

ORIGINAL RESEARCH

Pericytes improve locomotor recovery after spinal cord injury in male and female neonatal rats

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Abstract

Objective: It is not known how activation of the hypoxia-inducible factor (HIF) pathway in pericytes, cells of the microvascular wall, influences new capillary growth. We tested the hypothesis that HIF-activated pericytes promote angiogenesis in a neonatal model of spinal cord injury (SCI).

Methods: Human placental pericytes stimulated with cobalt chloride and naïve pericytes were injected into the site of a thoracic hemi-section of the spinal cord in rat pups on postnatal day three (P3). Hindlimb motor recovery and Doppler blood flow perfusion at the site of transection were measured on P10. Immunohistochemistry was used to visualize vessel and neurofilament density for quantification.

Results: Injection of HIF-activated pericytes resulted in greater vascular density in males but did not result in improved motor function for males or females. Injection of non-HIF-activated pericytes resulted improved motor function recovery in both sexes (males, 2.722 ± 0.31-fold score improvement; females, 3.824 ± 0.58-fold score improvement, $P < .05$) but produced no significant changes in vessel density.

Conclusions: HIF-activated pericytes promote vascular density in males post-SCI. Acute delivery of non-HIF-activated pericytes at the site of injury can improve motor recovery post-SCI.

KEYWORDS

angiogenesis, cell therapy, pericytes, sex differences, spinal cord injury

1 | INTRODUCTION

Capillaries, the smallest blood vessels in the body, are composed of endothelial cells and pericytes. During times of stress and tissue disruption, such as after spinal cord injury (SCI), the capillary bed must undergo reconstruction in order repair damaged capillary networks to meet tissue needs.¹ Reconstruction of capillaries is accomplished primarily through angiogenesis, the sprouting of new vessels from pre-existing vessels.² Functional angiogenesis is critical in ensuring

appropriate blood flow and recovery. The manipulation of angiogenesis is a potential therapeutic tactic to improve functional recovery after central nervous system (CNS) injury.³ How pericytes, vital components of capillary function, interact with the neurovascular environment after central nervous system (CNS) injury to regulate angiogenesis blood flow, and restore tissue function remains unclear.^{4,5}

Driving the hypoxia-inducible factor (HIF) pathway can stimulate pericytes to a pro-angiogenic state. In the highly hypoxic environment

of human fetal brain angiogenesis,⁶ pericytes were found to precede endothelial cells in the direction of growth.⁷ Similarly, in an angiogenic response to hypoxia, pericytes are some of the first vascular cells to migrate.^{8,9} The canonical hypoxia-driven cell signaling cascade is initiated by the stabilization of HIF- α .¹⁰ In previous studies, we have found that a chemical inducer of the HIF pathway can stimulate pericytes to a pro-angiogenic state in vitro.¹¹ Taking the evidence all together, within a hypoxic environment common after neuronal tissue injury, pericytes could interact with endothelial cells to induce a pro-angiogenic state and guide the remodeling of vascular beds.¹²⁻¹⁴ We do not know, however, how stabilizing the HIF pathway in pericytes would influence pericyte interaction with injured neuronal tissue in vivo. For example, would pericytes that have undergone HIF pathway activation, as compared to non-stimulated pericytes, alter vascular, and ultimately neurological function in the context of in vivo SCI? Based on previous work that stabilization of the HIF pathway can stimulate pericytes to a pro-angiogenic state, we tested the hypothesis that HIF pathway activation in pericytes would result in greater vessel density in the wounded tissue in both male and female rodents. We further assumed the greater vessel density would result in better neurological outcomes.

Traditionally, preclinical CNS research has focused on males.¹⁵ A mass of evidence has accumulated, however, that there are sex differences in CNS pathophysiology at both a functional and molecular level.^{16,17} There are also known sex differences in microvascular function,¹⁸ including angiogenesis post-CNS injury.^{19,20} For example, Huxley et al describe how endothelial cells are sexually dimorphic in regards to cell surface proteins that are involved in blood flow regulation, barrier function, and solute exchange.¹⁸ Jullienne et al found that seven days post-traumatic brain injury, male mice had greater vessel density and vessel bed complexity as compared to female mice.¹⁹ Given that pericytes are key regulators of vascular remodeling,^{12,13} these results highlight the possibility that there could be sex differences in pericyte/endothelial interactions as well. It is not known if there are sex differences in pericyte function during angiogenesis.¹⁸ Therefore, we decided to include both males and females in this study. Without any direct evidence of sex differences in pericyte function, we hypothesized that the effect of HIF stimulated pericytes on angiogenesis would be similar in males and females.

2 | MATERIAL AND METHODS

2.1 | Cell culture and reagents

Human placental pericytes are clinically available, scalable, and defined cell source.²¹ They have also been used in previous cell therapy studies related to muscle regeneration.²² We therefore chose to use human placental pericytes because of their potential for clinical translatability.^{23,24} One aliquot of primary human placental pericytes was purchased from Promo Cell (donor tissue was from a Caucasian female). The sex of the cells was chosen based on vendor availability at the time of purchase. One aliquot of rat brain endothelial cells

from a young adult female was purchased from ScienCell. Human placental cells were negative for isolectin GS-IB₄ 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. All cells were maintained in a humidified incubator (37°C, 5% CO₂, 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 CoCl₂ (400 μ mol/L, Cobalt chloride, MP Biomedicals) for 24 hours before use in experiments and are referred to in this study as *stimulated pericytes*. The use of CoCl₂ increases protein expression of HIF1 α , which then initiates the HIF1 α dependent cell signaling cascade. CoCl₂ is a widely used method of HIF1 α activation and induction of hypoxic conditions in vitro.²⁵⁻²⁷ The exact mechanisms of HIF α stabilization are still being investigated, and the relative role of HIF1 α and HIF2 α in the CoCl₂ stimulated cell pathway remains unclear.^{25,26} We have used a HIF1 α inhibitor in previous studies, however, which cancelled out the stimulating effect of CoCl₂ on pericytes. While we cannot confirm that CoCl₂ is only affecting the HIF α pathway in these cells, we do have evidence from previous work that the stimulating effect of CoCl₂ on pericytes is HIF1 α dependent.²⁸

