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In Vivo Remodelling of Vascularizing Engineered Tissues

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Abstract—A critical aspect of creating vascularized tissues is the remodelling that occurs *in vivo*, driven in large part by the host response to the tissue construct. Rather than a simple inflammatory response, a beneficial tissue remodelling response results in the formation of vascularised tissue. The characteristics and dynamics of this response are slowly being elucidated, especially as they are modulated by the complex interaction between the biomaterial and cellular components of the tissue constructs and the host. This process has elements that are similar to both wound healing and tumour development, and its features are illustrated by reference to the bottom-up generation of a tissue using modular constructs. These modular constructs consist of mesenchymal stromal cells (MSC) embedded in endothelial cell (EC)-covered collagen gel rods that are a few hundred microns in size. Particular attention is paid to the role of hypoxia and macrophage recruitment, as well as the paracrine effects of the MSC and EC in this host response.

Keywords—Hypoxia, HIF-1α, Angiogenesis, Mesenchymal stromal cells, Myeloid cells, Macrophages, M1, M2, Host response.

INTRODUCTION

There are now many experimental methods to form vascularized tissue engineering constructs.[21,33] Both top-down and bottom-up approaches have been exploited, using various combinations of cells, growth factors and biomaterials. We have focused on modular tissue engineering,[12,15,16,22] in which several thousand (for rodents) individual microtissues are injected in a space filling manner. In this method the EC form a network after implantation. The focus of this review is that regardless of how vessels are formed (bottom-up or top-down), there is a host response to the implant that results in remodelling of the tissue. The implant and the remodelling tissue become hypoxic, which alters the phenotype of the grafted cells and signals the recruitment of host cells (Fig. 1). Together, the host and donor cells form a "granulation" tissue that drives vascularization. Under favourable circumstances this "granulation" tissue becomes stabilized and matures into a functional tissue; alternatively, there is sustained chronic inflammation and ischemia resulting in cell death. Understanding how the host reacts to an engineered tissue is of critical importance.

Similar to tumour vessel formation, the net vascularization response of the tissue construct is dependent on a balance of pro- and anti-angiogenic factors.[42] Tissue engineered constructs are often poorly vascularised but are surrounded by normal, well-vascularised tissue. Pericyte stabilization of blood vessels inhibits new vessel formation in the host tissue, while the implanted tissue, lacking a well-defined vascular system, is hypoxic. This hypoxia is the initial driver of angiogenesis as it tips the local balance in favor of pro-angiogenic factors. Although many aspects of wound healing responses play a role in tissue engineering, we believe that there is merit in thinking about the parallels between tissue engineering and tumour models. Similar to tumours, the initial tissue hypoxia drives an angiogenic inflammatory response which is ultimately responsible for the anastomosis of the host vasculature to the implant's primitive vessels. Typically, wound healing begins with an acute injury causing both bleeding, leading to a clot for haemostasis, and a host inflammatory reaction.[65] The clot forms the first provisional matrix, which is growth factor and chemokine rich. This does not necessarily happen within an engineered tissue as the site of surgical injury may be remote from the tissue that is being remodeled. Also,

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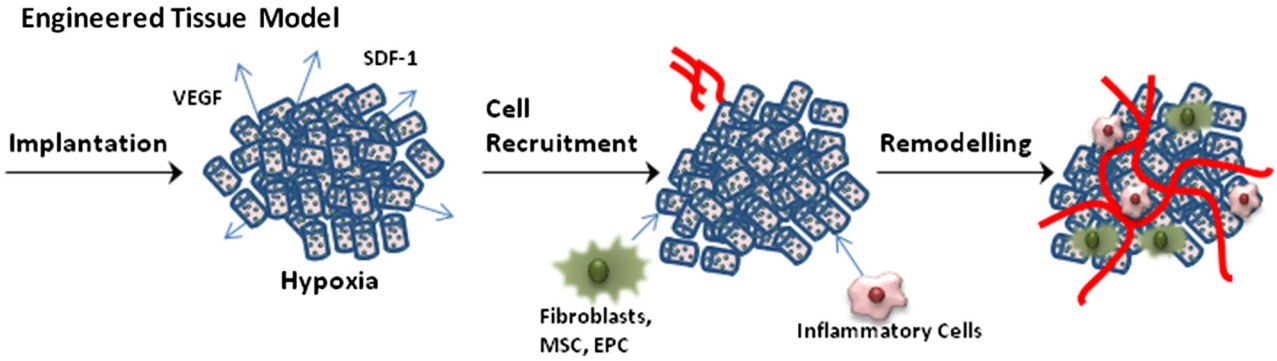

FIGURE 1. Models of the host response to an engineered tissue. Implantation of the construct causes the construct to become hypoxic and release hypoxia driven growth factors and cytokines. These factors recruit blood vessels, inflammatory (myeloid) cells, fibroblasts and bone marrow-derived cells that form a type of granulation tissue. This granulation tissue engrafts the engineered construct to the host.

the engineered tissue elicits a sterile inflammatory response which differs from that associated with acute wound healing and a pathogen response. In wound healing, cells migrate into the provisional matrix of the clot to drive angiogenesis and granulation tissue formation, which is then remodelled into functional tissue. At this point, wound healing and tissue engineering models begin to overlap as granulation tissue formation, inflammation and angiogenesis become the major forces that drive the successful remodelling of the newly formed tissue. However, the host cells that migrate to the engineered tissue and their responses to it can be different than those in wound healing due to the differences in the initial drivers of cell migration (i.e. the clot vs. hypoxia) and the type of inflammation.

This review will focus on the role of hypoxia as an initial driver of the inflammatory and angiogenic response to an engineered tissue implant. The hypoxic response of the implanted cells and the matrix in which the cells are implanted ultimately direct the host response to the tissue construct and understanding how changes to the design of an engineered tissue affect the reaction of the host is of great interest. Changes to the engineered tissue can have profound effects on engraftment and remodelling of the construct by altering cell survival and inflammatory outcomes in the tissue.

Our perspective is primarily from that of modular tissue engineering, a bottom-up methodology in which vessel formation occurs in parallel with remodeling. We believe that the major host response and angiogenesis drivers are similar in both bottom-up and top-down (i.e., gel plug or sheet) methods of tissue engineering, even though pre-formed vessels in top-down systems could potentially anastomose faster than those being generated in modular approaches. Certianly, using a top-down system, Chen *et al.* showed vessel-like structures by day 1 that contained red blood cells but the patency of the vessels was not determined using perfusion studies.[18] Kang *et al.* used tail-vein injections and ultrasound to study the formation and perfusion (but not leakiness) of capillary beds in Matrigel plugs. Capillary beds were allowed to form for 7 days in one host before being transferred to a second.[51] They showed that the vessels formed and were perfused within 5 days in the first host (bottom-up system) but that anastomosis was faster in the second with perfusion by day 3. However, by day 7 the vessel density of the implant in the second animal had not reached the density found on explants from the first animal. This suggests the transplanted pre-formed capillary beds must undergo extensive remodelling to allow perfusion, as the vessels are not correctly aligned with the vasculature of the recipients. This means that an *in vivo*-generated, top-down vasculature system may anastomose faster than a bottom-top system, but that remodelling of the vasculature still proceeds and this can decrease the vessel density. We would expect a similar result when using an *in vitro*-generated vasculature system. Our modular, bottom-up system shows similar rates of vessel formation (day 3) and perfusion by day 7; however, microCT shows that these vessels are still leaky.[22] There is a hierarchy of attributes relevant to new vessels: vascular structures, containing erythrocytes (by histology), perfusion, leakiness and finally function support of cells in constructs at high density.

