Exploring and Harnessing the Role of Hypoxia Inducible Factors in Vascularizing Modular Tissue Constructs

by

Gabrielle Chi Yan Lam

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials and Biomedical Engineering
University of Toronto

© Copyright by Gabrielle Chi Yan Lam 2018
Exploring and Harnessing the Role of Hypoxia Inducible Factors in Vascularizing Modular Tissue Constructs

Gabrielle Chi Yan Lam

Doctor of Philosophy

Institute of Biomaterials and Biomedical Engineering
University of Toronto

2018

Abstract

In my doctoral work, I identified hypoxia-inducible factor (HIF) as a primary mechanism driving modular tissue-induced vascularization, and harnessed it to enhance blood vessel formation in this platform. HIF orchestrates a range of cellular responses, but its role in driving vascularization of engineered tissues has yet to be specified. In the first pillar of my work, HIF was inhibited using pharmacological and genetic methods to define its contribution to vessel formation in modular tissues. Both inhibitory methods, systemic digoxin delivery and shRNA-mediated inhibition of HIF-1 in graft endothelial cells (EC), reduced vascularization and oxygenation of tissue constructs. Moreover, these methods elucidated different mechanisms underlying HIF-mediated vascularization. Systemic digoxin treatment reduced early recruitment of endothelial progenitor cells and monocytes/macrophages to implants. Inhibition of HIF-1 in graft EC reduced their angiogenic function in normal and serum-reduced conditions in vitro, highlighting the importance of HIF in graft-derived vascularization. After defining the significance of its role, we harnessed HIF to enhance rapid and mature vessel formation in modular tissues. In the second pillar of my work, microparticles releasing HIF-stabilizing deferoxamine (DFO) were incorporated into EC-only modular constructs, on the premise that local HIF stabilization would drive vascularization even in the absence of mesenchymal stromal
cells (MSC). Indeed, vessel densities were increased at day 7 and 21, corresponding with increased vessel maturation and perfusion at day 21. Furthermore, DFO-releasing microparticles increased the recruitment of neutrophils and alternatively activated macrophages to the implant. Although vessel formation was rescued to some degree, it was not comparable to that in MSC-containing modular constructs. Nonetheless, the mechanisms elucidated here could be harnessed to develop strategies that tune vascularization in modular tissues, and to improve engraftment of therapeutic cells.
Acknowledgments

I lay this at Your feet with a heart of thanks. Thank You –

For the opportunity to study a small thread of Your creation.

For the joys and challenges of this journey because, through them both, I saw Your goodness.

For a supportive and wise supervisor, who had my back in every step. For wonderful lab members, who helped me troubleshoot and stress-eat.

For the friendships You’ve given me – people with whom I’ve laughed to tears and shared meaningful conversations. Thank You, in particular, for those who were there regardless of distance or time.

For my parents. They are a special blessing from Your hands. How I could have pulled through without them, I wouldn’t know.

Thank You.
Table of Contents

Acknowledgments......................................................................................................................... iv
Table of Contents............................................................................................................................. v
List of Tables ........................................................................................................................................ x
List of Figures ....................................................................................................................................... xi
List of Appendices ............................................................................................................................. xiii
Chapter 1 ............................................................................................................................................. 1
  1 Thesis Overview............................................................................................................................... 1
     1.1 Hypotheses .................................................................................................................................. 1
     1.2 Specific Aims ............................................................................................................................... 1
     1.3 Thesis Structure ........................................................................................................................... 2
     1.4 Experimental Model .................................................................................................................... 3
        1.4.1 Modular tissue engineering ............................................................................................... 3
        1.4.2 Experimental Model ............................................................................................................. 5
        1.4.3 Research gap ......................................................................................................................... 7
     1.5 References ................................................................................................................................... 9
Chapter 2 ............................................................................................................................................. 11
  2 Background Literature ..................................................................................................................... 11
     2.1 Vascularization in Tissue Engineering ....................................................................................... 11
        2.1.1 Growth Factor Delivery ....................................................................................................... 11
        2.1.2 Implantation of Angiogenic Cells ....................................................................................... 13
     2.2 Hypoxia and Hypoxia Inducible Factors ..................................................................................... 15
        2.2.1 Hypoxic Induction of HIF ...................................................................................................... 15
        2.2.2 Regulation of HIF ................................................................................................................... 16
3.3 Results..................................................................................................................................................66
  3.3.1 HIF inhibition reduced vascularization and perfusion of modular tissue constructs ..........................................................66
  3.3.2 Digoxin and shRNA-mediated knockdown inhibited HIF-1 activation in modular implants .................................................68
  3.3.3 Digoxin inhibited recruitment of bone marrow-derived cells to the modular implant ..................................................69
3.4 Supplementary Materials ..........................................................................................................................72
  3.4.1 Supplementary Figures ......................................................................................................................72
  3.4.2 Supplementary Tables .......................................................................................................................80
3.5 Discussion .................................................................................................................................................81
3.6 Conclusion ..................................................................................................................................................84
3.7 Acknowledgements ..................................................................................................................................85
3.8 References ................................................................................................................................................86
Chapter 4 ..........................................................................................................................................................89
4 Hypoxia-inducible Factor Stabilization Induces Vascularization of Modular Tissue Constructs .........................89
  4.1 Introduction .............................................................................................................................................89
  4.2 Methods and Materials ..........................................................................................................................91
    4.2.1 Microparticle fabrication ....................................................................................................................91
    4.2.2 Microparticle characterization ..........................................................................................................91
    4.2.3 In vitro DFO treatment of HUVEC .................................................................................................92
    4.2.4 Module fabrication ............................................................................................................................93
    4.2.5 Animal surgeries ...............................................................................................................................94
    4.2.6 Immunohistochemistry ....................................................................................................................94
    4.2.7 Vascular perfusion ............................................................................................................................94
    4.2.8 Modular tissue digestion ....................................................................................................................95
    4.2.9 Flow cytometry .................................................................................................................................95
4.2.10 Statistical analyses ................................................. 96

4.3 Results ........................................................................ 97

4.3.1 Characterization of DFO-releasing microparticles ............... 97

4.3.2 DFO microparticles increased vascularization and vessel perfusion in modular tissues ......................................................................................................................... 98

4.3.3 DFO microparticles increased and prolonged HIF activation in modular tissues ........................................................................................................................................ 101

4.3.4 DFO microparticles augmented early recruitment of neutrophils and retention of M2-polarized macrophages to modular implants ............................................................................ 102

4.4 Supplementary Materials .................................................. 104

4.4.1 Supplementary Figures .................................................. 104

4.4.2 Supplementary Tables .................................................... 111

4.5 Discussion ....................................................................... 113

4.5.1 DFO microparticles partly rescued vascularization of modular tissues in the absence of MSC .......................................................................................................................... 113

4.5.2 DFO microparticles coordinated an angiogenic response from various cells in the subcutaneous space ........................................................................................................... 114

4.5.3 DFO release amplified the recruitment of inflammatory and anti-inflammatory cells in a temporal manner ............................................................................................................. 115

4.6 Conclusion ....................................................................... 117

4.7 References ....................................................................... 118

Chapter 5 .............................................................................. 122

5 Discussion and Future Directions ............................................. 122

5.1 Significance ..................................................................... 122

5.2 Limitations and Future Work ............................................. 124

5.3 Conclusion ..................................................................... 130

5.4 References ..................................................................... 131

Appendices ............................................................................ 133

Appendix I: DFO Controlled Release Profile ................................ 133
Appendix II: Statistical Analyses and Data Transformations.................................................................138
List of Tables

**Table 3-S1.** Summary of all statistically significant pairwise comparisons for HIF-1α+ nuclei density and HIF-1α+ nuclei proportion in digoxin experiments.

**Table 4-S1.** Summary of antibodies used for flow cytometry analyses of digested modular implants.

**Table 4-S2.** Summary of results of statistical analyses.
List of Figures

Figure 1-1. Modular tissues assemble into vascularized tissues when injected subcutaneously in immune-compromised mice.

Figure 2-1. The HIF-1 and HIF-2 pathways dominate different stages of vessel formation in response to hypoxic stress.

Figure 2-2. Post-translational regulation of HIF-1α by PHD and FIH.

Figure 2-3. Schematic diagram summarizing the various cellular responses and their associated genes that are transcriptionally regulated by HIF-1.

Figure 3-1. Inhibition of HIF reduced early vascularization of modular constructs.

Figure 3-2. Inhibition reduced HIF-1 nuclear localization in modular constructs at early times.

Figure 3-3. Systemic HIF inhibition affected the mobilization of bone marrow-derived cells and their recruitment to the modular construct.

Figure 3-S1. Effect of HIF inhibition on graft-derived (UEA-1+) vessel density and tissue oxygenation.

Figure 3-S2. Selection of HIF-1α target sequence for the design of lentiviral shRNA constructs, MOI for transduction, and confirmation of HIF-1α knockdown in cobalt chloride stimulated HUVEC.

Figure 3-S3. Effect of shRNA-mediated HIF-1α knockdown in HUVEC on sprout number and length in normal and reduced serum conditions.

Figure 3-S4. Effect of digoxin on HUVEC and adMSC viability and HIF-1α stabilization in vitro.

Figure 3-S5. Digoxin had no effect on the mobilization of leukocytes, neutrophils and hemangiocytes in peripheral blood, nor their recruitment to the modular tissue construct.
**Figure 3-S6.** Gating scheme for flow cytometry analyses of cells mobilized in peripheral blood.

**Figure 3-S7.** Gating scheme for flow cytometry analyses of cells recruited to modular tissue constructs.

**Figure 4-1.** In vitro characterization of DFO-loaded PLGA microparticles.

**Figure 4-2.** DFO-releasing microparticles enhanced vascularization of EC-only modular tissue constructs.

**Figure 4-3.** DFO-releasing microparticles enhanced maturation and perfusion of blood vessels in EC-only modular tissue constructs.

**Figure 4-4.** DFO-releasing microparticles increased HIF-1 and HIF-2 activation in EC-only modular tissue constructs.

**Figure 4-5.** DFO-releasing microparticles increased neutrophil and M2-polarized macrophage recruitment to EC-only modular tissue constructs.

**Figure 4-S1.** Graph of logarithm of cumulative fraction released versus logarithm of time.

**Figure 4-S2.** Schematic diagram summarizing the gating scheme used in flow cytometry analyses.

**Figure 4-S3.** Vessel structures in untreated and free DFO controls were poorly perfused.

**Figure 4-S4.** Number and proportion of cells recruited to EC-only modular tissue constructs with empty or DFO microparticles.

**Figure 4-S5.** Number of cells recruited to untreated EC-only modular tissue constructs.

**Figure 4-S6.** *In vitro* effects of DFO treatment on HUVEC sprouting.
List of Appendices

**Appendix I.** DFO Controlled Release Profile

**Appendix II.** Statistical Analyses and Data Transformations
The goal of this thesis was to elucidate the role of hypoxia-inducible factor (HIF) activation in driving module-induced vascularization, and to harness this mechanism to increase the rate, degree and maturity of vessel formation in modular tissue constructs. This work was undertaken to advance modular tissue engineering as a platform for enhancing therapeutic cell engraftment.

1 Thesis Overview

1.1 Hypotheses

**Hypothesis 1:** If HIF is inhibited, vascularization of modular tissue constructs will be suppressed, indicating that it is a critical driver of module-induced vascularization.

**Hypothesis 2:** If HIF is stabilized by the controlled release of deferoxamine from microparticles, rapid and mature vascularization will be induced in modular tissue constructs, even in the absence of MSC.

**Hypothesis 3:** If HIF is stabilized or inhibited, the recruitment of angiogenic bone marrow-derived cells, including endothelial progenitor cells (EPC) and macrophages, to the module implant will correspondingly increase or decrease, respectively.

1.2 Specific Aims

**Specific Aim 1:** Inhibit HIF in modular tissue constructs

A. Genetic knockdown of HIF-1α in HUVEC of modular tissues

B. Pharmacologic inhibition of HIF using digoxin
Specific Aim 2: Increase the rate of mature module-induced vascularization by controlled delivery of a pharmacological HIF stabilizer within modules, in the absence of MSC

A. Design and characterize microparticles for the encapsulation and controlled release of desferrioxamine (DFO)

B. Incorporate controlled releasing DFO microparticles in modular implants to increase the rate and maturation of module-induced vascularization in vivo

Specific Aim 3: Determine the effect of HIF on EPC mobilization into peripheral blood and recruitment to the modular implant

A. Monitor mobilization and recruitment of EPC in a pharmacological HIF inhibition model

B. Monitor mobilization and recruitment of EPC in modular implants with controlled release of DFO

1.3 Thesis Structure

This thesis compiles all experiments and findings that address the specific aims and hypotheses outlined above. A thorough review of literature is first presented in Chapter 2, encompassing pertinent studies on vascularization strategies in tissue engineering, the biology of HIF, strategies for harnessing HIF in tissue regeneration, and on the bone marrow-derived cell populations that contribute to angiogenesis.

Experimental findings are compiled into two scientific manuscripts. The first manuscript (Chapter 3) pertains to specific aims 1 and 3a, and seeks to define the role of HIF in driving module-induced vascularization using two inhibitory approaches. The second manuscript (Chapter 4) pertains to specific aims 2 and 3b, investigating the effect of prolonged HIF stabilization on rate and maturity of vascularization in EC-only modular tissues. Finally, the discussion of this thesis summarizes all important findings, highlights the significance and limitations of the work, and discusses some future work to be conducted.
1.4 Experimental Model

1.4.1 Modular tissue engineering

Modular tissue engineering is a strategy developed in the Sefton laboratory for the assembly of tissue constructs with integral vasculature. Because oxygen diffusion is limited to distances less than 100 μm (1), vascularization strategies are necessary for fabricating engineered tissues of clinically relevant size and cell loads. To this end, various strategies have been explored, including delivery of angiogenic growth factors (2–5) and cells (6, 7). A more extensive discussion is provided in Chapter 2. Modular tissue engineering not only addresses the challenge of vascularizing tissues, but is also amenable to a minimally invasive delivery format for the injection of microtissues by syringe and needle. For these reasons, modular tissue engineering is a promising platform for enhancing therapeutic cell engraftment.

Modular tissue constructs are formed from randomly assembled collagen cylinders (modules) of 500-600 microns length and 400 microns diameter. Modules are enveloped in a layer of endothelial cells (EC). When used to fill a space, they randomly pack to create a porous construct with EC-lined channels, as depicted in Figure 1-1. When first developed, the EC layer of modular tissues was shown to retain a non-thrombogenic phenotype that delayed clotting times when perfused with whole blood in vitro (8, 9). HepG2 cells were encapsulated within modules, demonstrating the feasibility of embedding other functional cell types within. Due to their modular format, tissue constructs could be fabricated in a scalable manner.

Modular tissue constructs have been injected subcutaneously in mice and into the omental pouch of rats to investigate their ability to induce vascularization in vivo. Importantly, subcutaneous delivery of EC-coated modules into immune-compromised mice resulted in apoptosis of graft-derived EC within the first 3 days of implantation, and poor vascularization of the implant (10). Apoptosis of human EC was partially attenuated by coating modules with fibronectin. It was later shown that co-implantation of mesenchymal stromal cells (MSC) within EC-coated modules induced robust vascularization (11, 12). In the presence of MSC, EC-lined channels remodeled and connected with host vessels to form a perfusable chimeric vasculature, as confirmed by micro-computed tomography scanning. It is postulated that graft-derived EC migrate away from module surfaces and connect with other EC-lined pores to form a perfusable vasculature. Moreover, transduction of implanted EC with an angiogenic extracellular matrix
protein, known as developmental endothelial locus-1 (Del-1), increased vessel density and vessel coverage with smooth muscle actin (SMA) at day 7 (13).

**Figure 1-1. Modular tissues assemble into vascularized tissues when injected subcutaneously in immune-compromised mice.** Schematic diagram of a module, containing MSC in type I collagen and enveloped with an EC layer (left). *In vivo*, modules pack randomly to form a porous construct (right, bottom) and EC-lined channels remodel over time to form perfusable blood vessels, as visualized by micro-CT (micro-CT image adapted from (12)).

Because modules can induce vascularization upon implantation, the platform has been used to construct various tissue types. Implantation of modular tissues embedded with adipose-derived MSC (adMSC) resulted in the formation of a vascularized fat pad in the subcutaneous space of severe-combined immunodeficient-beige mutation (SCID-bg) mice (12). Generation of adipose tissue, as assessed by Oil Red-O staining, was significantly enhanced by the presence of EC, and sustained vascularization and adipose tissue formation for up to 90 days post-implantation.

Additionally, implantation of cardiomyocyte-containing modules into the peri-infarct site in rats resulted in enhanced vascularization and formation of striated muscle-like structures (14). The modular tissue platform was also explored for the delivery of early outgrowth and late outgrowth bone marrow-derived cells in the treatment of chronic kidney disease (15). Using a sub-totally nephrectomized rat model, Kepecs et al. demonstrated that subcutaneous transplantation of both
early outgrowth and late outgrowth cells in modules enhanced reno-protective effects, relative to implantation of early outgrowth cells alone. Surprisingly, this effect was observed despite poor vascularization of the implant and efflux of cells, and was attributed to cell secretion of angiogenic and anti-fibrotic factors.

Modular tissue engineering was also explored for islet transplantation and treatment of diabetes. Syngeneic implantation of 2000 islets within endothelialized modules into streptozotocin-induced diabetic rats resulted in robust vascularization of the implant, and persistence of SMA\(^+\) vessels to 21 days (16). The presence of EC enhanced islet viability relative to uncoated modules. Using a subcutaneous implantation model in diabetic SCID-bg mice, Vlahos et al. showed that implantation of 750 rat islet equivalents into endothelialized modules resulted in maintenance of normoglycemia for 21 days, which was not otherwise observed with subcutaneous injection of 750 free islet equivalents (17). Co-implantation of islets with EC enhanced vascular integration of islets, as visualized using CLARITY imaging. Taken collectively, these studies show that the modular tissue engineering platform can be used to create vascularized tissues containing a therapeutic cell type of interest.

1.4.2 Experimental Model

1.4.2.1 Module system and fabrication

For experiments related to specific aim 1 and 3a, dual delivery of human umbilical vein endothelial cells (HUVEC) and human adMSC was chosen because the model yields robust vascularization with the cell loads used (11, 12, 18). Human cells were desirable to create a humanized platform for therapeutic cell engraftment, as well as for allowing visualization of the chimeric vasculature. While bone marrow-derived MSC are also commonly used, adipose tissue is a more easily accessible source. MSC contribute pro-angiogenic and anti-apoptotic effects that benefit EC survival and vessel formation. Additionally, MSC migrate from within the module and adopt a pericyte-like fate (SMA\(^+\)), supporting maturation of EC-based channels (11). Others have also shown that MSC increase the stability of vessels within the engineered tissue (6, 7).

For experiments pertaining to specific aim 2 and 3b, the HUVEC-only module platform was used. Previous work from the Sefton laboratory showed that implantation of EC alone were
insufficient for stimulating vascularization in the subcutaneous model used (10). We specifically used this vascularization-impaired module platform to investigate the effect of prolonged HIF stabilization on rescuing vessel formation. More importantly, we sought to develop a local HIF stabilization strategy that would simplify the modular tissue platform from using two cell types to a single cell type.

Modular tissues were fabricated using a procedure outlined previously (19). Each implant was prepared using 1.5 mL of purified bovine type I collagen (Advanced Biomatrix), with 10-times concentrated alpha Minimum Essential Media (Gibco, 128 µL/mL collagen), and 0.8 M sodium hydroxide (Sigma Aldrich, 14 µL/mL collagen). To fabricate modules containing adMSC, adMSC (Lonza) were cultured and used at passages 4-6, and suspended in the collagen solution at $1.5 \times 10^6$ adMSC/implant. adMSC-laden collagen or collagen solution without cells (for EC-only modules) were sectioned into cylindrical collagen pieces using an air-jet system. In this method (19), a pressurized air stream was used to shear the collagen solution, which was pushed towards a T-junction microchannel at a controlled volumetric rate. Collagen pieces were extruded into a gas-sterilized polyethylene tubing (Instech Laboratories, 760 µm diameter), and incubated at 37°C for one hour. Once gelled, the modules were flushed from the tubing. Modules containing adMSC were cultured overnight in a suspension culture petri dish containing 10 mL of medium (DMEM, Sigma Aldrich, supplemented with 10% FBS and 1% penicillin/streptomycin, Gibco). Module implants were seeded with $2 \times 10^6$ HUVEC (passage 3-6) with gentle shaking at 37°C for 1 hour. Modules were cultured in vitro prior to implantation either in a 50:50 mixture of adMSC and HUVEC media (for adMSC-containing modules) or in HUVEC media (EBM-2 supplemented with Bullet-kit, Lonza; for EC-only modules).

1.4.2.2 Animal and implantation model

SCID-bg mice (Jackson’s Laboratory) were used for all studies. SCID-bg mice lack T, B-lymphocytes, and have defective natural killer cells, rendering it an ideal recipient for human cells. Modules (0.1 mL implant volume, with or without MSC) were implanted subcutaneously in the dorsum of SCID-bg mice using a syringe and needle. This delivery format was chosen because it is minimally invasive, and demonstrates an advantage of the modular tissue engineering strategy. In addition to being easily accessible, the subcutaneous space is attractive
because it is poorly vascularized. Any observed vascularization can therefore be attributed to the angiogenic effect of modular tissues.

1.4.3 Research gap

While modular tissue engineering offers many advantages, our understanding of the major mechanisms governing their vascularization is limited. If elucidated, such mechanisms could be harnessed to better control module-induced vascularization, including rate, maturity and degree of vessel formation within the tissue constructs. That way, modular tissue engineering would be a more suitable platform for enhancing engraftment of therapeutic cells.

In previous work, I found that altering cell densities and implant volume of modular tissues affected the degree of vascularization induced in a subcutaneous transplantation model (18). Moreover, tuning these simple parameters corresponded to differences in activation of HIF-1 within implants. Large-volume implants and implants with high cell densities showed enhanced graft-derived vascularization, which corresponded to increased HIF-1 activation. In fact, many of the HIF-1α+ nuclei were identified as vessel-contributing EC. These initial observations not only point to a role of the HIF mechanism in driving modular tissue vascularization, but of its importance in EC assembly into vascular structures.

As it stands, modular tissue engineering involves the implantation of both EC and MSC to construct vascularized tissues. Implanted EC presumably assemble into vessel structures in the presence of paracrine signals and physical support from MSC, and which subsequently anastomose with host vasculature upon remodeling in vivo. Formation of a graft-derived vascular network is arguably beneficial for accelerating vascularization of the tissue construct, as opposed to reliance on ingrowth of host vessels into the implant. We and others have shown that implantation of EC or MSC alone does not give rise to robust vascularization (6, 7, 10, 12); this challenge is only addressed with co-delivery of both cell types. However, simplifying the modular tissue platform from a two-cell system to a one-cell system would be beneficial for its clinical translation.

The goal of the work presented herein is to elucidate the role of HIF activation in driving module-induced vascularization, with a particular focus on its effects on recruitment of
angiogenic bone marrow-derived cells. Another primary objective is to simplify modular tissues to a one-cell (EC-only) platform, and to harness HIF to increase the rate of mature vessel formation within these constructs.
1.5 References


Chapter 2

2 Background Literature

2.1 Vascularization in Tissue Engineering

Rapid and robust vascularization of engineered tissues is crucial to their application in supporting functional engraftment of therapeutic cells following implantation. To this end, various vascularizing strategies have been explored, and will be discussed in the following section. Modular tissue engineering, which was developed in the Sefton laboratory and discussed earlier, is one such strategy.

2.1.1 Growth Factor Delivery

The delivery of angiogenic growth factors within tissue scaffolds has been studied as a strategy for enhancing vascularization. Growth factors such as vascular endothelial growth factor (VEGF) have known and potent effects on vessel formation. However, solely incorporating these angiogenic growth factors into tissue scaffolds has been shown to be inefficient and oftentimes ineffective for inducing long-lasting vessels. Consequently, other strategies have been developed for the sequential delivery of multiple growth factors, as well as for the modification of delivered growth factors.

Temporally controlled delivery of angiogenic growth factors has been shown to be beneficial to inducing sprouting and maturation of vessel structures. Using a poly-lactide-co-glycolide (PLGA)-based scaffold, Brudno et al. demonstrated that rapid release of VEGF and angiopoietin-2 (Ang-2) induced greatest vessel density in a murine subcutaneous implantation model, which was suppressed by simultaneous release of platelet-derived growth factor (PDGF) and angiopoietin-1 (Ang-1) (1). Interestingly, the delayed release of PDGF and Ang-1 from PLGA microspheres, following rapid VEGF and Ang-2 delivery did not affect vessel density. Instead, it enhanced vessel maturation, as indicated by increased vessel coverage by α-smooth
muscle actin. In another study, VEGF was incorporated into PLGA nanofibers for its rapid release, and PDGF-loaded in nanoparticles integrated within the scaffold for its delayed release (2). When applied to full thickness cutaneous wounds in rats, sequential delivery of VEGF and PDGF accelerated wound closure and angiogenesis. Additionally, epithelialization was enhanced and persistence of granulation tissue reduced relative to wound treatment with a commercially available Hydrofera Blue® wound dressing. Together, these studies suggest the differing role of angiogenic growth factors in initiating vessel formation and in vascular remodeling, and to harness them in a temporally-controlled manner for driving vascularization in tissue constructs.

