epidural fat (D). * $p<0.05$. Scale bars = 100μm (A,C) and 50 μm (B-B''); SC= spinal cord.

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3.3 Prx1^{+} and Hic1^{+} MPC populations overlap: *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$ spines were stained for *Hic1* (**Figure 4 A-C, Supplementary Figure 5**) and *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ spines with for *Prx1* (**Figure 4 D-F, Supplementary Figure 5**). This was undertaken to determine if there was any overlap between the *Prx1* and *Hic1* MPC lineages during the timepoints examined (1-, 2- and 4-weeks post-tamoxifen induction). In *Prx1* lineage traced mice, only minimal overlap with *Hic1* immunostaining was observed regardless of the timepoint (**Figure 4 A-C**). This same pattern was observed in *Hic1* lineage traced mice with *Prx1* immunostaining (**Figure 4 D-F**). Furthermore, it did not appear that there was difference in co-localization of (*Prx1* with *Hic1* or vice versa) within the dura mater vs. epidural fat. Interestingly, we were able to identify instances of asymmetric cell division in which a lineage traced cell (in this case *Hic1*) gave rise to one cell expressing *Prx1* (or possibly the mother cell was *Prx1*$^{+}$) while the other did not (**Supplementary Figure 6**).

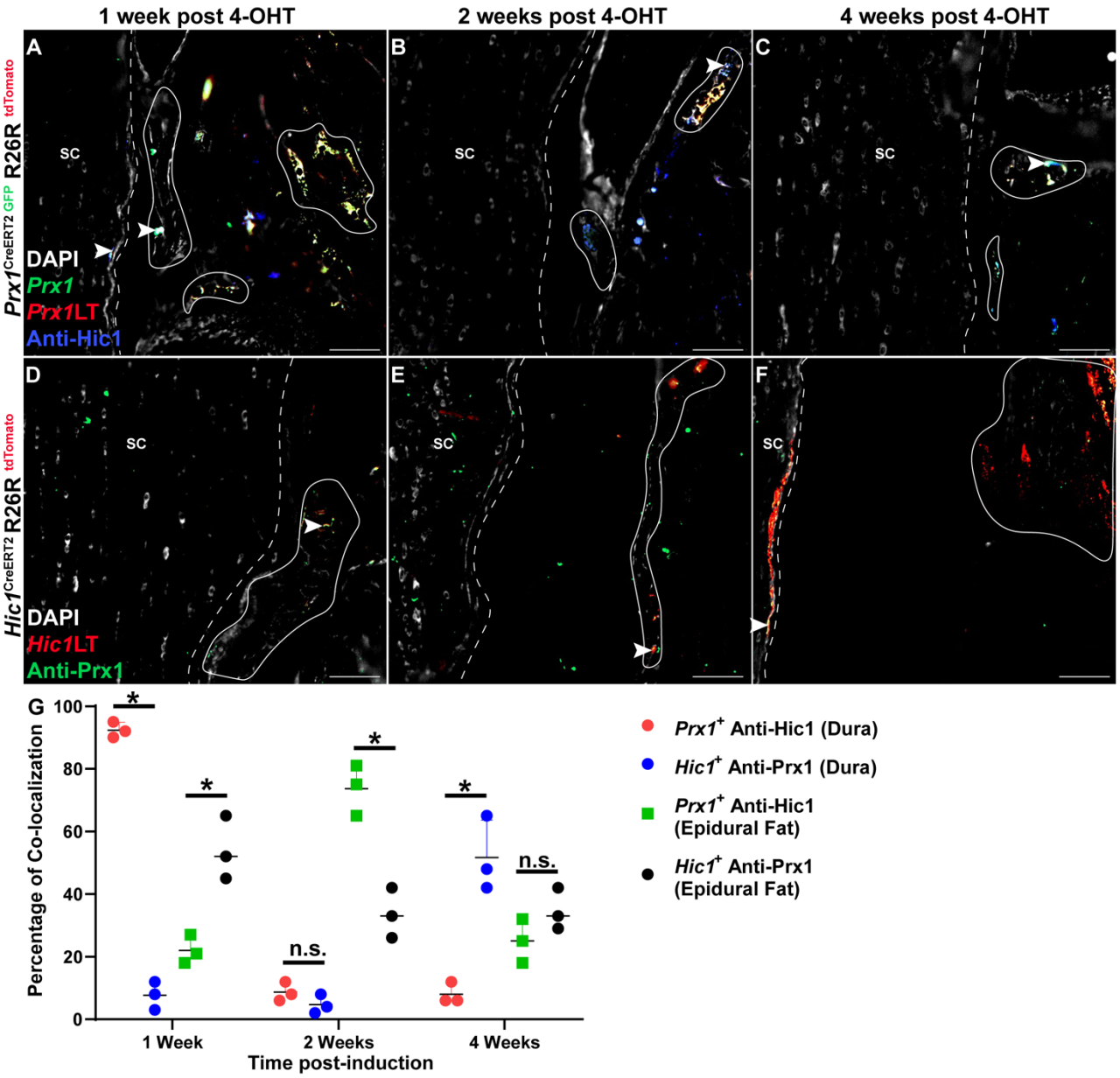


Figure 4. *Hic1* protein expression in *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$ mice (A-C) and *Prx1* protein expression in Hic*1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ mice (D-F) 1,2- and 4-weeks post-tamoxifen induction. The dura mater is outlined by the dashed line while the epidural fat is encircled by the solid line. Arrows indicate examples of colocalization between Prx1 and Hic1. Quantification of percentage of co-localized staining between *Prx1*$^{+}$ with anti-Hic1 staining and *Hic1*$^{+}$ with anti-Prx1 staining within the dura mater and epidural fat (G). * $p<0.05$. Scale bars = 100μm; SC= spinal cord.

3.4 Ablation of Prx1^{+} or Hic1^{+} MPCs results in a loss of α-SMA staining in the dura: While *Prx1^{+} and Hic1^{+}* MPCs were observed in the epidural fat and adjacent dural tissue, it remained unknown if these cell populations play a functional role in this micro-environment. Therefore, *Prx1*$^{CreERT2GFP+/-}$R26R$^{tdTomato+/-}$R26R$^{DTA+/-}$and *Hic1*$^{CreER2+/-}$R26R$^{tdTomato+/-}$R26R$^{DTA+/-}$ mice were employed to ablate these populations and α-SMA immunostaining was undertaken to determine if the loss of these populations has a negative effect on the dura mater and/or epidural fat (**Figure 5**). In the normal dura mater, α-SMA staining is fairly ubiquitous throughout (**Figure 5 A,D)**, however, when *Prx1^{+}* or *Hic1^{+}* MPCs were ablated, nearly all α-SMA staining was lost in the dura mater (**Figure 5 B,E,G)**. However, since genetic ablation is not 100% efficient, there were areas within the epidural fat and/or dura mater which retained *Prx1^{+}* or *Hic1^{+}* MPCs (**Figure 5 C,F,G)**. In these cases, α-SMA expression was observed in the dura mater, however the staining pattern was punctate and discontinuous (**Figure 5 C,F)**. Furthermore, the moderate amount of α-SMA in the dura mater of these animals was only observed in close proximity to remaining *Prx1^{+}* or *Hic1^{+}* MPCs (**Figure 5 C,F)**.

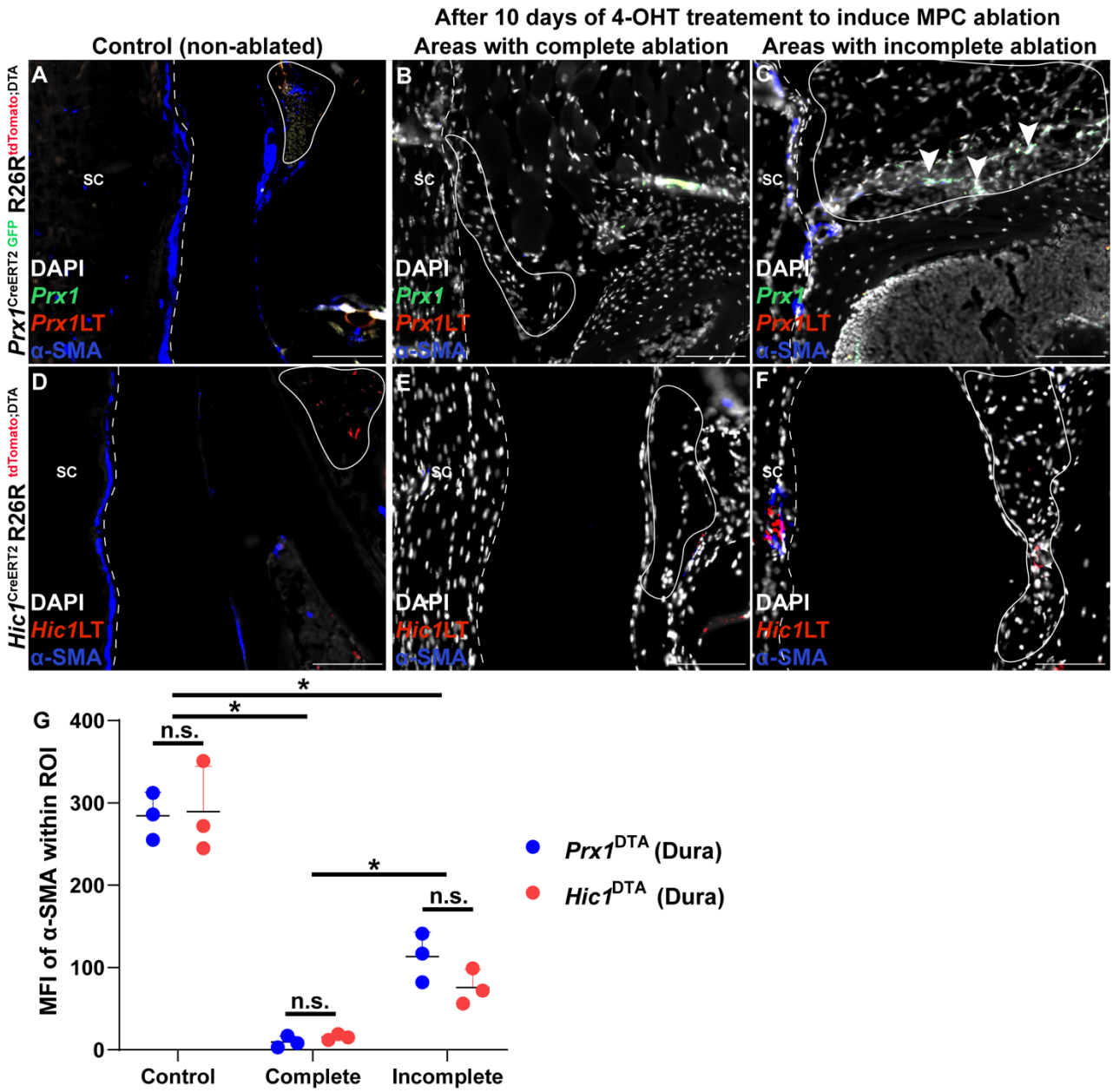


Figure 5. α-SMA expression in the dura mater of wild-type controls (A,D) and after *Prx1* (B,C) or *Hic1* (E,F) MPCs are ablated following 10 days of tamoxifen induction. The dura mater is outlined by the dashed line while the epidural fat is encircled by the solid line. Arrows indicate examples of $Prx1^{+}$ and $Hic1^{+}$ MPCs in the epidural fat. Quantification of mean fluorescent intensity (MFI) of α-SMA expression in $Prx1^{+}$ and $Hic1^{+}$ mice within the dura mater (G). * $p<0.05$. Scale bars = 100μm; SC= spinal cord.

3.5 Prx1^{+} and Hic1^{+} MPCs are found at the site of dural injury: The area of injury was determined by examining Safranin-O-stained slides to identify granulation tissue that resulted due to the surgery (**Figure 6 A,D**). Two weeks post-injury, *Prx1*$^{+}$ MPCs were found within the dural lesion. These MPCs remained in an undifferentiated state since they retained expression of *Prx1* (GFP^{+}) (**Figure 6 B,C**). These *Prx1*$^{+}$ MPCs were non-proliferative as they expressed Ki67 (**Figure 6 B**). However, while α-SMA staining was observed within the area of dural injury, it was not produced by these MPCs as α-SMA staining did not co-localize with GFP expression (*Prx1*$^{+}$ MPCs) (**Figure 6 C**). Similarly, proliferating (Ki67^{+}) *Hic1*$^{+}$ MPCs were also found at the site of injury (**Figure 6 E**), yet these cells also did not express α-SMA staining as no co-localization was observed with tdTomato expression (*Hic1*$^{+}$ MPCs) (**Figure 6 F**). There were no differences in the number of *Prx1*$^{+}$ vs. *Hic1*$^{+}$ MPC within the injury site (**Figure 6 G**).

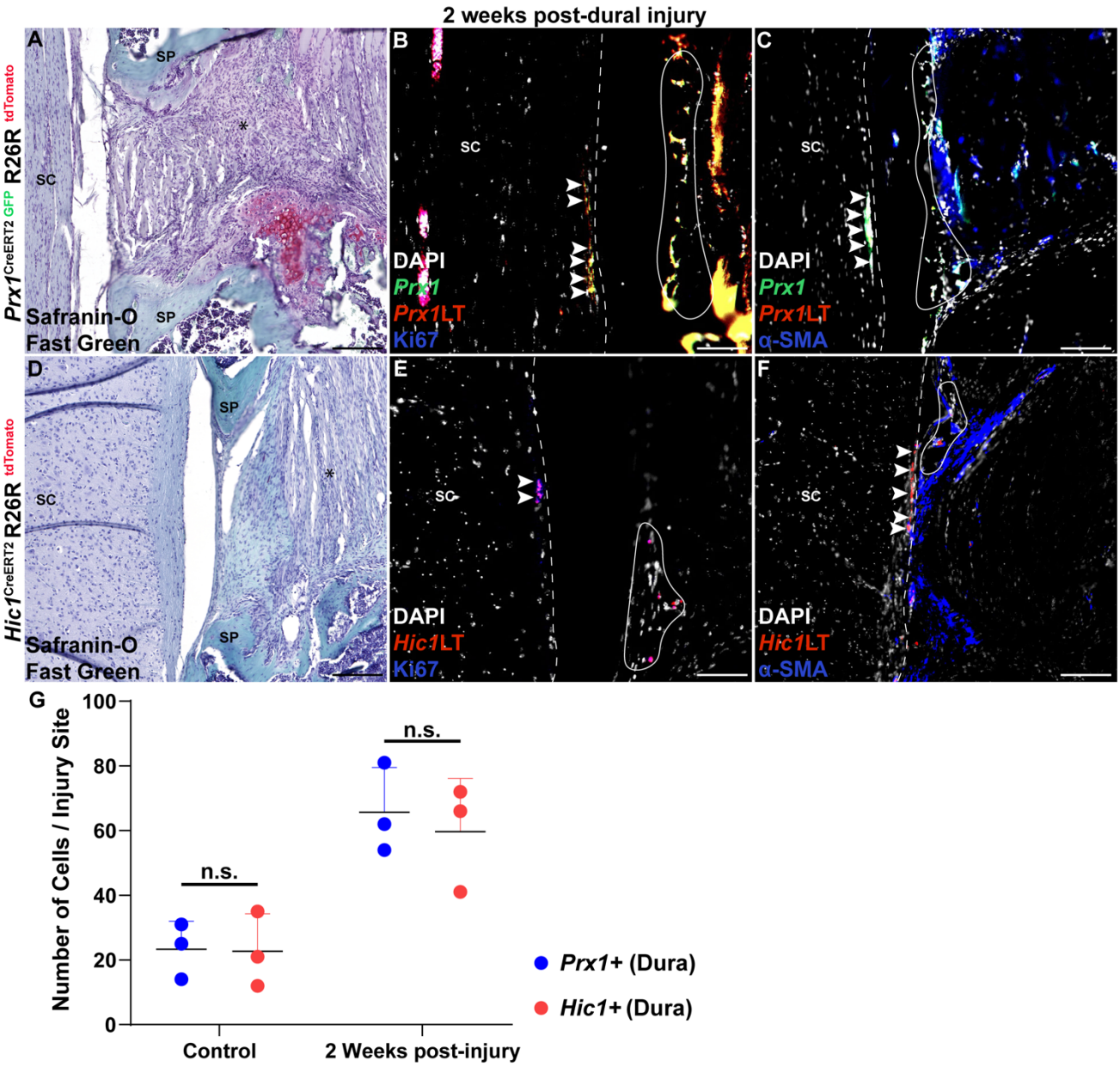


Figure 6. Endogenous repair of injured dura mater in the *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$ (A-C) and *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ mice (D-F). Histological (A,D) and corresponding Ki67 (B,E) and α-SMA (C,F) images are presented. The dura mater is outlined by the dashed line while the epidural fat is encircled by the solid line. Asterisks indicate granulation tissue adjacent to the dural injury (A,D). Arrows indicate examples of *Prx1*$^{+}$ and *Hic1*$^{+}$ MPCs within the dural injury site. Quantification of *Prx1*$^{+}$ and *Hic1*$^{+}$ MPCs within the dural injury site (G). * $p<0.05$. Scale bars = 100μm; SC= spinal cord, SP= spinous process.

4. Discussion

Epidural fat is a low-density white adipose tissue found within the spinal canal (49). Previously hypothesized functions of epidural fat centre around its mechanical properties (such as shock absorption) (55), however these paradigms are not widely accepted by clinicians. Therefore, epidural fat is commonly discarded during surgical procedures. Adjacent to epidural fat is the dura mater, the outermost protective membrane surrounding the spinal cord, which contains the cerebrospinal fluid and plays a crucial role in anchoring and protecting the central nervous system (75). Although we know that *Prx1*$^{+}$ cells are present within epidural fat from lineage tracing experiments (57), the biological relevance of these cells *in vivo* has not been explored. In this study, we have expanded upon the contributions of two MPC populations within the epidural fat / dural micro-environment.

