Hypoxia and matrix viscoelasticity sequentially regulate endothelial progenitor cluster-based vasculogenesis

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Vascular morphogenesis is the formation of endothelial lumenized networks. Cluster-based vasculogenesis of endothelial progenitor cells (EPCs) has been observed in animal models, but the underlying mechanism is unknown. Here, using O2-controllable hydrogels, we unveil the mechanism by which hypoxia, co-jointly with matrix viscoelasticity, induces EPC vasculogenesis. When EPCs are subjected to a 3D hypoxic gradient ranging from <2 to 5%, they rapidly produce reactive oxygen species that up-regulate proteases, most notably MMP-1, which degrade the surrounding extracellular matrix. EPC clusters form and expand as the matrix degrades. Cell-cell interactions, including those mediated by VE-cadherin, integrin-β2, and ICAM-1, stabilize the clusters. Subsequently, EPC sprouting into the stiffer, intact matrix leads to vascular network formation. In vivo examination further corroborated hypoxia-driven clustering of EPCs. Overall, this is the first description of how hypoxia mediates cluster-based vasculogenesis, advancing our understanding toward regulating vascular development as well as postnatal vasculogenesis in regeneration and tumorigenesis.

INTRODUCTION

Functional vasculature is critical for tissue homeostasis. Thus, the formation of neovascularure, vascular morphogenesis, is a hallmark of tissue development and regeneration, as well as cancer growth and metastasis. An in-depth understanding of the mechanisms governing vascular morphogenesis is critical to the identification of previously unidentified therapeutic targets and refinement of therapeutic strategies. Numerous studies have elegantly uncovered many key regulators of angiogenesis and vasculogenesis. A mechanistic understanding of “classical” single-cell vasculogenesis has been defined and refined over the last two decades by using intricately designed in vivo models, including those in both chick and mouse embryos (1–4), as well as zebrafish (5–7). Hydrogels, which can mimic the native three-dimensional (3D) extracellular matrix (ECM), have been used successfully to analyze and control vascular regeneration from human cells (8–12) and to understand classical single-cell vasculogenesis from human cells (11, 12). Briefly, classical single-cell vasculogenesis commences when endothelial cells (ECs) establish interactions with their surrounding ECM through integrin engagement (13–15). These cells then respond to an array of proangiogenic cues, including biological factors and physical factors, such as matrix stiffness (16, 17), to reorganize their cytoskeleton to form intracellular void spaces, termed vacuoles. These vacuoles then coalesce intercellularly and intracellularly to form lumen (7, 13–15). Sprouting and branching concurrent with ECM degradation and ECM remodeling culminate in the formation of perfusable, nascent vasculature (12–15). Both membrane-type and soluble matrix metalloproteinases (MMPs) are crucial throughout this process for matrix degradation and cell migration (11, 18). While these studies have uncovered many details of vascular morphogenesis that have transformed the understanding of how blood vessels form and reform, only a handful of studies describe another vasculogenic mechanism, termed “clusters-based” vasculogenesis herein.

Cluster-based vasculogenesis from circulating endothelial progenitor cells (EPCs) has been documented in animal models, but the underlying mechanism has not been studied. EPC cluster-based vasculogenesis has only been observed in hypoxic settings in vivo, including during development (19), ischemia (20), and tumor vascularization (21), suggesting the importance of low oxygen (O2) tension as a cue for this previously unknown mechanism. Here, circulating EPCs are mobilized and home to hypoxic and ischemic tissue through cytokine and growth factor gradients, particularly of stromal-derived factor 1 (SDF-1), as well as gradients of O2 (22). Recruited EPCs then attach to the activated endothelium local to the defect through hypoxia–up-regulated integrin-β2 (ITG-β2) and intercellular adhesion molecule–1 (ICAM-1) (23, 24). Last, EPCs extravasate through the endothelium to the interstitial tissue, form clusters, and then sprout to anastomose with existing blood vessels, thus revascularizing the local microenvironment (20, 21). While the recruitment of circulating EPCs to regions of hypoxia and ischemia has been well defined, it is not understood how clusters form and what drives subsequent vascular sprouting.

Hypoxia, defined as low O2 tension (<5%), has become recognized as a potent regulator of cell function and morphogenesis. Hypoxic microenvironments, accompanied by hypoxic gradients, exist throughout embryonic development (25), in tissue regeneration (26), and in tumor angiogenesis and progression (27), and are thus a critically important parameter to consider to fully understand these processes. Hypoxia stabilizes the transcription factors hypoxia-inducible factors (HIFs), which can up-regulate numerous proangiogenic factors, including vascular endothelial growth factor and MMPs (28–31). Further, hypoxia can act in a HIF-independent manner to promote production of reactive oxygen species (ROS) (32, 33). ROS can influence vascular morphogenesis and endothelial fate commitment (34, 35), thus providing a basis for their role in cluster formation. In addition, ROS are present in the inflammatory microenvironment in which neovessel formation is common (36).

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Current approaches to studying the effect of hypoxia on EPCs fail to recapitulate the range of $O_2$ tensions present in proangiogenic microenvironments, and thus may not capture the same responses observed in vivo. We developed oxygen-controllable hydrogels, which provide a means to establish and study the effects of hypoxic gradients in vitro (31, 37–40). These hydrogels provide a 3D system to examine EPC responses to a controllable microenvironment that recapitulates key parameters of the preregenerative milieu in which ECs establish new blood vessels. These hydrogels have enabled us to elucidate a previously unknown two-step mechanism for cluster-based vasculogenesis, including EPC hypoxic cluster formation and stabilization, followed by vascular sprouting into the stiffer, undergraded matrix and network formation.

