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Stimulating A Microvascular Cell For Cell Therapy Applications: Pericyte Potential For Manipulating Central Nervous System Vasculature

by
Jamie Nadine Mayo

A dissertation

submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy in the Department of Biological Sciences

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To the Graduate Faculty:

The members of the committee appointed to examine the dissertation of Jamie Nadine Mayo find it satisfactory and recommend that it be accepted.

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Animal Welfare Research Committee Approval Page

July 18, 2014

Shawn Bearden, Ph.D.
MS 8007
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Pocatello, ID 83209

RE: Your application dated 7/14/2014 regarding study number 722: Vascular Cell Therapy

Dear Dr. Bearden:

I have reviewed your request for approval of the new study listed above. This is to confirm that I have approved your application with the condition that BSL2 and Aseptic training be completed before the birth of animals to be used in the study, 8/7/2014.

Notify the IACUC of any adverse events. Serious, unexpected adverse events must be reported in writing within 10 business days.

An annual review for this study should be submitted by 7/16/2015.

You may conduct your study as described in your application effective immediately. The study is subject to renewal on or before 7/16/2017, unless closed before that date.

Please note that any changes to the study as approved must be promptly reported and approved. Some changes may be approved by expedited review; others require full board review. Contact Tom Bailey (208-282-2179; fax 208-282-4723; email: anmlcare@isu.edu) if you have any questions or require further information.

Sincerely,

Curt Anderson, PhD
IACUC Chair

Dedication

To Marc Christopher Mayo; my love, you helped me find the will to persist when I would have given up.

To Mark Cameron Bowles; my beautiful son, you are my motivation to always strive for a better version of myself.

Acknowledgements

I acknowledge Sierra Harris from Dr. Michele Brumley's lab for all of her hard work and excellence in performing surgeries on 342 rat pups. I also acknowledge Marc Mayo for scoring the hindlimb movements of 136 rat pups from over 5 hours of videos, thank you.

Thank you, Dr. Bearden, for your mentorship. Thank you to the Department of Biological Sciences at Idaho State University for providing the environment of my education and thanks to the IDeA Network of Biomedical Research Excellence for the pre-doctoral funding that supported me throughout my Ph.D program.

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List of Abbreviations

α-sma; Alpha smooth muscle actin

BBB; Basso, Beattie, and Bresnahan Motor Scale

BDNF; Brain-derived neurotrophic factor

CNS; Central nervous system

$CoCl_2$: Cobalt chloride

CST; Cortio-spinal tract

DMSO: Dimethyl Sulfoxide

EM: Endothelial culture media

HIF: Hypoxia-inducible factor

IHC; Immunohistochemistry

Kir6.1; Inwardly rectifying potassium channel, subfamily J, member 8

NG2; Nerve/glial antigen two

P#; Postnatal day #

PDGF; Platelet-derived growth factor

PDGFR-β; Platelet derived growth factor receptor-beta

PBS: Phosphate-buffered solution

PFA: Paraformaldehyde

PM: Pericyte culture media

S1P; Sphingosine-1-phosphate

RGS5; Regulator of G protein signaling 5

SCI; Spinal cord injury

TGF-β1; transforming growth factor beta one

VEGF; vascular endothelial growth factor

Abstract

Novel therapeutic options are needed to improve recovery after spinal cord injury. Cell therapies that are directed at improving both vasculature and neuronal outcomes will likely have the best success in promoting tissue health. Pericytes are microvascular cells that can engage in and guide new vessel growth (angiogenesis). The mechanisms by which pericytes induce angiogenesis are not known. While pericytes have been shown to be effective as a cell therapy in other disease models, it is not known if they are effective at improving outcomes in the central nervous system. The goal of this research project was 1) to discover if pericytes could be stimulated to induce angiogenesis in vitro, and 2) to determine if pericytes were an effective cell therapy for improving hindlimb motor recovery across sexes in a pediatric model of spinal cord injury. Experimental systems were utilized both in vitro and in vivo. In vitro cell culture of pericytes, endothelial cells, and spinal cord tissue was used to delineate a mechanism by which pericytes could be stimulated to a pro-angiogenic state. A dorsal thoracic hemi-section of the spinal cord in neonate rat pups was used to identify if pericytes utilized as a cell therapy could improve hindlimb motor recovery across sexes. This study identified that pericytes are pro-angiogenic with hypoxia-inducible factor pathway activation and can be used to support growth of microvascular networks. Pericyte promotion of greater vascular density is regulated by exosomal communication between endothelial cells and pericytes. There are sex specific vascular outcomes after spinal cord injury and the mechanisms of recovery in response to therapeutic interventions could differ between sexes. This study also determined that pericytes improve hindlimb motor recovery following spinal cord injury and are a novel cell type for potential use in cell therapy applications.

1. Introduction

Spinal cord injuries (SCI) and the resulting pathological outcomes are currently not curable. The introduction of an exogenous cell population to the site of tissue injury or manipulation of an endogenous cell type to improve tissue recovery has emerged as a promising therapeutic option for previously untreatable central nervous system (CNS) pathologies (1-3). With the noted potential of stem cells to increase tissue recovery, cell therapies have become an attractive treatment option for SCI (4). Cell therapy is presently limited in scope and adaptability to clinical practice because of the ethical concerns of harvesting embryonic stem cells and complications in the safety and efficacy of the cell types currently in use (5, 6). In order to effect the clinical translation of cell therapies, a major task will be to determine and dissect the mechanisms by which an endogenous cell type can be stimulated to enhance tissue recovery in a stem cell-like manner without adverse side effects. Optimally, the cell type would be easily harvested, undergo minimal in vitro manipulation, and be able to interact with multiple components of the neuronal environment, driving overall CNS recovery (4, 7).

A large majority of the basic research concerning CNS injury has been conducted in males (8, 9). Relatively little is known about the pathological/physiological responses that occur after CNS injury, or in response to treatment, in females. Determining if there are sex specific differences after injury and/or in response to treatment will be imperative to developing novel therapies that are clinically translational.

In this study, the aim was to elucidate the potential of perivascular cells, *pericytes*, to be activated for cell therapy applications in both sexes. A secondary aim for this study was to further elucidate if there are sex differences in vascular responses after injury or in the context of treatment following injury. This first chapter provides a review of the

literature, a list of the research aims, and briefly describes the experimental approaches utilized. The second chapter outlines a mechanism by which pericytes can be stimulated to promote greater vessel density in the CNS for cell therapy applications; the chapter is presented in the same form as it was for publication. The third chapter provides evidence that pericytes can be used effectively as a cell therapy to improve hindlimb motor recovery in a pediatric model of SCI; the chapter is in preparation for publication. The fourth chapter discusses the conclusions and future directions of this work.

1.1. Pericytes as a target to manipulate angiogenesis for cell therapy applications

1.1.1. Definition of a pericyte

Pericytes have emerged as novel candidates for cell therapy mediated tissue recovery (7). Pericytes are cells that are embedded in the basement membrane of capillary walls and come in close contact with endothelial cells, often encircling a vessel (Figure 1.1) (10, 11). Pericytes were first identified in 1873 by Rouget and were then extensively described in 1922 by Vimtrup (12, 13). In general, endothelial to pericyte ratios are thought to be 1:1 in the retina, 2:1 in the CNS, and greater than 5:1 in the periphery (10, 11, 14). The criterion for definitively identifying a cell as a pericyte is the identification of the presence of a perivascular cell within the vascular basement membrane matrix by electron microscopy (Figure 1:1C). When this is not practical, as for example during angiogenesis when remodeling vasculature can lack a defined basement membrane, it is common, though less definitive, to use at least two tissue appropriate pericyte markers for immunohistochemistry (IHC) coupled with visual conformation of a perivascular position (15).

In some of the first work describing pericyte-endothelial interactions, Tilton et al. hypothesized that pericyte physical interaction with endothelial cells, and consequently

pericyte effect on capillary function, might differ between tissues (11). Tilton et al. found differences in pericyte structure and density between tissue beds of the heart (in rat tissue) and skeletal muscle (in rat and human tissue) in that pericyte cell body extensions covered the vasculature of the skeletal muscle to a greater extent and had a greater degree of interdigitation with endothelial cells. Axons were closely associated with pericytes in the heart but not in skeletal muscle. These differences in structure and organization are consistent with the hypothesis that endothelial cells and pericytes interact with one another and that this interaction might vary resulting capillary function depending upon the tissue bed (11). Overall, it appears that pericyte function, much like the vasculature itself, could be specialized to meet the highly specific needs of the tissue milieu.

1.1.2. Developmental origin of pericytes

Recent evidence suggests that pericytes from different regions of the body are developmentally derived from different embryonic tissues. Peripheral pericytes originate from the mesoderm (16), head pericytes appear to originate from the neural crest (17, 18), and placental pericytes are from the trophoblast (19). With this being said, it has been proposed that in specific areas a vascular tree could contain pericytes from multiple developmental origins (15, 18).

1.1.3. Pericytes as a stem cell

Pericytes may have a unique ability to be activated to a stem cell-like state. In vivo, small populations of adult murine brain pericytes are multipotent (20). A sub-population of skeletal, pancreatic, adipose, and placental pericytes express mesenchymal stem cell markers (21). In vitro, rat brain microvascular pericytes are able to differentiate into cells with astroglial, oligodendroglial, and neuronal cell surface markers both in cell and capillary cultures (22, 23). Bovine retinal pericytes can also differentiate into

osteoblast-like cells and liver stellate cells can become liver cell progenitors (24, 25). Furthermore, pericytes can differentiate into chondrocytes, adipocytes, and fibroblasts (26). Human brain pericytes have also been induced in culture by viral transfection to differentiate into neuronal like cells (27).

While it is clear that a subset of pericytes are multipotent, it is also clear that most pericytes function in a differentiated manner to influence endothelial cells and do not display overt characteristics of stem cells (28). Whether these quiescent, "differentiated," or stable pericytes are stimulated in vivo to become more stem cell-like in times of injury or development is not known. There is evidence that after extensive in vitro manipulation, stable pericytes have multipotent capabilities (22).

Overall, a pericyte is a unique cell that has multiple developmental potentials and varying cell marker expression in response to environmental stimuli. Their ability to drive angiogenesis and unique cellular flexibility that persists into the adult animal could enable pericytes to participate in signal transduction between the neuronal environment and blood vessels, encouraging CNS recovery in response to the demanding needs of changing tissue conditions after injury. Elucidating a stimulus that efficiently activates human pericytes to a pro-angiogenic state would help to exploit these cells for use in CNS injury cell therapies by determining "priming" culture conditions before in vivo application in order to maximize positive therapeutic outcomes.

1.1.4. Mechanisms by which pericytes regulate angiogenesis and other vascular functions

Pericytes have been implicated as necessary constituents in multiple microcirculatory functions including angiogenesis, the sprouting of new vessels from preexisting capillaries (29-31). Several signaling molecules and pathways have been

identified as mechanisms through which pericytes regulate angiogenesis. Functionally, pericytes have been suggested to influence vessel stability, regulate basement membrane matrix formation, guide endothelial tubes, and induce endothelial cell migration.

Increased vessel stability is positively correlated with an increase in the ratio of pericyte to endothelial cell coverage (32, 33). PDGF (platelet-derived growth factor) deficient mice exhibited severe vessel deformities and hemorrhaging (29). Additionally, the capillaries from PDGF-B deficient mice's embryonic brains lacked pericyte coverage (33). Overall, PDGF-B participates in the recruitment and investment of pericytes to endothelial cells during angiogenesis and increases vessel stability (32, 34, 35). Sphingosine-1-phosphate (S1P) is implicated in establishing and strengthening the contacts between pericytes and endothelial cells (36). Pericytes also establish vessel stability by contributing to the formation of a basement membrane matrix (37). Adding to the evidence that pericytes help to establish a mature vascular network is the finding that pericytes engage in forming and maintaining the blood brain/spinal cord barrier during development and throughout adulthood (14, 38, 39).

Lindbloom et al. and Gerhardt et al. (34, 40) described PDGF-B as predominately expressed at the tip of sprouting endothelial tubes. This finding is consistent with early reports of pericytes found at the tip of endothelial tubes and even preceding endothelial cells during angiogenesis (30). A lack of pericyte vessel coverage by inhibition of PDGF-B, or its receptor PDGFR-β, induced a disorganized vascular pattern described by Benjamin et al. [17] as "irregularly shaped loops" and by Leveen et al. [104] as showing a "tortuous appearance of the capillaries that were irregularly spaced." Lindbloom et al. described the vasculature structure in PDGF-B deficient mice as having "less joining of vessels" and as having an "irregular," highly disorganized vascular pattern (34). In

human fetal brain angiogenesis, pericytes were found to precede endothelial cells in the direction of growth (41). Similarly, in an angiogenic response to hypoxia, pericytes are among the first vascular cells to migrate (42, 43). More recent evidence has also emerged for a role of pericytes in the earliest stages of angiogenesis (31). For example, nerve/glial antigen two (NG2; chondroitin sulfate proteoglycan 4) has been used as a marker of pericytes in both rodents and in humans and can be used to identify pericytes on growing capillary sprouts (41, 44). In rat mesentery, after inflammation-induced angiogenesis, desmin $^{+}$ α-sma^{-} pericytes participated in the earliest stages of vessel sprouting (45). These observations would suggest a guiding role for pericytes in the development of an organized and patterned vascular bed.

Approaching the question of the role of pericytes in establishing vessel architecture, previous studies have addressed how the interaction between pericytes and endothelial cells alters the motility patterns of both cell types. In wounds made on the dorsal flanks of hamsters, Crocker et al. observed the presence of primitive mesenchymal cells that surrounded the advancing tip cells of developing capillaries in the earliest stages of wound healing. These mesenchymal cells were eventually incorporated into the basement membrane of capillaries where they became elongated and changed the profile of their intracellular organelles, essentially becoming pericytes. Advancing endothelial tip cells surrounded by primitive mesenchymal cells were positively correlated with increases in capillary density and irregular lumen thickness. Incorporation of mesenchymal cells into capillary basement membranes was negatively correlated with a decrease in capillary number but positively correlated with an increase in mesenchymal/endothelial cell contacts and an increase of lumens of a uniform thickness (46). Birbrair et al. described two populations of pericytes; nestin $^{-}$ pericytes (type one

pericytes) and nestin $^{+}$ pericytes (type two pericytes). Nestin $^{-}$ pericytes were identified as collagen-producing cells (47) while nestin $^{+}$ pericytes were associated with angiogenic outcomes (48). These results suggest a dual role for pericytes, one in which they participate in the active proliferation and invasion of new capillaries and one in which they participate in the maturation of a stable vasculature.

Aside from participating in angiogenesis, pericytes also have a proposed role in regulating CNS blood flow. In culture preparations of the neonatal rat retina and cerebellar slices (sex unknown), pericytes initiated capillary constriction in response to stimuli and Peppiatt et al. postulated that pericytes could be "… modulators of blood flow in response to changes in neural activity"(49). In more recent work using brains of adult mice of both sexes, pericytes induced capillary constriction in vivo but the pre-capillary arterioles and penetrating arterioles were the determining factor in blood flow regulation in response to brain activity (50). In this work, Fernandez-Klett et al. hypothesized that pericyte constriction, while not pertinent under physiological conditions, might be of note during pathological conditions (50). Supporting this hypothesis, ischemia-induced pericyte constriction blocked blood flow in the adult mouse brain (sex unknown) after middle cerebral artery occlusion (51). Similarly, pericyte constriction was found to play a role in a model of traumatic brain injury in the adult male rat (52).

While there is mounting evidence that pericyte constriction/dilation plays a role in blood flow control in pathological conditions, whether pericytes are necessary for blood flow control in response to neuronal activity under physiological conditions is still controversial. Hall et al. provided convincing evidence that pericytes could participate in increases in blood flow in response to neuronal activity under physiological conditions in

a mouse model of whisker stimulation (adult animals of both sexes were included in the study)(53). There is still a lack of evidence of the importance of the role of pericyte modulation of blood flow in response to neuronal activity. For example, in pericyte deficient mice (PDGFR-$\beta^{+/-}$ mice at one month of age), there was a significant decrease in pericyte coverage and perfused capillary length but there were not significant impairments in behavior, a measurable functional outcome of neuronal activity (53). With a greater loss of pericyte vascular coverage over time (in animals 6-16 months of age), there was a greater decrease in perfused capillary length and concomitant impairments in behavior. It is unclear if the impairments in behavior in aged animals were because of a decrease in cerebral blood flow or because of toxicity from the breakdown of the blood-brain barrier (53). Still, overall, it appears that pericytes might participate at some level in the blood flow response to neuronal activity in both pathological and physiological situations. Taken together, evidence to date supports the larger concept that pericytes can act as an intermediate signal transducer between the neuronal environment and blood vessels, increasing their potential to be used as a therapeutic target to improve CNS tissue recovery after injury.

A noted advantage of pericytes as a cell therapy option is their ubiquitous presence throughout the microvasculature in all organs in the body and their "pre-programming" to influence angiogenesis and overall vascular function (31, 54). Dysfunctional or insufficient angiogenesis is the cause or perpetuating factor in many disease states (55). The end result of angiogenesis can not only meet the tissues' metabolic needs by an increase, redirection, or resumption of blood flow, but can also result in the generation of multiple guiding signals that growing capillary tubes themselves produce that ultimately aid tissue organization/regeneration (56-60).

Manipulating stable vasculature by either increasing or decreasing angiogenesis is therefore a therapeutic tactic to improve tissue recovery and increase neurogenesis (61-63). Activating pericytes to a pro-angiogenic state could increase their utility in a clinical setting.