HYPOXIA

Hypoxia is Induced on Implantation of Engineered Tissue

Because oxygen diffusion is limited to distances of ~150 μm,[13] vascularization has long been recognized

as an important challenge in the field of tissue engineering.[50] While ischemia can lead to failure of the engineered tissue, exposure to hypoxia is inevitable on transplantation and is necessary to initiate vascularization of the tissue. Hypoxia is typically defined as prolonged exposure to 1% Oxygen ($pO_2 =$ 7.6 mmHg). Since the idea of "central necrosis" in spheroidal culture and microencapsulated cells[85] has a long history in the tissue engineering literature, some studies have detailed the oxygen gradients through an engineered tissue *in vitro*. For example, Malda *et al.* measured oxygen levels as low as 2–5% in the centre of *in vitro* cultured engineered cartilage using glass microelectrodes.[62] Kellner *et al.* even identified anoxic conditions in engineered cartilage tissue and determined that oxygen tensions varied with cell density.[54] Engineered tissues experience similar or more severe hypoxic conditions *in vivo,* given that oxygen levels in culture is typically higher (pO_2 ~150 mmHg) than *in vivo* (pO_2 is $<$40 mmHg in tissue).

Direct measurements of hypoxia require invasive instrumentation, such as oxygen electrodes.[70,98] However, nitroimidazole stains, such as EF5 and pimonidazole, have been successfully used to visualize hypoxic regions in tumors.[41,48,83] These dyes may be useful in the context of tissue engineering with the caveat that, since the nitroimidazole stains are administered systemically, proper vascular perfusion of the engineered tissue is required to accurately identify hypoxic regions.

HIF Pathway Activation Mediates Cellular Response to Hypoxia

The cellular response to hypoxia is largely mediated by a family of transcription factors known as hypoxia-inducible factors (HIF), which activate gene expression related to anaerobic metabolism, angiogenesis and survival.[66,90,104] Examples of downstream gene targets include vascular endothelial growth factor (VEGF), VEGF receptor-2 (VEGFR2), insulin-like growth factor-2 (IGF2), transforming growth factor-alpha (TGFα), glucose transporter-1 (Glut1) and -3 (Glut3), lactate dehydrogenase A (LDHA) and Bcl-2 (Fig. 2).

The HIF transcription factor is a heterodimer composed of an oxygen-sensitive α-subunit (HIF1-α, HIF2-α, or HIF3-α) and a constitutively expressed HIF-β subunit. In normoxia, prolyl hydroxylase (PHD) hydroxylates prolyl residues in the oxygen-dependent degradation domain of the α-subunit, permitting interaction with the von Hippel-Lindau tumor suppressor protein (VHL). Once bound, the complex is ubiquitinated and degraded in the proteasome. In hypoxia, however, the α-subunit is not degraded, allowing it to translocate to the nucleus and dimerize with HIF-β to regulate transcription of downstream genes. While stability of the α-subunit is dependent upon proline modification, its transcriptional activity is regulated by factor inhibiting HIF (FIH)-1 *via* hydroxylation of an asparagine residue, which hinders protein–protein interaction.

Of the HIF transcription factors, HIF-1 and -2 have been studied most extensively (comparisons provided elsewhere[53,56]). Although the two isoforms share similar amino acid sequences, protein structure, and both dimers bind to the same conserved hypoxia responsive element (HRE) in DNA, HIF-1 and -2 have distinct gene targets. For instance, HIF-1 transactivates the genes of enzymes involved in glycolysis and apoptosis, such as phosphofructokinase and Bcl2, while HIF-2 induces transcription of genes involved in cellular invasion, such as matrix metalloproteinase (MMP) -2 and -13. Although HIF-1 and -2 can exert antagonistic effects, they also share common target genes like VEGF-A and Glut1. Due to regulator differences, HIF-1 is involved in the initial vasculogenesis of wounded or ischemic tissue, whereas HIF-2 becomes prominent in later stages of vessel anastomosis and remodelling.[53,56,94]

One of the essential pathways, besides angiogenesis, that is activated by hypoxia is the HIF1-mediated glycolytic switch. The glycolytic switch is a shift from aerobic to anaerobic metabolism to keep the cells alive in the hypoxic environment. The HIF-1 pathway upregulates the expression of glucose transporters Glut-1 and Glut-3, and glycolytic enzymes like LDHA, which contribute to anaerobic metabolism of glucose into lactate.[47,89] The HIF-1 pathway also activates transcription of pyruvate dehydrogenase kinase (PDK1), an enzyme responsible for inactivating pyruvate dehydrogenase, and thereby slowing mitochondrial processing of pyruvate.[55] This glycolytic switch is important for the initial survival of the implanted tissue until angiogenesis restores oxygen levels in the tissue.

Hypoxia Drives the Recruitment of Local Cells to the Tissue Construct

By upregulating pro-angiogenic factors, hypoxia sets up a paracrine signalling cascade, *via* secreted growth factors such as VEGF and bFGF, that drives the local migration and proliferation of endothelial cells (EC). Hypoxia also causes cells to release DAMP or "danger" signals, such as adenosine, that increases the proliferation and migration of EC,[68] as well as other local cascades, such as the release of MMP that locally degrades the ECM to allow the migration of cells. *In vitro* experiments have shown that without the activation of MT1-MMP EC cannot form angiogenic

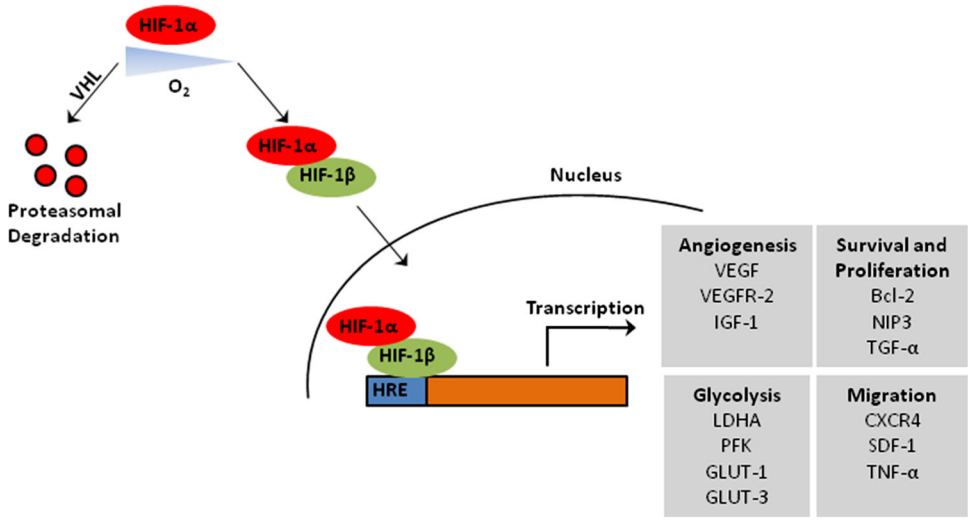

FIGURE 2. Hypoxic initiation of the angiogenic response to tissue constructs as mediated by HIF-1 and HIF-2 pathways. Cells are exposed to hypoxia upon implantation; HIF-1α and HIF-2α (not shown) subunits are stabilized in low oxygen environments and dimerize with the β-subunit. The complex translocates to the nucleus and binds to hypoxia responsive elements (HRE) activating transcription. HIF-1 and HIF-2 activation leads to increased cell survival *via* up-regulation of growth factor, cytokines, pro-survival and glycolytic genes.

sprouts.[20] It has also been shown that stromal cells from different sources drive angiogenesis *via* differing patterns of expression of MMP and other angiogenic factors,[35] leading to the release of different sets of cryptic peptides and growth factor signals driving different host responses.

