Assessing the Minimum Time-Period of Normoxic Preincubation for Stable Adipose Stromal Cell-Derived Vascular Networks

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Abstract

Introduction—Pre-vascularization of tissue engineered grafts is a promising strategy to facilitate their improved viability following *in vivo* implantation. In this process, endothelial cells (ECs) form capillary-like networks that can anastomose with host vasculature. Adipose-derived stromal cells (ASCs) are a commonly used cell population for tissue engineering and contain a subpopulation of ECs capable of assembling into robust vascular networks and anastomosing with the host. However, their initial vascular assembly is significantly impaired in hypoxic conditions (2% O_2). In this study, we explored the minimum period of normoxic (20% O_2) pretreatment required to enable the formation of stable vascular networks.
Methods—ASC-derived vascular structures were allowed to preassemble in fibrin hydrogels in normoxia for 0, 2, 4, or 6 days and then transplanted into hypoxic environments for 6 days. Total vascular length, pericyte coverage, cell proliferation, apoptosis rates, and ECM production was assessed.
Results—Vascular assembly increased with time over the 6 days of culture. We found that 4 days was the minimum period of time required for stable vascular assembly. We compared the major differences in cell behavior and network structure at Days 2 and 4. Neither proliferation nor apoptosis differed, however, the Day 4 time-point was associated with a significant increase in pericyte coverage (46.1 ± 2.6%) compared to Day 2 (24.3 ± 5.3%).
Conclusions—These data suggest oxygen tension may be a mediator of EC–pericyte interactions during vascular assembly. Pre-vascularization strategies should incorporate a normoxic period of to enable successful vascular formation and development.

Keywords—Oxygen tension, Hypoxia, Implantation, Endothelial cells, Pericytes, Preassembly.

INTRODUCTION

Tissue engineered implants have the potential to treat a number of large volumetric musculoskeletal disorders and defects[29] but are severely limited in scale by a lack of a perfused blood vessel networks.[44] The inclusion of functional vascular networks within tissue engineered constructs is a promising strategy when scaling from mouse- to human-sized applications in order to provide the oxygen, nutrients, and waste removal needed by tissues.[38] Neovascularization is a slow process with rates less than 1 mm/week[50] and cellular constructs with thicknesses greater than 400 μm become rapidly necrotic due to the diffusion limitation of oxygen.[20] Hence, the inability to provide sufficient vascularization has limited the clinical use of tissue engineered implants. Several approaches to create tissue engineered scaffolds with functional vascular networks have been reported. These include 3D-printing channels for vessel ingrowth,[32] releasing angiogenic growth factors from the scaffold,[6] and periods of *in vitro* culture to 'pre-vascularize' the construct before implantation.[11,28]

Pre-vascularization is a highly promising strategy in which endothelial cells (ECs) are stimulated to form nascent capillary-like vascular network structures that can anastomose with the host vasculature. In order to form stable networks, ECs are typically co-cultured with fibroblasts,[30] mesenchymal stem cells,[8,48] or pericytes,[49] which act as perivascular cells and stabilize the vascular networks. Recently, adipose-derived stromal cells (ASCs) from human lipoaspirate tissues have been shown to be a suitable source of ECs and perivascular cells. The vascular potential of ASCs arises, due to a sub-population of ECs at early passages[23,33] as well as their limited ability to differentiate into ECs.[3,13] ASCs

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also include a pericyte or pericyte-like population[1] and pericytes have potential to modulate the effect of angiogenic therapies.[27] When cultured in fibrin hydrogels, the EC sub-population within ASCs exhibit the potential to form extensive, interconnected vascular network structures[22,24] that survive *in vivo* implantation.[22]

The capacity of ECs and ASCs to assemble into vascular networks is highly oxygen-dependent. Specifically, hypoxic conditions ($< 5\%$ O_2) inhibit the self-assembly of vascular networks. Hypoxia (5% O_2) prevented the self-assembly of vascular networks in a co-culture of human endothelial colony forming cells and multipotent stromal cells.[16] Griffith and George have shown that in addition to inhibiting self-assembly, challenging constructs with hypoxia after a period of preassembly leads to the degradation of capillary networks.[17] More recently, it has been shown that hypoxia inhibits *de novo* assembly of ASC-derived vascular networks.[21] These reports run counter to the predominant narrative of hypoxia as a pro-angiogenic stimulus. In fact, while hypoxia drives new blood vessel formation *via* angiogenesis (and is a key mechanism underlying tumor vascularization), low oxygen tension is a known inhibitor of vascular assembly. Interestingly, while hypoxia inhibits the *de novo* vascular assembly of ASCs and their EC sub-populations, transferring pre-assembled ASC-vessels into hypoxic environments stimulated their growth.[21] Prevascularization strategies therefore require understanding the impact of oxygen on the kinetics of assembly and stabilization.

In this study, we aimed to determine the minimum amount of normoxic (20% O_2) preassembly time that is needed for ASC-vascular networks to stabilize so that they when transferred to hypoxic (2% O_2) conditions, the vessels would continue to elongate. To assess this, ASCs were cultured in fibrin hydrogels and allowed to pre-assemble into vascular network structures for 0, 2, 4, or 6 days. At the end of each of these pre-incubation periods, we split the cultures into two and transferred one group to continued normoxic cultures and the other group to hypoxic (2% O_2) culture conditions for a further 6 days (Fig. 1). We assessed geometric and cellular properties of the networks to establish the underlying mechanism mediating the switch in behavior of ECs to hypoxic environments. These studies suggest that the interactions between ECs and pericyte-like populations are strongly oxygen- and time-dependent. Further understanding of these interactions may be critical for exploiting the vascular capacity of ASCs in pre-vascularization strategies.

