Stem Cells Translational Medicine, 2022, **11**, 200–212
https://doi.org/10.1093/stcltm/szab014
Advance access publication 8 March 2022
Original Research

Prx1+ and *Hic1*+ Mesenchymal Progenitors Are Present Within the Epidural Fat and Dura Mater and Participate in Dural Injury Repair

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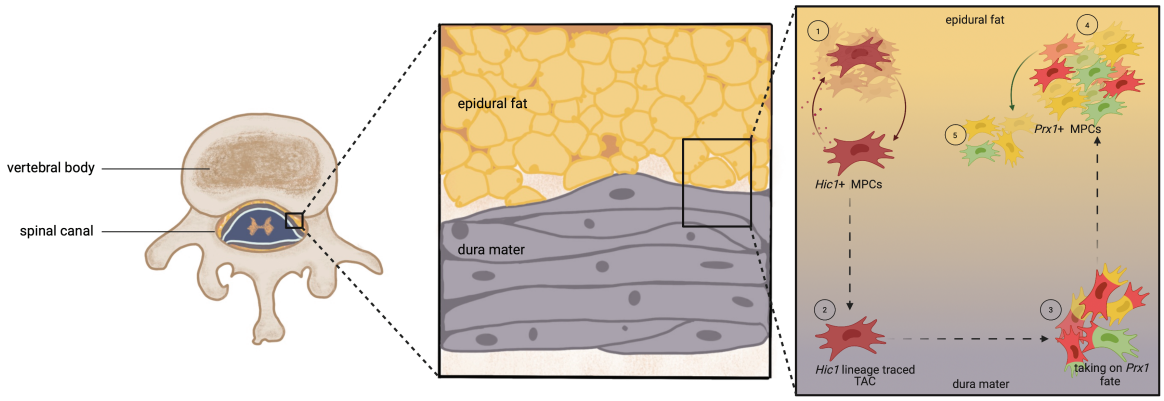
Abstract

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 the murine dura mater. This led us to believe that epidural fat may regulate homeostasis and regeneration in the vertebral microenvironment. 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.

Key words: regenerative medicine; epidural fat; stem cells; progenitor cells.

Graphical Abstract



Received: 12 April 2021; Accepted: 14 October 2021.

Significance Statement

The epidural fat is commonly discarded during surgical procedures involving the spine. Our study demonstrates that at least two progenitor populations exist in the epidural fat and/or dura mater and these cells contribute to tissue homeostasis and injury repair. This study further challenges the notion of epidural fat as an incidental/biologically inert tissue.

Introduction

Since their discovery in bone marrow by A.J. Friedenstein in 1976,[1] mesenchymal progenitor cells (MPCs) have been identified in almost all human tissues.[2] Although tissue of origin confers site-specific differences in MPC characteristics—such as morphology, fate commitment biases, and immune-phenotype,[3] 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.[4,5] 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.[6] MPCs have been identified and/or derived from most mesodermally derived tissues within the body.[7-9] Adipose tissue is an abundant source of MPCs, with less-invasive isolation methods and a higher MPC yield compared to bone marrow.[10] 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.[11-14] Adipose/fat is present throughout the body at varying quantities[15] and is typically harvested/discarded during cosmetic procedures and/or surgical interventions.[16] As fat is one of the largest organ systems in the body, and fat deposition typically increases,[17] adipose tissue is regularly used as a viable source of MPCs.[15,18] Furthermore, in vivo lineage tracing studies in mice have demonstrated that mesenchymal stem cells (MSCs) are present within white[19] and brown fat throughout the body.[20] While there is a substantial body of knowledge of adipose MPCs throughout the body, relatively little is known about the role of MPCs within epidural fat.

Epidural fat is found within the spinal canal[21] and is unevenly distributed along the spinal canal, increasing craniocaudally and posteriorly.[22] During development, epidural fat is found in greater abundance adjacent to the dural sac, whereas by adulthood, it becomes more discontinuous.[23] Epidural fat has a lower density versus subcutaneous fat, and this is thought to allow it to conform better to the epidural space.[21] While the localization of epidural fat is not associated with body size, the amount of epidural fat does vary in proportion with body size.[24-27] Commonly, epidural fat is considered to act as a shock absorber,[27] yet, little direct evidence supports this hypothesis. Clinically, however, it is commonly considered a space-filling tissue and is typically discarded during surgery to increase the operational field of view when present. This paradigm is being challenged by the recent discovery of MPC populations within human epidural fat.[28,29] Furthermore, using a *Prx1* lineage tracing mouse, it was also found that these epidural fat MPCs populated the dura mater.[28] 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.[30]

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[31] and are essentially master regulators of morphogenetic processes across species.[32] *Prx1* is a transcription coactivator highly enriched in developing mesodermal tissues (eg, limb buds),[33] is found in the ectomesenchyme of the face,[34] and is also expressed in adult tissues like the heart,[35] and regulates neural progenitors stemness.[36] 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.[37] *Prx1* expression has also been identified in MPCs capable of differentiating into bone,[38] cartilage,[39] and fat in vivo.[40] *Prx1* has been previously shown as a robust adipose MPCs marker in white adipose tissue.[40] 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.[41] Moreover, *Hic1* is transcriptionally regulated by many cell cycle genes such as *p53*,[42] *p21*,[43] and *E2F1*,[44] 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.[45] *Hic1*$^+$ MPCs give rise to transit-amplifying cells (TACs) that support regeneration post-injury.[45,46]

In a previous study by Krawetz and Lyons,[28] an adult 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.

Therefore, in this study, we 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.

Materials and Methods

Experimental Outcome

The experimental outline of the study is presented in Fig. 1. Briefly, *Prx1* and *Hic1* reporter mice were induced with 4 consecutive doses of tamoxifen to induce the expression of tdTomato, and mice were sacrificed at 1-, 2-, or 4-week post-induction to track the these MPC population (Fig. 1A). To ablate these MPC populations, *Prx1* and *Hic1* mice carrying a DTA (diphtheria toxin subunit A) transgene were induced