Prx1 (Paired related homeobox-1) is a robust adipose MPC marker (82) and it has previously been demonstrated that it marks presumptive MPCs within murine epidural fat (57). Recently, a novel potent MPC lineage marker, *Hic1* (Hypermethylated in cancer-1) was identified in skeletal/cardiac muscle- (149,150) and skin-derived cells (181). Interestingly, while *Prx1* is a transcription factor involved in early mesodermal fate commitment (136), *Hic1* is a transcriptional repressor (182,183) found to be expressed only in quiescent MPCs (149,184). These *Hic1*+ MPCs give rise to transit-amplifying cells (TACs) that support regeneration post-injury in the tissues examined to date (149,150,181). Since MPCs continue to demonstrate their vital importance in tissue homeostasis and regeneration, *Prx1* and *Hic1* reporter lineage tracing mice were employed in this study, and their contribution to the dural environment investigated. The current study has demonstrated epidural fat and/or dural MPCs contribute to the homeostasis of dural tissue over the course of normal growth and maturation, and that these MPCs are involved in tissue repair post-injury. We have demonstrated that there is an expansion of MPC populations during the growth/maturation period and when these animals reach skeletal maturity, there is a reduction of *Prx1*$^{+}$ MPCs, while *Hic1*$^{+}$ MPCs are maintained within the dura mater. When *Prx1*$^{+}$ and *Hic1*$^{+}$ MPCs are genetically ablated, there is a loss of dural tissue integrity (suggested by the loss of α-SMA expression). Moreover, when there is an injury in the dura mater, these MPCs are found within the injury site. These results strongly suggest that these cells are responsive to cues in the micro-environment and

participate in growth, repair and homeostasis. This behaviour fits with previous studies of MPCs in other tissues and demonstrates that the epidural fat and dura mater have reservoirs of MPCs. We hypothesize that the *Prx1*$^{+}$ MPCs are native to epidural fat (and not the dura) as *Prx1* expression remained consistent in the epidural fat over the time points examined, whereas there was an expansion followed by reduction of this population over time in the dura mater. Furthermore, our data suggests that these *Prx1*$^{+}$ MPCs are likely migrating from the epidural fat to the dura mater instead of proliferating within the dura. This is evidenced by the lack of Ki67 staining in the *Prx1*$^{+}$ MPCs within the dura mater, with the exception of the late timepoint (4 months) in which we believe these cells are slow cycling, which is consistent with MPC in other adult tissues, such as synovium (185). However, to be sure, BrdU/EdU labeling could be employed in future studies. This hypothesis is further supported by the appearance of the sparsely interspaced *Prx1*$^{+}$ MPCs in the dura mater at skeletal maturity; which mimics MPC patterns in other adult tissues wherein the percentage of MPCs is negligible in comparison to the mature, differentiated cells of the tissue (186). For example, long bone growth occurs at the growth plate located between the epiphyseal and metaphyseal bones. Progenitor populations divide in the growth plate and differentiate into chondrocytes that synthesize large amounts of extracellular matrix proteins. Most of these cells eventually undergo apoptosis (187), which is also a possible outcome that explains the loss of *Prx1*$^{+}$ MPCs in the dura by six months of age. Similarly, *Prx1*$^{+}$ MPCs in the epidural fat could be maintaining the dura mater throughout growth and acting as a reservoir in the adult mouse similar to MPCs within other tissues such as periosteum (188). Additional studies will be required to investigate whether *Prx1*$^{+}$ MPCs undergo apoptosis, terminally differentiate, and/or migrate away once the dural membrane reaches maturity.

Interestingly, the localization pattern of *Hic1*$^{+}$ MPCs was nearly inverse to that of *Prx1*$^{+}$ MPCs such that increased *Hic1* expression in the epidural fat was complemented by a decrease in expression in the dura mater over the 1-4-week post-Tamoxifen timepoints examined. Based on these results, it is plausible that these two populations of MPCs are spatially distinct in the spinal canal and possess different fate trajectories. However, at skeletal maturity, and distinct from what was observed in *Prx1* lineage traced animals, the dura mater was once again enriched with *Hic1*$^{+}$ MPCs. This led us to hypothesize that *Prx1* and *Hic1* do not mark distinct progenitor populations in the dural environment, and this was then supported by our finding of colocalization between

Prx1/Hic1 lineage traced cells and *Prx1/Hic1* protein expression. Our results clearly demonstrate that some (but not all) *Prx1*$^{+}$*/Hic1*$^{+}$ MPCs also express the other marker, and we have also seen evidence of asymmetrical cell division in these populations with mother/daughter cells expressing different combinations of *Prx1/Hic1*. However, this poses the question: is there a hierarchy between cells that express one of the two MPC markers and if so, which is the apex MPC marker in this case? Previous studies have shown that MPCs differentiate according to a discrete hierarchical model (6,189,190). In this case, does asymmetrical division in *Hic1*$^{+}$ cells result in the loss or gain of *Prx1* expression (and vice versa), and what significance does this hierarchical relationship hold to the anatomical region under study? Based on the pattern of *Prx1* and *Hic1* expression in the epidural fat and dura mater over time and that *Hic1* marks only quiescent MPCs, while *Prx1* marks committed progenitor populations, we propose a hypothetical model in which *Hic1* identifies MPC with greater potency than *Prx1* (**Figure 7**). In this model, quiescent *Hic1*$^{+}$ MPCs in the dura mater are activated in response to some biological cue (such as growth, injury, maintenance) and begin to proliferate. Once a sufficient TAC pool is obtained, these MPCs exit the cell cycle (**Figure 2F**) after which these TACs migrate to the epidural fat where they commit to a mesodermal fate and gain *Prx1* expression. With normal growth/maturation, these non-proliferative *Prx1*$^{+}$ MPCs migrate back to the dura mater where they remain non-proliferative until the mice reach skeletal maturity at which point, they take an apparent slow cycling phenotype, most likely to maintain this population within the dura mater. While our current data suggests migration, it does not prove that it occurs, therefore, it is also possible that these MPCs enter and leave quiescence in each respective tissue (dura vs. epidural fat) which would result in the absence/presence of *Hic1* expression while most retain *Prx1* expression due to their mesodermal fate commitment. With our current transgenic models, this hypothesis cannot be confirmed as the migration of *Prx1*$^{+}$ and *Hic1*$^{+}$ cells between tissues remains an assumption. However, tracing of transplanted *Prx1*$^{+}$*/Hic1*$^{+}$ MPCs into the epidural fat of a wild-type mouse could provide insight into this assumption.

The similar phenotype observed in the dura mater (loss of α-SMA staining) when both *Prx1*$^{+}$ and *Hic1*$^{+}$ MPCs were ablated also supports the ideas that *Prx1*$^{+}$ and *Hic1*$^{+}$ MPCs are not entirely unique populations and are at the least somewhat functionally similar as they contribute to maintaining the dura mater. Similarly, *Prx1*$^{+}$ and *Hic1*$^{+}$ MPCs both responded to the dural injury. However, the lack of α-SMA staining at the injury site suggests that these cells do not directly

reconstitute the dural tissue (as neither population gives rise to α-SMA^{+} cells), but instead likely play an immunomodulatory and cell-signaling role to promote repair and regeneration, as is widely accepted to be a role of MPCs *in vivo* (1,6,149,191–193). Moreover, *Hic1*+ cells in the wound were proliferative, which is supported by previous research demonstrating that these cells give rise to transit amplifying cells that support wound healing (149).

MSCs/MPCs delivered exogenously in preclinical trials of wound healing have been found to home to sites of injury as a chemotactic response to local influences such as inflammation and hypoxia (194–197). However, systemically delivered MPCs face mechanical barriers in small diameter vessels where they can passively arrest (198), and even when cleared from the blood, MPCs are commonly found entrapped in the lung (196). Specific to our study, the need to repair dural defects has led to the search for a functional substitute which possesses the same physiological characteristics, such as biomechanical properties and fiber architecture, as the dura mater, whilst ensuring biocompatibility and functional integration (76). Our study demonstrates that an endogenous cell source exists that participates in dural maintenance and repair; and that if this cell population could be mobilized, it may have the ability to stimulate repair in patients suffering from dural injuries/pathologies.

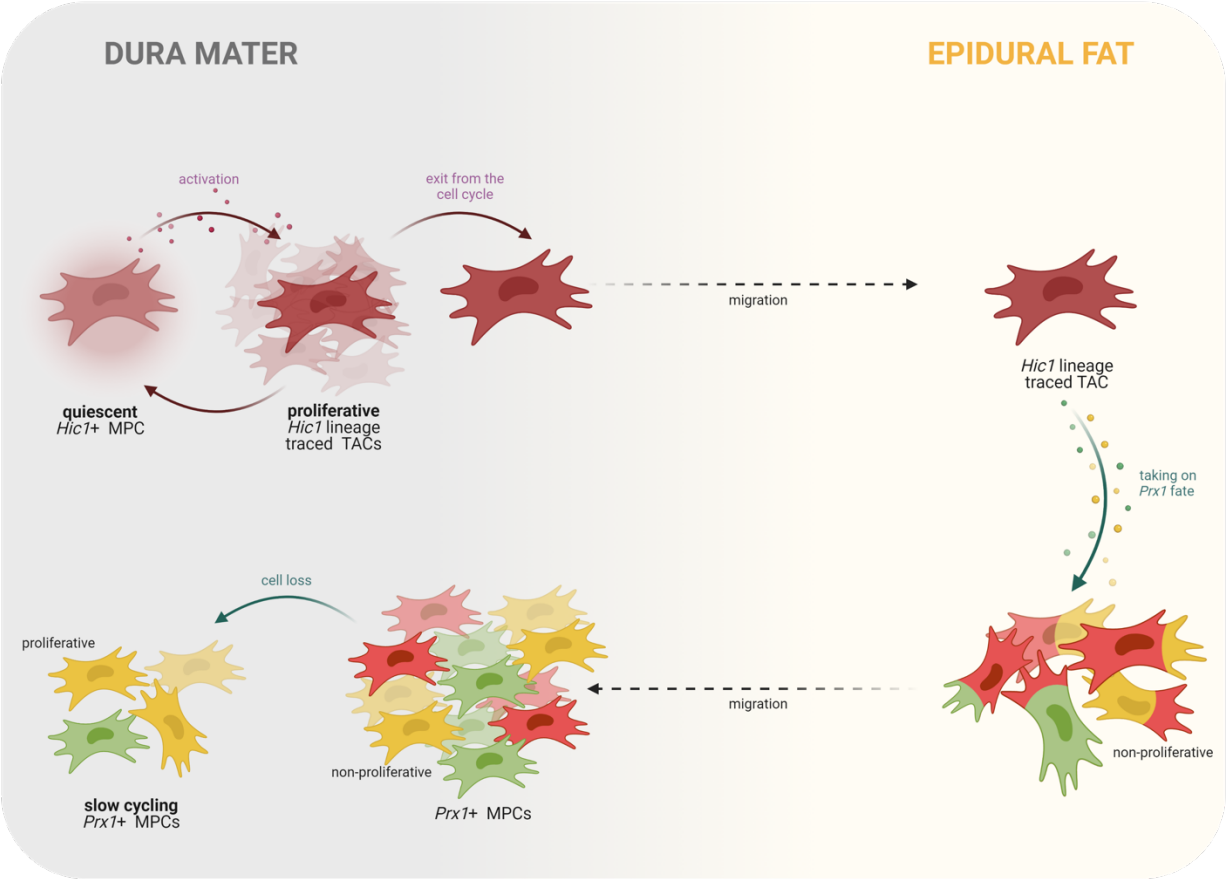
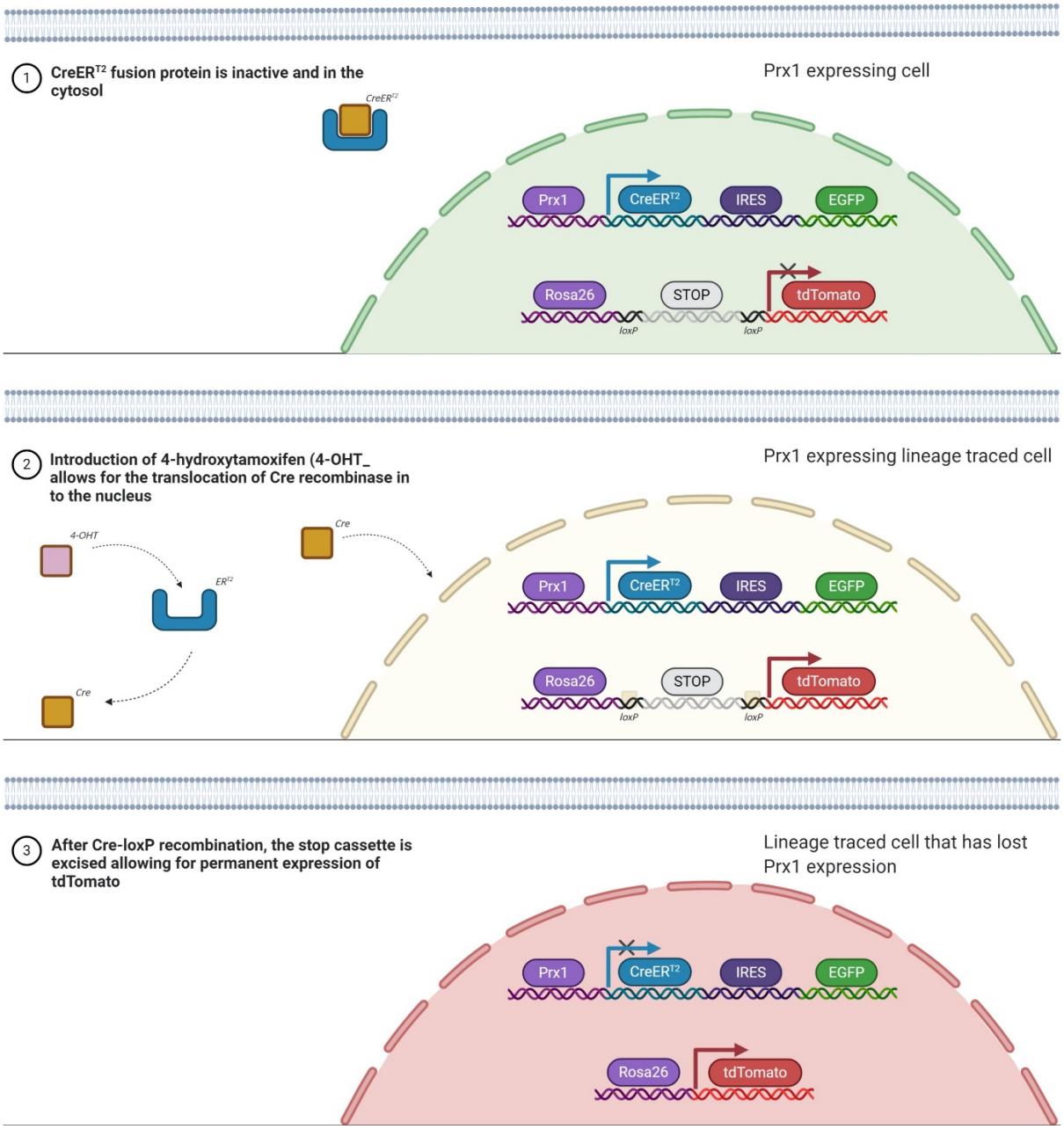


Figure 7. Hypothetical model proposed for the *Hic1* and *Prx1* hierarchy. *Hic1*$^{+}$ MPCs native to the dura mater proliferate in response to biological cues (**Figure 2 D,E**). These MPCs exit from the cell cycle while in dura mater (**Figure 2 F**) and then these *Hic1*$^{+}$ lineage traced TACs migrate to the adjacent epidural fat (**Figure 2 F**). In the epidural fat, these MPCs/TACs acquire a *Prx1*$^{+}$ mesodermal fate, as seen by the colocalization of *Hic1* and *Prx1* expression in cells (**Figure 4, Supplementary Figure 5**). These *Prx1*$^{+}$ MPCs migrate back to the dura mater, and do not re-enter the cell cycle. The expansion of *Prx1*$^{+}$ MPCs in the dura mater over-time (**Figure 2 A-C**) is a product of the migration of these cells from the epidural fat and not due to cell proliferation. At skeletal maturity, *Prx1*$^{+}$ MPCs in the dura mater are lost aside from a few interspaced MPCs, which are now proliferative (**Figure 3**) and most likely undergo slow cycling in this tissue to maintain homeostasis and respond to future activation cues/insults (**Figure 6**).

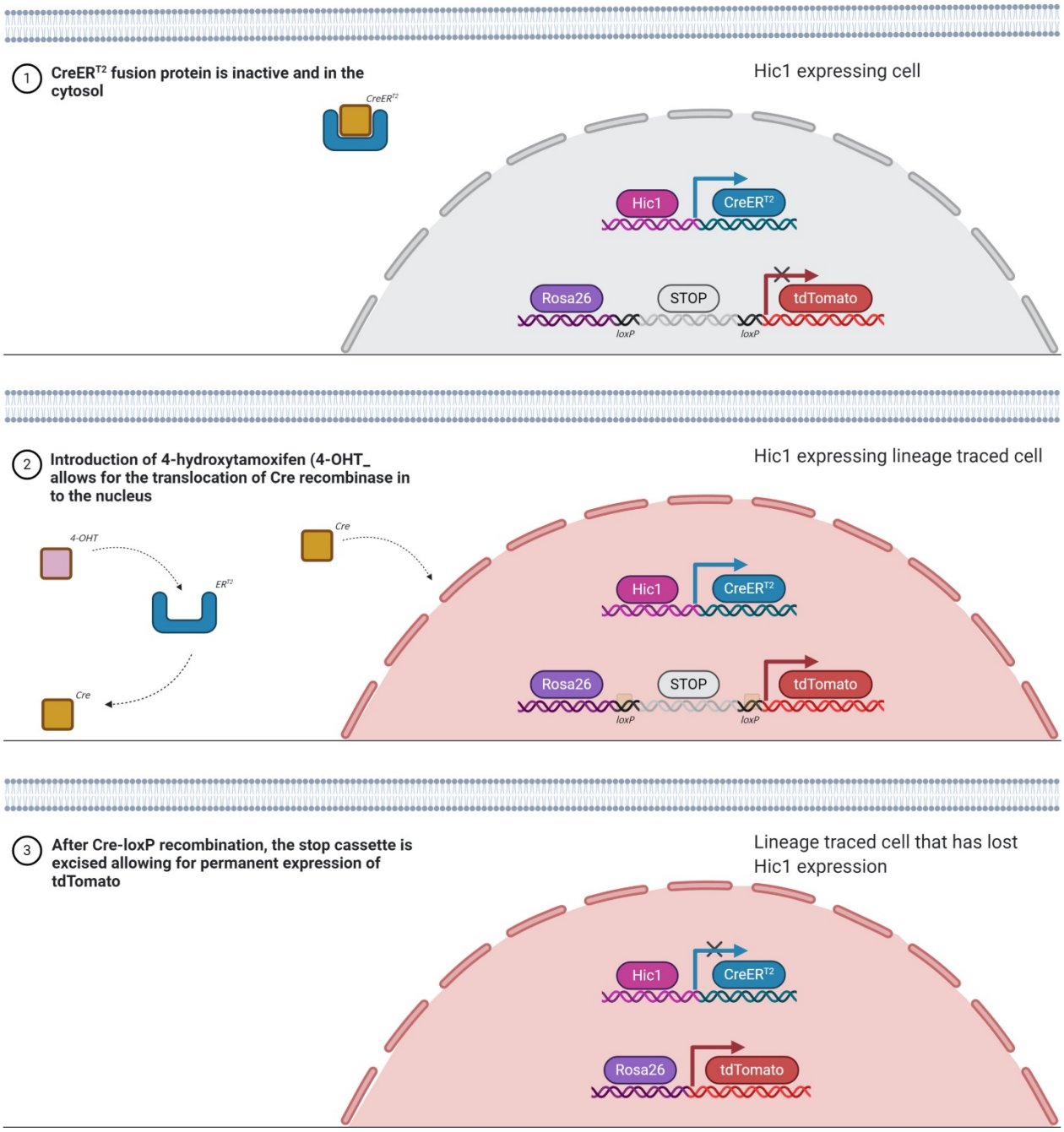
5. Conclusion

Only recently was a progenitor population in epidural fat identified (56,57), yet the role of these MPCs remained unknown. In the current study, we have demonstrated that MPCs within the dura mater and adjacent epidural fat are essential for the maintenance of the dura mater throughout growth and post-injury. Moreover, we have demonstrated partial overlap (marker expression and function) between *Prx1*$^{+}$ and *Hic1*$^{+}$ MPC populations. This finding opens new avenues of research into the hierarchical relationship between these two progenitor populations in the spinal micro-environment in addition to other tissues (such as bone, periosteum). This study further challenges the notion of epidural fat as an incidental/biologically inert tissue (51,57) and suggests additional research should be directed towards unveiling the molecular and functional differences between epidural fat and other types of white adipose tissue in specific regards to homeostasis and in disease states.