1.1.5. Pericytes in cell therapy

The large majority of studies characterizing pericytes for cell therapy applications have used peripheral pericyte populations. Human skeletal vasculature pericytes have been utilized in murine models of cardiac diseases and skeletal muscle regeneration but have yet to show utility in CNS injury (54, 64). Pericyte progenitors are involved in the CNS response to insults including ischemia and SCI, where they have the potential to increase tissue propensity for neurogenesis and angiogenesis (65-68). Injected pericytes had a protective effect in experimental autoimmune encephalomyelitis (22) and were both regenerative and reparative of skeletal muscle, bone, and cartilage in other models (69, 70). Injected bone marrow pericytes were mobilized by stress signals, able to differentiate, associated in a perivascular location with forming capillaries, and were interpreted to increase the potential for tissue repair by increasing the angiogenic response (68, 71, 72). Dental pulp stem cells took on a pericyte-like phenotype and enhanced an angiogenic response (73). Pericytes derived from adipose stem cells maintained a perivascular position, increased vessel growth, and improved outcomes in models of retinal vasculopathy (74). In ischemic heart disease, human fetal skeletal pericytes differentiated, maintained a perivascular position, facilitated repair, and increased angiogenic outcomes (54). Whether pericytes can be activated to maximize their ability to increase angiogenesis and tissue recovery, however, is unknown.

1.2. Exosome signaling and angiogenesis

Exosome signaling has recently been identified as a novel intercellular communication method that can either drive or suppress an angiogenic program. Exosomes are small (30-100 nm in diameter) membrane-based microvesicles that can transport a variety of signaling molecules including RNA, DNA, and proteins (75, 76). These microvesicles are released from cells and taken up by receiving cells by a variety of mechanisms including endocytosis (77). Exosomes can thereby participate in cell-to-cell communication to increase angiogenesis in response to varying environmental stimuli including hypoxia (78, 79). Whether pericytes and endothelial cells engage in this kind of intercellular communication is unknown.

1.3. Spinal cord injury as a model of central nervous system injury

SCI results in severe functional deficiencies. To date there are no clinical treatment options that completely cure these functional deficiencies. The vasculature of the spinal cord plays an active role in both the pathology and protective mechanisms that occur following injury. Additionally, spinal cord vasculature not only functions as a necessary conduit for nutrients and gas exchange, but can also participate in neuroregeneration, making it an attractive therapeutic target to enhance overall tissue health.

1.3.1. Vascular responses to spinal cord injury

After SCI, the primary injury (the initial mechanical injury) cannot always wholly account for the resultant cell death, tissue loss, and functional impairment. This observation led to the hypothesis of secondary injury (the sequelae of cellular cascades occurring after direct impact) through which, as a consequence of direct impact, additional tissue damage occurs (80, 81). The pathological changes that occur after SCI can be broken down and characterized by three temporal periods: an acute phase lasting

seconds to minutes after injury, a sub-acute phase occurring minutes to weeks after injury, and a chronic state lasting from months to years after injury (82). Given that therapeutic opportunities for intervention are limited within the acute phase, much of the research done to identify treatment strategies has focused on the mechanisms of secondary injury occurring during the sub-acute and chronic phases.

Early studies investigating SCI recognized the central role of vascular dysfunction in the pathological outcomes associated with injury (83). More recent studies have focused on the communication that occurs between the neuronal environment and the vasculature, ultimately discovering many neuroprotective roles for vascular cells in the later phases of injury. These observations depict dual vascular mechanisms of both injury and protection following SCI. Understanding the mechanisms that drive the interactions between the neuronal environment and the vasculature may help to delineate appropriate therapeutic manipulations to improve patient outcomes (84).

1.3.1.1. Pathological vascular responses to spinal cord injuries: treatment opportunities

Acute primary vascular responses to SCI include the tearing of blood vessels. Sub-acute to chronic secondary responses include: hemorrhage, reductions in blood flow, loss of autoregulation, vasospasm, ischemia, and blood-spinal cord barrier dysfunction (85). Allen described that massive hemorrhaging following a contusive SCI was one of primary factors in tissue necrosis aside from the direct mechanical damage of impact (83). Dohrmann et al. found that in monkeys, within the acute phase of a contusive injury, there was extensive hemorrhaging in the microvasculature. In the sub-acute phase, there was ischemic endothelial injury and neuronal dysfunction as evidenced by axonal degeneration and a loss of myelin sheath (81, 86). There are also significant reductions in

microcirculation and blood flow perfusion after SCI. Recovery of injured tissue is dependent on the functionality of the microcirculation (81, 87). Furthermore, axonal dysfunction is negatively correlated with a reduction in blood flow (88).

Injury to the spinal cord is followed by an immediate and progressive loss of function of the blood-spinal cord barrier that is persists up to 56 days after injury, contributing to the necrosis and tissue damage experienced during secondary injury (89, 90). Compounding these pathological vascular responses there is also a loss of the ability of the spinal cord to autoregulate blood flow over a range of arterial pressures (91, 92). As a whole, massive vascular dysfunction is a significant contributor to the pathology of SCI. Addressing vascular dysfunction after SCI provides a treatment opportunity to improve whole tissue recovery and it has been proposed that therapies that do not improve vascular function after CNS injury are unlikely to promote functional recovery (93, 94).

1.3.1.2. Vascular neuroprotective mechanisms: manipulating vasculature as a means to promote recovery after spinal cord injury

Angiogenesis is a major contributor to increases in tissue blood vessel density after injury or developmental stimuli. Vascular responses after SCI appear to be biphasic. Acute to sub-acute phases, vascular responses are predominately pathological while in the sub-acute to chronic phases angiogenesis has been proposed to be a neuroprotective vascular response (84, 85, 94). Specifically, increases in vessel density at the site of injury by targeted therapies are thought to add to the regenerative capacity of spinal cord tissue by the creation of an environment that encourages neuronal plasticity, axonal regeneration, and myelin sparing (4). Neuronal outgrowth was positively correlated and physically associated with an angiogenic response seven days after SCI (95). Recovery of

hindlimb motor performance in rat models of thoracic SCI is hypothesized to be due to plasticity of the cortical spinal tract (CST) to form compensatory networks with propriospinal neurons (96, 97). In an inflammatory model of SCI, angiogenesis proceeded and was positively correlated with the reorganization of the CST and improved hindlimb motor performance (63). Increased angiogenesis was also positively correlated with axonal regeneration into the lesion site of transected rat spinal cords after axonal growth inhibitor suppression (98).

Although the exact mechanisms of the creation of a neuroprotective milieu by the vasculature is unclear, it is known that neuronal and vascular development involve many of the same guidance molecules and are intricately connected throughout the life span of an organism (56, 99). Vascular cells can also provide trophic support of the neuronal environment. In the songbird, there is a causal relationship between angiogenesis and neurogenesis, driven by brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) (60). Following stroke, neurogenesis and neuronal migration was closely associated with angiogenesis and the vascular release of stromal derived factor one and angiopoietin one (59). In an in vitro model of endothelial and neuronal co-culture, Guo et al. described a neuroprotective role of endothelial cells. Specifically, the authors identified the endothelial derived soluble factor BDNF as a protective agent against neuronal damage (57). Corticospinal neurons also expressed the receptors for endothelial produced growth factors (58). In addition to BDNF, endothelial cell produced stromal derived factor one alpha, pleiotrophin, and insulin like growth factor two promoted postnatal rat corticospinal motor neuronal outgrowth and survival in culture. The expression of these growth factors was specific to CNS endothelial cells as compared to endothelial cells from other tissues (58). This work reveals a novel role for

vascular cells, not only in delivering nutrients and removing waste products as vascular tubes, but also as interactive cells that may contribute functionally to support neuronal recovery. Taken together, there is evidence that new vessel growth could act as a reservoir of trophic factors and act as a guiding scaffold for neurogenesis and neuronal remodeling in the CNS.

A potential complication in manipulating vascular density as a therapeutic mechanism, particularly in aiming to increase angiogenesis, is the inherent permeability of developing vessels and in the development of angiogenic tubes into mature vessels (94, 100). Additionally, there have been conflicting reports on the ability of a potent angiogenic stimulus, VEGF, to improve functional recovery after SCI. In one study, injection of VEGF alone was detrimental to functional recovery after SCI (100). Treatment with VEGF and PDGF-β (which can promote vessel stability) decreased lesion size and promoted axonal regrowth but did not improve motor recovery. Other researchers have seen no effect on functional motor recovery after using VEGF as a therapeutic strategy (101). Conversely, positive effects of VEGF treatment were observed in other studies (102, 103). The timing of therapeutic intervention to drive angiogenesis during a period that will be the most beneficial to tissue recovery will likely be critical in the development of new therapies. It is also possible that the injection of growth factors that participate in angiogenesis is not sufficient to stabilize pathological vascular responses, promote angiogenesis, and promote neuronal regeneration after certain types of SCI. Pericytes, as a cell therapy, might be useful in addressing these concerns. Pericytes are a cell type that can drive angiogenesis, participate in encouraging vessel stability, and secrete neuroprotective growth factors, introducing not only the necessary

growth factors for tissue recovery but also the cellular machinery to interact with the entire neuronal environment.

1.3.2 Cell therapy as a therapeutic tactic in spinal cord injury

Cell therapies currently utilize three basic cell types: embryonic stem cells (ES), bone marrow derived adult stem cells, and induced pluripotent stem cells (iPS) (4). Stem cells are defined as immature cells that can generate terminally differentiated cells while maintaining non-differentiated cells for self-renewal. Additionally, stem cells do not exhibit senescence (104). iPS cells are created by the genetic transfection or manipulation of multiple "stem cell factors" which include the up-regulation of Oct 3/4, Sox2, c-Myc, and Klf4 (105). Studies with ES, iPS, and adult stem cells have laid the ground work for establishing cell therapy as a viable candidate for CNS pathologies (106).

The mechanisms of stem cell induced tissue recovery have been attributed in part to the ability of these cells to increase blood vessel density at the site of injury and to fuse or differentiate into the cells of the target tissue (54, 104, 107). Specifically in SCI (as reviewed in (94)), co-implantation of neural stem cells and human umbilical endothelial cells rescued tissue hypoxia after SCI in rats (108). Bone marrow-derived endothelial progenitor cells promoted neovascularization that coincided with axonal growth in a mouse model of SCI (109). In a rat model of SCI, bone marrow stromal cells improved functional motor recovery, improved tissue retention, and promoted greater vessel density at the site of injury (110). Mesenchymal stem cells also improved functional motor recovery and promoted greater vascular density (111).

Despite encouraging results from the cell therapies currently employed in rodent models cell therapy is presently limited in scope and adaptability to clinical practice due to the ethical concerns of harvesting ES cells, the immune reaction between the injected

cells and the host, complications arising from the method of stem cell induction in iPS cells, and the residual epigenetic markers that iPS cells carry into the treatment environment (5, 6). Additionally, bone marrow derived adult stem cells have inconsistent efficacy in improving functional recovery, are limited in differentiation potential, and are derived from heterogeneous cell cultures (4, 7). ES, bone marrow derived adult stem cells, and iPS cells can also all have adverse effects such as inducing tumor development and metastases. Furthermore, cells therapies currently undergoing clinical trials have had limited success in improving motor recovery in humans after SCI (4). Ultimately, treatment of spinal cord injury will likely involve a multi-therapeutic approach and the clinical translation of cell therapies will necessitate the identification of multiple viable cell types and their various mechanisms of action (4, 94, 112).

1.3.3. Adaptive plasticity: neuronal recovery after spinal cord injury in younger mammals

Traditionally, SCI studies have been conducted in adult rats. There is a gap in knowledge concerning the mechanisms and processes of recovery in young animals. After a thorough review of the literature in 2011, it was concluded that

> "there is no evidence regarding the use of neuroprotective approaches for the treatment of SCI in children" (113).

It is known, however, that in young mammals the nervous system is still developing and displays increased adaptive plasticity to injury, although the question of whether this neuronal plasticity translates to human children is relatively unexplored (113, 114). Functional recovery of voluntary motor movement in young mammals after SCI is thought to occur through synaptic plasticity of remaining axonal tracts (115, 116). Plasticity in the adult nervous system is thought to be similar to developmental plasticity

in mechanism (117). Studying SCI and cell therapy integration in postnatal rat pups provides a unique opportunity to gain insight into therapeutic opportunities for the pediatric population and the mechanisms dictating plasticity for eventual translation into adult therapies (118).

The angiogenesis that occurs after spinal cord transection in the postnatal rat pup is an understudied phenomenon despite the known correlation of angiogenesis with tissue regeneration in the adult. During embryonic development, cross talk between neuronal cells and vascular cells determines CNS vascular patterning (56, 119). During the early postnatal period in both humans and rats, myelination of the spinal cord tracts is still in progress (120, 121). In the rat pup, spinal cord axon myelination and vessel patterning are still active processes, with myelination not complete until postnatal day 22 (P22) creating a very plastic and dynamic environment (114, 122-124). There is a positive correlation between vascularization and myelination suggesting a dependent relationship between these two events, although the mechanisms that dictate this relationship in the spinal cord are still unclear (120, 121). Additionally, extensive synaptic plasticity exists in the postnatal rat spinal cord (125). A large abundance of capillaries are associated with synapses in the central gray matter(126) and after injury in the adult angiogenesis is proposed to aid in synaptic plasticity (63). As a whole, there is evidence that CNS blood vessel architecture is patterned by an interaction with neuronal cells. The cellular machinery and physical juxtaposition of vascular cells and neuronal cells also insinuates a role of the vasculature to pattern aspects of neuronal growth.

1.3.4. Occurrence of spinal cord injury in the pediatric population

Approximately 5% of all SCI occur in the pediatric population (ages birth to 18) when the spinal cord is still developing. Around 1% of the injuries occur in children from

birth to 3 years of age. The majority of injuries in younger children occur from birth trauma, motor vehicle accidents, falls, and abuse. In older children the majority of injuries occur from motor vehicle accidents and sports injuries (127-130).

1.3.5. Sex differences in the epidemiology of spinal cord injury

Most studies report a higher incidence of SCI in males than in females in both younger and older pediatric patients (113, 127, 128). A case series in the adult population (14,433 patients over a 22-year period) done in 2004 concluded that greater neuronal recovery was found in women than in men but that men exhibited better functional recovery on a short term basis (131). In a similar study, done on a smaller population of patients (1,074 patients over a 10-year period) no effect of gender was found in functional outcome after SCI (132). Overall, sex differences in regards to neurological recovery after SCI are still controversial, although studies conducted on large populations appear to support that sex differences exist (112).

In rats, it has been proposed that differences in sex specific hormones lead to differences in neurological and functional outcomes after neonatal exposure to inflammatory insult. In these studies, females had a decreased sensitivity to painful stimuli in adulthood after neonatal injury as compared to males, but suffered enhanced sensitivity to pain after re-injury in adulthood(133, 134). While sex differences in the neurological response to injury have been hypothesized to be estrogen dependent (133), estrogen treatment following SCI in rats has been inconclusive (112). A study in rats that compared both males and females with SCI, however, did not find a neuroprotective role for estrogen, but did report that female rats had more spared tissue after injury in a non-estrogen dependent manner than did male rats (112).

Little is known concerning sex differences in vascular responses in humans after SCI except that females have a greater occurrence of deep venous thrombosis (135). With vascular responses in general, estrogen can interact with and affect multiple aspects of vascular and CNS physiology (136, 137). Furthermore, males and females might have different baseline potentials for angiogenesis based on their ability to produce key angiogenic molecules (138). Given the current evidence, it is possible that sex differences in responses to CNS injury could stem from both sex specific hormonal actions and from inherent genetic pre-dispositions. Taken together, the mechanisms of sex differences in the outcomes following SCI are likely complex and varied depending on age, ethnicity, and type of injury. Studying sex differences in a postnatal model of spinal cord injury allows for the quantification of sex-based differences following SCI in the absence of the compounding factors that are involved after sexual maturation.

Vascular function is a fundamental physiological process that is crucial for tissue health. Although there are documented sex differences in functional outcomes following SCI, it is possible that a single therapy that improves vascular function and/or vessel growth could improve gross functional outcomes across sexes. Further elucidating the molecular mechanisms of sex differences after SCI and in response to treatment will still be necessary, however, to effect the clinical translation of novel therapies.

1.4. Research aims

The overall aim in conducting this research was to determine the potential of a perivascular cell, pericytes, to be used for cell therapy applications across sexes and, to this end, maximize their pro-angiogenic effect on endothelial cells. A secondary aim for this research was to begin to determine if there are differences in vascular responses

following SCI and/or in response to treatment after injury. To address this goal, the study was broken into two aims.

Aim 1: Discover if pericytes could be stimulated to a pro-angiogenic state in vitro.

Aim 2: Quantify the effects of pericytes on functional motor recovery and CNS vessel density across sexes after injury in vivo.

Aim one is addressed in Chapter 2. Aim two is addressed in Chapter 3. The experimental systems used to accomplish these aims included human brain microvascular pericytes, placental pericytes, human brain microvascular endothelial cells, and a neonatal rat pup model of SCI.

1.5. Experimental systems and rationale

1.5.1. Cell culture

The use of cells in culture as a reductionist approach to study cell biology directly had its birth more than 100 years ago (139). This in vitro method of studying cell physiology in cultures that maintain in vivo qualities to varying degrees has the benefit of allowing cells to be directly manipulated by various methods. It also has the advantage of being able to remove compounding variables that exist when studying the whole animal. The results are then necessarily limited by the fact that the cells are being observed out of context of the tissue milieu.

With the harvesting and culture of the first human cell, HeLa cells, came the ability to study human cells in culture, expanding the knowledge of human cell physiology (140). Further developments in cell culture technique throughout the past 75 years have enabled the culture and study of non-cancerous human primary cells (139). Endothelial cells were first successfully cultured in 1973 from the human umbilical vein

by Jaffe et al.(141). Pericytes isolated from bovine, monkey, and human retinas were first cultured in 1975 (142). For this study, human brain microvascular pericytes, human placental pericytes, human brain microvascular endothelial cells, human brain smooth muscle cells, and adult rat brain microvascular endothelial cells were purchased commercially. Human brain endothelial cells and pericytes were originally harvested from aborted fetal tissue (20-24 weeks of gestation) without known medical conditions and isolated from capillary fractions.

Historically, cells derived from non-reproductive tissues have been thought of as asexual (9). Evidence is accumulating, however, that sex chromosomes can have an impact on cell physiology (143, 144). For example, one study has confirmed that seven murine genes have differential expression in male and female brains (145). As another example, dopaminergic neurons harvested from male and female rats are morphologically and functionally different in culture (9, 146). A recent study has concluded that liver cells have sex differences in their response to hepatotoxic drugs (147).