MATERIALS AND METHODS

ASC Isolation and Culture

Human subcutaneous adipose tissue was obtained in the form of lipoaspirate from three female Caucasian donors undergoing elective surgery and with written informed consent under the approval of the Johns Hopkins Medicine Institutional Review Board. ASCs were isolated as previously described.[9,21] Briefly, tissue was digested with collagenase (1 mg/mL; Worthington Biochemical Corp.) to isolate the stromal vascular fraction of cells. These cells were plated onto tissue culture plastic and were termed "passage 0 ASC" when they reached 80–90% confluence. ASCs were used at passage 2 for all experiments. Growth medium consisted of: high glucose DMEM (Gibco) with 10% fetal bovine serum (FBS; Atlanta Biologicals), 1% penicillin/streptomycin (Gibco), and 1 ng/mL basic fibroblast growth factor (FGF-2; PeproTech).

Cell Aggregation via Suspension Culture

Cells were trypsinized and resuspended at a concentration of 250,000 cells/mL in growth medium containing 0.24% (w/v) methylcellulose (Sigma). The cell suspension was pipetted into 10-cm Petri dishes coated with 2% (w/v) agarose to minimize cellular adherence to the dish. After overnight suspension culture, cellular aggregates were collected with a pipette, and then centrifuged before encapsulation procedures.

Aggregate Encapsulation and Culture

Pre-assembly Culture

Cell aggregates were suspended in fibrinogen (8 mg/mL final; Sigma) and thrombin (2 U/mL final; Sigma) at a final cell concentration of 2×10^4 cells/μL. Fibrin gels were formed by pipetting 12 μL of gel solution into 4-mm diameter wells and incubating at 37 °C for 30 min to allow complete gelation before adding medium. Each gel sample was fed with 1 mL of culture medium containing: endothelial basal medium-2 (EBM-2, Lonza), 10% FBS, and 1% penicillin/streptomycin. The media was not supplemented with growth factors beyond those naturally present in serum. To assess the effect of preassembly on future hypoxic cultures, freshly encapsulated cells were cultured in normoxia (20% O_2) for 0, 2, 4, or 6 days with the medial changed every other day to create different degrees of vascular networks.
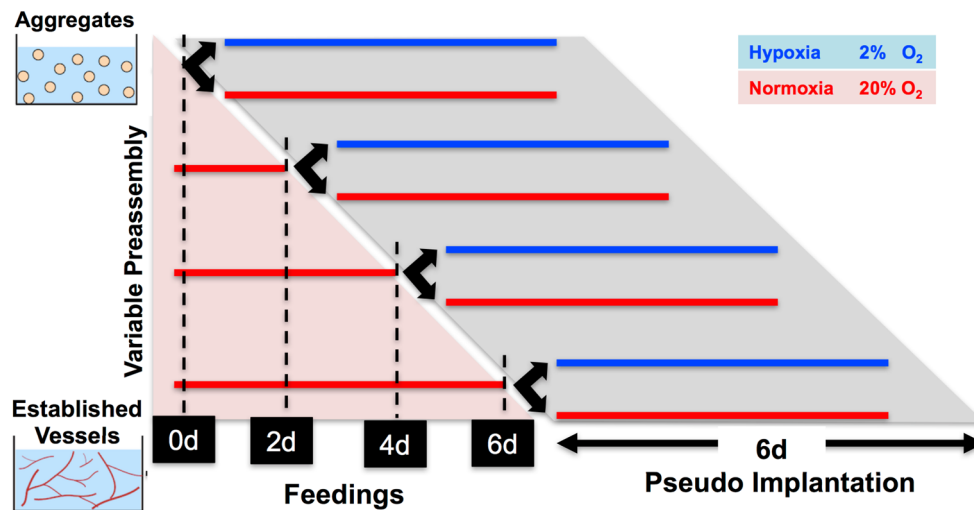
FIGURE 1. Schematic of the study gels of ASC aggregates were allowed to preassemble for 0, 2, 4, or 6 days in normoxia with regular feedings before moving into a pseudo-implant condition for 6 days.

Pseudo-implantation Culture

On the last day of preassembly, the samples were fed once more and then cultured in either normoxia or hypoxia (2% O_2) for an additional 6 days with no media changes (Fig. 1). Normoxic samples were maintained in a 37 °C incubator with 5% CO_2, 95% ambient air. Hypoxic samples were placed in a modular incubator chamber (Billups-Rothenberg) that was flushed every day with pre-mixed gas (2% O_2/5% CO_2/ N_2 balance) and placed in a 37 °C incubator.

Proliferation Labelling

Cells were incubated with bromodeoxyuridine (BrdU, Sigma) to detect proliferating cells. Briefly, 10 μM BrdU was pipetted into existing culture medium (i.e., medium was not changed), and samples were quickly returned to their appropriate oxygen environment (less than 5 min of normoxic exposure) for a 20-h incubation. Samples were then washed with PBS and fixed with 3.7% formaldehyde.

