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Oxygen-tunable endothelialized microvascular chip to assess hypoxia–reperfusion in sickle cell disease

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A better understanding of hypoxia reperfusion (H/R) injury is needed to gain deeper insight into the mechanisms driving sickle cell disease (SCD) pathophysiology. Existing *in vivo* and *in vitro* models have yet to fully explain H/R, which is typically associated with harmful inflammatory processes but has also been linked to a protective effect ameliorating subsequent severe vaso-occlusion. To address this need, we developed a novel microfluidic platform that includes three-dimensional endothelial-lined microchannels within an oxygen-tunable environment. These features enable simulation of H/R, red blood cell (RBC) sickling, and vaso-occlusion on-chip. The endothelial network cultured on-chip is physiologically relevant and expresses crucial microvascular features such as 3D lumen structure and expression of functional endothelial markers. We utilized this platform to perform an occlusion assay, evaluating the effects of hypoxic preconditioning on RBC-endothelial interactions contributing to occlusion. Our results demonstrate that both sustained mild hypoxia and cyclic hypoxia endothelial treatment reduce the likelihood of SCD occlusion on-chip. Specifically, average vaso-occlusion rates of 8.89% and 11.78% were observed among endothelialized devices preconditioned to cyclic and sustained hypoxia, respectively, compared to 57.93% and 55.05% for the control groups. Additionally, we leveraged RNA sequencing to identify differential regulation of specific genes contributing to this protective outcome. Of note, hypoxia preconditioning resulted in significant modulation of *CYBB*, *RELN*, and *SERPINA1*. These results offer a better understanding of the mechanistic changes affecting the endothelium during H/R and also offer potential targets for further exploration and therapeutic intervention in SCD.

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Introduction

Sickle cell disease (SCD) is an inherited autosomal recessive disorder caused by a mutation in the hemoglobin β gene, leading to hemoglobin polymerization, red blood cell (RBC) sickling, and a cascade of pathophysiological events culminating in painful vaso-occlusive crisis (VOC).^{1,2} Despite its discovery over a century ago, treatment options for the millions of people with SCD worldwide remain limited, highlighting the need for a better understanding of its

underlying mechanisms.^{3–5} Recently, increased attention has been paid to hypoxia reperfusion (H/R) injury as a driving mechanism of SCD pathology.^{6–9} In SCD, H/R injury starts with an initial vaso-occlusive event leading to tissue hypoxia and/or ischemia. Next, reperfusion of this tissue by oxygenation generates a cascade of signaling events culminating in a significant pro-inflammatory response. While H/R is classically thought to induce harmful inflammatory responses, mild or cyclic episodes of hypoxia may provoke a conditioning effect that protects against severe subsequent H/R injury.^{7,10} Previous attempts to identify mechanisms that initiate this pro-survival phenotype are incomplete and contradictory.^{6,7,10,11} Although some studies have begun to examine the effect of H/R preconditioning on various organs and tissue compartments,^{12–14} the effect on the microvasculature itself is largely unexplored. A better understanding of how H/R generates cytoprotective preconditioning of the endothelium will inform our overall understanding of SCD pathophysiology and offer insight towards therapeutic intervention, and microfluidic models are aptly suited to address these questions.

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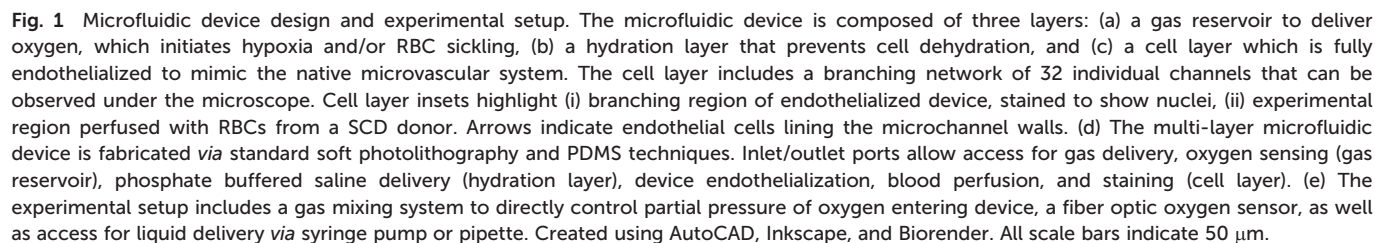
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The multi-layer microfluidic platform consists of (1) a gas reservoir to regulate oxygen tension in the device, initiating hypoxia and/or red cell sickling, (2) a hydration layer to prevent cell dehydration, and (3) a fully endothelialized cell layer to mimic a network of microvascular channels (Fig. 1).