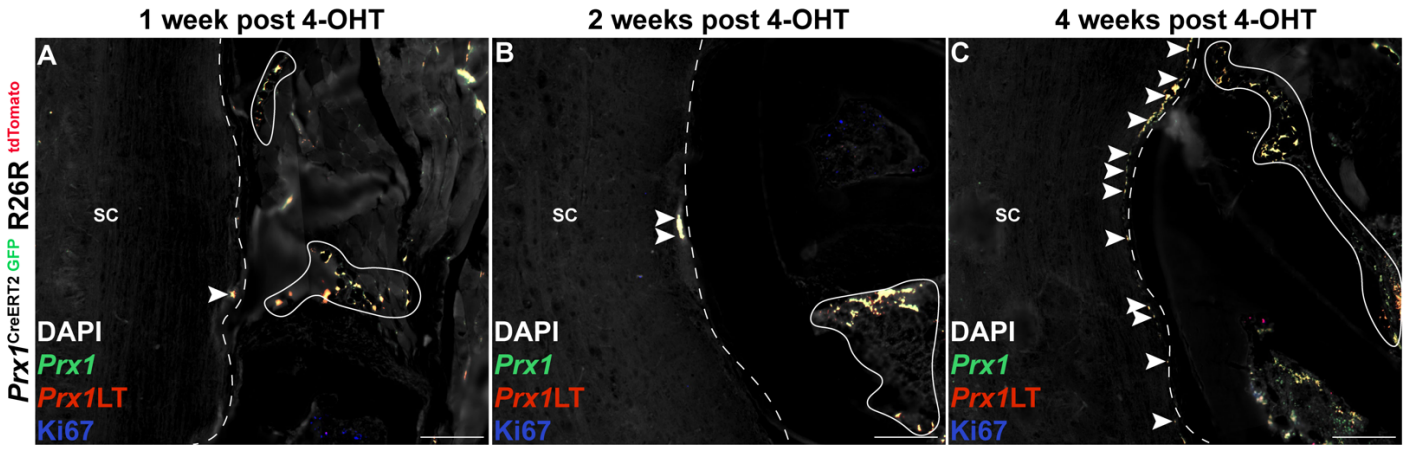
6. Supplementary figures



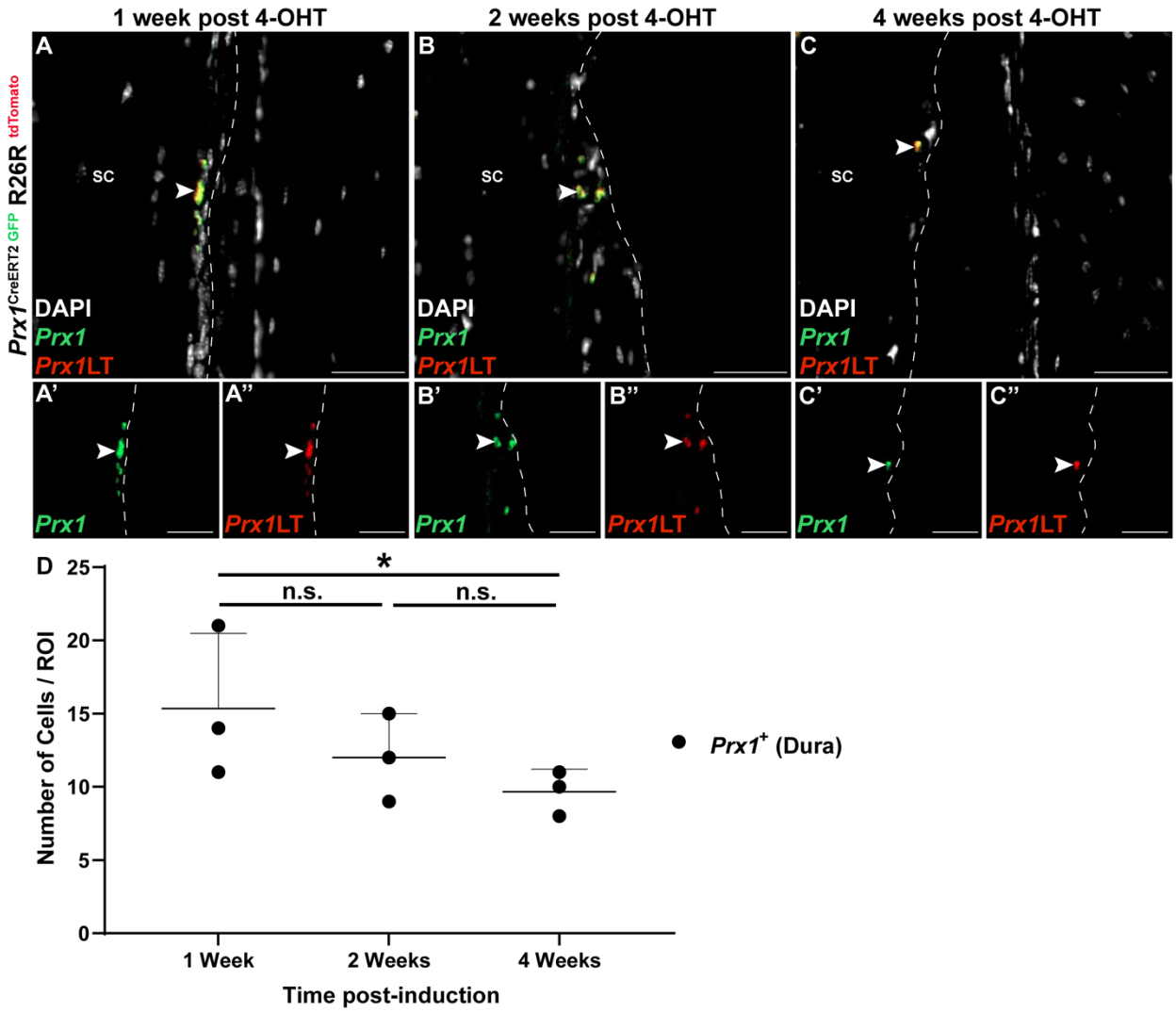
Supplementary Figure. 1. *Prx1* MPC lineage tracing overview within *Prx1*$^{\text{CreERT2-GFP+/+}}$R26R$^{\text{tdTomato+/+}}$ mice employed in the current study.



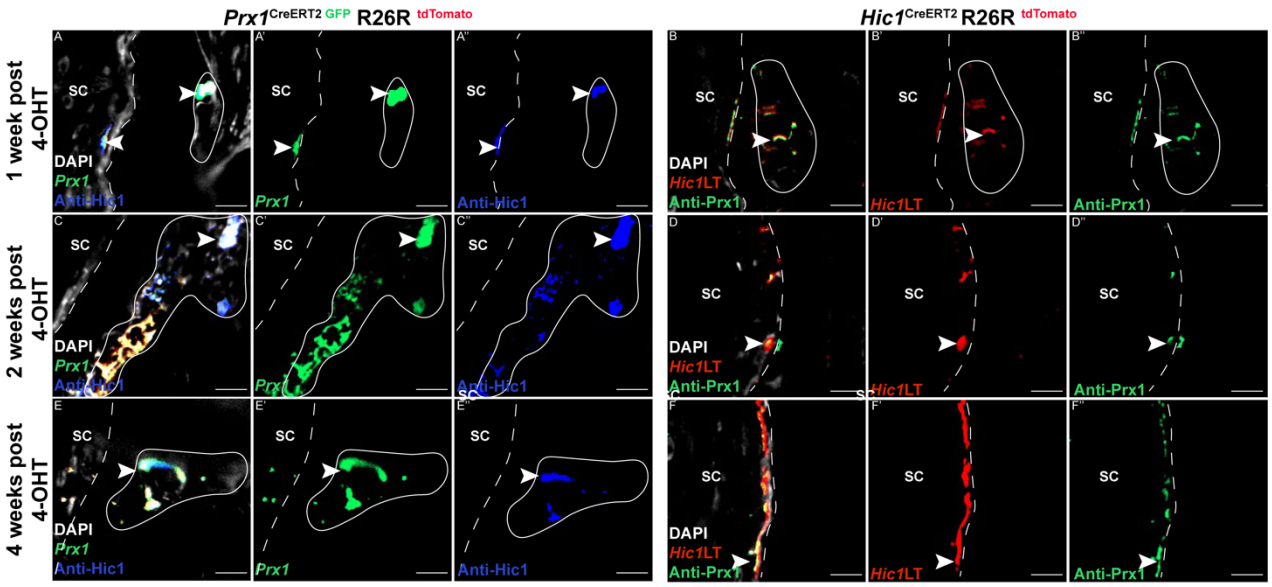
Supplementary Figure. 2. *Hic1* MPC lineage tracing overview within *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ mice employed in the current study.



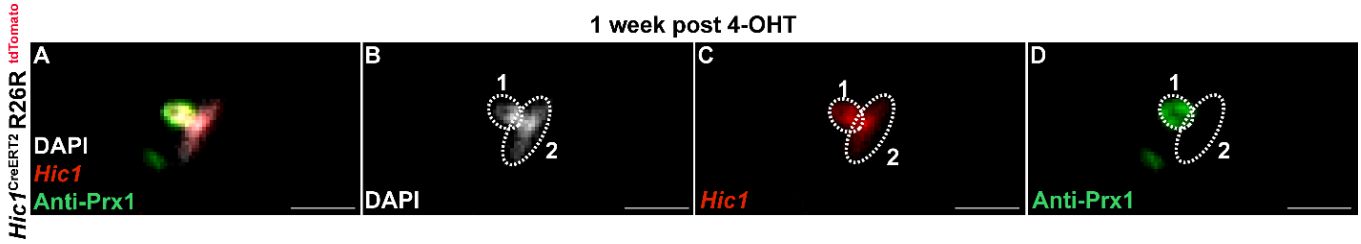
Supplementary Figure 3. *Prx1*$^{+}$ (A-C) MPC lineage tracing 1,2- and 4-weeks post-tamoxifen induction. *Prx1*$^{+}$ MPCs within the dura mater remained undifferentiated at the time points examined as they expressed both GFP (*Prx1*) and tdTomato (*Prx1*LT). The dura mater is outlined by the dashed line while the epidural fat is encircled by the solid line. Arrows indicate examples of *Prx1*$^{+}$ MPCs in the dura mater. Scale bars = 100μm; SC= spinal cord.



Supplementary Figure 4. *Prx1*$^+$ (A-C) MPC lineage tracing 1,2 and 4-weeks post-tamoxifen induction in an aged mouse (at 6 months). Individual GFP (*Prx1* - **A',B',C'**) and tdTomato (*Prx1*LT - **A'',B'',C''**) channel images are presented to demonstrate that the *Prx1*$^+$ lineage traced cells remain undifferentiated. The dura mater is highlighted by the dashed line. Arrows indicate examples of *Prx1*$^+$ MPCs within the dura mater. Quantification of *Prx1*$^+$ MPCs within the dura mater (D). * $p<0.05$. Scale bars = 50μm; SC= spinal cord.



Supplementary Figure 5. Examples of *Prx1* and *Hic1* colocalization with anti-Hic1 and anti-Prx1 respectively in the *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$ mouse (**A,C,E**) or Hic*1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ mouse (**B,D,F**) 1,2- and 4-weeks post-tamoxifen induction. Arrows indicate examples of colocalization between *Prx1*$^{+}$ and *Hic1*$^{+}$ MPCs (see **Fig. 6**). Scale bars = 20µm; SC= spinal cord.



Supplementary Figure 6. Asymmetrical cellular division in the dura mater (A) observed in a *Hic1*$^{+}$ lineage traced MPC (cells number 1 and 2; **B,C**) 1-week post-tamoxifen induction. *Prx1* antibody staining (**D**) reveals colocalization with *Hic1*$^{+}$ expression in one of the two dividing cells (**cell number 1**; **C,D**). Scale bars = 20µm.

CHAPTER 4: *Prx1*$^{+}$ mesenchymal progenitor cells display increased proliferation *in vivo* within the dura mater of *p21*$^{-/-}$ mice

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Abstract

The removal of epidural fat intraoperatively can lead to epidural fibrosis. The reconstruction of epidural fat using adipose-derived mesenchymal progenitor cells has shown promise in pre-clinical studies, however, such grafts do not take into account site-specific properties of mesenchymal progenitor cells. Epidural fat has been identified to possess a population of progenitors that integrate into the dura mater and are responsive to injury. This study used a *p21* knockout ($^{-/-}$) mouse model to determine if the contribution of these cells to the dura mater could be enhanced. The *p21*$^{-/-}$ mouse can regenerate certain tissues, with the repair characterized by increased cell proliferation. Therefore, it was hypothesized that the process by which epidural fat mesenchymal progenitors maintain the dura mater would be accelerated in *p21*$^{-/-}$ mice. Using transgenic mice designed to allow lineage tracing of mesenchymal progenitors that expressed *Prx1*, epidural fat progenitors were found to increase in the dura mater over time. However, by 3-weeks post tamoxifen induction, only a few progenitors remained in *p21*$^{-/-}$ mice, unlike the C57BL/6 mice where numbers continued to increase. These endogenous cells also localized to the dural injury lesion in both mouse strains, however, only *p21*$^{-/-}$ mice demonstrated increased proliferation. When epidural fat mesenchymal progenitors derived from *p21*$^{-/-}$ mice were transplanted into dural injuries in C57BL/6 mice, these cells homed to the injury site and proliferated *in vivo*. We have demonstrated epidural fat mesenchymal progenitors play a role in dural tissue maintenance. Our results suggest that these cells have potential to treat injuries and/or pathologies in tissues surrounding the spinal cord.

Keywords: mesenchymal progenitor cells, epidural fat, transgenic reporter mice, regeneration, *p21*$^{-/-}$

Introduction

The removal of epidural fat during spinal procedures, such as a laminectomy, has been identified as a contributing risk factor to epidural fibrosis such that a buildup of fibrotic tissue can adhere to the dura mater causing further complications (65). Although laminectomies are performed to alleviate spinal cord compression, epidural fibrosis can recompress the spinal cord and/or cauda equina. Moreover, the risk of durotomy increases during revision surgeries for patients with epidural fibrosis (77). Incidental durotomies are associated with poorer post-operative outcomes, longer lengths of stay in hospital, and increased re-operation rates(199). However, appropriate treatment can diminish long-term sequelae post-dural injury. The use of synthetic and biological grafts to treat insults to the dura mater began in the 20th century, including polymer sheets, glues, autografts, allografts, and even xenografts (76). To date however, a successful graft which mimics perfectly the collagen fiber arrangement and accordingly the biomechanical properties of the dura mater remain elusive.

Given that the loss of epidural fat can result in fibrosis in the epidural space, efforts to re-introduce epidural fat to its native place have been made including engineered fat from adipose derived mesenchymal progenitor cells (MPCs) (65), injectable extracellular matrices (73,200,201), and fat grafting (200,202–204). Attempts at preventing epidural fibrosis thus protecting the dura mater have also been made using biomaterials like DuraGen (66), and injection of nonsteroidal anti-inflammatory drugs to re-balance the micro-environment to take on a phenotype that promotes healing (205). Regardless of the multiple different techniques being explored, no treatment has gained wide clinical acceptance to prevent epidural fibrosis.

It has been established that epidural fat is home to a biologically active population of MPCs (Chapter 3)(56,57), and previously, we demonstrated that epidural fat MPCs contribute to dural maintenance and repair (Chapter 3). Therefore, in this study, we wanted to employ an animal model of enhanced tissue regeneration to see if epidural fat MPCs would demonstrate a differential wound healing response. We selected the *p21* knockout mouse as it is known to demonstrate increased cell proliferation and regeneration in a number of musculoskeletal tissues (161,206–210).

p21 is a cell cycle inhibitor that primarily regulates the G_1 checkpoint (159). p21 is involved in many cellular processes including regulation of gene transcription, modulation of apoptosis, and DNA repair (159). Recently, it has also been suggested that p21 is critical in maintaining stem cell

potential in numerous tissues (160,210–213), with the knockdown of p21 expression in MPCs resulting in increased cell proliferation and expression of stemness markers Nanog and Oct4 (214). The Heber-Katz group found that mice lacking p21 expression developed a regeneration ability similar to MRL mice and also possessed the same cellular phenotypes (DNA damage, G2/M checkpoint bias) (209). In the years since this study, many groups have been investigating the mechanisms by which p21 loss induces regeneration: modifying DNA damage checkpoints, reducing fibrotic stimulatory factors like TGF-β1, and supporting progenitor cell stability (161,215). With the deletion of a single gene, these mice have become a powerful model for the study of mammalian tissue regeneration.

As *p21*$^{-/-}$ mice demonstrate increased regeneration capacity (161,206–210,216,217), we wanted to further test the regenerative and/or reparative ability of epidural fat MPCs within this mouse model in response to normal growth cues and dural injury. Using lineage tracing reporter mice, we compared dural homeostasis in C57BL/6 vs. *p21*$^{-/-}$ mice and hypothesized that epidural fat MPCs in *p21*$^{-/-}$ mice would display increased cell proliferation thereby accelerating the process by which dural homeostasis is maintained.

Methods

Animal strains and sample size

All mice were handled in accordance with the Canadian Council on Animal Care Guidelines and the animal care protocol was approved by the University of Calgary Animal Care Committee (AC16-0043, AC20-0042). The *Prx1*$^{\text{CreERT2-GFP}}$ mouse line used in this study was kindly provided by Dr. Shunichi Murakami (Case Western Reserve, Cleveland, OH) (218). C57BL/6 and *p21*$^{-/-}$ (B6;129S2-*Cdkn1a*^{tm1Tyj}) were purchased from The Jackson Laboratory (Bar Harbor, ME). At least an n=3 was used per strain, per timepoint.