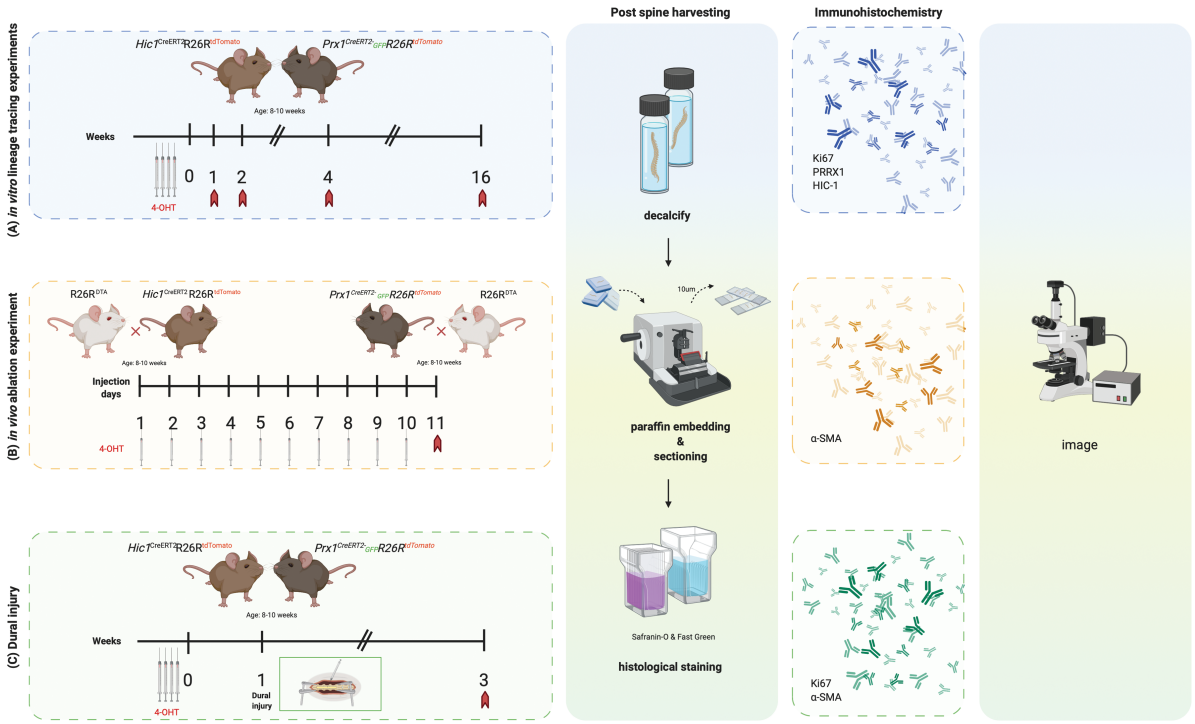


Figure 1. Schematic overview of the experimental design used in the current study. *Prx1* and *Hic1* reporter mice were induced with 4 consecutive doses of tamoxifen to induce the expression of tdTomato. Mice were sacrificed at 1-, 2-, or 4-week post-induction, and the spines were processed with histological and immunohistochemical analysis (A). *Prx1* and *Hic1* mice carrying a DTA transgene were induced with 10 consecutive doses of tamoxifen to specifically ablate the *Prx1*- and *Hic1*-positive cell populations. Mice were sacrificed at 11 days after the first tamoxifen injection and the spines were processed with histological and immunohistochemical analysis (B). *Prx1* and *Hic1* reporter mice were induced with 4 consecutive doses of tamoxifen to induce the expression of tdTomato. One week after the last tamoxifen injection, the mice underwent a dura injury. Mice were sacrificed at 2-week post-injury and the spines were processed with histological and immunohistochemical analysis (C). Abbreviations: DTA, diphtheria toxin subunit A; *Hic1*, hypermethylated in cancer 1; *Prx1*, paired related homeobox-1.

with 10 consecutive doses of tamoxifen. Mice were sacrificed at 11 days after the first tamoxifen injection (Fig. 1B). To determine if these MPC populations respond to dural injury, *Prx1* and *Hic1* reporter mice were induced with 4 consecutive doses of tamoxifen to induce the expression of tdTomato. One week after the last tamoxifen injection, the mice underwent a dura injury. Mice were sacrificed at 2-week post-injury (Fig. 1C). Spines from all mice were processed for histological and immunohistochemical analysis.

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). An n = 3 mice were used per group per time point, based on the total number of mice used, it was not possible to have equal numbers of males and females. However, every group contained male and female mice.

Lineage Tracing

Prx1$^{\text{CreERT2-GFP+/+}}$R26R$^{\text{tdTomato+/+}}$ (derived from stock no. 029211 and 007914 from The Jackson Laboratory; Supplementary Fig. 1) and *Hic1*$^{\text{CreERT2+/+}}$R26R$^{\text{tdTomato+/+}}$ (courtesy of Dr. T. Michael Underhill, University of British Columbia; Supplementary Fig. 2) reporter mice were used in this study. The active Z isomer of tamoxifen ((Z)-4-OHT, Sigma-Aldrich) was administered to both mice strains intraperitoneally (1 mg/injection) for 4 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-, and 4-week and at 4-month post-tamoxifen induction. Additionally, MPC lineage tracing was performed on aged mice (6 months) at 1-, 2-, and 4-week post-tamoxifen induction. Mice were sacrificed via CO_2 asphyxiation, and intact spines were removed and fixed for 7 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 4′,6-diamidino-2-phenylindole (DAPI) (emission wavelength 420-470 nm; Biotium) was applied to slides, and endogenous GFP and tdTomato fluorescence was assayed using an Axio Scan. Z1 Slide Scanner microscope (Carl Zeiss) outfitted with a Plan-Apochromat objective (10×/0.8 or 20×/0.8). The following filters were applied: DAPI (353 nm/465 nm), EGFP (493 nm/517 nm), DsRed (563 nm/581 nm).

Histology and Immunohistochemistry

Sections were deparaffinized with SlideBrite (Thermo Fisher Scientific) and then dehydrated with ethanol prior to being stained using safranin-O with fast green and hematoxylin counterstains to examine the general morphology of the spine as well as the presence of glycosaminoglycan. To prepare samples for immunostaining, serial sagittal paraffin sections (10 µm) were deparaffinized in CitriSolv (Thermo 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 coverslipped.

Cell Enumeration

Cells positive for specific markers were quantified within 2 regions of interest (area = 1.12×10^5 sq. µm): dura and epidural fat.[47,48] Briefly, $n = 3$ tissue sections per animal were counted for each fluorescent filter (eg, EGFP, R-PE, APC) and in combination when applicable. Two independent observers counted all images and their values were averaged. All data were analyzed with GraphPad Prism 8. All datasets containing 2 experimental groups were analyzed using a two-tailed unpaired parametric *t* test with a 95% confidence interval ($a = 0.05$). All datasets containing more than 2 experimental groups were analyzed using a one-way analysis of variance (ANOVA) with a 95% confidence interval ($a = 0.05$).