Interestingly, we find in different animal models (rat vs. mouse) that there is a difference in the need for MSC to be implanted with the EC. In the SCID/bg mouse, blood vessels did not form without the co-implantation of MSC, which become pericyte support cells and there was little ECM deposited as the implanted tissue remodeled.[12,22] However, in a immunosuppressed rat model, implantation of EC alone formed blood vessels which persisted for long periods of time but were leaky, and there was more granulation tissue that formed as the tissue remodeled.[15] The addition of MSC to the implanted tissue decreased the leakiness of the newly formed vessels.[16] It is unclear whether or not these differences are due to the different animals and cell types used in the two models, but regardless of cause, the difference shows that there can be drastic changes in the regulation of host cell migration into the tissue and/or survival and function of the implanted EC based on the model used.

At the same time as EC migration and vessel formation occurs, pericytes must be recruited to the tissue for the newly formed vessels to stabilize and mature. There are several factors, mostly produced by the EC,[1] which regulate the migration and proliferation of pericytes. The function of these factors differ depending on whether the vessel is pre-existing or nascent. For example, Ang2 is more involved in the loss of pericyte binding and sprouting from pre-existing vessels than in the formation of nascent vessels.[1] Other factors, including Sema3A and MMP-9, are thought to be involved in the recruitment of pericytes to nascent vessels.[17,40] The direct interaction of the pericyte with EC promotes decreased proliferation and increased survival of the EC.[3] It is well documented that if this pericyte-EC interaction does not happen there is regression of the blood vessels. *In vitro* assays of EC network formation and sprouting show that there is a faster regression of EC tubes without the presence of pericytes.[88] The pericyte cells stabilize the blood vessels by direct and indirect interaction with the EC *via* Tie2-Ang2 and N-cadherin.[3]

RECRUITMENT OF BONE MARROW-DERIVED CELLS

As well as the release of local factors that regulate the host response, there is also a hypoxia related release of systemic factors that mobilize leukocytes and other bone marrow-derived cell types including MSC and endothelial progenitor cells (EPC) to sites of ischemia. These cells play a crucial role in the vascularization of tumors, wound healing and re-vascularization of the ischemic limb.[44,71,79,99,107] For example, increased mobilization of MSC was observed in the peripheral blood of Sprague–Dawley rats housed in hypoxic chambers (10% oxygen).[59] This effect was abrogated by a potent HIF1α inhibitor, YC-1, showing that HIF1 activation is a key factor in MSC mobilization induced by hypoxia. Hypoxia is known to up regulate factors such as SDF-1. Ceradini, *et al.*,

showed that SDF-1 expression was proportional to oxygen tension in a tissue and levels of SDF-1 correlated with retention of injected EPCs.[14]

Hypoxia augments the profile of angiogenic factors secreted by EPC and MSC. For example, a microarray analysis of gene expression in bone marrow-derived MSC cultured in ambient (21% oxygen) vs. hypoxic (1% oxygen) conditions demonstrated up-regulation (at least three-fold increase) of secreted factors like VEGF-D, placental growth factor (PlGF), and MMP-9, in addition to genes associated with metabolism.[78] Conditioned media from hypoxia-preconditioned (1% oxygen) MSC enhanced the survival and tube formation of hypoxia-exposed EC, compared to conditioned media from normoxia-cultured MSC, *via* activation of the PI3 K-Akt pathway in EC.[46] When transplanted in a mouse model of hind limb ischemia, hypoxia-preconditioned MSC significantly increased the rate at which perfusion was restored compared to normoxia-cultured MSC.[45] Similar conclusions were drawn from studies in infarcted mouse hearts.[45] MSC are also well known to have anti-inflammatory and immune modulating properties that help the integration of the engineered tissue with the host (as reviewed in detail in[61,92]) in addition to their angiogenic activities.

Some EPC are peripheral blood mononuclear cells[8] that develop EC-like qualities and also contribute to revascularization and salvage of ischemic hind limbs.[4] *In vitro* characterization suggests that there are multiple subpopulations of EPC that are roughly defined as early and late outgrowth cells, or endothelial colony-forming cells (ECFC). As with MSC, there is no one well defined marker of the different EPC populations[43]; however, studies indicate the early outgrowth EPC are $CD14^+$ and are of myeloid lineage, whereas the late outgrowth EPC are $CD14^-$ and are of an endothelial lineage.[6] Interestingly, early outgrowth EPC do not reside in the damaged tissue long-term, nor do they physically integrate into the new endothelium, leading many researchers to presume that they are not endothelial cells and their main mode of action is *via* paracrine effects,[84] much like MSC. Early EPC secrete many pro-angiogenic cytokines, including HGF, IGF-1, bFGF and VEGF.[5,91] These signals, in turn, promote the proliferation and migration of EC[103] and subsequent vascularization of the hypoxic tissue. ECFC are a rare cell type that behave like mature EC and can integrate into newly forming blood vessels.[101] However, it was recently shown that they do not originate from the bone marrow and their site of origin is unknown.[101]

Myeloid Cell Mobilization and Response to Engineered Tissues

Of special interest with respect to bone marrow derived cell recruitment and angiogenesis are myeloid cells and especially neutrophils and macrophages.[97] Myeloid cells not only survive in hypoxic conditions, but migrate against oxygen gradients to areas of low oxygen tension. This response is in part mediated by VEGF and SDF-1 (CXCL12), downstream targets of HIF-1 and -2. When VEGF was conditionally and reversibly induced in the heart and liver of a transgenic mouse, its overexpression alone was sufficient to recruit myeloid ($CD45^+$) cells to the target organ.[39] Interestingly, in this study, retention of recruited cells in the perivascular space was only possible in the presence of SDF-1 produced by perivascular fibroblasts. SDF-1 is known to be involved in widespread leukocyte trafficking *via* CXCR4.[52,74] Several other HIF-regulated signals also control the recruitment of macrophages to hypoxic tissue[26,72]; two of these chemokines, SDF-1 and CCL2, have been shown to polarize macrophages to a pro-angiogenic state.[86,87]

Bone marrow-derived MSC secrete many factors that attract macrophages[19] and VEGFα and Ang1, were significantly up-regulated in MSC in response to hypoxia.[19] Demonstrating this recruitment, *in vivo* implantation of collagen modules containing MSC in a rat omental pouch led to migration of $CD68^+CD163^+$ (“M2”) macrophages into the tissue construct, compared with control modules lacking MSC, which did not affect macrophage migration.[16]

Once recruited to the tissue construct, myeloid cells can promote the formation of vascularised tissue. In studies of the tumour vasculature, it was shown that approximately half the angiogenic factors that drive vessel development come from myeloid cells,[58] including mast cells.[96] and neutrophils.[100] The latter are similarly recruited to implants within a few hours by ELR^+ CXC cytokines[67] and release angiogenic factors in response to TNFα but, interestingly, not in response to hypoxia.[96] This means that although neutrophils are attracted to the implant by hypoxic signalling, their angiogenic response is decoupled from the direct hypoxic response in the tissue. Neutrophil depletion impaired angiogenesis in a Matrigel plug,[9] as well as in a corneal model.[37] There is speculation that a variety of neutrophil sub-populations and activation states may exist, similar to those observed in macrophages.[57]

Macrophage Phenotype

Macrophages are the focal point for myeloid cell recruitment and subsequent remodelling. Macrophages are sentinels for the innate immune system, act as antigen-presenting cells, and respond in large numbers to inflammatory signals caused by injury and infection. As well, they carry out diverse tissue remodelling functions, and their role in angiogenesis is well established.[77]

Although yolk-sac derived tissue-resident populations are generally responsible for homeostatic and tissue-remodelling functions,[27,28] monocyte-derived macrophages supplement this population, particularly following inflammation and in response to allogeneic tissue transplants, such as engineered tissues.[36]