Cell culture

Microfluidic device endothelialization

Fluorescence staining and microscopy of endothelialized devices

Numerous fluorescent stains, probes, and antibodies were used to thoroughly characterize the endothelium, including Hoechst 33258 (Invitrogen), CellMask Deep Red (Invitrogen), CD31 conjugated monoclonal antibody (Bio-Rad), CD144 monoclonal antibody (Invitrogen), CD106 recombinant antibody (Invitrogen), Phalloidin CruzFluor 594 (Santa Cruz), and cell permeable DAF-2 DA (Santa Cruz). DAF-2DA staining was performed on live endothelial cells, whereas all other stains were performed on cells fixed with 4% paraformaldehyde (PFA). Endothelial confluency and alignment were visualized using the CellMask plasma membrane and Hoechst nuclear stains. Adhesion marker expression was verified using conjugated (CD31), primary (CD144 and CD106), and secondary antibodies (Alexa Fluor 555 and 647) which were prepared at 1:100–1:250 in blocking buffer and incubated in blocked endothelialized devices at 4 °C overnight. Phalloidin was used to label F-actin, and solution was made at 1:100 in blocking buffer and incubated in blocked endothelialized devices at 4 °C overnight. Nitric oxide production was visualized with the DAF-2 DA fluorescent probe. DAF-2 DA solution was prepared at a dilution of 1:250 in EGM-2 media and perfused *via*

syringe pump to live endothelialized devices at a rate of 60 μL per hour for 1 hour prior to imaging. Microscopy was performed on either a Zeiss Axio Observer widefield microscope or a Nikon AX R confocal microscope. The widefield scope was equipped with an incubation chamber that maintained the environmental temperature to 37 °C. Image processing was performed in Zen software (Zeiss), NIS Elements software (Nikon), and FIJI/ImageJ. Cell confluence was measured as the percent area of each microchannel fluorescently labeled with CellMask plasma membrane stain. Cell density was calculated as the number of Hoechst-stained nuclei per mm^2 within the microchannels.

Hypoxic preconditioning

Endothelial hypoxic preconditioning was achieved using a solenoid valve gas mixing system that has been previously described.^{32,34} Briefly, the gas mixer added compressed air (21% O_2 , 5% CO_2 , balance N_2) to compressed nitrogen (0% O_2 , 5% CO_2 , balance N_2) in a solenoid valve-controlled mixing chamber to achieve a user-specified oxygen concentration between 0 and 21%. Then, the gas at the desired oxygen concentration was perfused into the microfluidic device gas layer. Throughout experimentation, oxygen gas tension was monitored *via* a calibrated fiber optic oxygen sensor (NeoFox-GT, Ocean Optics). To maintain cell viability and alignment under flow, hypoxic preconditioning was performed in a 37 °C environmental chamber under consistent 60 $\mu\text{L h}^{-1}$ media flow.

Endothelialized devices were exposed, or “preconditioned”, to one of the following treatments for 60 minutes: (1) atmospheric control: 21% oxygen, (2) physiologic control: 12% oxygen, (3) cyclic hypoxia: alternating 12% oxygen and 5% oxygen at 10-minute intervals, or (4) sustained hypoxia: 5% oxygen. While most laboratory experiments are conducted at normal environmental oxygen levels (21% or 160 mmHg), it is important to note that this oxygen level is much higher than that of vasculature and tissue in the body. At sea level, arterial blood oxygen tension is typically between 75 and 100 mmHg, whereas venous blood oxygen tension is typically between 30 and 40 mmHg.^{38,39} Therefore, we also chose to include a “physiologic” control, which would precondition the endothelial cells to a 12% (~90 mmHg) normoxia level resembling *in vivo* conditions.^{38,39} For the hypoxia conditions, 5% oxygen level and 10-minute cycling intervals were based on preliminary experiments. Below 5% oxygen and more frequent (1 to 5-minute) cycling intervals negatively impacted endothelial cell viability and morphology. This parameter space may be further explored in subsequent work. After exposure to one of the four preconditioning treatments, endothelialized devices were returned the incubator (37 °C, 5% CO_2) and cultured under media flow for 4 additional hours. This window of time allowed the endothelium to respond to the preconditioning treatment and was chosen based on previous

studies on delay of genomic response in endothelial cells.^{11,40}

Blood sample collection and preparation

Blood samples from individuals with SCD (HbSS and HbSC) were collected in citrate-coated vacutainers from Massachusetts General Hospital (MGH), Children's Hospitals and Clinics of Minnesota, and the University of Minnesota Medical Center (UMN). All samples were collected and experiments were performed in accordance with the guidelines of Institutional Review Boards at the Massachusetts General Hospital, at the University of Minnesota, and Children's Hospital and Clinics of Minnesota under approved protocols 2006P000066 (MGH) and STUDY00003 (UMN and Children's). Informed consent (and assent, if minor) were obtained from all human participants of this study. Routine clinical measurements, including complete blood count and hemoglobin variant analysis were performed on the samples (Table S1). Prior to use, blood samples were stored at 4 °C for a minimum of 2 hours and a maximum of 3 days.