Growth factors have also been modified to enhance their angiogenic efficiency. Martino et al. identified a domain within platelet-derived growth factor (PDGF)-2 that was responsible for its strong interaction with various extracellular matrix proteins (3). This sequence was then fused to other growth factors to generate variants of VEGF and PDGF, and applied to murine full thickness cutaneous wounds within fibrin matrices. Compared to fibrin matrix alone, or the delivery of wildtype VEGF and PDGF, the growth factor variants accelerated wound closure, vessel formation and vessel maturation at days 7 and 10. In a similar study, it was hypothesized that rapid clearance of exogeneously delivered VEGF protein limited its angiogenic effect. A VEGF variant was designed, fused to a sequence from the α2-plasmin inhibitor, which allowed for its covalent crosslinking to a fibrin matrix, only to be released by enzymatic cleavage (4). When delivered within a fibrin matrix into the gastrocnemius muscle of SCID mice, the VEGF variant induced mature vessel formation within the concentration range of 0.01-5 µg/mL. When injected into the lower thigh muscle in a murine model of hindlimb ischemia, delivery of the VEGF variant at concentrations of 0.5 and 5 µg/mL enhanced limb perfusion 28 days post-treatment, compared to control gels. Chu et al. designed fibroblast growth factor (FGF)-2 polycation coacervates, which reduced its proteolytic degradation upon implantation (5). When injected subcutaneously, both free FGF-2 and FGF-2 coacervates induced vessel formation after one week. However, only FGF-2 coacervates increased α-smooth muscle actin (α-SMA) coverage of vessels after 2 weeks, which persisted 4 weeks post-implantation.
2.1.2 Implantation of Angiogenic Cells

Another well-studied strategy for vascularizing tissue constructs is the implantation of angiogenic cells. One of the first demonstrations of the creation of perfusable and persisting vasculature was by Koike et al. in 2004. In their seminal paper, HUVEC and mesenchymal precursor cells were implanted within a type 1 collagen-fibronectin scaffold into the cranial window chamber of SCID mice (6). Compared to implantation of EC alone, which gave rise to non-perfusable vessels that rarely survived past 60 days, co-delivery of mesenchymal precursor cells resulted in perfusable vessels that were stable for 4 months post-implantation. Some of the transplanted GFP-labelled mesenchymal precursors were found associated with vessels and expressing the mural marker α-SMA. Using the same murine cranial model, the authors showed that co-implantation of HUVEC and bone marrow-derived MSC in a type 1 collagen-fibronectin matrix also resulted in perfusable vessels that persisted to 130 days post-implantation (7). Like the previous study, bone marrow-derived MSC differentiated into perivascular cells that incorporated into vessel structures. Interestingly, implantation of bone marrow-derived MSC alone did not induce vascularization. In vitro, contact between EC and MSC resulted in endothelial upregulation of myocardin, an effect that was abolished in a transwell co-culture system (without cell contact). Using the same implantation model, authors demonstrated that EC and mesenchymal precursor cells derived from human induced pluripotent stem cells could generate a stable vasculature that persisted to 280 days in mice (8).

Others reported similar findings upon implantation of cord blood-derived endothelial progenitor cells (EPC) and adipose-derived MSC in a collagen-fibronectin matrix (9). Specifically, co-implantation of ASC reduced EPC apoptosis, and increased the formation of perfusable and α-SMA-lined vessels in a PDGF-dependent manner. Intravital imaging of implants using the mouse cranial window revealed that anastomosis of graft-derived vessel structures with host vasculature occurred by wrapping of graft EC around host vessels at the implant interface (10). Disruption of basement membrane in host vessels resulted from graft EC-produced metalloproteinases (MMP)-9 and -14, which allowed for redirection of blood flow into the chimeric vasculature and subsequent fortification by mesenchymal precursor cells.

Taking these strategies further, others have investigated the effect of genetically modified angiogenic cells to augment vascularization in engineered tissues. EPC and MSC were
transduced with adenoviral vectors to overexpress bone morphogenic protein-2 (BMP-2), and incorporated into calcium sulfate-alginate porous scaffolds (11). When implanted into a rat model of critical-sized calvarial bone defects, genetically modified cells enhanced bone and vessel formation relative to unmodified cells at 5 weeks post-implantation. In another study, bone marrow-derived MSC were transduced with a constitutively active form of the transcription factor HIF-1α and incorporated into a gelatin sponge scaffold for the repair of critical-sized calvarial defect in rats (12). Compared to wildtype cells, genetically modified bone marrow-derived MSC significantly enhanced bone and vessel formation 8 weeks after implantation. Moreover, these vessels were perfusable, as assessed by micro-computed tomography imaging.

Other cell-based vascularization strategies involve pre-assembling angiogenic cells into beneficial configurations. For example, Laschke et al. showed that organization of adipose-derived MSC into spheroids and their incorporation into porous polyurethane scaffolds enhanced vascularization upon implantation into a murine skinfold chamber model, compared to bare scaffolds and scaffolds seeded with individual MSC (13). In a different study, authors used a temperature-sensitive culture dish to fabricate cell sheets composed of cardiomyocytes and EC (14). The cardiac sheets were layered one atop the other and applied in a rat model of myocardial infarction. The incorporation of EC enhanced cardiac function and capillary density within the infarct zone 4 weeks after implantation. Miller et al. demonstrated that EC could be organized into cylindrical interconnecting networks, by seeding cells into a carbohydrate glass mold (15). These network channels could be perfused with blood and supported the in vitro culture of primary rat hepatocytes in the surrounding matrix.

Using the microtissue molding approach described above, vascular cords were fabricated by seeding EC and MSC within collagen gel into microchannel molds (16). When implanted into the parametrial fat pad in the intraperitoneal space of athymic mice, vascular cord-containing scaffolds enhanced vessel density of implants, with anastomosis of vessels observed as early as 3 days post-implantation. Furthermore, the vessels generated in these constructs enhanced function of hepatocyte aggregates over 20 days, as evidenced increased albumin promoter activity, relative to empty scaffolds and scaffolds with randomly seeded EC and MSC. Ectopic implantation of hepatocyte-fibroblast aggregates and EC patterned cords into the mesenteric fat of mice with chronic liver disease resulted in increased vascularization and expansion of hepatocytes (17). Interestingly, compared to implants with EC incorporated into hepatocyte-
fibroblast aggregates, those with EC cords enhanced hepatocyte function following liver injury, as measured by transferrin production. Zhang et al. demonstrated the micropatterning of parenchymal cells and EC-lined vascular channels in a platform called AngioChip (18). When cardiac AngioChip tissues were surgically anastomosed to the femoral vessels of Lewis rats, blood perfusion was established immediately and infiltration of mural cells observed after one week.

2.2 Hypoxia and Hypoxia Inducible Factors

2.2.1 Hypoxic Induction of HIF

Proper maintenance of oxygen tension is crucial for the survival and function of tissues. Physiological oxygen tensions vary depending on the tissue of interest, ranging from 150 mmHg (19.7%) in the trachea, to 41 mmHg (5.4%) in the liver, and 29 mmHg (3.8%) in muscle (19). When oxygen tensions reach below the physiological range of a tissue, such as from tissue injury or in pathological conditions, cells are stimulated to coordinate the restoration of tissue perfusion and homeostasis. Primary mediators of the hypoxic response are hypoxia inducible factors (HIF), heterodimers comprised of a constitutively expressed 91-94 kDa β-subunit (HIF-β), and a 120 kDa α-subunit. The α-subunit contains an oxygen-dependent degradation domain that renders the protein sensitive to oxygen tension.

There exist three isoforms of the HIF-α subunit: HIF-1α, -2α and -3α, of which the first two have been most extensively studied. Note that, except where specified in this document, the general abbreviation HIF refers to both HIF-1 and HIF-2 pathways. HIF-1α accumulates exponentially in low oxygen tensions, with increased stabilization at oxygen tensions below 40 mmHg (6%), half-maximal levels at 10 mmHg (1.5%) and maximal levels at 3 mmHg (0.5%) (20). Structurally, HIF-3α is similar to the other two isoforms in the basic helix-loop-helix and Per-Arnt-SIM domains, but does not have the C-terminal transactivation domain (TAD-C). HIF-1α and HIF-2α share similar protein structure, with 48% amino acid sequence identity, but have both overlapping and non-overlapping targets and mechanisms of regulation. Whereas HIF-1α is ubiquitously expressed, HIF-2α is restricted to specific tissues including endothelium, kidney,
lung, heart and small intestine. Generally speaking, HIF-1 activation is associated with the acute and early response to hypoxia (typically 2-24 hours), while HIF-2 activation governs remodeling in chronic hypoxia beyond 24-72 hours and in mild hypoxia (Figure 2-1, adapted from (19)).

Figure 2-1. The HIF-1 and HIF-2 pathways dominate different stages of vessel formation in response to hypoxic stress. Adapted from (19).

2.2.2 Regulation of HIF

Protein expression of the HIF α-subunit is regulated by several mechanisms, the most prominent being degradation by prolyl-4-hydroxylase (PHD) proteins PHD1, PHD2 and PHD3. PHD catalyzes post-translational hydroxylation of prolyl residues (Pro402 and Pro564 in HIF-1α; Pro405 and Pro531 in HIF-2α) in the oxygen-dependent degradation domain of the α-subunit. Once hydroxylated, the α-subunit interacts with the von Hippel-Lindau tumor suppressor protein (VHL), which recruits the E3-ubiquitin-ligase complex and tags the protein for degradation in the proteasome (21). The key enzyme in this process, PHD, requires oxygen and 2-oxoglutarate as substrates, as well as iron (II) as a co-factor for the hydroxylation of HIF-α. In hypoxia, the α-subunit is hydroxylated less extensively, causing its accumulation and translocation to the nucleus, where it dimerizes with HIF-β to regulate transcription of downstream genes. HIF-regulated genes contain a hypoxia responsive element (HRE) in the promoter region, to which the HIF transcription factor binds. The HRE is characterized by a core sequence five nucleotides long: RCGTG, where R is A/G (22).
While HIF-α protein stability is dependent upon proline modification, its transcriptional activity is regulated by factor inhibiting HIF (FIH)-1. Similar to PHD, FIH is a 2-oxoglutarate and iron (II)-dependent oxygenase; it hydroxylates an asparagine residue (Asp803 in HIF-1α, and Asp851 in HIF-2α) in the C-terminal activation domain, blocking subsequent interaction with p300/CBP coactivators (23). FIH-1 can also hydroxylate HIF-2α, although at a lower efficiency compared to HIF-1α (20). As with PHD, FIH-1 activity is reduced in low oxygen tension, resulting in binding of p300/CBP to HIF-1α and HIF-2α subunits and their subsequent transactivation. A schematic illustrating these major regulatory pathways is shown in Figure 2-2.

**Figure 2-2. Post-translational regulation of HIF-1α by PHD and FIH.** In normoxic conditions, two proline residues (Pro402 and Pro564) of HIF-1α are hydroxylated by PHD. PHD requires oxygen and 2-oxoglutarate as substrates and iron as a cofactor for its enzymatic activity. Once hydroxylated, HIF-1α interacts with VHL, is ubiquitinated by the E2 ligase and subsequently tagged for proteosomal degradation. FIH also regulates transcriptional activity of...
HIF-1 by hydroxylating an asparagine residue of HIF-1α. This interferes with its binding with transcriptional coactivators p300 and CREB. In hypoxic conditions, both PHD and FIH enzymatic activity is reduced, allowing HIF-1α to translocate into the nucleus, where it dimerizes with HIF-1β. Binding of transcriptional co-activators p300 and CBP allows the complex to regulate gene transcription.

VHL has been recognized as the primary regulator of HIF-α protein stability. However, other regulatory mechanisms have also been described, some of which are specific to the HIF-1α versus HIF-2α isoforms. One such regulator is the receptor of activated protein kinase C (RACK1), which is oxygen-insensitive and acts independently of VHL to promote HIF-α degradation by competing with heat shock protein-90 for binding to the PAS-A domain. RACK1 recruits binding of the E3 ubiquitin ligase and promotes degradation of HIF-1α (24). Additionally, human double minute 2 (Hdm2) was found to mediate proteosomal degradation of HIF-1α via its interaction with p53 (24).

Importantly, differential regulation of HIF-α isoforms gives rise to temporally controlled expression of HIF-1α and HIF-2α. Whereas PHD2 regulates HIF-1α preferentially over HIF-2α, PHD3 has greater influence over HIF-2α than HIF-1α stability (25). PHD and FIH-1 hydroxylation of HIF-2α occurs at a lower efficiency than HIF-1α, resulting in greater HIF-2α activity and stabilization at higher oxygen tensions compared to HIF-1α (26). Additionally, hypoxia-associated factor (HAF) differentially regulates HIF-α stability; it binds to and mediates ubiquitylation and degradation of HIF-1α, but enhances transactivation of HIF-2α under extended exposure to hypoxia (24).

2.2.3 Stimuli of HIF Activation

Hypoxia is a major stimulus of HIF activation, predominantly because PHD activity is dependent upon the availability of oxygen. However, there are other external stimuli that stabilize HIF, many of which are associated with the inflammatory environment. Under normoxic conditions, nitric oxide (NO) stabilizes HIF-1α by attenuating PHD activity, reducing its ubiquitination and impeding upon VHL binding to the HIF-1α subunit. A proposed mechanism by which this occurs is related to coordinate binding of NO and Fe(II) ions; since iron is a cofactor of PHD, its
enzymatic activity is consequently reduced. However, under hypoxic conditions, low concentrations of NO can actually destabilize HIF-1α by reducing its protein accumulation (27).

Another inflammatory stimulus that stabilizes HIF-1α is reactive oxygen species (ROS), which is produced by phagocytes like macrophages and neutrophils as part of their bactericidal activity. The antioxidant ascorbate is a crucial cofactor of PHD, as it maintains iron in its reduced form and to prevent inactivation of the enzyme. Accumulation of ROS can limit the availability of antioxidants like ascorbate in the biological system, resulting in reduced PHD activity and subsequent stabilization of HIF-1α.

In inflammation, various cytokines are produced that augment the recruitment and phagocytic activity of immune cells. It has been shown that the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) stabilize HIF-1α, although the exact mechanisms are not fully understood (28). It is proposed that TNF-α contributes to HIF-1α stabilization by stimulating the production of NO and ROS by cells (29), thereby attenuating PHD activity as described above. The effects of IL-1β have not been elucidated, but is presumed to increase HIF-1α translation.

Additionally, nuclear factor-κB (NF-κB) is also a necessary transcriptional activator of HIF-1α. NF-κB is a master transcriptional regulator in inflammation that is activated by binding to toll-like receptor, induced by hypoxia as well as the presence of bacterial cell wall components like lipopolysaccharides (LPS). Using mice deficient in IkappaB kinase-β (IκK-β), which controls activation of NF-κB, Rius et al. demonstrated that NF-κB activity is necessary for HIF-1α protein stabilization and expression of downstream targets like VEGF (30).

2.2.4 HIF Orchestrates the Angiogenic Response

HIF-1 was first discovered as a regulator of erythropoietin expression in hypoxia (31). Since then, HIF-1 has been found to orchestrate a wide range of cellular responses to hypoxia (and other stimuli, as discussed in Section 2.3), by controlling transcription of genes associated with angiogenesis, metabolism, growth, differentiation, survival and apoptosis (reviewed in (32)).
HIF-1 modulates the expression of many major angiogenic growth factors, including vascular endothelial growth factor (VEGF, (33)), stromal derived factor-1 (SDF-1, (34)), angiopoietin-1 (Ang1, (35)), angiopoietin-2 (Ang2, (36)), placental growth factor (PIGF, (37)), platelet-derived growth factor B (PDGFB, (38)) and stem cell factor (SCF, (35)). The cognate receptors for these growth factors are expressed on EC and vascular pericytes; once bound to their ligand, they direct and promote vessel sprouting from existing vessels (39). HIF-2 preferentially regulates the expression of delta-like ligand 4 (Dll4), which is involved in endothelial sprouting, and Ang2, involved in the remodeling of vasculature (27).

Much can be learned regarding HIF regulation of vascularization by understanding its role in directing embryonic vascular development. In the embryo, cells are subject to hypoxic gradients and other cues. Some of the dominant angiogenic signals in vascular development are VEGF-(A-D) and their receptors (VEGFR1-3), which play an important role in early vascular assembly. Angiopoietins 1-4 and their receptors Tie1 and Tie2 are more prominent in the later stages of vascular maturation (Figure 2-1, (19)).

Fong et al. generated embryos with mutation in the VEGFR-1 gene by homologous recombination, and observed that the targeted mutation did not affect endothelial cell differentiation. However, assembly of vascular structures was significantly impaired, suggesting that VEGFR-1 signaling may be important in EC interactions with other EC (40). In another study, Oladipupo et al. generated a murine model of inducible HIF-1 activation in keratinocytes in the absence of VEGF expression. In this model, induction of HIF-1 in keratinocytes did not result in neovascularization, despite the upregulation of downstream angiogenic targets like PIGF, angiogenin and PAI-1. In fact, endothelial sprouting was even enhanced in this model, suggesting that VEGF is necessary for the assembly of endothelial sprouts into vascular structures in HIF-mediated vascularization (41).

Specific deletion of HIF-2α in endothelial cells in a murine model resulted in vascular leakage, and impaired tumor angiogenesis. Endothelial cells with deletion of HIF-2α exhibited reduced expression of fibronectin, integrins, angiopoietin-2 and delta-like ligand 4 (Dll4), and demonstrated reduced adhesion to extracellular matrix proteins (41). In a murine model of hindlimb ischemia, HIF-2α deletion in EC did not affect vessel formation, but impaired arteriogenesis and perfusion of tissue. This phenotype was largely rescued by stimulating Dll4
expression in the muscle, suggesting the importance of Notch/Dll4 in the role of HIF-2 activation in EC (42). Embryos generated from HIF-2α/− embryonic stem cells died between E9.5 to E12.5. They still demonstrated vasculogenesis, but vessels showed abnormal fusion and assembly at later stages of development (43). Taken collectively, HIF-2 is important for the maturation of the vascular network, which involves recruitment of peri-endothelial support cells (19).

2.2.5 HIF Orchestrates Other Cellular Responses

In the previous section, we focused on HIF regulation of angiogenesis-related genes. However, it also orchestrates other cellular responses that are involved in restoring oxygen homeostasis in the tissue. These cellular responses include cell proliferation, cell survival, apoptosis, motility, cytoskeletal structure, cell adhesion, and pH regulation (Figure 2-3, and summarized in (32)).

One important category of cellular adaptations to hypoxia is metabolism, or more specifically, the metabolic switch to glycolysis in order to reduce oxygen consumption. Indeed, HIF-1α and HIF-2α both bind to the same HRE sequences of genes, thereby modulating the transcription of many overlapping genes like glucose transporter 1 (GLUT1) and VEGFA. However, HIF-1 preferentially regulates the transcription of genes that encode glycolytic enzymes, including phosphofructokinase, lactate dehydrogenase A, hexokinase-1, hexokinase-2, phosphoglycerate kinase 1 (19, 27).

Additionally, HIF-1 preferentially modulates the expression of genes related to pH regulation, such as monocarboxylate transporter 4 and carbonic anhydrase-9, as well as those promoting apoptosis, including BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3). On the other hand, HIF-2 preferentially regulates genes associated with cell invasion, such as matrix metalloproteinase (MMP) -2 and -13, and the stem cell factor OCT-3/4. It also differentially regulates the expression of erythropoietin and transforming growth factor-α. Interestingly, HIF-1 and HIF-2 modulate opposing effects is in nitric oxide production. In macrophages, HIF-1 activation is crucial to the expression of inducible nitric oxide synthase (iNOS), which is involved in the production of nitric oxide. By contrast, HIF-2 activation induces macrophage expression of arginase-1 (Arg-1), an enzyme that catabolizes and reduces L-arginine levels, which is otherwise necessary for nitric oxide production (27).
Figure 2-3. Schematic diagram summarizing the various cellular responses and their associated genes that are transcriptionally regulated by HIF-1. In addition to regulating genes associated with angiogenesis, HIF-1 regulates genes related to cell proliferation, survival, apoptosis, glucose metabolism, and extracellular matrix metabolism. Adapted from (32).

2.3 Harnessing Hypoxia for Regeneration

Because of its role as a master regulator of angiogenic responses, HIF has been recognized as a promising target for the treatment of various ischemic diseases. Targeting HIF yields an orchestrated response involving numerous growth factors, and is therefore considered advantageous over conventional methods of single or combinatorial growth factor delivery. This section focuses on genetic and pharmacological methods of stabilizing HIF for treatment of ischemic indications, as well as applications for its inhibition in pathological disease progression.
2.3.1 Genetic Stabilization of HIF-1α

2.3.1.1 Overexpression of HIF

Overexpression of the HIF-α isoforms has been explored as a method of driving vascularization and tissue regeneration in various models of ischemia. Niemi et al. delivered adenoviral vectors of HIF-1α and HIF-2α intramuscularly in a rabbit model of hindlimb ischemia (44). Although vessel densities and tissue perfusion were not enhanced to a level comparable to VEGF-A overexpression, HIF-1α and HIF-2α overexpression resulted in the formation of larger and non-leaky vessel structures, as evidenced by the absence of tissue edema.

Retroviral transduction of bone marrow-derived stromal cells with HIF-1α or HIF-2α increased their expression of endothelial markers, Tie2 and CD31, and their secretion of VEGF and SDF-1 (45). When applied in vivo in a corneal micropocket assay, cells transduced with HIF-2α induced greater vessel formation compared to both HIF-1α and control vectors. When overexpressed in endothelial cells, co-transfection of HIF-1α and HIF-2α enhanced activation and expression of downstream angiogenic proteins; these, in turn, enhanced migration of non-transfected endothelial cells (46). Additionally, implantation of endothelial cells with overexpression of both HIF-1α and HIF-2α in an in vivo Matrigel plug angiogenesis assay enhanced vessel formation. EC-specific overexpression of HIF-2α in transgenic mice increased tissue survival in a mouse skin flap model (47). Beneficial effects were related to the level of HIF-2α overexpression, as tissue survival was greater in high-copy versus low-copy transgenic mice. Enhanced tissue survival correlated with increased VEGF gene expression in the skin flap, and with increased vessel density 7 days post-operation.

A gelatin-based scaffold was used to deliver adenoviral HIF-1α in a rat model of alveolar bone defect (48). At 4 weeks post-treatment, rats treated with scaffolds containing adenoviral HIF-1α demonstrated enhanced mineralized bone formation, compared to control animals implanted with gelatin sponges alone or gelatin sponges loaded with empty adenoviral vectors. This corresponded with increased vessel formation, as well as high levels of VEGF and bFGF protein in the new tissue.
2.3.1.2 Overexpression of Oxygen-insensitive HIF-1α

Although exogenous delivery of wildtype HIF-α isoforms can induce vascularization \textit{in vivo}, as described above, they are ultimately susceptible to degradation in environments of high oxygen tension. Another genetic method of HIF-1α stabilization involves delivery of its constitutively active form, which is engineered to contain amino acid substitutes for proline residues in the oxygen-dependent degradation domain, or that lack the oxygen-sensitive domain altogether. Subcutaneous injection of a constitutively active HIF-1α (termed CA5) plasmid rectified wound healing in aged diabetic mice, an animal model which demonstrates reduced wound closure and expression of HIF-1α, VEGF-A, PI GF, PDGF, Ang1 and Ang2 (49). Compared to delivery of an empty vector control, mice receiving CA5 plasmids demonstrated enhanced wound closure, increased mobilization of circulating angiogenic cells in peripheral blood at day 3, and increased blood vessel density in the wound. In a rabbit model of critical limb ischemia, intramuscular injection of adenoviral CA5 (AdCA5) improved perfusion and motor score compared to delivery of a control vector (50). These effects were associated with increased expression of angiogenic growth factors (VEGF, SDF-1, PI GF, PDGF) in the ischemic site. In a similar study, delivery of two adenoviral constructs of constitutively active HIF-1α increased healing of diabetic wounds (51). This was concomitant with upregulation of angiogenic genes (VEGF-A, VEGFR-1, SCF, SDF-1) and heat shock protein-90, which mediates cell motility. Intramuscular AdCA5 delivery increased the mobilization of CD45-CD13+Flk+ circulating angiogenic cells in young mice with hindlimb ischemia, relative to the delivery of a control vector (52). Moreover, treatment with AdCA5 increased limb perfusion following femoral artery ligation in aged mice.