For this study, three aliquots of pericytes and endothelial cells from three different individuals were used. For the human brain endothelial cells, one aliquot was from a female and the other two aliquots were from tissue of unreported sex. For the human brain pericytes, one aliquot was from a male, one from a female, and one from a tissue of unreported sex. The human placental pericytes were from a female and the human brain smooth muscle cells were from a tissue of unreported sex. The rat brain endothelial cells were from an adult female. In culture studies, there was no statistical difference in the rate of wounding in co-cultures of endothelial cells and naïve pericytes or stimulated pericytes across different aliquots. Because of the lack of statistical difference between aliquots, the results from different aliquots of the same species were pooled.

Commercially purchasing these cells limited the selection to what was in stock at the company. For example, it was not possible to select only aliquots from females or males unless the buyer chose to wait for an undesignated amount of time until another female/male aliquot would became available. It also limited the type of cells from a specific tissue that could be obtained. Spinal cord cells, as opposed to brain cells, were not available for purchase. By choosing to use primary cells, the study was also limited to a certain number of cell passages (number of population doublings in culture) as cells that are not immortalized by genetic manipulation will stop proliferating and lose typical morphological characteristics after a certain number of passages. For the purposes of this study, cells were used between the passages of 4-11.

1.5.2. Priming conditions to stimulate pericytes

Hypoxia exposure has been used as a "priming" culture condition to increase the survival and differentiation of the stem cell populations used for cell therapy (148-150). Hypoxia treatment inhibited senescence in endothelial progenitor cells while maintaining their positive effect on therapeutic vessel growth (151). Stem cell function in general is thought to be regulated by oxygen availability, specifically by HIF1-α (152-154). Activation of HIF-1α in myeloid cells also increases angiogenesis (155).

In pericytes, a subpopulation isolated from fetal skeletal muscle increased migration and proliferation in response to hypoxia (156). Skeletal muscle pericytes have also been shown to increase VEGF, transforming growth factor (TGF-β1), and PDGF-β gene expression in response to hypoxia (54). Retinal pericytes decreased expression of extracellular superoxide dismutase and increased the production of intracellular reactive oxygen species in response to low oxygen availability (157). In human fetal brain angiogenesis, a highly hypoxic environment (152), pericytes were found to precede

endothelial cells in the direction of growth (41). Similarly, in an angiogenic response to hypoxia, pericytes are some of the first vascular cells to migrate (42, 43). The canonical hypoxia-driven cell signaling cascade is initiated by the stabilization of hypoxia inducible factor α (HIF-α) (158). Taken together, the evidence would support the hypothesis that driving the HIF pathway could stimulate pericytes to a pro-angiogenic state.

For this study, a chemical inducer of the HIF pathway was utilized to assess directly the role the HIF pathway in stimulating pericytes to a pro-angiogenic state. Cobalt (II) chloride ($CoCl_2$) is a chemical hypoxia mimic that acts by stabilizing the normally degraded HIFα isoforms, HIF1α and HIF2α in the absence of actual low oxygen availability. Chetomin is a chemical inhibitor of the HIF pathway that acts by preventing the binding of either HIF1α or HIF2α to necessary co-factors for transcription initiation after nuclear translocation (159). HIF1α is potently inhibited by the small molecule NSC134754 (160). Both chetomin and NSC134754 were used in this study to verify HIF pathway involvement after $CoCl_2$ stimulation.

$CoCl_2$ has been used in the concentration range of 50-600 µM, with the most common doses being between 100-250 µM (161, 162). Doses of 200 µM and 400 µM for 24 hours were chosen for human brain pericytes and human placental pericytes respectively based on preliminary studies. Briefly, for human brain pericytes a dose of 200 µM was initially chosen based on the current literature and observations of these cells in culture at various doses of $CoCl_2$. In early studies during this project, brain pericytes plated at high densities formed dense clusters of cells, which, for peripheral pericytes, have been reported to be an indication of multipotent activation. When dispersed and co-cultured with endothelial cells, these pericytes promoted a faster rate of wound healing. A dose of 200 µM was the minimum dose of $CoCl_2$ where pericytes

plated at confluence started to form these dense clusters of cells, but did not show signs of massive cell death. When then tested in co-culture with endothelial cells, pericytes stimulated with 200 µM $CoCl_2$ resulted in a 1.8 ± 0.17 fold faster rate of wound healing with no statistical difference of the rate of wound healing between different plating ratios of endothelial cells to pericytes (Figure 2.1). This dose of $CoCl_2$ was then used for all subsequent experiments. A dose of 400 µM optimized the promotion of a faster rate of wound healing (1.6 ± 0.1407 fold increase) when placental pericytes were co-cultured with endothelial cells in a wound healing assay (Figure 3.1) without massive cell death during pericyte stimulation.

1.5.3. Wound healing assay

Migration is an endothelial cell function that is necessary for angiogenesis. The wound healing assay is a well-established system used as a high-throughput in vitro assay to evaluate the angiogenic potential of different treatments before testing them in other 3D angiogenic assays (163). The wound healing assay, also known as the scrape migration assay, allows for the assessment of cell migration by producing a directional migration of cells into a denuded area created by scratching a confluent monolayer of cells, mimicking some of the features of cell migration in vivo (164).

1.5.4. Cord formation assay

A 3D in vitro assay of angiogenesis involves culturing endothelial cells alone or in co-culture in a collagen matrix (165). When embedded in a collagen matrix and stimulated with VEGF, endothelial cells will migrate and interact with each other to form cords (elongated, interconnected endothelial cells) which are thought to be precursory structures to vessel tubes during angiogenesis (165). Pericytes co-cultured with endothelial cells in this system will migrate to and influence cord formation (37). VEGF,

the most potent and ubiquitous angiogenic factor, was used as an angiogenic stimulus in the cord formation assay at a dose of 40 ng/ml based on the current literature (166).

1.5.5. Ex vivo culture model of spinal cord transection

While there are many benefits to studying cells in isolation, moving from cell culture to a more complex tissue culture model has translational benefits when the goal is ultimately to understand how cell therapies will incorporate into whole tissue function. Tissue from male postnatal pups at P10 was harvested and manipulated in culture to mimic a pediatric model of spinal cord transection. Data collected from this model was used as piloting data to justify progressing to an in vivo model of SCI. Although the methods used to harvest the tissue were intended to preserve the tissue as best as possible (167), cell death occurred over a period of time in culture. This loss of tissue viability overtime is consistent with tissue conditions after injury.

1.5.6. In vivo system of spinal cord injury in the postnatal rat pup

Given the gap in knowledge of how therapeutic treatments can be used in the pediatric population, a postnatal model of SCI was used to determine how pericytes would influence tissue recovery in the whole animal. To limit animal manipulation, rat pups were injected with pericytes at the time of injury. Subjects were harvested seven days after injury based on previous observations of the progression of angiogenesis and neuronal growth made in adult male rats (95). Both male and female postnatal pups were used in order to determine the utility of pericytes as a cell therapy for both sexes and to determine if there were sex differences in vascular responses following SCI.

Complete spinal cord transection is a severe form of SCI and is rarely seen in patients. A partial transection (hemi-section) is less severe, and while still rare in patients, has been used extensively in rodent models to assess cell therapy efficacy in promoting

greater axonal regeneration after injury (168). In this study, a dorsal bilateral hemi-section was used to assess pericyte potential to promote functional recovery after a severe injury. Using this model also allowed a rough comparison of results between this study and other studies using a similar injury to quantify cell therapy efficacy. A dorsal bilateral hemi-section, performed with irridectomy scissors, was used to create a lesion up to approximately the midline of the spine in the spinal cords of postnatal rat pups. Surgeries were performed on the lower thoracic regions, between T9 and T10, at postnatal day 3 (P3) in Sprague Dawley rat pups.

At the time of birth, litters were culled to between six and eight pups. All litters used for data collection purposes had an equal distribution of males and females at the time of surgery. All pups from one litter received the same treatment to avoid maternal care differences within the litter. To reduce variability further, all surgical animals were kept together until after recovery and returned to the mother at the same time.

In cell culture studies, human brain pericytes were used. While these studies were necessary in determining mechanisms by which endogenous pericytes could be activated to a pro-angiogenic state, human brain pericytes are not a practical option for injectable cell therapies due to ethical complications and the ability to harvest them in sufficient quantities. The identification and characterization of a cell population that avoids the ethical complications of harvesting fetal cells will be necessary for more eminent widespread clinical application. Placental pericytes are a population of pericytes that are easily harvestable and avoid ethical concerns, and can overcome immune system rejection. For these reasons, in the in vivo studies, human placental pericytes were used. The dose of $CoCl_2$ used for human placental pericyte stimulation was determined by

piloting experiments in the wound healing assay with rat brain endothelial cells (Figure 3.1).

Because this study involved injecting human cells into a rat, the animals needed to be on immunosuppressive drugs for the duration of the experiment. Pregnant dam cages were checked once daily for the presence of rat pups. Daily subcutaneous injections of the immunosuppressive drug cyclosporine A (10 mg/kg) and the anti-bacterial Baytril (50 mg/kg) were given starting on the first day that pups were observed in the cage with the mother (P0). These injections were given throughout the experiment. To avoid potential sickness and morbidity due to immunosuppression, animals were housed in microinsulator cages with autoclaved bedding, irradiated food, and sterilized water. Animal handlers also wore a mask when performing surgeries in addition to the smock, gloves, hairnets, and booties that are standard procedure. For humane considerations, immediately after surgery and for two days following the surgery Buprenex (pain medication, 0.1 ml of 0.04 mg/kg solution) and sterile saline (0.1 ml for fluid replacement) were administered.

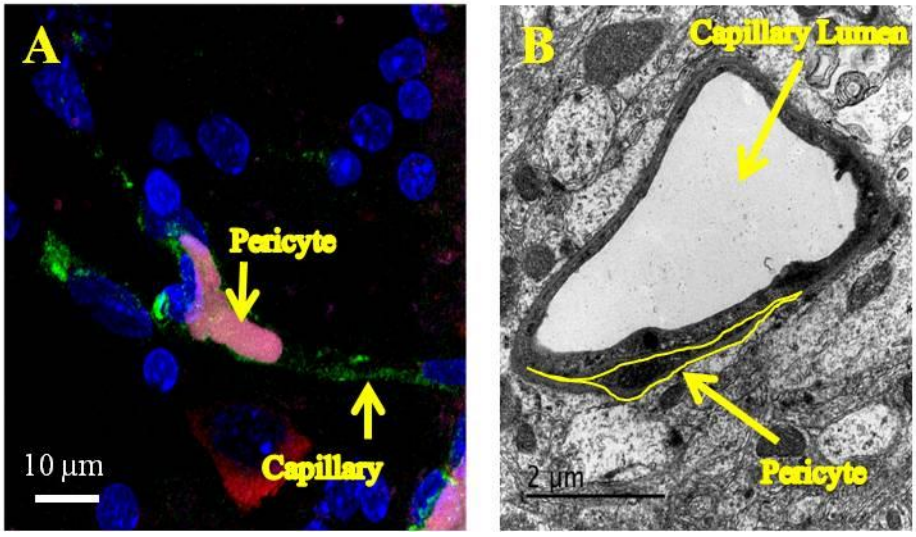

FIGURE 1:1:1. PERICYTES ARE PERIVASCULAR CELLS THAT ARE EMBEDDED IN THE BASEMENT MEMBRANE OF CAPILLARIES THROUGHOUT THE BODY.

(A) Immunohistochemical image of a capillary within the spinal cord of a neonatal rat pup. Capillaries (isolectin labeling) are green, smooth muscle cells (α-smooth muscle actin labeling) are red, pericytes (PDGFR-β labeling) are magenta, and nuclei (Hoechst labeling) are blue. **(B)** Transmission electron microscope image of a pericyte encircling a brain capillary taken by and used with the permission of Matthew Osbourne, ISU Department of Biological Sciences, 2014.

2. Driving the hypoxia inducible pathway in human pericytes promotes vascular density in an exosome dependent manner

Running Title: Activated Pericytes Promote Vascular Density

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2.1. Abstract

Objectives: The mechanisms involved in activating pericytes, cells that ensheath capillaries, to engage in the formation of new capillaries, angiogenesis, remain unknown. In this study, the hypothesis was tested that pericytes could be stimulated to promote angiogenesis by driving the hypoxia-inducible factor (HIF) pathway. **Methods:** Pericytes were stimulated with cobalt chloride ($CoCl_2$) to activate the HIF pathway. Stimulated pericytes were co-cultured with endothelial cells in a wound healing assay and in a 3D collagen matrix assay of angiogenesis. A culture system of spinal cord tissue was used to assess microvascular outcomes after treatment with stimulated pericytes. Pharmaceutical inhibition of exosome production was also performed. **Results:** Treatment with stimulated pericytes resulted in faster wound healing (1.92 ± 0.18 fold increase, $p < 0.05$), greater endothelial cord formation (2.9 ± 0.14 fold increase, $p < 0.05$) in cell culture assays and greater vascular density (1.78 ± 0.23 fold increase, $p < 0.05$) in spinal cord tissue. Exosome secretion and the physical presence of stimulated pericytes were necessary in the promotion of angiogenic outcomes. **Conclusions:** These results elucidate a mechanism that may be exploited to enhance features of angiogenesis in the central nervous system (CNS).

Keywords: Pericytes, endothelium, angiogenesis, exosomes, spinal cord, hypoxia inducible factor

2.2. Introduction

Pericytes are cells that are embedded in the basement membrane of capillaries (28). The current literature provides evidence for a dual role of pericytes: one in which they are pro-angiogenic, participating in the formation of new capillaries, and one in which they participate in the maturation of a stable vasculature (29-32, 41-43, 46). Mechanisms that drive their switch between a stable state and a pro-angiogenic state remain unknown.

Manipulating the process of angiogenesis, the sprouting of new vessels from pre-existing capillaries, is a therapeutic tactic to improve tissue recovery and promote neurogenesis throughout the CNS (61-63). Cell-directed therapies have shown the potential to improve patient outcomes (4). The mechanisms of tissue recovery following cell therapy have been attributed in part to the ability of these cells to promote angiogenesis at the site of injury (54, 104, 107).

Pericyte progenitors are involved in the CNS response to insults where they can increase tissue propensity for neurogenesis and angiogenesis (65-68). Pericytes also contribute to regeneration in skeletal muscle, bone, and cartilage (69, 70). Whether pericytes can be activated to maximize angiogenesis after CNS injury is unknown at this time. Conversion of pericytes to a pro-angiogenic cell type would present novel opportunities for designing tissue repair strategies after CNS injury (157, 169). The aim in conducting this study was to test the hypothesis that CNS pericytes could be converted to a pro-angiogenic state with meaningful outcomes for therapeutic potential.

2.3. Materials and methods

All procedures were approved by the Institutional Animal Care and Use Committee of Idaho State University and performed in accord with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (170).

2.3.1. Cell culture and reagents

Primary human CNS pericytes, endothelial cells, and smooth muscle cells, with their respective culture media, were obtained from ScienCell. Two factory aliquots of endothelial cells were used (sex of donors unknown; ages - fetal tissue at 24 weeks and 22 weeks of gestation). Three factory aliquots of pericyte were used (one aliquot from a female donor, one from a male donor, and one from an unknown donor; ages - fetal tissue at 20 weeks of gestation). One factory aliquot of smooth muscle cells was used (sex of donor unknown; age - unknown). Pericytes were negative for vascular endothelial cadherin (VEC) and isolectin GS-IB_4, and positive for platelet-derived growth factor (PDGFR)-β, smooth muscle alpha-actin (α-SMA), and nestin. Pericyte culture media (PM, ScienCell) was supplemented with 2% fetal bovine serum, pericyte growth supplement (PGS, ScienCell), and 1% penicillin/streptomycin. Endothelial culture media (EM, ScienCell) was supplemented with 5% fetal bovine serum, endothelial cell growth supplement (ECGS, ScienCell), and 1% penicillin/streptomycin. Medium was changed every 48 hours. Cells were maintained in a humidified incubator (37˚C, 5% CO_2, 95% room air). All cells were used between passages 5 and 11.

Cobalt chloride ($CoCl_2$, MP Biomedicals) was used to activate the HIF pathway in pericytes (162). To stimulate cells, pericytes were treated with 200 μM $CoCl_2$ for 24 hours before use in experiments and are referred to throughout this manuscript as stimulated pericytes. The pharmaceuticals chetomin (Cayman Chemicals), GW4869

(Cayman Chemicals), dynasore (Cayman Chemicals), and HIF-1α inhibitor (EMD Millipore) were dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific) for stock solutions and then diluted to the indicated doses in culture media. VEGF165aa mouse recombinant protein (VEGF, vascular endothelial growth factor, Millipore) was dissolved in sterile water as a stock solution and diluted in culture media to the indicated working concentrations.

Cell viability either directly following $CoCl_2$ stimulation or two hours after plating stimulated pericytes was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (1.2 mM, Research Products International). For western blotting, each PVDF membrane was probed using anti-nestin (1:500, Santa Cruz) and anti-PDGFR-β (1:500, Cell Signaling). Data were normalized to total protein for each lane by using β-actin as a loading control and presented as the fold increase from control pericytes.

The lipophillic dyes benzoxazolium, 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl]-, perchlorate (DiO) and 3H-Indolium, 5-[[[4-(chloromethyl)benzoyl]amino]methyl]-2-[3-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-ylidene)-1-propenyl]-3,3-dimethyl-1-octadecyl-, chloride (DiI) were used to label endothelial cells and pericytes (5 µl of dye/1 ml of culture media, Invitrogen). Z-stacked, sequential, high magnification images were obtained at the wound edge (at least three images per coverslip). For co-localization measurements, Olympus Fluoview software was used to calculate the Pearson coefficient on one Z-plane, which was similar across wells, for each image, with the limit of resolution at ~750 nm. BacMam Cell Light reagents were used to transduce respective cells to express a GFP or RFP indicator

specific for nuclei, mitochondria, or F-actin filaments (nuclei and mitochondria: 10 particles per cell (PPC), actin: 30 PPC, Life Technologies).