RESULTS AND DISCUSSION
Hypoxia mediates endothelial cluster formation
Gelatin-based hypoxia-controllable (Gel-HI) hydrogels are prepared by conjugating phenol-containing ferulic acid (FA) to a gelatin backbone, followed by enzymatic cross-linking using a laccase-mediated reaction that consumes $O_2$, resulting in hypoxic conditions within the hydrogel (31, 37–40). Gelatin, hydrolyzed collagen, provides intrinsic bioactivity, which enables cellular adhesion, migration, degradation, and remodeling, all of which are necessary for vascular regeneration (12). Because these hydrogels are analyzed in conventional incubators (5% $CO_2$, atmospheric $O_2$), $O_2$ diffusion from the atmosphere competes with the $O_2$-consuming cross-linking reaction to create a gradient throughout the hydrogel. Depending on the height of the hydrogel, the $O_2$ gradient can be varied to create both hypoxic and nonhypoxic conditions (fig. S1, A and B). Following completion of the cross-linking reaction, $O_2$ returns to atmospheric conditions as $O_2$ diffuses into the gel (fig. S1B).

We found that encapsulation of a subtype of EPCs, endothelial colony-forming cells (ECFCs), at a high concentration in Gel-HI hydrogels leads to cluster formation in hypoxic but not nonhypoxic hydrogels (Fig. 1, A to C). By 6 to 12 hours after encapsulation, ECFCs commence cluster formation (Fig. 1B). At this time point, when clusters begin to form, an $O_2$ gradient is present within the hydrogel (fig. S1, C and D). Thus, analyzing discrete $z$ positions within our hydrogels revealed that cluster formation was consistently initiated at specific $z$ positions, namely, at ~250 $\mu$m above the bottom of the plate, corresponding to ~1% $O_2$ (Fig. 1, D and E, and figs. S1D and S2). Through 24 hours and up to 48 hours, clusters increase in size under hypoxic conditions (in terms of number of “cells in clusters”) and fall toward the bottom of the hydrogel. Accordingly, the number of “single cells” decreases as the number of cells in clusters increases (Fig. 1E and fig. S2). We observed consistent cluster size up to 48 hours, suggesting that the clusters we observe are the requisite size for this previously unknown mechanism of cluster-based vasculogenesis. Cells that participated in cluster formation appear to remain spherical throughout the 48-hour experiment (movies S1 to S4). In this case, we postulated that encapsulated ECFCs degrade their surrounding matrix and passively migrate to the space voided by degradation. In nonhypoxic hydrogels, clusters do not form, and cells remain isolated as single cells with cell elongation and vascular sprout formation (Fig. 1, F and G, and movies S5 to S8). Movie S5 observations under nonhypoxic conditions at $z$ = 0 show classical endothelial sprout formation by 24 hours. A comparison of this mechanism with the mechanism governing cluster formation displays a clear distinction between the two methods for cell movement and morphology.

To ensure that nutrient deprivation is not the determining factor guiding cluster formation, we generated hypoxic conditions in nonhypoxic hydrogel geometries using a custom gas flush chamber with $O_2$ sensing. Exposing ECFCs encapsulated in hydrogels with nonhypoxic geometry to 1% $O_2$ resulted in similar kinetics of cluster formation and nearly identical $O_2$ measurements (fig. S3, A and C). ECFCs in nonhypoxic control hydrogels did not form clusters (fig. S3B). Quantification of percent area covered by clusters confirms that hypoxia, rather than nutrient deprivation, drives cluster formation (fig. S3D). To summarize, ECFCs exposed to hypoxic conditions form cell clusters after 6 to 12 hours, thereby recapitulating the first step of an alternative mechanism for vascular morphogenesis.

ECFC cluster formation is governed by ROS production and dependent on matrix stiffness
To determine whether HIFs play a role in cluster formation, we co-encapsulated ECFCs with either dimethyloxalylglycine (DMOG) or cobalt (II) chloride hexahydrate (CoCl$_2$). These potent prolyl-4-hydroxylase (PHD) inhibitors, which act to stabilize HIFs under nonhypoxic conditions, have been used extensively in vitro and in preclinical models (41–43). Neither DMOG nor CoCl$_2$ treatment resulted in cluster formation in nonhypoxic geometries (Fig. 2A and fig. S4A). ECFCs subjected to DMOG treatment remained rounded up to 48 hours, while those exposed to CoCl$_2$ exhibited classical sprouting morphology, although to a similar extent to those cells in control nonhypoxic hydrogels. Small interfering RNA (siRNA) studies further showed that suppression of HIF1α does not affect cluster formation (Fig. 2B). These experiments suggest that HIF1α does not play a major role in cluster formation. To determine whether ROS production induces cluster formation, we used a ROS inhibitor, diphenyleneiodonium chloride (DPI) (34, 35). Addition of DPI inhibited cluster formation in hypoxic hydrogels (Fig. 2C and fig. S4B). In nonhypoxic controls, DPI inhibited endothelial sprouting, as seen in previous studies in 3D hydrogels (fig. S4B) (34). In summary, hypoxia is a crucial factor, which acts to promote ECFC clustering through HIF-independent ROS production.

In addition to hypoxia, another important parameter that guides vascular morphogenesis is matrix stiffness (16, 17, 37). The oxygen-controllable hydrogels can be designed to vary initial matrix stiffness by adding a secondary cross-linker, microbial transglutaminase (mTG) (37). mTG is a biocompatible cross-linker that has been used in numerous other hydrogel applications (44, 45). By cross-linking unconjugated primary amines on the gelatin backbone (Fig. 2D), addition of mTG can significantly increase the initial matrix viscoelasticity (Fig. 2E). Encapsulating ECFCs within dual-cross-linked Gel-HI hydrogels yields a profound reduction in cluster formation in hypoxic hydrogels (Fig. 2, F and G). While we attribute a reduction in cluster formation to an increase in initial matrix stiffness, addition of mTG may also influence the matrix microstructure, including pore size, which may also affect cluster formation.