***Prx1*$^{\text{CreERT2-GFP+/+}}$R26R$^{\text{tdTomato+/+}}$; C57BL/6:** generated by crossing *Prx1*$^{\text{CreERT2-GFP+/+}}$ mice (C57BL/6 background) with R26R$^{\text{TdTomato+/+}}$ mice (C57BL/6 background; derived from stock no. 007914 from The Jackson Laboratory) and genotyped to generate *Prx1*$^{\text{CreERT2-GFP+/+}}$; R26R$^{\text{TdTomato+/+}}$. ***Prx1*$^{\text{CreERT2-GFP+/+}}$R26R$^{\text{tdTomato+/+}}$; *p21*$^{-/-}$:** a) Prx*1*$^{\text{CreERT2-GFP+/+}}$ mice were crossed with *p21*$^{-/-}$ mice (*Cdkn1a*^{tm1Tyj}/J; stock no. 003263 from The Jackson Laboratory, backcrossed with C57BL/6 mice for 10 generations prior to the start of the study) and genotyped to select *Prx1*$^{\text{CreERT2-GFP+/+}}$ *p21*$^{-/-}$ mice. b) R26R$^{\text{TdTomato+/+}}$ mice were crossed with *p21*$^{-/-}$ mice and genotyped to select R26R$^{\text{TdTomato+/+}}$ *p21*$^{-/-}$ mice. The two lines were then bred and genotyped to generate *Prx1*$^{\text{CreERT2-GFP+/+}}$R26R$^{\text{tdTomato+/+}}$; *p21*$^{-/-}$ mice.

Lineage tracing

In both backgrounds (C57BL/6, *p21*$^{-/-}$), *Prx*1 is identified by GFP expression. Mice were injected interperitoneally with tamoxifen (4 days; (Z)-4-OHT, *Sigma Aldrich*) to induce Cre-mediated recombination and permanently label *Prx1*-expressing cells with tdTomato. After the MPCs have differentiated, *Prx1* (and by extension GFP) expression is lost and cells that were expressing *Prx1* at the time of tamoxifen induction will only express tdTomato. *Prx1*$^{+}$ MPC lineage tracing was conducted 1-4-weeks post-tamoxifen induction in both strains of mice, and also at 4-months post-tamoxifen induction in the C57BL/6 background strain. Spines were removed from mice after CO_2 asphyxiation and fixed (10% NBF; *Fisherbrand*), decalcified (10% EDTA pH=7.4) and processed before paraffin embedding.

Dural injuries

Following induction of anaesthesia (isoflurane 3.0vol/vol% with 1L/min O2) and subcutaneous administration of Buprenorphine (0.05 mg/kg), the dorsal skin of the mice was shaved and disinfected (iodopovidone). The spinal column was then exposed below the thoracic region and an L3 laminectomy was performed. In the same location, the dura mater was punctured using a 30-gauge needle, followed by muscle repair and skin closure. 2-weeks post-injury, mice were sacrificed for histology and immunofluorescence. The injury site was identified by the presence of granulation tissue and disrupted paraspinal muscle.

Histology and immunofluorescence

Spine samples were sagittally sectioned (10 μm), deparaffinized (CitriSolv; *Fisher Scientific*), and rehydrated (graded ethanol to distilled water washes). For histology, slides were stained with Safranin-O and Fast Green to visualize collagens and proteoglycans within the dura membrane, respectively. For antigen retrieval, samples were submerged in 10 mM sodium citrate (pH 6.0) followed by blocking in goat serum for 1 hour (1:500 dilution; 200 μL goat serum: 100 mL TBST) before going through sequential washes (TBST, PBS) and conjugated antibody application steps. Ki-67 (AF647, Clone # SolA15, *eBioscience*), a non-histone nuclear marker for cell proliferation and α-SMA – AF647, Clone # 1A4, *Biolegend*), a dural marker, were applied to sections before cover slipping slides with EverBrite™ Hardset Mounting Medium with DAPI (*Biotium;* 353 nm/465 nm). MPC (*Prx1*$^{+}$: GFP; 493 nm/517 nm), differentiated MPC (*Prx1*$^{-}$: tdTomato; 563 nm/581 nm) and Ki-67 or α-SMA (650 nm/665 nm) expression were analyzed via immunofluorescence using an Axio Scan.Z1 Slide Scanner microscope (*Carl Zeiss*) outfitted with a Plan-Apochromat objective (10x/0.8 M27).

Epidural fat cell isolation

Murine epidural fat was harvested to isolate *Prx1*$^{+}$ cells that lacked expression of *p21*. Anesthetized *Prx1*$^{\text{CreERT2-GFP+/+}}$R26R$^{\text{tdTomato+/+}}$; *p21*$^{-/-}$ reporter mice were sacrificed via cervical dislocation and intact spines removed. Starting from the lumbar region, the spine was cut to access the hollow spinal canal where epidural fat was isolated. The fat was plated in 6-well plates with Mesencult™ Basal Medium (*Stemcell Technologies*) and incubated (37°C and 5% CO_2) until cells began proliferating. At 60% confluency, cells were washed with DPBS (*Lonza- BioWhittaker*)

and passaged with 0.05% Trypsin EDTA (*Gibco Life Technologies*) and then allowed to proliferate with media changes every 2 days. The cells were then assayed for GFP/tdTomato expression by fluorescence microscopy before being exogenously delivered (10,000 cells) to the dural injury site within a C57BL/6 (non-fluorescent) mouse.

Cell enumeration

Cell counting was performed using a methodology previously described (179,180). Briefly, cells positive for the reporter and/or antibodies of interest were enumerated within two regions of interest (Area = ~1.12 x 10^5 sq. μm): epidural fat and dura mater from n=3 animals at each data point. For each region of interest (per animal), a total of n=3 tissue sections were counted for each fluorescent filter (e.g., EGFP, R-PE, DsRed, APC) or in combination when applicable.

Statistical analyses

GraphPad Prism software (Version 7.0) was used to summarize the variables (mean ± SD) and perform standard descriptive statistics. Significance was reported at the level of $p \leq 0.05$. Statistical analysis was by one-way or two-way ANOVA (as appropriate) followed by Tukey's *post hoc* testing.

Results

Prx1^{+} MPC expression increases in the dura mater over time in both strains, however, there is a dramatic reduction in dural MPCs by 3-weeks in the *p21*$^{-/-}$ mice.

At 1- and 2-weeks post-tamoxifen induction in the *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; C57BL/6 mice, few *Prx1*$^{+}$ MPCs were observed within the dura (**Fig. 1A'-B',I-K**). By 3-weeks post-tamoxifen induction, a substantial portion of the dura was enriched for *Prx1*$^{+}$ MPCs, with the dura appearing almost completely comprised of *Prx1*$^{+}$ MPCs by 4-weeks post-tamoxifen induction (**Fig. 1D'**). Through fluorescent imaging, it was observed that the *Prx1*$^{+}$ MPCs within the dura were undifferentiated, as they expressed both GFP and tdTomato and were non-proliferative, as Ki67 (blue) staining was not co-localized with the GFP and/or tdTomato staining over the course of 4-weeks (**Fig. 1A'-D', Supplementary Fig. 1**). In the *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; *p21*$^{-/-}$ mice, increase in *Prx1*$^{+}$ MPCs within the dura was also observed over the course of the first two weeks of the experiment. Specifically, at week 1, the dura contained only few *Prx1*$^{+}$ MPCs, with numbers increasing at 2 weeks (**Fig. 1E'-1F',I-K**). However, at 3- and 4-weeks post-tamoxifen induction, rather than observing the dura become mainly *Prx1*$^{+}$ (as was observed in C57BL/6 mice), single *Prx1*$^{+}$cells expressing GFP, tdTomato and Ki67 were seen dispersed throughout the dura at intervals of ~100μm (**Fig. 1G'-H'**). There was also a dramatic difference in the number of proliferative *Prx1*$^{+}$ MPCs between the strains, as nearly every MPC in *p21*$^{-/-}$ mice was Ki67^{+}, while next to no Ki67^{+} staining was observed in the C57BL/6 mice (**Fig. 1A'-H',J-K, Supplementary Fig. 1**). To determine if this difference in *Prx1*$^{+}$ MPC localization between the strains was due to age/growth, C57BL/6 mice were examined at 4 months (16 weeks) post-tamoxifen induction, and we observed a very similar *Prx1*$^{+}$ MPC localization pattern as found in *p21*$^{-/-}$ mice at 3- and 4-weeks post-tamoxifen induction (**Supplementary Fig. 2**). Furthermore, it is also important to note that these MPCs were found to be proliferative as demonstrated by positive staining for Ki67.

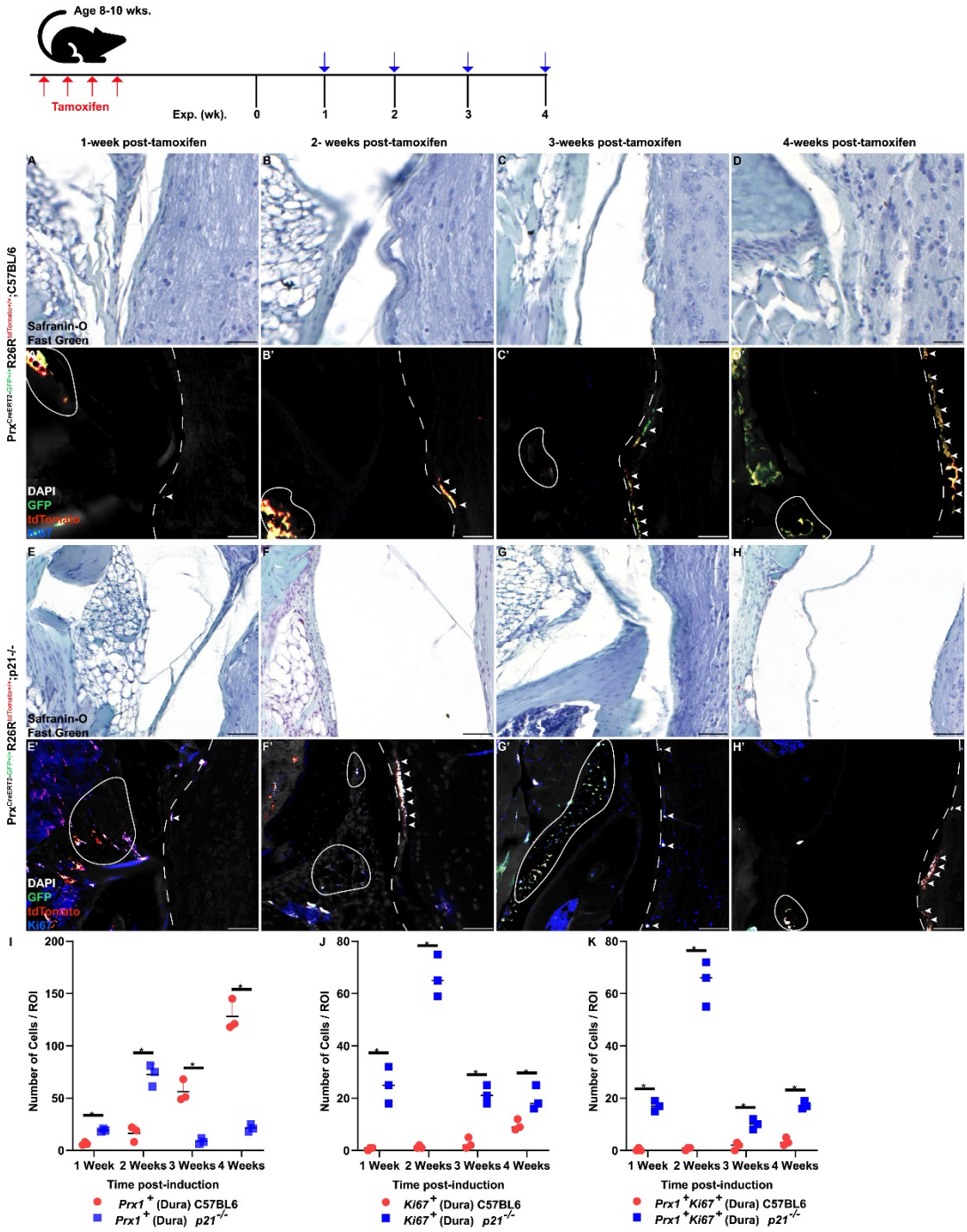


Figure 1. *Prx1*$^{+}$ MPC lineage tracing 1-4-weeks post-tamoxifen induction. A graphical representation of the experimental procedure (top panel). Histological images of Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; C57BL/6 (A-D) and Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; *p21*$^{-/-}$ (E-H) of the spinal column corresponding to the fluorescent images (A'-H') are presented. The dura is outlined by the dashed line, arrows indicate *Prx1*$^{+}$ MPCs in the dura, and *Prx1*$^{+}$ expression in the adjacent epidural fat is indicated by the solid line. Quantification of *Prx1*$^{+}$ MPCs (I), Ki67^{+} cells (J) and *Prx1*$^{+}$ Ki67^{+} MPCs (K). *p<0.05. Scale bars = 100μm.

Endogenous *Prx1*$^{+}$ MPCs localize to the site of dural injury.

Dural injuries were induced in C57BL/6 and *p21*$^{-/-}$ mice. At 2-weeks post-injury, *Prx1*$^{+}$ MPCs were found at the injury site in the *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; C57BL/6 mice (**Fig. 2B,G**). These *Prx1*$^{+}$ MPCs remained undifferentiated as they expressed both GFP and tdTomato, yet they were non-proliferative as Ki67 staining was absent (**Fig. 2B',H)**. In the *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; *p21*$^{-/-}$ mice, *Prx1*$^{+}$ MPCs were again found within the injury region (**Fig. 2E,G**). These *Prx1*$^{+}$ MPCs were in an undifferentiated state yet they were observed to be proliferative as Ki67 staining co-localized with GFP and tdTomato expression (**Fig. 2E',I**). We also examined the expression of α-SMA as it a dural marker (219), yet it was absent at the site of injury in both mouse strains and therefore did not colocalize with *Prx1*$^{+}$ MPCs (**Fig. 2C,F**).

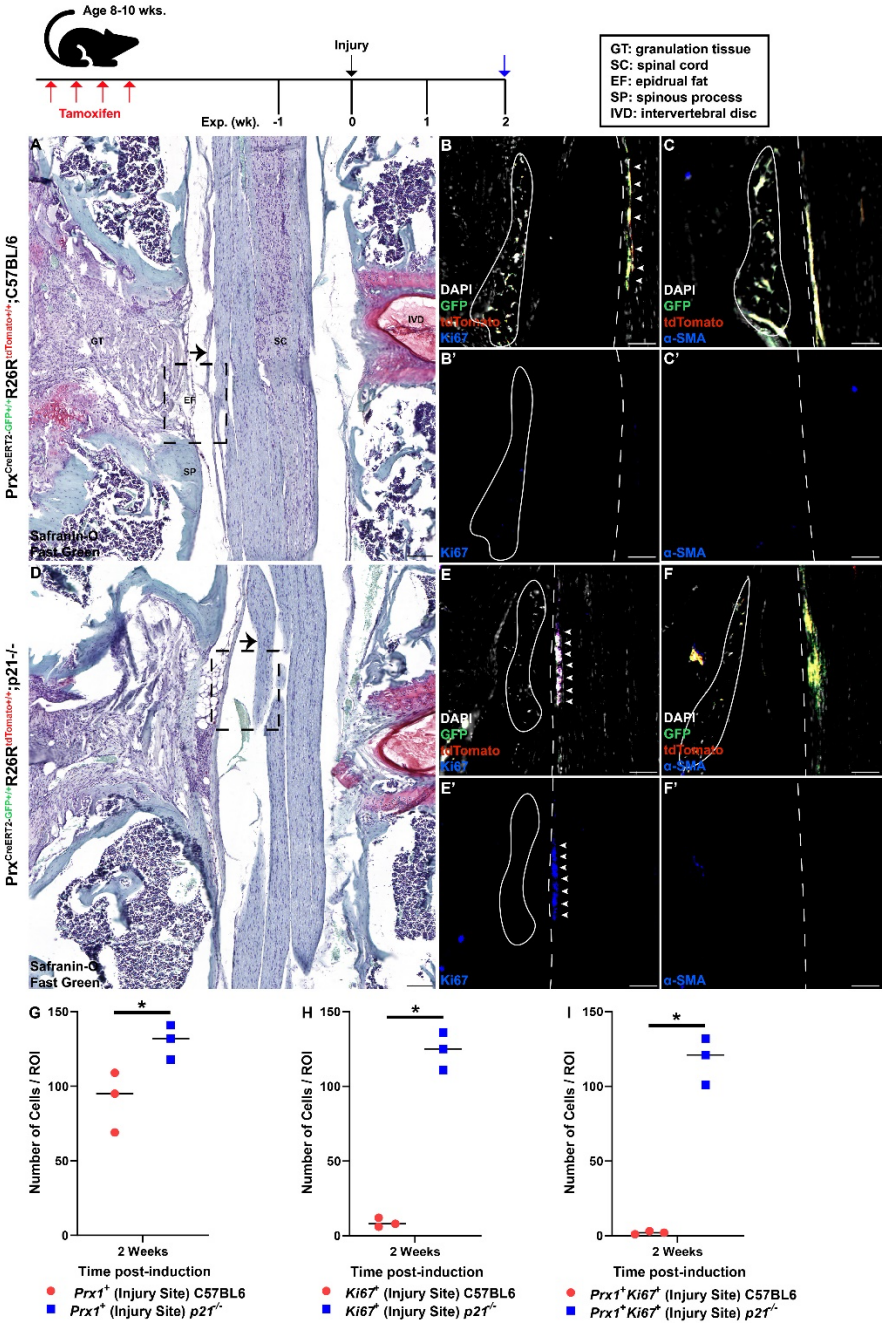


Figure 2. **Endogenous repair of dura injury.** A graphical representation of the experimental procedure (top panel). Histological (A,D) images of the injury site (identified by the presence of GT) in both mice backgrounds are presented alongside corresponding fluorescent Ki67 merged with *Prx1*$^{+}$ cell identifying channels (B,E) and individually (B' E') images. Fluorescent images of α-SMA expression merged (C,F) and individually (C',F') are also presented. The dura is outlined by the dashed line, arrows indicate *Prx1*$^{+}$ cells in the dura, and *Prx1*$^{+}$ expression in the adjacent epidural fat is indicated by the solid line. Quantification of *Prx1*$^{+}$ MPCs (G), Ki67^{+} cells (H) and *Prx1*$^{+}$ Ki67^{+} MPCs (I). *p<0.05. Scale bars = 200μm (A,C) and 100 μm (B-D).

Exogenously delivered epidural fat *Prx1*$^{+}$ MPCs from *p21*$^{-/-}$ mice localize to the injury site.