For wound healing assays, cells were co-cultured at the indicated ratios for 18-24 hours. The difference in wound area between initial wounding and six hours post-wounding was divided by the time elapsed to determine the average rate of growth into the denuded area. The average initial wound area was calculated from 200 wells across 10 replicate experiments (1.155 mm ± 0.007 mm) using cells from 2 different purchased cell batch aliquots. Because the relative standard deviation (RSD) was less than 10% (RSD=9.73%, standard deviation of 0.1125 mm), this average initial wound size was used to determine the rate of outgrowth in all subsequent experiments in order to improve the efficiency of the wounding procedure.

2.3.2. Conditioned media experiments

Conditioned media from co-cultures of endothelial and naïve pericytes or stimulated pericytes was removed after either 6 hours or 24 hours of co-culture and replaced with fresh media. The conditioned media was then used to plate co-cultures of endothelial cells and naïve pericytes for wound healing assays. Ultracentrifugation was used to isolate exosomes as previously described (171). Briefly, media from co-cultures of endothelial cells and stimulated pericytes was centrifuged for 30 minutes at 10,000 X g followed by a 70-minute 100,000 X g cycle. After the initial 100,000 X g cycle, the supernatant was removed (exosome depleted fraction) and the exosome pellet was resuspended in PBS (exosome fraction). Both fractions then underwent a second centrifugation at 100,000 X g. Both fractions were filtered through a 0.20 μm syringe filter (Corning) before being used in cell culture. For the wound healing experiments involving the exosome fractions, endothelial cells and stimulated pericytes were co-

cultured for 6 hours. The culture media was then removed and replaced by either the exosome depleted media or the exosome only media.

2.3.3. 3D collagen angiogenesis assay

Endothelial cells and pericytes were co-cultured in a collagen-media solution (2.5 mg/ml, collagen type I, rat tail, BD Biosciences; EM, ScienCell, pH to 7.0) at the indicated ratios in half-bottom 96-well plates (96-well half area, Corning). A concentration of 2 X 10^6 cells/ml was used (1 X 10^5 cells per well). VEGF, in endothelial cell media, was added on top of the solidified collagen (1 nM) as an angiogenic stimulus. Any other pharmaceutical treatments were also added to the media on top of the collagen matrices. Cord formation was quantified eight days after seeding. An Olympus FV1000 confocal laser-scanning microscope was used to collect a series of Z-stacked images of the collagen matrices with a 10X objective (N.A. 0.4). A visual count of all cords in one plane of the Z-stacked images was recorded. A cord was defined as at least two cells with continuous contact that extended more than 50 μm in length. Branches off a cord were counted as a separate cord if they contained two cells extending more than 50 μm from the branch point.

2.3.4. Organotypic system of spinal cord transection

Lower thoracic spinal cord segments were harvested from male Sprague Dawley rat pups on postnatal day 10 or 11. A total of 30 animals were used. For tissue samples with no treatment, harvested at day zero and day three n=3, for tissue samples treated with naïve pericytes n=6, for tissue samples treated with stimulated pericytes n=6, for tissue samples treated with GW4869+cells n=3. Harvested segments were placed in ice cold artificial cerebrospinal fluid (ACSF)(167). A vibratome was used to cut each

segment into frontal slices 200 µm thick. Three dorsal slices from each animal were used. Slices were seeded onto hanging culture inserts (12-well format, 8.0 µm pore size, BD Falcon). For cell treatment, 2 X 10^6 cells/ml were added to the inner chamber media (5 X 10^5 cells per insert). The pharmaceutical GW4869 was added to the inner chamber media.

Spinal cord slices were cultured in an incubator for three days and then fixed with 4% PFA for one hour before being processed for labeling with isolectin GS-IB_4 (1:500, Alexa Fluor 647, Life Technologies) or anti- endothelial cell antibody (RECA-1, 1:500, Abcam) and Hoechst 33258 (Sigma-Aldrich, 1 ng/ml). High-resolution images were obtained at the center of the slice (caudal to rostral) and across the slice (lateral to lateral) along a line 100 µm deep from the dorsal edge; three contiguous planes were imaged from which a Z-stack was produced.

Vessel density measurements were obtained by quantifying the area of isolectin or RECA-1 labeling per stacked image using ImageJ software (172). To obtain pericyte cell counts per field of view, cells were pre-incubated with DiI before treatment, as described above. After imaging, the number of DiI positive cells from one confocal plane every 20 µm dorsal to ventral was counted. Vessel counts were also made from one confocal plane every 20 µm dorsal to ventral. A vessel was defined as a continuous isolectin-positive structure longer than 150 µm or (depending on the orientation of the vessel in the imaged section) as having a diameter of at least 5 µm. In the case of vessels that branched, a branch was counted as a separate vessel if it extended more than 150 µm within the field of view.

The cell proliferation marker Ki67 (173) was used in cells (n=4) at 1:500 and in explants (n=3) at 1:250 (Abcam). YO-PRO-1 Iodide (Life Technologies) was used to assess endothelial apoptosis in explants (n=6). Both YO-PRO-1 and Ki67 were used in

conjunction with Hoechst 33258 (1 ng/ml) and/or isolectin in order to identify and quantify the number of endothelial cells positive for the label within each field of view.

2.3.5. Statistical analyses

Analyses were performed using GraphPad Prism 5.0 (Graph-Pad Software, San Diego, CA, USA), with alpha set at 0.05. One-way ANOVA was used to compare three or more groups with Tukey or Dunnett post hoc analysis. When comparing only two groups, Student's paired t-test was used. Data are presented as the mean ± standard error of the mean. Different letters were used to denote statistical difference between columns. For example, if one column was lettered as "a" and another column was lettered as "b," that would indicate that statistical difference was found between the two. Conversely, if both columns were lettered as "a," that would indicate that no statistical difference was found between the two columns. If three columns were lettered as "a," "b," and "c," respectively, then this would indicate that all three columns were statistically different from one another.

2.4. Results

2.4.1. Pericytes can be stimulated to a pro-angiogenic state

In monolayer co-culture assays, stimulated pericytes promoted faster rates of wound healing (Figure 2.1A). Under these conditions, the number of endothelial cells positive for Ki67 ($Ki67^{+}$) was not different whether cultured alone or with stimulated pericytes (Figure 2.1B). In a 3D collagen matrix model of angiogenesis, endothelial cells formed cords that contained lumens (Figure 2.1C). Stimulated pericytes and naïve pericytes homed to these cords (Figure 2.1C; insert). Naïve pericytes inhibited cord formation (Figure 2.1D). A higher density of cords was produced in matrices with

stimulated pericytes as compared to cultures with their naïve counterparts. This effect was independent of the ratio of endothelial cells to pericytes (Figure 2.1D).

To test the effect of stimulated pericytes on vessel density in a whole tissue environment, spinal cord explants were harvested from neonatal rats and cultured on tissue insert membranes for three days (Figure 2.2A). In culture, pericytes used during explant treatment did not label with isolectin. Vessel density decreased in spinal cord explants over time (Figure 2.2B-D), which is expected in culture as the tissue loses viability. This is consistent with the death of CNS tissue in the peri-lesion following many forms of trauma.

Tissues treated with stimulated pericytes yielded significantly higher vessel density (1.78 ± 0.23 fold increase in isolectin labeling, $p < 0.05$) with vessels whose architecture and morphology appeared more like vessels in explants at day zero (Figure 2.2B, and E-K. $p < 0.05$). Representative images of explant labeling are presented in Figure 2.2, panels E- J. To verify the quantified vessel density, a second endothelial marker was used (RECA-1). A similar fold increase in vessel density was observed in explants treated with stimulated pericytes (1.70 fold increase in RECA-1 labeling ± 0.23, $p < 0.05$).

The greater vessel density in explants containing stimulated pericytes was, in part, a result of endothelial cell proliferation (Figure 2.3A) as opposed to a decrease in endothelial apoptosis (Figure 2.3B). Stimulated pericytes were found to have migrated throughout the depth of the spinal cord tissue (Figure 2.3C). The largest difference in vessel count per field of view between groups was at a depth of 20 μm from the application surface (Figure 2.3D). The number of cells found within the explant tissue varied among explants and groups. Overall, explants treated with stimulated pericytes

contained more pericytes within the explant tissue, and those pericytes penetrated significantly deeper (1.5 cells ± .56 at 100 μm, $p < 0.05$, Figure 2.3C). Explants treated with naïve pericytes contained the fewest pericytes, which did not penetrate as deep (0.09 cells ± 0.09 at 100 μm, Figure 2.3C).

2.4.2. Pericyte stimulation is dependent on HIFα and is unique among mural cells

HIF1-α inhibitors applied during $CoCl_2$ pre-treatment inhibited the faster rate of wound healing (Figure 2.4A and B) verifying that pericyte stimulation was dependent on driving the HIFα pathway, as opposed to an off target effect of $CoCl_2$ treatment. Treatment with $CoCl_2$ decreased cell viability directly following stimulation (Figure 2.4C). Cell viability was not affected as compared to control pericytes two hours following treatment (Figure 2.4D). Additionally, stimulated pericytes were molecularly different from their naïve counterparts in that they had greater protein expression of nestin and PDGFR-β (Figure 2.4E and F).

Endothelial $CoCl_2$ pre-treatment also promoted faster wound healing (Figure 2.4G). Conversely, smooth muscle cell pre-treatment with $CoCl_2$ resulted in a slower rate of wound healing when compared to endothelial cells co-cultured with naïve smooth muscle cells (Figure 2.4H). There was no significant effect of co-culturing pre-treated endothelial cells with naïve smooth muscle cells (Figure 2.4H).

2.4.3. Stimulated pericytes and endothelial cells share membrane lipid components

In order to explore further possible mechanisms of stimulated pericyte action, the wound healing assay was utilized. In co-cultures of endothelial cells with naïve pericytes or endothelial cells with stimulated pericytes, both cell types populated the wound edge. In the stimulated pericyte co-culture, there were more membrane sharing events between

the two cell types (Figure 2.5A-F). When examined in 3D and at high resolution, cells with both membrane labels had one nucleus paired with concomitant DiO and DiI labeling throughout the cell (Figure 2.5G-I). Endothelial cells, stimulated pericytes, and cells with shared lipid components were all present at the wound edge. Additionally, both endothelial cells and stimulated pericytes received membrane components from the other cell type, i.e., bi-directional signaling (Figure 2.5J-K).

Co-cultures of endothelial cells and stimulated pericytes had a Pearson coefficient above 0.5, while co-cultures with naïve pericytes had a coefficient below 0.2 (Figure 2.5L). Membrane sharing events required activation of the HIF pathway (Figure 2.5M). Cultures of pre-treated endothelial cells with naïve pericytes also showed greater co-localization of DiO and DiI (Pearson coefficient of 0.34 ± 0.03, $p < 0.05$) than in control cultures (Pearson coefficient of 0.11 ± 0.04, Figure 2.5N). In co-cultures of endothelial cells with stimulated smooth muscle cells, the co-localization coefficient was smaller than in control co-cultures (EC: SMC; 0.44 ± 0.04, EC: SMC $CoCl_2$; 0.22 ± 0.05, $p < 0.05$). Co-cultures of pre-treated endothelial cells with naïve smooth muscle cells had higher co-localization coefficients ($ECCoCl_2$: SMC; 0.73 ± 0.03, $p < 0.05$) than control co-cultures (Figure 2.5O).

2.4.4. Conditioned media from endothelial cells co-cultured with stimulated pericytes is necessary but not sufficient for faster rate of wound healing

Removing the conditioned media from co-cultures of endothelial cells and stimulated pericytes six hours after co-culture and replacing it with fresh media inhibited the faster rate of wound healing (Figure 2.6A). The conditioned media was not sufficient, however, because it failed to produce faster wound healing in co-cultures of endothelial cells and naïve pericytes (Figure 2.6B-C). Removing the conditioned media and returning

the same conditioned media still resulted in faster wound healing (Figure 2.6B), which controls for the act of removing and adding media in the above experiments.

Exosome signaling between endothelial cells and stimulated pericytes could be transferred in conditioned media and account for both the faster rate of wound healing and the observed membrane sharing events. To test the hypothesis that exosomes were a necessary secreted factor in the conditioned media to promote faster wound healing in co-cultures of endothelial cells and stimulated pericytes, ultracentrifugation was used to create an exosome-free media fraction and an exosome-only media fraction. Removing exosomes from the conditioned media inhibited the faster rate of wound healing in co-cultures of endothelial cells and stimulated pericytes (Figure 2.6D).

2.4.5. Stimulated pericytes promote angiogenesis through exosome secretion

Chemical inhibition of ceramide-dependent exosome secretion and endocytosis-dependent membrane vesicle cycling was used to test the hypothesis further that exosomes were a necessary signaling mechanism. The rate of wound healing was not different in control co-cultures with or without inhibition of ceramide-dependent exosome secretion (Figure 2.7A). In contrast, the faster rate of wound healing in co-cultures of stimulated pericytes and endothelial cells required ceramide-dependent exosome secretion (Figure 2.7B). Moreover, the greater rate of wound healing produced by stimulated pericytes required membrane vesicle cycling (Figure 2.7C-D). In addition to slower wound healing, exosome cycling was required for membrane fusion events in co-cultures of stimulated pericytes and endothelial cells (Figure 2.7E). In the models of 3D cord formation and vessel density in whole tissue, the effect of stimulated pericytes was also dependent of exosome secretion (Figure 2.7F-G).

2.5. Discussion

Pericytes regulate tissue capillarity by stabilizing microvessels (32) and by promoting greater microvascular density through angiogenesis. Pericytes can contribute to angiogenesis by migrating from the basement membrane matrix (46, 67, 174) and assuming a leading position on sprouts after injury (30, 46) or during development (41). In this study, activation of the HIF pathway was used successfully as a molecular tool to convert pericytes to a pro-angiogenic state. Under these conditions, exosome-mediated signaling was a necessary mechanism in promoting wound healing in cell culture, cord formation in collagen matrices, and microvascular density in spinal cord explants.

The switch to activate pericytes to a pro-angiogenic state was specific to pericytes because the other mural cells, smooth muscle cells, responded to the same treatment with the opposite outcome (Figure 2.4G-H). Thus, it is possible that the stress of trauma and/or hypoxia drives smooth muscle cells to stabilize the vessels that regulate blood flow, arterioles, while activating pericytes to increase capillary density in the downstream tissue. Stimulation in response to driving the HIF pathway, while unique to pericytes as opposed to smooth muscle cells, was similar in endothelial cells and pericytes (Figure 2.4G and H). This could be important for an overall microvascular response to hypoxia, in that it allows for either cell type to initiate an angiogenic response to hypoxic stimulation, whether that stimulus is initiated from a luminal or abluminal vascular position. Though the present study focused on the possibility of using stimulated pericytes as an exogenous therapeutic tool, the results support the idea that the collective endogenous response of microvascular cells to hypoxia may be complementary by producing an expanded, flow-regulated capillary network.

There is an extensive involvement of exosomes in many types of cell-cell communication (175-177). Specifically, membrane-based microvesicles released from cells can promote angiogenesis in response to environmental stimuli. Endothelial to endothelial exosome-based communication can induce angiogenesis (175) and endothelial cells can incorporate delta-like ligand 4 into exosomes, inhibiting notch signaling in receiving endothelial cells (178). Hypoxia results in modification to the contents of the exosomes released by glioma cells and these exosomes induced angiogenesis (79). Exosomes produced under hypoxic conditions also promote angiogenesis by participating in communication between multiple myeloma cells and endothelial cells (179). Placental mesenchymal stem cells produce more exosomes under hypoxic conditions, which promote endothelial migration and sprout formation (180). Additionally, microvesicles released by endothelial progenitor cells encourage endothelial proliferation, survival, and the formation of vessels in subcutaneously implanted Matrigel® plugs (78). Ultimately, exosomes modulate angiogenesis by transporting signals in the form of proteins, DNA, and RNA (including microRNA) (181, 182). These signaling events are mechanisms by which pericytes could modulate an angiogenic program in response to changing tissue conditions.

This study provides evidence that the exosomal communication between endothelial cells and pericytes is likely complex. For example, both cell types received exosomes from the other (Figure 2.5J-K), which is consistent with bidirectional communication. In the absence of endothelial contact, human placental pericytes can secrete hepatocyte growth factor (31, 183) and bovine retinal pericytes can secrete VEGF (184) . Endothelial cells can also influence pericyte migratory behavior (79), survivability, and investment (33, 34) via paracrine factors. Although exosome formation

and endocytosis was necessary for the pro-angiogenic activity induced by stimulated pericytes (Figure 2.5, 2.6, and 2.7), unidentified soluble factors may also participate.

Endothelial cell and pericyte contact is an important element in vessel stability and maturity (32, 38, 185). Indeed, some of the first work performed with endothelial and pericyte co-cultures indicated that transforming growth factor- β (TGF-β)(186) in combination with the close proximity or cell-cell contact is required for the inhibitory effect of pericytes on endothelial migration. In a stable vascular bed, there is tight contact between endothelial cells and pericytes, with pericytes embedded in the basement membrane of vessels. In the developing retina, formation of an endothelial plexus precedes pericyte investment and pericyte investment is concomitant with vessel stability (32). After injury or during the later stages of development, however, there is rarely a complete absence of pericytes during angiogenesis. Instead, modifications to the extent of pericyte-endothelial contact are the regulated variable. After injury to the brain or spinal cord, while pericytes migrate from the endothelial basement membrane matrix, they are still found in a perivascular position (43, 67, 174). In the early stages of fetal human brain angiogenesis and in peripheral wound healing, pericytes lead tissue invasion of endothelium (41, 46). This work provides evidence of a paracrine interaction between stimulated pericytes and endothelial cells in order to promote an angiogenic program (Figure 2.6A,D and Figure 2.7). Both the physical presence of the stimulated pericytes and the exosomes released in the media were necessary factors in the current study (Figure 2.6B-C), however, highlighting a possible role for both cell-cell contact and exosome communication between pericytes and endothelial cells in promoting angiogensis. These results support the hypothesis that modulation of endothelial-pericyte

communication by contact (e.g., gap junctions) and paracrine factors, rather than the absence of pericytes, induces pro-angiogenic outcomes(187).

Naïve pericytes can limit sprout formation in collagen matrices (Figure 2.1D) and do not promote vessel growth appreciably when implanted with endothelial cells subcutaneously as compared to endothelial cells implanted alone(31). A pro-angiogenic effect of naïve pericytes introduced after ischemic heart injury has been reported. The authors postulated that the naïve peripheral pericytes injected after heart injury were activated to promote angiogenesis by being introduced to an ischemic environment (54).