MSC Ablation

Prx1$^{CreERT2-GFP+/+}$R26R$^{tdTomato+/+}$ and *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ mice were crossed with R26R$^{DTA+/+}$ mice (DTA; stock no. 010527 from The Jackson Laboratory) mice to generate the *Prx1*$^{CreERT2GFP+/-}$R26R$^{DTA+/-}$ and *Hic1*$^{CreERT2+/-}$R26R$^{DTA+/-}$ strains. (Z)-4-hydroxytamoxifen (1 mg/injection) was administered to mice (aged 2 months) intraperitoneally for 10 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.

Dural Injuries

The injury model was performed on both induced *Prx1* and *Hic1* reporter mice. Mice were anesthetized (isoflurane 3.0 vol/vol% with 1 L/min O_2), 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 was 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 2-week post-injury for histology (Safranin-O and Fast Green) and immunohistochemistry.

Results

Prx1+ Cells Enrich the Dura Mater Over Time

Post-tamoxifen induction in the *Prx1*$^{CreERT2-GFP+/+}$R26R$^{tdTomato+/+}$ model, if the cells continue to express *Prx1*, GFP, and tdTomato expression is co-localized (resulting in a yellow cell). Once *Prx1*$^+$ cells commit to a fate decision, these cells lose *Prx1* (and GFP) expression, yet retain tdTomato expression (resulting in a red cell). If a *Prx1*$^+$ cell did not under recombination it would solely express GFP (resulting in a green cell). At 1- and 2-week post-tamoxifen induction, few *Prx1*$^+$ cells were present within the dura mater (Fig. 2A, 2B), however, by 4-week post-induction, there was an enrichment of *Prx1*$^+$ cells within the dura mater (Fig. 2C). 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 (Fig. 2A-2C). It is also important to note that the *Prx1*$^+$ MPCs within the dura mater remained *Prx1*$^+$ as they retained the expression of GFP (Supplementary Fig. 3). These *Prx1*$^+$ MPCs were non-proliferative within the dural tissue as Ki67 staining (blue) was absent over the time points examined (Fig. 2A-2C). Interestingly, in the skeletally mature mouse (6 months old; 4-month post-tamoxifen induction), single *Prx1*$^+$ cells were observed interspaced every ~100 µm within the dura mater (Fig. 3). Similar to lineage traced *Prx1*$^+$ cells in the younger mice, these cells also retained GFP expression suggesting they retained expression of *Prx1* (Fig. 3). Yet, in contrast to the earlier time points, these cells expressed Ki67 (Fig. 3B, 3C), suggesting they were proliferative within the dural tissue (Fig. 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 Fig. 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 MPCs (GFP$^+$) remained in the dura mater (Supplementary Fig. 4).

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 1- and 2-week post-tamoxifen induction (Fig. 2D, 2E). However, by 4-week post-tamoxifen induction, the adjacent epidural fat was enriched with *Hic1*$^+$ MPCs (Fig. 2F). Ki67 staining revealed that nearly all the *Hic1*$^+$ MPCs within the dura mater were proliferative (Fig. 2D) with the exception of 4 weeks, where no Ki67 staining was present within *Hic1*$^+$ MPCs (Fig. 2F). At 4-month post-tamoxifen induction, *Hic1*$^+$ MPCs were once again observed within the dura mater, with minimal tdTomato expression observed within the epidural fat (Fig. 3D). Furthermore, these *Hic1*$^+$ MPCs within the dura mater were proliferative as evidenced by Ki67 expression (Fig. 3D).

When comparing the number of *Prx1*$^+$ or *Hic1*$^+$ lineage traced MPCs in the dura and epidural fat over time, an inverse relationship between the 2 MPC populations was observed (Fig. 2G). Specifically, at all 3 time points examined (1-, 2-, and 4-week post-induction), there were increased *Hic1*$^+$ versus *Prx1*$^+$ lineage traced MPCs present in the dura, but there were increased *Prx1*$^+$ versus *Hic1*$^+$ lineage traced MPCs in the epidural fat; except for 4-week post-induction, where there was no difference (Fig. 2G). At the skeletally mature time point (4-month post-induction), increased numbers of *Hic1*$^+$ versus *Prx1*$^+$ lineage traced MPCs were observed in both the dura and epidural fat (Fig. 3E).

Prx1+ and Hic1+ MPC Populations Overlap

Prx1$^{CreERT2-GFP+/+}$R26R$^{tdTomato+/+}$ spines were stained for Hic1 (Fig. 4A-4C, Supplementary Fig. 5) and *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ spines with for Prx1 (Fig. 4D-4F, Supplementary