In another study, intradermal injection of CA5 plasmid was combined with delivery of bone marrow-derived angiogenic cells pre-conditioned with HIF-stabilizing dimethloxyalgllycine (DMOG) in aged mice with burn wounds (53). Enhanced wound closure in treatment groups was concomitant with increased wound perfusion, vessel density at day 21 post-treatment, and vessel maturation, as evidenced by SMA coverage of vessel structures. Additionally, CA5 plasmid enhanced the recruitment of bone marrow-derived angiogenic cells to the wound site 48 hours following their delivery, relative to an empty vector control. Similar findings were observed when combination therapy was used in a murine model of hindlimb ischemia. Rey \textit{et al.} demonstrated that intramuscular injection of AdCA5 and co-administration of DMOG-treated bone marrow-derived angiogenic cells improved perfusion, motor score, and limb salvage in
aged mice (54). DMOG pre-conditioning increased the expression of β2 integrins on bone marrow-derived angiogenic cells, and their homing to the ischemic site with AdCA5 administration. Beneficial functional effects were abolished with co-delivery of the HIF inhibitor digoxin, or a β2 integrin neutralizing antibody.

Constitutively active HIF-1α has also been applied for vascularizing tissue engineered bone. Zou et al. transduced bone marrow-derived MSC with CA5 and delivered the cells within gelatin sponges to improve healing of critical-sized calvarial defects (55). CA5-transduced bone marrow-derived MSC increased the expression of VEGF, SDF-1, bFGF, Ang1, and PIGF at both the gene and protein levels, relative to cells transduced with wildtype HIF-1α or a control GFP vector. Moreover, their delivery in the gelatin sponge increased vessel density and bone formation 8 weeks post-implantation, relative to all control groups studied. The same authors also found that transduction of the mutant truncated form of HIF-1α into bone marrow-derived MSC also increased their expression of osteogenic genes, including BMP-2, osteocalcin (OCN), type 1 collagen, osteopontin (OPN), and alkaline phosphatase (39). When implanted within a calcium-phosphate cement scaffold into a rat model of critical-sized calvarial defect, CA5-transduced cells increased vessel density and bone formation relative to cells transduced with an empty vector.

Trentin et al. designed a peptide-based vector for the delivery of constitutively active HIF-1α, lacking the oxygen-dependent degradation domain, from a fibrin matrix (56). The peptide-based vector consisted of a binding domain for entrapment in fibrin, and a nuclear localization sequence for the delivery of DNA to cell nuclei. When applied to full thickness wounds in mice, constitutively active HIF-1α increased vessel formation to a degree comparable to delivery of VEGF-A. Compared to delivery of VEGF-A, stabilization of HIF-1α in wounds induced the formation of mature SMA+ vessels as early as 7 days post-treatment. Thiersch et al. also designed a biomaterial platform for the delivery of constitutively active HIF-1α, based on peptide-modified PLL-g-PEG polymers (57). The biomaterial scaffold, which delivered nano-condensates of mutant HIF-1α lacking the oxygen-dependent degradation domain, was applied to diabetic wounds in rats and increased the number of endothelial cells and smooth muscle cells in the tissue 7 days post-injury.
2.3.1.3 Knockdown of PHD

Another genetic method of stabilizing HIF involves silencing PHD using small interfering RNA (siRNA) or short hairpin RNA (shRNA). As discussed earlier, PHD are the primary enzymes that regulate stability of the HIF-α subunit in an oxygen-dependent manner. Loinard et al. tested the intramuscular delivery of shRNA plasmids targeting PHD-1, -2 or -3 in a murine model of critical limb ischemia (58). Compared to the delivery of control shRNA plasmid, all PHD-targeting shRNA plasmids enhanced the expression of VEGF-A and eNOS at the gene and protein levels. Functionally, delivery of shRNA plasmid targeting PHD-3 resulted in the greatest increase in vessel density and restoration of foot perfusion. This was dependent upon the upregulation of HIF-1α, as simultaneous delivery of a shRNA plasmid targeting HIF-1α dramatically decreased vessel density and restoration of perfusion. Moreover, the effects of PHD-3 silencing corresponded with increased gene expression of monocyte chemotactic protein-1 (MCP-1) and infiltration of macrophages into the ischemic tissue 14 days post-treatment. In a similar study, plasmids of shRNA targeting PHD-2 were delivered into full-thickness wounds of diabetic mice, or intramuscularly in a murine model of hindlimb ischemia (59). In the case of diabetic wound healing, treatment with shRNA targeting PHD-2 significantly increased the rate of wound closure and vessel density. When injected intramuscularly into mice following femoral artery ligation, shRNA targeting PHD-2 improved tissue perfusion and limb salvage compared to delivery of shRNA targeting a scramble control sequence. Systemic infusion of siRNA targeting PHD-2 reduced infarct size in mice with ischemia/reperfusion-induced myocardial infarction, relative to saline controls and mice receiving siRNA targeting a scramble sequence (60). Interestingly, treatment with siRNA targeting PHD-2 in iNOS knockout mice did not yield any beneficial effects, suggesting the importance of iNOS in cardiac protective effects.

Gene silencing of PHD has also been explored in combination with cell therapy. Intramuscular injection of bone marrow-derived MSC transfected with siRNA targeting PHD-2 was investigated in a diabetic mouse model of critical limb ischemia (61). Knockdown of PHD-2 in these cells enhanced VEGF-A expression and cell survival when delivered into the ischemic site. Compared to bone marrow-derived MSC transfected with control siRNA, those with PHD-2 knockdown enhanced vessel density and restoration of foot perfusion 14 days post-treatment. In both studies described above (58, 61), effects were abrogated with co-delivery of shRNA or siRNA targeting HIF-1, suggesting that functional benefits to neovascularization were HIF-
dependent. In a similar study, a lentiviral vector of shRNA targeting PHD-2 was transduced in adipose-derived stem cells, which were subsequently implanted into the infarct following coronary artery ligation (62). Cells transduced with shRNA targeting PHD-2 reduced the infarct size, improved cardiac function and reduced apoptosis of cardiomyocytes relative to cells transduced with an empty control vector. Beneficial effects were abrogated by simultaneous transduction with shRNA targeting HIF-1α or with the delivery of IGF neutralizing antibody. This suggests that HIF-1α-dependent induction of IGF played an important role in cardiac tissue regeneration following myocardial infarction.

2.3.2 Pharmacological Stabilization of HIF

In addition to genetic methods of HIF activation, pharmacological means have also been explored. In this section, we will focus on two, dimethyloxalylglycine (DMOG) and desferrioxamine (DFO), which have been extensively studied. Both drugs act by inhibiting PHD-mediated degradation of HIF. Whereas DFO is an FDA-approved iron chelator which has been used for treatment of patients with thalassemia, DMOG acts as a competitive inhibitor of α-ketoglutarate. Like any pharmacological method, they are associated with off-target effects, which are explored more thoroughly in a later section.

2.3.2.1 Dimethyloxalylglycine

Therapeutic efficacy of DMOG has been demonstrated in vivo in various models. Importantly, inhibition of PHD using DMOG also affects collagen assembly (63). In a murine ischemic brain injury model, intraperitoneal injection of DMOG following induction of stroke significantly increased functional outcome, as measured by ischemic infarct volume, blood flow and behavioral deficits (64). Inhibition of PHD and subsequent HIF-1 stabilization correlated with reduced apoptosis in ischemic tissue 24 hours following distal occlusion of the middle cerebral artery. Of note, beneficial effects were abrogated by the HIF-1α pharmacological inhibitors digoxin and acriflavine. Takaku et al. also showed that DMOG treatment 48 hours prior to creation of ischemic skin flaps in the dorsum of mice increased surviving flap area and vessel density in the ischemic tissue (65). DMOG preconditioning reduced apoptosis of cells in the skin flap, and increased mobilization of CD34+CD133+ endothelial progenitor cells into peripheral
blood 1 day post-operation. Skin flap survival was impaired in heterozygous HIF-1α-deficient mice, highlighting the importance of HIF-1 activation in DMOG-associated protective effects. In a murine femur fracture model, injection of DMOG directly into the fracture site improved vascularization 2 weeks following treatment and callus formation 28 days post-treatment (66).

While DMOG can be administered systemically or directly to the site of injury, others have explored strategies that incorporate the drug in engineered tissue to induce bone regeneration. DMOG pre-conditioning of adipose-derived stem cells significantly improved vascularization and bone closure upon delivery within a commercially available hydrogel in a rat model of critical sized calvarial defects (67). Treatment of adipose-derived stem cells with DMOG not only enhanced expression of HIF-1α and VEGF-A protein, but also increased expression of osteogenic genes like RUNX-2, osteocalcin and alkaline phosphatase. Conditioning bone marrow-derived angiogenic cells with DMOG increased their proliferation, expression of glycolytic genes (GLUT-1, LDHA, MCT4), and enzyme activity associated with pH regulation (carboxylic acid reductase) (68). Interestingly, bone marrow-derived angiogenic cells isolated from HIF-1α+/– haplodeficient mice did not demonstrate increased glycolytic metabolism, suggesting that HIF-1 activity plays a key role in metabolic changes induced by DMOG.

In a recent study by Wu et al., DMOG was incorporated into mesoporous bioactive glass scaffolds to increase angiogenic (VEGF) and osteogenic (OCN, OPN, ALP) gene expression in bone marrow-derived MSC in vitro (69). DMOG release from mesoporous bioactive glass scaffolds was sustained over 4 weeks, and repaired critical-sized calvarial defects in rats 8 weeks post-implantation (70).

2.3.2.2 Desferrioxamine

Another pharmacological inhibitor of PHD and stabilizer of HIF is DFO, and has been shown to exert therapeutic benefits in vivo. DFO is advantageous in that it also stabilizes the binding of p300 coactivator to the C-terminal transactivator domain of HIF, thereby enhancing its transcriptional activity in addition to stabilizing HIF-1α protein (71). Intraperitoneal DFO injection significantly improved tissue survival 14 days following creation of a skin flap in the dorsum of diabetic mice (72). DFO treatment of skin flaps significantly increased VEGF protein levels in the tissue 3 days post-surgery, and vessel density at 7 days, which resulted in greater tissue perfusion. DFO treatment also enhanced the mobilization of VEGFR-1+CD11b−
endothelial progenitor cells into peripheral blood, relative to untreated diabetic mice. In another study, delivery of DFO pre-conditioned CD34\(^+\) hematopoietic stem cells \textit{in vivo} improved blood perfusion and functional performance of hindlimbs, 14 days following femoral artery ligation (73). This corresponded to enhanced retention of cells in the ischemic site up to 14 days post-transplantation, compared to untreated controls. Effects of DFO on transplanted cell retention in the ischemic tissue was abolished by co-delivery of an inhibitor of CXCR4 or of PI3K. \textit{In vitro}, DFO conditioning enhanced gene expression of VEGF, SDF-1, eNOS and phosphorylated-AKT in CD34\(^+\) hematopoietic cells, and increased tube formation in an \textit{in vitro} assay.

In addition to conventional methods of drug administration, several groups have designed novel delivery methods for various applications. A transdermal DFO delivery system was developed by Duscher and colleagues, which was comprised of DFO-encapsulated micelles dispersed within an ethyl cellulose layer. Its application significantly improved healing of pressure-induced ulcers in a diabetic mouse model, as assessed by wound closure, dermal thickness and neovascularization (74). Surprisingly, prophylactic treatment of mouse skin with the DFO-loaded patch 48 hours prior prevented pressure-induced ulcer formation, and was associated with reduced cell apoptosis relative to the control patch. In another study, DFO was encapsulated within lecithin nanoparticles and applied topically to tracheal transplants at the time of surgery (75). DFO treatment of tracheal transplants significantly improved graft perfusion and reduced cell apoptosis following transplantation in BALB/C mice. DFO-loaded nanoparticles also increased gene expression of PlGF and SDF-1, as well as levels of phosphorylated eNOS and Ki67 in EC of trachea. Intramuscular delivery of DFO-loaded gelatin-based hydrogel significantly increased total and SMA\(^+\) vessel density 7 days post-surgery in a mouse model of critical hind limb ischemia, when compared to delivery of the scaffold alone or a bolus injection of DFO (76).

### 2.3.2.3 Other Stabilizers

Apart from treatment with DMOG and DFO, Zhang \textit{et al.} developed an injectable hydrogel platform for the sustained release of a PHD inhibitor, 1,4-dihydrophenonthrolin-4-one-3-carboxylic acid (1,4-DPCA) over a period of 4 to 10 days (77). Peripheral injections of the drug-releasing PEG-based hydrogel in a mouse model of ear hole injury resulted in enhanced wound closure. This was concomitant with HIF-1\(\alpha\) stabilization and increased expression of stem cell
markers in fibroblasts within the surrounding tissue. In a study by Sham et al., the PHD inhibitor pyridine-2,4-dicarboxylic acid (PDCA) was conjugated to a gelatin sponge scaffold, which increased vessel density once implanted into the perirenal fat tissue of rats for 8 days (78). In vitro culture of fibroblasts on PDCA-loaded gelatin scaffolds increased fibroblast infiltration and enhanced their secretion of VEGF.

Another method that has been studied for the stabilization of HIF is delivery of cobalt ions. Cobalt ion release was achieved in mesoporous glass scaffolds, and found to enhance VEGF secretion from bone marrow-derived stromal cells in vitro (79). Similar effects were observed when bone marrow-derived cells were cultured on mesoporous bioactive glass scaffolds that were designed to release copper ions (80). In another study, a collagen-alginate scaffold was designed for the release of cobalt chloride and BMP-2 to stimulate both angiogenesis and bone formation upon implantation (81). When implanted into critical calvarial defects in rats, dual delivery enhanced new bone formation 6 weeks post-implantation, relative to naked scaffolds or delivery of cobalt ions or BMP-2 alone.

2.3.3 Oxygen-manipulating Biomaterials

In the strategies discussed above, cellular responses to hypoxia were manipulated using genetic or pharmacological methods. Recently, there has been increasing focus on developing biomaterial scaffolds that, when implanted, alter the oxygen content within the tissue microenvironment. These biomaterial strategies have been explored to support engraftment of therapeutic cells and to develop engineered tissue constructs.

One class of these materials is oxygen releasing materials, which gradually release or generate oxygen into their surroundings. Gradual release of oxygen is important to minimize cell death, which can be otherwise induced by rapid oxygen release and subsequent formation of free radicals. Additionally, a gradual and steady release of oxygen is important for supporting cell viability and for vascularization of the engineered tissue. A polydimethylsiloxane (PDMS)-encapsulated calcium peroxide disk was designed for the slow release of oxygen over 6 weeks (82). Hydrophobic PDMS reduced penetration of water into the scaffold, allowing oxygen to be released gradually via a hydrolytic reaction. Culture of rat pancreatic islets within a PDMS-
calcium peroxide disk significantly reduced apoptosis and maintained glucose-responsive insulin secretion relative to untreated controls, even upon exposure to hypoxia. When rat pancreatic islets were transplanted with PDMS-calcium peroxide disks into an extrahepatic site of diabetic rats, non-fasting blood glucose levels were restored over a period of 40 days post-transplantation, and glucose was effectively cleared following an intravenous glucose tolerance test (83). Importantly, the oxygen-generating disc supported the long-term survival of insulin-producing β-cells. Calcium peroxide was also incorporated into a poly(d-l-lactide-co-glycolide) (PLGA) scaffold for the generation of oxygen over 10 days (84). When fibroblasts were cultured on oxygen generating PLGA scaffolds in hypoxic conditions (1% oxygen), cells were more proliferative than when cultured on PLGA scaffolds alone.

Hydrogen peroxide has also been incorporated into material systems to generate oxygen. It is decomposed into water and oxygen by the catalytic enzyme catalase. However, the accumulation of hydrogen peroxide can increase the production of free radicals and lead to reduced cell viability. A PLGA microsystem was designed for the encapsulation of hydrogen peroxide, which contained alginate-immobilized catalase in order to convert any free hydrogen peroxide into oxygen (84). In another study, an injectable thermosensitive hydrogel was developed for oxygen generation and to support engraftment of cardiac progenitor cells (85). This system contained hydrogen peroxide and poly(2-vinylpyrridione)-encapsulated PLGA microspheres within a N-isopropylacrylamide-based hydrogel, and sustained the release of oxygen over 14 days. In vitro culture of cardiosphere-derived cells within the oxygen-generating hydrogel enhanced cell viability, proliferation and differentiation in hypoxia (1% oxygen), compared to control hydrogels. A similar biomaterial scaffold was designed, containing PLGA microparticles encapsulating hydrogen peroxide within a collagen sponge and fibrin-conjugated heparin/VEGF, for the delivery of pancreatic islets (86). Upon transplantation into the omental pouch of streptozotocin-induced diabetic mice, a suboptimal islet mass (250 islet equivalents) significantly improved glucose levels and graft revascularization over a period of 30 days, relative to all material controls studied.

Unlike the oxygen generating materials discussed above are oxygen consuming materials, which lower the oxygen tension in the local microenvironment. Park and Gerecht designed a gelatin-based scaffold conjugated with ferulic acid, which consumes oxygen via a lactase-mediated reaction (87). Encapsulation of endothelial progenitor cells into the gel resulted in cell exposure
to prolonged low oxygen tensions for 50 hours. It stabilized HIF-1α and HIF-2α expression, and increased formation of vascular structures over 3 days, compared to a non-hypoxic control gel. Moreover, subcutaneous implantation of the gel resulted in vascularization and perfusion of the implant as early as 3 days post-implantation. Other cell types have also been encapsulated in this oxygen-consuming hydrogel, including tumor cells and EC, and used to study the effects of tunable oxygen gradients on cell behavior (88).
2.4 Recruitment of Host-derived Angiogenic Cells

Vascularization is a process that involves the coordinated response of various cell types. Indeed, EC play a key role in this process, as they themselves physically assemble to form vascular structures. However, with tissue damage or injury, many cells are in fact recruited from the bone marrow and contribute significantly to blood vessel formation. Bone marrow-derived cells can alter the microenvironmental milieu by producing cytokines and growth factors, as well as by physically incorporating into blood vessels. In the following section, we highlight three bone marrow-derived populations that are crucial to vascularization: neutrophils, monocytes (and from them, differentiated macrophages), and endothelial progenitor cells (EPC). In addition, we discuss the role of HIF activation in modulating their recruitment to damaged tissue and their angiogenic function.

2.4.1 Neutrophils

2.4.1.1 Role in Vascularization

Neutrophils are crucial mediators of tissue injury, and among the first cells to be recruited to sites of inflammation. When EC become activated, through direct pattern-recognition receptor-mediated detection of pathogens or through stimulation by inflammatory mediators produced by leukocytes, they upregulate the expression of P-selectin and E-selectin which increase neutrophil adhesion (89). Once recruited, neutrophils remove pathogens by phagocytosis, the release of granule contents, production of reactive oxygen or nitrogen species, and generation of neutrophil extracellular traps (89).

Additionally, neutrophils produce factors that initiate vascularization. Depletion of neutrophils by intraperitoneal delivery of neutralizing Ly6G antibody impaired vascularization of islets transplanted into the cremaster muscle of mice (90). The same authors used intravital imaging to observe that neutrophils recruited to hypoxic islets localized to the sprouting tip of neovessels. Nozawa et al. similarly found that neutrophils penetrated into the islet core in a mouse model of pancreatic islet carcinogenesis, and that their depletion using Gr-1 neutralizing antibody impaired angiogenesis (91). Neutrophil-derived factors that initiate angiogenesis include VEGF (92), Bv8 (93) and MMP-9 (94–96).
2.4.1.2 Neutrophil Polarization

While neutrophils have been traditionally associated with inflammation, there is growing evidence that they are also involved in its resolution. Similar to the delineation of polarized macrophage phenotypes is the recognition of different neutrophil phenotypes. Characterization of neutrophils in methicillin-resistant Staphylococcus aureus (MRSA)-resistant and MRSA-sensitive mice showed marked differences in the expression of pro- and anti-inflammatory factors. Neutrophils from MRSA-resistant mice presented a pro-inflammatory phenotype, with increased expression of IL-12 and MIP-3; those of MRSA-susceptible mice were anti-inflammatory in nature, with increased expression of IL-10 and MCP1 (97). Tumor growth factor-β (TGF-β) is an important factor in polarizing neutrophils toward an anti-inflammatory phenotype, as its blockade increased the presence of cytotoxic neutrophils and their secretion of inflammatory cytokines in ectopic flank tumors in mice (98).

2.4.1.3 HIF-mediated Neutrophil Function

The hypoxic environment is a characteristic of inflamed tissue. It is therefore not surprising that various functions of neutrophils are affected by hypoxia and dependent upon HIF activation. Foremost, the lifespan of neutrophils is prolonged in low oxygen, in a manner dependent upon HIF-1 and HIF-2. Specifically, HIF-1-dependent activation of NFκB was found to be required for prolonged neutrophil survival in hypoxia, and was concomitant with increased production of the pro-survival factor macrophage inflammatory protein-1β (MIP-1β) (99). Pharmacological stabilization of HIF-1 and overexpression of an oxygen-insensitive form of HIF-1α in neutrophils decreased their apoptosis and increased their retention in a zebrafish larvae tail transection model (100). Using gain-of-function and loss-of-function studies, Thompson et al. demonstrated that HIF-2 activation also governs neutrophil survival (101). Overexpression of HIF-2α in human neutrophils increased their survival in ex vivo culture, while myeloid-specific deletion of HIF-2α reduced neutrophil recruitment and inflammation in a LPS-induced model of acute lung injury in mice.

Furthermore, others demonstrated the importance of HIF-1 activation in neutrophil function via genetic depletion of HIF-1α in myeloid cells (including neutrophils, monocytes and macrophages) using a transgenic mouse model with cre-recombinase expression driven by the lysozyme M promoter (102). In this study, knockout of HIF-1α in the myeloid lineage
significantly reduced the ATP pool in neutrophils, reduced intracellular killing of bacteria, and subdued inflammation in models of cutaneous inflammation and arthritis. In a similar study, HIF-1α deletion in myeloid cells impaired neutrophil killing of Gram-positive pathogen group A Streptococcus (GAS), and led to larger necrotic lesions in a GAS-induced soft tissue infection model (103).

Importantly, neutrophils are not only responsive to hypoxia, but also alter the oxygen tension in their microenvironment via oxygen consumption. Transmigration of neutrophils across epithelium reduces local oxygen tension, and causes stabilization of HIF-1α in epithelial cells. Additionally, in an in vivo model of colitis, stabilization of HIF in mucosal cells is dependent upon oxygen consumption by neutrophils during the respiratory burst (104).

2.4.2 Monocytes and macrophages

2.4.2.1 Monocyte Recruitment

Monocytes are a population of cells resident in the bone marrow, which are beckoned to sites of injury by various signals. A major source of these signals is platelets, which are released upon damage of blood vessels and are a major component of blood clots. Platelets release a plethora of factors, including CCL5, VEGF, TGF-β and PDGF (105). CCL5 is a primary chemokine that attracts monocytes to the site of injury. Platelets also release thrombin, which promotes clot formation by converting soluble fibrinogen to insoluble fibrin. In addition to its role in clot formation, thrombin also has pro-inflammatory effects, stimulating the release of inflammatory cytokines like CCL2, IL-6 and IL-8 by EC, which in turn, mediate monocyte recruitment. Thrombin also induces the release of inflammatory cytokines from monocytes, including IL-6, IFN-γ, IL-1β and TNF-α, which are believed to induce monocyte differentiation into classically activated macrophages.

Monocytes are the precursors of macrophages and dendritic cells, and they themselves exhibit different phenotypes. In humans, three phenotypes have been defined, according to their relative expression of the surface markers CD14 and CD16. Classical monocytes (CD14<sup>hi</sup>CD16<sup>-</sup>) comprise the majority of all monocytes, while intermediate monocytes (CD14<sup>hi</sup>CD16<sup>+</sup>) and non-classical monocytes (CD14<sup>+</sup>CD16<sup>hi</sup>) express elevated CD16 expression (106, 107). In mice, two
general populations of monocytes have been recognized, according to their expression of CCR2, CX3CR1 and Gr-1: inflammatory monocytes are CCR2⁺CX3CR1⁻Gr1⁺ and resident monocytes are CCR2⁻CX3CR1⁺Gr1⁻ (108).

2.4.2.2 Macrophage Polarization

Upon recruitment to the damaged tissue, monocytes can differentiate into macrophages in response to microenvironmental cues. Macrophages are plastic cells that take on various phenotypes and functions. It is important to mention that although macrophages may be derived from monocytes recruited from the bone marrow, there also exist macrophages that reside in native tissue, termed tissue-resident macrophages. Traditionally, macrophage phenotypes were understood as spanning a linear spectrum, with the classically activated (M1) and alternatively activated (M2) states representing the two extremes (reviewed in (109–111)). These states mirror and are intrinsically linked with the T helper type 1 (Th1) and T helper type 2 (Th2) responses of T cells. M1 macrophage differentiation is stimulated by IFNs and TLR signaling via STAT1, while the M2 macrophage phenotype is induced by IL-4 and IL-13 via STAT6 (111).