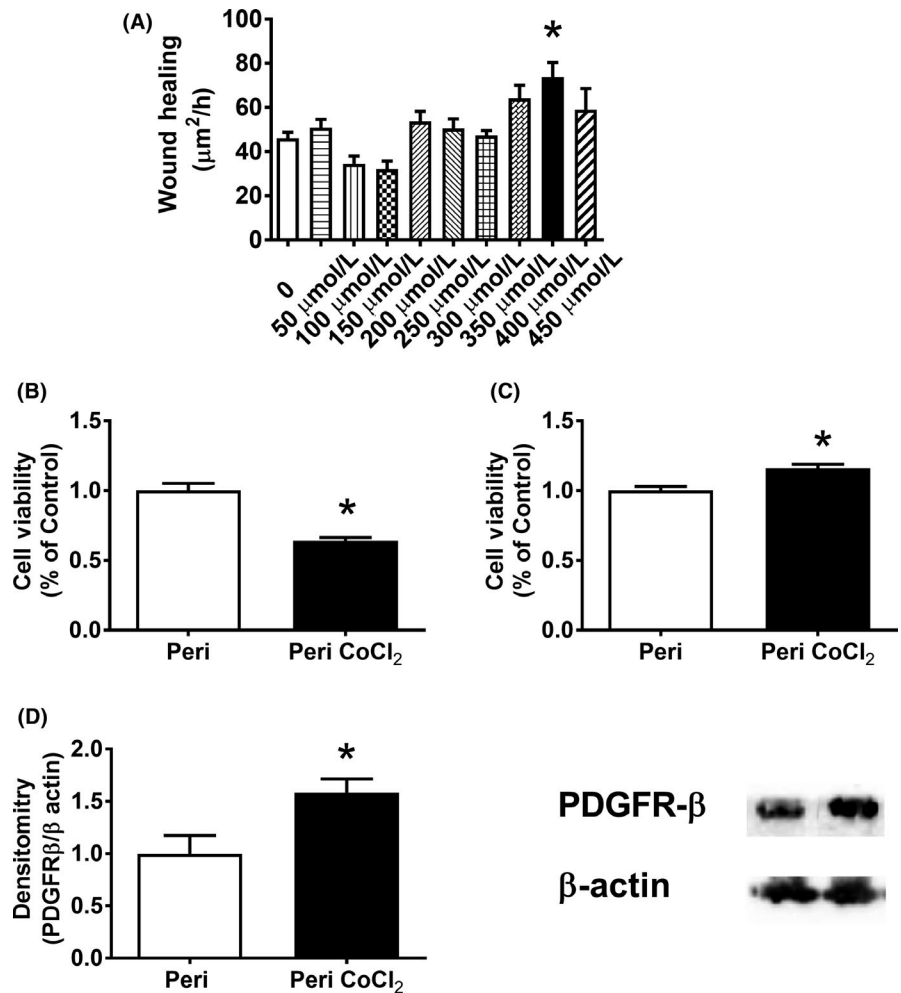
2.2 | Cell viability and harvesting for injection

It is possible that CoCl₂ could cause unknown effects to the cell beyond HIF1 α stabilization that are beyond the scope of this study, with the biggest concern being a lack of cell viability after CoCl₂ dosing.²⁵ To ensure that CoCl₂ treatment did not reduce the viability of pericytes after removal of the treatment media, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (1.2 mmol/L, Research Products International) was used to determine cell viability either directly following CoCl₂ stimulation (Figure 1B) or two hours after plating stimulated pericytes (Figure 1C). All cells were used between passages ≥ 4 and ≤ 11 . 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). For stimulated pericytes, pericytes were stimulated with CoCl₂ for 24 hours, rinsed with media, and then harvested for injection.

2.3 | Wound healing

The co-culture of pericytes and endothelial cells, and subsequent wound healing assay, was performed, characterized, and validated in our previous study evaluating the effect of pericyte stimulation in vitro.¹¹ Based on previous findings that pericytes can be stimulated to promote pro-angiogenic activity,¹¹ a scrape wound assay

FIGURE 1 Human placental pericytes can be stimulated to a pro-angiogenic state. (A) Rat brain endothelial cells and human placental pericytes 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 compared to pericytes with no CoCl_2 stimulation prior to co-culture with endothelial cells (one-way ANOVA with Dunnett pos hoc analysis, $P < .05$, $n \geq 10$ for each group). (B-C) Cell viability of stimulated pericytes (MTT assay) after a 24-hour treatment with $400 \mu\text{M}$ of CoCl_2 (B) or 2 hours after plating stimulated pericytes (C). * indicates significant difference; Student's t test, $P < .05$, $n = 30$. (D) Densitometry of PDGFR β labeling in pericytes and stimulated pericytes, $P < .05$



was used to determine the appropriate dose to stimulate placental pericytes in order to maximize the rate of wound healing of co-cultured endothelial cells. Briefly, cells were cultured at a 2:1 endothelial to pericyte ratio for 18-24 hours. Endothelial cells and stimulated pericytes were co-cultured after removal of CoCl_2 from stimulated pericytes and allowed to sit for 18-24 hours before wound healing. The difference in wound area between initial wounding and 6 hours post-wounding was divided by the time elapsed to determine the average rate of growth into the denuded area.