Protease-mediated matrix degradation is required for hypoxic cluster formation and stabilization
Proteolytic degradation is a key mediator of cell migration and morphogenesis. To understand the role of matrix degradation in cluster formation, we established an assay using DQ-gelatin, which fluoresces as it is degraded by proteolysis and can identify a broad
range of protease activity with high sensitivity (46). We began by encapsulating fluorescent microspheres in our Gel-HI hydrogels, with or without MMP (collagenase IV) and tracked cluster formation and relative fluorescence with time-lapse microscopy (fig. S5, A to D). Clusters formed in the MMP-supplemented hydrogels only (fig. S5, C and D), where clusters began to form concurrent with increases in fluorescence (fig. S5, B to D). It is important to note that the microspheres used here may interact with one another through the hydrophobic effect, suggesting that this is not simply a random degradation-clustering event, but rather a more complex phenomenon requiring interactions between participating particles. As cells are not hydrophobic, this further highlights the importance of cell-cell interactions throughout the cluster formation process. We next used the DQ-gelatin assay in the ECFC–Gel-HI hydrogels. We observed cluster formation in hypoxic but not in nonhypoxic hydrogels (fig. S5, E and F), with a significant increase in fluorescence under hypoxic conditions after 24 hours, indicating the importance of protease production for cluster formation (Fig. 3A). It should be noted that an increase in fluorescence under nonhypoxic conditions supports the importance of protease production in classical vasculogenesis (11, 18). Moreover, fluorescence steadily increases starting at approximately 6 hours after encapsulation and continues to

Fig. 1. ECFC clusters form only under hypoxic conditions. (A) Schematic for hypoxic and nonhypoxic cell encapsulation. (B) Bright-field images of cell morphology in hypoxic and nonhypoxic hydrogels up to 48 hours. Hypoxic hydrogels exhibit cluster morphology starting at approximately 6 to 12 hours in culture. Clusters expand up to 48 hours. ECFCs encapsulated in nonhypoxic hydrogels do not form clusters but rather branch and sprout. (C) O$_2$ tension, measured at the bottom of the gel over 4 days in culture. (D) Time-lapse microscopy and (E) subsequent quantification of number of cells in clusters (top) and single cells (bottom) confirm observations of cluster formation kinetics in hypoxic hydrogels. (F and G) Clusters do not form in nonhypoxic hydrogels.
increase until it reaches saturation at approximately 18 hours, corroborating our observations of cluster formation at 6 to 12 hours after encapsulation (Fig. 3B). Rather than quantitatively identifying concentrations of proteases present, the trend of increased fluorescence informs our understanding of the temporal regulation of cluster formation via matrix degradation.

As matrix degradation is critical for cluster formation, we next sought to identify which proteases specifically regulate this process. We used a proteome profiler human protease array to identify relative quantities of soluble proteases in media collected from hypoxic and nonhypoxic hydrogels. Many proteases were present in the media under both conditions, including a bevy of cathepsins and MMPs, as well as ADAMTS1 and others (Fig. 3C and fig. S6A). Both cathepsins and MMPs have been inextricably linked to vasculogenesis (12, 47). Only one protease, MMP-1, was significantly more prevalent under hypoxic than under nonhypoxic conditions. 

To confirm the importance of MMP-1, we added a broad-spectrum inhibitor of MMPs, GM6001, and saw inhibition of clusters in the presence of this inhibitor (Fig. 3D and fig. S6B). It should be noted that a high concentration (1 mM) of GM6001 was required to inhibit cluster formation. Lower concentrations, closer to those previously published (5 to 500 μM) (11), did not inhibit cluster formation.

There are a number of potent upstream regulators of MMP, but perhaps most notably, ROS can rapidly induce MMP production in ECs (48). This rapid induction is of particular interest here, as cluster formation commences at 6 to 12 hours after encapsulation. To identify the contribution of ROS to MMP up-regulation, we performed the same experiment, but with control (no treatment) hypoxic hydrogels and hypoxic hydrogels with the addition of DPI.
Fig. 3. MMP-1–mediated matrix degradation is required for cluster formation and stabilization. (A) DQ quantification along culture period. Increasing fluorescence indicates the presence of proteases. H, n = 6; NH, n = 4. (B) Representative quantification of fluorescence signal over the course of the experiment. (C) Proteome profiler protease array showing that only MMP-1 is increased in hypoxic compared to nonhypoxic conditions. Results are presented as the mean pixel density of two arrays. (D) Media supplemented with 1 mM GM6001 inhibited cluster formation in hypoxic hydrogels. (E) Exogenous MMP supplementation facilitates dose-dependent cluster formation in nonhypoxic hydrogels. (F) Cluster stabilization is shown under hypoxic conditions by vascular endothelial cadherin (VE-cad) localization between some, but not all, cells in clusters, indicating cell-cell interactions (arrowheads). F-actin phalloidin staining localized at the cell membrane shows structures resembling filopodia (arrows). Clusters are rare in nonhypoxic hydrogels, but some cellular sprouting is present. (G) ICAM-1 and ITG-β2 are present at the cell membrane within hypoxia-induced clusters, indicating cell-cell interactions. Only a few cell-cell interactions are observed under nonhypoxic conditions. Scale bars, 20 µm. Graphical data in (A), (D), and (E) are reported as means ± SD. *P < 0.05, and ****P < 0.0001.
We observed cluster formation in control hydrogels, but not in DPI-treated hydrogels (fig. S6C), alongside a reduction in the fluorescence of DPI-treated hydrogels, suggesting that MMP up-regulation is at least partially regulated by ROS production (fig. S6C). A significant increase in fluorescence under both conditions indicates that protease production is not completely blocked by DPI.