After three days of culture, the vessel density of tissue treated with stimulated pericytes did not appreciably exceed the amount of vasculature that was present at the time of culture (Figure 2.2D and K). Therefore, the promotion of greater vessel density after three days in culture with stimulated pericyte treatment, as compared to untreated explants or explants treated with naïve pericytes (Figure 2.2D and K), could indicate either improved maintenance of vessel viability, the promotion of an angiogenic program, or both. Endothelial proliferation (Figure 2.3A) in combination with a promotion of greater vascular density (Figure 2.2K) and little change in endothelial apoptosis (Figure 2.3B) is consistent with a system that is experiencing an increase in angiogenesis as opposed to the maintenance of established vasculature. Collectively, these results demonstrate the utility of stimulated pericytes in promoting vessel outgrowth in the context of spinal cord trauma.

Stimulated pericytes did not directly promote endothelial cell proliferation in vitro (Figure 2.1B) but were facilitative of endothelial proliferation in whole tissue (Figure 2.3A), implicating complex interactions between stimulated pericytes and the tissue milieu that promote endothelial cell migration and angiogenesis. For example, it is known

that cell-matrix interactions can directly regulate angiogenesis (187, 188). Pericyte cell-cell contact, pericyte-endothelial contact, or cell-extracellular matrix interactions can influence pericyte synthesis of extracellular matrix proteins (37, 189). Pericyte activation by environmental stimuli can also alter extracellular matrix composition (190). In this study, it is likely that stimulated exogenous pericytes delivered to damaged spinal cord tissue promoted endothelial cell proliferation through tissue-dependent factors that were not present in basic cell co-culture (Figure 2.3A). These factors could include differences in the composition and organization of the extracellular matrix as well as the possible ability of pericytes to interact with these components.

This study provides direct evidence that CNS pericytes can be stimulated to a pro-angiogenic state and used to promote vessel density in CNS tissue. It also identifies critical signaling events that underlie these outcomes. Ultimately, pericytes may be a target of directed therapies aimed at manipulating angiogenesis.

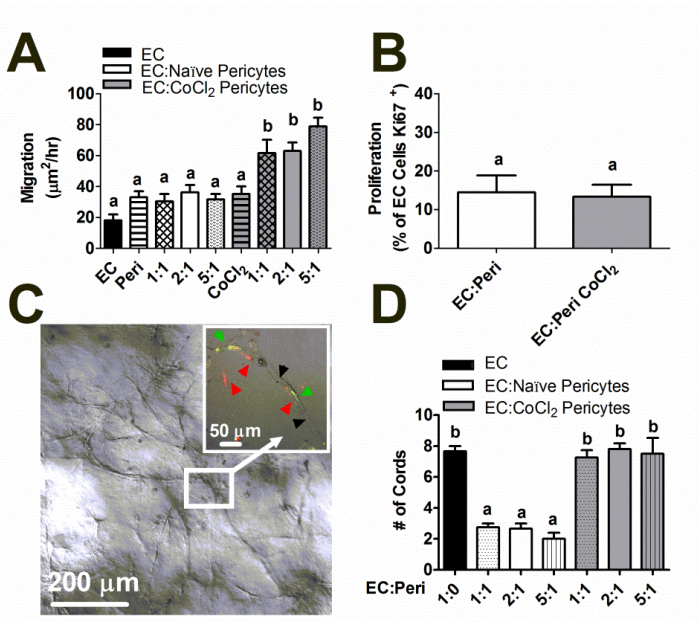

FIGURE 2:1. PERICYTES CAN BE STIMULATED TO A PRO-ANGIOGENIC STATE

(A) Pericytes were stimulated for 24 hours with 200 μM $CoCl_2$ before co-culturing at the indicated ratios with endothelial cells for a wound healing assay. Ratios indicate relative numbers of endothelial cells to pericytes. (B) $Ki67^+$ endothelial cells at the wound edge, in co-cultures of endothelial cells and pericytes, were counted to assess the effect of stimulated pericytes on endothelial proliferation. (C) Representative images of cord formation on a single z-plane from a collagen matrix. A representative cord at higher resolution that has formed a lumen (black arrows), comprises endothelial cells labeled with DiO (green arrows) and pericytes labeled with DiI that have homed to the cord (red arrows) (C insert). (D) Quantification of cord formation on a single z-plane of the collagen matrix plugs from co-cultures at the indicated ratios after eight days of co-culture. Ratios indicate relative numbers of endothelial cells to pericytes. Different letters indicate significant differences between respective groups, $p < 0.05$.

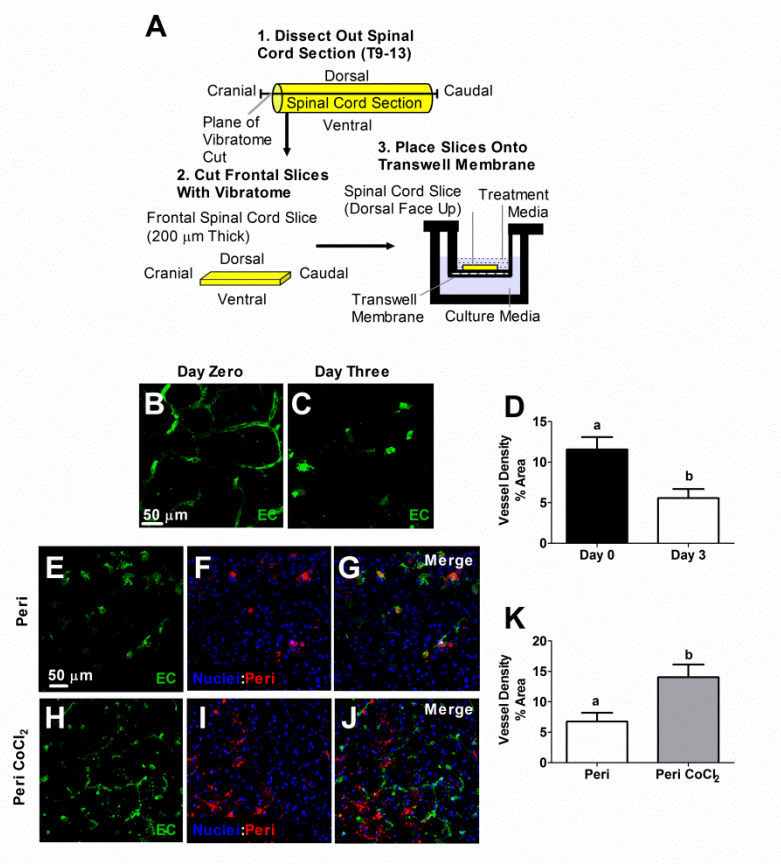

FIGURE 2:2. STIMULATED PERICYTES PROMOTE ANGIOGENESIS IN SPINAL CORD TISSUE

(A) Schematic illustrating the steps involved in dissecting out and culturing spinal cord slices onto transwell membranes. (B-C) Representative images of isolectin labeling in explants with no treatment at the time of culture (day zero) and three days after culture. (D) Spinal cord slice vessel density immediately after harvest (day zero) and three days after culture. (E-J) Representative images of spinal cord slice labeling three days after culture with indicated treatments. (K)Vessel density in explants treated with or without stimulated pericytes added directly to the treatment media in the inner chamber of the transwell, three days after culture. Pericytes were stimulated for 24 hours with 200 µM $CoCl_2$. Different letters indicate statistical differences between groups, $p < 0.05$.

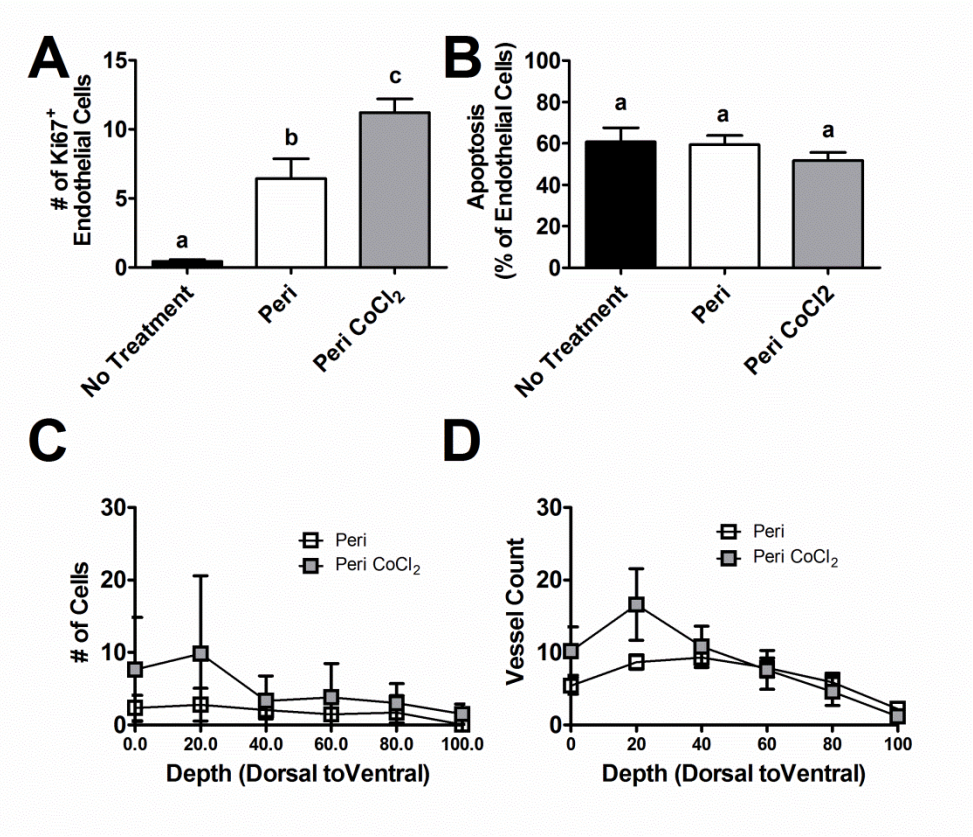

FIGURE 2:3. STIMULATED PERICYTES MIGRATE INTO SPINAL CORD TISSUE

(A) Number of $Ki67^{+}$ endothelial cells per field of view in spinal cord slices after three days of incubation with indicated treatments. (B) Percent of endothelial cells per field of view in spinal cord slices that were positive for YO-PRO-1 labeling after three days of incubation with indicated treatments. (C) Number of cells per field of view from a single plane 0-100 µm deep into the tissue, from dorsal to ventral, three days after culture. (D) Vessel count per field of view from a single plane 0-100 µm deep into the tissue, from dorsal to ventral, three days after culture. Different letters indicate statistical differences between groups, $p < 0.05$.

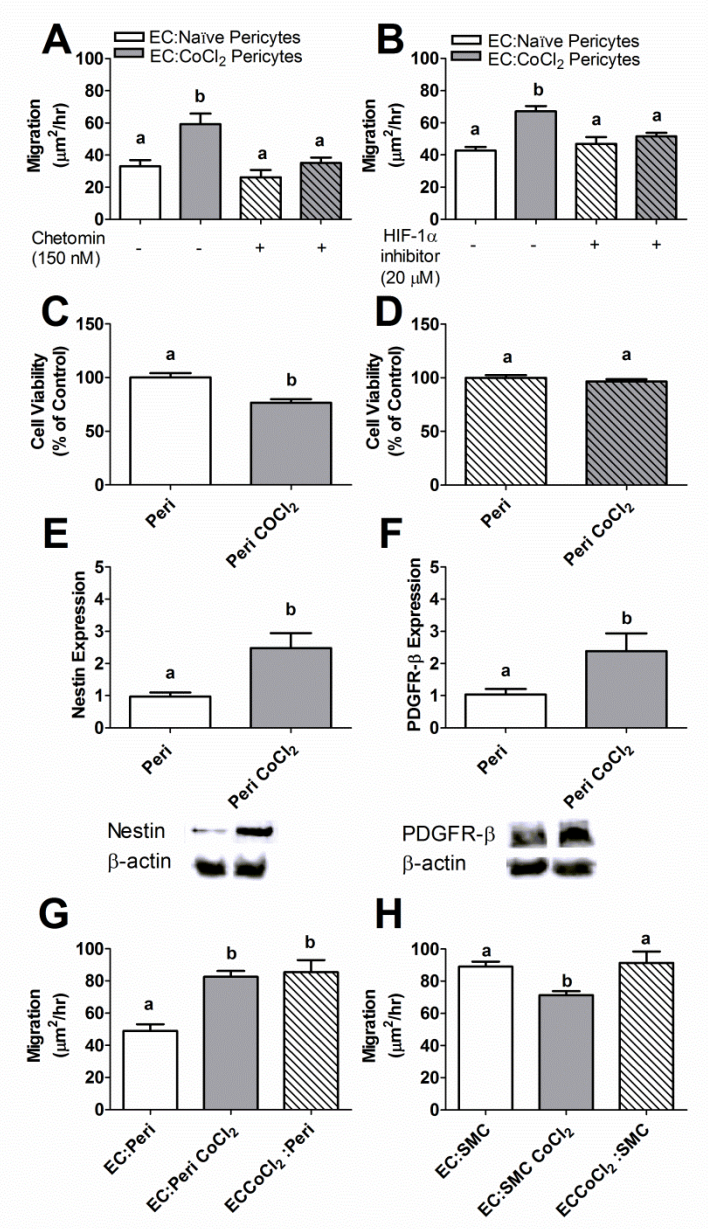

FIGURE 2:4. PERICYTE STIMULATION IS DEPENDENT ON THE HIF PATHWAY AND THE FUNCTIONAL OUTCOMES OF STIMULATION ARE SPECIFIC TO PERICYTES AND ENDOTHELIAL CELLS

(A-B) Chetomin (150 nM) and the HIF-1α inhibitor (20 μM) were used prior to co-culture, during the 24 hours of pericyte stimulation.(C-D) MTT assay performed immediately after stimulation (C) and two hours after culture following stimulation (D). (E-F) Western blot of nestin and PDGFR-β protein expression immediately after stimulation in either naïve pericytes or stimulated pericytes. (G-H) All co-cultures were plated at a 2:1 ratio. Cells were stimulated with 200 μM $CoCl_2$, following the same procedure as for pericytes. Different letters indicate significant differences between respective groups, $p < 0.05$.

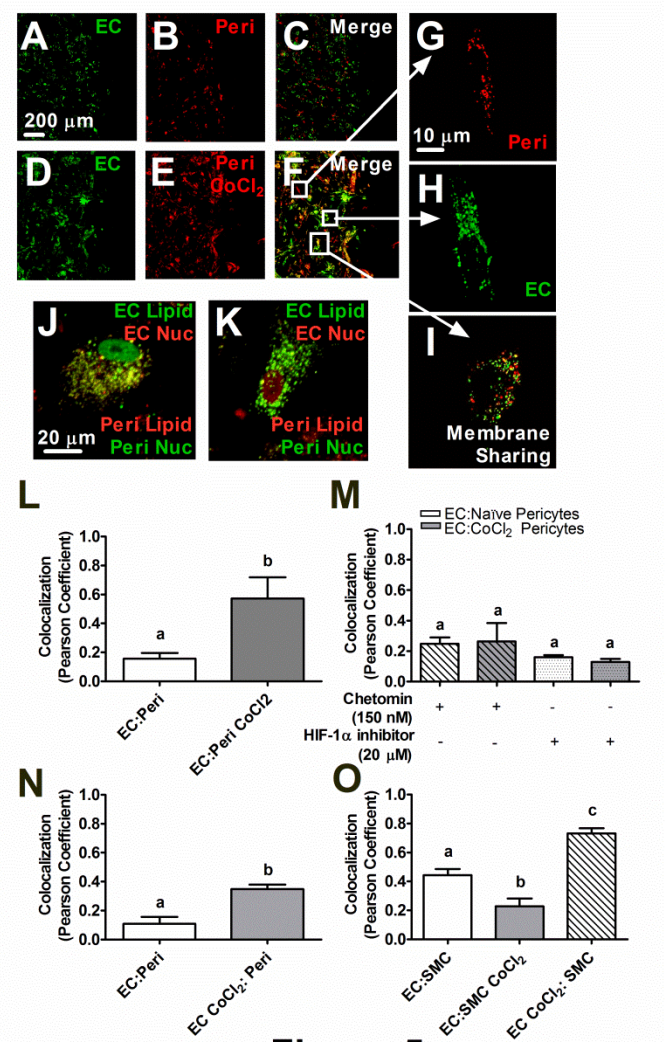

FIGURE 2:5. STIMULATED PERICYTES AND ENDOTHELIAL CELLS SHARE MEMBRANE LIPID COMPONENTS

(A-I) Representative images of the wound edge in co-cultures of endothelial cells and naïve or stimulated pericytes. EC were labeled with DiO and pericytes were labeled with DiI prior to co-culture. (G-I) High-resolution representative images of pericytes with only DiI label (G), EC with only DiO label (H), and a cell that has shared membrane components from both cell types (I). (J-K) Representative images of cells at the wound edge in co-cultures of endothelial cells and stimulated pericytes, labeled with DiO and DiI. Nuclear transduction was performed before co-culture with either a GFP tag for pericyte nuclei (J) or an RFP tag for EC nuclei (K). (L-O) Pearson co-localization coefficient for the membrane labels DiO and DiI in co-cultures of endothelial and mural cells in the indicated experimental conditions. Different letters indicate significant differences between respective groups, $p < 0.05$.

