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Driving the Hypoxia Inducible Pathway in Human Pericytes Promotes Vascular Density in an Exosome Dependent Manner

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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 (CoCl₂) 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

Introduction

Pericytes are cells that are embedded in the basement membrane of capillaries[6]. 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[9,12,15,27,31,38,43,52]. 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 [10,24,36]. Cell-directed therapies have shown the potential to improve patient outcomes [35]. 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 [3,13,42].

Pericyte progenitors are involved in the CNS response to insults where they can increase tissue propensity for neurogenesis and angiogenesis [8,22,28,37]. Pericytes also contribute to regeneration in skeletal muscle, bone, and cartilage [17,39]. 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 [2,29]. 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.

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* [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 were used (sex of donor unknown; age - unknown). Pericytes were negative for vascular endothelial cadherin (VEC) and isolectin GS-IB₄, 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% CO₂, 95% room air). All cells were used between passages 5 and 11.

Cobalt chloride (CoCl₂, MP Biomedicals) was used to activate the HIF pathway in pericytes [53]. To stimulate cells, pericytes were treated with 200 μ M CoCl₂ 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-1 α 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 CoCl₂ 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 lipophilic 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 μ l 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 $\sim$ 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 $\pm$ 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.

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 [48]. Briefly, media from co-cultures of endothelial cells and stimulated pericytes was centrifuged for 30 minutes at 10,000 $\times$ g followed by a 70 minute 100,000 $\times$ g cycle. After the initial 100,000 $\times$ 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 $\times$ 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.

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 2×10^6 cells/ml was used (1×10^5 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 2 cells with continuous contact that extended more than 50 μm in length. Branches off of a cord were counted as a separate cord if they contained two cells extending more than 50 μm from the branch point.

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)[34]. 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, 2×10^6 cells/ml were added to the inner chamber media (5×10^5 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-IB₄ (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 [41]. 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) that had 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 [25] 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.

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 $\pm$ 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.

Results

Pericytes Can Be Stimulated To A Pro-angiogenic State

In mono-layer co-culture assays, stimulated pericytes promoted faster rates of wound healing (Figure 1A). Under these conditions, the number of endothelial cells positive for Ki67 (Ki67⁺) was not different whether cultured alone or with stimulated pericytes (Figure 1B). In a 3D collagen matrix model of angiogenesis, endothelial cells formed cords that contained lumens (Figure 1C). Stimulated pericytes and naïve pericytes homed to these cords (Figure 1C; insert). Naïve pericytes inhibited cord formation (Figure 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 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 2A). In culture, pericytes used during explant treatment did not label with isolectin. Vessel density decreased in spinal cord explants over time (Figure 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 2B, and E–K. $p < 0.05$). Representative images of explant labeling are presented in Figure 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 $\pm$ 0.23, $p < 0.05$).

The greater vessel density in explants containing stimulated pericytes was, in part, a result of endothelial cell proliferation (Figure 3A) as opposed to a decrease in endothelial apoptosis (Figure 3B). Stimulated pericytes were found to have migrated throughout the depth of the spinal cord tissue (Figure 3C). The largest difference in vessel count per field of view between groups was at a depth of 20 μ m from the application surface (Figure 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 $\pm$ .56 at 100 μ m, $p < 0.05$, Figure 3C). Explants treated with naïve pericytes contained the fewest pericytes, which did not penetrate as deep (0.09 cells $\pm$ 0.09 at 100 μ m, Figure 3C).

Pericyte Stimulation is Dependent on HIF α and is Unique Among Mural Cells

HIF1- α inhibitors applied during CoCl₂ pre-treatment inhibited the faster rate of wound healing (Figure 4A and B) verifying that pericyte stimulation was dependent on driving the HIF α pathway, as opposed to an off target effect of CoCl₂ treatment. Treatment with CoCl₂ decreased cell viability directly following stimulation (Figure 4C). Cell viability was not affected as compared to control pericytes two hours following treatment (Figure 4D). Additionally, stimulated pericytes were molecularly different from their naïve counterparts in that they had greater protein expression of nestin and PDGFR- β (Figure 4E and F).

Endothelial CoCl₂ pre-treatment also promoted faster wound healing (Figure 4G). Conversely, smooth muscle cell pre-treatment with CoCl₂ resulted in a slower rate of wound healing when compared to endothelial cells co-cultured with naïve smooth muscle cells (Figure 4H). There was no significant effect of co-culturing pre-treated endothelial cells with naïve smooth muscle cells (Figure 4H).

Stimulated Pericytes and Endothelial Cells Share Membrane Lipid Components

In order to further explore 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 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 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 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 5L). Membrane sharing events required activation of the HIF pathway (Figure 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 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 CoCl₂; 0.22 ± 0.05 , $p < 0.05$). Co-cultures of pre-treated endothelial cells with naïve smooth muscle cells had higher co-localization coefficients (ECCoCl₂: SMC; 0.73 ± 0.03 , $p < 0.05$) than control co-cultures (Figure 5O).

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 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 6B–C). Removing the conditioned media and returning the same conditioned media still resulted in faster wound healing (Figure 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 6D).