To generate clusters under nonhypoxic conditions, we supplemented the cell media with MMP and found that with increasing concentrations of MMP, cellular clusters formed more rapidly (Fig. 3E and fig. S6D). With the highest concentration (100 μg/ml), the hydrogels were almost completely degraded by 24 hours. The reduction in area covered by clusters between 24 and 48 hours is due to an increase in cell cluster density between those two time points. In other words, cells are packed more tightly, thus covering a decreased percentage of the field of view. With the intermediate concentration (10 μg/ml), the kinetics and morphology of clusters more closely resembled hypoxic conditions. The lowest concentration of MMP (1 μg/ml) yielded sprouting reminiscent of classical single-cell vasculogenesis but no cluster formation. These results suggest that an unprecedented concentration of MMPs is required for cluster formation compared with those analyzed from patient samples and those typically associated with classical single-cell vasculogenesis (49–53).

Through this process, cell clusters remain intact as they grow in size and migrate through the ECM, indicating an active interaction between cells. ICAM-1 and ITG-β2 are vital toward attachment of size and migrate through the ECM, indicating an active interaction vasculogenesis (52–54). – samples and those typically associated with classical single-cell vasculogenesis compared with those analyzed from patient samples and those typically associated with classical single-cell vasculogenesis (49–53).

To evaluate the relevancy of our proposed model in vivo, we used a plug assay, using the oxygen-controllable hydrogel system. As EPC recruitment to ischemic tissues has been well defined (20, 22–24), we focused on cell behavior in a controlled environment after recruitment. Using this approach, we were able to mask the variable effects of cell recruitment as dependent on O2, by injecting hydrogels loaded with SDF-1α (58, 59). To determine early stages of EPC behavior in the hypoxic environment, we also injected green fluorescence protein (GFP)–tagged ECFCs via intracardiac injection (Fig. 5A) to follow their organization once recruited to the hydrogels. To control the O2 microenvironment in vivo, we injected the hydrogels with either calcium peroxide (CaO2), an O2-releasing molecule, for nonhypoxic hydrogels, or calcium hydroxide [Ca(OH)2], a decomposition product of CaO2, for hypoxic hydrogels (fig. S10A) (31). Within 12 hours, we could detect GFP-ECFCs recruited to both hypoxic and nonhypoxic hydrogels. O2 measurements in vivo confirmed differences between the two conditions up to the 12-hour time point (fig. S10B). GFP-ECFCs recruited to the nonhypoxic hydrogels appeared more singular, with some adopting a spindle-like shape (Fig. 5B, i) and some remaining rounded (Fig. 5B, ii). In contrast, GFP-ECFCs recruited to the hypoxic hydrogels were more often grouped in clusters with some already sprouting (Fig. 5B, iii and iv).
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Mouse lectin (GS-IB4) was used to identify host cells recruited to the hydrogels. Lectin-positive cells did not cluster as often in nonhypoxic hydrogels (Fig. 5B, i and ii) as in hypoxic hydrogels (Fig. 5B, iii and iv). The number of GFP cells in both conditions was similar, suggesting that differences in morphology arise from changes in O$_2$ (Fig. 5C). The area covered by clusters was slightly higher, and a statistically significant increase in the mean size of clusters was observed in the hypoxic hydrogels compared to nonhypoxic hydrogels (Fig. 5, D and E).
Fig. 5. Gel-HI hydrogels facilitate ECFC cluster formation in vivo. (A) Intracardiac injection of GFP-ECFCs, followed by injection of SDF-1α loaded nonhypoxic and hypoxic hydrogels into the flank of nu/nu mice. (B) Twelve hours after injection, GFP-ECFCs exhibited single-cell spindle (i) or rounded (ii) morphology in nonhypoxic hydrogels. Host cells (GS-IB4 lectin) were also present as isolated, rounded cells. Under hypoxic conditions, GFP-ECFCs were present in clusters (iii and iv), with some clusters containing sprouting cells (iii). Host cells also exhibited cluster morphology under hypoxic conditions (iii and iv). (C) Quantification revealed a similar number of cells under both conditions and (D) a slight increase in percent area covered by clusters under hypoxic compared to nonhypoxic conditions. (E) Clusters were larger under hypoxic than under nonhypoxic conditions. (F) Intracardiac injection of GFP-ECFCs followed by injection of SDF-1α loaded hypoxic (ctl) and hypoxic (DPI) hydrogels into the flank of nu/nu mice. (G) Twelve hours after encapsulation, GFP-ECFCs were present as both single cells (ii and iii) and clusters (i and iv) under both conditions. (H) An increased number of GFP+ cells were present in the control group, and (I) the percent area covered by clusters was increased in the control versus DPI-treated group. (J) Mean cluster sizes between the two groups were not statistically significantly different. n = 6 nu/nu mice per experiment. Graphical data in (C), (D), (H), and (I) are reported as box and whisker plots from minimum to maximum. Graphical data in (E) and (J) are reported as means ± SD, with all points denoted by dots. *P < 0.05.
Inhibition of ROS with DPI resulted in complete blocking of cluster formation under hypoxic conditions in vitro (Fig. 2C). Thus, we co-encapsulated 20 μM DPI within hypoxic hydrogels and then injected the gels into the mouse flank. DPI-treated hydrogels were compared to hypoxic (ctl) hydrogels. GFP-ECFCs were again injected via intracardiac injection and tracked 12 hours after injection (Fig. 5F). GFP-ECFCs were recruited to both DPI-treated and hypoxic (ctl) hydrogels (Fig. 5G). Under both conditions, cells exhibited both single-cell (Fig. 5G, ii and iii) and cluster morphology (Fig. 5G, i and iv). However, clusters were more prevalent in hypoxic (ctl) hydrogels than in DPI-treated hydrogels. DPI appeared to have a multipotent effect, both reducing the total number of GFP⁺ cells recruited (Fig. 5H) and reducing the total area covered by clusters (Fig. 5I). While the percent area covered by clusters was decreased by the addition of DPI, the mean cluster size was not significantly different between the two groups (Fig. 5J). Although DPI does not completely block cluster formation in vivo, it does have a significant effect on both cell number and area covered by clusters, confirming the role of ROS in cluster formation, even in a more complex in vivo microenvironment. This result supports the highly biomimetic nature of our in vitro platform as a method to identify regulators of complex biological phenomena.