Whole-Mount Immunostaining

Whole-mount immunostaining of fibrin gels was performed as previously described.[24] Briefly, samples were fixed with 3.7% formaldehyde for 3 h at 4 °C, washed with PBS, and blocked with 5% normal goat serum/0.2% Triton X-100/PBS for 3 h at 4 °C. Antibodies were incubated overnight at 4 °C, followed by three 1-h washes in PBS with 0.1% Tween. Primary antibodies included: mouse anti-human CD31 (4 μg/ mL, Sigma), mouse anti-human collagen IV (20 μg/ mL, Santa Cruz Biotech), and Cy3-conjugated mouse anti-alpha smooth muscle actin (αSMA; 7 μg/mL,

Sigma). Secondary antibodies used included: DyLight 488-conjugated goat anti-mouse (3.75 μg/mL, Jackson ImmunoResearch), cy3-conjugated donkey anti-mouse (7 μg/mL, Jackson ImmunoResearch), biotin-conjugated goat-anti mouse (5.5 μg/mL, Jackson ImmunoResearch) and fluorescein-conjugated strep-tavidin (4.5 μg/mL, Jackson ImmunoResearch). Prior to secondary labelling for incorporated BrdU, samples were stained for all other antigens and post-fixed with 3.7% formaldehyde for 30 min to preserve the stain. Samples were then denatured with 2 N HCl/0.5% Triton X-100 for 45 min at room temperature, washed, re-blocked, and then incubated with AlexaFluor 647-conjugated mouse anti-BrdU (4 μg/mL, Invitrogen) overnight at 4 °C. Cell nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Sigma).

Apoptosis Staining

The APO-BrdU TUNEL assay kit (Invitrogen) was used following manufacturer protocol before staining for other antigens. Briefly, samples were fixed in 70% ethanol at − 20 °C for 1 week, rinsed in wash buffer for 20 m twice, incubated with DNA-labelling solution for 4 h in a shaking water bath, washed with rinse buffer twice for 20 m at 4 °C, and stained with Alex-aFlour 488-conjugated mouse anti-BrdU overnight at 4 °C. Samples were co-stained with DAPI.

Imaging and Analysis

Immunostained gels were mounted on glass slides and imaged using a Zeiss LSM 510 confocal microscope (×5 and ×20 objectives). Confocal z-stacks were z-projected and thresholded for quantification.

AngioQuant[36] software was used to quantify total vessel length (sum of the lengths of all vessel branches within a gel). Matlab (Mathworks) was used for all other image analysis. Pericyte coverage was defined as αSMA+ area within at least 5 μm of the abluminal face of vessel networks. Briefly, vessel networks were selected in the CD31 channel of thresholded image composites. Selections were enlarged by 5 μm at all edges and applied to the αSMA channel. αSMA+ area fraction within the selected area was measured and displayed as "Percent SMA Coverage". BrdU+ nuclei were counted and counts from the whole gel indicate overall proliferation within the culture (displayed as "# BRDU/CD31 (#/mm^2)". To assess proliferation within the vessels only, CD31$^+$ vessel area was selected and applied to the BrdU channel prior to counting within the selected area. This count was normalized to the CD31$^+$ vessel area to account for differences in vessel density and is displayed as "#BRDU in CD31".

Statistical Analysis

Quantitative data are expressed as mean ± standard error. Statistical analyses were performed using GraphPad Prism 5 software. Statistical significance was determined by one-way ANOVA with Tukey's post-test and is denoted as *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

RESULTS

Preassembly-Mediated Vascular Assembly

ASC aggregates were heterogenous in size. They underwent vascular morphogenesis and developed a highly-branched vascular morphology when cultured in the fibrin hydrogel for 6 days in normoxic conditions (Fig. 2a). The ASCs used in this study are isolated *via* plastic adherence and contain a population of 0.6–1.1% CD31$^+$ cells,[21,23] which we understand to be the building block of the vascular network, rather than ASCs differentiating into ECs. The vascular lengths at 2, 4, and 6 days were (30.9 ± 7.7), (43.6 ± 10.9), and (61.2 ± 15.3 mm), respectively (Fig. 2b). With 0 days pre-assembly, subsequent culture in either normoxia or hypoxia for 6 days without media changes (pseudo-implantation model) resulted in no visible vascular assembly, demonstrating the nutrient-starving nature of the pseudo-implant condition alone is sufficient to impair vascular assembly. However, with 2 days of preassembly, ASCs in the normoxic pseudo-implant condition assembled into preliminary branching structures with significantly greater vessel length (67.2 ± 16.8 mm) than the hy-

poxic pseudo-implant (38.4 ± 9.6 mm). After 4 days of preassembly, transplantation of the vascular structures into either normoxic or hypoxic pseudo-implantation conditions for 6 days resulted in continued vascular development and the formation of interconnected networks (72.3 ± 17.8 vs. 75.4 ± 18.5 mm, respectively). Similarly, with a full 6 days of preassembly ASC vascular networks continued to branch and develop into highly interconnected and dense vascular networks after pseudo-implantation in normoxia (77.4 ± 19.3 mm) and hypoxia (92.8 ± 23.2 mm). Hence, with 0 or 2 days of preassembly in a favorable, normoxic environment, transfer to hypoxic microenvironments had detrimental effects on vascular morphogenesis. However, following 4 or 6 days of pre-assembly, transfer to hypoxic microenvironments appeared to be supportive of subsequent vascular assembly. Thus, we observed a time-dependent change in the effect of hypoxia on ASC-derived vascular networks between Days 2 and 4 of pre-assembly.