2.4 | Thoracic spinal cord hemi-section in neonatal rats

All animal procedures were reviewed and 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*.²⁹ To determine the angiogenic effect of pericyte HIF pathway activation in the context of SCI, pericytes and stimulated pericytes were acutely injected in a neonatal model of SCI. It was hypothesized that pericyte treatment would result in a greater density of the vascular bed that corresponded with improved hindlimb motor recovery. A model

of dorsal thoracic bilateral hemi-section in neonatal rats was chosen for this initial test of pericyte potential to improve outcomes in a developing system. Because SCI occurs in males and females,³⁰ both sexes were included in this study. Furthermore, a neonatal model allowed us to eliminate the confounding factor of urinary tract infection that often occurs in adult male rodents post-SCI. In the neonate, the dam's actions void the pups bladder, regardless of SCI status, and therefore makes including males and females more feasible. When we use the term sex, it applies to biological sex as defined by secondary sex organ verification of rat pups.

Sprague-Dawley rats of both sexes received a thoracic spinal cord dorsal bilateral hemi-section on postnatal day three (P3), and then were behaviorally tested on P10 and subsequently euthanized. Immediately after injury, rats were injected with a vehicle control (collagen-media solution), pericytes with the collagen-media solution, or stimulated pericytes with the collagen-media solution. For the male control group, $n = 8$ across 6 litters, and for the female control 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.

A total of 48 animals were used. Litters were culled on P0 (within 24 hours of birth) to between six and eight pups, with equal numbers

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

The spinal cord hemi-section applied was adapted from Kao et al.³¹ and Strain et al.³² Briefly, rats were anesthetized by hypothermia. A laminectomy was then performed, exposing the spinal cord from T8 to T10. By using a combination of landmarks in the surrounding tissue and on the iridectomy scissors, a consistent one segment lesion was created between T8 and T10 that extended to approximately the midline of the spinal cord. Directly after the spinal cord was cut, the rats were injected with 30 μ L of respective treatments, on top of the spinal cord tissue between T8 and T10. For cell treatments, the final concentration of injected cells was 1×10^6 cells. The muscle and skin on the back were then sutured, and pups were returned to the dam. Subcutaneous injections of buprenorphine hydrochloride (pain medication, 0.1 mL of 0.04 mg/kg solution) and sterile saline solution (for fluid replacement) 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 (as noted by visual inspection during dissection) at P10 were excluded (< 3% of animals harvested). To maintain consistency in the lesion that was created across groups and over time, the same surgeon and surgery team were used for all surgeries. Animals were euthanized at P10 by carbon dioxide overdose.

2.5 | BBB locomotor scale

The Basso, Beattie, and Bresnahan (BBB) locomotor scale was used to assess motor performance following injury.³³ The BBB scale ranges from 0 to 21, where 0 is complete paralysis and 21 is normal adult locomotion. The scores presented are the locomotor scores averaged across hindlimbs. 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. Coordinated locomotion develops over time in neonatal rat pups with a score of 21 being typical around P21.³⁴ 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.

At P10, rats were recorded freely moving for two minutes in an open field. The videos were then scored by three examiners unaware of the experimental conditions. Examiners gave an average score of

both hindlimbs for each subject. The final score was calculated from an average of the three examiners scores. To assess inter-rater reliability, the standard deviation of scores across different examiners was compared. To assess intra-rater reliability, each examiner scored the same set of videos on multiple occasions. 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 the original study that evaluated the validity and reliability of using the BBB motor scale.³³

2.6 | 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.

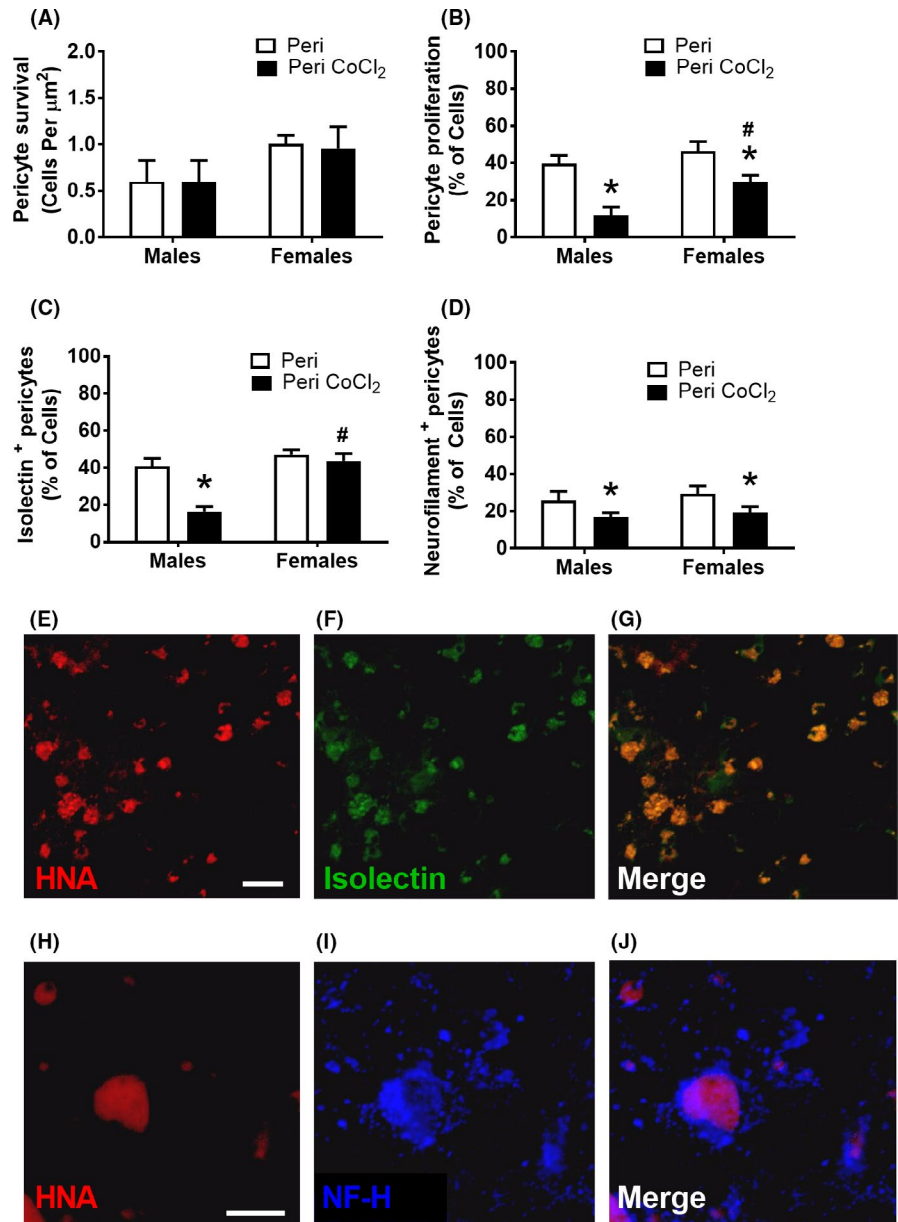
2.7 | Immunohistochemistry and histology