The existence of cluster-based vasculogenesis has been documented in numerous animal development and disease models (19–21). While each of these animal model systems was different in a variety of ways, they all shared the important characteristic of a hypoxic microenvironment in which ECs interacted to form neovessels. By establishing a controllable environment to study cluster-based vasculogenesis in vivo, we were able to observe cluster formation at a similar time scale in vivo to that which we observed in vitro, as well as confirm the importance of a key regulator of the mechanism.

**Defining a new mechanism for cluster-based vasculogenesis**

The bulk of the literature describing postnatal vasculogenesis has focused on the study of single-cell vasculogenesis. In this mechanism, cells initiate interactions with their surrounding ECM and then proceed to form vacuoles and lumen through cytoskeletal rearrangements in response to an assortment of angiogenic growth factors. Cell-mediated local matrix degradation then permits vascular branching and sprouting to form vascular networks (12). This mechanism has informed countless studies ranging from those in vascular biology to the clinic and should never be discounted or neglected. However, an understudied, parallel mechanism appears to contribute to vasculogenesis in hypoxic microenvironments, which has been documented in numerous animal development and disease models (19–21). An understanding of this mechanism is critical to expand upon our understanding of vascular morphogenesis, as hypoxia is a key regulator in most niches that induce vascular growth. To reestablish vasculature and aid tissue regeneration, EPCs are recruited along O₂ and chemokine gradients to regions in need of neovascularization (22). These EPCs leave the preexisting vasculature to occupy the hypoxic niche (Fig 6A). From here, we have defined a mechanism by which EPCs (ECFCs) form vasculature in response to their surrounding hypoxic microenvironment. EPCs rapidly respond to the new hypoxic microenvironment and produce ROS that up-regulates proteases, which degrade the surrounding ECM to facilitate EPC cluster formation (Fig. 6B). As clusters continue to degrade the matrix, the number of cells per cluster increases, while clusters are stabilized through ITG-β2, ICAM-1, and VE-cad (Fig. 6C). Last, EPCs in clusters engage with the surrounding stiffer ECM to sprout and form vascular networks (Fig. 6D). Overall, using an oxygen-controllable 3D environment, we were able to observe cluster formation at a similar time scale both in vitro and in vivo, allowing us to recapitulate and understand...
the hypoxic conditions that activate the signaling pathway of cluster-based vasculogenesis.

**MATERIALS AND METHODS**

Gelatin (Gtn, from porcine skin gel strength 300, Type A; G2500), trans-4-hydroxy-3-methoxycinnamic acid (FA; 128708), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC; E6383), N-hydroxysuccinimide (NHS; 56480), dimethyl sulfoxide (DMSO; 276855), laccase (lyophilized powder from mushroom, ≥4.0 U/mg; 75117), DMOG (D3695), CoCl2 (C8661), DPI (D2926), CaO2 (466271), Ca(OH)2 (239232), 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; 10236276001), bovine serum albumin (BSA; A3059), paraformaldehyde (PFA; P6148), and Hoechst Stain solution (Hoechst; H6024) were purchased from Sigma-Aldrich (St. Louis, MO) and used as obtained without purification. Microbial transglutaminase (mTG) (Activa-TI) was obtained from Ajinomoto Inc. Dulbecco’s phosphate-buffered saline (DPBS; 14190250), tryspin-EDTA 0.05% (25300120), DQ Gelatin from Pig Skin Fluorescein Conjugate (DQ-gelatin; D12054), Collagenase Type IV powder (17104019), Alexa Fluor 488 phalloidin (phalloidin; A21283), Isolectin GS-IB4 Alexa Fluor 568 Conjugate (I21412), Halt Protease and phosphatase inhibitor cocktail (100×) (78440), formaldehyde 37% by weight (formaldehyde; F79), Triton X-100 (85111), Tween-20 (BP337), Donkey anti-Goat Secondary Antibody Alexa Fluor 488 (gt488; A11055), Donkey anti-Mouse Secondary Antibody Alexa Fluor 546 (ms546; A10036), Antibody diluent (003218), and BD Lo-Dose U-100 insulin needles (28 gauge) (BD329461) were all purchased from Thermo Fisher Scientific (Waltham, MA). Dialysis membranes (molecular mass cutoff = 3500 Da) (132724) were purchased from Spectrum Laboratories (Rancho Dominguez, CA). Endothelial growth media–2 (EGM2; CC-3162) was purchased from Lonza (Walkersville, MD) and supplemented with additional characterized HyClone FBS from GE Healthcare Life Sciences (Hyclone FBS; SH30071.03) (Logan, UT) and used to culture ECFCs (provided by M. Yoder, Indiana University School of Medicine) on collagen I, rat tail (Col 1; 354236) from Corning (Corning, NY) coated cell culture plates. O2 (1%) was purchased from Airgas (Radnor, PA). Envy Green fluorescent microspheres (PS065F) were purchased from Bangs Laboratories Inc. (Fishers, IN). GM6001 InSolution (GM6001; 364206) and GM6001 (364205) were purchased from EMD Millipore (Burlington, MA). VE-cadherin antibody (sc-9989) was purchased from Santa Cruz Biotechnology (Dallas, TX). ICAM-1 antibody (BBA3), integrin-β2 antibody (AF1730), SDF-1α (350-NS-010), and the Human Protease Proteome Profiler Array Kit (ARY021B) were purchased from R&D Systems (Minneapolis, MN). SMARTpools:siGENOME HIF1A siRNA (M-004018-05-005), siGENOME Non-Targeting siRNA 1 (D-001210-01-05), and DharmaFECT2 (T-2002-02) were purchased from Dharmacon Inc. (Lafayette, CO).