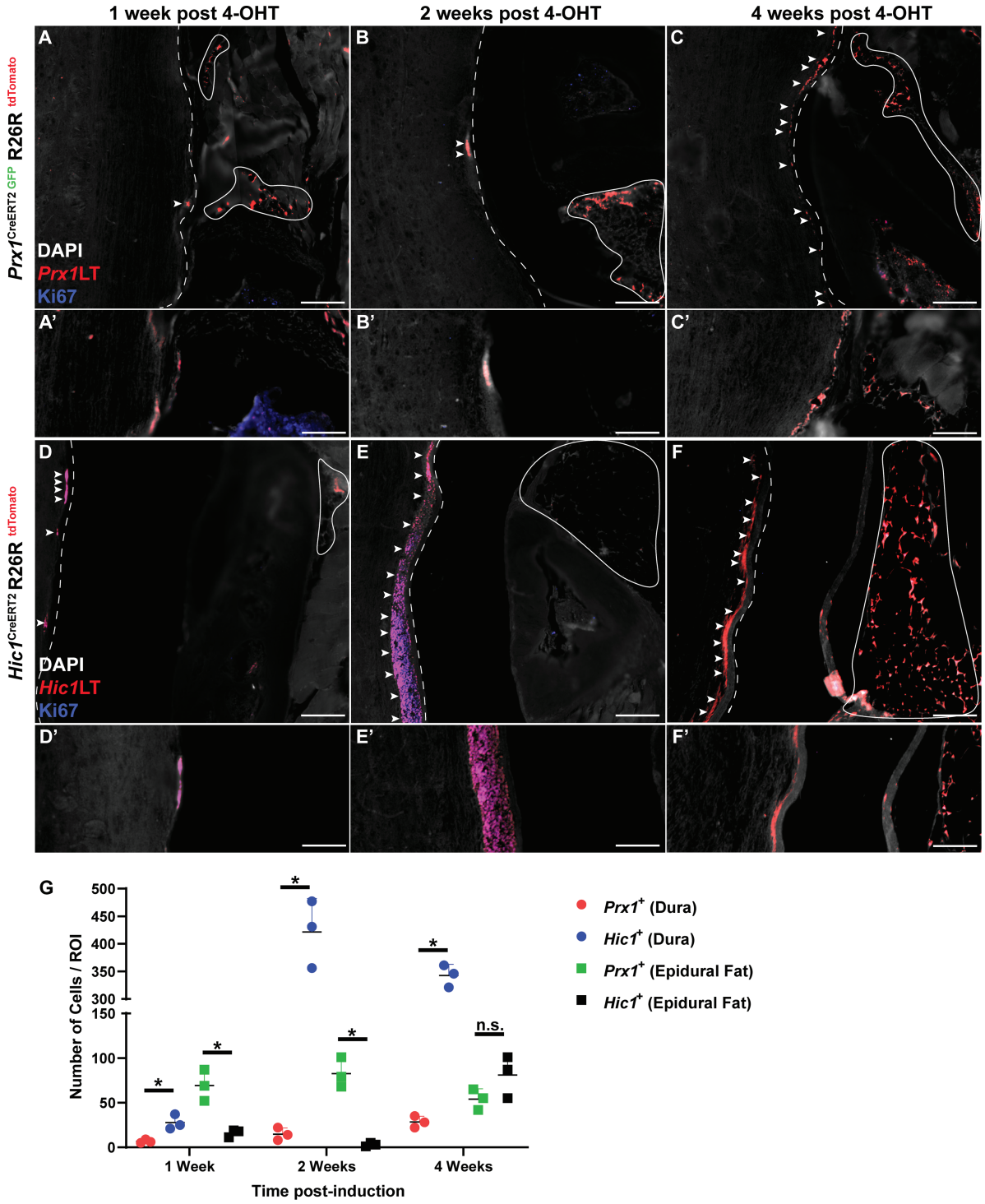


Figure 2. *Prx1*⁺ (A-C) and *Hic1*⁺ (D-F) MPC lineage tracing 1-, 2-, and 4-week post-tamoxifen induction. Sections were also stained with Ki67 to identify proliferative cells (A-F). The dura mater is highlighted by the dashed line while the epidural fat is encircled by the solid line. Arrows indicate examples of *Prx1*⁺ and *Hic1*⁺ cells within the dura mater. Quantification of *Prx1*⁺ and *Hic1*⁺ MPCs within the dura mater and epidural fat (G). An $n = 3$ mice were used per group per time point. $*P < .05$. Scale bars = 100 μm. Abbreviations: *Hic1*, hypermethylated in cancer 1; MPCs, mesenchymal progenitor cells; *Prx1*, paired related homeobox-1.

Fig. 5). This was undertaken to determine if there was any overlap between the *Prx1* and *Hic1* MPC lineages during the time points examined (1-, 2-, and 4-week post-tamoxifen induction). In the dura of *Prx1* lineage traced mice, ~90% of *Prx1*⁺ MPCs also expressed Hic1 at 1-week post-induction, however, this level decreased substantially at 2- and 4-week post-induction (~10%) (Fig. 4A-4C). This inverse pattern was observed in the dura of *Hic1* lineage traced mice with *Prx1* immunostaining (Fig. 4D-4F). Specially, minimal overlap was observed at 1- and 2-week post-induction (~10%), and this increased to ~50% by 4-week post-induction (Fig. 4D-4F).

In the epidural fat of *Prx1* lineage traced mice, ~20% of *Prx1*⁺ MPCs also expressed Hic1 at 1- and 4-week