Endothelial Cell Proliferation

EC proliferation was assessed by monitoring the incorporation of the thymidine analog BrdU into CD31$^+$ cells during mitosis over the final 20 h of culture (Fig. 3). Proliferation predominantly occurred within CD31$^+$ areas. We hypothesized that ECs might be in a more proliferative state with increasing amounts of preassembly. While this hypothesis is supported by the large increase in number of BrdU+ nuclei within CD31$^+$ regions at 6 days of preassembly, only a slight increase was observed between 2 and 4 days of preassembly (Fig. 3b, Days 2 and 4: 22.8 ± 9.2 and 32.7 ± 4.6, Day 6: 99.3 ± 49). However, this trend is not present when the number of BrdU+ nuclei was normalized to the CD31$^+$ area (Fig. 3c). The spike in #BrdU/CD31 at 2 days of pre-assembly (2295.9 ± 276.2 #/mm^2) is due in part to the very small area fraction of the gel that is CD31$^+$ at that time, resulting in division by a very small number. After pseudo-implantation, proliferation is greater in normoxic than hypoxic groups with zero (hypoxic 870.0 ± 381.1, normoxic 1201.7 ± 340.1 #/mm^2) and 2 days of preassembly (hypoxic 270.0.0 ± 38.8, normoxic 995.1 ± 143.1 #/mm^2), and then switches to be increased in hypoxic groups with 4 (hypoxic 1038.6 ± 226.6, normoxic 594.4 ± 132.7 #/mm^2) and 6 days of preassembly (hypoxic 1304.7 ± 341.6, normoxic 437.9 ± 68.0 #/mm^2). These results are similar to our total vessel length analysis, indicating that cultures with four or more days of pre-assembly entering a more proliferative state after exposure to hypoxia.
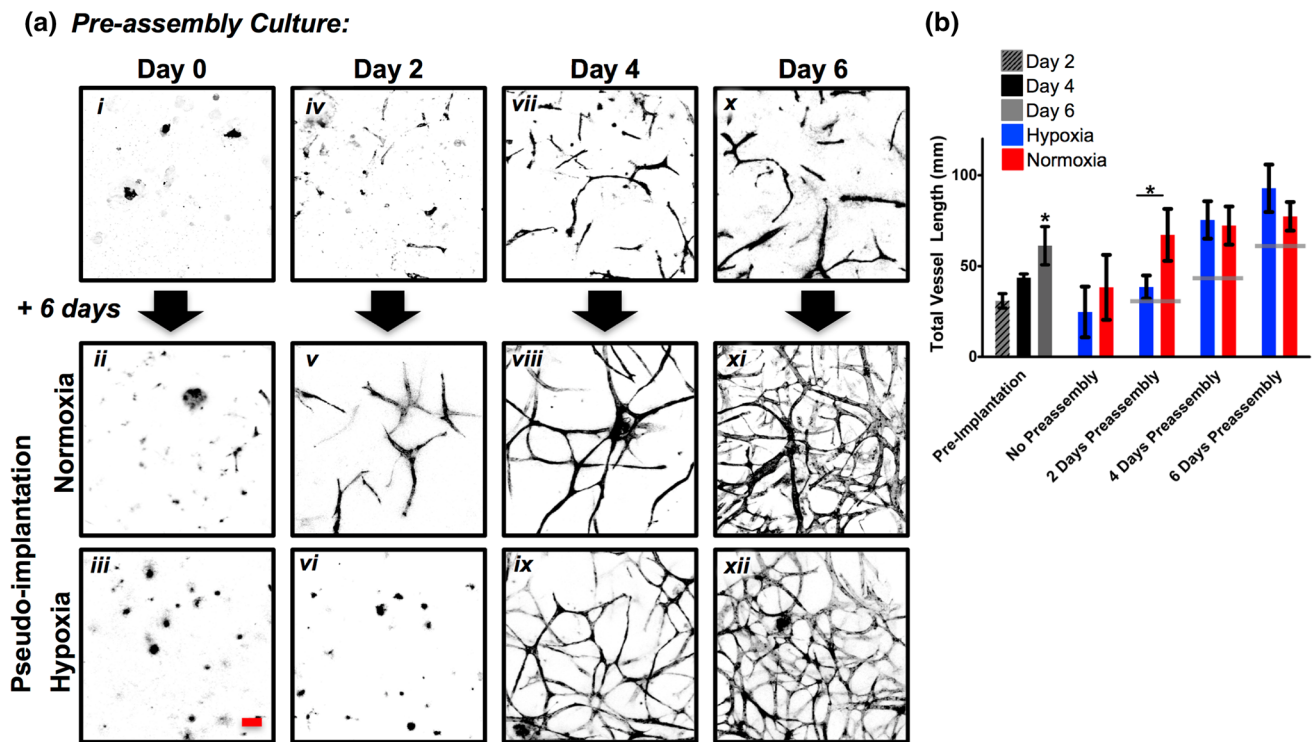
FIGURE 2. Assessing vascular length. (a) ASCs grown in fibrin hydrogels for 6 days sprouted into vascular network structures that stained positively for CD31. At 0, 2, 4, and 6 days ASCs were transferred to either normoxic or hypoxic pseudo-implantation conditions for a subsequent 6 days. (b) Measurements of total vessel length. Gray lines indicate preimplantation length. *$p < 0.05$, $n = 4$. Scale bar = 100 μm.