To ensure that cells contributing to dural repair post-injury were in fact *Prx1*$^{+}$ epidural fat MPCs, epidural fat MPCs harvested from a *Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; *p21*$^{-/-}$ mouse were exogenously delivered to the site of dural injury in a C57BL/6 (wild-type) mouse (**Figure 3)**. Two weeks post-transplant, these *Prx1*$^{+}$ MPCs were found localized at the site of the dural injury (**Fig. 3B**). These exogenously delivered epidural fat *Prx1*$^{+}$ MPCs remained undifferentiated as they expressed both GFP and tdTomato. They were also proliferative *in vivo* as they expressed Ki67 (**Fig. 3B'**). Furthermore, α-SMA staining co-localized with the epidural fat MPC expression within the dural lesion (**Figure 2C,C'**). While we also planned on transplanting *Prx1*$^{+}$ epidural fat MPCs from C57BL/6 mice, we observed that these cells became hypertrophic and non-proliferative by 30 days in culture, while cells from *p21*$^{-/-}$ mice increased in size but retained the ability to expand in culture past the 30-day mark (**Supplementary Fig. 3**).

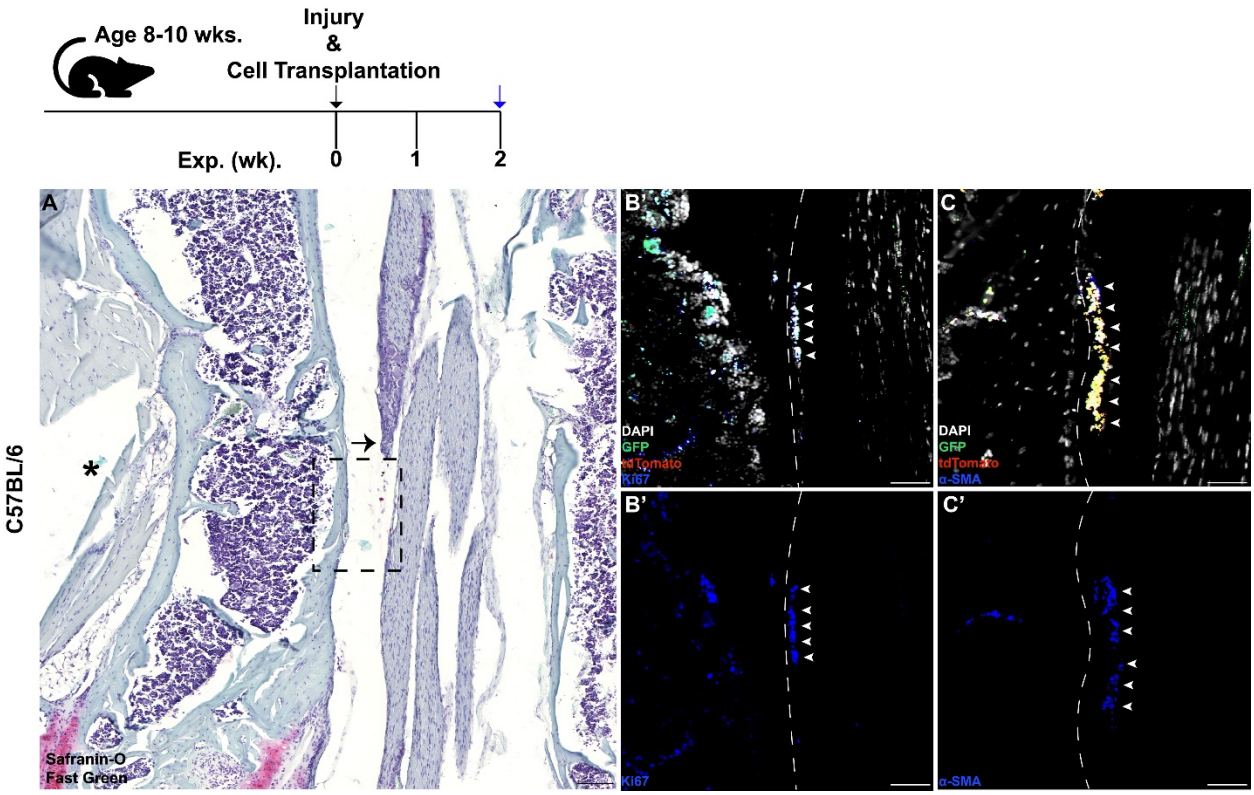


Figure 3. Exogenous delivery of *p21*$^{-/-}$ *Prx1*$^{+}$ epidural fat cells to the site of dural injury in a C57BL/6 mouse. A graphical representation of the experimental procedure (top panel). Histological (A) image of the injury site (identified by disrupted paraspinal muscle and indicated by the asterisk) is presented alongside corresponding fluorescent Ki67 merged with *Prx1*$^{+}$ cell identifying channels (B) and individually (B') images. Fluorescent images of α-SMA expression merged (C) and individually (C') are also presented. The dura is outlined by the dashed line and arrows indicate *Prx1*$^{+}$ cells in the dura. Scale bars = 200μm (A) and 100 μm (B).

Discussion

Historically, epidural fat has been thought of as a space filling material and/or shock absorber (49). Therefore, it is not uncommon for this tissue to be discarded during surgical procedures (178). However, there is a growing body of evidence suggesting that epidural fat is necessary to maintain the dural microenvironment providing an agile reservoir of progenitor cells capable of responding to local and systemic signals, and that the absence of epidural fat can thus lead to worse post-operative outcomes (60). Recognizing its importance, studies using adipose-derived MPCs within scaffolds and matrices have attempted to reconstruct epidural fat and when these tissues were implanted, reduced adherence of fibrotic tissue to the dura mater was observed (65,73). However, the effects of such engineered tissue on the long-term maintenance of the dura mater have not been assessed. Moreover, since MPCs display characteristics specific to their native anatomic location and maintain homeostasis in the tissue wherein they reside (24), the regenerative properties inherently present in epidural fat MPCs (as opposed to MPCs derived from other adipose sources) were not considered in these previous studies. While potential roles of endogenous epidural fat MPCs in the dural microenvironment have been hypothesized (Chapter 3)(57,178); their contribution to the homeostasis of the dura mater during and after growth as well as their role in tissue repair post-injury still remains elusive. To follow these MPCs *in vivo*, we employed a *Prx1* reporter mouse since *Prx1* is an osteoprogenitor and adipose progenitor marker (82), that has previously been shown to identify MPCs in the epidural fat (57,178). We further employed this lineage tracking method on a $p21^{-/-}$ background to identify if there was a change in behaviour of these MPCs *in vivo* with the deletion of *p21*.

Over the course of 4-weeks in a mouse still undergoing skeletal maturity (between 10 and 14 weeks of age), an expansion of the $Prx1^{+}$ MPC population within the dura was observed. This pattern was also observed in $p21^{-/-}$ mice, however, by 3-weeks the expansion halted and instead, the dura became sparsely populated with $Prx1^{+}$ cells interspaced at apparently regular intervals of ~100 μm. Interestingly, this pattern of MPC localization matches with our previous findings in skeletally mature C57BL/6 mice, but when the mice were 4-months post-tamoxifen induction (~18 weeks of age) (Chapter 3, also shown in **Supplementary Figure 2**). Although this finding is supported by the fact that many tissues present with a decrease in progenitor cells from infancy to adulthood (186), the 3-week post-tamoxifen induction timepoint in $p21^{-/-}$ mice cannot be considered as skeletally mature. However, it is important to note that $p21^{-/-}$ mice demonstrate increased cell

proliferation (209,214,220) and have increased regeneration capacity (161), therefore, it is possible that their tissues may reach a level of cellular maturity (balance between progenitor vs. terminally differentiated populations) faster than the wild-type mice. This would explain the sparse MPCs in the dura starting at 3-weeks in the *p21*$^{-/-}$ mice rather than at 4-months post-tamoxifen induction in the C57BL//6 mice and would also account for the overall increased Ki67 staining observed throughout the epidural/dural microenvironment. The influx of MPCs seen in earlier timepoints followed by a cease in expansion at skeletal maturity can also be explained by the fact that the spinal cord does not grow past birth. It reaches its final position within the spinal canal, typically between the first and second lumbar vertebrae, due to the growth of the axial skeleton (171). Therefore, MPCs from the epidural fat could be sustaining this initial growth, which would account for why we observe a decrease in *Prx1*$^{+}$ MPCs overtime. Moreover, if *p21*$^{-/-}$ mice are reaching spinal maturity faster than C57BL/6 mice, this would also account for the differences observed in *p21*$^{-/-}$ MPC kinetics within the dural microenvironment.

There have been several previous studies that have explored the link between cell proliferation and regeneration. For instance, mice lacking *p21* present with regeneration of appendages (ear holes) (209); enhanced bone healing and fracture repair (207,208); and have the ability to survive severe liver injury due to continuous hepatocyte proliferation (220). In response to injury, *p21*$^{-/-}$ and C57BL/6 mice displayed a regenerative response with the localization of *Prx1*$^{+}$ MPCs to the dural lesion, however, we are not able to quantify the level of injury repair in this model, so it is unclear if *p21*$^{-/-}$ mice had an increased repair response. Yet, it was observed that only endogenous *p21*$^{-/-}$ *Prx1*$^{+}$ MPCs (vs. C57BL/6 *Prx1*$^{+}$ MPCs) proliferated *in vivo* in response to dural injury, which is again consistent with previous studies (216). When delivered exogenously, these *p21*$^{-/-}$ *Prx1*$^{+}$ MPCs maintained proliferating ability *in vivo*, but also appeared to express α-SMA within the dural injury site post-transplantation. This is particularly interesting since we did not observe α-SMA expression from the endogenous *p21*$^{-/-}$ *Prx1*$^{+}$ MPCs. It is well known that *in vitro* culture can induce aberrant and/or artefactual behaviour in cells due to the attachment to plastic and/or exposure to animal/bacterial derived proteins(221–223). Yet, it is important to note that although the C57BL/6 and *p21*$^{-/-}$ *Prx1*$^{+}$ MPCs homed to the dural injury site, only *p21*$^{-/-}$ *Prx1*$^{+}$ MPCs proliferated at the site. Therefore, further studies will be required to tease out the functional consequences of endogenous proliferative vs. non-proliferative cells on dural repair and for this to occur, a dural repair grading system needs to be developed. The identification and characterization

of additional dural markers will also be beneficial in determining repair vs. regeneration. Although α-SMA is known to be expressed in the dura (219), it is more commonly employed as a myo-fibroblast marker which is typically associated with fibrotic tissue/scar (224). Additional markers may shed light on the nature of the MPC repair response (e.g., direct vs. indirect). As α-SMA in MPCs was only observed in the cultured and exogenously delivered *p21*$^{-/-}$ epidural fat MPCs, a transcriptomic and proteomic analysis could reveal the changes in gene/protein expression of these cells thereby explaining their ability to reconstitute the dura mater after injury, a response the endogenous cells did not display. Furthermore, this type of analysis might explain why we were not able to keep the C57BL/6 epidural fat MPCs expanding in culture beyond 1 month while this was not an issue in *p21*$^{-/-}$ epidural fat MPCs.

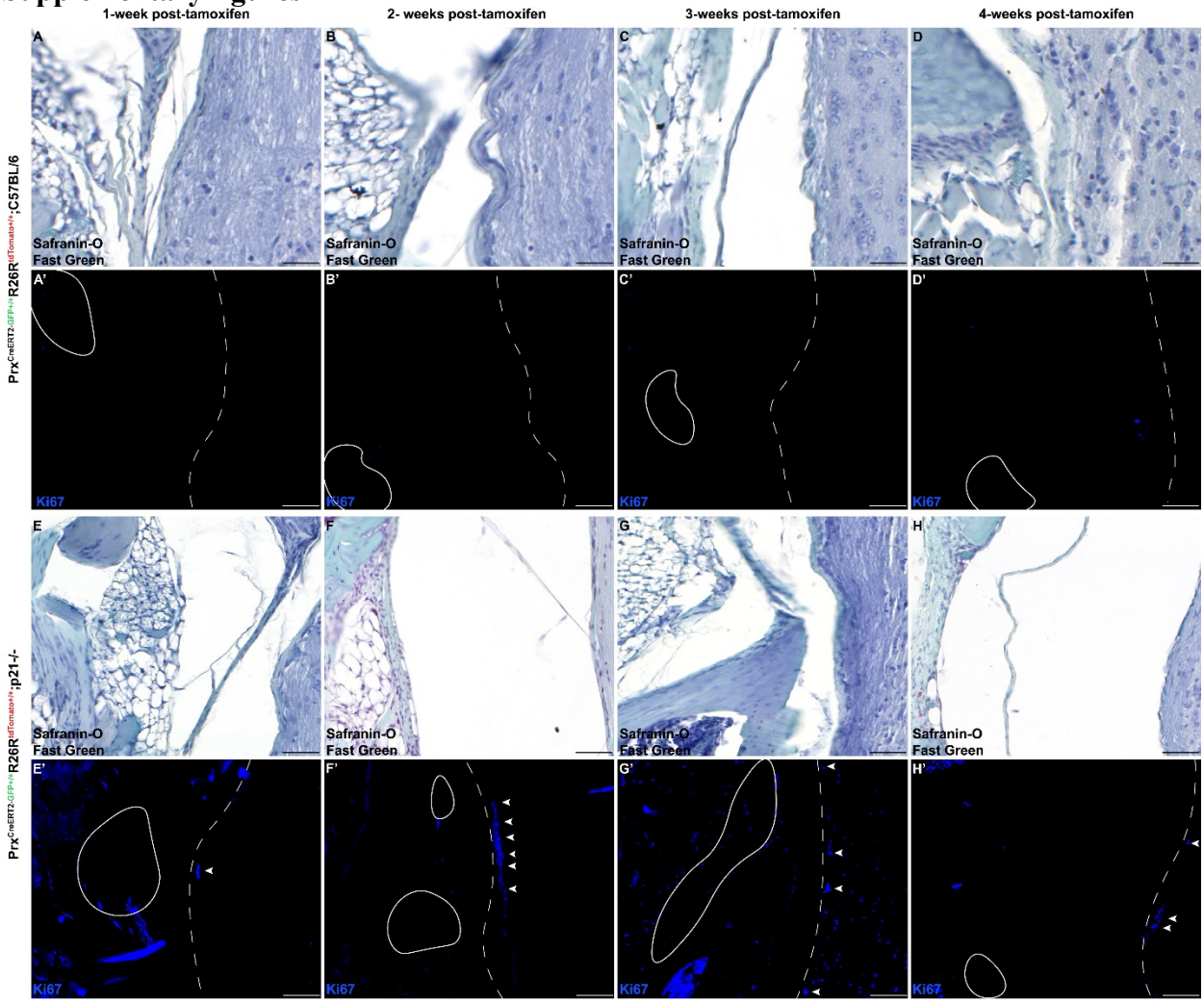
While this study reveals *p21*$^{-/-}$ leads to an increase in cell proliferation in the dura mater, the role of inflammation cannot be ignored when looking at repair and regeneration of tissue post injury. It has previously been hypothesized that factors such as inflammation can activate cell proliferation (225). Moreover, inflammation has been held responsible for the recruitment of MPCs and immune cells to initiate repair and regeneration after injury (226,227). Although this study demonstrates the increased proliferative capacity of *p21*$^{-/-}$ MPCs outside the realm of inflammation (i.e., over the course of normal skeletal growth), we cannot definitively say that this proliferation is also responsible for the enhanced repair of the dura mater after injury, an environment where inflammation is present.

Nonetheless, we have demonstrated that epidural fat MPCs play a role in dural tissue maintenance, validating our previous findings (Chapter 3)(57), and have also shown that exogenously delivered *p21*$^{-/-}$ epidural fat MPCs can directly contribute to dural injury repair. This study reiterates the notion that epidural fat should be maintained as much as feasible intraoperatively, and that in cases where the fat cannot be left intact, epidural fat MPCs may be a viable cell source for tissue engineering strategies to replace this tissue and prevent epidural fibrosis. Moreover, in the event of iatrogenic or traumatic durotomy, epidural fat may provide the critical cell population necessary to drive dural repair.

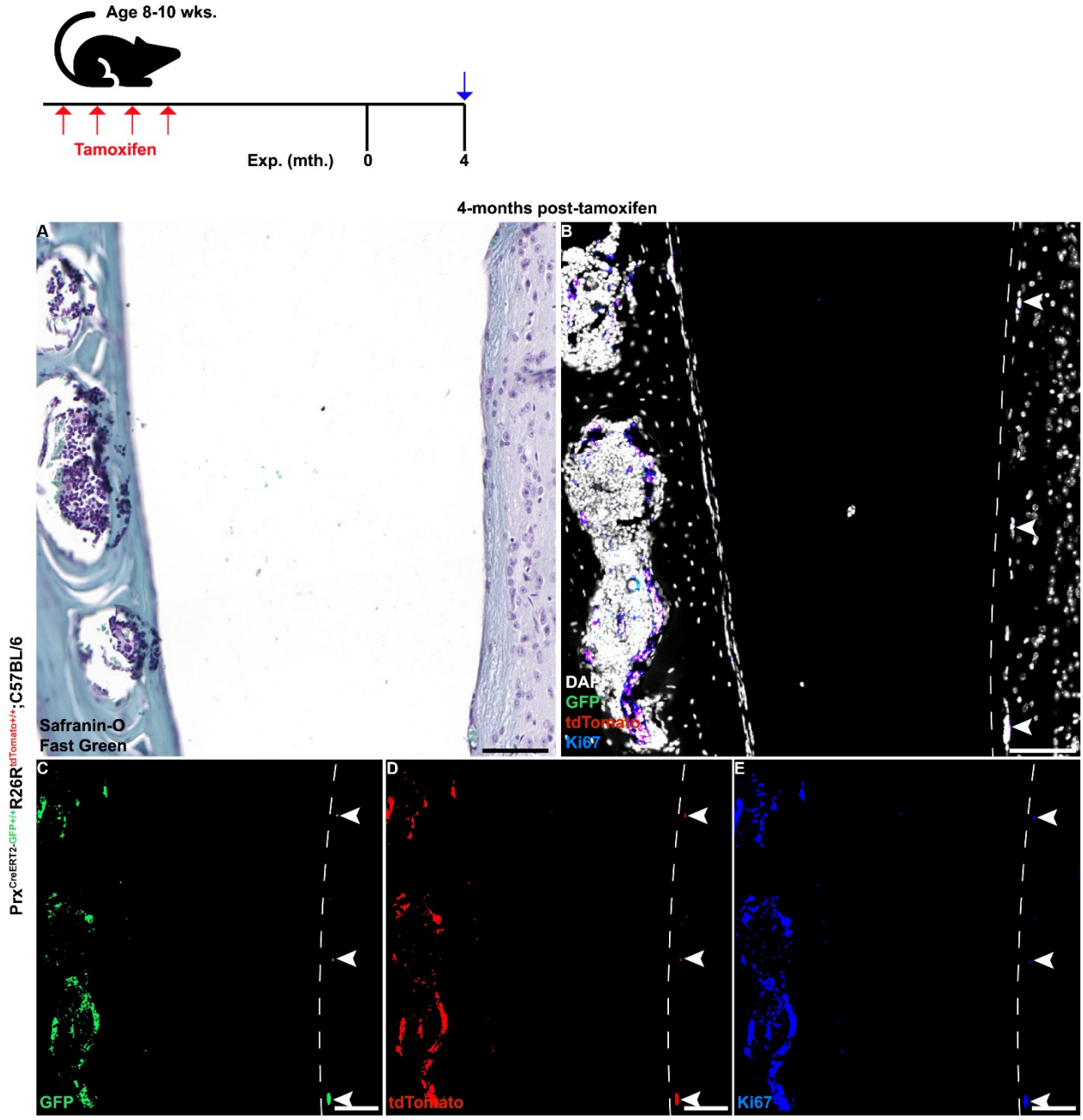
Conclusion

We hypothesized that the process by which epidural fat MPCs maintain the dura mater through growth and after injury would be accelerated in *p21*$^{-/-}$ mice. In this study, we have shown that *p21* knockout leads to increased epidural fat MPC proliferation within the dura mater. However, additional experimentation is needed to determine the effects of *p21*$^{-/-}$ on dural reconstitution after injury as the effects of inflammation and *in vitro* culture artefacts cannot be ignored. Although this project is focused on dural tissue in homeostasis and post-injury, it is important to consider that the research and clinical impact of this adult MPC population, and the mechanism by which p21 modulates its proliferation, extends past the dura mater, epidural fibrosis, and incidental durotomies.