After tissue fixation in formalin (10%), spinal cords were dissected, frozen, and sectioned into 20 μ m 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-IB₄ (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 (NA 0.75) with a limit of optical resolution at 0.409 μ m. Three contiguous planes were imaged per slice, each image within a field of view that was 660 \times 660 μ m. 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 μ m rostral and one 660 μ m 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 positive for isolectin labeling per stacked image was quantified using ImageJ software.³⁵ 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

FIGURE 2 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. (B) Percent of pericytes positive for the proliferation marker Ki67 in indicated groups. (C) Percent of injected pericytes positive for isolectin. (D) Percent of injected pericytes positive for the neuronal body marker, neurofilament. * indicates significant differences across treatments within sex; two-way ANOVA, $P < .05$. # represents significant differences between sexes within the same treatment; one-way ANOVA, $P < .05$. Stimulated pericyte injected females, $n = 6$; stimulated pericyte injected males, $n = 6$. Pericyte injected females ($n = 8$) and males ($n = 7$). Collagen injected females ($n = 7$) and males ($n = 5$). (E-J) Representative images of pericytes positive for isolectin (E-G) or for neurofilament (H-J). (E-G) Scale bar = $30 \mu\text{m}$. (H-J) Scale bar = $10 \mu\text{m}$



view were quantified with ImageJ software and divided by total area 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 co-positive for isolectin and neurofilament 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.

2.8 | Statistical analyses

Analyses were performed using GraphPad Prism 5.0 (GraphPad Software), with alpha set at 0.05. All data analyzed were normally distributed. Two-way ANOVA with Bonferroni analysis was used to assess statistical differences across treatment groups within sex. There is accumulating evidence, however, that males and females respond differently to CNS injury and can have different angiogenic outcomes after injury.^{17,36} To account for possible differences between sexes, sex was treated as an independent variable. Because of the significant effects and/or interactions of sex and treatment found, one-way ANOVA with Tukey's post hoc analysis was also performed to assess sex differences within treatment groups.³⁷ Student's *t* test was performed when comparing only two groups. Data are presented as the mean \pm standard error of the mean.