Apoptosis Analysis

To determine if cells were undergoing apoptosis at an increased rate in hypoxia—as opposed to proliferating at an increased rate—a TUNEL assay was used to label the nicked ends of nuclear DNA as it was reduced into 200 bp fragments by the apoptotic cascade (Fig. 4). There was an increase in the number of apoptotic cells during the preassembly period (Days 2, 4, 6: 52.5 ± 4.2–129.0 ± 20.3–228.2 ± 10.1), and in the normoxic groups after the pseudo-implantation period relative to parallel hypoxic conditions (228.0 ± 68.7–1120.0 ± 333.6 with 4-day preassembly; 688.5 ± 55.9–1164.0 ± 293.9 with 6 days preassembly). Therefore, it is more likely that cells are simply not proliferating in hypoxic conditions instead of undergoing apoptosis.

Pericyte and Collagen IV Coverage Analysis

Pericyte coverage of vessels was analyzed by staining for αSMA fibers co-localized within 5 μm of CD31^{+} areas (Fig. 5 and Supp. Fig. 1). Pericyte coverage was significantly increased from 2 to 4 days of preassembly (24.3 ± 5.3–$46.1 \pm 2.6\%$; $p < 0.005$). αSMA coverage in normoxic groups post-pseudo implantation with 4 and 6 days of preassembly was greater than parallel hypoxic groups. In fact, despite the hypoxic groups increasing total vascular length with time, they demonstrate decreasing αSMA coverage post-pseudo-implantation from 2 to 4 to 6 days of preassembly (51.9 ± 7.2–25.2 ± 4.8–$9.8 \pm 1.9\%$). This decrease in αSMA coverage might be due to pericytes migrating away from their classical position on the abluminal wall of a tubule to facilitate greater growth in hypoxia.[39]

Therefore, we hypothesized that a more mature vascular structure with greater pericyte coverage was surviving and thriving in the hypoxic environment, and tested by using a pharmaceutical inhibitor of pericyte coverage. Additionally, one of the functions of the pericytes is to deposit collagen IV into a basement membrane which can provide supportive signaling to ECs during hypoxia.[46] Pericytes were inhibited with AG1295 and the vascular assembly was contrasted with co-localization of collagen IV. AG1295 significantly inhibited vascular assembly at Days 4 and 6 of preassembly relative to untreated controls (Fig. 6a, Day 4: 6.22 ± 0.55–2.57 ± 0.26; Day 6: 7.73 ± 0.55–3.57 ± 0.52 mm/mm^2) and almost totally eliminated collagen IV deposition throughout the construct (Fig. 6b, day 6: 20.7 ± 2.80–$2.2 \pm 0.13\%$, $p < 0.05$). Within CD31^{+} regions, AG1295 significantly reduced collagen IV deposition at all timepoints (Fig. 6c, $p < 0.005$).
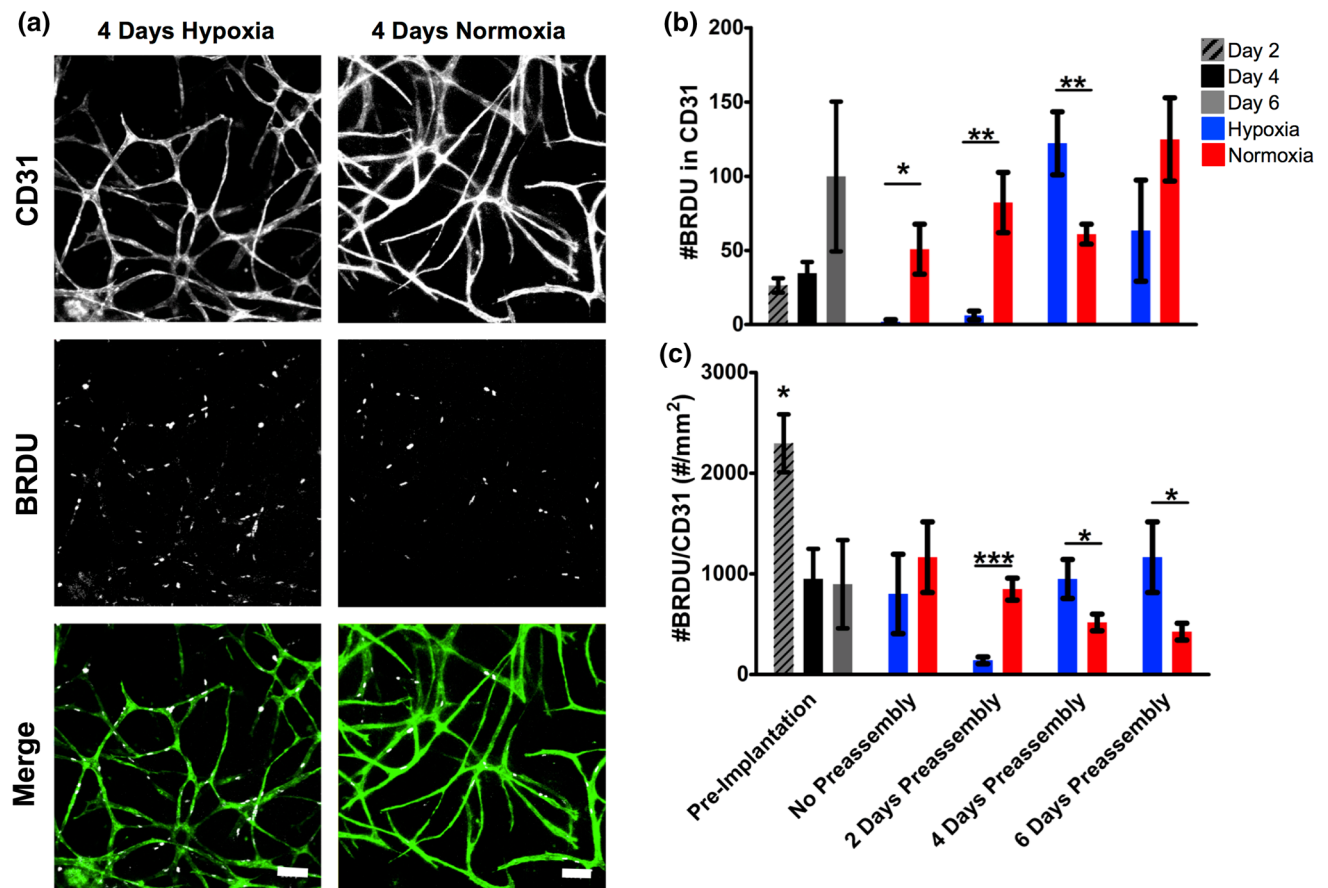
FIGURE 3. Proliferation analysis. (a) Total number of BrdU+ nuclei within CD31 in each gel. (b) Number of BrdU+ nuclei within CD31$^+$ areas in each gel. (c) Density of BrdU+ nuclei within the area of CD31 in each gel. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, $n = 5$. Scale bar = 100 μm.
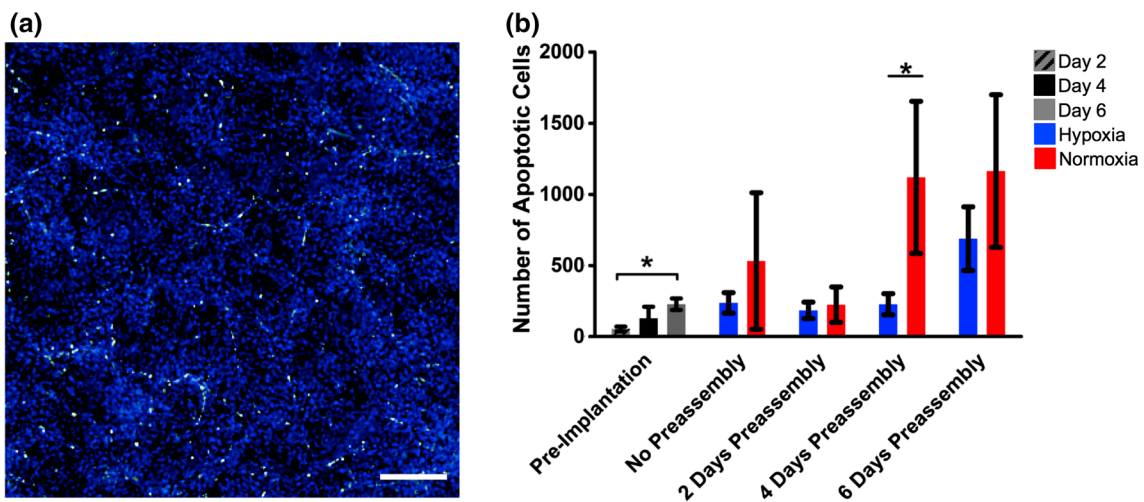