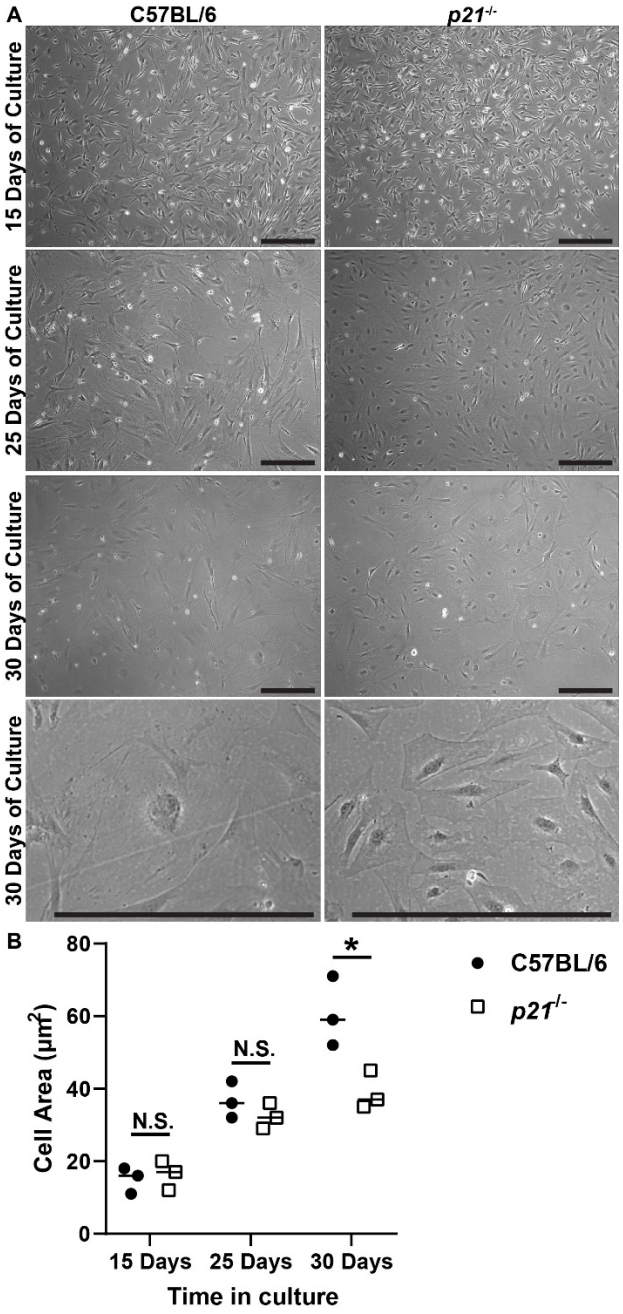
Supplementary figures



Supplementary Figure 1. Ki67^{+} staining colocalization with Prx1^{+} MPCs 1-4-weeks post-tamoxifen induction. Histological images of Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; C57BL/6 (A-D) and Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; p21-/- (E-H) of the spinal column corresponding to the individual Ki67 fluorescent images (A'-H') are presented. The dura is outlined by the dashed line, arrows indicate Ki67^{+} cells in the dura, and encircled is the adjacent epidural fat. Scale bars = 100μm.



Supplementary Figure 2. *Prx1*$^{+}$ MPC lineage tracing in a skeletally mature Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; C57BL/6 mouse. A graphical representation of the experimental procedure (top panel). Histological and fluorescent (A-B) images of the dura are presented. Individual channels are used to show the lineage traced cells are undifferentiated (GFP^{+}-C) and proliferative (Ki67^{+}-E). The dura is outlined by the dashed line and arrows indicate *Prx1*$^{+}$ cells in the dura. Scale bars = 100μm.



Supplementary Figure 3. Expansion of epidural fat MPCs *in vitro*. MPCs from C57BL/6 and *p21*$^{-/-}$ mice demonstrate proliferation *in vitro* until day 30 of culture (A). MPCs from C57BL/6 demonstrated increased hypertrophy and lost the ability to proliferate shortly after 30 days in culture. Scale bars = 40 µm).

CHAPTER 5: Discussion

Overview

Epidural fat is routinely discarded during surgical procedures of the spine to increase the operational field of view. However, as discussed throughout this thesis, the discovery of MPCs in this tissue prompted us to believe it is not an incidental, space-filling tissue; rather, it serves a critical biological purpose in the vertebral microenvironment. Therefore, this thesis sought to explore the role of epidural fat MPCs in maintaining homeostasis within the dura mater throughout normal skeletal growth and in response to injury. Transgenic reporter mice models were employed allowing for the lineage tracing of two MPC markers *in vivo*. *Prx1* is a robust adipose MPC marker (82), has been identified in MPCs capable of trilineage differentiation *in vivo* (82,141,142), and its expression has been observed in murine dura mater and epidural fat (143). *Hic1* is a more recent MPC marker however, it has been shown to a robust marker identifying MPCs in many tissues, yet it is only expressed in quiescent MPCs. These *Hic1*$^{+}$ MPCs have also been found to give rise to TACs which support regeneration post-injury (149,150,181). These previous studies provided the rationale for using *Prx1* and *Hic1* as MPC lineage markers within this project. Using these models, I have demonstrated that epidural fat and the MPCs endogenous to this tissue are drivers of repair and regeneration in this microenvironment. This thesis has advanced our knowledge of MPCs within the epidural fat and identified that these cells play functional roles in this microenvironment, however, future studies will be needed to unveil molecular differences between epidural fat and other adipose tissues/MPCs to truly understand if dural maintenance/repair is specific to epidural fat MPCs. If MPCs from other adipose tissues can substitute for epidural fat MPCs, then this could open new areas for the treatment of dural injuries. If other MPCs do not have this property, then this could be an indication as to why subcutaneous fat transplantation to the site of injury in the spinal canal is not interchangeable with endogenous epidural fat. These results hold the potential of directly impacting clinical care. In the section below, I will revisit the hypotheses presented within my thesis and discuss how the data supports/refutes each in more detail.

The first hypothesis was: *Prx1* and/or *Hic1* expression in the epidural fat marks a population of MPCs *in vivo* which maintain and/or contribute to the growth of the dura mater in mice.

In relation to the Aims presented in this thesis, the completion of Aims 1 - 4 suggests that this hypothesis was correct.

In Chapter 3, it was demonstrated that there is an expansion of MPC populations in the dura mater during normal growth indicating that these cells are responsive to cues in the microenvironment and participate in growth of the dura mater. A reduction in the number of *Prx1*$^+$ MPCs was observed in the dura mater at the point of skeletal maturity, while the numbers of *Hic1*$^+$ MPCs were maintained. The reduction of *Prx1*$^+$ MPCs and maintenance of potentially quiescent *Hic1*$^+$ MPCs suggests that the epidural fat and dura, like other adult tissues (188,228–230), have slow-cycling or quiescent reservoirs of MPCs which can respond to non-homeostatic signals/cues and injury. This is also consistent with the fact that MPCs are typically rare cell populations within adult tissue (186), and can undergo apoptosis after contributing to the growth of the tissue wherein they reside, as seen in bone (187). Moreover, ablation of *Prx1*$^+$ or *Hic1*$^+$ MPCs resulted in a loss of α-SMA expression in the dura mater, implying a disruption of dural tissue phenotype and even possibly tissue integrity (if α-SMA relates to function), further supporting the hypothesis that these MPCs maintain the dura mater.

Although it was initially hypothesized that *Prx1* and *Hic1* would mark distinct MPC populations *in vivo*, the localization pattern of *Prx1*$^+$ and *Hic1*$^+$ MPCs in the epidural fat and dura mater over time prompted a revisitation of the hypothesis and instead, it was found that *Prx1* and *Hic1* do not mark completely distinct MPC populations in the dural environment. The similar phenotype upon ablation of both MPC populations suggests that *Prx1* and *Hic1* are not functionally distinct MPC populations and share characteristics allowing them to maintain the dura mater. Colocalization between *Prx1*$^+$ and *Hic1*$^+$ lineage traced cells and *Prx1/Hic1* protein expression was also observed suggesting that these *Prx1*$^+$ and *Hic1*$^+$ MPC phenotypes are related. Hence, a hypothetical model in which *Hic1* identifies MPC with greater potency than *Prx1* was proposed (see **Figure 7** in Chapter 2). Quiescence (cell cycle arrest in G_0) is a prolonged state in which most stem cells persist, however, this is reversed in response to various stimuli such as tissue injury (231). Stem cell quiescence is essential in maintaining tissue homeostasis and regeneration as extended expansion can lead to stem cell exhaustion (232). This rationalizes why more apex stem cells are commonly quiescent and will undergo asymmetrical cellular division to give rise to transit amplifying cells and progenitor populations; These cells are then responsible in increasing the cell pool and proceed towards terminal differentiation. If the apex stem cell fully committed to

differentiation and high cell turnover was seen, stem cell/progenitor exhaustion would occur resulting in decreased maintenance/repair/regeneration in tissues (233). *Hic1* is a robust marker of quiescent MPCs, which further reinforces the hypothetical model wherein *Hic1* is more potent and takes on *Prx1* expression once it exits quiescence. *Prx1* in turn is a mesodermal fate lineage marker. It is important to consider however that if *Prx1*$^+$ cells were to re-enter quiescence, they could regain *Hic1* expression. In this case, depending on the point of tamoxifen induction, *Hic1*$^+$ potency would be seen cycling above and below *Prx1*$^+$ (**Figure 1**). Moreover, our model assumes MPC migration and acquisition of *Prx1* expression in different tissue, which is yet to be proven.

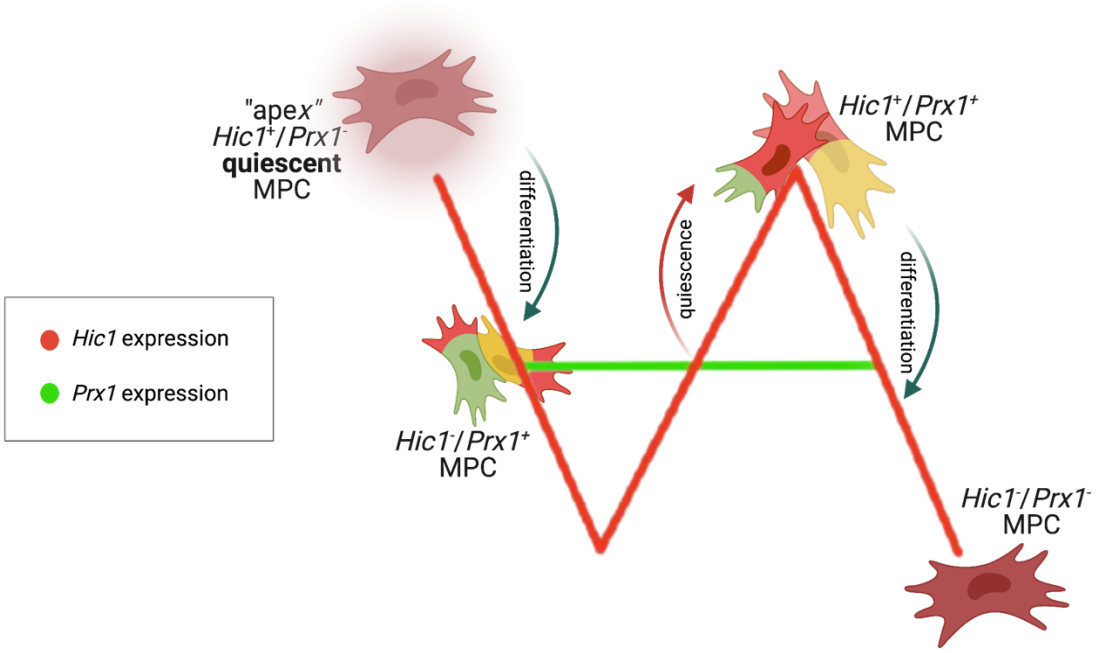


Figure 1. Expansion on the hypothetical model proposed for the *Hic1* and *Prx1* hierarchy (Figure 7 in Chapter 3). Depending on the point of tamoxifen induction, *Hic1*$^+$ expression could be observed in the presence or absence of *Prx1* expression, until terminal differentiation into a cell that is negative for both markers.

The second hypothesis was: *Prx1* and/or *Hic1*$^+$ MPCs (and/or their differentiated progeny) respond to dural injury.

In relation to the Aims presented in this thesis, the completion of Aim 5 suggests that this hypothesis was correct.

In chapter 3, *Prx1*$^{+}$ and *Hic1*$^{+}$ MPCs were shown to be present within the site of dural injury. These results strongly suggest that dura mater has an endogenous repair response and that these MPCs are recruited during this process. However, because these cells did not express α-SMA in the injury site, it is believed that these MPCs are not directly differentiating into connective tissue cells that make up the dura mater. This is not completely unexpected as MPCs can regulate the secretion of inflammatory factors and act on immune cells of both the innate and adaptive system, hence playing an immune-modulatory role (16,149,191,234,235). *Hic1*$^{+}$ MPCs have been found to be recruited in large wounds and directly contributing to neodermal regeneration by acquiring a fibroblast-like fate (181). However, previous studies also demonstrate an indirect repair response such that stage-specific immunomodulation and trophic/mechanical support is provided by *Hic1*$^{+}$ MPCs for skeletal muscle regeneration (149). Similarly, *Prx1*$^{+}$ have been found to play a functional role in the repair of bone fractures with contribution to the callus (236), however in another study looking at periodontal regeneration, it remained elusive whether *Prx1*$^{+}$ cells maintain their stemness in the process or whether their unipotent osteo-competent progeny was the actual contributor to regeneration (237). As such, it is not unlikely that *Prx1*$^{+}$ and *Hic1*$^{+}$ MPCs can play an immune-modulatory role in the wound healing phenotype.

The final hypothesis was: *Prx1*$^{+}$ MPCs in a *p21*$^{-/-}$ mouse model display increased cell proliferation *in vivo.*

In relation to the Aims presented in this thesis, the completion of Aims 1 and 5 suggests that this hypothesis was correct.

In Chapter 4, I demonstrated that there is an expansion of *Prx1*$^{+}$ MPCs within the dura mater over skeletal growth in *p21*$^{-/-}$ mice. However, by 3-weeks post-Tamoxifen induction (11 weeks of age), the expansion appeared to have halted, and the dura was only sparsely populated with *Prx1*$^{+}$ cells. This pattern of MPC localization matched the one observed within skeletally mature C57BL/6 mice in Chapter 3. Although 11-weeks is not skeletally mature, this finding can be supported by the observation that *p21*$^{-/-}$ mice demonstrate increased cell proliferation and tissue regeneration (162,214,238) and may be reaching a level of cellular/tissue maturity faster than the C57BL/6 mice.

In response to dural injury, endogenous *Prx1*$^{+}$ MPCs in the *p21*$^{-/-}$ mice were found within the dural lesion and displayed proliferation as evidenced by Ki67 expression, which was not found in the

C57BL/6 mice. The regenerative response and functional consequences of dural repair by proliferative vs. non-proliferative cells remain to be investigated, implying the need to develop a dural injury grading system and/or method of quantifying repair. Exogenous delivery of *p21*$^{-/-}$ putative epidural fat MPCs localized to the site of injury, proliferated, and also expressed α-SMA suggesting direct reconstitution of the dura mater, which was not observed in the endogenous *p21*$^{-/-}$ *Prx1*$^{+}$ MPCs. It is believed this may be due to anomalous behaviour induced in cells due to *in vitro* culture (221,222). Putative MPCs were isolated from both C57BL/6 and *p21*$^{-/-}$ mice, however only MPCs from *p21*$^{-/-}$ mice continued to proliferate after 30 days in culture as the C57BL/6 cells underwent hypertrophy (**Supplementary Figure 3** in Chapter 4). As such, only MPCs from *p21*$^{-/-}$ mice were delivered exogenously to the site of dural injury, and an exogenous C57BL/6 MPC control is still required. Despite the use of stem cell media which has been successful in maintaining the growth of murine adipose derived MPCs, epidural fat MPC proliferation was subpar. This leads us to believe that epidural fat MPCs require additional factors *in vitro* not required by other anatomical sources of murine adipose tissue derived MPCs.

Significance

Dural injuries can arise due to primary trauma/disease or because of post-surgical complications. If dural ruptures go unnoticed and proper care is not administered, serious health issues can follow (239,240). Therefore, synthetic or biological grafts to treat dural injuries and/or complications are commonly studied (241,242). However, a biocompatible graft with correct fiber arrangement and biomechanical properties of the dura mater has yet to be developed. Tissue engineered epidural fat using MPCs derived from other adipose sources are an option and has been used in pre-clinical models (243,244) however, due to our incomplete understanding of the role of epidural fat within the local microenvironment, these solutions may not satisfy all the roles/function of the native epidural fat, such as the ability of epidural fat to contribute to sliding of the adjacent tissues in the microenvironment. Moreover, exogenous delivery of any material into the body requires caution to avoid an inflammatory/rejection response and ensure that the 'cure' is not potentially more harmful than the disease (242).

This thesis highlights the presence of MPCs within the epidural fat and dura as an endogenous source of repair and regeneration in the spinal canal. Studying the *in vivo* role of a novel source(s) of MPCs in the epidural environment has provided new insights regarding the maintenance of the dura mater. This line of research will allow for further investigation on how to overcome dural complications including post-operative issues such as dural fibrosis and ruptures. Additionally, my research suggests that epidural fat be minimally disturbed from its original location as it may have the potential to generate clinically impactful results and positively impact endogenous repair post-surgery. While it may be necessary to remove the fat to be able to clearly see the operational site, the fat need not be discarded and could be replaced before the incision is closed. Although this thesis was focused on dural tissue in homeostasis and post-injury, it is important to consider that the research and clinical impact of this adult stem/progenitor cell population may be important to the homeostasis of other tissue in this microenvironment in addition to the dura mater.