Macrophages are broadly defined as $CD11b^+F4/80^+$ cells, although individual markers differ among tissues and can change substantially in response to local cytokine signaling. Classically-activated (''M1'') macrophages are distinguished from alternatively-activated ''(M2'') macrophages, although this is a somewhat conceptual distinction reflecting differences that can be observed *in vitro*, but that may not be relevant *in vivo*. The former are thought to be activated in response to pathogens or injury, specifically by exposure to interferon gamma (IFN-γ) from T helper 1 (T_H1) and natural killer (NK) cells, tumour necrosis factor (TNF) from antigen-presenting cells, and binding of toll-like receptors by bacterial lipopolysaccharide (LPS).[73] They are thought to have an inflammatory role and are responsible for microbial destruction and clearance of apoptotic cells. Alternatively-activated (''M2'') macrophages are thought to arise in response to cytokines, such as interleukin-4 (IL-4) and IL-13, from T_H2 cells and granulocytes, and IL-10 from such sources as regulatory T cells.[25,64] They encompass a number of sub-phenotypes which are collectively termed ''alternative'' not necessarily due to their similarities, but because they are thought to be anti-inflammatory and thus distinguishable from macrophages of the classical ''M1'' activation pathway.[64] ''M2'' macrophages are often further subdivided into ''M2a'', ''M2b'', and ''M2c'' subsets,[63] based largely on the cytokines used to polarize the cells *in vitro*.

Macrophages Regulate Angiogenesis and Change Phenotype During Remodelling

The manner in which macrophages regulate angiogenesis (Fig. 3) has been thoroughly reviewed.[77] They are recruited to target tissues by chemotactic factors such as HIF-mediated CCL2, VEGF, and SDF-1 signalling, and secrete pro-angiogenic mediators (*e.g.*, VEGF, FGF2, IL-1β, IL-8) and MMP critical for ECM degradation and tissue remodelling. In embryonic development, wound-healing, and tumour vascularization, macrophages share similar ''M2''-like phenotypes[77,82] and play important roles in the regulation of angiogenesis.[10,75,81] Their depletion generally leads to severely impaired vessel formation.[69,102,106] For example, Melero-Martin *et al.* showed that $CD11b^+$ cells were recruited to implanted Matrigel plugs containing EPC and MSC, and were necessary to achieve the formation of blood vessels from the implanted cells.[69] In an adipose tissue engineering system, also Matrigel-based, a four-fold decrease in $CD31^+$ staining was seen in response to clodronate liposome treatment.[29]

Wound healing studies have indicated that macrophage phenotype and their role in the healing process both change over time. Lucas *et al.* showed that depletion of macrophages during the early stage of healing (days 0–5) caused a reduction in vascularization of granulation tissue, hemorrhaging during the middle stage (days 4–9), but had no effect during the late stage (days 9–14).[60] Furthermore, early depletion prevented the typical ''M1'' to ''M2'' shift over the course of healing; late stage macrophages failed to express alternative activation markers FIZZ1 and YM1 following early stage depletion. Similarly, Willenborg *et al.* demonstrated that macrophages derived from classical $Ly6C^+CCR2^+$ circulating monocytes were critical early drivers of angiogenesis.[105] When myeloid-specific CCR2 was deleted, monocyte recruitment was significantly impaired, leading to decreased VEGF levels and decreased vascularization. VEGF in wound tissues was overwhelmingly macrophage-derived at early time points (days $\leq$4), even though only 19% of macrophages present expressed VEGF. By day 7 this level decreased and non-myeloid VEGF was as prevalent as macrophage-derived VEGF, and by day 14 nearly all VEGF was non-myeloid in origin. Over the same period there was a general shift toward expression of ''M2''-associated genes (e.g., IL-10, CD206, CD163), although the initial inflammatory population expressed a mix of ''M1'' and ''M2'' markers. However, deletion of myeloid-specific VEGF alone was sufficient to cause significant reduction in vascularization, indicating the small population of inflammatory, VEGF-producing early macrophages is critical for the induction of angiogenesis.

These findings are at odds with the standard inflammatory, non-angiogenic ''M1'' and anti-inflammatory, angiogenic ''M2'' dichotomy, and there is growing evidence that multiple, overlapping populations are involved in complex ways during tissue remodelling. In a comparison of the tissue remodelling potential of 14 decellularized matrix-based biologic meshes, it was observed that the most efficient remodelling occurred in meshes into which large macrophage populations were recruited early, and had high proportions of $CD206^+$ (''M2'') macrophages. The presence of $CCR7^+$ (''M1'') macrophages in those populations did not impair remodelling, provided that the ratio of CD206+:CCR7+ cells remained high[11] and this ratio at early timepoints (day 14) was a strong predictor of remodelling efficiency at later timepoints (day 35). Another study of scaffold vascularization found that ''M1'', ''M2a'', and

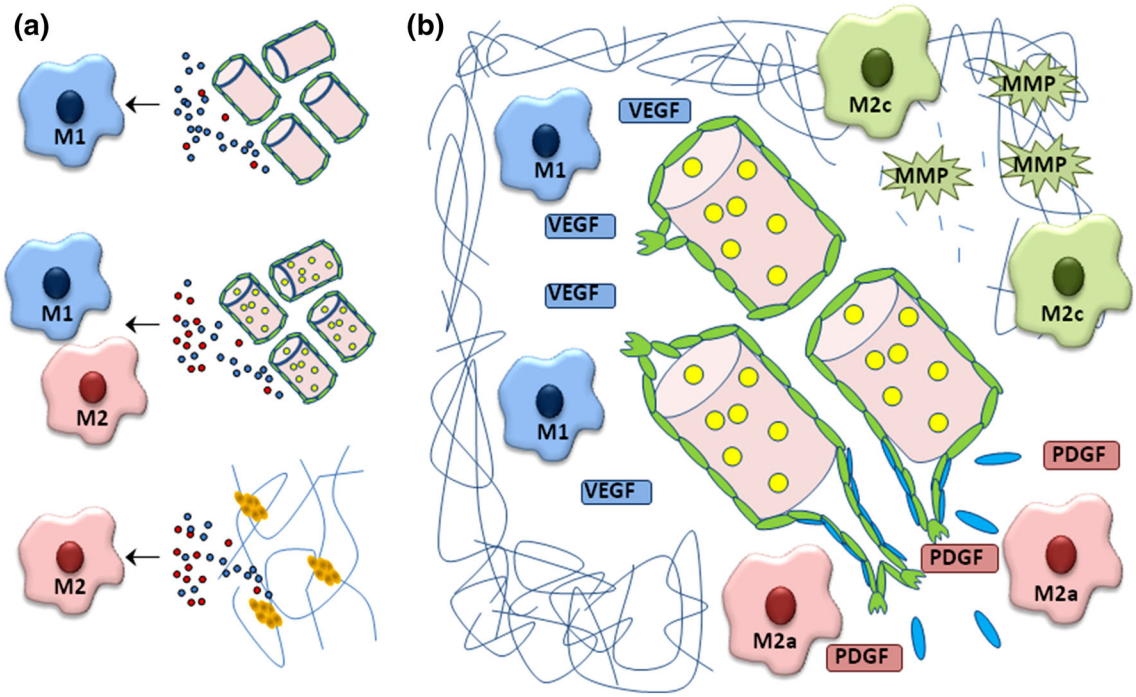

FIGURE 3. Engineered tissue characteristics are hypothesized to direct recruited macrophage phenotype and subsequent remodelling outcome. (a) Implanted tissues recruit macrophages through the secretion of hypoxia associated chemokines (e.g., VEGFa, SDF-1 (CXCL12), CCL2). Polarization of recruited cells is modulated both by implanted cells and the type of the matrix. Dependent on the nature of the construct (e.g., with or without embedded MSC or different biomaterials), different macrophage polarization states result (M1, M2 or a mixture of M1 and M2). Three hypothetical examples are illustrated. (b) Various subsets of polarized macrophages perform specialized functions necessary for angiogenesis and efficient remodelling. M1 macrophages express high levels of VEGF, driving EC migration and sprouting. M2c macrophages secrete MMP which degrades ECM and is critical for remodelling. M2a macrophages support vessel maturation through PDGF-mediated perictye recruitment and promoting vessel anastomosis.