Generally speaking, M1 macrophages are IL-12high, IL-23high, IL-10low, and produce inflammatory cytokines like IL-1β, TNF and IL-6, thereby contributing to the Th1 response. On the other hand, M2 macrophages are IL-12low, IL-23low, IL-10high, and express a high level of scavenger, mannose and galactose-type receptors (109). In fact, cell surface receptors have been used as markers of M1 and M2 macrophage activation (112). Markers of M1-associated activation in mouse macrophages include major histocompatibility complex class II (MHCII) and nitric oxide synthase 2 (NOS2). Those indicative of an M2 state include resistin-like-α (also called FIZZ1), arginase-1, and mannose receptor 1 (CD206). In humans, however, M2-polarized macrophages do not express FIZZ1 or arginase 1, but instead upregulate indoleamine 2,3-dioxygenase expression.

Functionally, M1 macrophages rely on anaerobic glycolysis to navigate hypoxic microenvironments in the early stages of acute inflammation, while M2 macrophages primarily use oxidative glucose metabolism to carry out functions in tissue remodelling and repair (109). The two macrophage polarization states are also distinct in their production of nitric oxide species. M1 polarized macrophages produce nitric oxide as part of their anti-microbial activity.
By contrast, M2 macrophages do not produce nitric oxide, and instead, express arginase-1 which catalyzes the production of polyamines for collagen synthesis and cell proliferation (109).

There is now overwhelming acknowledgement that the understanding of macrophage polarization along a linear spectrum is too limited a description. For example, others have defined that the alternatively activated M2 state actually encompasses a number of phenotypes that express distinct gene expression profiles, which are termed M2a, M2b, M2c and M2d (113, 114). Mosser and Edwards proposed that the spectrum of macrophage phenotypes was best depicted as a color wheel, anchored by three macrophage classifications (analogous to the three primary colors of the color wheel, (115)). According to their description, classically activated macrophages respond to the combination of IFN-γ and TNF signals, and secrete pro-inflammatory cytokines to mediate microbicidal and tumoricidal functions. Wound healing macrophages are primarily stimulated by IL-4, which induces arginase activity and subsequent collagen synthesis for remodelling of extracellular matrix. The third macrophage phenotype described by Mosser and Edwards are regulatory macrophages, which produce large amounts of the immunosuppressive cytokine IL-10 in response to prostaglandins, apoptotic cells, and sphingosine 1-phosphate.

2.4.2.3 HIF-mediated Macrophage Polarization

Macrophage polarization has been shown to be linked to differential expression of HIF-1α and HIF-2α isoforms. Specifically, the Th1 cytokines IFN-γ and LPS increased HIF-1α gene and protein expression, while the Th2 cytokine IL-4 exclusively induced HIF-2α gene and protein expression. Macrophage regulation of nitric oxide levels was also differentially regulated, with HIF-1α knockout in myeloid cells impairing iNOS expression and HIF-2α knockout significantly reducing arginase-1 expression (116). *In vitro* co-culture of HIF-1α−/− macrophages with tumor spheroids led to macrophage polarization towards an M2 phenotype, as observed by increased expression of CD206 and stabilin-1, and reduced expression of inflammatory TNF-α, iNOS and IL-6 (117). *In vivo*, knockout of HIF-1α in macrophages using the Cre/LoxP system driven by the LysM promoter led to decreased neointimal thickening and macrophage recruitment in a femoral artery injury model (118). Moreover, this correlated with reduced TNF-α and IL-6 expression in the tissue, consistent with *in vitro* results described above.
However, other studies suggested that regulation of M1-M2 macrophage polarization by activation of HIF-1 versus HIF-2 may be an oversimplification. PHD-2 preferentially regulated HIF-1 stability over HIF-2 (105). HIF-1 stabilization in PHD-2+/− haplodeficient mice resulted in reduced ischemia and increased density of large α-SMA+ vessels in the early stages following ligation of both the femoral and coronary arteries (119). Interestingly, PHD-2+/− macrophages showed upregulation of M2-associated genes and concomitant downregulation of M1-associated genes; these cells induced increased migration and proliferation of smooth muscle cells. Using three tumor mouse models, Colegia et al. demonstrated that tumor-derived lactic acid polarized macrophages towards an M2 or tumor-promoting state, with increased expression of VEGF and Arg-1 (120). In fact, polarization was dependent upon HIF-1 activation, as specific deletion of HIF-1α in myeloid cells impaired VEGF and Arg-1 induction upon macrophage treatment with tumor-conditioned media.

TGF-β, a target of HIF-1, is also a key mediator of macrophage polarization towards an M2 phenotype. The supernatant of apoptotic cells induced HIF-1 activation in macrophages, in a manner dependent upon TGF-β and sphingosine-1-phosphate (121). In vitro exposure of macrophages to exogenous TGF-β increased their expression of M2-associated genes, and was largely attributed to enhanced activity of the TGF-β-sensitive transcription factor SNAIL (122).

Taken collectively, results suggest that regulation of macrophage polarization is related to activation of the HIF pathways, but that the direction of polarization cannot be simply attributed to one HIF-α isoform over the other. Instead, the process is more likely sensitive to the specific in vivo context in question, as well as the interactions between HIF and other pathways. Another likely explanation for apparent discrepancies is that activation of HIF pathways regulates polarization of macrophages along a spectrum of phenotypes, which is poorly captured by assaying expression of a few genes, cytokine production, or even cell surface receptors typical of defining M1 and M2 phenotypes.

2.4.2.4 Role in Vascularization

The angiogenic role of macrophages has been well established, and attributed to the factors they secrete (reviewed (123)). Metalloproteases, for instance, are secreted by macrophages and remodel the extracellular matrix to release sequestered angiogenic factors (basic fibroblast growth factor, transforming growth factor-β, granulocyte-macrophage colony stimulating factor)
originally bound to heparin-like glycosaminoglycans. Macrophages also secrete various other factors that induce EC migration, proliferation and tube formation, including VEGF, IL-8, angiotropin, PDGF, IL-6, TGF-β, TGF-α, IGF-1 and human angiogenic factor. In a recent study, intravital imaging of an in vivo angiogenic assay of islets transplanted into the cremaster muscle of mice revealed that macrophages also physically integrate into new vasculature (90). They were found in close association with neutrophils and sprouting endothelium in the early stages of vascularization, and then later migrated to perivascular sites.

Using a temporally controlled model of macrophage depletion, in which administration of diphteria toxin targeted cells expressing Cre-recombinase under the LysM promoter, Lucas et al. demonstrated a crucial role of macrophages in various stages of wound healing (124). Early depletion of macrophages led to reduced wound closure and decreased numbers of alternatively activated macrophages (Fizz1+ and Ym1+) at later time points. Macrophage depletion during the mid-stage of wound healing resulted in reduced vascular stability and hemorrhage, as a result of EC apoptosis.

With an increased vocabulary for describing different macrophage subsets, researchers have sought to differentiate their respective roles in angiogenesis. There is some evidence suggesting that alternatively activated macrophages contribute to vascularization. Jetten et al. transplanted IFN-γ (M1), IL-4 (M2a) or IL-10 (M2c) -stimulated macrophages within Matrigel plugs in mice, and found that M2-polarized macrophages induced vascularization to the greatest degree (125). However, there is growing recognition that classically activated macrophages that are first recruited to sites of injury are also necessary for tissue vascularization. Using a CCR2-eGFP reporter mouse, authors identified a large influx of CCR2+Ly6C+ cells to the wound, of which a subset strongly expressed VEGF-A that was necessary for vascularization and wound closure (126). Characterization of in vitro polarized macrophages revealed that M1 macrophages expressed the highest levels of VEGF, compared to M2a and M2c-polarized macrophages (127). More importantly, the study showed that transplantation of glutaraldehyde-crosslinked scaffolds led to robust vascularization, and unlike other scaffolds, was infiltrated by high levels of both M1 and M2 macrophages. The results suggest that both macrophage populations, and not one, are necessary for vascularization.
2.4.3 Endothelial Progenitor Cells

2.4.3.1 Defining Endothelial Progenitor Cells

Endothelial progenitor cells (EPC) were first described by Asahara et al. (128). In their seminal work, CD34+ mononuclear blood cells were isolated from human peripheral blood, plated onto fibronectin-coated dishes and cultured to give rise to a population of cells that stained positively for UEA-1 and were able to uptake acetylated low-density lipoprotein (ac-LDL). In addition to forming tubular structures in vitro, EPC physically incorporated into the walls of new vessels when injected systemically into mouse and rabbit models of hind limb ischemia. However, since their discovery, there has been some controversy in isolating and defining the putative EPC population, which has led to other methods of culture and expansion (reviewed in (129)). For one, contaminating platelets in peripheral blood can release membrane particles that bind to the receptors of adherent cells, and give rise to false positive platelet-protein markers. Secondly, monocytes have also been shown to adhere to fibronectin-coated plates and upregulate expression of various endothelial markers (130). The method described by Asahara and colleagues, which identifies EPC based upon adhesion of mononuclear cells to fibronectin-coated plates, is therefore prone to contaminating monocytes.

For these reasons, other methods for defining EPC have been explored. Human EPC isolated from mononuclear cells of peripheral blood have generally been recognized to express CD34, AC133 and KDR (129). However, these markers are also expressed by hematopoietic stem and progenitor cell, and even when additional identification markers are considered (CD117, CD45, CD105, CD106, CD144), it is difficult to differentiate EPC from hematopoietic cells. Apart from EPC identification by cell surface receptor expression, others have established colony forming assays to culture circulating endothelial cells with proliferative potential (131). In this method, adult peripheral blood cells are plated onto type 1 collagen-coated plates and cultured for 2-3 weeks to form colonies. Some of these cells demonstrated high clonal potential upon replating, and enhanced vascularization when delivered into a model of hind limb ischemia (131). Others have further characterized the cells generated from this method, termed late outgrowth EC, to describe a CD34+ population with a progenitor-like and angiogenesis-inducing phenotype. A CD34+ population appeared to display a mature EC phenotype (132).
In the flow cytometry experiments conducted in this work, EPC were identified according to a panel of cell surface receptors, as described previously (133). Specifically, EPC were defined as non-leukocytes (CD45−) that co-express CD34, VEGFR-2 (receptor of VEGF) and CD117 (receptor for SCF).

2.4.3.2 Role in Vascularization

Despite some of the challenges and controversies involved in defining EPC, their role in enhancing vascularization has been well demonstrated. EPC can directly integrate into new vasculature in gliomas (134) and hepatocellular carcinomas (135), and have been shown to be involved in the angiogenic switch in tumors (reviewed in (136–138)). Apart from physically integrating into the vasculature, there is evidence that EPC predominantly exert their effects by secreting angiogenic factors like VEGF, HGF, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (139).

Implantation of EPC and MSC in Matrigel plugs into the subcutaneous space of athymic nu/nu mice resulted in the formation of perfusable vasculature, with GFP-labelled EPC incorporated into luminal positions of vessel structures (140). Similar findings were observed when the authors transplanted EPC with smooth muscle cells in the in vivo Matrigel plug assay (141). Interestingly, the in vivo angiogenic potential of EPC decreased with increased duration of ex vivo culture, and corresponded to increased maturation, as indicated by morphological changes and proliferation in response to angiogenic factors. Yoder et al. also demonstrated that transplantation of EPC (to be distinguished from endothelial cell colony-forming units) in collagen/fibronectin gels into the flank of immune-compromised mice resulted in the formation of erythrocyte-containing vessels, as examined by histology (131). Intramuscular injection of EPC and late outgrowth EC in a murine model of hind limb ischemia enhanced vascularization and limb salvage, even compared to the injection of a single cell population (142).

2.4.3.3 HIF-mediated EPC Functions

EPC must be responsive to hypoxic cues in order to contribute to restoring tissue vascularization and oxygenation; many of these responses are mediated by HIF signalling. Foremost, expression of CXCR4 (receptor of SDF-1) in EPC was found to be regulated by HIF-2, as knockdown of HIF-2α using short hairpin RNA reduced its expression (143). Overexpression of CXCR4 in
EPC, which exhibited low aldehyde dehydrogenase activity and correspondingly low angiogenic potential, increased their contribution to vascularization in an ischemic skin flap model. Moreover, Chang et al. showed that impaired healing of ischemic skin flaps in aged mice was due to decreased HIF-1α expression and its downstream targets SDF-1 and VEGF, which subsequently reduced the recruitment of EPC to the ischemic site (144). Systemic delivery of DFO, a pharmacologic which chelates iron and stabilizes HIF, increased EPC mobilization, vascularization and survival of ischemic skin flaps in aged mice. Another mechanism by which HIF mediates EPC recruitment to ischemic sites is by upregulating integrin-linked kinase (ILK) in EC, which increases their expression of SDF-1 and ICAM-1, and subsequent EPC recruitment (145). That is, blockade of ILK by adenovirus delivery of a dominant negative form of ILK significantly impaired vascularization in a hindlimb ischemia model, while its overexpression by adenovirus delivery of ILK enhanced vascularization in an in vivo Matrigel plug assay.

In addition to mediating the recruitment of EPC to ischemic sites, HIF also affects their angiogenic function. Overexpression of HIF-1α in EPC using recombinant adenovirus increased their proliferation, migration and their formation of vessel structures in vitro (146). Similarly, others found that transfection of EPC with HIF-1α plasmid increased their differentiation towards an endothelial-like phenotype (as assessed by CD31 expression), VEGF secretion, as well as their recruitment to and contribution to vascularization in a murine model of hind limb ischemia (147). It was also shown that SCF, a downstream target of HIF-1, enhanced in vitro tube formation and in vivo vessel formation in a Matrigel plug upon subcutaneous transplantation, as compared to mature EC. This effect was dependent upon expression of the SCF receptor, c-kit (148). Moreover, exposure of EPC to hypoxia induced the expression of Dll4, Hey1 and Hey 2 via activation of HIF-1α and Notch signalling, which mediated EPC differentiation towards an arterial cell fate (149).
2.5 Challenges and Implications

From a review of the literature, HIF was identified as a potential target for enhancing rate and maturity of vascularization in modular tissue constructs. Because of its role in modulating transcription of numerous angiogenic genes, targeting HIF effects a holistic angiogenic response. Not only does HIF regulate transcription of the well-known angiogenic target VEGF, but also those contributing to blood vessel maturation (Ang1 and PDGF-B) and chemokines modulating recruitment of angiogenic bone marrow-derived cells (SDF-1 and SCF). Stabilizing HIF arguably drives formation of mature vessels, as opposed to the many leaky vessels produced from specifically targeting VEGF expression. Although its effects in the dysregulated contexts of cancer progression and wound healing have been studied, its role in vascularizing tissue engineered constructs has yet to be specified. Elucidating its role and underlying mechanisms would advance methods for harnessing HIF to control vascularization of modular tissue constructs.

In Section 2.3, I reviewed different strategies for stabilization and overexpression of HIF in models of tissue repair following severe injury, including critical hind limb ischemia, wound healing and critical-sized calvarial defects. However, there have been few efforts investigating the effect of HIF stabilization in the context of minimal tissue damage, such as subcutaneous injection of vascularizing tissue constructs. This offers opportunities to harness HIF, specifically via controlled and prolonged stabilization, for tuning vascularization in modular tissue constructs.
2.6 References


34. Ceradini DJ, et al. (2004) Progenitor cell trafficking is regulated by hypoxic gradients


279(37):38458–38465.


Chapter 3

3 Hypoxia-inducible Factor Drives Vascularization of Modularly Assembled Engineered Tissue

3.1 Introduction

Hypoxia is a well-recognized challenge in tissue engineering, to be overcome by vascularization and integration with the host vascular network. Different strategies have been explored to enhance vascularization of tissue constructs, including the delivery of vascular cells in biomaterial scaffolds (1–4). Vascularizing engineered tissues have also been used to probe mechanistic questions related to endothelial cell (EC) biology and angiogenesis, elucidating the role of genes like Bcl-2 (5, 6), platelet-derived growth factor-B (PDGF-B) (7), and developmental endothelial locus-1 (Del-1) (8).

An emerging paradigm sees hypoxia as a tool to resolve the challenge of vascularizing engineered tissue constructs. Hypoxia-inducible factor-1 (HIF-1) is a primary regulator of the cellular response to hypoxia, orchestrating a program of genes related to metabolism, survival and proliferation (9). In particular, HIF-1 modulates angiogenesis by controlling the transcription of many genes, including VEGF, Ang-1, and PDGF (10). Unlike VEGF, whose overexpression results in the formation of leaky vessels, HIF-1 overexpression induces mature vessel formation (11). It thereby offers a promising avenue to enhance vascularization and perfusion of tissue constructs.

Despite growing interest (12–16), a causal relationship between HIF activation and the vascularization of engineered tissues in vivo has yet to be established. Using modularly assembled collagen-based engineered tissues, we inhibited HIF globally by systemic delivery of digoxin. Digoxin is a cardiac glycoside used for treatment of cardiac arrhythmia, and has been shown to inhibit HIF-1 and HIF-2 protein levels in a manner independent of mTOR, Na⁺/K⁺ ATPase or the PHD-VHL-proteosome pathway. Daily intraperitoneal injections significantly reduced vascularization and tumor growth in a xenograft mouse model (17). The specific role of HIF-1 activation in graft EC was then studied using a lentiviral construct containing short hairpin
RNA (shRNA) targeting HIF-1α; graft EC were transduced and incorporated into engineered tissues.

Modular tissue engineering is based on subcutaneous assembly of micro-tissues called modules, collagen cylinders filled with adipose-derived mesenchymal stromal cells (adMSC) and enveloped by human umbilical vein EC (HUVEC) (4). Modules not only remodel *in vivo* into a perfusable tissue, but are injectable and scalable due to their modular design. We hypothesized that HIF is central to initiating vascularization of modular constructs, through its effects on cell recruitment and on the activity of graft EC.
3.2 Methods and Materials

3.2.1 Cell culture and modular tissue fabrication

Primary adMSC and HUVEC were obtained commercially (Lonza) and cultured in supplemented DMEM (Sigma, with 10% FBS and 1% penicillin-streptomycin) and EGM-2 (Lonza), respectively. Media was changed every 2-3 days and cells used at passages 3-5. Modular tissues were fabricated using an air-jet method (18). Implants (0.1 mL, ~ 1.7×10^3 contracted modules) consisted of 1.5 × 10^6 adMSC and 3.9 × 10^5 HUVEC, and were cultured for two days in vitro prior to implantation.

3.2.2 Pharmacological inhibition study

Daily intraperitoneal injections of digoxin (2 mg/kg, Sandoz, 0.25 mg/mL) or saline (vehicle, 0.9% sodium chloride) were administered for 9 days, beginning 2 days prior to module implantation, and continuing to 7 days post-implantation. Modules were cultured in medium with digoxin (100 nM) or without (control) for 4 hours prior to implantation. Explants were subsequently analyzed by immunohistochemistry and flow cytometry.

3.2.3 Genetic inhibition study

Lentiviral shRNA constructs were provided by Dr. Jeffery Medin (University Health Network Vector Core facility in Toronto; now at Medical College of Wisconsin), and designed with a bidirectional promoter configuration (gene map in Supplementary Figure S2). HUVEC (P2) were transduced with lentiviral particles containing sh-scr or sh-HIF1α at a multiplicity of infection (MOI) of 10. The optimum HIF-1α target sequence and MOI for transduction were determined as described below. Transduced HUVEC were coated onto modules prior to implantation, according to an established protocol (19). The effect of shRNA-mediated inhibition of HIF-1α or a scramble control sequence on EC sprouting was demonstrated in an in vitro sprouting assay (Supplementary Figure S3).
3.2.3.1 Optimization of shRNA target sequence

HEK-293T cells were transfected at 50-80% confluence by lipofection (Lipofectamine LTX with PLUS Reagent, Life Technologies). shRNA plasmids targeting a scramble sequence (control) or five different HIF-1α sequences were diluted in Opti-MEM Reduced Serum Medium (Life Technologies) at a concentration of 2.5 µg DNA in 500 µL media. DNA-Lipofectamine complexes were created as per the manufacturer’s protocol, and incubated with cells for 24 hours prior to assaying for transgene transfection. One transfection was conducted per plasmid tested (n=3).

Total RNA was isolated using the Qiagen RNeasy Mini Kit, and cDNA synthesized using the Invitrogen SuperScript III First-Strand Synthesis Supermix. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assay HIF-1α gene expression relative to 18S gene expression with the Bio-Rad Ssofast Evagreen PCR Reaction Mix (Supplementary Figure S2B). The following primers were used: 5’- AGG CCG CTC AAT TTA TGA AT -3’ (HIF-1α forward); 5’- TTT GGC AAG CAT CCT GTA CT -3’ (HIF-1α reverse); 5’ - AGG AAT TGA CGG AAG GGC AC - 3’ (18S forward); 5’ - GGA CAT CTA AGG GCA TCA CA - 3’ (18S reverse). The most efficient targeting sequence was identified as that which yielded greatest knockdown of HIF-1α gene expression.

3.2.3.2 Optimization of lentiviral shRNA MOI

A lentiviral vector was subsequently constructed (Supplementary Figure S2A) using this sequence and transduced into HUVEC at a range of MOI. Briefly, HUVEC (P2-3) were grown to 70% confluence in 6-well plates, and incubated with lentivirus-containing media for 24 hours before media was changed. HIF-1α and GLUT-1 gene expression were assayed by qRT-PCR (Supplementary Figure S2C). Additionally, cells were assayed for GFP expression using flow cytometry (Supplementary Figure S2D). One transduction was conducted for each MOI tested, in both gene expression and flow cytometry analyses (n=3).

To confirm knock-down, HUVEC (P2-3) were transduced with shRNA constructs targeting HIF-1α or a scramble sequence at a MOI of 10, and assayed by Western blot (Supplementary Figure S2E) and qRT-PCR (Supplementary S2F) at P5-6. In both assays, transduced HUVEC were stimulated with 300 µM cobalt chloride for 6 hours (n=3 per experimental condition). Nuclear
HIF-1α protein was extracted using the Nuclear Cytoplasmic Extraction Kit (Thermo Scientific). Nuclear protein (12 µg) was loaded in each well. After protein transfer, the nitrocellulose membrane was incubated with primary antibody overnight at 4°C. HIF-1α primary antibody (Novus Biologics, NB100-479) was used at 1:1000 dilution, while β-actin (Novus Biologics, NB600-503H) was used at 1:2000 dilution, and incubated with goat anti-rabbit secondary horse radish peroxidase antibody (Santa Cruz) for 1 hour at 1:5000 dilution. In the qRT-PCR assay, RNA was isolated and cDNA synthesized from transduced HUVEC as described above (n=3 per experimental group). The following primer sequences were used for GLUT-1: forward primer 5’- CAC CAC CTC ACT CCT GTT ACT T -3’; reverse primer 5’- CAA GCA TTT CAA AAC CAT GTT TCT A -3’.

3.2.4 Animals and surgery

SCID-bg mice (Charles River) were operated on at 6-7 weeks of age. All surgeries were performed using sterile procedures, in accordance with guidelines by the Faculty of Medicine Animal Care Committee at the University of Toronto. In one cohort of mice, modular tissues were collected for immunohistochemical analysis. In a separate cohort, tissue explants were digested or peripheral blood was collected for flow cytometry analyses. A sample size of n=5 was used in all in vivo experiments.

3.2.5 Immunohistochemistry

Mice were sacrificed at day 7 and 14 post-implantation (n=5 per experimental group and time point), and modular tissue explants were fixed in 10% neutral buffered formalin prior to immunohistochemical processing. Tissue sections were stained for CD31, biotinylated Ulex Europaeus Agglutinin I (UEA-1), HIF-1α and HIF-2α. Briefly, samples were embedded in paraffin wax, dewaxed with xylene and rehydrated with graded alcohol. Sections 4 µm thick were stained for Masson Trichome, anti-CD31 (Santa Cruz Biotechnology Inc. SC-1506-R, 1:2000, rabbit polyclonal antibody), biotinylated Ulex Europaeus Agglutinin I (UEA1, Vector Laboratories B1065, 1:400; stains human EC only), anti-HIF1α (Novus Biologicals NB100-479,
1:2000, rabbit polyclonal antibody), and anti-HIF2α (Novus Biologicals NB100-122, 1:1000, rabbit polyclonal antibody).

For EF5 experiments, a subset of animals (n=3 per experimental group and time point) received a tail vein injection of EF5 (0.25 mL, 10 mM in 5% dextrose and 2.4% ethanol, provided by the National Cancer Institute. Animals were sacrificed 3 hours later, and tissue sections stained with anti-EF5 conjugated to Cy5 (1:100, University of Pennsylvania) and anti-CD31 (1:1000, Santa Cruz).

Histology slides were scanned using ScanScope XT (Aperio Technologies) in the Advanced Optical Microscopy Facility (University Health Network), with one tissue section analyzed per implant (n=5 per experimental group per time point). Digital images were analyzed using the Aperio ImageScope software (Leica). Densities of vessels (containing a lumen), HIF-1α+ and HIF-2α+ nuclei were quantified using previously described methods (19). Immunofluorescent sections were scanned using AxioScan Z.1 (Carl Zeiss) in the Advanced Optical Microscopy Facility (University Health Network), and images captured using the Zen LE (Blue) Lite software.