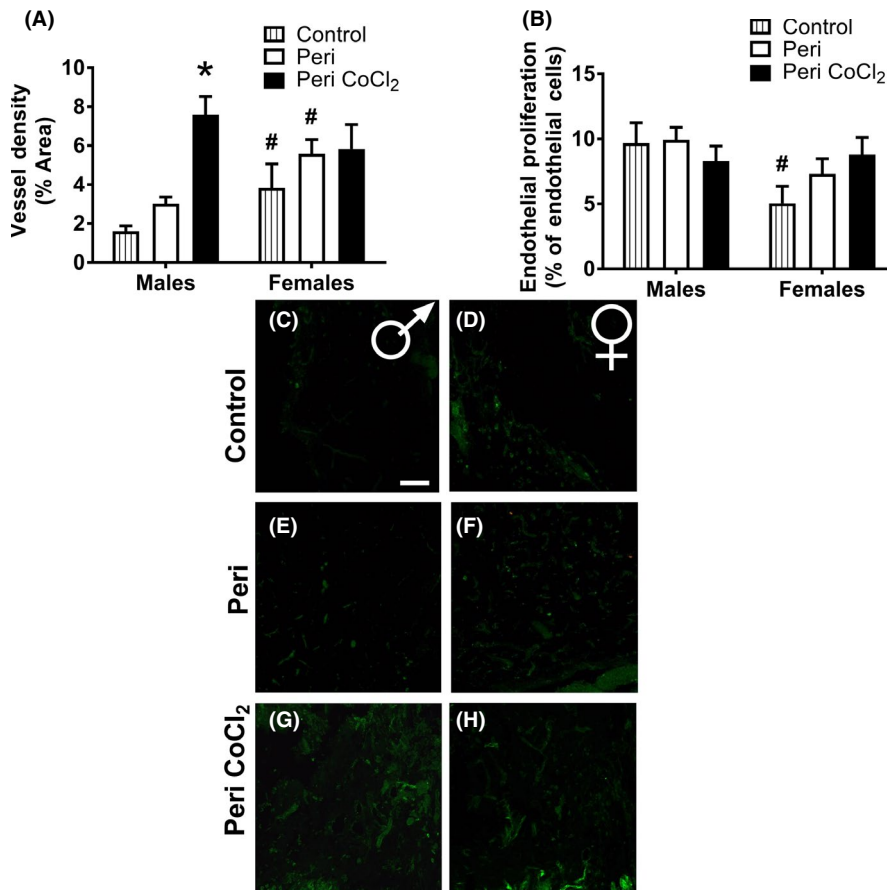


FIGURE 3 Vessel density after spinal cord injury is sex-specific. (A) Total vessel density of the wounded area, the area rostral to the center of the wounded area, and the area caudal to the center of the wounded area across groups. (B) Percent of endothelial proliferation as calculated by percent of vessel labeling that was positive for the proliferation marker, Ki67. There were no statistical differences across treatments within sex as determined by two-way ANOVA but sex was found to be a significant source of variation, $P < .05$. Stimulated pericyte injected females ($n = 6$) and males ($n = 6$). Pericyte injected females ($n = 8$) and males ($n = 6$). Collagen injected females ($n = 7$) and males ($n = 5$). (C-H) Representative images of isolectin labeling in the different groups from the wounded area. Scale bar = 100 μm . * indicates significant differences across treatments within sex; two-way ANOVA, $P < .05$. # represents significant differences between sexes within the same treatment; one-way ANOVA, $P < .05$

3 | RESULTS

3.1 | Human placental pericytes can be stimulated to induce pro-angiogenic activity in vitro

Pericytes treated with CoCl_2 (400 $\mu\text{mol/L}$) promoted a faster rate of wound healing when co-cultured with CNS endothelial cells (Figure 1A). Based on these results, a dose of 400 $\mu\text{mol/L}$ was used in all subsequent experiments to stimulate placental pericytes. The viability of placental pericytes when stimulated at this dose of CoCl_2 was below that of their naïve counterparts initially (Figure 1B) but CoCl_2 treatment did not result in more long-term reduction of cell viability in vitro (Figure 1C) or in vivo (Figure 2A). Stimulated pericytes remained positive for PDGFR- β , a common pericyte marker, after CoCl_2 treatment (Figure 1D).

3.2 | Placental pericytes survive and proliferate within the host tissue

Placental pericytes were identified within the tissue of all groups that were injected with cells for the full seven days of the experimental period (Figure 2A). There was not a significant effect of treatment or sex on the survival of injected cells ($P > .05$). There

was an effect of both sex and treatment ($P < .05$) on the rate of proliferation of the injected cells that remained seven days after injury. Injected naïve pericytes, as opposed to stimulated pericytes, had the highest rates of proliferation (Figure 2B). For example, $39.6\% \pm 1.3\%$ of naïve placental pericytes were proliferating in male tissue and $46.0\% \pm 1.9\%$ in female tissue. Additionally, stimulated pericytes proliferated more in female than in male tissue ($30.0\% \pm 4.9\%$ vs $12.0\% \pm 0.8\%$, respectively). Pericytes and stimulated pericytes also migrated throughout the host tissue. Both types of pericytes were found 660 μm rostrally and caudally from the wounded area. They also were located at 400 μm laterally, from the midline.

Injected pericytes and stimulated pericytes were positive for isolectin in vivo (Figure 2C). The percent of pericytes that labeled positive for isolectin was effected by sex, treatment, and interactions between sex and treatment for ($P < .05$). Stimulated pericytes injected into male tissue had the lowest percent of pericytes positive for isolectin. Injected naïve pericytes and stimulated pericytes were also positive for neurofilament in vivo (Figure 2D). While there was a significant effect of treatment on the percent of injected cells positive for neurofilament ($P < .05$), there was not a significant effect of sex ($P > .05$). Representative images of pericytes positive for isolectin and neurofilament are presented in Figure 2, panels E-J.

3.3 | Vessel density after spinal cord injury is sex-specific

The images of the Isolectin labeling presented in Figure 3C-H are from an image capture that was centered in all directions around the wounded area, capturing both the tissue spared by the initial cut and the cut area. This is an area that was completely transected between T8 and T10 on a three-day-old rat pup. The Isolectin labeling is faint in this area, as to be expected, as the images are from directly over the wounded site, where we would expect decreased tissue mass and decreased blood flow as compared to other tissue sites. For example, if we look at the control (Figure 3C), where the rat pup was cut and only received collagen injections, we see that only 2% of the tissue area was positive for Isolectin labeling. Even in the stimulated pericyte group, where we see the most Isolectin labeling, we only quantified around 8% of tissue area that was positive for Isolectin. There were still, however, identifiable and significant effects of treatment and interactions between treatment and sex in vessel density after SCI ($P < .05$) within this traumatized area. In the vehicle control group, females had higher vessel density seven days after injury than males ($P < .05$). This pattern persisted following pericyte injection ($P < .05$), and the average vessel density was not different than their sex-matched controls ($P > .05$). With stimulated pericyte injection, males had a greater vessel density than sex-matched controls ($P < .05$) and no longer had lower vessel density than females (Figure 3A). These vessels, however, appeared disorganized compared to vessels within the other groups. Representative images of vessel labeling are presented in Figure 3. Sex was a significant source of variation ($P < .05$) for endothelial proliferation. Within the vehicle control group, there was less endothelial proliferation in female tissue than there was in male tissue ($P < .05$, Figure 3B).

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

Naïve pericyte injection did not promote higher blood flow in either male or female rats, as measured by micro-Doppler. There was a significant interaction between sex and treatment ($P < .05$); stimulated pericytes promoted more blood flow perfusion in male rats compared to sex-matched controls ($P < .05$) but did not promote more blood flow in female rats (Figure 4).