**Synthesis of gelatin–g–ferulic acid**

Gelatin–g–ferulic acid (Gtn-FA) was synthesized using EDC and NHS as coupling reagents. A mixture of DMSO and distilled (DI) water (1:1 volume ratio) was prepared as a solvent. Gtn (1.0 g) was dissolved in 50 ml of the solvent at 40°C. FA (0.777 g, 4.0 mmol) was dissolved in 20 ml of the solvent and reacted with EDC (0.92 g, 4.8 mmol) at room temperature for 15 min and then with NHS (0.64 g, 5.6 mmol) at room temperature to activate the terminal carboxyl groups of FA (carboxyl/EDC/NHS = 1:1.2:1.4). The activated solution was then added to the Gtn solution, and a conjugative reaction was conducted at 40°C for 24 hours. Following completion of the reaction, the solution was washed against DI water for 5 days (molecular mass cutoff = 3500 Da) and then lyophilized.

**Characterization of hydrogel precursor solutions**

The degree of substitution (DS) of FA was measured using a UV/vis spectrometer (SpectraMax; Molecular Devices, Sunnyvale, CA). Gtn-FA polymer (10 mg) was dissolved in 1 ml of the DMSO/DI water (1:1) solvent, and the absorbance was measured at 320 nm. The concentration of conjugated FA molecules was calculated from a calibration curve given by monitoring the absorbance of known concentrations of FA.

**Preparation of hypoxia-inducible hydrogels**

Hydrogel precursor solutions (Gtn-FA, laccase, and mTG) were prepared in DPBS. Enzymes were used at final concentrations of 25 U/ml (laccase) (31, 37) and 0.3 U/ml (mTG) (37). Hypoxia-inducible (HI) hydrogels were prepared by mixing aqueous Gtn-FA polymer with laccase and/or mTG solutions. Hydrogels were prepared in a 3:1 polymer/enzyme ratio. Polymer solutions [Gtn-FA, 4.0 wt% (weight %) and enzyme solutions [laccase (100 U/ml) and/or mTG (1.2 U/ml)] were mixed by pipetting to form hydrogels. All gels were formed at 37°C.

**O2 measurements**

We measured dissolved O2 (DO) levels noninvasively in both acellular and cell-encapsulated hydrogels at the bottom of hydrogels using commercially available sensor patches (Oxygen Sensor Spot; SP-Pst3) and a multichannel fiber-optic oxygen meter (OXY-4 mini) from Presens (Regensburg, Germany). To measure O2 levels at the bottom of hydrogels, the hydrogels were added on top of the sensors, which were immobilized in each well of a 96-well plate. All experiments were conducted in a controlled environment at 37°C and 5% CO2 in a standard incubator.

O2 gradients were measured in preformed cell-encapsulated hydrogels at specified time points (continuous gradient measurements are not possible with our O2 sensors). Commercially available needle-type oxygen microsensors (Oxygen Microsensor; NTH-Pst1) and a microfiber optic oxygen transmitter (Microx TX3) from PreSens were used to measure O2 gradients. Sensors were calibrated using atmospheric and anoxic (N2 flush) conditions at 37°C. O2 sensors were precisely controlled using a Manual Micromanipulator MM (PreSens). Starting at the bottom of the hydrogel, measurements were recorded (when the reading stabilized) every 250 μm within the hydrogel and every 500 μm or 1 mm within the media.

**Measurement of viscoelastic properties**

Rheological analysis of the Gel-HI hydrogels was performed using a rheometer (AR1500ex; TA Instruments, New Castle, DE) with an 8-mm parallel plate geometry. For analysis of viscoelastic properties of preformed hydrogels in their equilibrium swelling state, hydrogel discs were prepared and swelled in DPBS. For dynamic time sweep experiments, we monitored the elastic modulus (G′) and viscous modulus (G″) at 1% strain and a frequency of 0.1 Hz at 37°C with a 1.3-mm gap.
CoCl$_2$ were made fresh before each use in diH$_2$O [according to the manufacturer and previous literature (41)]. Stock solutions of commercially available O$_2$ sensors (PreSens). In particular, 1% O$_2$ added to nonhypoxic hydrogels, and cells were cultured under standard cell culture conditions (37°C, 5% CO$_2$) for up to 3 days. The culture medium was replaced daily. Bright-field images were captured at predetermined time points to monitor cell morphology using an Olympus IX50 (Olympus; Center Valley, PA). Additional details regarding encapsulation in Gel-HI hydrogels can be found in (39). ECFCs in Gel-HI were observed via time-lapse microscopy for up to 48 hours using a Nikon Eclipse Ti (Nikon; Melville, NY) or a Zeiss AXIO Observer Z.1 (Carl Zeiss; Oberkochen, Germany) under standard cell culture conditions (37°C, 5% CO$_2$).

**Mimicking hypoxic conditions in nonhypoxic geometries (1% O$_2$ flush, DMOG, CoCl$_2$)**

To mimic hypoxic conditions in nonhypoxic geometries, a custom hypoxia chamber was designed using AutoCAD (Autodesk; San Rafael, CA) and machined at the Johns Hopkins University Whiting School of Engineering Machine Shop. This custom hypoxia chamber facilitated flow of mixed gases and real-time monitoring of O$_2$ using commercially available O$_2$ sensors (PreSens). In particular, 1% O$_2$ (Airgas; Radnor, PA) was continually flushed through the chamber commencing immediately following ECFC encapsulation, and O$_2$ was monitored along the culture period. Bright-field images were captured at predetermined time points to monitor cell morphology (Olympus IX50).

DMOG and CoCl$_2$ act as PHD inhibitors, which up-regulate HIFs. Stock solutions of DMOG were made in diH$_2$O [according to the manufacturer and previous literature (41)]. Stock solutions of CoCl$_2$ were made fresh before each use in diH$_2$O [according to the manufacturer and previous literature (43)]. DMOG (1 mM) or CoCl$_2$ (200 μM) was co-encapsulated with ECFCs. Bright-field images were captured at predetermined time points to monitor cell morphology (Olympus IX50). ImageJ (public domain) was used to quantify cell clusters. Graphs were generated using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA).