Prior to an experiment, the whole blood sample was fractionated in a centrifuge (400g, 5 minutes $\times$ 3 wash cycles). The plasma and buffy coat layers were removed and replaced with PBS without calcium or magnesium to generate washed RBCs. Washed RBCs were gently loaded into a syringe with attached 0.015" inner diameter tubing (BD Intramedic) and mounted on a syringe pump (Harvard Apparatus). Washed RBCs were used in this experiment to isolate just the contribution of the endothelium and RBCs in vaso-occlusion, decoupling variability from platelets, leukocytes, *etc.* Samples were loaded into new syringes for each microfluidic trial to limit the effect of red cell packing.

Occlusion assay

After the 4-hour incubation window, a preconditioned microfluidic device was placed in the 37 °C temperature-controlled microscope chamber equipped with a gas mixing system in preparation for the occlusion assay. The device preparation involved: (1) oxygen mixing system calibration, (2) PBS injection into hydration layer, (3) delivery of 12% oxygen into gas layer *via* solenoid mixing system, and (4) oxygen tension validation *via* NeoFox oxygen sensor. Once complete, washed RBCs from a SCD donor were perfused through the endothelialized cell layer at a rate of 60 $\mu\text{L h}^{-1}$ using a syringe pump (Harvard Apparatus). All microfluidic devices underwent 10 minutes of RBC perfusion at physiologic oxygen levels (12% O_2) followed by 10 minutes of perfusion under hypoxia (5% O_2). During the experiment, bright field microscopy was monitored at 5–10 $\times$ by scanning for clot formation and occlusion in each of the 32 parallel microchannels. Microchannel occlusion was defined as complete blockage/stasis of flow and occlusion incidence was measured at 5-minute intervals, quantified as percentage of the 32 microchannels fully obstructed within each device.



correlative analysis, Spearman testing was performed in GraphPad Prism software. Correlation significance was indicated *via* p-value, in which * $p < 0.05$ and ** $p < 0.01$. In multiple statistical analyses, subgroup analysis was also performed to evaluate any potential effect due to transfusion by removing the three donor data sets who had a recent history of transfusion therapy (donor ID numbers 2, 3, and 12).

Results & discussion

Development of an endothelialized, oxygen-tunable microfluidic model to study SCD

A multi-layer platform was constructed to both control oxygen tension and culture endothelial cells on the same device, combining multiple aspects of SCD pathophysiology that have not previously been combined on-chip (Fig. 1). These features enable simulation of blood flow through microvascular channels, hypoxia-induced RBC sickling and H/R, and observation of RBC vaso-occlusion on a single device. The first layer acts as a gas reservoir to regulate oxygen tension, facilitating initiation of hypoxia, induction of RBC sickling, and evaluation of H/R injury (Fig. 1a). The second layer provides humidity and prevents cell dehydration (Fig. 1b). The third layer consists of a perfusable, fully endothelialized network of channels to mimic a microvascular network (Fig. 1c). The branching vascular network resolves into 32 individual channels, which permits observation of a large number of microchannels in parallel, similar to other vessel-on-chip geometries.^{16,19–21,36} The size-scale (60 by 50 μm in the experimental region) and microchannel geometries were designed to mimic postcapillary venules—low oxygen microvasculature that are often associated with cell sickling, adhesion, and vaso-occlusion in SCD.^{19,41,42}

Due to the challenging nature of the branching vessel geometry and small size scales, established protocols¹⁹ for device endothelialization were incapable of fully endothelializing this geometry's thin branching network without clogs or bare regions. We refined a protocol to generate a confluent, aligned microvasculature system that recapitulates small vessels *in vivo* (see Methods). In particular, key advancements included: (1) increasing HUVEC seeding density to 15×10^6 cells per mL from commonly used ranges of $0.5\text{--}2 \times 10^6$ cells per mL, (2) repeated, intermittent seedings *via* pipette tip instead of *via* syringe pump to improve cell contact time with microchannel walls, and (3) 90-minute incubation period before adding flow to allow cells to fully attach and spread throughout channels. This method is versatile; preliminary experiments also demonstrated successful culture of microvascular endothelial cells (HMEC-Ls) in device microchannels (Fig. S1). Overall, our microfluidic device design and experimental setup effectively enables cell culture of 3D endothelial vessel-structures in an oxygen-tunable environment. (Fig. 1d and e). Additionally, the design supports perfusion of donor RBCs and the

Statistical analyses

ANOVA statistical testing was performed using GraphPad Prism version 10.3.1. Occlusion incidence significance results were determined *via* one-way or two-way ANOVA testing, where appropriate. Geisser–Greenhouse correction was used to account for lack of sphericity/unequal variability of differences as recommended. A Tukey test for multiple comparison analysis was performed at a confidence interval of 95%/alpha threshold of 0.05. For

investigation of RBC-endothelial cell interactions driving SCD adhesion and vaso-occlusion.