3.5 | Pericyte treatment promotes greater neurofilament density after spinal cord injury

There was a significant effect of treatment ($P < .05$) but not sex ($P > .05$) on neurofilament density. Naïve pericyte treatment resulted in more neurofilament density within the spinal cord tissue in both males and females than their sex-matched controls ($P < .05$, Figure 5A). With naïve pericyte treatment, males and females had

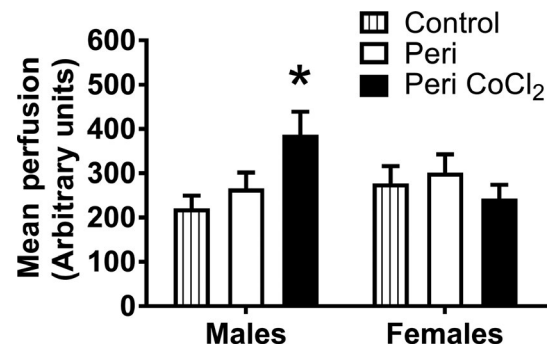


FIGURE 4 Blood flow perfusion after treatment with stimulated pericytes is sex dependent. Male and female blood flow perfusion at the site of transection on P10. Stimulated pericyte injected females ($n = 7$) and males ($n = 6$). Pericyte injected females ($n = 7$) and males ($n = 8$). Collagen injected females ($n = 7$) and males ($n = 7$). *indicates significant differences across treatments within sex as determined by two-way ANOVA, $P < .05$. No statistical differences between sexes within the same treatment were found; one-way ANOVA, $P > .05$

neurofilament that spanned the wounded area. Representative images from females are shown in Figure 5B, C.

3.6 | Acute placental pericyte injection improves hindlimb motor recovery after spinal cord injury

When injected into neonatal rat pups after SCI, pericytes but not stimulated pericytes, promoted more hindlimb motor recovery in both males and females as assessed by the Basso, Beattie, and Bresnahan (BBB) motor scale on P10, seven days after injury and cell injection (Figure 6). The significant effect of treatment regarding hindlimb motor recovery ($P < .05$) was independent of sex ($P > .05$). Supplementary Figure 1A-D include sample videos of typical hindlimb movement seen from a P10 rat pup with no intervention, a control surgery P10 rat pup, and P10 rat pups treated with pericytes and stimulated pericytes at the time of surgery.

4 | DISCUSSION

In this study, we discovered a potential role of pericytes in motor function recovery post-SCI. We also elucidated a mechanism by which pericytes can induce a pro-angiogenic state in vivo. The major findings are that naïve pericytes injected acutely into the site of injury post-SCI can help to improve motor recovery, that HIF pathway activation in pericytes can promote angiogenic activity in vivo, and that this pro-angiogenic activity was sex dependent.

This work provides proof of concept that naïve pericytes may be useful in improving outcomes after SCI.³⁸ The positive effect of pericyte injection on locomotor recovery is consistent with results of pericyte cell therapy in peripheral and retinal pathologies. Injected naïve pericytes isolated from human fetal skeletal muscle vasculature improved tissue recovery in a model of ischemic heart

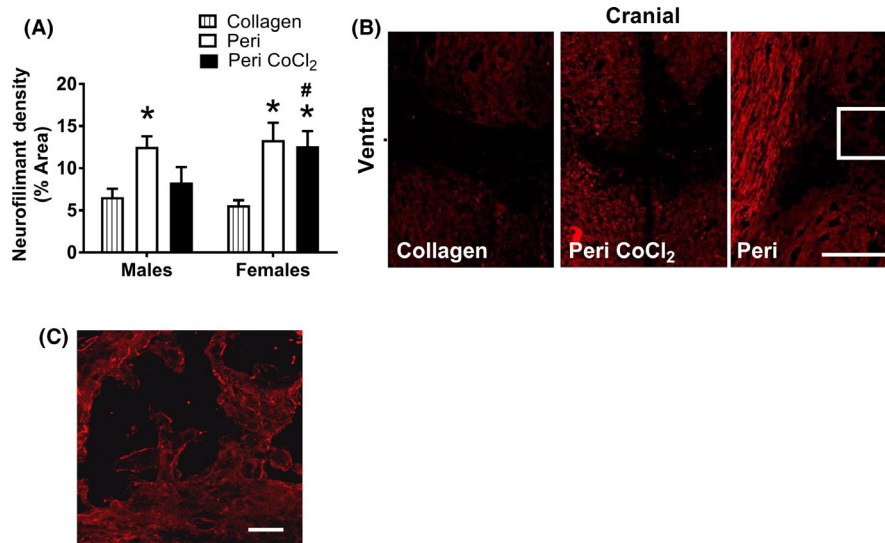


FIGURE 5 Pericyte treatment results in more neurofilament density within the tissue. (A) Neurofilament density across groups. * indicates significant differences across treatments within sex; two-way ANOVA, $P < .05$. # represents significant differences between sexes within the same treatment; one-way ANOVA, $P < .05$. Stimulated pericyte injected females ($n = 6$) and males ($n = 6$). Pericyte injected females ($n = 8$) and males ($n = 7$). Collagen injected females ($n = 7$) and males ($n = 5$). (B) Representative images of neurofilament density at the wounded site seven days post-injury, across groups, scale bar = 200 μm . The red color is neurofilament (NF). These are sagittal sections, centered around the wounded area, rostral is towards the top of the image, caudal towards the bottom, right side of the image is dorsal. (C) Magnified boxed area in B, highlighting neurofilament density spanning the wounded area, scale bar = 20 μm