FIGURE 4. Quantification of apoptotic cells. (a) TUNEL staining of apoptotic cells in the fibrin hydrogel. TUNEL = green, DAPI = blue. (b) Quantification of apoptotic cells during preassembly and post-pseudo implantation. *$p < 0.05$, $n = 4$. Scale bar = 400 μm.
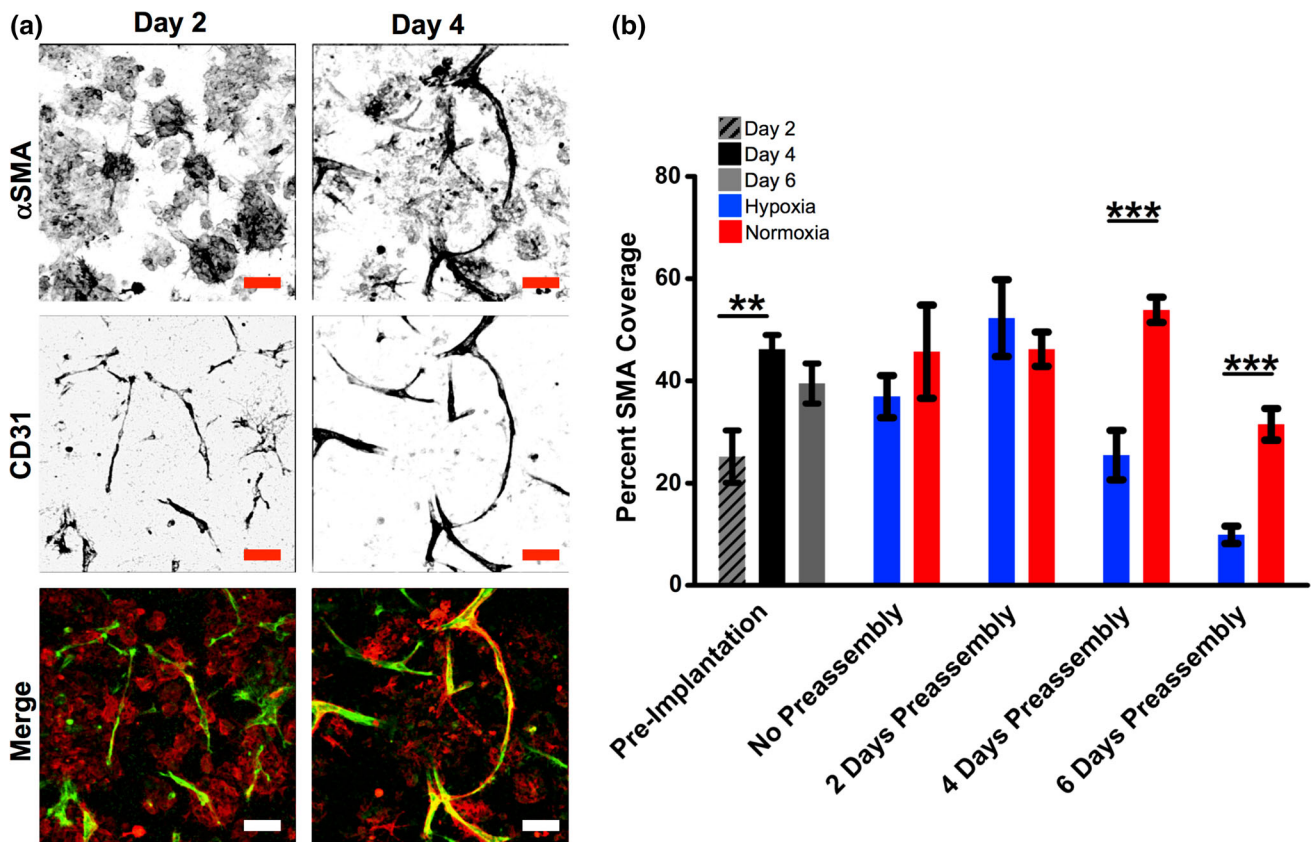
FIGURE 5. Analysis of pericyte coverage. (a) Constructs at Days 2 and 4 of preassembly stained for pericytes (αSMA, red) and endothelial cells (CD31, green). (b) Quantification of αSMA+ coverage of CD31$^+$ vessels during vascular assembly of ASCs during preassembly and following transfer to pseudo-implant conditions. **$p < 0.005$, $n = 5$. Scale bar = 100 μm.
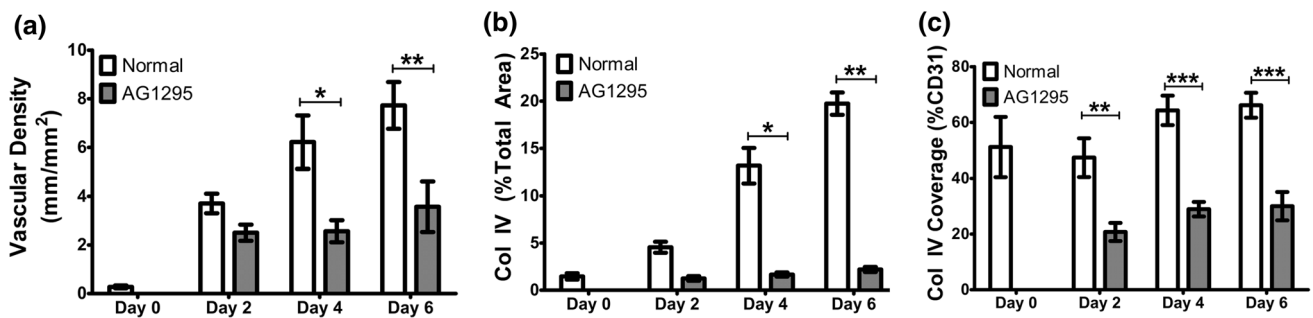