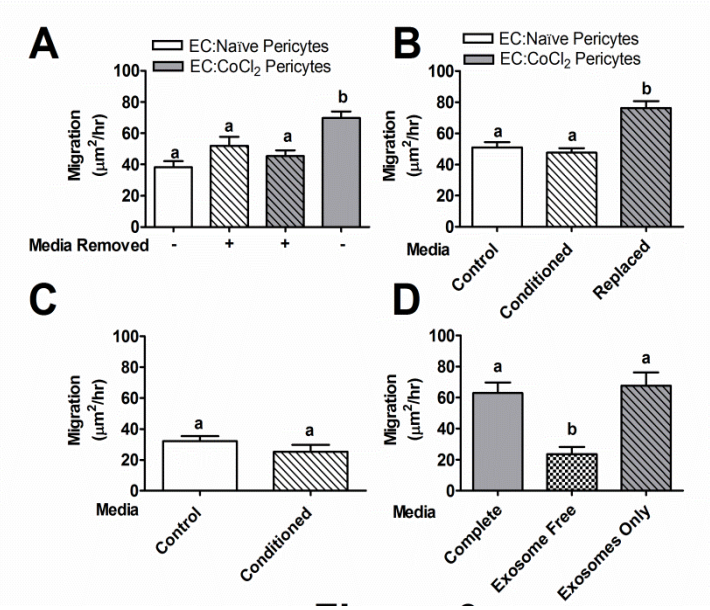

FIGURE 2:6. CONDITIONED MEDIA FROM ENDOTHELIAL CELLS CO-CULTURED WITH PERICYTES IS NECESSARY BUT NOT SUFFICIENT FOR THE FASTER RATE OF WOUND HEALING

(A-D) Endothelial cells were co-cultured with naïve or stimulated pericytes for a wound healing assay. (A) After six hours, media was removed from co-cultures and replaced with fresh media. Co-cultures were then allowed to incubate for 18 additional hours before scrape wounding. (B-C) Conditioned media from co-cultures of endothelial cells and naïve or stimulated pericytes was removed either 6 (B) or 24 (C) hours after co-culture. Conditioned media was then used to plate co-cultures of endothelial cells and naïve pericytes for wound healing assays. Conditioned media was also removed from a co-culture of endothelial cells and stimulated pericytes six hours after co-culture and then replaced with the same conditioned media. (D) Media from co-cultures of endothelial cells and naïve or stimulated pericytes was replaced after six hours of co-culture and replaced with the indicated media fractions. Different letters indicate significant differences between respective groups, $p < 0.05$.

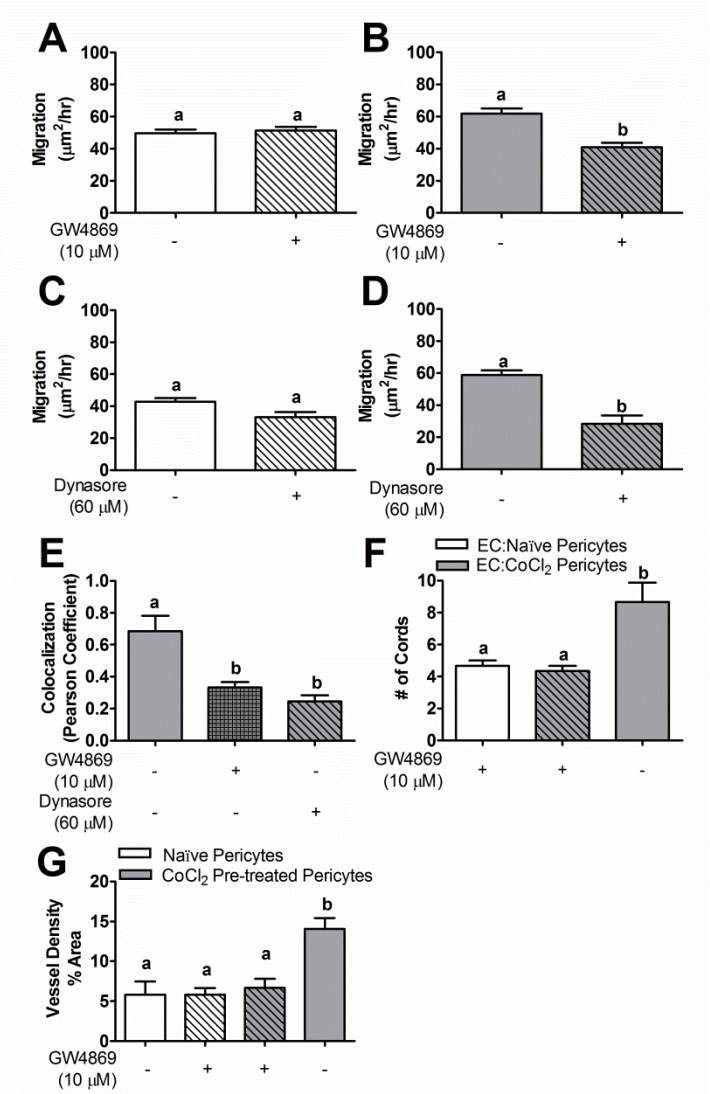

FIGURE 2:7. STIMULATED PERICYTES PROMOTE ANGIOGENIC POTENTIAL IN AN EXOSOME DEPENDENT MANNER

(A-G) Endothelial cells and naïve or stimulated pericytes were co-cultured for a wound healing assay. (A-B) Co-cultures were treated with GW4869 (10 μM) or the vehicle control (DMSO at 1:1000) during the 24-hour co-culture period prior to scrape wounding. (C-D) Co-cultures were treated with dynasore (60 μM) or the vehicle control (DMSO at 1:1000) during the 24-hour co-culture period prior to scrape wounding. (E) Pearson co-localization coefficient for the membrane labels DiO and DiI in co-cultures of endothelial cells and stimulated pericytes treated with the indicated pharmaceuticals. (F) Quantification of cord formation with GW4869 treatment, eight days after co-culture. (G) Vessel density in explants treated with or without GW4869 (10 μM) three days after culture. Pericytes were stimulated for 24 hours with 200 μM $CoCl_2$.The pharmaceutical GW4869 was used at a concentration of 10 μM and added directly to the treatment media in the inner

chamber of the transwell. Different letters indicate significant differences between respective groups, $p < 0.05$.

3. Human placental pericytes improve functional motor recovery across sexes in a pediatric model of spinal cord injury

Running Title: Pericytes Improve Functional Motor Recovery After Spinal Cord Injury

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3.1. Abstract

Recent evidence has shown that pericytes can be stimulated to induce pro-angiogenic activity (the activities associated with and including the formation of new capillaries). This study tested the hypothesis that human placental pericytes stimulated to a pro-angiogenic state would improve functional motor recovery across sexes in a pediatric model of spinal cord injury. **Methods:** Human placental pericytes were stimulated with cobalt chloride ($CoCl_2$). Naïve pericytes and stimulated pericytes were injected acutely into neonatal rats that underwent a thoracic dorsal hemi-section of the spinal cord on postnatal day 3 (P3). On P10, hindlimb motor recovery was assessed using the Basso, Beattie, and Bresnahan motor score scale. Doppler blood flow perfusion at the site of transection was also measured at P10. Immunohistochemistry was used to determine injected pericyte survival, vessel density, neurofilament density, and endothelial proliferation within the injured tissue. **Results:** Treatment with pericytes but not stimulated pericytes resulted in improved hindlimb motor recovery in both sexes (pericyte injected males, 2.722 ±0.3093 fold motor score improvement from control; pericyte injected females, 3.824 ± 0.5803 fold motor score improvement from control, $p < 0.05$). Both pericytes and stimulated pericytes were found in the tissue on P10 across sexes. Pericyte treatment maintained the relative vessel density and blood flow perfusion found in males and females while improving the retention of neurofilament density as compared to controls (pericyte males, 1.9± 0.09151 fold increase in neurofilament density; pericyte females, 2.031 ±0.1553 fold increase in neurofilament density). **Conclusions:** Human placental pericytes improve hindlimb motor recovery after spinal cord injury across sexes in the pediatric population by supporting an organized, flow-regulated vascular bed and promoting greater neurofilament density.

Keywords: Pericytes, angiogenesis, spinal cord injury, cell therapy

3.2. Introduction

There are no FDA approved treatments for spinal cord injury (SCI). Early studies have identified vascular dysfunction as a major contributor to pathology after SCI (83). Improving vascular function and promoting angiogenesis have become promising therapeutic strategies to drive overall central nervous system (CNS) recovery following injury (63, 107). Identifying ways to limit vascular dysfunction and promote an increase in vessel growth might elucidate novel ways to improve motor recovery after SCI.

The introduction of an exogenous cell population to the site of tissue injury has emerged as a promising therapeutic tactic to affect central nervous system (CNS) vasculature positively and ultimately improve tissue recovery (4, 94). Cell therapy is presently limited in adaptability to clinical practice due to the ethical concerns of harvesting embryonic stem cells and complications in the safety and efficacy of the cell types currently in use (4). Recent evidence has emerged that pericytes, an endogenous CNS microvascular cell, can be stimulated to a pro-angiogenic state by in vitro manipulation (191). While pericytes have the ability to influence angiogenesis (31), can direct oligodendrocyte progenitor cell migration (192), and have shown promise as an effective cell therapy in other disease models (54, 64), it is not known whether pericytes or stimulated pericytes can improve hindlimb motor recovery after spinal cord injury.

Pediatric SCI accounts for approximately 5% of all SCI cases (118). There is a gap in knowledge concerning appropriate treatment options for pediatric patients with SCI (113). The basis for motor recovery after spinal cord injury at any age is thought to be due to the reorganization of neuronal connections (116, 193, 194). Very little is known about the mechanisms of injury and recovery in the pediatric population other than that there is some evidence indicating an improved potential for synaptic plasticity and

thereby improved motor recovery (113, 118). Determining the efficiency of therapies in a pediatric model could ultimately aid in the development of novel treatment options for these injuries and elucidate ways to improve recovery in the adult.

There is evidence of sex differences in humans (131) and in animals models of SCI (112), although the mechanisms that dictate these sex differences remain unclear. While the mechanism of response and recovery to injury might differ between sexes, it is possible that a single therapy targeting a basic physiological response (blood vessel growth and function) could improve gross outcomes of functional recovery in both sexes. In this study the hypothesis was tested that stimulated pericytes acutely injected as a cell therapy would improve hindlimb motor recovery across sexes in a pediatric model of SCI. Ultimately, however, a thorough understanding of the sex differences that exist after SCI will be necessary in order to develop a clinically translational treatment for both males and females (8). It is unknown if there are sex differences in the angiogenic response after SCI. Therefore, a secondary aim for this study was to determine if there are sex differences in angiogenic measures after SCI.

3.3. Materials and methods

All animal procedures were approved by the Institutional Animal Care and Use Committee of Idaho State University and performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (170).

3.3.1. Cell culture and reagents

One aliquot of primary human placental pericytes was purchased from Promo Cell (donor tissue was from a Caucasian female). The cells were negative for isolectin GS-IB_4 and platelet endothelial adhesion molecule-1 (PECAM-1). They were positive for

platelet-derived growth factor (PDGFR)-β, smooth muscle alpha-actin (α-SMA), and nestin. One aliquot of rat brain endothelial cells from a young adult female was purchased from ScienCell. All cells were used between passages 4 and 11.

Cells were maintained in a humidified incubator (37˚C, 5% CO_2, 95% room air). Pericyte cell culture medium (PM, ScienCell) was supplemented with 2% fetal bovine serum, pericyte growth supplement (PGS, ScienCell), and 1% penicillin/streptomycin. Endothelial culture medium (EM, ScienCell) was supplemented with 5% fetal bovine serum, endothelial cell growth supplement (ECGS, ScienCell), and 1% penicillin/streptomycin. To stimulate cells pericytes were treated with the indicated dose of $CoCl_2$ (Cobalt chloride, MP Biomedicals) for 24 hours before use in experiments and are referred to in this manuscript as stimulated pericytes. To harvest cells for injection, pericytes or stimulated pericytes were trypsinized and then combined with a collagen-media, pH balanced solution (2.5 mg/ml, collagen type I, rat tail, BD Biosciences; pH to 7.0).

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (1.2 mM, Research Products International) was used to determine cell viability either directly following $CoCl_2$ stimulation or two hours after plating stimulated pericytes. For western blotting, anti-PDGFR-β (1:500, Cell Signaling) was used. Data were normalized to total protein for each lane by using β-actin as a loading control and presented as the fold increase from control pericytes.

3.3.2. Wound healing

Based on previous findings that pericytes can be stimulated to promote an increase in pro-angiogenic activity (191), a scrape wound assay was used to determine the appropriate dose to stimulate placental pericytes in order to maximize the rate of

wound healing when co-cultured with endothelial cells. For this assay, cells were cultured at a 2:1 endothelial to pericyte ratio for 18-24 hours. The difference in wound area between initial wounding and six hours post-wounding was divided by the time elapsed to determine the average rate of growth into the denuded area.

3.3.3. Thoracic spinal cord hemi-section in neonatal rats

Sprague-Dawley rats of both sexes received a thoracic spinal cord hemi-section on postnatal day three (P3) and were then euthanized on P10. A total of 48 animals were used. All rats were injected with collagen-media solution (vehicle control), pericytes with the collagen-media solution, or stimulated pericytes with the collagen-media solution directly after injury. For the male collagen injected group n= 8 across 6 litters and for the female collagen injected group n=8 across 7 litters. For the male pericyte injected group n=8 across 6 litters and for the female pericyte injected group n=8 across 6 litters. For both the male and female stimulated pericyte injected group n=8 across 5 litters.

Litters were culled on P0 to between six and eight pups, with equal numbers of males and females in each litter. Beginning on P0, pups received daily subcutaneous injections of the immunosuppressant cyclosporine A (10 mg/kg) and the anti-bacterial Baytril (50 mg/kg). All pups from one litter received the same treatment to control for possible variances in maternal behavior between treatments. Animals were housed on a 12-hour light cycle in microinsulator cages with autoclaved bedding, irradiated food, and sterilized water.

The spinal cord transection method applied was adapted from that used by Kao et al. (195) and Strain et al. (196). Briefly, rats were anesthetized by hypothermia. A laminectomy was performed, exposing the spinal cord from T8 to T10. By using a combination of landmarks in the surrounding tissue and on the irridectomy scissors, a

consistent lesion was created between T8 and T10 that extended to approximately the midline of the spinal cord. To maintain consistency in the lesion that was created across groups and over time, the same surgeon and surgery team was used for all surgeries. Directly after the spinal cord was cut, the rats were injected with 30 μl of the indicated treatments, into and around the space created by the lesion. For cell treatments, the final concentration of injected cells was $1X10^6$ cells. The muscle and skin on the back was then sutured and pups were returned to the dam. Buprenex (pain medication, 0.1 ml of 0.04 mg/kg solution) and sterile saline solution (for fluid replacement) subcutaneous injections were given immediately after the surgery and for two days following the surgery. Any animals with cuts made above or below T8 or T10 respectively were excluded at the time of tissue dissection (P10). Additionally, animals with completely severed spinal cords at P10 were excluded (< 3% of animals harvested).

3.3.4. BBB motor scale

At P10, the Basso, Beattie and Bresnahan (BBB) motor scale was used to assess hindlimb motor recovery (197). The BBB scale ranges from 0 to 21, with 0 being complete paralysis and 21 being normal adult locomotion. Scores 1-8 focus on joint movement and weight support. Rats receiving these scores have no to slight hindlimb joint movement and/or cannot support their own weight. Scores of 9-13 represent rats that can stand, take steps, and coordinate hindlimb and forelimb movements to various degrees. Rats that receive scores of 14-21 exhibit progressive improvements in foot placement, tail position, trunk stability, and toe clearance. Rats were recorded freely moving for two minutes in an open field. The videos were then scored by an examiner blinded to the experimental conditions. Coordinated locomotion develops over time in neonatal rat pups with a score of 21 being typical around P21. In piloting studies,

typically developing male rats given daily injections of cyclosporine and Baytril were found to score a 13 ± 0.0 at P10. Female rats were found to score 14 ± 1.41 at P10.

To assess the inter-rater reliability of the individual examiner ultimately used for examining videos of test subjects, the standard deviation of scores across two examiners was assessed in piloting studies. To assess intra-rater reliability, the examiner scored the same set of videos at two different time points. In these piloting studies, for both inter-rater reliability and intra-rater reliability, the mean scoring variability was within one scoring category (± 1.06). This variability is consistent with previous reports (197).

3.3.5. Blood flow perfusion

At P10, rat pups were anesthetized with isoflurane and the site of spinal cord transection was exposed. A Doppler flow micro-probe was placed just above the dorsal surface of the spinal cord midline at the transection site and at approximately a 90˚ angle to the tissue surface. Perfusion measurements were acquired over a two-minute interval with Powerlab (8SP) at 100 Hz (Chart 5; ADInstruments). Chart 5 software was then used to calculate a mean perfusion for each animal. Animals were then euthanized.

3.3.6. Immunohistochemistry and histology

After tissue fixation in formalin (10%) and then dissection, spinal cords were frozen down and then sectioned into 20 μm thick sagittal slices. Slices were then prepped for either hematoxylin and eosin staining or for immunohistochemistry (IHC). For IHC, anti- Ki67 (1:500, abcam) was used as a proliferation marker, anti-human antigen (1:500, Millipore) as a marker of human placental pericytes, anti-neurofilament 200 (1:200, Sigma) as a neuronal body marker, and isolectin GS-IB_4 (1:500, Alexa Fluor 647, Life Technologies) as a vessel label. An Olympus FV1000 confocal laser-scanning

microscope was used to collect a series of Z-stacked images of the spinal cord slices with a 20X objective (N.A. 0.75) with a limit of optical resolution at 0.409 μm. Three contiguous planes were imaged per slice, each image within a field of view that was 660 μm X 660 μm. One image was taken approximately centered in all directions around the wounded area, capturing both the tissue spared by the initial cut and the cut area. This is referred to as the wounded area. One image was then taken 660 μm cranially (area cranial to the wounded area) and one 660 μm caudally (area caudal to the wounded area). For each animal, one slice was imaged from the area to the left of the sagittal midline, one from the area around the midline, and one from the area to the right of the sagittal midline.

For endogenous vessel density measurements, the percent of tissue area with isolectin labeling per stacked image was quantified using ImageJ software (172). To exclude human placental pericytes that were positive for isolectin from this measurement, the percent of human nuclear antigen labeling that overlapped with isolectin labeling , as calculated using Olympus Fluoview software, was subtracted from the total percent of isolectin area. Human placental pericyte counts (human nuclear antigen^{+} cells) per field of view were quantified with Image J software and divided by area in order to determine the number of cells per μm^2. Placental pericyte proliferation was determined by calculating the percent of human nuclear antigen labeling that overlapped with Ki67 labeling using Olympus Fluoview software. Following the same procedure, the percent of placental pericytes positive for isolectin and neurofilament labeling was determined. Endothelial proliferation was determined by calculating the total percent of isolectin labeling that overlapped with Ki67 labeling and then subtracting the percent of human nuclear antigen labeling that overlapped with Ki67 and isolectin. To calculate

endogenous neurofilament density, the percent of tissue area with neurofilament labeling per stacked image was quantified using ImageJ software and then the percent of human nuclear antigen labeling that overlapped with neurofilament labeling was subtracted from the total.