Future Directions

Additional research focused on the phenotypic identification and biological function of these epidural fat/dural MPCs would provide a clearer understanding of their normal function *in vivo* and potentially how this function is disrupted if the epidural fat is removed during surgical procedures of the spine. For example, when treating spinal cord injuries or to surgically remove tumours, all three meningeal layers may be disrupted. It would be necessary to observe how the tissue resident MPC populations respond to these varying insults in the contexts of having the epidural fat present vs. absent. Therefore, in a broader context, there is a real potential of these MPCs playing significant roles in many spinal conditions and surgical procedures. Moreover, a transcriptomic analysis of epidural fat vs. other adipose-derived MPCs would tease out the molecular similarities/differences between these cells. This may provide insight on how (molecularly) epidural fat MPCs regulate homeostasis and if non-epidural fat MPCs share enough similarities to act as a surrogate in the case of tissue engineered epidural fat replacements.

It is important to note that although I have demonstrated that epidural fat/dural MPCs respond to injury by homing to the lesion site, these cells are likely not directly repairing the tissue (e.g., differentiation into new dura) as was indicated by the lack of colocalization of these cells with α-SMA expression. Therefore, additional studies are needed to determine if these cells are playing an immunomodulatory/ cell recruitment role and if so, what factors are these MPCs secreting/recruiting to accomplish this. If the bio-reactive factor(s) can be identified, then they alone might be sufficient to induce repair in place of the cells. Given the ability of MPCs to modulate tissue repair and regeneration via paracrine effects (245), in particular the secretion of soluble factors and extracellular vesicles, MPC derived exosomes are often called nano-therapeutic agents as they have tremendous therapeutic potential in disease and wound repair (246,247). As mediators of MPC paracrine work, MPC-exosomes have been found to immune-regulate and regenerate tissue (246). As such, if epidural fat/dural MPCs are not directly repairing the dura and indeed play a cell secretory role instead, MPC-derived exosomes may be a credible candidate for the response observed. If these MPCs are recruiting cells of the immune system and/or regulation the inflammatory microenvironment of the injury site, then staining for markers such as TNFα or IL1β in addition to pro/anti-inflammatory macrophage phenotypes, may shed light on this function. Based on the literature, MPCs can also initiate angiogenesis (248–250), and

therefore, staining for angiogenic markers such as vascular endothelial growth factor (VEGF) may also reveal if these MPCs play a role in neovascularization of the damaged tissue.

Another interesting future study that will provide further insight into the biological relevance of epidural fat is to determine if it contains any beige adipocytes, or if MPCs within the epidural fat can be induced to take on beige adipocyte fate/phenotype. Given progenitor cells in WAT give rise to beige adipocytes (104), these cells are assumed to behave differently in response to normal physiological cues and insult. Therefore, studying the existence of beige adipocytes in epidural fat might highlight the dynamic roles of epidural fat MPCs in returning homeostasis to injured tissues. This would also introduce new avenues of research in MPCs giving rise to beige fat outside the realm of obesity and thermogenesis.

Finally, much of the literature on the dura mater is centered around cranial dura. Specifically, it has been found that cranial dura mater regulates both the genesis and migration of neural progenitors/neurons during fetal development and plays a vital role in brain development (251). Then postnatally and over the course of infancy, neural crest-derived cells of the dura mater have been found sequestered in niches, which is characteristic of stem/progenitor cells (165,251). Studies that investigate the cellular activity within the dural sac and whether these cells also release soluble factors which coordinate the development of surrounding tissues and bones would give insights in to whether MPCs within the dura mater regulate events in the underlying spinal cord/nerves.

Limitations and Challenges

One limitation in this study has been acquiring mice and therefore obtaining a similar sample size between experiments (**Table 1**). This was due to issues around mouse breeding and in part due to the loss of animals post-tamoxifen administration. Tamoxifen was administered into the peritoneal cavity of the mice as this is a common method used to administer larger volumes of fluid safely in smaller rodents where intravenous injections are difficult (252). Although effective, intraperitoneal injections require competency as inadvertent puncturing of blood vessels in the vicinity or injection into organs can lead to death of the mouse. It took time and practice until I achieved expertise in this procedure, and this resulted in some loses of mice early in my thesis.

Another limitation is that the reporter gene expression in the mice models employed was limited to *Prx1* and *Hic1* expressing cells. It is entirely possible that other MPC markers are enriched in epidural fat/ dural MPCs such as *Sca1*, *CD140a*, *LeptinR*, and C*D44* among others (253–257); and if employed, may have provided different results than *Prx1* or *Hic1*. While it was not possible to use additional reporter mice in this thesis, it would be beneficial to examine the localization of MPCs in the dural microenvironment with some other markers to determine the reproducibility/generalizability of my results. Similarly, α-SMA was the only marker used to identify dura mater in fluorescence imaging. α-SMA is conventionally used as a marker of fibrotic tissue (myofibroblasts) (258,259), therefore it remains unclear whether the dura is repaired post-injury via MPC immune-modulation or through a fibrotic response.

A significant limitation of this study is the lack of *in vitro* experimentation. Epidural fat isolation from mice and subsequent characterization of MPCs was attempted numerous times. The spinal canal in mice is exceptionally small and epidural fat is found unevenly distributed. Harvesting a sufficient amount of tissue whilst avoiding bacterial/fungal contamination during the collection process remains a challenge. As seen in Chapter 4, even when cell seeding was successful, proliferation only lasted for a short period of time before cells underwent hypertrophy. Therefore, optimization of *in vitro* work is required to corroborate functionality of MPCs. This was an interesting observation since we used commercial media that had been tested and validated for mouse adipose derived MPCs. This in itself suggests that epidural fat MPCs require unique cell culture conditions that will need to be optimized.

Finally, as spines were removed from mice *en bloc*, multiple tissues were harvested at once such as bone, cartilage, muscle, fat, ligaments, nerves and blood vessels. Processing, fixation and decalcification times however could not be optimized for every tissue within the spine; therefore, limitations exist in the histology and immunohistochemistry of the spines. These limitations can present themselves as difficulty in sectioning, artifacts in staining or in altered antigen detection. At the beginning of this thesis, Cal-Ex™ (*Fisher Scientific*) was being used as the decalcifying agent, however the detection and clarity in DAPI nuclear staining was severely reduced. Rather than seeing nuclei, fluorescent images displayed smearing. It is believed Cal-Ex™ may be denaturing double-stranded DNA such that intercalating dyes like DAPI cannot detect DNA. Following other studies, samples were then decalcified using 10% EDTA (pH=7) and the visualization of distinct nuclei was made possible (using mounting medium with DAPI). Optimization of nuclear staining on the Cal-Ex™ decalcified samples was attempted by additionally staining with DAPI antibody, histone antibody, Hoechst dye, and TOTO™ -3 Iodide (**Figure 2**). Nuclear staining was only achieved using TOTO™ -3 Iodide, which has high sensitivity for nucleic acid and doesn't require double stranded DNA as other intercalating dyes (such as DAPI) do for binding to DNA for fluorescent detection.

Table 1. Summary of experimental groups and sample size.

Experiment	Mouse strain	n=	
Epidural fat/dura MPC localization *in vivo* over 4 weeks	*Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$	1wk (post induction)	3
		2wk	3
		3wk	4
		4wk	5
	Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; *p21*$^{-/-}$	1wk	3
		2wk	3
		3wk	5
		4wk	3
	Hic1$^{CreERT2+/+}$R26R$^{tdTomato+/+}$	1wk	3
		2wk	4
		3wk	-
		4wk	3
Epidural fat/ dura MPC localization and migration *in vivo* in a skeletally mature mouse model (6 months of age)	*Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$	4m	5
	Hic1$^{CreERT2+/+}$R26R$^{tdTomato+/+}$	4m	5
Dural injuries	*Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$	8	
	Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; *p21*$^{-/-}$	5	
	Hic1$^{CreERT2+/+}$R26R$^{tdTomato+/+}$	4	
	C57BL/6	2	
MPC ablation	*Prx1*$^{CreERT2GFP+/-}$R26R$^{tdTomato+/-}$ R26R$^{DTA+/-}$	4	
	Hic1$^{CreER2+/-}$R26R$^{tdTomato+/-}$R26R$^{DTA+/-}$	3	
Pilot project: delivery of human epidural fat MPCs to an immune compromised dural injury mouse	*Rag1*^{tm1Mom} (*Rag1*$^{-/-}$)	7	
Harvesting epidural fat MPCs	*Prx1*$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$	9	
	Prx1$^{CreERT2\text{-}GFP+/+}$R26R$^{tdTomato+/+}$; *p21*$^{-/-}$	2	
	Hic1$^{CreERT2+/+}$R26R$^{tdTomato+/+}$	6	
	C57BL/6	15	

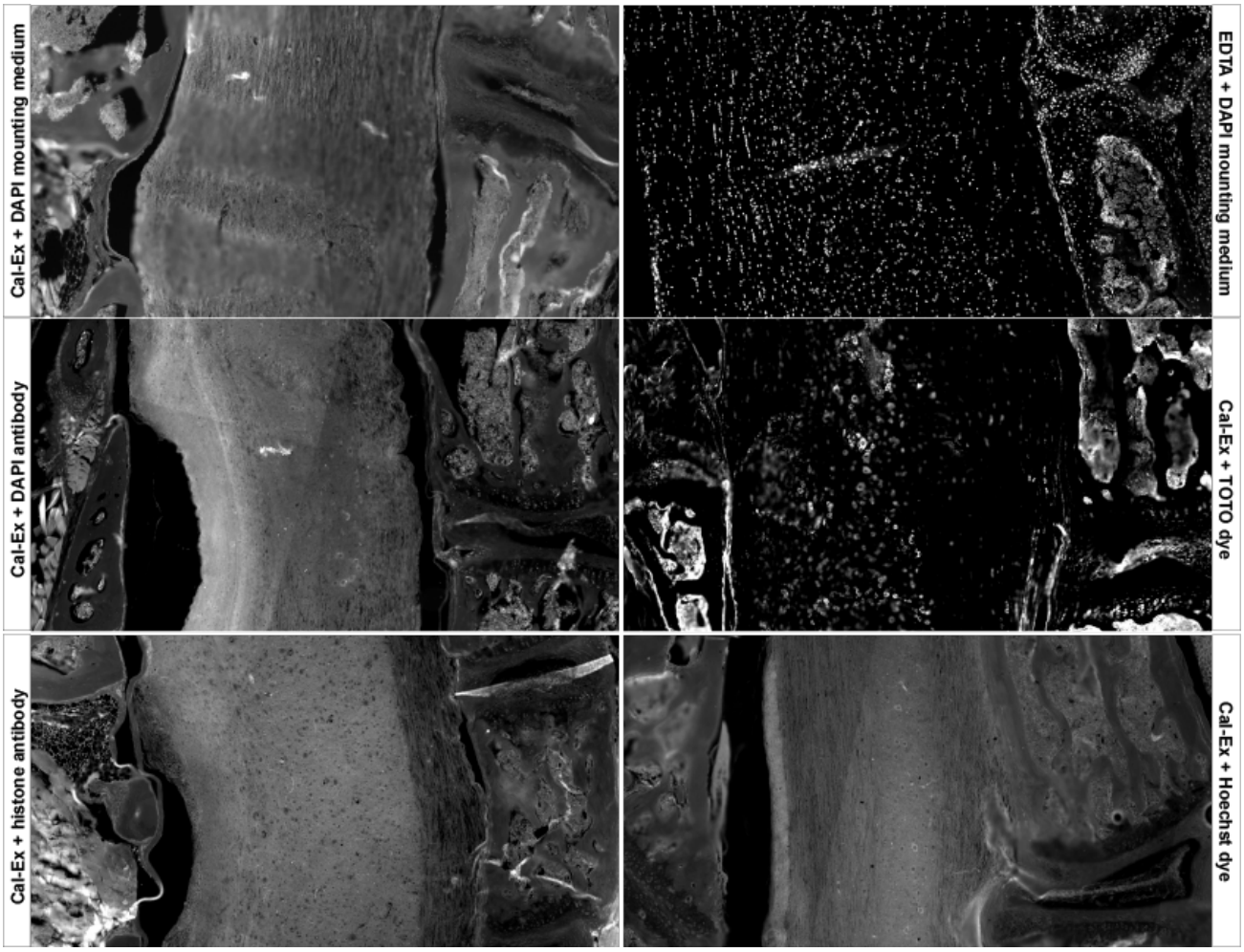


Figure 2. **Optimization of nuclear staining**. To preserve nuclear staining in histological samples, 10% EDTA is preferred. In samples treated with Cal-Ex™, only TOTO™ -3 Iodide staining was able to identify nuclei as opposed to DAPI, Hoechst and an anti-Histone H1 antibody.

APPENDIX A: Pilot Project

Studying the effects of human epidural fat MPCs on dural injury repair

As murine epidural fat isolation proved difficult in this project, a functional analysis of epidural fat MPCs at the molecular level was not possible. Therefore, MPCs were derived from human epidural fat and xeno-transplanted to the site of dural injury in an immune compromised mouse model. This pilot project served to corroborate the role of epidural fat MPCs after injury and determine whether this function is conserved in humans.

Mouse model

Millions of antibodies are generated in the body by a fractional number of genes; This diversity seen in antibody and T-cell receptors is due to a process known as V(D)J (variable-diversity-joining) recombination occurring in lymphocytes (260), which essentially cleaves and recombines V(D)J genes to generate an array of permutations. A common recombinase is believed to developmentally control recombination in both B-cell and T-cell precursors (261). Recombination activating gene-1 (*Rag1*) is a gene which has been found to activate and/or catalyze V(D)J recombination (262) in lymphocytes, hence playing an essential role in the generation of mature B- and T-cells of the adaptive immune system. Deletion of the *Rag1* gene resulted in an immature-stage halt of lymphocyte differentiation leading to small lymphoid organs and no mature B- or T-cells (263). This is similar to the phenotype seen in severe combined immunodeficiency (*scid*) mice (264), however *scid* mice are deemed "leaky" as they can successfully generate B- and T-cell clones by 10-14 months of age (265). The mouse model used for this experiment was the $Rag1^{tm1Mom}$ ($Rag1^{-/-}$) mouse (stock no. 002216 from the Jackson Laboratory). Due to their nonleaky nature, $Rag1^{-/-}$ mice were suitable for this study as human-derived cells were introduced into the spinal canal. Moreover, the *scid* gene mutation is known to have pleiotropic effects (266).

General methodology

Two human epidural fat cell lines with full MSC potential (143) were expanded in human stem cell media (Mesencult, *Stemcell Technologies*). $Rag1^{-/-}$ mice (aged 2 months) were subjected to the dural injury model described in Chapter 2 and 3. The dura was focally punctured with a 30-gauge needle that was loaded with human epidural fat MSCs (20,000 in 10 µl DPBS) and FluoSpheres® carboxylate fluorescent beads (*Life Technologies;* to verify injury site under

fluorescent light). Mice were sacrificed two weeks post-injury for histology (Safranin-O and Fast Green) and immunohistochemistry. To detect the cells *in vivo*, samples were stained with human nuclear antigen (HNA– AF647; Clone # 235-1, *Abcam*), and the dural marker α-SMA – AF647 (Clone # 1A4, *Biolegend*).

Human epidural fat MSCs localize to the site of dural injury and generate fat

When the *Rag1*$^{-/-}$ mouse was subjected to the dural injury without administration of any epidural fat MSCs, α-SMA expression within the dura became discontinuous and was absent within the dural lesion (**Figure 1B**). When human epidural fat MSCs were xeno-transplanted into the site of injury in the *Rag1*$^{-/-}$ mice, fluorescent bead and HNA expression were found within the dural lesion indicating successful delivery of the cells to the site of injury (**Figure 2C)**. HNA expression in what appears to be reconstituted dura mater at the site of injury suggests that human epidural fat MSCs also play a role in the repair/regeneration of human dural tissue *in vivo* (**Figure 2D**). Moreover, α-SMA staining was found within the dura mater at the site of injury, indicating that at least some of the transplanted human MSCs were directly involved in repair by differentiating into connective tissue cells of the dura mater (**Figure 3C**). Previously, α-SMA expression in the dura mater post-injury was only seen when mouse epidural fat MPCs were exogenously delivered to the site of injury (Chapter 4). The same has been found in this study, however it still remains unknown whether human epidural fat MSCs are involved in the direct repair of the dura mater due to aberrant behaviour of cells as a result of *in vitro* expansion (221,222). Interestingly, fat tissue was observed at the site of injury in all animals injected with human epidural fat MSCs (**Figure 2,3 A**). Although epidural fat is present in the spinal canal, its presence and localized abundance adjacent to the injury site was not observed under any other circumstances other than when human epidural fat MSCs were xeno-transplanted. This may suggest that human epidural fat MSCs have a greater bias towards generating fat vs. dura mater. This is not surprising since cultured adipose-derived MSCs can differentiate into fat (38), and have been found to generate epidural fat-like tissue in a transplant-scaffold study (65). As human epidural fat MSCs were found contributing to the restoration of epidural fat, this serves as evidence to refute the common paradigm of epidural as biologically insignificant. This study shows that mouse and human epidural fat MPCs share a similar functional phenotype *in vivo*, yet *in vitro* there are issues with expansion of mouse cells that were not observed with human cells. This is of interest since human and mouse specific MSC

media was used that is validated for adipose derived MPCs in each species. Yet, mouse epidural MPCs underwent hypertrophy/senescence while this was not seen in human epidural fat MPCs. This strongly suggests further media optimization is required for mouse epidural fat MPCs.