3.2.6 Flow cytometry

Peripheral blood was collected from mice via cardiac puncture at day 0 and 1. Untreated animals (without ip injections or module tissue implants) served as a baseline. Peripheral blood (70 µL) from each animal was used to prepare fully stained samples and fluorescent-minus-one (FMO) controls, according to an established protocol (20). Modular tissues were explanted from animals at day 1 and 3, with surrounding connective tissue trimmed. An entire implant was isolated from each animal, and digested in buffer containing collagenase I, collagenase XI, DNase and hyaluronidase, as detailed previously (18). In both experiments, samples (n=5 per experimental group and time point) were stained on ice with limited light exposure. Following fixation in 2% paraformaldehyde, flow cytometry was run using the BD LSR Fortessa X-20. All cells from peripheral blood and modular tissue isolates were analyzed. In modular tissue experiments, the number of cells were normalized to the implant mass, as determined prior to tissue digestion. Gating schemes are provided in Supplementary Figures S6 and S7.
3.2.7 Sprouting assay

shRNA-transduced HUVEC were seeded onto Cytodex beads and embedded within fibrin gel at a density of 200 beads/well of a 24 well plate (0.5 mL fibrinogen solution). In reduced serum experiments, HUVEC-coated beads were cultured in reduced serum DMEM (1% FBS) for 2 days, followed by restoration in fully supplemented (10% FBS) EGM-2 medium at day 2. In other experiments, HUVEC-coated beads were cultured in fully supplemented EGM-2 medium for 5 days. Beads were imaged using a bright field microscope (Zeiss Axiovert 135). The number and length of sprouts were quantified using ImageJ software. At specified time points, live and dead cells were stained using calcein and ethidium bromide (Life Technologies), and visualized by confocal microscopy (Nikon Eclipse Ti).

3.2.8 Statistical analysis

Statistical analyses were performed using two-way analysis of variance (ANOVA, JMP Pro 13), with Tukey’s honest significant difference post-hoc test. The assumption of normally distributed residuals was tested using the Shapiro-Wilk W goodness-of-fit test. In cases where the assumption was not met, the data was transformed using logarithmic, square root or cube root functions (tested in that order). Differences were considered statistically significant at p<0.05.
3.3 Results

3.3.1 HIF inhibition reduced vascularization and perfusion of modular tissue constructs

Systemic delivery of digoxin markedly reduced total vessel density (Figure 1A; p<0.01) and human-derived (UEA-1+) vessel density at day 7 (Supplementary Figure S1A; p<0.01). By day 14, one week after cessation of the drug regimen, differences were no longer significantly different. However, greater EF5 staining denoted hypoxic regions in digoxin-treated implants persisting to day 14 (Figure 1B). Digoxin therefore reduced vascularization during its administration, and adversely affected tissue oxygenation one week after cessation of digoxin administration.

**Figure 1. Inhibition of HIF reduced early vascularization of modular constructs.** (A) Systemic delivery of digoxin (ip, 2 mg/kg per day for 9 days beginning on day -2) resulted in a significant decrease in total CD31+ vessel densities at day 7 (but not at day 14), relative to saline controls. Representative histology images at day 7 are shown (CD31, scale bar is 200 µm). (B) Increased staining of the hypoxia marker EF5 was observed in digoxin-treated implants 14 days post-implantation, indicating persistent hypoxia (left column: EF5, right column: merged stains with CD31 in red and DAPI in blue, scale bar is 100 µm). EF5+ cells were likely host-derived cells or adMSC, not EC, since adjacent tissue sections showed little staining for CD31. (C)
Knockdown of HIF-1α in HUVEC via transduction of shRNA targeting HIF-1α reduced total CD31+ vessel density upon their implantation in modules at day 7 (but not at day 14), compared to implants containing HUVEC transduced with shRNA targeting a scramble control sequence. Representative histology images at day 7 are shown (CD31, scale bar is 200 μm). (D) Some regions showed increased staining of the hypoxia marker EF5 was observed in sh-HIF1α HUVEC implants 7 days post-implantation (left column: EF5, right column: merged stains with CD31 in red and DAPI in blue, scale bar is 100 μm). EF5+ cells were EC (CD31+) as well as host-derived cells. n=5; error bars are standard error of the mean (sem); *p<0.05 and **p<0.01.

Since systemic delivery of a pharmacologic agent can have widespread and off-target effects, shRNA was transduced in graft EC. Previously, we observed that increasing cell densities and implant volume were associated with HIF-1α nuclear localization in EC (19). We postulated that implanted EC played a central role in driving vascularization of modular tissues, and proceeded to inhibit HIF-1α in HUVEC by lentiviral transduction of shRNA targeting HIF-1α (sh-HIF1α) or a scrambled sequence control (sh-scr). Lentiviral sh-HIF1α knocked down HIF-1α at the mRNA and protein levels in vitro (Supplementary Figure S2). Moreover, in an in vitro sprouting assay, it reduced EC sprouting in both normal and reduced serum conditions, the latter mimicking the nutrient-deprived in vivo environment of the poorly vascularized subcutaneous space (Supplementary Figure S3).

Implantation of sh-HIF1α HUVEC reduced total vessel density at day 7 (Figure 1C: 14 ± 4.4 vs. 26 ± 3.9 CD31+ vessels/mm²; p<0.05), but no effect was observed by day 14. This was presumably due to host vessel ingrowth, as vessels in the sh-HIF1α group were predominantly UEA-1- at day 14 (Supplementary Figure S1). Some hypoxic regions (positive EF5 staining) were identified in module implants containing sh-HIF1α HUVEC at day 7 (Figure 1D); EC-specific inhibition therefore reduced vascularization and tissue oxygenation at this time.
3.3.2 Digoxin and shRNA-mediated knockdown inhibited HIF-1 activation in modular implants

Systemic digoxin treatment reduced HIF-1α+ nuclei density at day 1 (Figure 2A: 460 ± 40 vs. 660 ± 60 HIF-1α+ nuclei/mm²; p<0.05) and day 3 (550 ± 70 vs. 880 ± 30 HIF-1α+ nuclei/mm²; p<0.01), despite the persistence of hypoxia in the implant (positive EF5 staining at day 14, Figure 1B). This is consistent with the expectation that digoxin inhibits HIF-1 mediated activity. *In vitro* findings support this, as digoxin inhibited HIF-1α protein stabilization in cobalt chloride-stimulated HUVEC and adMSC (Supplementary Figure S4). EC-specific inhibition also reduced HIF-1α+ nuclei density throughout the implant. The difference was statistically significant at day 7 (Figure 2B: 430 ± 10 vs. 670 ± 70 HIF-1α+ nuclei/mm²; p<0.05), whereas with digoxin treatment, HIF-1α+ density was not significantly lower at day 7, but only at day 3. Moreover, EC-specific inhibition of HIF-1α at day 7 was accompanied by an increase in HIF-2α+ nuclei density throughout the implant (Figure 2C: 140 ± 10 vs. 50 ± 10 HIF-2α+ nuclei/mm²; p<0.01), pointing to HIF-2 as a compensatory mechanism for EC-specific HIF-1 inhibition.

![Figure 2. Inhibition reduced HIF-1 nuclear localization in modular constructs at early times.](image-url) 

(A) Systemic delivery of digoxin significantly reduced the density of HIF-1α+ nuclei within the modular construct at day 1 (p<0.05) and day 3 (p<0.01), but differences were no longer observed at 7 and 14 days post-implantation. Select statistically significant pairwise
comparisons between experimental groups are shown graphically. All other significant differences are summarized in Supplementary Table S1. Representative histology images at day 1 and 3 are shown (scale bar is 60 µm). HIF-1α+ cells were likely host-derived inflammatory cells. (B) Implantation of sh-HIF1α HUVEC significantly reduced the density of HIF-1α+ nuclei within modular implants at day 7, although differences were not observed by day 14. HIF-1α+ cells at day 7 were likely host-derived inflammatory cells, and not EC. The density of HIF-2α+ nuclei was markedly increased in modular constructs containing sh-HIF1α HUVEC at day 7. HIF-2α+ cells were likely host-derived cells and EC. Representative histology images at day 7 are shown (scale bar is 60 µm). n=5; ±sem; *p<0.05 and **p<0.01.

3.3.3 Digoxin inhibited recruitment of bone marrow-derived cells to the modular implant

Since HIF modulates the expression of chemokines governing recruitment of bone marrow-derived cells, we investigated the effect of systemic HIF inhibition on mobilization of these cells into peripheral blood and their recruitment to the modular implant.

Digoxin treatment reduced the mobilization of CXCR4+CD45+ leukocytes into peripheral blood at day 0 and 1, relative to saline and baseline controls, although not at statistically significant levels. However, it markedly increased the mobilization of CD45 CD34+CD117+VEGFR2+ cells, here termed endothelial progenitor cells (EPC, (21)) in peripheral blood at day 0 (two days following drug administration, prior to module injection) compared to baseline controls (although not statistically significant). Following module injection, the number of these cells in peripheral blood decreased markedly (p<0.05). We hypothesized that this was due to their migration to the explants. However, when modular tissues were enzymatically digested, there were many more EPC in saline controls than with digoxin treatment at day 3 (Figure 3B: 6.0 ± 1.0 vs. 18 ± 2.0 cells/mg implant; p<0.01); there was no significant difference at day 1.
Figure 3. Systemic HIF inhibition affected the mobilization of bone marrow-derived cells and their recruitment to the modular construct. (A) Peripheral blood analysis: Systemic digoxin delivery reduced the mobilization of CXCR4+ leukocytes (CD45+CXCR4+) into peripheral blood, albeit not at a statistically significant level. Digoxin treatment increased endothelial progenitor cell (EPC, CD45−CD34+CD117+VEGFR2+) mobilization into peripheral blood at day 0 (prior to module injection, but 2 days after beginning of digoxin administration), relative to baseline controls (although not statistically significant). However, their numbers decreased markedly 1 day following module implantation (p<0.05), to a level similar to saline and baseline controls. (B) Explant analysis: Systemic digoxin delivery significantly reduced the
recruitment of EPC (p<0.01) and monocytes/macrophages (CD45^+CD11b^+Ly6G^{low}, not statistically significant) to the modular tissue construct relative to saline controls at day 3. In saline controls, the number of EPC and monocytes/macrophages increased from day 1 to day 3. In order for residuals to be normally distributed for subsequent statistical analyses by ANOVA, the data sets were transformed logarithmically (monocyte/macrophages in tissue) or using the cube root function (EPC in tissue and EPC in peripheral blood). n=5; ±sem; *p<0.05 and **p<0.01.

In addition to its effects on EPC, digoxin reduced recruitment of monocytes/macrophages to modular implants at day 3 (Figure 3B: 1.4 ± 0.3 vs. 3.2 ± 0.9 \times 10^3 \text{ cells/mg implant}; albeit not statistically significant). It did not, however, affect the total number of leukocytes (CD45^+), neutrophils (CD45^+CD11b^+Ly6G^{high}), or hemangiocytes (CD45^+CXCR4^+VEGFR1^+) in peripheral blood at day 0 and 1, nor their recruitment to the modular implant at day 1 or 3 post-implantation (Supplementary Figure S5).
3.4 Supplementary Materials

3.4.1 Supplementary Figures

**Figure S1. Effect of HIF inhibition on graft-derived (UEA-1+) vessel density and tissue oxygenation.** (A) Digoxin treatment reduced UEA1+ vessel density at day 7 (p<0.01), but had no effect by day 14. Representative histology images at day 7 are shown to the right (scale bar is 200 μm). (B) Genetic knockdown of HIF-1α by lentiviral transduction of shRNA in HUVEC had no significant effect on UEA1+ vessel density at either day 7 or 14. Representative histology images at day 7 are shown to the right (scale bar is 200 μm). n=5; ±sem; **p<0.01.
Figure S2. Selection of HIF-1α target sequence for the design of lentiviral shRNA constructs, MOI for transduction, and confirmation of HIF-1α knockdown in cobalt chloride stimulated HUVEC. (A) A schematic of the shRNA construct (7579 nucleotides) shows the shRNA sequence (6179-6248) under the control of the U6 promoter (5922-6178), and the eGFP gene (5767-5051) expressed by the mini CMV promoter (5894-5786) in a bidirectional configuration. Other plasmid elements are the central polypurine tract (Cppt, 4458-4581), the Rev response element (RRE, 3734-3975), pUC19 (1-1999), CMV IE-1 promoter (2217-2889), phage F1 origin (7121-7579), SV40polyA oriR (6754-6959), and polyA (4851-4596). (B) shRNA plasmids targeting 5 different HIF-1α sequences were transfected in HUVEC and assayed for HIF-1α mRNA knockdown, with plasmid 3 yielding the most effective knockdown. This sequence was subsequently used to construct lentiviral vectors for the delivery of shRNA. Lentiviral sh-HIF1α vectors were transduced in HUVEC at varying MOI, and assayed via (C) mRNA expression analysis and (D) flow cytometry analysis for GFP expression. An MOI of 10 was selected for transduction of HUVEC in subsequent experiments, as it yielded a transduction
efficiency >85%. (E) Knockdown of HIF-1α in stably transduced sh-HIF1α HUVEC reduced nuclear HIF-1α protein upon stimulation with the hypoxia mimetic cobalt chloride (300 µM, 6 hours). (F) Knockdown of HIF-1α in sh-HIF1α HUVEC inhibited induction of GLUT-1 mRNA expression, a downstream target of the HIF-1 pathway, upon stimulation with cobalt chloride (300 µM, 6 hours), as well as HIF-1α gene expression under stimulated and non-stimulated conditions. Relative expression was normalized to the untreated sh-scramble HUVEC group.
Figure S3. Effect of shRNA-mediated HIF-1α knockdown in HUVEC on sprout number and length in normal and reduced serum conditions. (A) In normal serum conditions (10% FBS), sh-HIF1α HUVEC produced fewer sprouts than sh-scramble HUVEC, but differences were not significantly different except at day 2 (p<0.01). sh-HIF1α HUVEC sprouts were markedly shorter than those of sh-scr HUVEC at day 2, 4 and 5 (p<0.01). Staining for live (green) and dead cells (red) revealed no differences in cell viability at day 5. (B) sh-HIF1α HUVEC produced fewer sprouts (p<0.01 at day 4 and 5) and shorter sprouts than sh-scramble HUVEC (p<0.01 at day 1, 4 and 5) after being exposed to low serum conditions (1% FBS) for 2 days, and even upon return to normal serum conditions. Staining for live (green) and dead cells (red) revealed that reduced serum negatively impacted cell viability at day 2. n=9; ±sem; ##p<0.01 relative to sh-scr control.
**Figure S4. Effect of digoxin on HUVEC and adMSC viability and HIF-1α stabilization in vitro.** (A) Digoxin did not affect cell viability when cells were cultured as a monolayer (100 nM, 6 hours; left: adMSC, middle: HUVEC) or when adMSC were embedded within HUVEC-coated modules (right). (B) Digoxin reduced nuclear HIF-1α protein stabilization in both HUVEC and adMSC when stimulated with the hypoxia mimic, cobalt chloride (100 nM digoxin and 300 µM cobalt chloride, 6 hours). A comparison of positive HIF-1α pixel area in western blots are shown for cobalt chloride-stimulated HUVEC (C) and adMSC (D).
Figure S5. Digoxin had no effect on the mobilization of leukocytes, neutrophils and hemangiocytes in peripheral blood, nor their recruitment to the modular tissue construct.

(A) Peripheral blood analysis: No significant differences were observed in the mobilization of leukocytes (CD45$^+$), neutrophils (CD45$^+$CD11b$^+$Ly6G$^\text{high}$) and hemangiocytes (CD45$^+$CXCR4$^+$VEGFR1$^+$) in the peripheral blood of digoxin-treated animals, compared to saline treated groups and baseline controls, at either day 0 or 1. (B) Explant analysis: No significant differences were observed in the recruitment of leukocytes, CD45$^+$CXCR4$^+$ leukocytes, neutrophils and hemangiocytes to modular tissue constructs of digoxin-treated animals, compared to the saline-treated group, at both days 1 and 3. In order for residuals to be normally distributed for subsequent statistical analyses using ANOVA, the data sets were transformed using logarithmic (CXCR4$^+$ leukocytes) and cube root functions (hemangiocytes). n=5; ± sem.
Figure S6. Gating scheme for flow cytometry analyses of cells mobilized in peripheral blood. Cellular debris was first excluded (gating on FSC-A vs. SSC-A), followed by singlet selection (FSC-H vs. FSC-A). A gate was then created on live cells, which were segregated into CD45+ and CD45− populations. Hemangiocytes (CXCR4+VEGFR1+) and neutrophils (Ly6G+) were identified from the CD45+ leukocyte population. Endothelial progenitor cells (CD117+VEGFR2+) were selected from the CD45− cell population. All gate boundaries were defined using fluorescence-minus-one (FMO) controls.
Figure S7. Gating scheme for flow cytometry analyses of cells recruited to modular tissue constructs. Cellular debris was first excluded (gating on FSC-A vs. SSC-A), followed by singlet selection (FSC-H vs. FSC-A). A gate was then created on live cells, which were segregated into CD45+ and CD45− populations. CD45+ leukocytes were further separated into neutrophils (CD11b+Ly6G\text{high}), monocytes/macrophages (CD11b+Ly6G\text{low}), and hemangiocytes (CXCR4+VEGFR1+). Endothelial progenitor cells were identified as CD34+CD117+VEGFR2+ cells within the CD45− population. All gate boundaries were defined using fluorescence-minus-one (FMO) controls.
3.4.2 Supplementary Tables

Table S1. Summary of all statistically significant pairwise comparisons for HIF-1α+ and HIF-2α+ nuclei densities. Notation used: digoxin (d) and saline (s) in digoxin experiments; sh-HIF1α (h) and sh-scr (s) in shRNA experiments; the number following each experimental group designation refers to the time point of interest (e.g., d3 is digoxin treatment at day 3). Significant differences between treatment groups within a time point are highlighted in bold.

<table>
<thead>
<tr>
<th>Data set</th>
<th>p&lt;0.01</th>
<th>p&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α+ nuclei density</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Digoxin experiment)</td>
<td>d3 vs. d14</td>
<td>d1 vs. s1</td>
</tr>
<tr>
<td></td>
<td>d3 vs. s3</td>
<td>d7 vs. d14</td>
</tr>
<tr>
<td></td>
<td>s1 vs. s7</td>
<td>s1 vs. s14</td>
</tr>
<tr>
<td></td>
<td>s1 vs. s14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>s3 vs. s7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>s3 vs. s14</td>
<td></td>
</tr>
<tr>
<td>HIF-1α+ nuclei density</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>(shRNA experiment)</td>
<td>h7 vs. h14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>h7 vs. s7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>h7 vs. s14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>h14 vs. s7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>s7 vs. s14</td>
<td></td>
</tr>
<tr>
<td>HIF-2α+ nuclei density</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>(shRNA experiment)</td>
<td>h7 vs. h14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>h7 vs. s7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>h7 vs. s14</td>
<td></td>
</tr>
</tbody>
</table>
3.5 Discussion

As expected, HIF is a critical driver of vascularization in modular tissue constructs. Both drug-mediated systemic inhibition and shRNA-mediated EC-specific inhibition crippled early (day 7) vessel formation, but vessel density normalized by day 14. Despite apparent similarities in the vascularization pattern, the hypoxia marker EF5 showed marked differences. Whereas systemic HIF inhibition had enduring effects on tissue hypoxia, with EF5+ regions observed even one week after cessation of digoxin administration (day 14), EC-specific inhibition only affected implant oxygenation to day 7.

Firstly, results demonstrate the value of using EF5 to assess construct oxygenation. With it, we identified hypoxic regions in digoxin-treated implants, even though vessel densities were similar to that of saline-treated implants at day 14. The nitroimidazole is commonly used to study tumor oxygenation; we recommend that it be used more readily in tissue engineering.

Secondly, differences in construct oxygenation (EF5 staining) highlight different mechanisms governing the two inhibitory methods used. Systemic HIF inhibition led us to identify two vascularization-related cell populations, EPC (CD45-CD34+CD117+VEGFR2+) and monocytes/macrophages (CD45+CD11b+Ly6Glow), whose recruitment to the construct was HIF-dependent. The roles of both populations in vessel formation have been well established (22, 23). Notably, effects on recruitment were most pronounced at day 3, corresponding to a peak in HIF-1 activation in control implants. Others observed a similar time course, in which myeloid cell (CD45+CD11b+) infiltration peaked 2 days after implantation of cell-laden Matrigel plugs (24). In macrophages, hypoxia stimulated an angiogenic gene and protein expression profile that was mediated by HIF-1 and -2 (25). Hypoxia was shown to increase EPC recruitment in a soft tissue ischemia model via HIF-mediated upregulation of SDF-1 (26), stimulating EPC proliferation and vasculogenesis (27) as a result of c-kit activation (28). Rather than recruitment, it is conceivable that digoxin affected generation of these circulating cells. Studies in support of this notion are limited: digoxin reduced colony forming units of granulocyte-macrophage progenitors in an in vitro assay (29).

Because EPC numbers in peripheral blood decreased one day following module implantation in digoxin-treated mice, we sought to determine whether there was greater recruitment to the implant. In fact, digoxin markedly reduced EPC numbers in the construct at day 3. Taken
together, we propose that digoxin mobilized EPC into peripheral blood by inhibiting downstream targets SCF and SDF-1 in the bone marrow niche. Gene expression of both chemokines is known to be regulated by HIF and involved in EPC recruitment (26, 28). With reduced niche signals, EPC migrated from the bone marrow into peripheral blood. This is consistent with others’ findings showing that EPC numbers in peripheral blood increased after administration of the CXCR4 antagonist AMD3100 (30), and due to inhibition of the SCF receptor c-kit (31). These cells would have been beckoned from the blood and recruited to the implant, had systemic HIF inhibition not also reduced HIF-modulated chemokines in the implant.

Ultimately, digoxin experiments were limited by off-target effects typical of the use of any pharmacologic. There is indication that digoxin can cause cell cycle arrest in tumor cells (17) and induce apoptosis of human EC (32). With this understanding, a genetic method of HIF-1 inhibition was pursued to complement pharmacologic studies. EC-specific inhibition had a less enduring effect on tissue oxygenation than did systemic inhibition, presumably because activation of HIF-1 in cells other than graft EC also contributed to ingrowth of host vessels. Moreover, increased HIF-2α+ nuclei density pointed to HIF-2 as a likely compensatory mechanism for EC-specific HIF-1 inhibition. HIF-1α and HIF-2α share high homology in protein structure; both dimerize with Arnt and bind hypoxia responsive elements to regulate expression of overlapping and non-overlapping genes (reviewed elsewhere (33)). NF-κB is another likely compensatory mechanism, as its expression is also stimulated by hypoxia (34). NF-κB can contribute to vascularization by regulating HIF-1α protein accumulation in bone-marrow derived macrophages exposed to hypoxia (35), and by affecting delta-like ligand 4 and Notch signaling in EC (36).

However short-lived the effects of EC-specific inhibition, one must appreciate that knockdown of HIF-1α in graft EC alone was sufficient to reduce vascularization and tissue oxygenation to day 7. Not only does this underline the importance of graft EC in vascularizing modular tissues, but of HIF-1 in governing their angiocrine function. This was reinforced by our results from an *in vitro* sprouting assay (Supplementary Figure S3) and by others, who showed that HIF-1 is crucial to EC survival in hypoxia, reduced serum, and in the presence of oxidative stress (37). Intriguingly, HIF-1α knockdown in EC led to shorter sprouts even in normal serum conditions. This effect on baseline function (normal serum levels) was also reported by others, who showed that aortic rings dissected from ArntloxP/loxP mice had fewer and significantly shorter sprouts in an
*in vitro* angiogenesis assay, even in normoxic and serum-rich conditions (38). Modular tissues were constructed with HUVEC, which are derived from large vessels, and arguably functionally different from EC that line capillaries and micro-vessels. However, prior work showed little difference in degree of vascularization between HUVEC and human microvascular EC derived from adipose tissue (4), or with late outgrowth EC (unpublished). It remains to be seen whether there are differences at the level of the hypoxic response.

In this study, we used two inhibitory approaches to tease out the role of HIF in vascularizing engineered tissues *in vivo*. Both methods confirmed HIF as a rate limiting factor in vascularizing collagen-based modular constructs, while highlighting two underlying mechanisms at play. HIF drives formation of functional vessels through recruitment of EPC and monocytes/macrophages to the construct, and through EC-specific effects. Furthermore, our study revealed a role of HIF in the early stage (day 3) of construct remodeling, as its inhibition significantly affected HIF-1α+ nuclei density and cell recruitment at this time point. Results also demonstrated that graft EC are crucial in construct vascularization, and that their angiogenic function is largely governed by HIF-1. Going forward, methods targeting either or both HIF-mediated mechanisms may be exploited to drive vascularization of engineered tissue constructs and, in turn, be used to design tissue engineering platforms for therapeutic application.
3.6 Conclusion

Strategies for vascularizing engineered tissues are crucial to their functional engraftment following implantation. However, many strategies, like the harnessing of target genes, have been derived from contexts wholly different from that of tissue construct implantation. Here, we demonstrate that injectable, modularly assembled tissue constructs harness the oxygen-sensing cellular machinery, HIF, to drive vessel formation via multiple mechanisms.