Endothelialized microfluidics replicate key biological features of microvasculature

Fluorescence microscopy was used to evaluate the endothelium, including visualization of the vascular network and also confirmation of key physiologic attributes (Fig. 2). It was critical that our model system exhibited confluency along the microchannel, presence of a perfusable lumen, and alignment in the direction of flow.⁴³ Confocal microscopy was utilized to verify that endothelial cells in our microfluidic device effectively coated the top, bottom, and side walls of each microchannel while supporting a central lumen (Fig. 2a). Observation of the endothelium in the Z-direction also effectively confirmed endothelial confluency as well as alignment under flow. Cell confluence and density were quantified after 24- and 48-hour incubation windows to more thoroughly characterize the endothelium (Fig. S2). Endothelial cell

confluence and density improved with longer incubation under flow; in particular, density increased from 878 ± 110 to 1067 ± 122 nuclei per mm^2 between 24 and 48 hours of incubation (Fig. S2b).

Beyond confluency, lumen structure, and alignment, it was critical that the vascular network within the microfluidic device displayed key functional characteristics. These include expression of adhesion molecules, the ability to synthesize essential biological factors, the development of an organized cytoskeleton, and the ability to tolerate fluctuations in oxygen. Fluorescence microscopy was used to characterize the endothelium and identify the presence of physiologically relevant components. Adhesion molecules such as platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Fig. 2c) and vascular endothelial cadherin (VE-cadherin/CD144) (Fig. 2d) are associated with formation and maintenance of tight junctions between endothelial cells. Resultantly, these adhesion molecules contribute to barrier function and help maintain vascular homeostasis.⁴⁴ Vascular cell adhesion molecule-1 (VCAM-1/CD106) (Fig. 2e) also

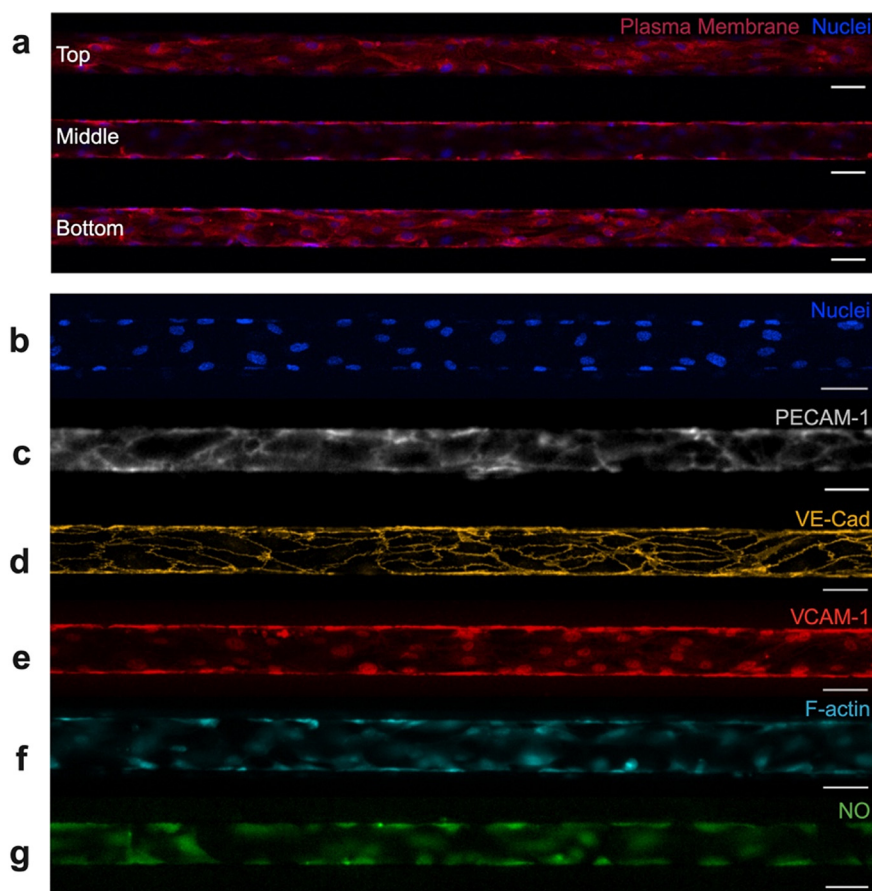
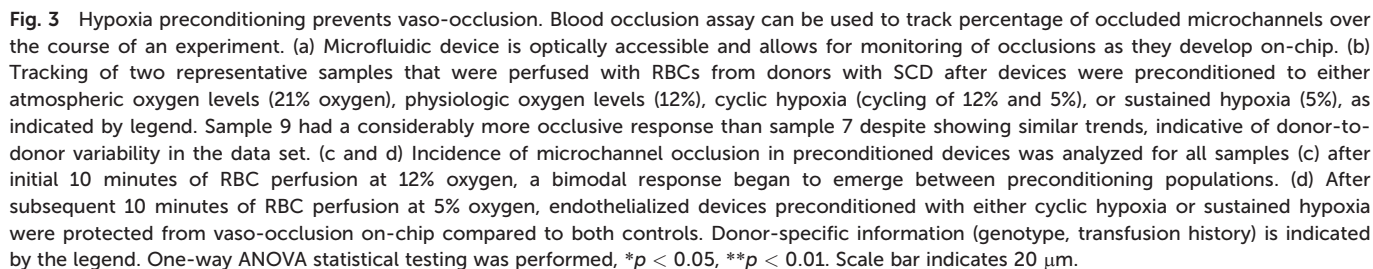