"M2c" subsets all expressed strong, yet distinct, angiogenic properties *in vitro*. When implanted *in vivo*, scaffolds designed to elicit an "M2" response were less effective than those that produced a mixed "M1"–"M2" response in vascularization of the scaffold.[95]

This dynamic aspect of macrophage phenotype has important implications for engineered tissues, as, in addition to the normal phenotypic fluctuations, the composition and morphology of a biomaterial strongly affect the polarization of macrophages with which it interacts, which could interfere with the optimal polarization.[7,34] For example, human peripheral blood monocytes cultured for seven days on 2D surfaces coated with poly(lactic-*co*-glycolic acid) (PLGA) showed increased expression of an "M1" marker 27E10, while those cultured on poly(ethylene oxide)-based (NCO-sP(EO-*stat*-PO)) showed increased "M2" marker CD163; differences were seen with nanofibre networks of the same polymers.[7] Biomaterials must be carefully chosen to avoid upsetting the critical inflammatory balance and to maximize the benefit obtained from its manipulation, although the exact parameters of this balance remain to be identified (Fig. 3).

One of the first demonstrations that polarized macrophages can be effectively used to promote angiogenesis *in vivo* examined subcutaneously injected Matrigel plugs in a mouse model, supplemented with polarized macrophages. At day 14, "M2" pre-polarized macrophages showed increased endothelial cell counts by $CD31^+$ staining compared with "M1", "M0", and macrophage-free controls.[49] This was supported by similar results using *in vitro* tube formation assays, which also suggested that direct contact between EC and "M1" or "M0" macrophages may itself inhibit tube formation. Supporting evidence for the importance of direct EC-macrophage contact comes from studies of the developing zebrafish hindbrain, in which tissue macrophages were shown to promote anastomosis of vessel sprouts by interacting directly with endothelial tip cells and guiding their fusion to form vascular networks.[32]

EXTRACELLULAR MATRIX

As the host cells (myeloid and non-myeloid) migrate into the hypoxic tissue construct they lay down a

matrix loosely similar to that of late-stage wound healing. In wound healing this matrix typically starts as a fibronectin, and hyaluronic acid granulation tissue that is remodelled into collagen-rich ECM by macrophages and fibroblasts.[93] From the tumour and wound healing literature it is known that hypoxia and several cytokines and growth factors influence the amounts and types of matrix deposited. For example, both EC and fibroblasts are known to up-regulate transforming growth factor beta (TGFβ) expression in hypoxia[2,31] and TGFβ is known to induce fibroblasts into a myofibroblast phenotype.[30] Fibroblasts exposed to TGFβ increase their expression of several ECM proteins (fibronectin, elastin, collagen type 1, collagen type 4, decorin, vitronectin) leading to the formation of a granulation tissue.[80] *In vitro* experiments also demonstrate the role of fibroblast-secreted ECM proteins in angiogenesis, where several secreted proteins (PCOLCE, Col1A1, SPARC, IGFBP7, and βig-h3) are critical for lumen formation but not sprout elongation in fibrin gel bead assays.[76] TGFβ is also important for switching classical, "M1", macrophages to alternatively activated, "M2", macrophages, which dampens the initial inflammation and allows tissue healing to progress.[38] The Badylak group has proposed that the ratio of "M2" to "M1" macrophages must be tipped towards "M2" for proper regeneration of tissue defects when decellularized matrix is implanted.[11] The swing from nominally "M1" to "M2" macrophages could be due to the release of bound growth factors, such as TGFβ, from the decellularized matrix.

The extracellular matrix is both an outcome of the host response and a mediator of other outcomes. Differences noted above, between rats and mice can be related to the differences in amount and type of matrix that was formed. Perhaps, the larger amount of ECM in the rat granulation tissue supports an environment for blood vessel development which is not achieved in the mouse model. We have shown that changes to ECM components can have a beneficial impact on EC survival and angiogenesis in the tissue construct. The addition of fibronectin to collagen microtissues increased the survival of implanted HUVEC and caused a corresponding increase in vessel density (in the absence of additional MSC) in mice.[24] More interestingly, when the HUVEC where transduced to express a pro-angiogenic ECM protein, Del-1, there was an increase in the number of blood vessels that formed in the implanted tissue in mice, when adMSC were present to limit initial apoptosis.[22,23] In both of these cases the bulk of the ECM was type 1 collagen for structural support of the tissue, but by effectively doping it with small amounts of accessory ECM there were changes in the amount of vessel development. There are opportunities for further manipulation of the deposited extracellular matrix to beneficially impact experimental outcomes.

Summary

The host response to an engineered tissue is driven and influenced by multiple factors. Initially the implanted tissue is hypoxic and the cells are stressed. This causes the cells in the implant to upregulate the production of several cytokines and growth factors, including VEGF and SDF-1 and release danger signals, like adenosine, that recruit pro-inflammatory and pro-angiogenic cells to the tissue. The balance of these functions determines the survival of the tissue. If the pro-inflammatory response outweighs the pro-angiogenic response then the tissue fails to engraft. Small changes to the secretion profile of the implanted cells or the implanted ECM can have dramatic effects on the balance of this response. A greater understanding of how the hypoxic response of implanted tissue drives the subsequent recruitment and activation of cells (non-myeloid and myeloid) influence this balancing act are among the crucial needs for the development of the field.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

REFERENCES

[1]Aguilera, K. Y., and R. A. Brekken. Recruitment and retention: factors that affect pericyte migration. *Cell. Mol. Life Sci.* 71:299–309, 2014.

[2]Akman, H. O., H. Zhang, M. A. Siddiqui, W. Solomon, E. L. Smith, and O. A. Batuman. Response to hypoxia involves transforming growth factor-beta2 and smad proteins in human endothelial cells. *Blood* 98:3324–3331, 2001.