From digoxin experiments, we identified EPC and monocytes/macrophages to be two host-derived cell populations that play a crucial role in the vascularization of modular tissue constructs. Moreover, effects of systemic HIF inhibition were most pronounced at day 3. Results therefore suggest that both the cell type recruited and the temporal profile of their recruitment are important for robust vascularization. From shRNA experiments, we were surprised to find that HIF-1 inhibition in graft EC alone was sufficient to affect vessel formation and implant oxygenation. Results not only highlighted the role of HIF-1 in the angiogenic function of EC, but also the particular role of graft EC in driving vessel formation in modular tissues.

Studies are currently underway to harness HIF for driving vascularization in modular tissue constructs. Indeed, HIF is already expressed by cells within the construct immediately following implantation into the poorly vascularized subcutaneous space. Strategies to augment and prolong HIF stabilization in graft EC, potentially in combination with methods to enhance early recruitment of host-derived angiogenic cells, may be promising strategies to drive rapid, robust and mature vascularization in engineered tissues. This, in turn, would advance our understanding and use of engineered tissue platforms for therapeutic applications.
3.7 Acknowledgements

The authors acknowledge Chuen Lo for performing the animal surgeries. We thank Drs. Jeffrey Medin and Monty McKillop (University Health Network Vector Core facility in Toronto; now at Medical College of Wisconsin) for providing the lentiviruses necessary for genetic inhibition experiments, and Dionne White (Faculty of Medicine Flow Cytometry Facility, University of Toronto) for assistance with flow cytometry experiments. We acknowledge financial support from the Canadian Institute for Health Research (CIHR, MOP-142406) and the University of Toronto Medicine by Design initiative, by the Canada First Research Excellence Fund, as well as graduate student support from CIHR for GL.
3.8 References


30. Shepherd RM, et al. (2006) Angiogenic cells can be rapidly mobilized and efficiently


Chapter 4

4 Hypoxia-inducible Factor Stabilization Induces Vascularization of Modular Tissue Constructs

4.1 Introduction

Engineered tissues are a promising solution to address the shortage of donor organs, but the lack of rapid and robust vascularization upon implantation limits their engraftment. Various vascularization strategies have therefore been devised, one of which involves the implantation of vascular cells like endothelial cells (EC).

We and others have shown that delivery of EC alone does not initiate robust vascularization, due to their apoptosis shortly after implantation (1, 2). EC-based vascularization strategies require co-delivery of support cells, such as mesenchymal stromal cells (MSC, (1, 3–6)), which give rise to mature and functional blood vessels that persist for several weeks. For example, modular tissue constructs containing MSC and EC demonstrated increased vessel density, smooth muscle lining of vessel structures, and functional perfusion of new vasculature (5, 6); without MSC, EC apoptose upon implantation, and few vessels are formed (2).

The beneficial effects of MSC can be attributed to the plethora of trophic factors they produce, which enhance EC survival and angiogenesis (7, 8). Additionally, MSC stabilize vasculature via physical contact with EC (3, 9), which is partly mediated by VE-cadherin/β-catenin signaling (10, 11). We hypothesized that prolonged activation of hypoxia-inducible factors (HIF) in engineered tissue constructs would obviate the role of MSC in initiating rapid and mature vascularization.

HIF are master regulators of cellular response to hypoxia, orchestrating the expression of genes related to angiogenesis, metabolism and proliferation (12). Targeting HIF can thus be advantageous to delivering a single growth factor like VEGF, as it orchestrates a complex response, inducing both vessel formation and maturation (13). Once normal oxygen levels are
restored, the HIF-α subunit is degraded, due to its hydroxylation by prolyl hydroxylase (PHD) and subsequent degradation in the proteosome (14), rendering HIF transcriptionally inactive. Some pharmacologics can stabilize HIF-α even in the presence of oxygen. One such compound is deferoxamine (DFO), which chelates iron, a co-factor of PHD, thereby inhibiting PHD activity so that the HIF-α subunit can translocate into the nucleus to enable gene transcription, even in the presence of normoxia. Recently, others have shown that delivery of DFO from nanoparticles can drive vascularization in an orthotopic tracheal transplant model (15). Likewise, controlled release of DFO from reverse micelles improved closure of diabetic wounds in a mouse model of pressure-induced wounds (16).

In this study, we prepared polylactide-co-glycolide (PLGA) microparticles for the controlled release (over 3 weeks) of DFO in modular tissues constructs. Modular tissue engineering is a platform for creating vascularized tissues via the random assembly of sub-millimeter-sized collagen-based tissues containing EC and MSC (5, 6). We hypothesized that local HIF stabilization, through the controlled release of DFO, could drive vascularization even in the absence of a support cell like MSC. This, in turn, could lead to simplifying the modular tissue platform, which currently requires a minimum of two cell types, to a one-cell vascularization strategy.
4.2 Methods and Materials

4.2.1 Microparticle fabrication

Microparticles were fabricated using a procedure based on a previous study (17). DFO (Sigma) was dissolved in PBS (Gibco) at 50 mg/mL, and vortexed vigorously. A solution of 50:50 polylactide-co-glycolide (PLGA, 24,000-38,000 g/mol with ester end-capping, Sigma) in dichloromethane was prepared at 0.13 mg/mL without further purification. Aqueous DFO (50 µL) was added to the PLGA solution (450 µL), vortexed at maximum speed for 10 seconds, and homogenized for 15 seconds at 20% amplitude (Sonics VibraCell). The primary emulsion was added to 25 mL of 2.5% polyvinyl alcohol (PVA, Sigma) in 10% sodium chloride and homogenized for 60 seconds at 4000 rpm (Brinkmann Polytron PT 3000). The secondary emulsion was transferred to 75 mL of PVA (2.5%, 10% sodium chloride) and stirred at low speed for 4 hours at room temperature. Microparticles were collected by centrifugation (1600 rpm, 5 minutes), rinsed three times with distilled water (Gibco), and lyophilized overnight. All procedures were performed under sterile conditions in a laminar flow hood, using sterile techniques and equipment.

4.2.2 Microparticle characterization

4.2.2.1 Scanning electron microscopy

Microparticles were imaged using a scanning electron microscope (FEI XL30 ESEM at the Nanoscale Biomedical Imaging Facility, Mount Sinai Hospital, Toronto). Briefly, lyophilized microparticles were mounted onto a sample stub using double-coated carbon tape. Samples were sputter-coated with gold prior to imaging.

4.2.2.2 Size distribution

The size distribution of microparticles was determined using the Mastersizer 2000 (Malvern Instruments). Four microparticle preparations were pooled, since a minimum number of microparticles are required for size measurement. The size distribution and volume weighted average diameter were determined.
4.2.2.3 Controlled release profile

The controlled release profile of DFO-encapsulated PLGA microparticles was assessed as follows: two microparticle preparations were added to 10 mL PBS and incubated at 37°C on an orbital shaker. Supernatant was collected periodically (0.5 mL per sample) and replenished with fresh PBS. All samples were stored at -20°C until analysis. DFO content was assessed using high performance liquid chromatography-mass spectrometry (LC-MS; Applied Biosystems API 4000 at the Advanced Instrumentation for Molecular Structure, University of Toronto). The LC-MS method used was adapted from a previous study (16), using a mobile phase consisting of 40% methanol and 60% water with 0.1% formic acid, run isocratically at 200 µL/min and using a 10 µL injection volume. The liquid chromatography column used (Kinetex, 00D-4462-AN) had the following specifications: 2.6 µm, C18, 100 mm by 2.1 mm. A calibration curve (0-100 ng/mL DFO dissolved in PBS) was used to determine the DFO concentration in diluted samples.

The cumulative fraction released was plotted as a function of time. The data was fit to the model chosen using the Solver analysis tool in Microsoft Excel, and the unknown coefficients determined by minimizing the sum of squared error.

4.2.2.4 Encapsulation efficiency

The encapsulation efficiency of DFO was determined by dissolving two freshly prepared microparticle preparations in 50 mL dichloromethane and incubated for 1 hour. DFO was then extracted into PBS within a separation funnel using five volumes of 50 mL PBS, for a total aqueous volume of 250 mL. Aliquots were stored at -20°C prior to analysis. The concentration of DFO was determined using the LC-MS method described above. The encapsulation efficiency was determined by comparing the amount recovered with that used in preparing the microparticles.

4.2.3 In vitro DFO treatment of HUVEC

4.2.3.1 Effect of DFO on HIF target gene expression in HUVEC

HUVEC were seeded onto 6-well plates and cultured in fully supplemented EGM-2 (Lonza) with varying concentrations of DFO (0, 20, 50 and 100 µM). At 24 and 48 hours post-treatment, total
RNA was isolated using the Qiagen RNeasy Mini Kit. cDNA was synthesized using the Invitrogen SuperScript III First-Strand Synthesis Supermix. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assay gene expression, using the Bio-Rad Ssofast Evagreen PCR Reaction Mix.

The following primers were used: 5’- CAA CAT CAC CAT GCA GAT TAT GC -3’ (VEGF forward); 5’ - GCT TTC GTT TTT GCC CCT TTC -3’ (VEGF reverse); 5’- CAC CAC CTC ACT CCT GTT ACT T -3’ (GLUT-1 forward); 5’- CAA GCA TTT CAA AAC CAT GGT TCT A -3’ (GLUT-1 reverse); 5’ - AGG AAT TGA CGG AAG GGC AC - 3’ (18S forward); 5’ - GGA CAT CTA AGG GCA TCA CA - 3’ (18S reverse). Expression of target genes was normalized to 18S gene expression.

4.2.3.2 Effect of DFO on HUVEC Sprouting

HUVEC were seeded onto Cytodex beads and embedded within fibrin gel at a density of 200 beads/well in a 24-well plate. HUVEC-coated beads were cultured under two conditions. In normal serum experiments, HUVEC-coated beads were cultured in fully supplemented EGM-2 (Lonza) in the presence of 0 or 50 µM DFO. In reduced serum experiments, HUVEC-coated beads were first cultured in reduced serum DMEM (1% FBS) for 2 days, and returned to fully supplemented EGM-2 at day 2. Beads were imaged (Zeiss Axiovert 135) every day to day 5, and the number and length of sprouts quantified using ImageJ software.

4.2.4 Module fabrication

Collagen modules were fabricated using an air-jet system, wherein a channel of collagen solution (PureCol, type 1 bovine collagen) intersects a channel of pressurized air at an orthogonal junction (18). The resulting collagen modules were collected in polyethylene tubing and gelled at 37°C. Modules were coated with human umbilical vein endothelial cells (HUVEC, Lonza) at a density of 1.5×10⁶ per implant in 10 mL complete media on an orbital shaker for one hour. Unlike previous studies (6, 19), mesenchymal stromal cells were not used. HUVEC were used at passages 3-6 and cultured with complete endothelial growth media (Lonza, EGM-2). HUVEC-coated modules were cultured over four days, with media changed after two days, prior to implantation.
4.2.5 Animal surgeries

Severe combined immunodeficient mice with the beige mutation (SCID-bg, Charles River) were operated on at 6-7 weeks of age. All procedures were performed using sterile techniques at the University of Toronto, in accordance with ethical guidelines of the Faculty of Medicine Animal Care Committee. Mice were anaesthetized by isofluorane inhalation, and hair on the dorsum removed with hair removal cream (Nair).

Modules were rinsed three times with PBS (Gibco) and delivered subcutaneously using an 18-gauge needle, as before (6, 19). HUVEC-coated modules were delivered in 0.4 mL PBS with DFO-loaded microparticles or empty microparticles (control). In other groups, modules were delivered with 50 µM DFO in PBS (free DFO) or PBS without microparticles (untreated control). An n=5 was used per experimental group and time point.

4.2.6 Immunohistochemistry

Modular tissues were explanted at day 3, 7, and 21 post-implantation, and fixed in 10% neutral buffered formalin for at least 24 hours prior to immunohistochemical processing. Briefly, samples were embedded in paraffin wax, dewaxed with xylene and rehydrated with graded alcohol. Sections 4 µm thick were stained with the following antibodies: anti-CD31 (Santa Cruz Biotechnology Inc. SC-1506-R, 1:2000, rabbit polyclonal antibody), biotinylated Ulex Europaeus Agglutinin I (UEA1, Vector Laboratories B1065, 1:400; stains human EC only), anti-α Smooth Muscle Actin (SMA, Sigma-Aldrich A5228, 1:200, mouse monoclonal antibody), anti-HIF1α (Novus Biologicals NB100-479, 1:2000, rabbit polyclonal antibody), and anti-HIF2α (Novus Biologicals NB100-122, 1:1000, rabbit polyclonal antibody).

4.2.7 Vascular perfusion

In a separate cohort, mice were implanted with HUVEC-coated modules along with empty or DFO-loaded microoparticles (n=3 per experimental group and time point). At day 7 and 21, mice were given intravenous injections of 0.25 mL of 25 mg/mL Texas Red dextran (ThermoFisher,
lysine fixable and 70,000 MW) half an hour prior to sacrifice. Tissues were embedded in OCT compound (Tissue-Tek), snap frozen and stained with CD31 (BD Pharmingen 550274, 1:100) with secondary antibody (Invitrogen Life Technologies A11006, 1:100, goat anti-rat), and DAPI (Roche 10-236276-001, 1:100). Immunofluorescent tissue sections were scanned using the AxioScan Z.1 (Carl Zeiss) at the Advanced Optical Microscopy Facility (University Health Network). The densities of Texas Red dextran-containing vessels were quantified and images captured using the Zen LE (Blue) Lite software.

4.2.8 Modular tissue digestion

Tissues were homogenized in 500 µL of digestion buffer (100 µL 450 U/mL Collagenase I, 100 µL 125 U/mL Collagenase XI, 100 µL 60U/mL DNase I, 100 µL 60 U/mL Hyaluronidase, 100 µL 1.0 M HEPES buffer and 4.5 mL HBSS) using the OctoMACS dissociator (Miltenyi) as before (20). Samples were incubated at 37°C for 1 hour on a shaker plate, and homogenized again using the OctoMACS dissociator. The lysate was passed through a 40 µm filter to obtain a single cell suspension suitable for analysis by flow cytometry.

4.2.9 Flow cytometry

Single cell suspensions were incubated with Fc block (BD Pharmingen, rat anti-mouse CD32/16) and stained with a panel of antibodies (Supplementary Table S1). Samples were processed on ice with minimal light exposure. Fluorescence-minus-one (FMO) controls were prepared to define gate boundaries. All samples were diluted in 0.2 mL FACS buffer (3 g EDTA, 2 g BSA in 1 L PBS) and assayed on a BD Fortessa X20 flow cytometer (Faculty of Medicine Flow Cytometry Facility, University of Toronto) at low speed. Single-stained compensation controls were prepared using compensation beads (BD Biosciences), and all analyses conducted in FlowJo version 10.

The gating scheme is summarized in Figure (Supplementary Figure S1), and adapted from previous work (21, 22). Cellular debris was first excluded, singlets isolated, and live cells isolated. The resulting cells were separated into CD45+ leukocytes and CD45- cells. EPC were
gated from CD45⁻ cells, based on triple-positive staining of CD34, CD117 and VEGFR2. Macrophages were identified as CD45⁺CD11b⁻Ly6G⁻F4/80⁺ cells, and further segregated into M2-polarized (CD206⁺MHCII⁻) and M1-polarized (MHCII⁺CD206⁻) populations.

4.2.10 Statistical analyses

Statistical analyses were performed using two-way ANOVA (JMP Pro 13), and significant interactions tested using the Tukey Honest Significant Difference test. With each statistical analysis, the assumption of normally distributed residuals was tested using the Shapiro-Wilk W goodness-of-fit test. In cases where the assumption was not met, the data was transformed using logarithmic, square root or cube root functions (tested in that order), so that the distribution of residuals was normal. A summary of the data transformations applied to each data set, and the statistically significant pairwise comparisons are given in Supplementary Table S2. Results are presented as the average ± standard error of the mean. Differences were statistically significant at p<0.05.
4.3 Results

4.3.1 Characterization of DFO-releasing microparticles

DFO-loaded PLGA microparticles were spherical, with a volume weighted mean diameter of 48 μm (Figure 1). The cumulative release of DFO from PLGA microparticles was characterized using HPLC-MS, and modelled according to the following equation (23):

\[ \frac{M_t}{M_\infty} = k t^n \]

The exponential relationship describes the Fickian and non-Fickian release of drug from a polymeric release device, where \( \frac{M_t}{M_\infty} \) is the cumulative fraction of solute released, \( t \) is time, \( k \) is a constant (here, \( k=0.10 \)), and \( n \) is a diffusional exponent (here, \( n=0.34 \)). \( M_\infty \) is the total encapsulated DFO amount, which was determined experimentally to be 0.5 mg per implant preparation, with a corresponding encapsulation efficiency of 82%. Following rapid release of the drug within the first 100 hours, the release rate was relatively constant between 100-500 hours and estimated to be approximately 0.45 μg/hour per implant according to the model used.
Figure 1. In vitro characterization of DFO-loaded PLGA microparticles. (A) Representative scanning electron microscopy images of empty and DFO-loaded PLGA microparticles (scale bar is 20 µm). (B) The single emulsion fabrication process yielded microparticles with a volume weighted mean average diameter of 48 µm. (C) The controlled release profile of DFO-loaded microparticles was determined by HPLC-MS, and modelled using a power function (curve fit evaluated in Supplementary Figure S2). The release profile is presented as the fraction of solute release \( \frac{M_t}{M_\infty} \) as a function of time.

4.3.2 DFO microparticles increased vascularization and vessel perfusion in modular tissues

Co-delivery of DFO-releasing microparticles with HUVEC-coated modules enhanced total (CD31\(^+\)) vessel formation at day 7 (not statistically significant) and day 21 (Figure 2, \( p<0.01 \)), relative to all control groups. However, even so, vessel numbers were low relative to previous studies with MSC co-delivery (19, 24). Of note, prolonged DFO release from microparticles was beneficial to vascularization, as subcutaneous delivery of modules in 50 µM DFO solution (free DFO control) did not yield similar levels of vascularization. Enhanced vascularization coincided with some increase in graft-derived (UEA1\(^+\)) vessel density at day 21 (\( p<0.05 \) relative to untreated controls) as shown in histology images of Figure 2. No differences in UEA1\(^+\) vessel density were observed among groups at other time points.
Figure 2. DFO-releasing microparticles enhanced vascularization of EC-only modular tissue constructs. (A) Implantation of DFO-releasing microparticles increased total (CD31+) vessel density at day 7 (although not statistically significant) and at day 21, relative to all other groups. (B) DFO also increased graft-derived vascularization at day 21, relative to untreated controls. (C) Representative histology images of tissue sections stained with CD31 and UEA-1 at day 7 and 21 are shown (scale bar is 200 µm). The CD31+ vessel density data set was transformed using the square root function, and the UEA-1+ vessel density data set was transformed using the cube root function so that residuals were normally distributed for statistical analysis using two-way ANOVA. Only pairwise comparisons between experimental groups within a time point are shown; all other statistically significant differences are listed in Table S2. n=5; error bars are standard error of the mean; *p<0.05 and **p<0.01.

Additionally, DFO microparticles increased the density of SMA+ vessels relative to empty microparticles at day 21, although differences were not statistically significant (Figure 3). Vessels were also perfusable, with Texas Red dextran present within many CD31-lined structures, which was not otherwise observed with control microparticles at day 21. At day 7,
however, vessels were mostly non-perfused in both groups. Vessel structures were non-perfusable (absence of Texas Red dextran) in controls (untreated and free DFO) at all time points studied (Supplementary Figure S3).

Figure 3. DFO-releasing microparticles enhanced maturation and perfusion of blood vessels in EC-only modular tissue constructs. (A) Implantation of DFO-releasing microparticles increased the density of α-SMA⁺ vessels at day 21, relative to empty microparticles (although not statistically significant). (B) Representative histology images at day 21 with staining for α-SMA are shown for empty and DFO-releasing microparticle groups. (C) Representative immunofluorescent images of modular tissue constructs show that few vessels are perfusable (contain intravenously delivered Texas Red dextran) at day 7, but that there are more perfusable vessels in tissues with DFO-releasing microparticles at day 21 compared to empty microparticles (scale bar is 50 μm). Large images show merged channels, with individual stains.
on the right (DAPI: top, CD31: middle, Texas Red dextran: bottom). n=5; error bars are standard error of the mean.

4.3.3 DFO microparticles increased and prolonged HIF activation in modular tissues

Controlled release of DFO increased HIF-1α+ nuclei density within implants relative to empty microparticles at day 7 and 21 (Figure 4, p<0.01). Increased HIF-2α+ nuclei density was also observed at day 21, although the difference relative to the control was not statistically significant. Findings were consistent with the DFO release profile defined in vitro, which was characterized by steady drug release after the first four days.

**Figure 4.** DFO-releasing microparticles increased HIF-1 and HIF-2 activation in EC-only modular tissue constructs. (A) Implantation of DFO-releasing microparticles increased HIF-1α+ nuclei density within implants relative to empty microparticles at both day 7 and 21. (B) An increase in HIF-2α+ nuclei density was also observed at day 21, although the difference relative to control microparticles was not statistically significant. (C) Representative histology images of
tissue sections stained with HIF-1α and HIF-2α at day 7 and 21 are shown (scale bar is 200 µm). The HIF-2α+ vessel density data set was transformed using the square root function, so that residuals were normally distributed for statistical analysis using two-way ANOVA. Only pairwise comparisons between experimental groups within a time point are shown; all other statistically significant differences are listed in Table S2. n=5; error bars are standard error of the mean; *p<0.05 and **p<0.01.

4.3.4 DFO microparticles augmented early recruitment of neutrophils and retention of M2-polarized macrophages to modular implants

Controlled release of DFO increased the proportion of recruited neutrophils at day 3, compared to empty microparticles (Figure 5, p<0.05). Neutrophil numbers were not significantly different between groups (Supplementary Figure S4D). Moreover, controlled release of DFO increased recruitment of M2-polarized (CD206+MHCII-) macrophages to the modular tissue implant at day 7, relative to empty microparticles (Figure 5B; p<0.05). This was reflected in the corresponding ratio of M2-to-M1 macrophages in the tissue, although the difference in ratios was not statistically significant. No difference in M1-polarized (MHCII+CD206-) macrophage recruitment was observed between DFO-loaded and empty microparticles at all time points studied (Figure 5C). Unfortunately, the number of endothelial progenitor cells recruited to module implants containing empty or DFO-loaded microparticles was too low to be detected (data not shown).