Fig. 2 Characterization of endothelium. Numerous fluorescent probes and techniques were used to visualize the endothelial cells and examine their physiologic relevance. (a) Confocal microscopy was used to verify 3D lumen structure and confluent microchannel lining. Top, middle, and bottom sections of the microchannel were fluorescently imaged after staining for nuclei (blue) and plasma membrane (red). HUVECs adequately cover the top, bottom, and side walls of the microchannel while maintaining a central lumen. Cells also align with flow in the horizontal direction. Fluorescent imaging of the device's experimental region highlights (b) confluency, via nuclear staining (blue), (c) PECAM-1 expression, (d) VE-cadherin expression, (e) VCAM-1 expression, (f) F-actin structures, and (g) nitric oxide production. All scale bars indicate 50 μm .



produce nitric oxide, another indicator of vascular function (Fig. 2g). Nitric oxide is synthesized by endothelial cells in the body, which is thought to modulate vascular homeostasis, vascular tone, and even protect blood vessels from injury.⁴⁷ Lastly, endothelialized devices underwent preliminary hypoxia testing, which validated that no loss of cells, detachment from microchannel walls, or significant morphological change occurred due to short-term hypoxia exposure (Fig. S3). Together, confirmation of adhesion molecule expression, cytoskeletal development, synthesis of biological factors, and tolerance to fluctuations in oxygen level demonstrate that the endothelium in our microfluidic devices morphologically and functionally resemble that of *in vivo* microvasculature.



Hypoxic preconditioning reduces likelihood of SCD RBC microchannel occlusion

Other groups have recently pioneered the use of microfluidics to measure occlusion incidence on-chip as an indicator of clinical severity, a means of evaluating response to therapy, or as a tool for mechanistic study.^{26,27,48,49} Our occlusive assay is unique in its functionality: incorporation of fully endothelialized microfluidic channels, ability to trigger H/R and hypoxia-mediated red cell sickling in real-time, as well as use of biologically relevant size scales/shear. Therefore, to test the effect of hypoxic preconditioning on likelihood of microchannel occlusion, RBCs from donors with SCD ($n = 13$) were perfused through endothelialized channels that had been preconditioned to one of four conditions: atmospheric control (21% O₂), physiologic control (12% O₂), cyclic hypoxia (cycling of 12% and 5% O₂), or mild sustained hypoxia (5% O₂). During an experiment, development of occlusive events was monitored in real-time (Fig. 3a), with percentage of device microchannel obstruction calculated each 5-minute interval (Fig. 3b; Fig. S4).

Across samples, compared to endothelialized devices conditioned at atmospheric or physiologic oxygen levels, devices preconditioned with either cyclic or sustained hypoxia exhibited a lower likelihood of microchannel occlusion. After the first phase of the experiment (during which the device was exposed to the physiologic level of 12% O₂), hypoxic preconditioned devices trended towards reduced microchannel occlusion (Fig. 3c). After the second phase of the experiment (during which the device was exposed to the hypoxic level of 5% O₂), devices preconditioned to hypoxia exhibited significantly lower microchannel occlusion compared to controls ($p < 0.01$) (Fig. 3d). Overall, we observed average microchannel occlusion rates of 8.89% and 11.78% after cyclic and sustained hypoxic preconditioning compared to 57.93% and 55.05% after atmospheric and physiologic controls, respectively. This result supports the hypothesis that mild or cyclic episodes of hypoxia can provoke a conditioning effect that protects against endothelial injury or vaso-occlusion. There was no significant difference between control conditions or, separately, between the two hypoxic conditions. As some donors had recently undergone transfusion therapy, we performed subgroup analysis excluding those donors which did not significantly affect the results or interpretation (Fig. S5). A reproducibility analysis was also performed by evaluating microchannel occlusion of a single SCD donor sample across five independently fabricated and endothelialized microfluidic devices, which indicated minimal chip-to-chip variability and a low coefficient of variation at later experimental timepoints (CV of 2.83% at the end of the experiment) (Fig. S6).