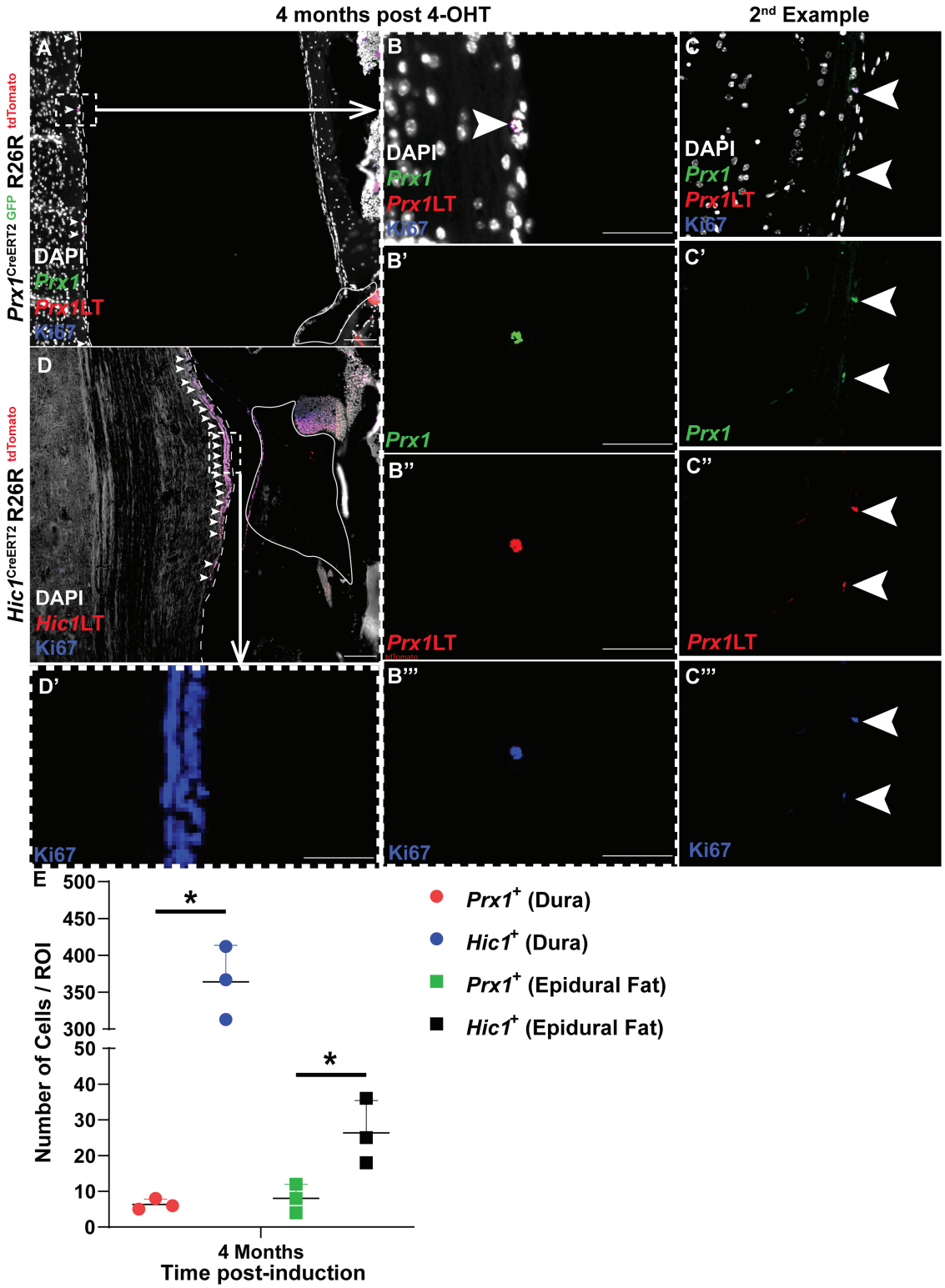


Figure 3. *Prx1*+ (A-C) and *Hic1*+ (D) MPC lineage tracking (LT) in skeletally mature mice (4-month post-tamoxifen induction). Individual GFP (*Prx1*), Tomato (*Prx1*LT), and Ki67 channel images (B/C', B/C'', B/C''') are presented to demonstrate that the *Prx1*+ LT cells remain undifferentiated. The 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. An individual Ki67 channel image (D) is presented to demonstrate that the *Hic1*+ LT cells remain proliferative. The dura mater is outlined by the dashed line while the epidural fat is encircled by the solid line (C). Quantification of *Prx1*+ and *Hic1*+ MPCs within the dura mater and epidural fat (E). An $n = 3$ mice were used per group per time point. $*P < .05$. Scale bars = 100 μm (A, C) and 50 μm (B-B''). Abbreviations: GFP, green fluorescent protein; *Hic1*, hypermethylated in cancer 1; MPCs, mesenchymal progenitor cells; *Prx1*, paired related homeobox-1.

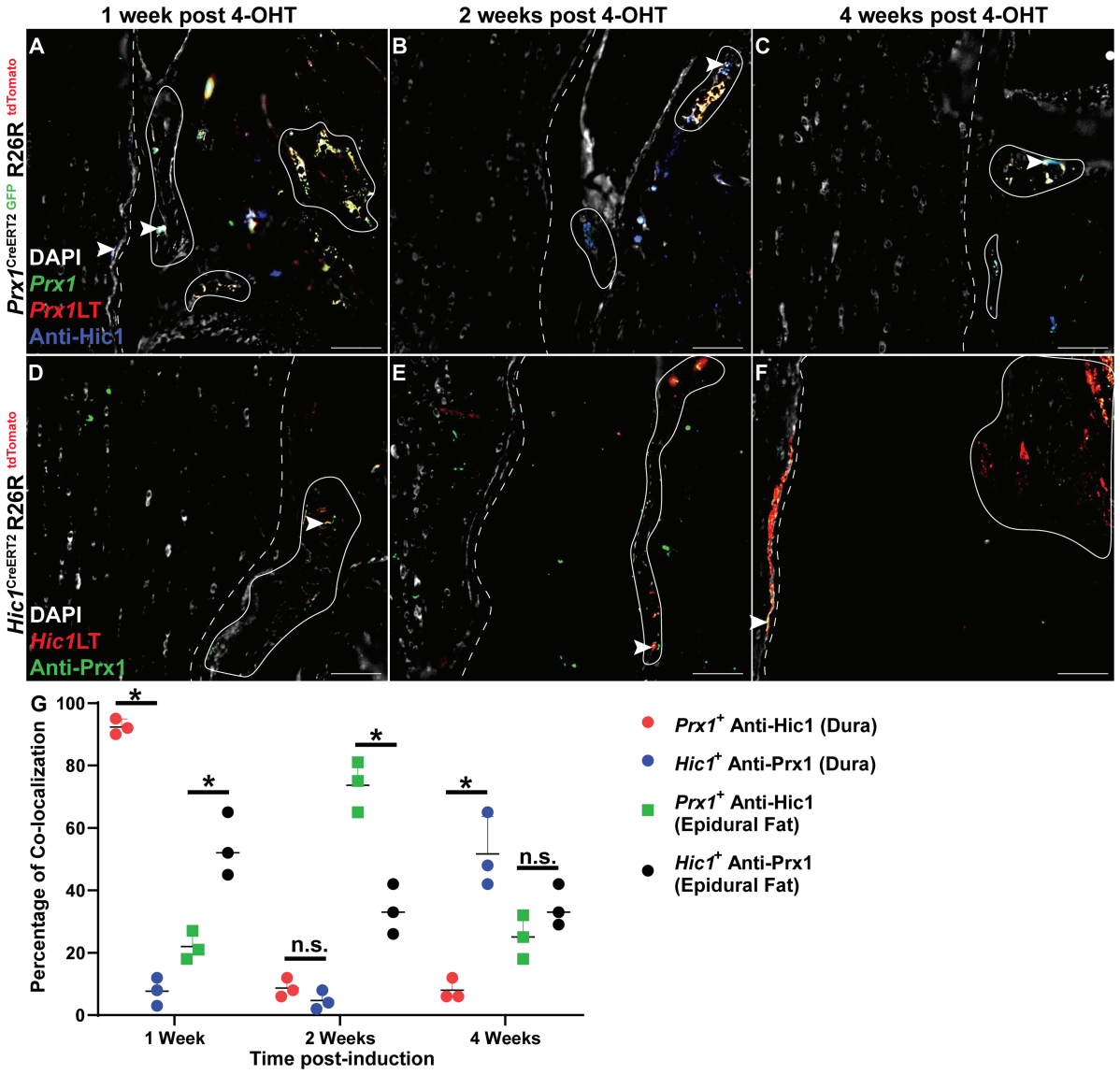


Figure 4. *Hic1* protein expression in *Prx1*$^{CreERT2-GFP+/+}$R26R$^{tdTomato+/+}$ mice (A-C) and *Prx1* protein expression in *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ mice (D-F) 1-, 2-, and 4-week post-tamoxifen induction. The dura mater is outlined by the dashed line while the epidural fat is encircled 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). An $n = 3$ mice were used per group per time point. $*P < .05$. Scale bars = 100 μm. Abbreviations: *Hic1*, hypermethylated in cancer 1; *Prx1*, paired related homeobox-1.

post-induction, however, this level temporality increased at 2-week post-induction (~75%) (Fig. 4A-4C). Once again, an inverse pattern was observed in the epidural fat of *Hic1* lineage traced mice with *Prx1* immunostaining (Fig. 4D-4F). Specially, ~50% overlap between *Hic1* and Prx1 staining was observed at 1-week post-induction, and this decreased to ~30% by 2- and 4-week post-induction (Fig. 4D-4F).