Interestingly, neutrophil recruitment was significantly less in untreated EC-only modules at days 3 and 7 (Supplementary Figure S5) than in empty and DFO-loaded microparticle groups. The recruitment of M1-polarized (MHCII+CD206-) macrophages was also less in untreated EC-only modules compared to both microparticle groups. As with microparticle groups, too few EPC were recruited to the implant to be detected (data not shown).
Figure 5. DFO-releasing microparticles increased neutrophil and M2-polarized macrophage recruitment to EC-only modular tissue constructs. Flow cytometry analyses showed that implantation of DFO-releasing microparticles increased (A) the proportion of neutrophils at day 3 relative to empty microparticles, and (B) recruitment of M2-polarized (CD206^+MHCII^-) macrophages at day 7 to the modular tissue construct. Effects on (C) M1-polarized (MHCII^+CD206^-) macrophages and (D) M2-M1 ratio were not statistically significant between treatments at all time points investigated. (E) Representative dot plots and (F) histograms for polarized macrophages are shown. The M2 macrophage number data set was transformed using the cube root function, the M1 and CD206^+MHCII^+ macrophage data sets were transformed logarithmically, and the M2/M1 data set transformed using the square root function so that residuals were normally distributed for statistical analysis using two-way ANOVA. Only pairwise comparisons between experimental groups within a time point are
shown; all other statistically significant differences are listed in Table S2. n=5; error bars are standard error of the mean; *p<0.05

4.4 Supplementary Materials

4.4.1 Supplementary Figures

Figure 4-S1. Graph of logarithm of cumulative fraction released versus logarithm of time.
Figure 4-S2. Schematic diagram summarizing the gating scheme used in flow cytometry analyses. Cellular debris was first excluded using the FSC-A vs. SSC-A plot, singlets isolated using the FSC-A vs. FSC-H plot, and live cells selected by exclusion of the live-dead stain. Live cells were subsequently segregated into CD45+ and CD45− populations. EPC were defined from the CD45− population as CD34+VEGFR2+CD117+ cells. Neutrophils were defined from the CD45+ population, expressing CD11b+Ly6G+. Macrophages were also identified from the CD45+ population as Ly6G−CD11b+F4/80+. Further discrimination of macrophages was applied to define M1-polarized macrophages (MHCII+CD206−) and M2-polarized macrophages (MHCII−CD206+). All gate boundaries were defined using fluorescence-minus-one (FMO) controls.
Figure 4-S3. Vessel structures in untreated and free DFO controls were poorly perfused. Representative immunofluorescent images of modular tissue constructs show that vessel structures are poorly assembled and non-perfusable (absence of intravenously delivered Texas Red dextran) at day 7 and 21 in controls untreated or DFO-treated without microparticles (scale bar is 50 µm). Large images show merged channels, with individual stains on the right (DAPI: top, CD31: middle, Texas Red dextran: bottom).
Figure 4-S4. Number and proportion of cells recruited to EC-only modular tissue constructs with empty or DFO microparticles. Number of CD45^+ leukocytes (A), CD45^+CD11b^+Ly6G^-F4/80^- macrophages (B), and MHCII^+CD206^- macrophages (C) and CD45^+CD11b^+Ly6G^- neutrophils (D) are shown normalized to implant mass. The proportion of leukocytes (E) and macrophages (F) are shown, as a percentage of live cells. The proportion of M2-polarized MHCII^+CD206^- macrophages (G), M1-polarized MHCII^+CD206^- macrophages (H), and MHCII^+CD206^- macrophages (I) are shown, as a percentage of total macrophage number. Data sets were transformed using the logarithmic function (leukocyte number, leukocyte proportion, neutrophil number, CD206^+MHCII^+ macrophage proportion), and square root function (macrophage number) so that residuals were normally distributed for statistical analysis using two-way ANOVA. Only pairwise comparisons between experimental groups within a time
point are shown; all other statistically significant differences are listed in Table S2. n=5; error bars are standard error of the mean; *p<0.05 and **p<0.01.
Figure 4-S5. Number of cells recruited to untreated EC-only modular tissue constructs. The number of CD45⁺ leukocytes (A), CD45⁺CD11b⁺Ly6G⁻F4/80⁺ macrophages (B), CD45⁺CD11b⁺Ly6G⁺ neutrophils (C), M1-polarized MHCII⁺CD206⁻ macrophages (D), M2-polarized MHCII⁺CD206⁺ macrophages (E), and MHCII⁺CD206⁺ macrophages (F) in untreated implants are shown at day 3 and 7, normalized to implant mass. n=5; error bars are standard error of the mean.
Figure 4-S6. *In vitro* effects of DFO treatment on HUVEC gene expression and sprouting. DFO increased HUVEC expression of HIF-1 target genes, GLUT-1 (A) and VEGF-A (B) in a dose-dependent manner (0-50 μM) following 24-hour treatment. HUVEC-coated Cytodex beads were used to assess sprouting *in vitro* under normal serum conditions (10% fetal bovine serum), with corresponding changes in sprouts per bead (C) and sprout lengths (D) shown. In another set of experiments, HUVEC-coated beads were exposed to reduced serum conditions (1% fetal bovine serum) for two days, prior to re-exposure to normal serum conditions for the remaining period. Corresponding measurements of sprouts per bead (E) and length of sprouts (F) are shown. n=5; error bars are standard errors of the mean; #p<0.05, ##p<0.01.
4.4.2 Supplementary Tables

Table 4-S1. Summary of antibodies used for flow cytometry analyses of digested modular implants. The table includes the fluorophore conjugate, the excitation laser, detection wavelength and sourcing for each antibody.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugate</th>
<th>Excitation</th>
<th>Detection (nm)</th>
<th>Brand and Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live/dead</td>
<td>Fixable near-IR</td>
<td>Red</td>
<td>780</td>
<td>ThermoFisher (L34975)</td>
</tr>
<tr>
<td>CD11b</td>
<td>PE-CF594</td>
<td>Yellow-green</td>
<td>612</td>
<td>BD Bioscience (562287)</td>
</tr>
<tr>
<td>CD206</td>
<td>PE-Cy7</td>
<td></td>
<td>785</td>
<td>eBioscience (25-2061-80)</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>PE</td>
<td></td>
<td>578</td>
<td>BD Bioscience (555308)</td>
</tr>
<tr>
<td>MHCII</td>
<td>PerCP-e710</td>
<td>Blue</td>
<td>710</td>
<td>eBioscience (46-5321-80)</td>
</tr>
<tr>
<td>CD34</td>
<td>FITC</td>
<td></td>
<td>520</td>
<td>BD Bioscience (553733)</td>
</tr>
<tr>
<td>CD45</td>
<td>BV 711</td>
<td>Violet</td>
<td>711</td>
<td>Biolegend (103147)</td>
</tr>
<tr>
<td>Ly6G</td>
<td>V450</td>
<td></td>
<td>448</td>
<td>BD Bioscience (560603)</td>
</tr>
<tr>
<td>CD117</td>
<td>BV605</td>
<td></td>
<td>602</td>
<td>BD Bioscience (563146)</td>
</tr>
<tr>
<td>F4/80</td>
<td>BUV395</td>
<td>Ultra-violet</td>
<td>395</td>
<td>BD Bioscience (565614)</td>
</tr>
</tbody>
</table>
**Table 4-S2. Summary of results of statistical analyses.** This table summarizes the transformations applied to each data set, such that the residuals were normally distributed for statistical analysis by two-way ANOVA. The assumption of normally distributed residuals was tested using the Shapiro-Wilk W goodness-of-fit test. In cases where the assumption was not met in the untransformed data, the data was transformed using the logarithmic, square root or cube root functions. All statistically significant pairwise differences at p<0.01 and p<0.05 are listed. Notation used: untreated control (un), free DFO control (fd), empty microparticle (c) and DFO-loaded microparticle (d); the number following each experimental group designation refers the time point of interest (eg. un3 is untreated control day 3). Significant differences between experimental groups within a time point are highlighted in bold.

<table>
<thead>
<tr>
<th>Data set</th>
<th>Transformation</th>
<th>p&lt;0.01</th>
<th>p&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31+ vessel density</td>
<td>x^12</td>
<td>c3 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. d21</td>
<td>d7 vs. un3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c21 vs. d21</td>
<td>d21 vs. d3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d3 vs. d7</td>
<td>d7 vs. un3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d7 vs. d21</td>
<td>d21 vs. un3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c21 vs. un3</td>
<td>un3 vs. un7</td>
</tr>
<tr>
<td>UEA1+ vessel density</td>
<td>x^12</td>
<td>c3 vs. d7</td>
<td>d7 vs. un3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. d21</td>
<td>d21 vs. d3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c21 vs. d21</td>
<td>d21 vs. un3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. d21</td>
<td>d21 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c21 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c21 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td>HIF-1α+ nuclear density</td>
<td>-</td>
<td>c3 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. d21</td>
<td>d21 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c21 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c21 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td>HIF-2α+ nuclear density</td>
<td>x^12</td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. d7</td>
<td>c7 vs. d21</td>
</tr>
<tr>
<td>Leukocyte number</td>
<td>log(x)</td>
<td>c7 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d21</td>
</tr>
<tr>
<td>%Leukocyte</td>
<td>log(x)</td>
<td>c7 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td>Neutrophil number</td>
<td>log(x)</td>
<td>c7 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td>%Neutrophil</td>
<td>-</td>
<td>c7 vs. d7</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c7</td>
<td>c7 vs. d7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Data set</th>
<th>Transformation</th>
<th>p&lt;0.01</th>
<th>p&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage number</td>
<td>x^12</td>
<td>c1 vs. c3</td>
<td>c3 vs. c21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>d7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. c21</td>
<td>d7 vs. d7</td>
</tr>
<tr>
<td>%Macrophage</td>
<td>-</td>
<td>c1 vs. c3</td>
<td>c3 vs. c21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. c21</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td>M2 Macrophage number</td>
<td>x^12</td>
<td>c1 vs. c3</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. c21</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td>%M2 Macrophage</td>
<td>-</td>
<td>c1 vs. c3</td>
<td>c3 vs. c21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. c21</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td>M1 Macrophage number</td>
<td>log(x)</td>
<td>c1 vs. c21</td>
<td>c1 vs. c7</td>
</tr>
<tr>
<td>%M1 Macrophage</td>
<td>-</td>
<td>c1 vs. c7</td>
<td>c7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. c21</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td>%MHCIIC°/CD206° Macrophage</td>
<td>log(x)</td>
<td>c3 vs. c21</td>
<td>d1 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c7 vs. c21</td>
<td>d7 vs. d21</td>
</tr>
<tr>
<td>%MHCIIC°/CD206°</td>
<td>-</td>
<td>c3 vs. c21</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td>M2/M1 ratio</td>
<td>x^12</td>
<td>c1 vs. c21</td>
<td>c21 vs. c7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c7 vs. d7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c3 vs. c21</td>
<td>c7 vs. d7</td>
</tr>
</tbody>
</table>
4.5 Discussion

4.5.1 DFO microparticles partly rescued vascularization of modular tissues in the absence of MSC

Controlled release DFO microparticles were incorporated into modular tissues, on the premise that prolonged HIF stabilization would replace the role of MSC in driving vascularization. We and others have shown that implantation of EC alone does not induce robust vascularization, as EC apoptose in the early stages following implantation (1, 2). Here, we observed increased vessel density and perfusion in modular implants containing DFO-releasing microparticles, demonstrating that HIF stabilization partly, but not entirely, rescued vascularization in the absence of MSC.

Nonetheless, vessel densities achieved with co-delivery of DFO-releasing microparticles were lower than that of constructs containing 1.5×10^6 adMSC at day 7 (25 CD31^+ vessels/mm^2, (19)) and at day 21 (approximately 30 CD31^+ vessels/mm^2, (24)). This is not surprising, since implanted adMSC can produce an overwhelming quantity of angiogenic factors that promote EC survival, assembly into vessels, and that modulate the immune response (25, 26). Some MSC-secreted factors may not be regulated by HIF, such as neutrophil activating protein-2. Moreover, MSC can physically integrate into the vasculature (3, 4), taking on a pericyte-like role to promote angiogenesis through direct contact with EC (reviewed in (27)). These are two mechanisms by which MSC support vascularization, and which likely cannot be replicated by local HIF stabilization alone.

When used with conventional modular tissues containing both EC and adMSC (1.5×10^6 adMSC/implant), DFO releasing microparticles did not further increase vessel densities at day 7 or 14 (data not shown). The effect of DFO on HIF stabilization may be overwhelmed by the effect of adMSC. It is possible that DFO releasing microparticles would have an effect on modular implants containing a reduced dose of adMSC, although this was not explored in the present study. Ultimately, HIF stabilization by DFO microparticles could not enhance the rate, degree or maturity of vessels in modular tissues above that normally seen in the EC and MSC-containing platform. This suggests that HIF stabilization by DFO in the early stages of vascularization, during which there is low oxygen tension within the tissue, does not enhance vessel formation in the presence of MSC. However, HIF stabilization in presumably normoxic
tissues prolonged vascularization, as seen with the persistence of vessels in EC-only constructs at day 21.

4.5.2 DFO microparticles coordinated an angiogenic response from various cells in the subcutaneous space

Partial rescue of modular tissue vascularization may be attributed to effects of HIF activation on various cell types within the subcutaneous space. Foremost, HIF activation in EC enhances their angiogenic potential. Others showed that overexpression of a constitutively active HIF-1α mutant in EC induced expression of angiogenic growth factors (VEGF, IGFBP3, PDGFB, PGF, ANGPTL4), and accelerated tube formation in Matrigel (28). DFO treatment also increased endothelial expression of phosphorylated endothelial nitric oxide synthase (eNOS), enhanced EC proliferation in a mouse orthotopic tracheal transplant model (15) and vascularization in a mouse ischemic hindlimb model (29).

In our hands, DFO (50 µM) induced HUVEC expression of HIF-1 targets VEGF-A and GLUT-1 (Supplementary Figure S6A, B). DFO also increased initial sprouting of HUVEC in normal serum conditions (Supplementary Figure S6C, p<0.05 at day 1). This is similar to others’ findings, which showed DFO-treated EC increased network formation in an in vitro Matrigel assay after one day (29). We also found that DFO increased the length of sprouts upon re-exposure to normal serum after two days’ culture in reduced serum conditions (Supplementary Figure S6F, p<0.05 at day 3). However, there were no marked effects on sprouting at longer time points. The in vitro sprouting model oversimplifies the in vivo context, since mural cells (not included in vitro) in subcutaneous tissue can enhance vascularization as a result of HIF stabilization. Indeed, we observed some persistence of graft-derived vessels at day 21 in vivo with co-delivery of DFO-releasing microparticles. However, except when compared to untreated controls, these differences were not statistically significant. Thus, local HIF stabilization predominantly contributed to increased ingrowth of host vessels into modular tissues and, to a lesser extent, increased retention of graft-derived vessels.

Local HIF stabilization can also coordinate an angiogenic response from various cells in the subcutaneous space. Knockdown of HIF-1α in fibroblasts reduced vascularization of ischemic
skin flaps and subcutaneous wounds in a transgenic mouse model (30). Its deletion in smooth muscle cells reduced thickening of murine aortal wall even upon induction with angiopoietin II (31). Others showed, using a transgenic mouse model with inducible keratinocyte activation of HIF-1 without concomitant VEGF production, that vascularization was impaired (31). Without VEGF production by keratinocytes, neovascularization was not enhanced, even though other angiogenic downstream targets of HIF-1 were upregulated. Taken together, we suggest that HIF stabilization in the subcutaneous space can induce an angiogenic response from a variety of cells including fibroblasts, keratinocytes and smooth muscle cells, not just graft-associated HUVEC.

4.5.3 DFO release amplified the recruitment of inflammatory and anti-inflammatory cells in a temporal manner

DFO release also increased the retention of neutrophils at day 3, presumably due to HIF stabilization in these cells. Using transgenic mice, others showed that HIF-1 and HIF-2 activation increased neutrophil longevity at sites of inflammation and hypoxia (32, 33). HIF-1α deletion in myeloid cells also impaired glycolysis, reduced their ATP pool, cell motility and invasion (34), pointing to mechanisms by which HIF activation in neutrophils could prolong their retention in modular implants. Once recruited, neutrophils can initiate vascularization by secreting VEGF (34) and Bv-8 (35).

At the same time, controlled release of DFO increased M2-polarized macrophages in implants at day 7, which could be attributed to HIF-1 -dependent upregulation of SDF-1 (36). Indeed, polarization of macrophages toward an alternatively activated state is associated with vascular remodeling and maturation, through secreting VEGF, bFGF, IL-8 and MMP-9 in a HIF-1 -dependent manner (37). To our surprise, a large number of M2-polarized macrophages was present in untreated implants at day 3 (Supplementary Figure S5), but the number plummeted by day 7. This suggests that one of the features of DFO releasing microparticles is their ability to retain M2-polarized macrophages at day 7, which is likely important to subsequent vascularization.

Implantation of PLGA microparticles also increased recruitment of M1-polarized macrophages at day 3 and 7, compared to untreated controls (Supplementary Figure S5). However, effects
were not specific to DFO delivery, as differences were not observed between empty and DFO-loaded microparticle groups. Increased M1-polarized macrophage presence may be attributed to the effects of PLGA, as cell contact with PLGA can induce an inflammatory profile in macrophages (38) and monocyte-derived dendritic cells (39, 40), in a manner partially dependent upon NF-κB signaling.

Taken together, DFO-induced vascularization in modular constructs is characterized by early neutrophil recruitment, followed by polarization of macrophages towards an alternatively activated state. Vascularization is not only dependent on the number and cell types recruited, but is temporally controlled, requiring an initial inflammatory phase and a later anti-inflammatory phase. This is also reflected in the sequential polarization of M1 and M2 macrophages (41). Controlled release of DFO amplified both phases relative to controls. Increased neutrophil recruitment at day 3 was likely mediated by downstream targets of HIF, SDF-1 (36, 42) and PDK1 (43). Macrophage polarization at day 7 has been shown to be regulated by HIF-1α/HIF-2α (44, 45). Since DFO stabilizes both HIF-1α and HIF-2α subunits by chelating iron, its effects likely coordinated with a plethora of microenvironmental cues, including nitric oxide production by neutrophils and M1 macrophages, which can polarize macrophages towards an alternatively activated state (reviewed in (46)).

The near absence of EPC (CD45−CD34+VEGFR2+CD117+) in EC-only modular implants at all time points studied was unexpected. We hypothesized that DFO release would increase EPC recruitment, since previous work (unpublished) showed their distinct presence in MSC-containing modular implants. Instead, the number of EPC within EC-coated module groups was below the detection limit, suggesting that MSC-secreted factors may be important for their recruitment. Indeed, others demonstrated that application of MSC-conditioned medium to full excisional wounds promoted healing and EPC recruitment (47).
4.6 Conclusion

We demonstrated that controlled release of DFO from microparticles partly rescued vascularization of modular tissues in the absence of MSC. Our initial objective of simplifying modular engineered tissues to a one-cell system could not be achieved with DFO-releasing microparticles alone. However, flow cytometry studies pointed to potential strategies to further tune and drive vascularization in modular tissue constructs.

Local HIF stabilization prolonged the retention of neutrophils and alternatively activated macrophages within implants. Not only so, an intriguing observation was the near absence of EPC in EC-containing modular tissue constructs, pointing to their recruitment as a key element of MSC-driven vascularization. Going forward, systemic and/or local delivery methods that augment recruitment of these cell types in a temporally controlled manner may drive rapid and mature vascularization. Previously, when EPC (also termed early outgrowth EC) were incorporated into modular tissues, we observed their efflux from constructs and little induction of vascularization, although renal function was enhanced in a rat model of chronic kidney disease (48). In future studies, it would be valuable to investigate whether local stabilization of HIF from DFO releasing microparticles could increase retention of implanted EPC and thereby drive vessel formation.

Not surprisingly, local stabilization of HIF from DFO releasing microparticles could not entirely replace the function of MSC in modular tissue constructs. Nonetheless, the incorporation of controlled release microparticles in modular tissue constructs provides a platform to study and tune vessel formation. It can ultimately be used to design vascularized tissue constructs that enhance engraftment of therapeutic cells.
4.7 References


Chapter 5

5 Discussion and Future Directions

5.1 Significance

In the work presented herein, we identified HIF as a key driver of vascularization in modular tissue constructs, as both systemic and graft EC-specific HIF inhibition impaired vessel formation and tissue oxygenation. However, local HIF stabilization alone, as achieved with incorporation of controlled release DFO microparticles, was insufficient for fully rescuing vascularization of EC-only modular tissues.

The role of HIF has been investigated in wound healing and in the dysregulated context of tumor growth. However, to our knowledge, inhibitory methods have not been employed to specify its effects in the context of vascularizing engineered tissue constructs. The results of our inhibition experiments were consistent with findings of studies investigating the effects of HIF inhibition on tumor progression (1–3). In these studies, systemic delivery of pharmacological HIF inhibitors or genetic HIF inhibition in tumor cells reduced tumor growth in mice; these effects were partly attributed to impaired tumor vascularization. Moreover, findings related to HIF-mediated recruitment of host-derived angiogenic cells, including EPC, neutrophils and alternatively activated macrophages, were also in line with the existing literature.

Having specified the role of HIF in driving module-induced vascularization, we sought to harness it to enhance mature vascularization in the absence of MSC. Unfortunately, DFO releasing microparticles rescued vascularization to some extent, but not to a degree comparable with implants containing MSC. DFO-releasing microparticles could not entirely replace MSC in module-induced vascularization, which may be related to effects of MSC-EC contact, as discussed earlier (Chapter 4). Therefore, there appear to be HIF-independent mechanisms that govern vascularization of MSC and EC-containing modular tissues. For instance, co-culture of MSC and EC increased the expression of extracellular matrix proteins, including collagen IV, fibronectin, and perlecan I, relative to the culture of either cell types alone (4). Adhesion of EC to extracellular matrix proteins, such as fibronectin, was shown to enhance EC survival via
interaction with α5β1 integrin (5), and may be related to MSC-EC specific effects. Interestingly, Merfeld-Clauss et al. showed that co-culture of EC and adMSC yielded robust vascular network formation in an in vitro culture model, and was superior to co-culture of EC with dermal fibroblasts or smooth muscle cells (4). Although the addition of conditioned medium from EC-adMSC co-cultures increased vascular network formation of EC cultured with dermal fibroblasts or smooth muscle cells, it was not to the same degree as that seen with EC co-culture with adMSC. This intriguing comparison suggests that there are angiogenesis-inducing mechanisms that are unique to direct contact between EC and MSC. These remain to be elucidated in order to fully rescue vascularization of EC-only tissue constructs.

At first glance, our findings appear inconsistent with others’ more promising results. HIF stabilization by DFO delivery improved the perfusion of tracheal transplants (6), enhanced healing and even prevented formation of pressure-induced ulcers in diabetic mice (7). Furthermore, release of a HIF stabilizing pharmacologic also improved healing and vascularization of tissue in a murine model of ear hole injury (8). It should be emphasized that these studies were undertaken in models of significant tissue injury and inflammation. This is unlike modular tissue implantation, which induces minimal tissue damage via delivery with syringe and needle. It is possible that damage-related responses synergized with local HIF stabilization strategies to enhance healing and vascularization. It is also conceivable that, applied to models of tissue injury or damage, modular tissues incorporating controlled release DFO microparticles could induce a therapeutically significant effect on vascularization and tissue regeneration.

Taken together, this work confirms the importance of HIF in driving vascularization of modular engineered tissue. Harnessing HIF, via local and prolonged stabilization, resulted in some rescue of vascularization in EC-only constructs. In the end, HIF stabilization by DFO could not enhance the rate, degree or maturity of vessels in modular tissues beyond that normally observed in the EC and MSC-containing platform. This is likely due to low oxygen tension already present in the implant during the early stage of vascularization; HIF stabilization during this time does not enhance vessel formation in the presence of MSC. However, its stabilization in presumably normoxic tissue increased the persistence of vascular structures, even without MSC, as observed with increased vascularization in EC-only modules at day 21. Results also suggest that other mechanisms are also crucial to vascularization of MSC and EC-containing modular tissues, and
remain to be elucidated. Moving forward, the combination of controlled release microparticles (of molecules modulating other rate limiting pathways) and engineered tissue constructs can serve as a platform to study other potential mechanisms. This understanding can, in turn, be applied to devise strategies that enhance the degree, rate and maturity of blood vessels generated.

5.2 Limitations and Future Work

The conclusions discussed above were drawn based on experiments that were designed to address the hypotheses outlined (Section 1.1). Inevitably, there are limitations inherent in the methods and experimental designs chosen, which I discuss in this section. These will be explored according to each specific aim. Unexplored topics of interest will also be highlighted, and opportunities for future work presented.

In the first pillar of my doctoral thesis, the role of HIF in driving vascularization of modular tissues was probed using inhibitory methods. Conclusions made from these studies were therefore affected by limitations of the techniques chosen. Digoxin, used to study the effects of systemic HIF inhibition, is associated with off-target effects inherent in the use of any pharmacologic. As a cardiac glycoside that has been used clinically for the treatment of cardiac arrhythmia, digoxin inhibits sodium-potassium ATPase in addition to its known effects on HIF inhibition. In vitro treatment of tumor cells with digoxin caused cell cycle arrest, and induced apoptosis of human EC. Such effects are partially related to HIF inhibition, since forced expression of constitutively active HIF-1α in tumor cells rescued proliferation to some extent. Nonetheless, the implications of sodium-potassium ATPase inhibition must be acknowledged. Because sodium-potassium ATPase maintains the cell membrane potential and ion concentration gradient, via transporting sodium ions out of the cell and potassium ions into the cell, its inhibition can affect cell osmolarity, contraction of cardiac and vascular smooth muscle cells, as well as signal transduction by the MAPK pathway and generation of mitochondrial ROS. These effects, and not HIF inhibition alone, cumulatively contributed to the observed effects on construct vascularization.

Recognizing the off-target effects of digoxin, we designed experiments involving genetic inhibition of HIF-1α in graft EC to complement earlier studies. shRNA-mediated inhibition was
chosen to study specific effects of HIF-1 inhibition (as opposed to inhibition of both HIF-1 and HIF-2 by digoxin) in implanted HUVEC, which are believed to play a key role in inducing modular tissue vascularization. However, shRNA-mediated gene knockdown can also lead to off-target effects. Others showed that as little as an eleven nucleotide match can result in off-target knockdown by siRNA (10), leading to undesirable and unpredictable effects. Additionally, recognition of siRNA can lead to sequence-independent induction of type 1 interferons in immune and non-immune cells alike (11, 12). This is regulated by dsRNA-activated protein kinase, toll-like receptors, as well as activation of transcription factors like NF-κB (reviewed in (10)). Considering these limitations, one must appreciate that other effects may be contributing to effects observed from shRNA-mediated knockdown.

An alternative tool for genetic manipulation is the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas)9 system. Using custom guide RNAs and the Cas9 endonuclease, CRISPR/Cas9 relies on homologous recombination within the cell for site-specific genetic insertions and deletions. Among other things, this tool is advantageous in its specificity and amenability for the knockdown of multiple target genes. From the results of shRNA experiments, we identified HIF-2 activation as a potential compensatory mechanism of genetic HIF-1 knockdown in graft EC. This hypothesis could be further investigated by simultaneous knockdown of HIF-1α and HIF-2α in EC using CRISPR/Cas9, which would be otherwise difficult to accomplish using shRNA technology. Although genetic manipulation by CRISPR/Cas9 offers its advantages, it is also associated with off-target effects, which may have a role in recognizing viral nucleic acids or plasmid DNA (13, 14). Nonetheless, it would be a valuable tool for probing multiple gene modifications, and for studying their effects on vascularization of modular tissue constructs.