**siRNA transfection**

ECFCs were transfected with SMARTpool:siGENOME HIF1A and Non-Targeting siRNA 1 (scr) according to the manufacturer’s protocol and previous literature (31). Briefly, cells were seeded on a six-well plate and treated with 50 nM siRNA. We confirmed knockdown via quantitative real-time fluorescence polymerase chain reaction after 24 hours and used transfected cells in experiments. ImageJ (public domain) was used to quantify cell clusters. Graphs were generated using GraphPad Prism 6 (GraphPad Software Inc.).

**ROS inhibition (DPI)**

DPI is a potent inhibitor of ROS (citations). DPI stock solutions were prepared at 3.5 mM in DMSO. ECFC medium (EGM2) was supplemented with 20 μM DPI, and we captured bright-field images at predetermined time points to monitor cell morphology (Olympus IX50). ImageJ (public domain) and a custom MATLAB script (MathWorks) were used to quantify cell clusters. Graphs were generated using GraphPad Prism 6 (GraphPad Software Inc.).

**Altering matrix stiffness with mTG**

To increase matrix stiffness, mTG was added as a secondary cross-linker to laccase stock solutions to a concentration of 1.2 U/ml, as described previously (37) to achieve a final working concentration of 0.3 U/ml. ECFCs were encapsulated within these stiffer gels and monitored by both bright-field imaging (Olympus IX50) and time-lapse microscopy (Nikon Eclipse Ti or a Zeiss AXIO Observer Z.1) under standard cell culture conditions (37°C, 5% CO$_2$). ImageJ (public domain) was used to quantify cell clusters.

**Dynamic matrix stiffening**

At predetermined time points (24 hours), media changes were accompanied by supplemental addition of mTG (0.6 to 2.4 U/ml). Rheological analysis (AR1500ex; TA Instruments) was performed as described above using an immersion cup. We assume uniform secondary cross-linking by the addition of supplemental mTG. While diffusion of mTG into the existing matrix potentially leads to a nonuniform matrix stiffness, we assume uniformity through a measurement of bulk matrix properties. ECFCs encapsulated in Gel-HI were monitored up to day 3 by capturing bright-field images (Olympus IX50), at which point they were fixed and stained with phalloidin and DAPI as described above. Vascular networks and lumen were observed using confocal microscopy (LSM 780; Zeiss). Networks with and without the addition of mTG were analyzed.

**Protease activity assay**

DQ-gelatin (Thermo Fisher Scientific) fluoresces in the presence of proteases. Stock solutions of DQ-gelatin were made according to the manufacturer’s instructions (1 mg/ml in diH$_2$O). DQ-gelatin was co-encapsulated with Gel-HI hydrogels to a final concentration of 100 μg/ml. Fluorescent microspheres (Bangs Laboratories Inc.) or ECFCs were encapsulated in Gel-HI, and cluster formation was monitored via time-lapse microscopy (Zeiss AXIO Observer Z.1). DQ-gelatin fluorescence (488/515 nm excitation/emission) signal was continuously monitored in fluorescent microsphere experiments and quantified using ImageJ (public domain). Cell experiments required different hydrogel geometries; thus, fluorescence was measured at time 0 (T0) and time 24 hours (T24) using a SpectraMax Gemini XPS (Molecular Devices; Sunnyvale, CA) and graphed using GraphPad Prism 6 (GraphPad Software Inc.).

**Proteome profiler array**

Medium was collected after 24 hours of culture from two hypoxic and four nonhypoxic hydrogels per experiment to normalize for cell number. Immediately after collection, protease inhibitors (Halt Protease and phosphatase inhibitor cocktail) were added to the medium, and then the medium was stored at −80°C until use. Arrays were prepared according to the manufacturer’s protocol. Films were exposed for 20 min and then analyzed using ImageJ (public domain).
and graphed using GraphPad Prism 6 (GraphPad Software Inc.). Values of mean pixel density were reported.

**Addition of exogenous MMP**

Following ECFC encapsulation in Gel-HI, ECFC medium (EGM2) was supplemented with exogenous MMP (collagenase type IV; Thermo Fisher Scientific) at a range of concentrations. Bright-field images were captured at predetermined time points to monitor cell morphology (Olympus IX50). ImageJ (public domain) and a custom MATLAB script (MathWorks) were used to quantify cell clusters. Graphs were generated using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA).

**Observation of cell-cell interactions**

After 24 hours in culture, ECFCs within Gel-HI hydrogels were fixed with 2% FA for 20 min at room temperature. Hydrogels were then washed three times with 1× DPBS with 10 min in between each wash. Encapsulated ECFCs were then permeabilized with 1% Triton X-100 for 15 to 20 min and then washed three times with 1× DPBS with 10 min in between each wash. Next, hydrogels were incubated in 5% BSA blocking solution for 1 hour at room temperature and then washed with 0.05% Tween-20 with 1× DPBS three times with 10 min in between. Hydrogels were then stained with primary antibody in antibody diluent solution overnight at 4°C and then washed with 0.05% Tween-20 in 1× DPBS three times with 10 min in between. Hydrogels were then incubated in secondary antibody in antibody diluent solution for 2 hours at room temperature and then washed with 0.05% Tween-20 in 1× DPBS three times with 10 min in between. Last, hydrogels were incubated in DAPI solution for 15 min at room temperature and then washed with 1× DPBS three times with 10 min in between. Hydrogels were analyzed using confocal microscopy (LSM 780; Zeiss). Primary antibodies anti-ICAM-1 and ITG-β2 (R&D Systems) were diluted in sterile DBPS to 500 and 200 μg/ml, respectively, and were used at a final concentration of 15 μg/ml in antibody diluent (Thermo Fisher Scientific), according to the manufacturer’s protocol. VE-cadherin antibody (Santa Cruz Biotechnology) was used at a 1:50 dilution in antibody diluent. Secondary antibodies, gt488 and ms 546, were used at 1:250 in antibody diluent and phalloidin was used at 1:500 in antibody diluent.