[3]Armulik, A., A. Abramsson, and C. Betsholtz. Endothelial/pericyte interactions. *Circ. Res.* 97:512–523, 2005.
[4]Asahara, T., T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, and J. M. Isner. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275:964–967, 1997.
[5]Awad, O., E. I. Dedkov, C. Jiao, S. Bloomer, R. J. Tomanek, and G. C. Schatteman. Differential healing activities of CD34+ and CD14+ endothelial cell progenitors. *Arterioscler. Thromb. Vasc. Biol.* 26:758–764, 2006.
[6]Balaji, S., A. King, T. M. Crombleholme, and S. G. Keswani. The role of endothelial progenitor cells in postnatal vasculogenesis: implications for therapeutic neovascularization and wound healing. *Adv. Wound Care (New Rochelle)* 2:283–295, 2013.
[7]Bartneck, M., K. H. Heffels, Y. Pan, M. Bovi, G. Zwadlo-Klarwasser, and J. Groll. Inducing healing-like human primary macrophage phenotypes by 3d hydrogel coated nanofibres. *Biomaterials* 33:4136–4146, 2012.
[8]Basile, D. P., and M. C. Yoder. Circulating and tissue resident endothelial progenitor cells. *J. Cell. Physiol.* 229:10–16, 2014.
[9]Benelli, R., M. Morini, F. Carrozzino, N. Ferrari, S. Minghelli, L. Santi, M. Cassatella, D. M. Noonan, and A. Albini. Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. *FASEB J.* 16:267–269, 2002.
[10]Brancato, S. K., and J. E. Albina. Wound macrophages as key regulators of repair: origin, phenotype, and function. *Am. J. Pathol.* 178:19–25, 2011.
[11]Brown, B. N., R. Londono, S. Tottey, L. Zhang, K. A. Kukla, M. T. Wolf, K. A. Daly, J. E. Reing, and S. F. Badylak. Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials. *Acta Biomater.* 8:978–987, 2012.
[12]Butler, M. J., and M. V. Sefton. Cotransplantation of adipose-derived mesenchymal stromal cells and endothelial cells in a modular construct drives vascularization in SCID/bg mice. *Tissue Eng. Part A* 18:1628–1641, 2012.
[13]Carmeliet, P., and R. K. Jain. Angiogenesis in cancer and other diseases. *Nature* 407:249–257, 2000.
[14]Ceradini, D. J., A. R. Kulkarni, M. J. Callaghan, O. M. Tepper, N. Bastidas, M. E. Kleinman, J. M. Capla, R. D. Galiano, J. P. Levine, and G. C. Gurtner. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat. Med.* 10:858–864, 2004.
[15]Chamberlain, M. D., R. Gupta, and M. V. Sefton. Chimeric vessel tissue engineering driven by endothelialized modules in immunosuppressed sprague-dawley rats. *Tissue Eng. Part A* 17:151–160, 2011.
[16]Chamberlain, M. D., R. Gupta, and M. V. Sefton. Bone marrow-derived mesenchymal stromal cells enhance chimeric vessel development driven by endothelial cell-coated microtissues. *Tissue Eng. Part A* 18:285–294, 2012.
[17]Chantrain, C. F., H. Shimada, S. Jodele, S. Groshen, W. Ye, D. R. Shalinsky, Z. Werb, L. M. Coussens, and Y. A. DeClerck. Stromal matrix metalloproteinase-9 regulates the vascular architecture in neuroblastoma by promoting pericyte recruitment. *Cancer Res.* 64:1675–1686, 2004.
[18]Chen, X., A. S. Aledia, S. A. Popson, L. Him, C. C. Hughes, and S. C. George. Rapid anastomosis of endothelial progenitor cell-derived vessels with host vasculature is promoted by a high density of cotransplanted fibroblasts. *Tissue Eng. Part A* 16:585–594, 2010.
[19]Chen, L., E. E. Tredget, P. Y. G. Wu, and Y. Wu. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* 3:e1886, 2008.
[20]Chun, T. H., F. Sabeh, I. Ota, H. Murphy, K. T. Mc Donagh, K. Holmbeck, H. Birkedal-Hansen, E. D. Allen, and S. J. Weiss. MT1-MMP-dependent neovessel formation within the confines of the three-dimensional extracellular matrix. *J. Cell. Biol.* 167:757–767, 2004.
[21]Ciucurel, E. C., M. D. Chamberlain, and M. V. Sefton. The modular approach. In Biofabrication: Micro- and Nano-fabrication, Printing, Patterning and Assemblies, edited by G. Forgacs and W. Sun. Oxford: Elsevier Inc., 2013, pp. 119–148.
[22]Ciucurel, E. C., and M. V. Sefton. DEL-1 overexpression in endothelial cells increases vascular density in tissue-engineered implants containing endothelial cells and adipose-derived mesenchymal stromal cells. *Tissue Eng. Part A* 20:1235–1252, 2014.
[23]Ciucurel, E. C., A. E. Vlahos, and M. V. Sefton. Using del-1 to tip the angiogenic balance in endothelial cells in modular constructs. *Tissue Eng. Part A* 20:1222–1234, 2014.
[24]Cooper, T. P., and M. V. Sefton. Fibronectin coating of collagen modules increases in vivo HUVEC survival and vessel formation in SCID mice. *Acta Biomater.* 7:1072–1083, 2011.
[25]Couper, K. N., D. G. Blount, and E. M. Riley. Il-10: the master regulator of immunity to infection. *J. Immunol.* 180:5771–5777, 2008.
[26]Cursiefen, C., L. Chen, L. P. Borges, D. Jackson, J. Cao, C. Radziejewski, P. A. D'Amore, M. R. Dana, S. J. Wiegand, and J. W. Streilein. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J. Clin. Investig.* 113:1040–1050, 2004.
[27]Davies, L. C., S. J. Jenkins, J. E. Allen, and P. R. Taylor. Tissue-resident macrophages. *Nat. Immunol.* 14:986–995, 2013.
[28]Davies, L. C., M. Rosas, S. J. Jenkins, C.-T. Liao, M. J. Scurr, F. Brombacher, D. J. Fraser, J. E. Allen, S. A. Jones, and P. R. Taylor. Distinct bone marrow-derived and tissue-resident macrophage lineages proliferate at key stages during inflammation. *Nat. Commun.* 4:1886, 2013.
[29]Debels, H., L. Galea, X. L. Han, J. Palmer, N. van Rooijen, W. Morrison, and K. Abberton. Macrophages play a key role in angiogenesis and adipogenesis in a mouse tissue engineering model. *Tissue Eng. Part A* 19:2615–2625, 2013.
[30]Desmouliere, A., A. Geinoz, F. Gabbiani, and G. Gabbiani. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J. Cell Biol.* 122:103–111, 1993.
[31]Falanga, V., S. W. Qian, D. Danielpour, M. H. Katz, A. B. Roberts, and M. B. Sporn. Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. *J. Invest. Dermatol.* 97:634–637, 1991.
[32]Fantin, A., J. M. Vieira, G. Gestri, L. Denti, Q. Schwarz, S. Prykhozhij, F. Peri, S. W. Wilson, and C. Ruhrberg. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood* 116:829–840, 2010.
[33]Fitzpatrick, L. E., A. Lisovsky, E. C. Ciucurel, and M. V. Sefton. Scaffold vascularization. In Scaffolds for Tissue