SCD donor clinical variables are correlated with microchannel occlusion on-chip

Phenotypic heterogeneity between SCD subjects with the same genotype is poorly understood.^{1,50,51} Likewise, in our

microvascular occlusion assay, there was notable inter-donor variation (Fig. S4). To further investigate this heterogeneity, we examined the correlation between occlusion outcomes and the donor clinical data set (Table S1). First, we performed a statistical analysis to determine if samples from either male ($n = 8$) or female ($n = 5$) donors were more likely to occlude. Based on our limited cohort, samples from male donors exhibited higher occlusion likelihood on-chip (Fig. S7). In the case of physiologic control, samples from male donors were significantly more likely to occlude than female donors ($p = 0.05$ for all samples, or $p < 0.05$ when excluding transfused samples). These results are consistent with the literature: In pediatrics, males exhibit higher pain crisis frequency than females (1.6 vs. 0.6 per year).⁵² Moreso regardless of age, male SCD subjects experience more severe infectious and cardiovascular complications,⁵² and overall worse SCD-associated morbidity and mortality rates.⁵³ Additional samples are needed to further validate this finding, but these initial conclusions prompt significant interest for future studies.

Next, we performed a nonparametric Spearman correlation to investigate the relationship between routine clinical measurements and percent occlusion in response to each type of hypoxic preconditioning treatment (Fig. 4; Fig. S8). Once again, statistical testing was conducted on all samples, then on non-transfused samples only. Across both analyses, a number of significant trends were observed. Hemoglobin and hematocrit demonstrated a weak inverse correlation with occlusion percentage across all groups but the atmospheric control. Generally, HbS, mean corpuscular volume (MCV), white blood cell count (WBC), and platelet count (PLT) exhibited moderate correlation with occlusion percentage across all groups. When analyzing the entire population sample set ($n = 13$), the results show significant correlation between sickle hemoglobin percentage (HbS) and percent occlusion in the 5% preconditioning group ($r = 0.769$, $p = 0.003$), between PLT and percent occlusion in the 5% preconditioning group ($r = 0.735$, $p = 0.006$), and between white WBC and percent occlusion in the 5% and 12% oxygen preconditioning groups ($r = 0.696$, $p = 0.01$, and $r = 0.612$, $p = 0.03$, respectively). When analyzing only non-transfused samples, we found a stronger correlation between both MCV and red cell distribution width (RDW) with occlusion, but a weaker trend between HbS and occlusion. In particular, a significant correlation was observed in the 5% preconditioning group between MCV and percent occlusion ($r = 0.714$, $p = 0.024$), between RDW and percent occlusion ($r = 0.689$, $p = 0.028$), and between WBC and percent occlusion ($r = 0.702$, $p = 0.085$). These results indicate that individuals with high levels of WBC, PLT, HbS, MCV, or RDW, as well as low levels of hemoglobin or hematocrit may exhibit a more occlusive, severe SCD phenotype. It was noted that the 5% preconditioning group exhibited a much stronger correlation with certain clinical parameters than other preconditioning groups. This finding may have been a result of the 5% preconditioning treatment more appropriately simulating the *in vivo* oxygen conditions. This result