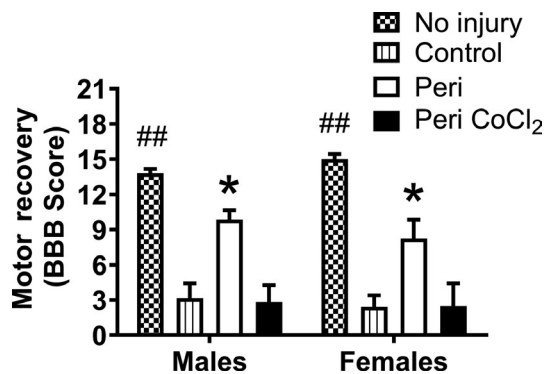


FIGURE 6 Pericyte injection improves hindlimb motor recovery. BBB locomotor scale scores averaged across hindlimbs for the indicated groups at P10. The labels along the x-axis identify the type of treatment each group received at the time of injury. * indicates significant differences across treatments within sex; two-way ANOVA, $P < .05$. ** indicates that the no injury group ($n = 5$ for males and 6 for females) was significantly different from all other groups; one-way ANOVA, $P < .05$. No statistically significant differences were found between sexes within the same treatment; one-way ANOVA, $P > .05$. Stimulated pericyte injected females ($n = 6$) and males ($n = 6$). Pericyte injected females ($n = 8$) and males ($n = 7$). Collagen injected females ($n = 7$) and males ($n = 6$)

disease.³⁹ Similarly, adipose stem cells proposed to be pericyte progenitors improved vascular outcomes in models of retinal vasculopathy.⁴⁰ In contrast to these studies,^{39,40} injection of naïve pericytes did not result in more vascular density within the wounded area in this study. This could be related to differences in models, tissues, and injury mechanism. Overall pericytes seem to improve functional

recovery in several disease models and tissue types. The ultimate effect of pericytes on vessel density, however, appears to be highly regulated by environmental stimulus and varies depending on the tissue, disease state, sex, and HIF pathway activation.

A common idea in the current literature is that promoting vascular density through the process of angiogenesis can result in improved outcomes post-CNS injury.² In an adult rodent model of multiple sclerosis, angiogenesis induced neuronal remodeling that improved motor recovery.⁴¹ Similarly, after spinal cord contusion injury in the adult rat, new blood vessels apparent 3 days after injury were associated with new axon outgrowth by 14 days after injury and were positively correlated with improved behavioral outcomes.⁴² Xiong et al found that greater vessel density and concurrent neurogenesis was associated with neurobehavioral recovery⁴³ and has proposed that inducing angiogenesis could be a key mechanism to improving outcomes post-CNS injury.³ In this work, while stimulated pericytes promoted greater vascular density in males, this did not result in increased NF density or improved BBB scores. The process of angiogenesis can result in an initially unstable and immature vascular bed.^{7,44} An acute vascular pathology of clinical concern in SCI is hemorrhage and blood-spinal cord barrier disruption.^{45,46} The increase in blood flow and vascular density seen in male rats injected with stimulated pericytes could negatively affect a tissue in which the blood-spinal cord barrier is not intact. Therefore, the timing and the pairing of active angiogenesis with the creation of a functional endothelial barrier will be critical to the success of future interventions targeting angiogenesis.

The mechanisms by which pericytes could have improved hindlimb recovery remain unknown. One hypothesis is that pericyte

treatment improved locomotor outcomes by limiting vascular dysfunction. Pericytes are thought to aid in the creation of a stable, flow-regulated mature vascular bed.⁴⁷ Therefore, it is possible that acutely injected naïve pericytes were able to improve blood-spinal cord barrier function and thereby stabilize and promote a more functional vascular bed.

The basis for motor recovery after SCI at any age is thought to be due to the reorganization of neuronal connections.^{48,49} Naïve pericytes could have contributed to motor function recovery by influencing the neuronal environment. In contrast to stimulated pericyte and collagen treatment, pericyte-treated males and females produced neurofilament that spanned the wounded area (Figure 5). Similarly, tissue bridges across the wounded area have been associated with improved recovery after SCI in humans.⁵⁰ The rescue of neurofilament density as compared to controls and maintenance of blood flow perfusion in female rats injected with stimulated pericytes, however, did not lead to functional motor recovery in the current study. This could indicate that a flow-regulated vascular bed must be coupled with other processes of the reorganization of neuronal connections beyond the capacity of stimulated pericytes.

Select pericyte populations can have stem cell-like properties in that, under certain conditions, they can take on characteristics of other types of cells within the tissue bed.^{4,51} Nakagomi et al found that pericytes from ischemic tissue beds expressed surface markers of neural and vascular cells when cultured under ischemic conditions *in vitro*.⁵² In this study, we found that a percentage of both stimulated and non-stimulated pericytes were positive for isolectin and/or neurofilament *in vivo* (Figure 2 C and D). We did not find evidence of these cells morphologically “becoming” other types of cells, or evidence that the injected pericytes took up a perivascular position at the time point of tissue harvest. What the function of these cells is, how they contribute to vessel density, influence blood flow, or interact with the neuronal environment remains unclear.

In contrast to Nakagomi et al.'s ischemic conditions, direct HIF pathway activation of pericytes tended to decrease the percentage of cells that then were positive for vascular or neuronal cell surface markers (Figure 2 C and D). This highlights how critical environmental cues are to pericyte expression of cell surface markers—how slight differences in the cell's experience of hypoxia can change cell surface marker expression. Our work is consistent with the *in vitro* findings of Nakagomi et al, however, in that a subset of pericytes can change cell surface markers under differing environmental stimulus.