Stimulated Pericytes Promote Angiogenesis Through Exosome Secretion

Chemical inhibition of ceramide-dependent exosome secretion and endocytosis-dependent membrane vesicle cycling was used to further test the hypothesis 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 7A). In contrast, the faster rate of wound healing in co-cultures of stimulated pericytes and endothelial cells required ceramide-dependent exosome secretion (Figure 7B). Moreover, the greater rate of wound healing produced by stimulated pericytes required membrane vesicle cycling (Figure 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 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 7F–G).

Discussion

Pericytes regulate tissue capillarity by stabilizing microvessels [9] and by promoting greater microvascular density through angiogenesis. Pericytes can contribute to angiogenesis by migrating from the basement membrane matrix [15,20,28] and assuming a leading position on sprouts after injury [15,38] or during development [52]. 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 cell, smooth muscle cells, responded to the same treatment with the opposite outcome (Figure 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 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 [14,40,50]. Specifically, membrane based microvesicles released from cells can promote angiogenesis in response to environmental stimuli. Endothelial to endothelial exosome-based communication can induce angiogenesis [50] and endothelial cells can incorporate delta-like ligand 4 into exosomes, inhibiting notch signaling in receiving endothelial cells [46]. Hypoxia results in modification to the contents of the exosomes released by glioma cells and these exosomes induced angiogenesis [30]. Exosomes produced under hypoxic conditions also promotes angiogenesis by participating in communication between multiple myeloma cells and endothelial cells [23]. Placental mesenchymal stem cells produce more exosomes under hypoxic conditions, which promote endothelial migration and sprout formation [44]. Additionally, microvesicles released by endothelial progenitor cells encourage endothelial proliferation, survival, and the formation of vessels in subcutaneously implanted Matrigel® plugs [18]. Ultimately, exosomes modulate angiogenesis by transporting signals in the form of proteins, DNA, and RNA (including microRNA) [19,51]. 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 5J–K), which is consistent with bidirectional communication. In the absence of endothelial contact, human placental pericytes can secrete hepatocyte growth factor [4,12] and bovine retinal pericytes can secrete VEGF [16]. Endothelial cells can also influence pericyte migratory behavior [30], survivability, and investment [32,33] via paracrine factors. Although exosome formation and endocytosis was necessary for the pro-angiogenic activity induced by stimulated pericytes (Figure 5, Figure 6, and Figure 7), unidentified soluble factors may also participate.

Endothelial cell and pericyte contact is an important element in vessel stability and maturity [7,9,26]. Indeed, some of the first work performed with endothelial and pericyte co-cultures indicated that transforming growth factor- β (TGF- β)[5] 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 [9]. 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 [20,27,28]. In the early stages of fetal human brain angiogenesis and in peripheral wound healing, pericytes lead tissue invasion of endothelium [15,52]. This work provides evidence of a paracrine interaction between stimulated pericytes and endothelial cells in order to promote an angiogenic program (Figure 6A,D and Figure 7). Both the physical presence of the stimulated pericytes and the exosomes released in the media were necessary factors in the current study (Figure 6B–C), however, highlighting a possible role for both cell-cell contact and exosome communication between pericytes and endothelial cells in promoting angiogenesis. 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[21].

Naïve pericytes can limit sprout formation in collagen matrices (Figure 1D) and do not promote vessel growth appreciably when implanted with endothelial cells subcutaneously as compared to endothelial cells implanted alone[12]. 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 [13].

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 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 2D and K), could indicate either improved maintenance of vessel viability, the promotion of an angiogenic program, or both. Endothelial proliferation (Figure 3A) in combination with a promotion of greater vascular density (Figure 2K) and little change in endothelial apoptosis (Figure 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 1B) but were facilitative of endothelial proliferation in whole tissue (Figure 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 [21,49]. Pericyte cell-cell contact, pericyte-endothelial contact, or cell-extracellular matrix interactions can influence pericyte synthesis of extracellular matrix proteins [11,47]. Pericyte activation by environmental stimuli can also alter extracellular matrix composition [45]. 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 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.

Perspectives

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.

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List of Abbreviations

CNS	Central nervous system
CoCl₂	Cobalt chloride
DMSO	Dimethyl Sulfoxide
EM	Endothelial culture media
HIF	Hypoxia-inducible factor
PBS	Phosphate buffered solution
PFA	Paraformaldehyde
PM	Pericyte culture media
VEGF	Vascular endothelial growth factor

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Figure 1. Pericytes can be stimulated to a pro-angiogenic state

(A) Pericytes were stimulated for 24 hours with 200 μM CoCl₂ 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. 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 μ M CoCl₂. Different letters indicate statistical differences between groups, $p < 0.05$.

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Figure 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 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-1 α 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 CoCl₂, following the same procedure as for pericytes. Different letters indicate significant differences between respective groups, $p < 0.05$.

Figure 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, $p < 0.05$.

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Figure 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 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 μ M) three days after culture. Pericytes were stimulated for 24 hours with 200 μ M CoCl₂. The pharmaceutical GW4869 was used at a concentration of 10 μ M and added directly to the treatment media in the inner chamber of the transwell. Different letters indicate significant differences between respective groups, $p < 0.05$

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