Engineering: Bioogical Design, Materials and Fabrications, edited by C. Migliaresi and A. Motta: Pan Stanford, 2014, pp. 173–217.
[34]Garg, K., N. A. Pullen, C. A. Oskeritzian, J. J. Ryan, and G. L. Bowlin. Macrophage functional polarization (M1/M2) in response to varying fiber and pore dimensions of electrospun scaffolds. *Biomaterials* 34:4439–4451, 2013.
[35]Ghajar, C. M., S. Kachgal, E. Kniazeva, H. Mori, S. V. Costes, S. C. George, and A. J. Putnam. Mesenchymal cells stimulate capillary morphogenesis via distinct proteolytic mechanisms. *Exp. Cell Res.* 316:813–825, 2010.
[36]Ginhoux, F., and S. Jung. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* 14:392–404, 2014.
[37]Gong, Y., and D.-R. Koh. Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an in vivo corneal model. *Cell Tissue Res.* 339:437–448, 2010.
[38]Gong, D., W. Shi, S. J. Yi, H. Chen, J. Groffen, and N. Heisterkamp. Tgfbeta signaling plays a critical role in promoting alternative macrophage activation. *BMC Immunol.* 13:31, 2012.
[39]Grunewald, M., I. Avraham, Y. Dor, E. Bachar-Lustig, A. Itin, S. Jung, S. Chimenti, L. Landsman, R. Abramovitch, and E. Keshet. Vegf-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 124:175–189, 2006.
[40]Gu, C., and E. Giraudo. The role of semaphorins and their receptors in vascular development and cancer. *Exp. Cell Res.* 319:1306–1316, 2013.
[41]Gulliksrud, K., I. K. Vestvik, K. Galappathi, B. Mathiesen, and E. K. Rofstad. Detection of different hypoxic cell subpopulations in human melanoma xenografts by pimonidazole immunohistochemistry. *Radiat. Res.* 170:638–650, 2008.
[42]Hanahan, D., and J. Folkman. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86:353–364, 1996.
[43]Hirschi, K. K., D. A. Ingram, and M. C. Yoder. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler. Thromb. Vasc. Biol.* 28:1584–1595, 2008.
[44]Hu, M., D. Ludlow, J. S. Alexander, J. McLarty, and T. Lian. Improved wound healing of postischemic cutaneous flaps with the use of bone marrow-derived stem cells. *Laryngoscope* 124:642–648, 2014.
[45]Hu, X., S. P. Yu, J. L. Fraser, Z. Lu, M. E. Ogle, J. A. Wang, and L. Wei. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. *J. Thorac. Cardiovasc. Surg.* 135:799–808, 2008.
[46]Hung, S. C., R. R. Pochampally, S. C. Chen, S. C. Hsu, and D. J. Prockop. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells* 25:2363–2370, 2007.
[47]Iyer, N. V., L. E. Kotch, F. Agani, S. W. Leung, E. Laughner, R. H. Wenger, M. Gassmann, J. D. Gearhart, A. M. Lawler, A. Y. Yu, and G. L. Semenza. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev.* 12:149–162, 1998.
[48]Jankovic, B., C. Aquino-Parsons, J. A. Raleigh, E. J. Stanbridge, R. E. Durand, J. P. Banath, S. H. MacPhail, and P. L. Olive. Comparison between pimonidazole binding, oxygen electrode measurements, and expression of endogenous hypoxia markers in cancer of the uterine cervix. *Cytometry B Clin. Cytom.* 70:45–55, 2006.
[49]Jetten, N., S. Verbruggen, M. J. Gijbels, M. J. Post, M. P. De Winther, and M. M. Donners. Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. *Angiogenesis* 17:109–118, 2013.
[50]Johnson, P. C., A. G. Mikos, J. P. Fisher, and J. A. Jansen. Strategic directions in tissue engineering. *Tissue Eng.* 13:2827–2837, 2007.
[51]Kang, K. T., P. Allen, and J. Bischoff. Bioengineered human vascular networks transplanted into secondary mice reconnect with the host vasculature and re-establish perfusion. *Blood* 118:6718–6721, 2011.
[52]Karin, N. The multiple faces of CXCL12 (SDF-1) in the regulation of immunity during health and disease. *J. Leukoc. Biol.* 88:463–473, 2010.
[53]Keith, B., R. S. Johnson, and M. C. Simon. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. *Nat. Rev. Cancer* 12:9–22, 2012.
[54]Kellner, K., G. Liebsch, I. Klimant, O. S. Wolfbeis, T. Blunk, M. B. Schulz, and A. Gopferich. Determination of oxygen gradients in engineered tissue using a fluorescent sensor. *Biotechnol. Bioeng.* 80:73–83, 2002.
[55]Kim, J. W., I. Tchernyshyov, G. L. Semenza, and C. V. Dang. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab.* 3:177–185, 2006.
[56]Koh, M. Y., and G. Powis. Passing the baton: the HIF switch. *Trends Biochem. Sci.* 37:364–372, 2012.
[57]Kolaczkowska, E., and P. Kubes. Angiogenic neutrophils: a novel subpopulation paradigm. *Blood* 120:4455–4457, 2012.
[58]Lin, E. Y., J. F. Li, L. Gnatovskiy, Y. Deng, L. Zhu, D. A. Grzesik, H. Qian, X. N. Xue, and J. W. Pollard. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res.* 66:11238–11246, 2006.
[59]Liu, L., Q. Yu, J. Lin, X. Lai, W. Cao, K. Du, Y. Wang, K. Wu, Y. Hu, L. Zhang, H. Xiao, Y. Duan, and H. Huang. Hypoxia-inducible factor-1alpha is essential for hypoxia-induced mesenchymal stem cell mobilization into the peripheral blood. *Stem Cells Dev.* 20:1961–1971, 2011.
[60]Lucas, T., A. Waisman, R. Ranjan, J. Roes, T. Krieg, W. Muller, A. Roers, and S. A. Eming. Differential roles of macrophages in diverse phases of skin repair. *J. Immunol.* 184:3964–3977, 2010.
[61]Ma, S., N. Xie, W. Li, B. Yuan, Y. Shi, and Y. Wang. Immunobiology of mesenchymal stem cells. *Cell Death Differ.* 21:216–225, 2013.
[62]Malda, J., J. Rouwkema, D. E. Martens, E. P. Le Comte, F. K. Kooy, J. Tramper, C. A. van Blitterswijk, and J. Riesle. Oxygen gradients in tissue-engineered PEGT/PBT cartilaginous constructs: measurement and modeling. *Biotechnol. Bioeng.* 86:9–18, 2004.
[63]Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25:677–686, 2004.
[64]Martinez, F. O., L. Helming, and S. Gordon. Alternative activation of macrophages: an immunologic functional perspective. *Annu. Rev. Immunol.* 27:451–483, 2009.
[65]Martins-Green, M., M. Petreaca, and L. Wang. Chemokines and their receptors are key players in the orchestra that regulates wound healing. *Adv. Wound Care (New Rochelle)* 2:327–347, 2013.