3.3.7. Statistical analyses

Analyses were performed using GraphPad Prism 5.0 (Graph-Pad Software, San Diego, CA, USA), with alpha set at 0.05. One-way ANOVA with Dunnett post hoc analysis was used to compare three or more groups for cell culture data. To compare two groups for the cell culture data, a Student's t-test was used. Two-way ANOVA with Bonferroni analysis was used to assess statistical differences across treatment groups within sex. Because of the significant effects and/or interactions of sex and treatment found, one-way ANOVA with Tukey post hoc analysis was also performed to assess sex differences within treatment groups (198). Data are presented as the mean ± standard error of the mean.

3.4. Results

3.4.1. Human placental pericytes can be stimulated to induce pro-angiogenic activity in vitro

Pericytes treated with 400 μM of $CoCl_2$ promoted a faster rate of wound healing when co-cultured with CNS endothelial cells (Figure 3.1A). Based on these results, a dose of 400 μM was used in all subsequent experiments to stimulate placenta pericytes. The viability of placental pericytes when stimulated at this dose of $CoCl_2$ was below that of their naïve counterparts initially (Figure 3.1B) but stimulated pericytes had more viability then their naïve counterparts over time (Figure 3.1C). Stimulated placental

pericytes were also molecularly different from their naïve counterparts in that they had more protein expression of a common pericyte marker, PDGFR-β (Figure 3.1D).

3.4.2. Acute placental pericyte injection improves hindlimb motor recovery after spinal cord injury

An example of tissue histology from each group seven days after injury is presented in Figure 3.2, panels A-F. When acutely injected into neonatal rats pups after spinal cord injury, pericytes but not stimulated pericytes promoted more hindlimb motor recovery in both males and females (Figure 3.2G-H). While there was a significant effect of treatment in regards to hindlimb motor recovery ($p < 0.05$), there was not a significant effect of sex ($p > 0.05$).

3.4.3. Placental pericytes survive and proliferate within the host tissue

After seven days within the host tissue, there were still surviving placental pericytes within the tissue of all groups injected with cells (Figure 3.3A). There was not a significant effect of treatment or gender on the survival of injected cells ($p > 0.05$). There was an effect of both gender and treatment ($p < 0.05$) in regards to the rate of proliferation of injected cells. The highest rates of proliferation of the cells that remained were found in tissues injected with pericytes as opposed to stimulated pericytes (Figure 3.3B). For example, 39.62% ± 1.272% of naïve placental pericytes were proliferating in male tissue and 46% ± 1.944% in female tissue. Additionally, stimulated pericytes fared worse in terms of proliferation in male tissue than in female tissue (11.96%±0.7896% and 29.98% ±4.941% respectively). Pericytes and stimulated pericytes also migrated throughout the host tissue. Both types of pericytes were found 660 μm cranially and

caudally from the wounded area. They also were found at a distance of 400 μm out from both left and right from the midline

Although negative for the endothelial marker isolectin in culture and in previous studies done in organotypic culture (191), injected pericytes and stimulated pericytes were positive for isolectin in vivo (Figure 3.3C). There were significant effects of sex, treatment, and interactions between sex and treatment in regards to pericyte isolectin labeling ($p < 0.05$). Stimulated pericytes injected into male tissue had the lowest percent of pericytes positive for isolectin. Injected pericytes and stimulated pericytes were also positive for neurofilament in vivo (Figure 3.1D). While there was a significant effect of treatment on the percent of injected cells positive for neurofilament ($p < 0.05$), there was not a significant effect of sex ($p > 0.05$). Representative images of pericytes positive for isolectin and neurofilament are presented in Figure 3.3, panels E-J.

3.4.4. Blood flow perfusion changes in response to stimulated pericyte treatment after spinal cord injury are sex-specific

Naïve pericyte injection did not promote more blood flow in either male or female rats. There was a significant interaction between sex and treatment ($p < 0.05$) in that stimulated pericytes promoted more blood flow perfusion in male rats as compared sex matched controls ($p < 0.05$) but did not promote more blood flow in female rats (Figure 3.4A and B).

3.4.5. Vessel density after spinal cord injury is sex-specific

There were significant effects of treatment and interactions between treatment and sex in regards to vessel density after SCI ($p < 0.05$). In the vehicle control group, females had a higher vessel density seven days after injury than males (collagen alone, Figure

3.5A, $p < 0.05$). This pattern persisted with pericyte injection ($p < 0.05$) and the average vessel density was not significantly higher than their sex matched controls ($p > 0.05$). With stimulated pericyte injection, males had more vessel density then their sex-matched controls ($p < 0.05$) and no longer had lower vessel density then females (Figure 3.5A). These vessels, however, appeared disorganized as compared to vessels within the other groups. Representative images of vessel labeling are presented in Figure 3.5, panels C-H. Endothelial proliferation was not different between the cell treatment groups seven days after injury ($p > 0.05$) although sex was a significant source of variation ($p < 0.05$). In the vehicle control group, however, there was less endothelial proliferation in female tissue than there was in male tissue ($p < 0.05$, Figure 3.6).

3.4.6. Pericyte treatment promotes more neurofilament density after spinal cord injury

There was a significant effect of treatment ($p < 0.05$) but not sex ($p > 0.05$) on neurofilament density. Pericyte treatment resulted in more neurofilament density within the spinal cord tissue in both males and females than their sex-matched controls ($p < 0.05$, Figure 3.7A-B). In pericyte treated groups, in both males and females, neurofilament was also found to span the wounded area (Figure 3.7C-G). Representative images from females are shown in Figure 3.7, panels D-G.

3.5. Discussion

A major finding of this study is that placental pericytes improve hindlimb motor recovery in both sexes (Figure 3.2G and H). The placenta is a tissue that is readily harvestable and avoids the ethical concerns of harvesting embryonic stem cells. Harvesting placental pericytes for cell therapy applications also avoids the extensive in vitro manipulation involved in creating induced pluripotent stem cells and the associated

risks of tumor formation (4). Moreover, pericytes are cells that can interact with both the vasculature and the neuronal environment with potential to interact positively with multiple components of the neuronal environment, possibly leading to an improvement in overall tissue health. While this work focused on utilizing pericytes as an exogenous cell therapy, pericytes are cells that are endogenous to the CNS. The pharmaceutical or genetic manipulation of endogenous cells within the CNS is becoming more feasible. For example, a recent study identified a pharmaceutical approach to stimulate endogenous oligodendrocytes in order to promote more remyelination in a murine model of multiple sclerosis (199). A potential long-term future application of this work could be in manipulating endogenous pericytes to promote tissue recovery, avoiding the need to inject exogenous cells altogether.

The initial hypothesis that drove this work was that stimulated pericytes would improve hindlimb motor recovery after spinal cord injury based on previous in vitro evidence that stimulated pericytes induced pro-angiogenic activity (191). The evidence did not support this hypothesis (Figure 3.2). Stimulated pericytes did, however, interact differently than their naïve counterparts in regards to motor recovery (Figure 3.2), vessel density (Figure 3.5), blood flow perfusion (Figure 3.4), and neuronal density (Figure 3.7) within the tissue. Although recent evidence has demonstrated that human brain pericytes can be stimulated to induce pro-angiogenic activity when co-cultured with endothelial cells, this work demonstrates that pericytes from a different embryological origin can also be stimulated in the same way. Taken together, this supports the overall hypothesis that driving the hypoxia inducible pathway in pericytes fundamentally changes their functional interaction within the neuronal environment.

Naïve pericytes are thought to aid in the creation of a stable, flow-regulated mature vascular bed (32). Conversely, angiogenic pericytes can regulate and guide vessel growth that can result in an initially unstable and immature vascular bed (31, 41). The major acute vascular pathology in SCI injury includes hemorrhage and blood-spinal cord barrier disruption (83, 94). The increase in blood flow seen in male rats injected with stimulated pericytes (Figure 3.4) could negatively affect a tissue in which the blood-spinal cord barrier is not intact, negatively affecting neuronal density, as seen in Figure 3.7. It is possible that, in this work, injected naïve pericytes were able to stabilize and promote a functional flow-regulated vascular bed (Figures 3.5-7) while stimulated pericytes were not.

Although males injected with stimulated pericytes exhibited the highest levels of vessel density, they did not have more endothelial proliferation as would be expected with the initiation of an angiogenic program. Stimulated pericytes could have initiated an angiogenic program and endothelial proliferation at an earlier time point not captured by this study that resulted in the higher vessel density and more blood flow perfusion. Because female rats injected with stimulated pericytes did not show an increase in blood flow perfusion, or the concomitant loss of neuronal density seen in male rats, females could have intrinsic mechanisms that controlled blood flow perfusion even in the presence of the stimulated pericytes. This rescue of neuronal density in female rats injected with stimulated pericytes, however, did not lead to functional motor recovery suggesting that a flow-regulated vascular bed must be coupled with other neurological processes of which stimulated pericytes were not capable in order to affect whole tissue recovery.

This work has identified that there are sex differences in vascular outcomes following SCI (Figure 3.5A). Within the vehicle control group, female rats had more vessel density then males (Figure 3.5) but less endothelial proliferation (Figure 3.6) supporting the hypothesis that females may be better able to maintain vessel viability after initial injury. There was also a significant effect of sex on endothelial proliferation across treatment groups. Taken together, it's possible that the capacity for the initiation of an angiogenic program following CNS injury differs between males and females. These effects could be because of genetically based mechanisms (112), and/or because of maternal care differences between sexes (200). Although there were differences in vessel density between sexes, there were not differences in functional outcomes (Figure 3.2 and 3.7). These results highlight the possibility that even though males and females might reach the same gross functional end measurement that they could do so by different molecular mechanisms.

Evidence is accumulating that subsets of pericytes can be multipotent (23). In this study, both pericytes and stimulated pericytes were positive for isolectin and neurofilament in vivo. Isolectin positive pericytes did not appear to create vascular-like tubes (Figure 3.3E-G). Males and females injected with pericytes had a greater percentage of pericytes that were positive for neurofilament then their sex-matched controls which was positively correlated with hindlimb motor recovery (Figure 3.2 and 3.3). If pericytes can differentiate into host tissue cells that functionally incorporate into neuronal tissue, however, is not known.

In contrast to stimulated pericyte and collagen treatment, pericyte treated males and females had neurofilament that spanned the wounded area. Pericytes have been found to guide oligodendrocyte progenitor cells during corticogenesis through transforming

growth factor-β signaling (192). Similarly, injected pericytes could promote neurofilament migration across the wounded area by a mechanism of which stimulated pericytes are not capable. Ultimately, this work identifies a novel cell therapy that can improve hindlimb motor function in a pediatric model of SCI by promoting the retention of neurofilament density and an organized, flow-regulated vasculature.

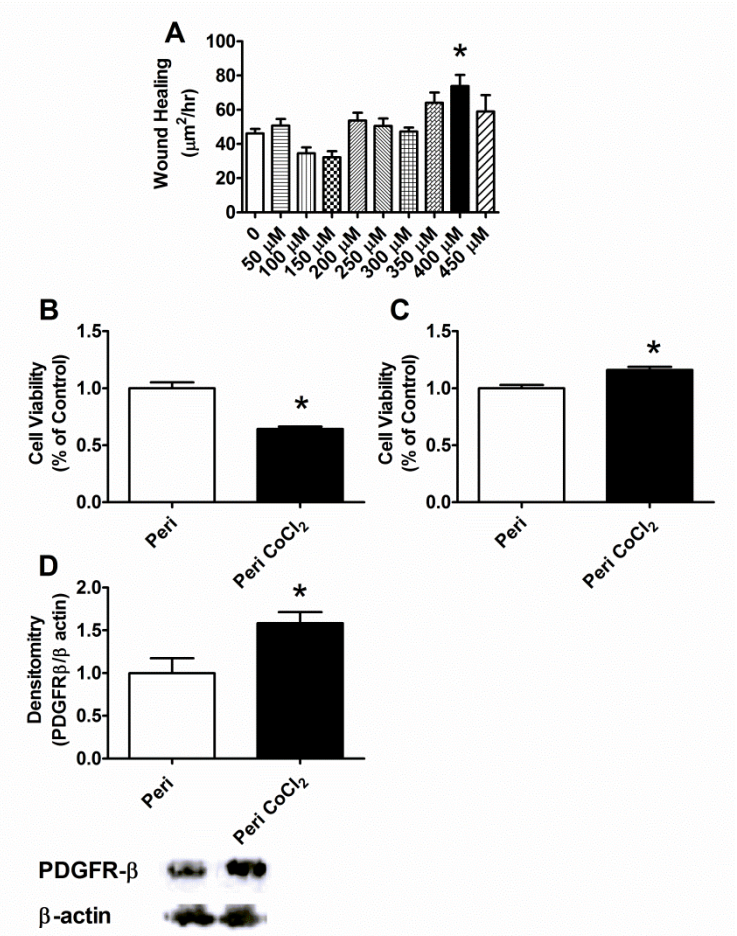

FIGURE 3:1. HUMAN PLACENTAL PERICYTES CAN BE STIMULATED TO A PRO-ANGIOGENIC STATE. (A) Rat brain endothelial cells and pericytes and co-cultured at a 2:1 ratio for a wound healing assay. Dose of $CoCl_2$ used during 24 hour pericyte stimulation prior to co-culture is listed on the x-axis. * indicates significant difference as compared to pericytes with no $CoCl_2$ stimulation prior to co-culture with endothelial cells, $p < 0.05$. (B-C) Cell viability of stimulated pericytes as determined by a MTT assay directly after a 24-hour treatment with 400 μm of $cocl_2$ (b) or 2 hours after plating stimulated pericytes (C). (D) Western blot of PDGFR-β protein expression immediately after stimulation in either naïve pericytes or stimulated pericytes. * indicates significant differences between respective groups, $p < 0.05$.

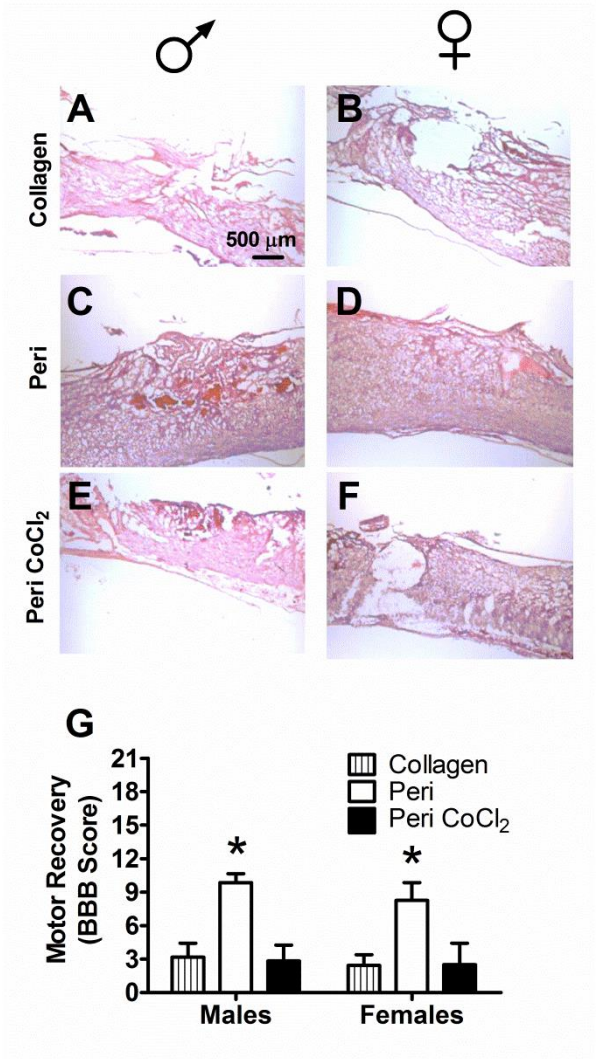

FIGURE 3:2. PERICYTE INJECTION IMPROVES HINDLIMB MOTOR RECOVERY

(A-F) Representative H&E staining of sagittal sections of the wounded area from the indicated treatment groups seven days after injury. For each image, the dorsal side of the animal is at the top and the cranial end towards the left. (G-H) BBB motor scale scores for the indicated groups at P10. The labels along the x-axis identify the type of treatment each group received at the time of injury. * indicates significant differences across treatments within sex, $p < 0.05$.