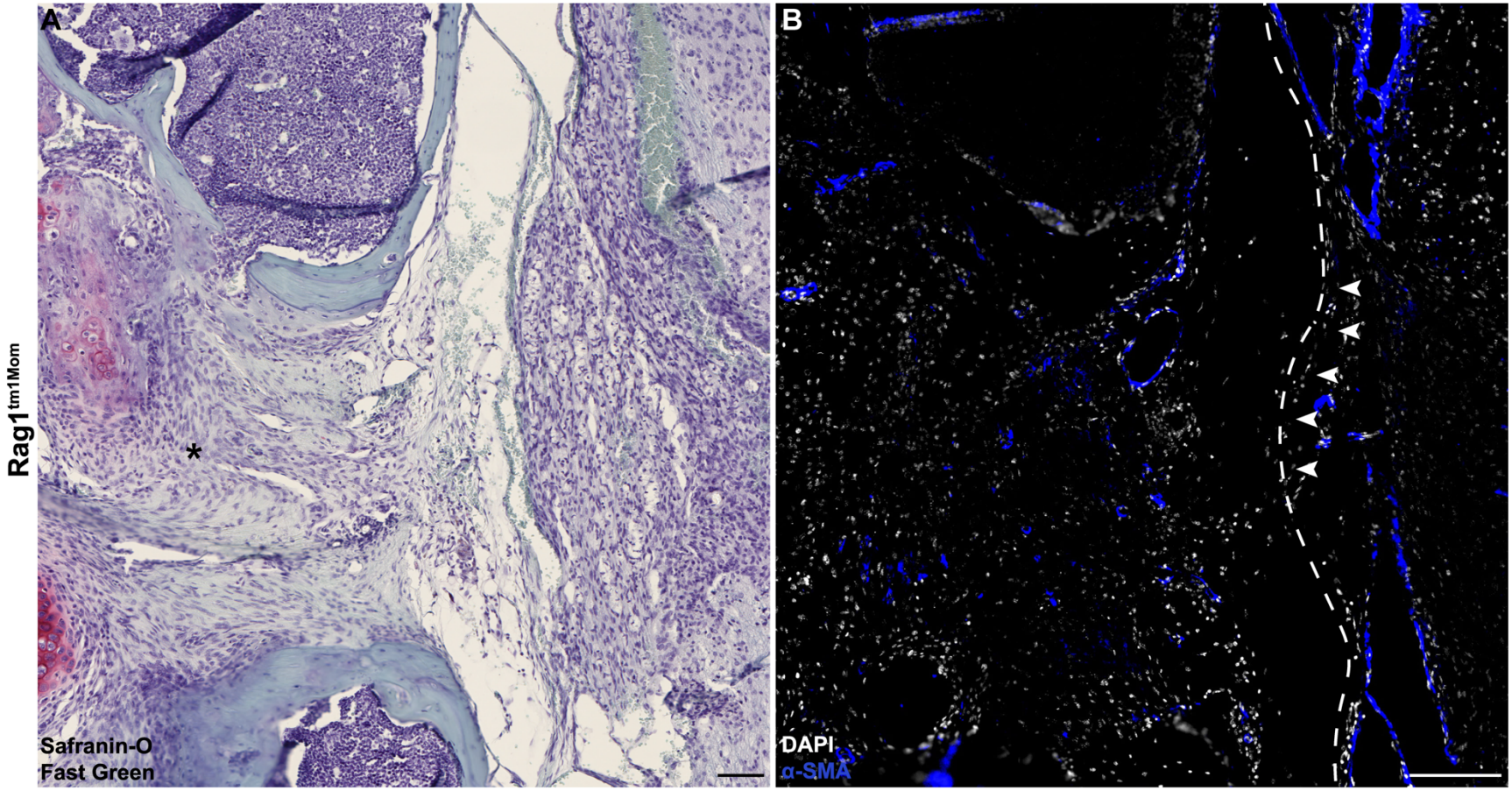


Figure 1. Loss of α-SMA expression seen within the dural lesion. Histological (A) image of the injury site (identified by granulation tissue) is presented alongside corresponding fluorescent α-SMA staining (B) image. The asterisk indicates granulation tissue adjacent to the dural injury and arrows indicate lack of α-SMA staining in the dura mater. No cells were injected in this mouse. Scale bars = 200μm.

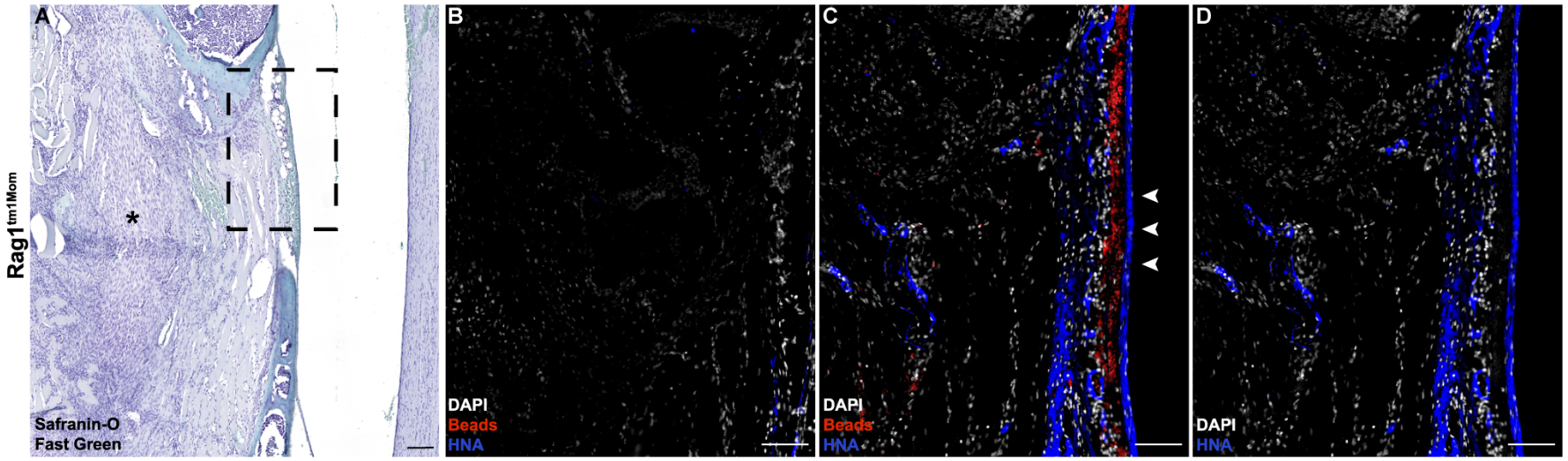


Figure 2. Fluorescent bead and HNA expression were localized to the dural lesion. Xeno-transplantation of human epidural fat MSCs to the site of dural injury in a *Rag1*$^{-/-}$ mouse**.** Histological (A) image of the injury site (identified by granulation tissue and epidural fat) is presented alongside corresponding fluorescent control (B), beads merged with HNA identifying channels (C) and individual HNA staining (C) images. The asterisk indicates granulation tissue adjacent to the dural injury and arrows indicate **f**luorescent bead and HNA expression staining in the epidural fat/dura mater. Scale bars = 200µm (A) and 100 µm (B-D).

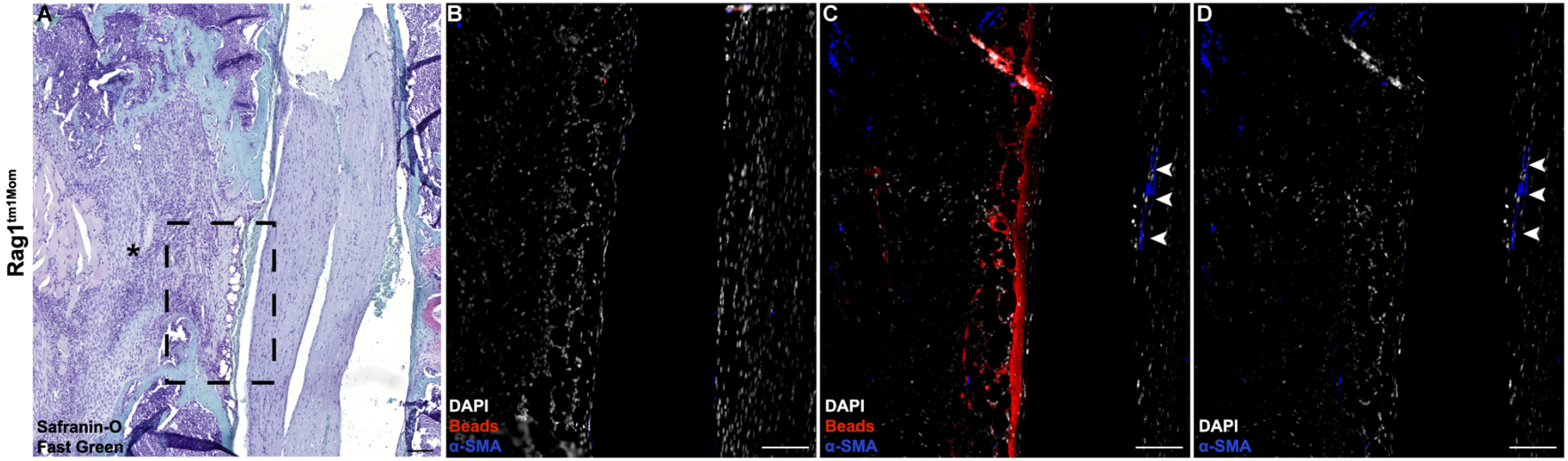


Figure 3. Fluorescent bead and α-SMA expression were localized to the dural lesion. Xeno-transplantation of human epidural fat MSCs to the site of dural injury in a *Rag1*$^{-/1-}$ mouse**.** Histological (A) image of the injury site (identified by granulation tissue and epidural fat) is presented alongside corresponding fluorescent control (B), beads merged with α-SMA identifying channels (C) and individual α-SMA staining (C) images. The asterisk indicates granulation tissue adjacent to the dural injury and arrows indicate **f**luorescent bead and α-SMA expression staining in the epidural fat/dura mater Scale bars = 200μm (A) and 100 μm (B-D).

APPENDIX B: Detailed Protocols

Tamoxifen Preparation

A few days prior to use,

1. Place 50 mg of (Z)-4-hydroxytamoxifen powder (Sigma) in a 15 ml falcon tube
2. Wash tamoxifen vial with 500 µl of 100% ethanol and add to 15 ml falcon tube
3. Sonicate solution in 37°C water bath for 5 minutes. Note: tamoxifen is light sensitive. Therefore, cover the sonicator when samples are inside
4. Remove, slightly mix and add 1 ml of sunflower oil
5. Sonicate for 25 minutes
6. Remove, slightly mix and add 4 ml of sunflower oil

Make sure the total amount in the tube is ~5.5ml as sunflower oil can be lost when pipetting

7. Sonicate for 20 minutes
8. Remove, and ensure the solution is homogenous
9. Aliquot solution in small Eppendorf tubes

Mice receive 1mg/injection therefore 100 µl of the above solution is administered intraperitoneally to the mice.

Tissue fixation, decalcification, processing and embedding

1. Place harvested spine sections in 10% NBF for 7 days
2. Remove NBF and decalcify samples in 10% EDTA (pH=7) with changes every second day for at least 7 changes, or until samples are decalcified (sample can slightly bend)
3. Place samples in cassettes and wash with dH_2O
4. Process samples overnight:
    a. H_2O
    b. 80% ethanol x1 – 1 hour
    c. 95% ethanol x 2 – 1 hour
    d. 100% ethanol x 3 – 1 hour
    e. Xylene substitutions x 3 – 1 hour
    f. Paraffin x 2 – 1 hour
5. Place samples in vacuum for 1 hour and turn on cold plate
6. Attach labelled rings to histology trays and fill with molten wax
7. Place spine within the tray and leave on the cold plate for 1 hour
8. Remove block from the tray and store in a sealed bag
9. Cut blocks using a microtome at 10 µm

Histological Staining: Safranin-O/ Fast Green

1. Citrasolv x 3 - 10 mins
2. 100% ethanol x 2 - 5-10 mins
3. 95% ethanol x 2 - 5-10 mins
4. 70% ethanol x 1 - 5-10 mins
5. dH_2O x 1 - 5 mins

} Rehydrate sections

***Citrasolv**: solvent degreaser; deparaffinizes*

***Hematoxylin**: deep blue-purple, positive ly charged basic dye; binds acidic and negatively charged tissue components like DNA, RNA*
***Tap water**: allows stain to develop*

6. Hematoxylin - 8 mins
7. Room temperature running tap water - 15 mins

8. Acid Alcohol - 1 min
9. H_2O - 1 min
10. Ammonia Water - 1 min
11. Fast Green - 7 mins
12. 1% Acetic Acid - 1 min MAX
13. dH_2O (replace every time)- 1 min
14. Safranin-O - 1 min 45 sec
15. 70% ethanol - 30 sec
16. 95% ethanol - 1 min
17. 100% ethanol x 3 - 3 mins

} Clear excess staining

***Acid Alcohol**: to de-stain*
***Ammonia water**: bluing step; converts the initial soluble red color within the nucleus to an insoluble blue color*
***Fast Green**: counterstain; stains cytoplasm, collagen and mucus green*
***Acetic acid**: weak acid; de-stain excess non-specific stains*
***Saf-O**: red, positively charged basic dye; stains cartilage (proteoglycans), mucins and mastocyte granules red*

18. Citrasolv (X, Y, Z, ZZ) x 4 - 5 mins
19. Dry slides with Kimwipe
20. Place 1 drop of Flo-Texx Mounting Medium on each tissue sample
21. Coverslip (covering all tissue)
22. Place slides in metal tray and leave in the fume hood to dry overnight

***Citrasolv**: remove any remaining wax*

Cartilage (proteoglycans) → RED
Cells → BLUE
Background → GREEN

Immunohistochemistry

Deparaffinize and rehydrate slides:

1. Citrasolv x 2 - 10 mins
2. 100% ethanol x 2 - 10 mins
3. 95% ethanol x 2 - 10 mins
4. 70% ethanol x 1 - 10 mins
5. dH_2O x 1 - 5 mins

***1M sodium citrate**: dissolve 129.03g of sodium citrate powder in 500 ml dH2O*

***10mM sodium citrate**: add 5 mL of 1 M sodium citrate stock in 495 mL of dH2O*

Begin step 6 when samples are in dH_2O

Antigen retrieval:

6. Microwave 10mM sodium citrate (pH = 6.0) for 5 minutes or until temperature reaches 60-65°C
7. Place slides in sodium citrate and let cool at room temperature for 1 hour
8. dH_2O x 2 - 10 mins

Block non-specific binding:

9. Place slides in a 1:500 dilution of goat serum for 1 hour at room temperature

Primary antibody:

10. TBST - 5 mins
11. Add 20 µl of antibody solution to samples
12. Cover slides in a humidity chamber (no coverslip)
13. Incubate overnight at 4°C

***Goat serum**: 200 µl serum in 100 ml TBST*

Following day:

Secondary antibody (if required):

14. TBST x 2 - 10 mins
15. PBS x 1 - 10 mins
16. Add 20 µl of 2° antibody solution to samples
17. Incubate at room temperature for 1 hour
18. Repeat wash steps 14/15
19. Dry slides and add DAPI mounting medium
20. Coverslip
21. Let slides dry at room temperature for ~2 hours
22. Refrigerate in slide tray (protected from light) until scanning

***TBST**: combine 50 ml 20x TBS, 5 ml 10% Tween & Triton stock, and 945 ml dH2O*

Epidural Fat MPC Isolation

1. Sacrifice anesthetized (isoflurane 3.0vol/vol% with 1L/min O2) mice via cervical dislocation
2. Remove intact spines and place in a DPBS (*Lonza- BioWhittaker*) and antibiotic-antimycotic (*Thermo Fisher Scientific*) solution to prevent contamination
3. Starting from the lumbar region, cut the spine to access the spinal canal, from where epidural fat can be isolated.
4. Plate the fat in 6-well plates with Mesencult™ Basal Medium (*Stemcell Technologies*) and incubate at 37°C and 5% CO_2 until cells begin proliferating.
5. At 60% confluency, wash cells with DPBS and passage with 0.05% Trypsin EDTA (*Gibco Life Technologies*) and then allow to proliferate with media changes every two days.
6. After sufficient proliferation of putative epidural fat MPCs, cell differentiation in to osteogenic, adipogenic and chondrogenic lineages can be induced as follows:

Table 1. **Basal medium for cell differentiation** (all from *Thermo Fisher Scientific*).

	Volume (ml)	**Final Concentration**
DMEM/ F-12 HEPES	500	-
+fetal bovine serum (FBS)	50	10%
+Antibiotic- Antimycotic	5	1%
+MEM non-essential amino acids (MEM-NEAA)	5	1%

Osteogenic differentiation

1. Seed cells as a monolayer (minimum 2.5 x10^4 cells/well in a 48 well plate) in basal medium (**Table 1**) along with osteogenic agents (**Table 2**). Total volume in wells should not exceed 0.5mL
2. Perform media changes every second day, for a total of 21 days

Table 2. **Osteogenic differentiation media stock** (all from *Sigma Aldrich*)

	Volume per 50ml of stock (µl)	**Final Concentration**
Dexamethasone (Dex; 10^{-4} M)	50	1nM
L-Ascorbic Acid	50	50µg/mL
β-glycerophosphate (B-GP)	1000	10mM

Adipogenic differentiation

1. Seed cells as a monolayer (minimum 2.5 x10^4 cells/well in a 48 well plate) in basal medium (**Table 1**) and adipogenic agents (**Table 3**). Total volume in wells should not exceed 0.5mL
2. Perform media changes every second day, for a total of 21 days

Table 3. Adipogenic differentiation media stock (all from *Sigma Aldrich*).

	Volume per 50ml of stock (µl)	**Final Concentration**
Isobutyl methylxanthine (IBMX)	100	500µM
Indomethacin (IM)	100	200µM
Dex (10^{-2} M)	5	10nM
Insulin	500	10µM

Chondrogenic differentiation

1. Culture cell pellets (minimum 2.5 x10^4 cells) in 1.5mL Eppendorf tubes in basal medium (**Table 1**) and chondrogenic agents (**Table 4**). Total volume in tubes should not exceed 0.15mL
2. Perform media changes every second day, for a total of 21 days.

Table 4. **Chondrogenic differentiation media stock** (BMP-2 and TGF- β3 from *PeproTech Inc*., Dex and L-Ascorbic Acid from *Sigma Aldrich*, sodium pyruvate from *Thermo Fisher Scientific*, and ITS from *Lonza-BioWhittaker*).

	Volume per 50ml of stock (µl)	**Final Concentration**
Bone morphogenic protein 2 (BMP-2)	25	500ng/mL
Transforming growth factor β-3 (TGF- β3)	50	10ng/mL
Dex (10^{-4} M)	5	100µM
L-Ascorbic Acid	50	50µg/mL
MEM-NEAA	1240	-
Sodium Pyruvate	455	-
Insulin transferrin selenium (ITS)	500x= 100 **or** 100x= 500	-
Sodium hydroxide (NaOH)	200	-

Appendix C: Permission Letter and Manuscript License

2021-04-15

Dear Sathvika Mudigonda, Dr. Paul Salo, Dr. Alim Mitha, and Dr. Roman Krawetz,

I am in the process of writing my master's thesis and would like your permission to include the following published manuscript (on which you are co-authors) in the thesis. This thesis will be added to the institutional repository at the University of Calgary and the Library and Archives Canada.

University of Calgary Theses Repository – The Vault http://theses.ucalgary.ca/ Library and Archives Canada http://collectionscanada.gc.ca/obj/s4/f2/frm-nl59-2-e.pdf

Title: Epidural fat mesenchymal stem cells: Important microenvironmental regulators in health, disease, and regeneration: Do EF-MSCs play a role in dural homeostasis/maintenance?

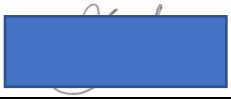
Authors: Sophia Shah, Sathvika Mudigonda, Alim P Mitha, Paul Salo, and Roman J Krawetz. Journal: Bioessays
Year: 2021

Volume/Issue: 43(2)

doi: 10.1002/bies.202000215. Epub 2020 Nov 16.

Your consent to my request would be appreciated. If you have any questions, please contact me. If you agree, please sign below after reading the statement of consent.

I give permission for Sophia Shah to use the publication highlighted above in her master's thesis at the University of Calgary.

Name	Signature
Sathvika Mudigonda	
Dr. Paul Salo	
Dr. Alim Mitha	
Dr. Roman Krawetz	

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