FIGURE 6. Assessment of vascular structure following AG1295 inhibition. (a) Vascular density of CD31$^+$ networks. (b) Total area fraction of Col IV in the gel. (c) Area fraction of CD31 positive for Col IV. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, $n = 3$.

DISCUSSION

The hypoxic microenvironments within volumetric tissue defects provide serious challenges to cell-based regenerative strategies. Hypoxia impairs cell survival and hypoxia vascular assembly of transplanted ECs used to rapidly vascularize engineered grafts.[16,17,21] The data in the current study confirm that hypoxia is inhibitory to the earliest stages of vascular assembly. To overcome this limitation in the therapeutic application of ASCs, one solution is to preassemble the vascular networks before implantation. A preassembly period allows for a number of organizational and construction steps in vascular assembly to take place in a metabolically favorable environment. However, preassembly approaches have regulatory and manufacturing drawbacks. In this study, we modeled preassembly using a culture period with abundant nutrients and oxygen. Additionally, we investigated several parameters of vascular assembly during the preassembly period to characterize the tissue features that would support further growth in hypoxia. The

data herein suggest that at minimum, 4 days of normoxic and nutrient-rich conditions are required before implantation into a hypoxic or ischemic environment. Such a period allows for a sufficient amount of pericyte and collagen IV coverage to develop, and supports the growth and proliferation of ECs in a future hypoxic and nutrient-starved environment.