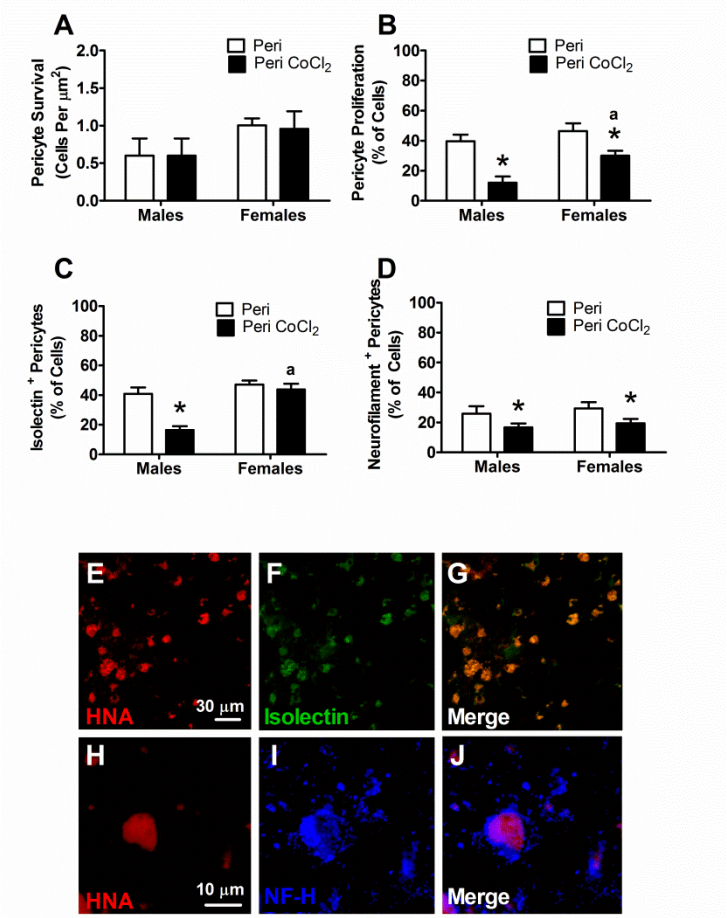

FIGURE 3:3. PERICYTES AND STIMULATED PERICYTES SURVIVE IN THE HOST TISSUE SEVEN DAYS AFTER INJECTION

(A) Pericyte counts per µm^2 for indicated treatment groups. The labels along the x-axis identify the type of treatment each group received at the time of injury. An "M" designates males and an "F" designates females. (B) Percent of pericytes positive for the proliferation marker Ki67 in indicated groups. (C) Percent of injected pericytes positive for the endothelial marker, isolectin. (D) Percent of injected pericytes positive for the neuronal body marker, neurofilament. * indicates significant differences across treatments within sex, $p < 0.05$. Letters represent significant differences between sexes within the same treatment, $p < 0.05$. (E-J) Representative images of pericytes positive for isolectin (E-G) or for neurofilament (H-J).

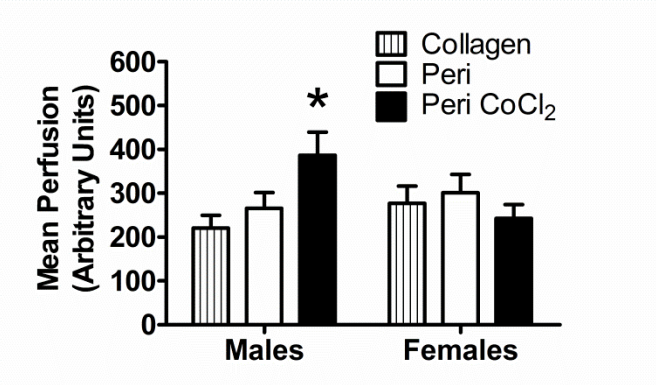

FIGURE 3:4. BLOOD FLOW PERFUSION AFTER TREATMENT WITH STIMULATED PERICYTES IS SEX DEPENDENT

(A-B) Male and female blood flow perfusion at the site of transection on P10. The labels along the x-axis identify the type of treatment each group received at the time of injury. * indicates significant differences across treatments within sex, $p < 0.05$.

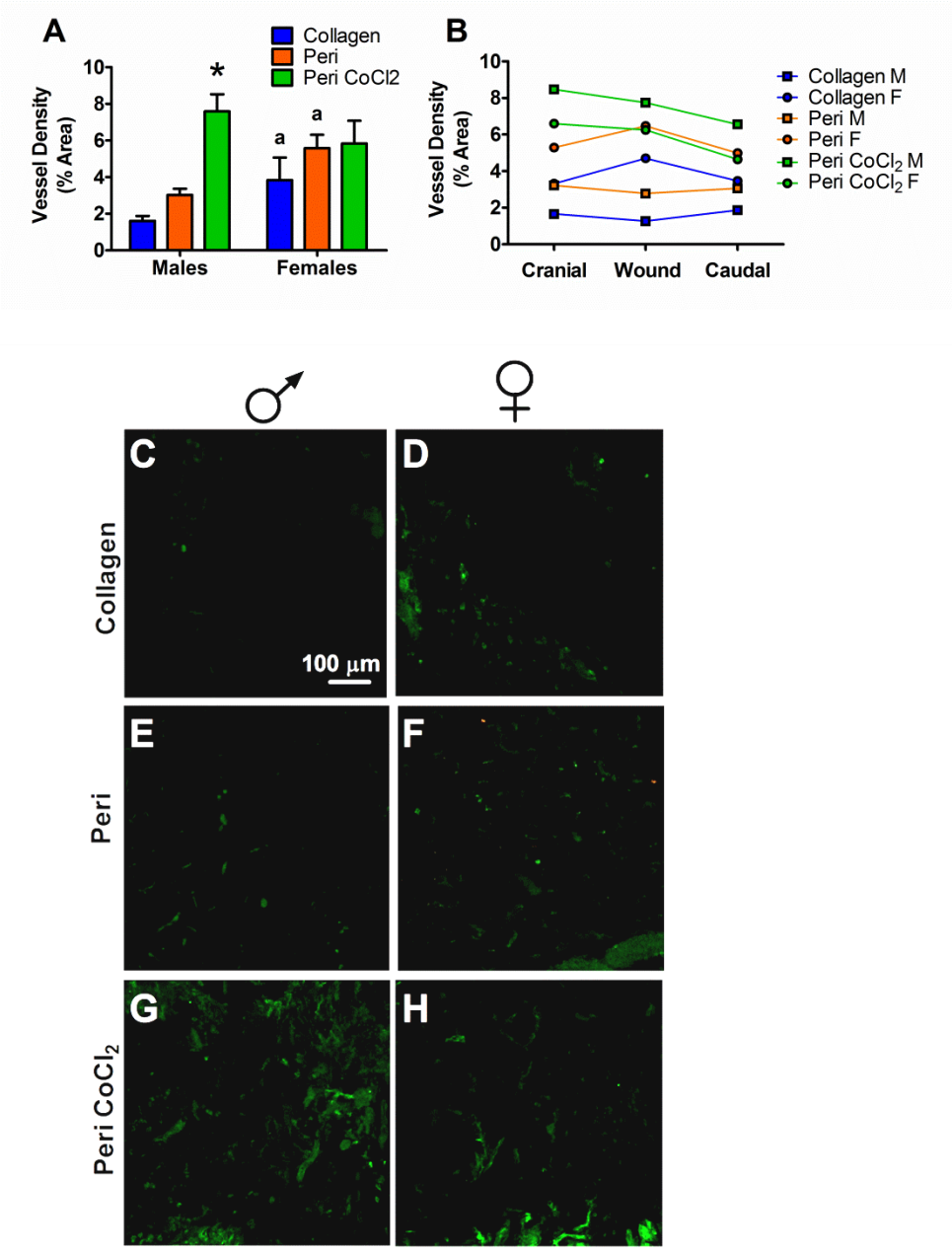

FIGURE 3:5. VESSEL DENSITY AFTER SPINAL CORD INJURY IS SEX-SPECIFIC

(A) Total vessel density from the wounded area, the area cranial to the center of the wounded area, and the area caudal to the center of the wounded area across groups. (B) Vessel densities at the areas indicated across groups. * indicates significant differences across treatments within sex, $p < 0.05$. Letters represent significant differences between

sexes within the same treatment, $p < 0.05$. (C-H) Representative images of isolectin labeling in the different groups from the wounded area.

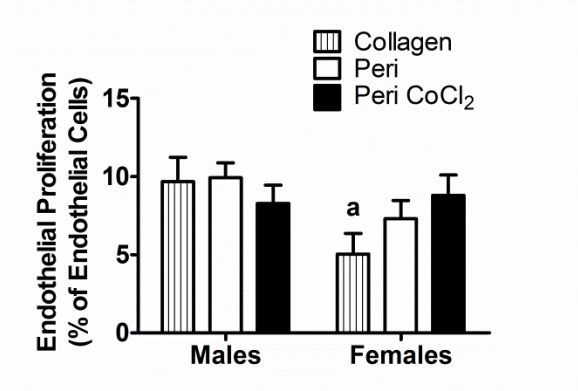

FIGURE 3:6. ENDOTHELIAL PROLIFERATION AFTER SPINAL CORD INJURY IS SEX-SPECIFIC

Percent of endothelial proliferation as calculated by percent of vessel labeling that was positive for the proliferation marker, Ki67. Letters represent significant differences between sexes within the same treatment, $p < 0.05$.

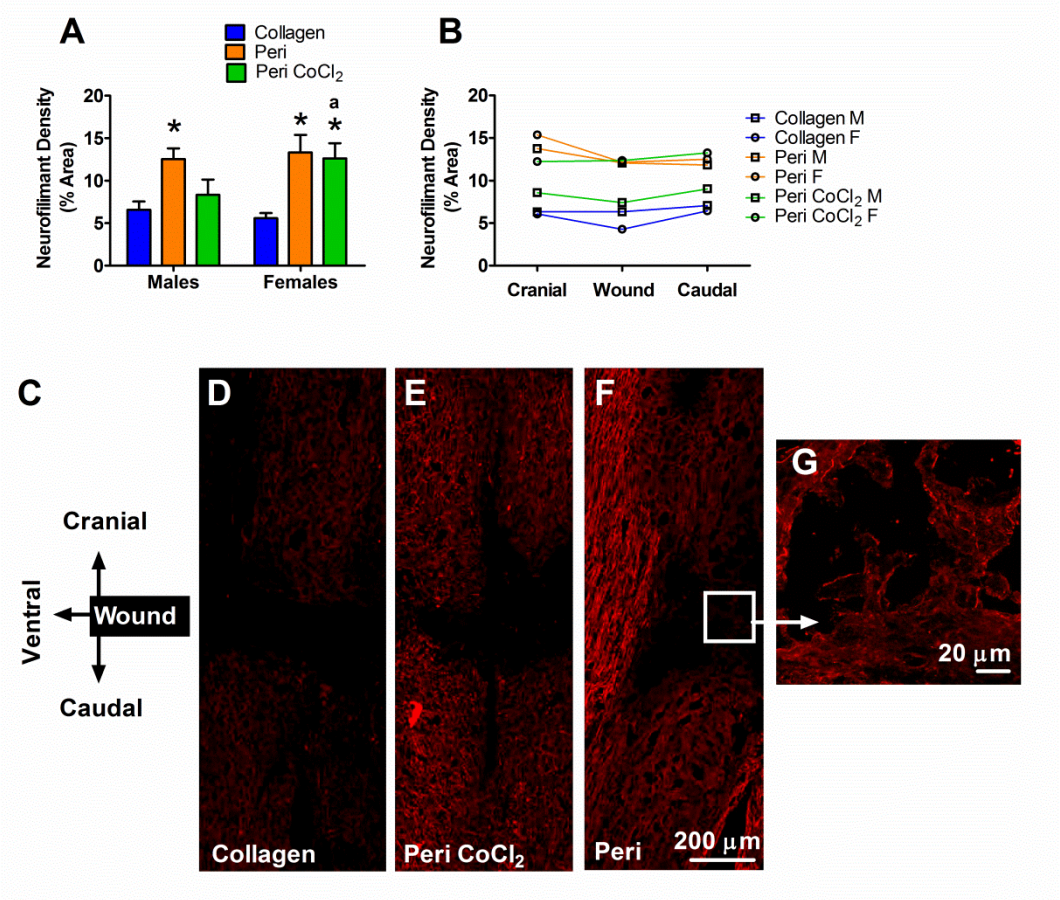

FIGURE 3:7. PERICYTE TREATMENT RESULTS IN MORE NEUROFILAMENT DENSITY WITHIN THE TISSUE

(A) Total neurofilament density from the wounded area, the area cranial to the center of the wounded area, and the area caudal to the center of the wounded area across groups. * indicates significant differences across treatments within sex, $p < 0.05$. Letters represent significant differences between sexes within the same treatment, $p < 0.05$. (B) Neurofilament density at the areas indicated across groups. (C) Directional orientation for the images of sagittal sections in D-F. (D-G) Representative images of neurofilament labeling from females.

4. Future directions and conclusions

4.1 Future directions

While this research has identified pericytes as a cell type that could be used in cell therapies, it has not dissected the molecular mechanisms by which pericytes promote greater hindlimb motor recovery after spinal cord injury. TGF-β signaling is a potential molecular bridge between neuroregeneration and the vasculature in that both systems actively participate in and depend on this signaling pathway. From the neuronal perspective, TGF-β signaling has been found to be a major mechanism in adult neurogenesis (201). TGF-β signaling is a regulator of CNS development and injury (202-204). It has also been found to have neuroprotective functions after spinal cord injury (205, 206). From the vascular perspective, TGF-β signaling is necessary for vascular development and the maintenance of a stable vasculature. Additionally, differential TGF-β signaling can regulate angiogenesis (207). Pericytes can secrete TGF-β (186) and TGF-β production from pericytes has been found to dictate the migration of oligodendrocytes during brain development (192).

In preliminary studies, done with IHC on the spinal cords of the neonatal rats used in this work, tissue TGF-β signaling altered with pericyte injection. This result was sex specific in that males had a decrease in TGF-β signaling after pericyte injection while females had an increase in TGF-β signaling (Figure 4.1). A future direction of this work could be to address the hypothesis that TGF-β secreted by injected pericytes is necessary for the observed improvement in hindlimb motor recovery. One approach would be to inject pericytes with a genetic knockdown of TGF-β and in another group inject pericytes that were genetically manipulated to overexpress TGF-β. By analyzing the results of hindlimb motor recovery, vessel density, and neuronal density in both sexes within this

experimental set up, it would be possible to determine the role of pericyte secreted TGF-β in pericytes ability to improve hindlimb motor function.

4.2 Conclusions

This study focused on driving the HIF pathway solely as an in vitro tool to manipulate pericytes (Chapter 2). Hypoxia, however, is a common physiological and pathological environmental stimulus. While a direct comparison between in vivo hypoxic conditions and the dose of $CoCl_2$ used to stimulate pericytes is not possible based on the results of this study, it is valid to postulate that if endogenous pericytes experienced a similar induction of the HIF pathway, they could respond in a similar manner. It is likely that pericyte stimulation is not a simple on-off switch in vivo, but rather a gradation, where some stimulation is positive for overall tissue health but an extreme in either direction could cause pathological outcomes. For example, this work is one of the first to report exosomal communication, regulated by HIF pathway activation, between endothelial cells and pericytes that engages an angiogenic program. On the other hand, after an ischemic insult, there is evidence that CNS pericytes undergo massive cell death (53). One level of HIF stimulation might be necessary to initiate an angiogenic program but another, such as an extreme ischemic insult, could result in pericyte death. Pericyte dysfunction is thought to be a major contributor to CNS pathology (38, 51, 52). Ultimately, regulating the HIF pathway to varying extents in endogenous pericytes could be a way to manipulate vascular outcomes.

Multiple studies have reported finding different populations of pericytes that can be separated into groups based on differing molecular markers (23, 48, 208). While it is feasible that these different subsets of pericytes originate from different precursor cells, it is also possible that these different subsets of pericytes represent pericytes from one type

of precursor cell that are in different stages of stimulation based on their specific environmental stimuli.

Studies that have approached sex differences after CNS injury have predominantly focused on measures of gross functional outcomes and the effects of sexual hormones (8). Just as important toward efficacious drug development as differences in gross functional outcomes, however, are the molecular mechanisms by which the different sexes arrive at those outcomes. This work provides evidence that there are both vascular (Chapter 3) and molecular (Chapter 4) differences between males and females that are present prior to sexual maturation, both in controls and in response to treatment, despite similar gross functional results. Continuing to elucidate these sex differences following CNS injury will be imperative in developing treatments to improve patient outcomes across sexes.

In the in vivo model of SCI, this work provides direct evidence that pericytes can influence neuronal outcomes on a molecular and a functional level (Chapter 3). Injected pericytes induced greater neurofilament density and the presence of neurofilament across the wounded area. Whether this effect was a consequence of improved vascular function or a direct interaction with the neuronal environment is unknown. It is possible, however, that both options are true. Pericytes could independently regulate vascular function while guiding neuronal migration.

Manipulating vasculature has become a promising therapeutic tactic in various CNS pathologies including cancer, stroke, and traumatic CNS injury. Novel therapeutic strategies are needed, however, to address the limitations of currently used vascular-based therapies and to meet the need for treatment options for traumatic CNS injury. While endothelial cells have been extensively studied under this context, another vascular

cell that mediates vessel function, pericytes, have been largely overlooked until recent years. The key findings in this work are as follows:

- Pericytes are pro-angiogenic with hypoxia-inducible factor pathway activation and can be used to support growth of microvascular networks.
- Pericyte promotion of greater vascular density is regulated by exosomal communication between endothelial cells and pericytes.
- There are sex specific vascular outcomes following spinal cord injury and the mechanisms of recovery in response to therapeutic interventions could differ between sexes.
- Pericytes are a novel cell type for potential use in cell therapies to improve hindlimb motor recovery following spinal cord injury.

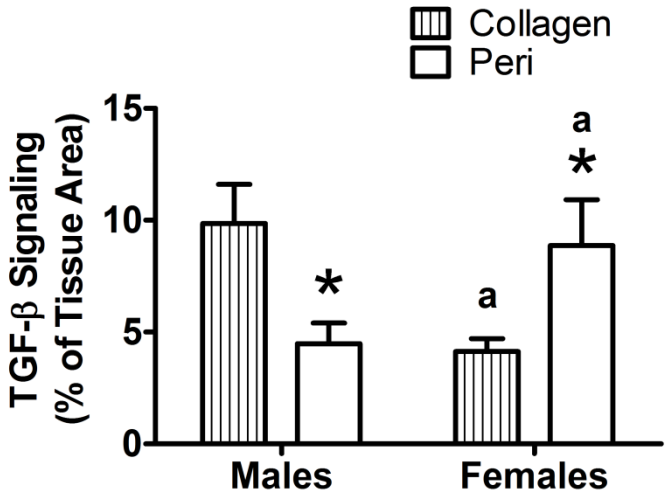

FIGURE 4:1. TGF-B SIGNALING IS ALTERED IN A SEX-SPECIFIC MANNER WITH PERICYTE INJECTION

TGF-β signaling, as identified by phosphorylated Smad5, was quantified in the spinal cord tissue of P10 pups, seven days after injury, by IHC. This data represents total TGF-β signaling from the wounded area, the area cranial to the center of the wounded area, and the area caudal to the center of the wounded area across groups. * indicates significant differences across treatments within sex, $p < 0.05$. Letters represent significant differences between sexes within the same treatment, $p < 0.05$.

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