Interestingly, we were able to identify instances of asymmetric cell division (~1 instance per 2 or 3 sections) in which a lineage traced cell (in this case *Hic1*) gave rise to one cell expressing *Prx1* (or possibly the mother cell was *Prx1*-positive) while the other did not (Supplementary Fig. 6).

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 microenvironment. Therefore, *Prx1*$^{CreERT2GFP+/-}$R26R$^{DTA+/-}$ and *Hic1*$^{CreER2+/-}$R26R$^{DTA+/-}$ mice were used 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 (Fig. 5). In the normal dura mater, α-SMA staining is fairly ubiquitous throughout (Fig. 5A, 5D), however, when *Prx1*$^{+}$ or *Hic1*$^{+}$ MPCs were ablated, nearly all α-SMA staining was lost in the dura mater (Fig. 5B, 5E, 5G). However, since genetic ablation is not 100% efficient, there were areas within the epidural fat and/or dura mater that retained *Prx1*$^{+}$ or *Hic1*$^{+}$ MPCs (Fig. 5C, 5F, 5G). In these cases, α-SMA expression was observed in the dura mater, however, the staining pattern was punctate and discontinuous (Fig. 5C, 5F). 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 (Fig. 5C, 5F).

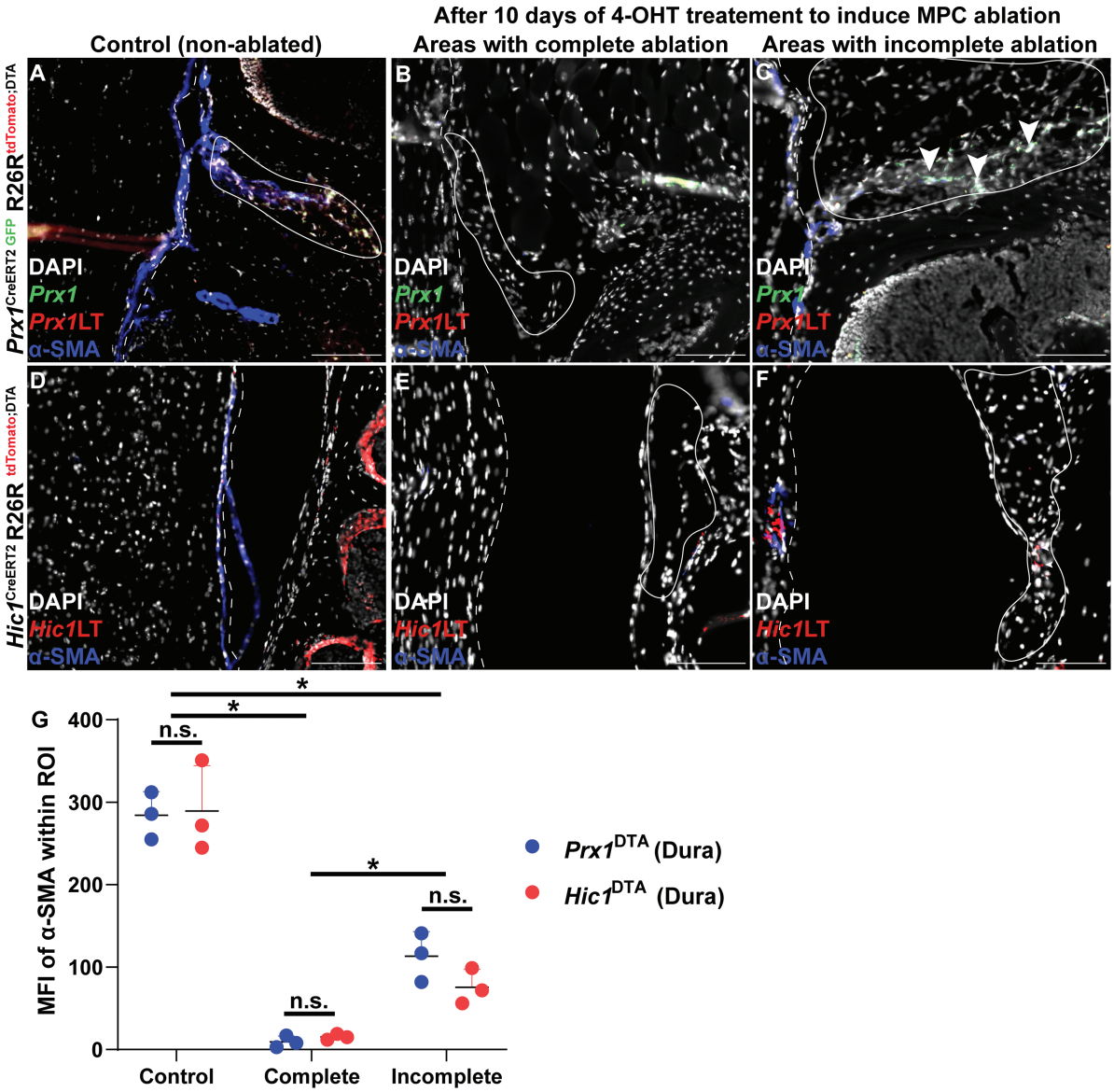


Figure 5. α-SMA expression in the dura mater of wild-type controls (A, D) and after *Prx1* (B, C) or *Hic1* (E, F) MPCs are ablated following 10 days of tamoxifen induction. The dura mater is outlined by the dashed line while the epidural fat is encircled by the solid line. Arrows indicate examples of *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). An $n = 3$ mice were used per group per time point. $*P < .05$. Scale bars = 100 μm. Abbreviations: *Hic1*, hypermethylated in cancer 1; MPCs, mesenchymal progenitor cells; *Prx1*, paired related homeobox-1; α-SMA, alpha-smooth muscle actin.

Prx1+ and Hic1+ MPCs Are Found at the Site of Dural Injury

The area of injury was determined by examining Safranin-O-stained slides in *Prx1* and *Hic1* reporter mice to identify granulation tissue that resulted due to the surgery (Fig. 6A, 6D). Two weeks post-injury, *Prx1*$^+$ MPCs were found within the dural lesion. These MPCs retained the expression of *Prx1* (GFP$^+$) (Fig. 6B, 6C). These *Prx1*$^+$ MPCs were also proliferative as they expressed Ki67 (Fig. 6B). 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) (Fig. 6C). Similarly, proliferating (Ki67$^+$) *Hic1*$^+$ MPCs were also found at the site of injury (Fig. 6E), yet these cells also did not express α-SMA staining as no co-localization was observed with tdTomato expression (*Hic1*$^+$ MPCs) (Fig. 6F). There were no differences in the number of *Prx1*$^+$ versus *Hic1*$^+$ MPC within the injury site (Fig. 6G).