Males injected with stimulated pericytes exhibited the highest levels of vessel density. They did not present with greater markers of endothelial proliferation compared to sex-matched controls as might be expected with the initiation of an angiogenic program (Figure 3B). At the same time, they did not appear to have the organized vasculature that would be expected with the maintenance of pre-injury vasculature (Figure 3G). Therefore, stimulated pericytes may have initiated an angiogenic program and endothelial proliferation at an earlier time point not captured by this study, which resulted in the higher vessel density and more blood flow perfusion (Figure 4).

The timing of, or capacity for, the initiation of an angiogenic program and maintenance of vessel stability following CNS injury might differ between males and females. For example, the male controls had greater endothelial proliferation than females (Figure 3B). Female rats, on the other hand, had greater vessel density than males (Figure 3A). It is possible that females may be better able to maintain vessel viability after initial injury, even before sexual maturity. Additionally, an increase in blood flow perfusion was seen in males treated with stimulated pericytes but not females. Females might therefore possess intrinsic mechanisms that control blood flow perfusion even in the presence of the stimulated pericytes. This is consistent with clinical findings that there are sex differences in endothelial function, including vasodilation and blood pressure.⁵³

Stimulated pericytes interacted differently than their naïve counterparts within the host tissue in regard to motor recovery, vessel density, blood flow perfusion, and neurofilament density (Figures 3–6). There is evidence from the current literature that initiation of the HIF pathway can change pericyte behavior and interaction with the neuronal environment.^{8,9,11,54} These results support the idea that the HIF pathway fundamentally regulates the functional interaction of pericytes within the neuronal environment.⁵⁵

The intensity of HIF pathway activation in pericytes might be critical to the ultimate pro-angiogenic activity vs anti-angiogenic behavior induced. Hall et al. noted that in brain slice culture, 90% of pericytes died within one hour of chemically induced oxygen-glucose deprivation.⁵⁶ In the current study, driving a hypoxia based signaling pathway in pericytes with CoCl_2 resulted in greater cell viability than naïve pericytes (Figure 1C). It is possible that the level of HIF pathway activation in pericytes differed between these systems. For example, pericytes only promoted a greater rate of wound healing when co-cultured with endothelial cells at a specific dose of pericyte CoCl_2 pre-treatment (Figure 1A). One level of HIF activation could therefore stimulate pericytes to enhance vessel density, as in this study, while another could induce pericyte cell death.⁵⁶ Taken together, varying degrees of HIF pathway activation could dictate pericyte response and act as a mechanism by which pericytes respond to changing environmental conditions. A limitation of these results is that we do not know what the effects of inflammation are in the modulation of the pericyte and endothelial cell population during SCI recovery.

We did not directly measure endothelial cell and pericyte interactions. For this first study, we focused on how HIF-activated pericytes influenced important outcome measures such as vessel density, NF density, and movement post-SCI. Future studies could examine sex-specific endothelial/pericyte interactions within the context of this model.

A limitation of this study is that we could not make definitive cause and effect conclusions about the interactions between treatment, vascular density, and NF density. For example, while stimulated pericytes did drive vascular density, we did not see concurrent increases in NF density. Whether this is related to direct interaction with stimulated pericytes vs. non-stimulated pericytes and neurons, a consequence in response to changes in vessel density caused by

treatment, an issue with treatment timing, or some other unknown cause, is unclear. It is possible that since only with naïve pericyte treatment did we see neurofilament span the wounded area (indicative of axonal presence across the wounded area) something about the presence of naïve pericytes influences neuronal maintenance/remodeling post-injury in a way that is not possible with stimulated pericytes. What the specific mechanism could be is a question that future studies could address.

A further limitation is that we only had access to female human placental pericytes. This made it so that the injected pericytes were not sex matched to the subject. It is possible that the sex differences noted in this study could be influenced by this aspect of the study's design. These results, however, are in line with previous reports in adults that males and females can have different vascular outcomes after injury or during pathology.^{36,57} There is also evidence of sex differences in functional outcomes in humans⁵⁸ and in animals models of SCI.⁵⁹ The mechanisms that dictate these sex differences, however, remain unclear.

Despite the sex differences noted in this study, naïve pericyte injection improved hindlimb motor recovery in both sexes. This is consistent with the hypothesis that a single therapy targeting a basic physiological response could improve gross outcomes of functional recovery in both sexes. The results from this study highlight the importance of appreciating that even when males and females reach the same gross functional end measurements, they might do so by different structural and molecular mechanisms.⁶⁰

Consistent with our initial hypothesis, HIF-activated pericytes did increase vascular density in the wounded area. This is also consistent with our previous in vitro work that stimulating the HIF pathway in pericytes drives them to a pro-angiogenic state.¹¹ Contrary to our initial hypothesis, greater vessel density did not result in greater neurological recovery post-SCI. Above and beyond greater vessel density, recovering neuronal tissue is dependent on a highly functional microvascular system. Interventions that focus on creating a functional and healthy microvascular tissue bed will likely be more successful in improving neurological recovery than those just focusing on driving greater vessel density. Overall, the timing and the pairing of active angiogenesis with the creation of a functional endothelial barrier will be critical to the success of future interventions targeting angiogenesis. Manipulation of the HIF pathway in pericytes might be helpful in creating these functional vascular beds post-SCI.

5 | PERSPECTIVES

Pericytes influence the neurovascular environment post-CNS injury. Environmental cues that instigate pericyte HIF signaling could act as initiation codes, switching the pericyte role from that of stabilization to the task of tissue remodeling. How the molecular interactions between pericytes, endothelial cells, and neurons are altered by HIF pathway activation in vivo and ultimately mediate neurological outcomes is still an open discussion. Furthermore, as we learn more

about sex differences in microcirculation, elucidating how pericytes contribute to sex differences in angiogenesis, blood flow regulation, and neuronal remodeling will likely be critical for clinical translation of microcirculatory based therapies.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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