**Plug assay in vivo**

Acellular hydrogels were injected into the flank of nu/nu female mice (8 to 10 weeks old) (nu/nu 088 homozygous; Charles River) using insulin needles (28 gauge). Hydrogel (200 μl) was injected with CaO2 (nonhypoxic) or Ca(OH)2 (hypoxic), as described previously (31). Briefly, CaO2 decomposes and releases O2 when it comes into contact with H2O. As such, we mixed CaO2 as an O2-releasing compound for nonhypoxic hydrogels. For hypoxic hydrogels, we mixed Ca(OH)2, a by-product of CaO2 decomposition, to minimize the differences in hydrogel composition between the two groups. We mixed sterilized CaO2 or Ca(OH)2 with the polymer solution immediately before hydrogel injection to achieve a final concentration of 0.02 wt% CaO2 or Ca(OH)2. In addition, GFP-ECFCs (provided by M. Yoder and transfected by K. Eisinger and K. Pak; University of Pennsylvania) were injected via intracardiac injection (1 million cells per 100 μl of injection) with insulin needles (28 gauge). SDT-1α (100 ng/ml; R&D Systems) was co-encapsulated within hydrogels to enhance recruitment of circulating EPCs, including host cells and injected GFP-ECFCs (58, 59). DPI (20 μM) or vehicle control (DMSO) was co-encapsulated where indicated. At 12 hours, mice were euthanized and hydrogels were fixed overnight in 2% FA. Following fixation, hydrogel was washed with 1× DPBS three times, stained with Isol lectin GS-IB4 Alexa Fluor 568 Conjugate (1:200 in antibody diluent) (Thermo Fisher Scientific) for 2 hours at room temperature, and then washed with 1× DPBS three times with 10 min in between. Hydrogels were then incubated in Hoechst solution (1:2000 in DPBS) for 10 min at room temperature and then washed with 1× DPBS three times with 10 min in between. Stock solutions of Isol lectin GS-IB4 and Hoechst were prepared according to the manufacturer’s instructions. Recruited cells were analyzed using confocal microscopy (LSM 800; Zeiss). All animal studies were performed following approval by the Johns Hopkins University Institutional Animal Care and Use Committee (MO15A145, MO18A244).

**O2 measurements in vivo**

Commercially available needle-type oxygen microsensors (Oxygen Microsensor; NTH-Pst1) and a microfiber optic oxygen transmitter (Microx TX3) from PreSens were used to measure in vivo O2. After 12 hours, needle-type sensors, controlled by a micromanipulator and concurrent with temperature readings using a manufacturer-provided thermometer, were inserted into the hydrogel, and O2 was recorded when the reading stabilized. All animal studies were performed following approval by the Johns Hopkins University Institutional Animal Care and Use Committee (MO15A145, MO18A244).

**Image quantification**

Analysis of cells and clusters during time-lapse experiments was performed using ImageJ (public domain) and a custom MATLAB script (MathWorks; Natick, MA). Analysis of vessel characteristics was performed using ImageJ (public domain) and MetaMorph (Universal Imaging, Downingtown, PA). Analysis of stress fibers was performed using ImageJ (public domain) and a custom MATLAB script developed in the laboratory (MathWorks; Natick, MA) (60). Analysis of GFP-ECFCs in vivo was performed using Imaris (Bitplane, Belfast, UK) and ImageJ (public domain). All graphs were made using GraphPad Prism 6 (GraphPad Software Inc.).

**Statistical analysis**

We performed statistical analysis using GraphPad Prism 6 (GraphPad Software Inc.). We also used this software to perform t tests to determine significance. Replicates are indicated throughout the figure captions. All graphical data are reported as means ± SD. Significance levels were set at *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. All graphical data were reported.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/3/eaau7518/DC1

Fig. S1. Hydrogel height controls O2 gradients.

Fig. S2. Cluster size over time.

Fig. S3. Low O2 tension, rather than diffusional limitations or nutrient deprivation, facilitates cluster formation.

Fig. S4. Cluster formation is HIF independent and ROS-mediated.

Fig. S5. Experimental setup for time-lapse monitoring of increases in fluorescence upon proteolytic degradation of DQ-gelatin.

Fig. S6. Protease activity and inhibition.

Fig. S7. Vascular sprouting from clusters.
Fig. S8. Dynamic matrix stiffening accelerates vascular network formation.

Fig. S9. Increased matrix viscoelasticity influences vascular network formation.

Fig. S10. In vivo O2 measurements.

Movie S1. Time lapse of ECFCs encapsulated under hypoxic conditions at z = 0 µm above the bottom of the plate.

Movie S2. Time lapse of ECFCs encapsulated under hypoxic conditions at z = 100 µm above the bottom of the plate.

Movie S3. Time lapse of ECFCs encapsulated under hypoxic conditions at z = 200 µm above the bottom of the plate.

Movie S4. Time lapse of ECFCs encapsulated under hypoxic conditions at z = 300 µm above the bottom of the plate.

Movie S5. Time lapse of ECFCs encapsulated under nonhypoxic conditions at z = 0 µm above the bottom of the plate.

Movie S6. Time lapse of ECFCs encapsulated under nonhypoxic conditions at z = 100 µm above the bottom of the plate.

Movie S7. Time lapse of ECFCs encapsulated under nonhypoxic conditions at z = 200 µm above the bottom of the plate.

Movie S8. Time lapse of ECFCs encapsulated under nonhypoxic conditions at z = 300 µm above the bottom of the plate.

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