Discussion

Although we know that *Prx1*$^+$ cells are present within epidural fat from lineage tracing experiments,[28] the biological relevance of these cells in vivo has not been explored. In this study, we have expanded upon the contributions of 2 MPC populations within the epidural fat/dural microenvironment.

Prx1 (paired related homeobox-1) is a robust adipose MSC marker[40] and it has previously been demonstrated that it marks presumptive MPCs within murine epidural fat.[28] Recently, a novel potent MPC lineage marker, *Hic1* (hypermethylated in cancer 1) was identified in skeletal/cardiac muscle-[45,46] and skin-derived cells.[49] Interestingly, while *Prx1* is a transcription

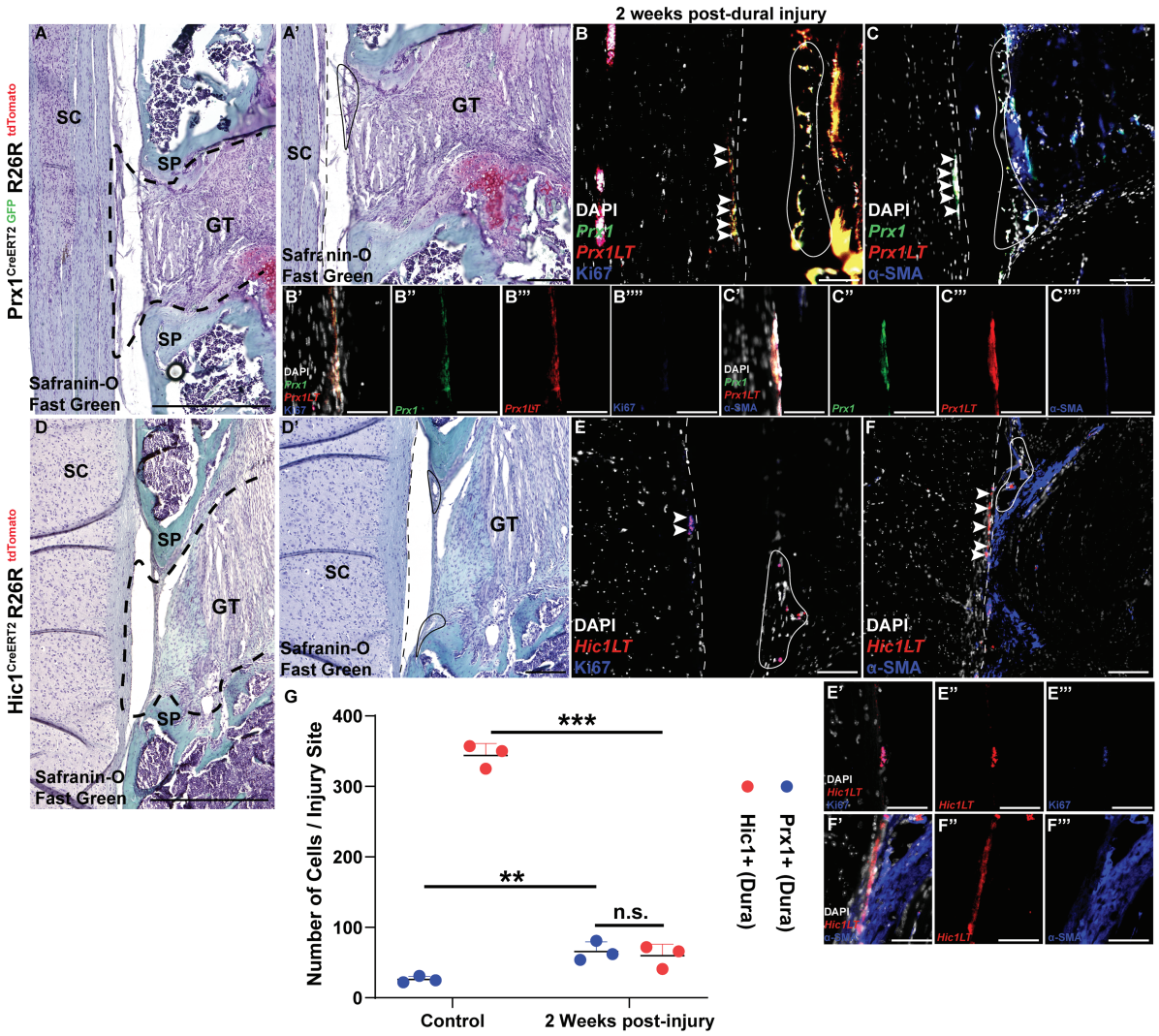


Figure 6. Endogenous repair of injured dura mater in the *Prx1*$^{CreERT2-GFP+/+}$R26R$^{tdTomato+/+}$ (A-C) and *Hic1*$^{CreERT2+/+}$R26R$^{tdTomato+/+}$ mice (D-F). Histological (A, D) and corresponding Ki67 (B, E) and α-SMA (C, F) images are presented. The dura mater is outlined by the dashed line 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). An $n = 3$ mice were used per group. $^{**}P < .01$, $^{***}P < .001$. Scale bars = 100 μm. Abbreviations: *Hic1*, hypermethylated in cancer 1; MPCs, mesenchymal progenitor cells; *Prx1*, paired related homeobox-1; α-SMA, alpha-smooth muscle actin.

factor involved in early mesodermal fate commitment,[33] *Hic1* is a transcriptional repressor[50,51] found to be expressed only in quiescent MPCs.[45,52] These *Hic1*$^{+}$ MPCs give rise to TACs that support regeneration post-injury in the tissues examined to date.[17,18,45] Since MPCs continue to demonstrate their vital importance in tissue homeostasis and regeneration, *Prx1* and *Hic1* reporter lineage tracing mice were used in this study, and their contribution to the dural environment was 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 microenvironment and participate in growth, repair, and homeostasis. This behavior fits with previous studies of MPCs in other tissues[39,45,49] 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 suggest 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 time point (4 months) in which we believe these cells are slow cycling, which is consistent with MPC in other adult tissues, such as synovium.[53] However, to be sure, BrdU/EdU labeling could be used 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.[54] 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,[55] which is also a possible outcome that explains the loss of *Prx1*$^+$ MPCs in the dura by 6 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.[56] 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- to 4-week time points examined. Based on these results, it is plausible that these 2 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 1 of the 2 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.[57-59] 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* (Fig. 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 (Fig. 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 suggest 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.[5,45,57,60-62] Moreover, *Hic1*$^+$ cells in the wound were proliferative, which is supported by previous research demonstrating that these cells give rise to TACs that support wound healing.[45] In vitro experiments using purified progenitors focusing on proliferation and/or wound healing (eg, scratch assays) could begin to address differences in underlying mechanisms governing behavioral differences between these MPC types. Furthermore, additional transgenic mice carrying fibroadipose[63,64] markers such as Sca1 and/or CD140a could be used to better understand the progenitor landscape in the epidural fat and dura mater.