We found that pericyte-like cells in ASC cultures are essential stabilizers of vascular structures. Inhibition of pericyte-like cell recruitment *via* blocked PDGF-BB signaling closely mimics inhibition of vascular assembly in hypoxia. This suggests that hypoxia delays the contributions of pericyte-like cells during vascular assembly. Previous work identified pericyte-like ASCs as an important component of ASC-vascular assembly (*via* heterotypic cell assembly).[23] Pericytes have well defined functions in microvascular systems controlling EC proliferation, sprouting, and stabilization[43] *via* factor signaling, regulation of the basement membrane ECM, and cell–cell contact signaling *via* Notch pathways.[2,42] Indeed, pericyte–EC interactions are required for proper basement membrane ECM formation with fibronectin, laminin, vitronectin and collagen IV proteins, which together tune vascular tube formation *via* EC integrin interactions.[35,46] Further, EC integrin sensing of vitronectin drives expression of the anti-apoptotic protein Bcl-w and autocrine expression of vascular endothelial growth factor-A (VEGF-A).[15,25]

Hypoxia inhibits vascular assembly of individual ECs but promotes angiogenesis of ASC-vessels that have at least 4 days of preassembly. In general, hypoxia at physiologic (20 mmHg in bone vs. 160 mmHg in atmospheric air) or slightly lower levels causes an increased amount of EC proliferation and angiogenesis.[14] However, EC-vasculature also regresses *via* apoptosis in response to severe hypoxia (1% or less)[10,51] in contrast to the positive angiogenic effects of less severe hypoxia. The idea of a sufficient amount of preassembly is required for hypoxic function supplants the idea that exposure to hypoxia supports vascular growth, *via* increased expression of VEGF and increased tubule formation.[40] These hypoxic vascular-boosting effects have traditionally been observed with vascular explants or other structures that have already been assembled, and not with individual ECs.

These findings suggest that direct implantation of a vascular population may not be sufficient to provide assembled vasculature to the construct before the development of anoxia and associated necrosis. However, this 4 day period of assembly might be achievable *in vivo* using approaches that can deliver oxygen and other nutrients during the first 4 days of implantation.[12] One such approach utilizes oxygen-releasing materials in 3D-printed bone scaffolds, and can sustain delivery periods up to 48 h long.[7]

While the time-scale of vasculogenesis in fibrin hydrogels depends on the cell types and growth media, the assembly of a robust vascular network can commonly be observed in as little as 6 days in a number of systems.[5,34,46] Chen *et al.* compared vascular fibrin constructs with and without 1 week of preassembly *in vivo* and found that preassembly accelerated anastomosis with host vasculature, increased proliferation of implanted cells, and increased production of ECM, confirming that a period of preassembly is critical to *in vivo* use of vascular hydrogels.[5] However, it is important to limit the amount of preassembly as vessels may regress without perfusion and other physiological maturation cues.[4] Additionally, there are potential benefits to limiting culture time as studies have shown that culture on tissue culture plastic could induce cell fate plasticity.[18,45] Perfusion of vascular networks drives their maturation and remodeling[26,47] and was not included in the current study. The increased apoptotic rate observed in normoxia might be due to natural pruning/regression as they have matured without a hypoxic or fluid shear stress signal. Remodeling is further driven by the transport demands of the surrounding tissue exceeding the ability of diffusion.[14]

The distance between individual ECs impacts the paracrine gradient and might have an impact on the rate of vascular assembly. Previous experience[23] revealed higher seeding densities lead to a more rapid assembly of vascular networks, perhaps due to the close positioning of ECs to each other at high seeding densities. MacGabhann *et al.* demonstrated local gradients of VEGF have an effect on vascular assembly in skeletal muscle over distances as short as 10 μm.[31] Other groups[19,41] have modeled tip cell interaction by pairing computational and experimental models, but highlight that there are additional complicating factors beyond tip distance and VEGF gradients (such as Notch and EC–PC signaling) which add complexity to the system. This gradient sensing and directed vascular growth is increasingly important as systems become more complicated with anatomic geometries and physical barriers to vascular networks, avascular border regions between implant scaffold and host vasculature, and approaches where the cell density is limited.

ECs are highly sensitive to changes in oxygen levels through the hypoxia induced factor family of transcription factors, metabolism, hypoxia-regulated microRNAs, causing upregulation of VEGF and its receptors.[14] VEGF is a potent mitogenic for ECs, which supports network assembly *via* angiogenesis or vasculogenesis.[37] While we did not measure variable production of growth factors as a function of preassembly here, previous studies found VEGF-A production by ASC-vessels was only upregulated in severe

(0.2% O_2) hypoxia after normoxic preassembly for 6 days.[21] Such induction of VEGF-A expression might be useful in large scale applications, where despite vascular preassembly, anastomosis and blood flow to interior regions might be delayed, result in network regression, and the associated VEGF-A aiding those regions in undergoing revascularization.

CONCLUSION

This study sought to elucidate the processes active during ASC-vascular assembly that might support the beneficial response of ASC-vasculature morphogenesis in hypoxia. It builds upon previous work that *de novo* assembly of ASC-vasculature is inhibited in hypoxia. In ASC-fibrin constructs with less than 4 days of preassembly, hypoxia drove disassembly of vascular structures. However, with a minimum of 4 days preassembly before exposure to hypoxia resulted in robust vasculature network formation, whereupon hypoxia drove increased network formation. The main difference observed between 2 and 4 days of preassembly was a significant amount of pericyte coverage, and inhibition of that coverage similarly inhibited vascular formation. This suggests the interactions between ECs and PCs in *de novo* vascular assembly is oxygen-mediated.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (https://doi.org/10.1007/s12195-018-0539-6) contains supplementary material, which is available to authorized users.

CONFLICT OF INTEREST

Ethan Nyberg and Warren Grayson declare no conflict of interest.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the Ethical Standards of the Institutional Research Committee and with the 1964 Helsinki Declaration and its later amendments. No animal research was performed in this study.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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