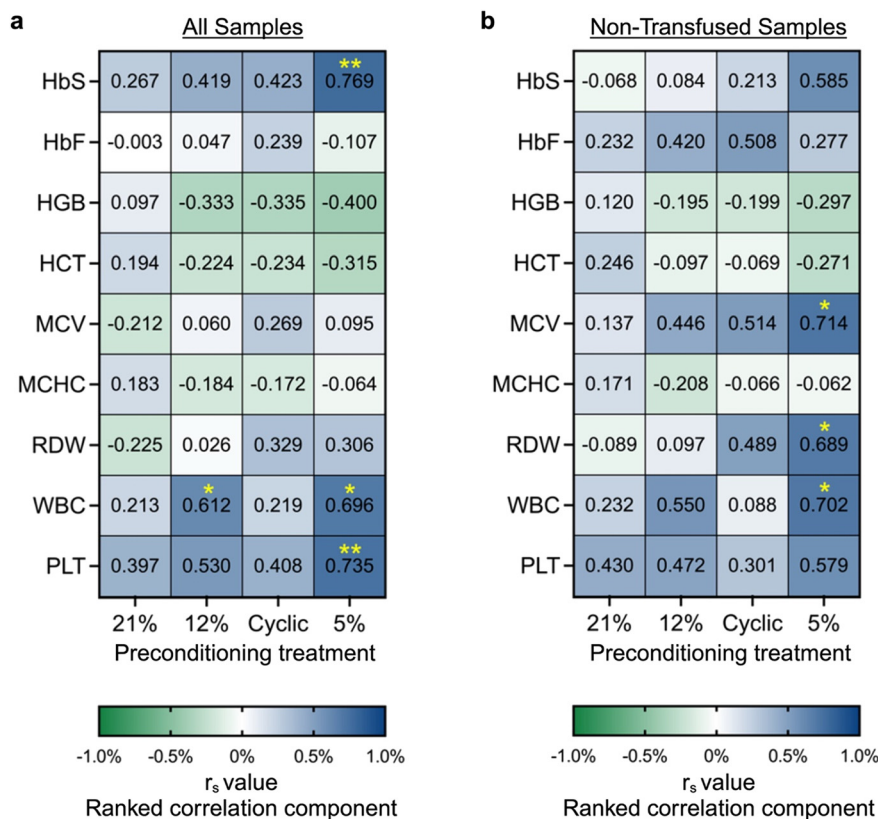


Fig. 4 Spearman correlation of routine clinical measurements with occlusion. A nonparametric spearman correlation was used to quantify correlation between routine clinical measurements and percent microchannel occlusion after various hypoxia preconditioning treatments. Spearman correlation r value was calculated for (a) all samples ($n = 13$) and (b) only non-transfused samples ($n = 10$). Clinical metrics were obtained from hemoglobin variant analysis and complete blood count testing, and consist of hemoglobin S (HbS), hemoglobin F (HbF), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), RBC distribution width (RDW), white blood cell count (WBC), and platelet count (PLT). Ranked correlation component " r_s " ranges from -1 to 1 as indicated in the legend, with 0 indicating that the metrics do not vary together, 1 indicating perfect correlation, and -1 indicating perfect inverse correlation. Significance is indicated by asterisks, $*p < 0.05$, $**p < 0.01$.

highlights the importance of integrating physiologically similar environmental conditions and stimuli when developing biological models.

Evidence of a correlation between occlusion and non-RBC related indices (WBC and PLT) is particularly intriguing since the occlusion assay was performed using only RBCs. While others have shown that WBC and PLT counts are significantly elevated during VOC and high leukocyte counts have also been positively correlated with a number of SCD risk factors including early death, ACS, and stroke,⁵⁴ our study suggests that RBCs from SCD donors with leukocytosis and thrombocytosis exhibit higher occlusion capacity, even in hypoxia preconditioned endothelium. As leukocytosis and thrombosis may reflect systemic inflammation, further studies to assess how systemic inflammation in SCD may overcome the benefits of endothelial H/R preconditioning are warranted.

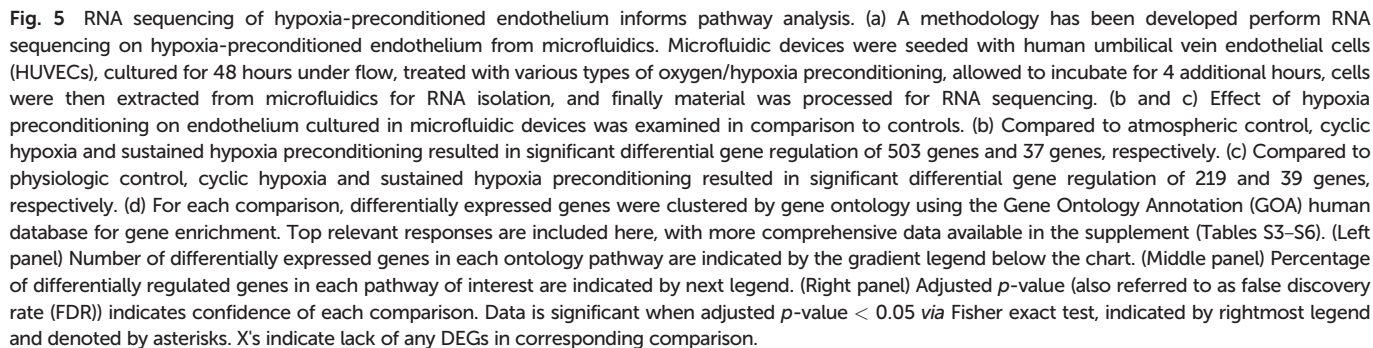
Hypoxic preconditioning causes transcriptional changes associated with vaso-occlusion