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.[65-68] However, systemically delivered MPCs face mechanical barriers in small diameter vessels where they can passively arrest,[69] and even when cleared from the blood, MPCs are commonly found entrapped in the lung.[67] Specific to our study, the need to repair dural defects has led to the search for a functional substitute that possesses the same physiological characteristics, such as biomechanical properties and fiber architecture, as the dura mater, while ensuring biocompatibility and functional integration.[70] 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.

Conclusion

Only recently, a progenitor population within epidural fat has been identified,[28,29] yet the role of these MPCs remains 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 2 progenitor populations in the spinal microenvironment in addition to other tissues (such as bone, periosteum). The findings of the current study further challenge the notion that epidural fat is an incidental/biologically inert tissue[23,28]

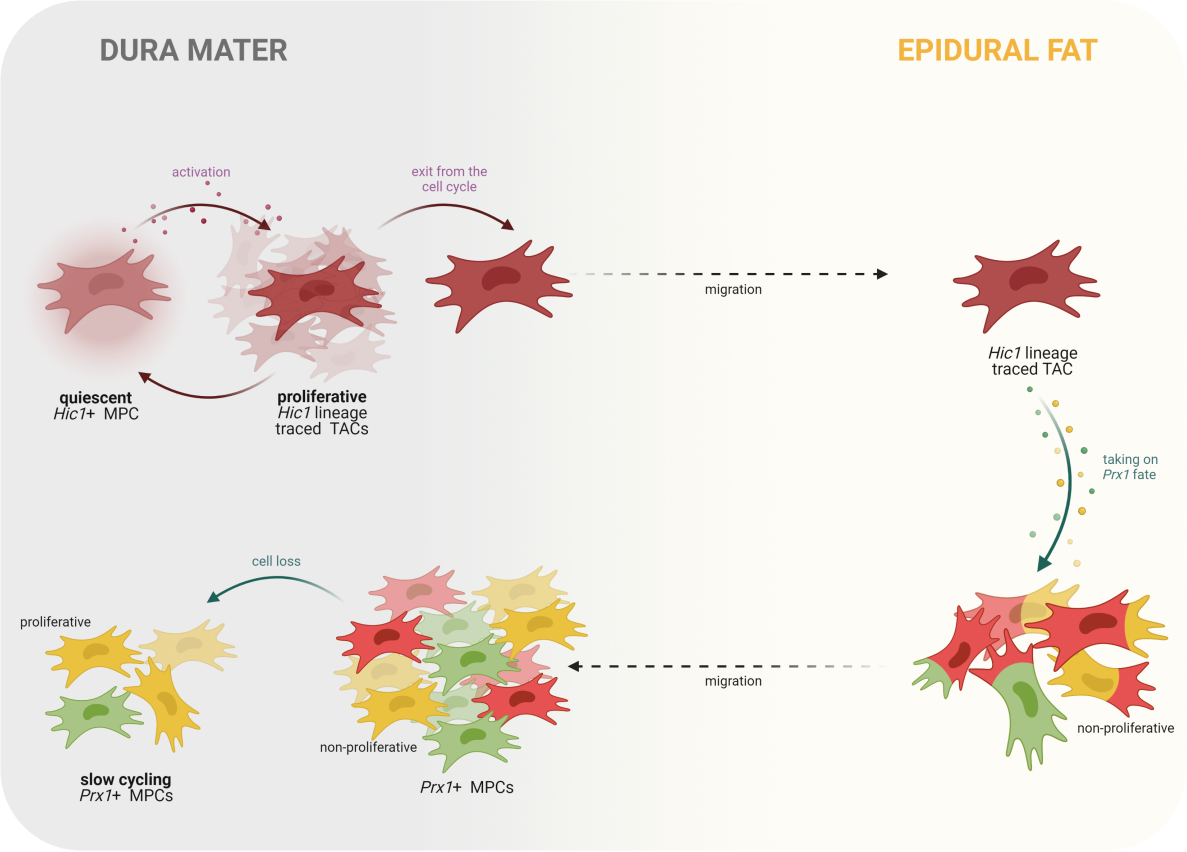


Figure 7. Hypothetical model proposed for the *Hic1* and *Prx1* hierarchy. *Hic1*+ MPCs native to the dura mater proliferate in response to biological cues (Fig. 2D, 2E). These MPCs exit from the cell cycle while in the dura mater (Fig. 2F) and then these *Hic1*+ lineage traced TACs migrate to the adjacent epidural fat (Fig. 2F). In the epidural fat, these MPCs/TACs acquire a *Prx1*+ mesodermal fate, as seen by the colocalization of *Hic1* and *Prx1* expression in cells (Fig. 4; Supplementary Fig. 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 (Fig. 2A-2C) 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 (Fig. 3) and most likely undergo slow cycling in this tissue to maintain homeostasis and respond to future activation cues/insults (Fig. 6). Abbreviations: *Hic1*, hypermethylated in cancer 1; MPCs, mesenchymal progenitor cells; *Prx1*, paired related homeobox-1; TACs, transit-amplifying cells.

and suggest additional research should be directed toward 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.

Funding

S.S. is supported by an NSERC CGSM Studentship. S.M. is supported by an O'Brien Undergraduate Studentship. R.J.K. is supported by a Tier 2 Canada Research Chair. This work from supported by grants from the Alberta Spine Foundation, The McCaig Institute for Bone and Joint Health, and the University of Calgary Section of Orthopedics.

Conflict of Interest

A.P.M. declared patent holder and stock ownership with Fluid Biotech Inc., Advisory role with Cerus Endovascular, and Research funding from Stryker Neurovascular. All of the other authors declared no potential conflicts of interest.

Author Contributions

S.S., P.T.S., R.J.K.: conception and design. S.S., S.M., R.J.K.: analysis and interpretation of the data. S.S.: drafting of the article. S.S., S.M., T.M.U., A.P.M., P.T.S., R.J.K.: critical revision of the article for important intellectual content. S.S., S.M., T.M.U., A.P.M., P.T.S., R.J.K.: final approval of the article. T.M.U., R.J.K.: provision of study materials. P.T.S., R.J.K.: obtaining of funding. S.S., S.M., P.T.S., R.J.K.: collection and assembly of data.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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