[66]Maxwell, P. H., C. W. Pugh, and P. J. Ratcliffe. Activation of the HIF pathway in cancer. *Curr. Opin. Genet. Dev.* 11:293–299, 2001.
[67]Mehrad, B., M. P. Keane, and R. M. Strieter. Chemokines as mediators of angiogenesis. *Thromb. Haemost.* 97:755–762, 2007.
[68]Meininger, C. J., M. E. Schelling, and H. J. Granger. Adenosine and hypoxia stimulate proliferation and migration of endothelial cells. *Am. J. Physiol.* 255:H554–H562, 1988.
[69]Melero-Martin, J. M., M. E. De Obaldia, P. Allen, A. C. Dudley, M. Klagsbrun, and J. Bischoff. Host myeloid cells are necessary for creating bioengineered human vascular networks in vivo. *Tissue Eng Part A* 16:2457–2466, 2010.
[70]Menon, C., G. M. Polin, I. Prabakaran, A. Hsi, C. Cheung, J. P. Culver, J. F. Pingpank, C. S. Sehgal, A. G. Yodh, D. G. Buerk, and D. L. Fraker. An integrated approach to measuring tumor oxygen status using human melanoma xenografts as a model. *Cancer Res.* 63:7232–7240, 2003.
[71]Mikami, S., A. Nakashima, K. Nakagawa, T. Maruhashi, Y. Iwamoto, M. Kajikawa, T. Matsumoto, Y. Kihara, K. Chayama, K. Noma, M. Ochi, M. Nishimura, K. Tsuji, Y. Kato, C. Goto, and Y. Higashi. Autologous bone-marrow mesenchymal stem cell implantation and endothelial function in a rabbit ischemic limb model. *PLoS One* 8:e67739, 2013.
[72]Mojsilovic-Petrovic, J., D. Callaghan, H. Cui, C. Dean, D. B. Stanimirovic, and W. Zhang. Hypoxia-inducible factor-1 (HIF-1) is involved in the regulation of hypoxia-stimulated expression of monocyte chemoattractant protein-1 (MCP-1/CCl2) and mcp-5 (CCL12) in astrocytes. *J. Neuroinflamm.* 4:12, 2007.
[73]Mosser, D. M., and J. P. Edwards. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8:958–969, 2008.
[74]Murdoch, C. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* 104:2224–2234, 2004.
[75]Murdoch, C., M. Muthana, S. B. Coffelt, and C. E. Lewis. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat. Rev. Cancer* 8:618–631, 2008.
[76]Newman, A. C., M. N. Nakatsu, W. Chou, P. D. Gershon, and C. C. Hughes. The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Mol. Biol. Cell* 22:3791–3800, 2011.
[77]Nucera, S., D. Biziato, and M. De Palma. The interplay between macrophages and angiogenesis in development, tissue injury and regeneration. *Int. J. Dev. Biol.* 55:495–503, 2011.
[78]Ohnishi, S., T. Yasuda, S. Kitamura, and N. Nagaya. Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. *Stem Cells* 25:1166–1177, 2007.
[79]Peng, L. H., Z. Y. Mao, X. T. Qi, X. Chen, N. Li, Y. Tabata, and J. Q. Gao. Transplantation of bone-marrow-derived mesenchymal and epidermal stem cells contribute to wound healing with different regenerative features. *Cell Tissue Res.* 352:573–583, 2013.
[80]Pickup, M., S. Novitskiy, and H. L. Moses. The roles of TGFbeta in the tumour microenvironment. *Nat. Rev. Cancer* 13:788–799, 2014.
[81]Pollard, J. W. Trophic macrophages in development and disease. *Nat. Rev. Immunol.* 9:259–270, 2009.
[82]Pucci, F., M. A. Venneri, D. Biziato, A. Nonis, D. Moi, A. Sica, C. Di Serio, L. Naldini, and M. De Palma. A distinguishing gene signature shared by tumor-infiltrating Tie2-expressing monocytes, blood "Resident" Monocytes, and embryonic macrophages suggests common functions and developmental relationships. *Blood* 114:901–914, 2009.
[83]Rademakers, S. E., J. Lok, A. J. van der Kogel, J. Bussink, and J. H. Kaanders. Metabolic markers in relation to hypoxia; staining patterns and colocalization of pimonidazole, HIF-1alpha, CAIX, LDH-5, GLUT-1, MCT1 and MCT4. *BMC Cancer* 11:167, 2011.
[84]Richardson, M. R., and M. C. Yoder. Endothelial progenitor cells: quo vadis? *J. Mol. Cell. Cardiol.* 50:266–272, 2011.
[85]Roberts, T., U. De Boni, and M. V. Sefton. Dopamine secretion by PC12 cells microencapsulated in a hydroxyethyl methacrylate–methyl methacrylate copolymer. *Biomaterials* 17:267–275, 1996.
[86]Rose, S., A. Misharin, and H. Perlman. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry Part A* 81A:343–350, 2012.
[87]Sanchez-Martin, L., A. Estecha, R. Samaniego, S. Sanchez-Ramon, M. A. Vega, and P. Sanchez-Mateos. The chemokine CXCL12 regulates monocyte-macrophage differentiation and runx3 expression. *Blood* 117:88–97, 2011.
[88]Saunders, W. B., B. L. Bohnsack, J. B. Faske, N. J. Anthis, K. J. Bayless, K. K. Hirschi, and G. E. Davis. Coregulation of vascular tube stabilization by endothelial cell timp-2 and pericyte timp-3. *J. Cell Biol.* 175:179–191, 2006.
[89]Seagroves, T. N., H. E. Ryan, H. Lu, B. G. Wouters, M. Knapp, P. Thibault, K. Laderoute, and R. S. Johnson. Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. *Mol. Cell. Biol.* 21:3436–3444, 2001.
[90]Semenza, G. L. Hypoxia-inducible factors in physiology and medicine. *Cell* 148:399–408, 2012.
[91]Sieveking, D. P., A. Buckle, D. S. Celermajer, and M. K. Ng. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. *J. Am. Coll. Cardiol.* 51:660–668, 2008.
[92]Singer, N. G., and A. I. Caplan. Mesenchymal stem cells: mechanisms of inflammation. *Annu. Rev. Pathol.* 6:457–478, 2011.
[93]Singer, A. J., and R. A. Clark. Cutaneous wound healing. *N. Engl. J. Med.* 341:738–746, 1999.
[94]Skuli, N., A. J. Majmundar, B. L. Krock, R. C. Mesquita, L. K. Mathew, Z. L. Quinn, A. Runge, L. Liu, M. N. Kim, J. Liang, S. Schenkel, A. G. Yodh, B. Keith, and M. C. Simon. Endothelial HIF-2alpha regulates murine pathological angiogenesis and revascularization processes. *J. Clin. Investig.* 122:1427–1443, 2012.
[95]Spiller, K. L., R. R. Anfang, K. J. Spiller, J. Ng, K. R. Nakazawa, J. W. Daulton, and G. Vunjak-Novakovic. The role of macrophage phenotype in vascularization of tissue engineering scaffolds. *Biomaterials* 35:4477–4488, 2014.
[96]Stockmann, C., D. Schadendorf, R. Klose, and I. Helfrich. The impact of the immune system on tumor: Angiogenesis and vascular remodeling. *Front Oncol* 4:69, 2014.
[97]Stockmann, C., D. Schadendorf, R. Klose, and I. Helfrich. The impact of the immune system on tumor: angiogenesis and vascular remodeling. *Front Oncol.* 4:69, 2014.

[98]Sun, C. J., C. Li, H. B. Lv, C. Zhao, J. M. Yu, G. H. Wang, Y. X. Luo, Y. Li, M. Xiao, J. Yin, and J. Y. Lang. Comparing ct perfusion with oxygen partial pressure in a rabbit VX2 soft-tissue tumor model. *J. Radiat. Res.* 55:183–190, 2014.

[99]Takahashi, T., C. Kalka, H. Masuda, D. Chen, M. Silver, M. Kearney, M. Magner, J. M. Isner, and T. Asahara. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat. Med.* 5:434–438, 1999.

[100]Tazzyman, S., C. E. Lewis, and C. Murdoch. Neutrophils: key mediators of tumour angiogenesis: neutrophils in tumour angiogenesis. *Int. J. Exp. Pathol.* 90:222–231, 2009.

[101]Tura, O., E. M. Skinner, G. R. Barclay, K. Samuel, R. C. Gallagher, M. Brittan, P. W. Hadoke, D. E. Newby, M. L. Turner, and N. L. Mills. Late outgrowth endothelial cells resemble mature endothelial cells and are not derived from bone marrow. *Stem Cells* 31:338–348, 2013.

[102]van Amerongen, M. J., M. C. Harmsen, N. van Rooijen, A. H. Petersen, and M. J. van Luyn. Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. *Am. J. Pathol.* 170:818–829, 2007.

[103]Van Belle, E., B. Witzenbichler, D. Chen, M. Silver, L. Chang, R. Schwall, and J. M. Isner. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. *Circulation* 97: 381–390, 1998.

[104]Weidemann, A., and R. S. Johnson. Biology of HIF-1alpha. *Cell Death Differ.* 15:621–627, 2008.

[105]Willenborg, S., T. Lucas, G. van Loo, J. A. Knipper, T. Krieg, I. Haase, B. Brachvogel, M. Hammerschmidt, A. Nagy, N. Ferrara, M. Pasparakis, and S. A. Eming. CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. *Blood* 120:613–625, 2012.

[106]Zeisberger, S. M., B. Odermatt, C. Marty, A. H. Zehnder-Fjallman, K. Ballmer-Hofer, and R. A. Schwendener. Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. *Br. J. Cancer* 95:272–281, 2006.

[107]Zhu, H., Q. Shao, X. Sun, Z. Deng, X. Yuan, D. Yu, X. Zhou, and Y. Ding. The mobilization, recruitment and contribution of bone marrow-derived endothelial progenitor cells to the tumor neovascularization occur at an early stage and throughout the entire process of hepatocellular carcinoma growth. *Oncol. Rep.* 28:1217–1224, 2012.