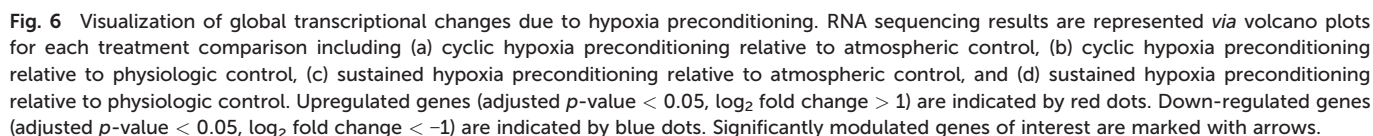
The ability of hypoxic preconditioning to prevent microvascular occlusion incidence in our devices led us to

investigate the hypoxia-induced gene-level changes to the endothelium. We developed a protocol to extract RNA from hypoxia-preconditioned endothelial cells cultured on-chip and then performed RNA-sequencing analysis (Fig. 5a). Despite the complexity of decellularizing the microfluidic devices, high cell viability was maintained which resulted in high quality and integrity RNA (Table S2). Compared to physiologic or atmospheric controls, both types of hypoxic preconditioning resulted in significant endothelial differential gene expression (Fig. 5b and c). Compared to sustained hypoxia, cyclic hypoxia resulted in more abundant differential gene modulation. We next performed gene ontology clustering to identify relevant enriched gene pathways modulated by hypoxic preconditioning (Fig. 5d; Tables S3–S6). We note significant modulation in critical pathways involving hypoxia sensitivity, blood vessel modification, coagulation, inflammation, adhesion, and endothelial cell functional behavior. Taken together, these changes in pathways of interest may contribute to the protective effect garnered by hypoxic preconditioning.

Next, we assessed volcano plots and heat maps (Fig. 6; Fig. S9), drawing attention to specific genes of interest. Cyclic







Gene ontology and volcano plots also highlight the interaction between hypoxic preconditioning and numerous genes involved in coagulation and inflammation. In particular, *SERPINA1* was significantly upregulated in 3 of 4 comparisons (on average, a 6.00-fold change in differential gene expression, $p < 0.01$ for all 3 comparisons). The *SERPINA1* gene encodes the alpha 1-antitrypsin (AAT) protein, which inhibits inflammatory proteases.⁵⁶ Mutation in *SERPINA1* is correlated with venous thromboembolism and a number of pulmonary disorders.^{57,58} AAT has also been shown to be elevated in SCD and positively correlated with

Additionally, a single adhesion-specific gene – *RELN*—was up-regulated across all 4 comparisons (on average, a 1.60 ± 0.48 -fold change in differential gene expression, $p < 0.05$ for all comparisons). The *RELN* gene encodes the protein reelin, which has been shown to influence morphology, membrane permeability, and membrane resistance of vascular endothelial cells as a means of vessel wall integrity regulation.⁵⁹ More specifically, reelin increases endothelial cell expression of pro-adhesive VCAM-1, ICAM-1, and E-selectin, resulting in increased monocyte-endothelial activation, inflammation, and adhesion.⁶⁰ To date, reelin has not been explored in SCD. However, our data suggests that upregulation of endothelial cell *RELN* expression in response to hypoxic preconditioning may contribute to a pro-adhesive environment. This is notable, as inhibition or reduction of circulating reelin has been proposed a possible approach for the prevention of cardiovascular disease due to its protection against atherosclerosis.⁶⁰ Therefore, further studies exploring reelin within the context of SCD are warranted in order to

ascertain if reelin inhibition may also be useful in protection against vaso-occlusion in SCD.

Conclusion

Our novel microfluidic platform fulfills an unmet need in microvascular research. Using sickle RBCs, we leveraged our platform to investigate the likelihood of donor blood samples to occlude on-chip and also to explore hypoxia-induced endothelial gene modulation. Collectively, these results offer a better understanding of the mechanistic changes affecting the endothelium during H/R and also supports the theory that in certain types of hypoxia conditioning may offer a protective effect against vaso-occlusion in SCD.

Author contributions

SRS designed and performed experiments, analyzed data, and wrote the manuscript. JDB assisted with data interpretation and revised the manuscript. WAL aided in experimental design and edited the manuscript. DKW assisted with experimental design, data interpretation, and edited the manuscript.

Conflicts of interest

J. D. Beckman receives research funding from Bayer and Novartis independent from work herein.

Data availability

Supplementary information is available: The SI contains Supplementary Figures and Supplementary Tables. The Tables provide information about blood samples used in this study and additional RNA sequencing information. See DOI: <https://doi.org/10.1039/D5LC00211G>.

Data supporting this article has been included as a part of the SI.

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