In studies pertaining to HIF stabilization, we found that DFO-releasing microparticles partly rescued vascularization of modular constructs in the absence of MSC. Ultimately, we did not achieve the original objective of this work – to simplify the modular tissue platform to a single cell type. Not explored herein was the incorporation of DFO-releasing microparticles to enhance vascularization of modular tissues containing adMSC. In a pilot study, we observed that delivery of DFO microparticles with modules containing EC and the conventional load of adMSC (1.5×10^6 adMSC/implant) did not further enhance vessel density at day 7 or 14. It was hypothesized that the overwhelming quantity of angiogenic growth factors produced by adMSC
had masked any beneficial effects of HIF stabilization. However, vessel formation was not assessed at earlier time points; HIF stabilization may have enhanced early vascularization, resulting in no apparent differences by day 7. It is also conceivable that DFO-releasing microparticles would enhance vascularization in the presence of a reduced adMSC dose. Testing this hypothesis would require titration of adMSC density within implants to determine a critical dose, below which vessel density is significantly impaired. The proposed experiments should be pursued to further tease out strategies to enhance the rate of mature vessel formation in modular tissue constructs.

That said, the objective of simplifying the modular tissue platform to one cell type is still a worthy pursuit. In this study, it could not be achieved with primary EC, but there are other cell types that could be investigated. An obvious candidate is EPC, which were found to be recruited to adMSC-containing modular tissues but absent in EC-only constructs. In a previous study (Yarden Gratch, Master of Applied Science thesis, University of Toronto 2016), we found that modular tissues containing bone marrow-derived outgrowth EC and HUVEC induced vessel densities comparable to that seen with implants containing adMSC and HUVEC. Additionally, Kepecs et al. showed that delivery of EPC (there termed early outgrowth EC) and late outgrowth EC in modular tissues improved renal function in a rat model of chronic kidney disease (15).

Contrary to expectation, implanted cells migrated from the construct and did not induce vascularization. It would be important to explore whether local stabilization of HIF, such as by the controlled release of DFO from microparticles, would impact retention of EPC within modular implants and their subsequent vascularization. Moreover, DFO-releasing microparticles may be used in combination with strategies that increase the recruitment and retention of host-derived angiogenic cells, such as systemic delivery of a pharmacologic that increases mobilization of EPC and monocytes/macrophages from the bone marrow.

Based on results from flow cytometry experiments, we identified EPC, neutrophils and alternatively activated macrophages as three cell populations whose presence in the implant play an important role in construct vascularization. As mentioned previously, a surprising finding was the near absence of EPC in all EC-only modular tissue constructs. Although it was not further explored in the present study, it would be valuable to investigate whether EPC recruitment is a key aspect of vascularization in the presence of MSC. Supporting this hypothesis is the observation that inhibition of HIF by systemic delivery of digoxin reduced EPC recruitment to
adMSC-containing implants. The effect of digoxin treatment on serum levels of SDF-1 or SCF was not elucidated in our studies. Identifying the specific chemokines regulating their recruitment from the bone marrow would be valuable for devising strategies to enhance their recruitment to modular tissues. Future efforts may also be invested in characterizing the time course of EPC recruitment to implants (beyond 3 days). Better understanding of the role of EPC in modular tissue vascularization could be used to devise strategies to enhance and/or prolong EPC recruitment, and to drive vessel formation.

Another limitation of the cell recruitment studies conducted in this work was the reliance on flow cytometry analysis to define cell populations. Indeed, cell surface receptors have been well studied and used to distinguish immune cell populations, including neutrophils, monocytes and macrophages. However, resolving subtleties in phenotypes within a cell population, such as macrophage polarization states, is limited when relying on flow cytometry alone. It has been recognized that polarized macrophage states exist along a spectrum, with classically activated (M1) and alternatively activated (M2) states representing two characteristic phenotypes. In our study, however, the definition of these phenotypes was simplified to the binary detection of CD206 and MHCII. While results pointed to some marked differences in their expression, other differences may not have been fully appreciated using the method chosen. In future studies, it would be valuable to supplement flow cytometry experiments with gene expression analyses of implants, to better characterize phenotypes of macrophages recruited to modular tissues. Profiling the gene expression of implants over time would also be useful for capturing global changes, and may allow us to identify other cell populations of interest.

In addition to complementing flow cytometry analyses, gene expression analyses of implants would also be useful for identifying the activation of molecular pathways within tissue constructs. In our studies, we used immunohistochemistry to determine the activation of HIF-1 and HIF-2 in cells, as quantified by HIF-1α+ and HIF-2α+ nuclei density. Although this method provides information regarding spatial organization and distribution of HIF-1α+ and HIF-2α+ cells within the implant, it is also limited to the two-dimensional plane. Assessing the induction of downstream genes would confirm observations from immunohistochemistry techniques. Another useful tool for studying HIF-1 activation in vivo is the bioluminescent reporter mouse (Jackson Laboratory), which has the firefly luciferase gene fused to the C-terminal of the HIF-1α oxygen-dependent degradation domain. This transgenic mouse model would allow for
longitudinal studies of HIF-1 activation in host cells following tissue construct implantation. Nonetheless, these methods do not address an important gap in our work – identifying the particular cell types that upregulate HIF-1 or HIF-2. This could be achieved using co-staining methods in immunohistochemistry, albeit tediously.

Thus far, we have focused on the limitations pertaining to each specific aim, and have yet to discuss those that apply at a broader level. Because my primary object of investigation is vascularization, the measures used to assess blood vessel formation must be carefully evaluated. Here, we have focused on immunohistochemistry to quantify vessel density, to visualize vessel perfusion and hypoxic regions within the implant. Although immunohistochemistry is a well-established tool, it also limits our understanding of three-dimensional tissue constructs to a two-dimensional plane. In future work, it will be important to image the construct in three dimensions by processing the implant using the CLARITY technique (16), so to visualize vessel formation in the entire modular tissue construct. CLARITY involves infusing hydrogel monomers into the tissue of interest to create a tissue-hydrogel hybrid, followed by clearance of lipids using an ionic detergent-based solution. Upon immersion into a refractive index homogenization solution, the tissue-hydrogel hybrid is rendered transparent. An important advantage of the CLARITY method is its amenability to be stained with multiple rounds of molecular probes. For example, it could be used in combination with perfused Texas Red dextran or EF5 to visualize perfused vessels and hypoxic regions in relation to CD31-lined structures within the entire construct. The clarified tissue construct could then be washed and stained for other markers of interest. The Sefton laboratory has already begun to use this tool to image vessel formation induced by modular tissue constructs containing pancreatic islets (17), as well as those induced by methacrylic acid beads (18). An important step going forward is developing methods for quantifying these measures from three-dimensional constructions.

Although CLARITY is a powerful tool for interrogating the tissue construct in three dimensions, it is inevitably limited to end point studies. Longitudinal assessment of vessel formation could be conducted in parallel, using ultrasound imaging of microbubble-perfused vessels, as demonstrated previously (19). Other groups have also implemented the dorsal window chamber model for longitudinal assessment of vessel formation within biomaterial constructs, and for simultaneous tracking of blood monocyte recruitment (20). Longitudinal studies of vessel perfusion, in conjunction with three-dimensional imaging of tissue constructs, would be
powerful methods of assessing the rate and maturity of vessel formation in engineered tissues. Nonetheless, the ultimate assessment of modular tissue-induced vascularization is the ability of the platform to support engraftment of therapeutic cell types. To do so would require vascularizing tissue platforms that drive rapid and mature vascularization, so that functional engineered tissues could be created for the treatment of disease.
5.3 Conclusion

The following encapsulates the major findings of my doctoral work:

- HIF activation is crucial to inducing modular tissue vascularization, as its inhibition impaired vessel formation in modules containing both adMSC and EC.

- HIF stabilization by local, prolonged release of DFO rescued vascularization in EC-only modular implants, but not to an extent comparable with adMSC co-implantation.

- HIF inhibition or stabilization altered the profile of host-derived cells to the implant. Systemic HIF inhibition reduced the recruitment of EPC and monocytes/macrophages to the modular tissue construct. Local release of DFO augmented the recruitment of neutrophils and alternatively activated macrophages in EC-only implants. Undetectable EPC recruitment to EC-only modules may contribute to the inferior vascularization of modules in the absence of adMSC.

The following is a list of scientific contributions from my doctoral work:

1. Lam GC, Sefton MV (submitted for review) Hypoxia-inducible factor drives vascularization of modularly assembled engineered tissue. *Biomaterials*.


5.4 References


Appendices

Appendix I: DFO Controlled Release Profile

To determine the controlled release profile of DFO-loaded microparticles using measurements obtained from HPLC-MS experiments, a calibration curve was constructed. The table below (left) summarizes the peak area measurements for standards of DFO in PBS, with known concentrations between 0-100 ng/mL. The calibration curve below (right) was constructed in Excel, and a linear model fitted to the data with corresponding $R^2=0.95$.

<table>
<thead>
<tr>
<th>Standard Concentration (ng/mL)</th>
<th>Peak Area (cps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00E+00</td>
</tr>
<tr>
<td>5</td>
<td>1.82E+04</td>
</tr>
<tr>
<td>10</td>
<td>3.32E+04</td>
</tr>
<tr>
<td>20</td>
<td>5.79E+04</td>
</tr>
<tr>
<td>40</td>
<td>1.00E+05</td>
</tr>
<tr>
<td>60</td>
<td>1.40E+05</td>
</tr>
<tr>
<td>80</td>
<td>1.29E+05</td>
</tr>
<tr>
<td>100</td>
<td>1.87E+05</td>
</tr>
</tbody>
</table>

Using the linear model defined above, the DFO concentration in each sample was determined. The table below summarizes results of these calculations. Columns 1-4 detail the properties of each sample, including sample name (column 1), time point of sampling (column 2), dilution factor applied to the original sample to obtain a concentration within the linear range of the calibration curve (column 3) and the peak area readings obtained from HPLC-MS (column 4). A sample calculation for sample concentration (column 5) is provided below, using data from sample d_505h_250:

\[ y = 1727.9x + 15143 \]
\[
x = \frac{y - 15143}{1727.9} = \frac{62900 - 15143}{1727.9} = 27.6 \text{ ng/mL}
\]

<table>
<thead>
<tr>
<th>1</th>
<th>Sample</th>
<th>2</th>
<th>Time point (h)</th>
<th>3</th>
<th>Dilution factor</th>
<th>4</th>
<th>Peak Area (cps)</th>
<th>5</th>
<th>Concentration (ng/mL)</th>
<th>6</th>
<th>DFO content (ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d_505h_250</td>
<td>505</td>
<td>250</td>
<td>6.29E+04</td>
<td>27.64</td>
<td>72.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_460h_250</td>
<td>460</td>
<td>250</td>
<td>7.41E+04</td>
<td>34.12</td>
<td>89.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_392h_250</td>
<td>392</td>
<td>250</td>
<td>5.95E+04</td>
<td>25.67</td>
<td>67.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_360h_250</td>
<td>360</td>
<td>250</td>
<td>7.53E+04</td>
<td>34.82</td>
<td>91.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_335h_250</td>
<td>335</td>
<td>250</td>
<td>5.48E+04</td>
<td>22.95</td>
<td>60.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_320h_250</td>
<td>320</td>
<td>250</td>
<td>6.80E+04</td>
<td>30.59</td>
<td>80.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_291h_250</td>
<td>291</td>
<td>250</td>
<td>6.14E+04</td>
<td>26.77</td>
<td>70.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_264h_250</td>
<td>264</td>
<td>250</td>
<td>7.09E+04</td>
<td>32.27</td>
<td>84.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_240h_250</td>
<td>240</td>
<td>250</td>
<td>7.25E+04</td>
<td>33.19</td>
<td>87.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_222h_250</td>
<td>222</td>
<td>250</td>
<td>6.19E+04</td>
<td>27.06</td>
<td>71.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_152h_250</td>
<td>152</td>
<td>250</td>
<td>9.86E+04</td>
<td>48.30</td>
<td>127.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_120h_250</td>
<td>120</td>
<td>250</td>
<td>9.85E+04</td>
<td>48.24</td>
<td>127.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_102h_250</td>
<td>102</td>
<td>250</td>
<td>1.20E+05</td>
<td>60.68</td>
<td>159.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_96h_500</td>
<td>96</td>
<td>500</td>
<td>6.19E+04</td>
<td>27.06</td>
<td>142.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_71h_500</td>
<td>71</td>
<td>500</td>
<td>8.56E+04</td>
<td>40.78</td>
<td>214.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_54h_500</td>
<td>54</td>
<td>500</td>
<td>7.08E+04</td>
<td>32.21</td>
<td>169.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_32h_500</td>
<td>32</td>
<td>500</td>
<td>9.46E+04</td>
<td>45.98</td>
<td>242.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_30h_500</td>
<td>30</td>
<td>500</td>
<td>9.09E+04</td>
<td>43.84</td>
<td>230.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_24h_500</td>
<td>24</td>
<td>500</td>
<td>7.21E+04</td>
<td>32.96</td>
<td>173.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_8h_500</td>
<td>8</td>
<td>500</td>
<td>1.27E+05</td>
<td>64.74</td>
<td>340.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_6h_500</td>
<td>6</td>
<td>500</td>
<td>7.79E+04</td>
<td>36.32</td>
<td>191.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_4h_500</td>
<td>4</td>
<td>500</td>
<td>8.07E+04</td>
<td>37.94</td>
<td>199.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_2h_1000</td>
<td>2</td>
<td>1000</td>
<td>3.17E+04</td>
<td>9.58</td>
<td>100.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d_1h_1000</td>
<td>1</td>
<td>1000</td>
<td>2.37E+04</td>
<td>4.95</td>
<td>49.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In order to determine the actual DFO amount in each original sample, one must account for the total volume of PBS in which the microparticles were suspended (10 mL), the dilution factor applied for HPLC-MS measurement (column 3), and the dilution factor associated with replenishing 0.5 mL fresh PBS after sampling (0.95). A sample calculation for DFO content (column 6) is given below using data from sample d_505h_250:

\[
DFO \text{ content} (\mu g) = \left( 27.64 \frac{ng}{mL} \right) \times 10 \text{ mL} \times 250 \times \left( \frac{1}{0.95} \right) \times \left( \frac{\mu g}{1000 \text{ ng}} \right) = 72.7 \text{ ng DFO}
\]
Note that the dilution factor of 0.95 was not applied to the first sample (d_1h_1000), since a 0.5 mL sample was taken directly from the original sample.

Using the DFO amounts determined earlier, the cumulative release profile was defined. The cumulative fraction released was determined by normalizing to the total DFO amount encapsulated. This was determined in another experiment using standards and creating a calibration curve anew, as outlined above. The total encapsulated DFO amount was determined to be 2.05 mg per microparticle preparation. The table below summarizes the cumulative release and cumulative fraction release.

<table>
<thead>
<tr>
<th>Time point (h)</th>
<th>Cumulative release (mg/preparation)</th>
<th>Cumulative fraction released</th>
<th>Model</th>
<th>Squared error</th>
</tr>
</thead>
<tbody>
<tr>
<td>505</td>
<td>1.62</td>
<td>0.79</td>
<td>0.714841</td>
<td>0.005873</td>
</tr>
<tr>
<td>460</td>
<td>1.59</td>
<td>0.77</td>
<td>0.698098</td>
<td>0.005721</td>
</tr>
<tr>
<td>392</td>
<td>1.54</td>
<td>0.75</td>
<td>0.670311</td>
<td>0.006647</td>
</tr>
<tr>
<td>360</td>
<td>1.51</td>
<td>0.74</td>
<td>0.655971</td>
<td>0.006303</td>
</tr>
<tr>
<td>335</td>
<td>1.46</td>
<td>0.71</td>
<td>0.644092</td>
<td>0.00475</td>
</tr>
<tr>
<td>320</td>
<td>1.43</td>
<td>0.70</td>
<td>0.636643</td>
<td>0.0038</td>
</tr>
<tr>
<td>291</td>
<td>1.39</td>
<td>0.68</td>
<td>0.62147</td>
<td>0.003269</td>
</tr>
<tr>
<td>264</td>
<td>1.36</td>
<td>0.66</td>
<td>0.606292</td>
<td>0.003044</td>
</tr>
<tr>
<td>240</td>
<td>1.31</td>
<td>0.64</td>
<td>0.591795</td>
<td>0.002397</td>
</tr>
<tr>
<td>222</td>
<td>1.27</td>
<td>0.62</td>
<td>0.580194</td>
<td>0.001541</td>
</tr>
<tr>
<td>152</td>
<td>1.23</td>
<td>0.60</td>
<td>0.526988</td>
<td>0.005639</td>
</tr>
<tr>
<td>120</td>
<td>1.17</td>
<td>0.57</td>
<td>0.496286</td>
<td>0.005594</td>
</tr>
<tr>
<td>102</td>
<td>1.11</td>
<td>0.54</td>
<td>0.476222</td>
<td>0.004082</td>
</tr>
<tr>
<td>96</td>
<td>1.03</td>
<td>0.50</td>
<td>0.468947</td>
<td>0.001038</td>
</tr>
<tr>
<td>71</td>
<td>0.96</td>
<td>0.47</td>
<td>0.434367</td>
<td>0.001028</td>
</tr>
<tr>
<td>54</td>
<td>0.85</td>
<td>0.41</td>
<td>0.405204</td>
<td>7.89E-05</td>
</tr>
<tr>
<td>32</td>
<td>0.76</td>
<td>0.37</td>
<td>0.35479</td>
<td>0.000322</td>
</tr>
<tr>
<td>30</td>
<td>0.64</td>
<td>0.31</td>
<td>0.349023</td>
<td>0.001247</td>
</tr>
<tr>
<td>24</td>
<td>0.53</td>
<td>0.26</td>
<td>0.329796</td>
<td>0.005238</td>
</tr>
<tr>
<td>8</td>
<td>0.44</td>
<td>0.22</td>
<td>0.249513</td>
<td>0.001184</td>
</tr>
<tr>
<td>6</td>
<td>0.27</td>
<td>0.13</td>
<td>0.231936</td>
<td>0.009986</td>
</tr>
<tr>
<td>4</td>
<td>0.18</td>
<td>0.09</td>
<td>0.209244</td>
<td>0.015341</td>
</tr>
<tr>
<td>2</td>
<td>0.08</td>
<td>0.04</td>
<td>0.175475</td>
<td>0.019264</td>
</tr>
<tr>
<td>1</td>
<td>0.02</td>
<td>0.01</td>
<td>0.147155</td>
<td>0.018246</td>
</tr>
</tbody>
</table>
The change in cumulative fraction released over time was first modeled using the following equation (power function):

\[
\frac{M_t}{M_\infty} = k t^n
\]

The Solver algorithm in Microsoft Excel was used to model the data, by minimizing the sum of squared error (column 5). Applying this tool yielded \( k=0.10 \) and \( n=0.34 \), with the following controlled release profile:

In order to verify the curve fit, a logarithmic-logarithmic plot of the data was produced:
Based on the linear regression analysis ($R^2 = 0.89$), the relationship between cumulative fraction released and time can be described by a power function.

Because the rate of release is relatively constant after 100 h, the release rate between 100 and 500 h was determined as follows:

\[
M_{100h} = \frac{\left(2.05 \frac{mg}{preparation}\right) \times (0.10 \times (100)^{0.34})}{4 \text{ implants/preparation}} = 0.25 \text{ mg/implant}
\]

\[
M_{500h} = \frac{\left(2.05 \frac{mg}{preparation}\right) \times (0.10 \times (500)^{0.34})}{4 \text{ implants/preparation}} = 0.42 \text{ mg/implant}
\]

\[
\text{Rate of release} = \frac{M_{500h} - M_{100h}}{400 \text{ h}} = \frac{0.42 - 0.25 \frac{mg}{implant}}{400 \text{ h}} = 0.45 \mu g/h
\]
Appendix II: Statistical Analyses and Data Transformations

This appendix details the calculations and statistical tests employed to analyze data sets. The two-way analysis of variance (ANOVA) was used with experiments involving two variables, such as time and treatment. To demonstrate sample calculations, the M2-polarized macrophage recruitment data set from flow cytometry experiments will be used. All statistical analyses were performed using JMP Pro 13.

A two-way ANOVA is first performed on the raw data, with time, treatment and time-treatment interaction input as predicted effects. The normality of the distribution of residuals was then examined. From the M2-polarized macrophage data set, the following results were obtained:

Results from the goodness-of-fit Shapiro-Wilk W Test indicate that residuals are not normally distributed (p<0.01). The data must therefore be transformed to obtain normally distributed residuals, before two-way ANOVA can be applied. A cube root transformation of the M2-polarized macrophage data set was applied, and the normality of residual distribution tested once again. The following table summarizes the raw data, cube root-transformed data, including their associated residuals:
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
<th>CD206</th>
<th>Residuals(CD206)</th>
<th>cbrt(CD206)</th>
<th>Residuals(cbrt(CD206))</th>
</tr>
</thead>
<tbody>
<tr>
<td>c</td>
<td>1</td>
<td>718</td>
<td>307</td>
<td>8.95</td>
<td>1.748</td>
</tr>
<tr>
<td>c</td>
<td>1</td>
<td>111</td>
<td>-300</td>
<td>4.81</td>
<td>-2.392</td>
</tr>
<tr>
<td>c</td>
<td>1</td>
<td>460</td>
<td>49</td>
<td>7.72</td>
<td>0.518</td>
</tr>
<tr>
<td>c</td>
<td>1</td>
<td>371</td>
<td>-40</td>
<td>7.19</td>
<td>-0.012</td>
</tr>
<tr>
<td>c</td>
<td>1</td>
<td>395</td>
<td>-16</td>
<td>7.34</td>
<td>0.138</td>
</tr>
<tr>
<td>d</td>
<td>1</td>
<td>382</td>
<td>-1</td>
<td>7.25</td>
<td>-0.01</td>
</tr>
<tr>
<td>d</td>
<td>1</td>
<td>350</td>
<td>-33</td>
<td>7.05</td>
<td>-0.21</td>
</tr>
<tr>
<td>d</td>
<td>1</td>
<td>389</td>
<td>6</td>
<td>7.3</td>
<td>0.04</td>
</tr>
<tr>
<td>d</td>
<td>1</td>
<td>413</td>
<td>30</td>
<td>7.45</td>
<td>0.19</td>
</tr>
<tr>
<td>c</td>
<td>3</td>
<td>1359</td>
<td>3.4</td>
<td>11.08</td>
<td>0.044</td>
</tr>
<tr>
<td>c</td>
<td>3</td>
<td>1747</td>
<td>391.4</td>
<td>12.04</td>
<td>1.004</td>
</tr>
<tr>
<td>c</td>
<td>3</td>
<td>1135</td>
<td>-220.6</td>
<td>10.43</td>
<td>-0.606</td>
</tr>
<tr>
<td>c</td>
<td>3</td>
<td>1376</td>
<td>20.4</td>
<td>11.12</td>
<td>0.084</td>
</tr>
<tr>
<td>c</td>
<td>3</td>
<td>1161</td>
<td>-194.6</td>
<td>10.51</td>
<td>-0.526</td>
</tr>
<tr>
<td>d</td>
<td>3</td>
<td>1017</td>
<td>-262.6</td>
<td>10.06</td>
<td>-0.748</td>
</tr>
<tr>
<td>d</td>
<td>3</td>
<td>1200</td>
<td>-79.6</td>
<td>10.63</td>
<td>-0.178</td>
</tr>
<tr>
<td>d</td>
<td>3</td>
<td>1082</td>
<td>-197.6</td>
<td>10.27</td>
<td>-0.538</td>
</tr>
<tr>
<td>d</td>
<td>3</td>
<td>1318</td>
<td>38.4</td>
<td>10.96</td>
<td>0.152</td>
</tr>
<tr>
<td>d</td>
<td>3</td>
<td>1781</td>
<td>501.4</td>
<td>12.12</td>
<td>1.312</td>
</tr>
<tr>
<td>c</td>
<td>7</td>
<td>347</td>
<td>-15.8</td>
<td>7.03</td>
<td>0.038</td>
</tr>
<tr>
<td>c</td>
<td>7</td>
<td>441</td>
<td>78.2</td>
<td>7.61</td>
<td>0.618</td>
</tr>
<tr>
<td>c</td>
<td>7</td>
<td>168</td>
<td>-194.8</td>
<td>5.52</td>
<td>-1.472</td>
</tr>
<tr>
<td>c</td>
<td>7</td>
<td>258</td>
<td>-104.8</td>
<td>6.36</td>
<td>-0.632</td>
</tr>
<tr>
<td>c</td>
<td>7</td>
<td>600</td>
<td>237.2</td>
<td>8.44</td>
<td>1.448</td>
</tr>
<tr>
<td>d</td>
<td>7</td>
<td>948</td>
<td>117.2</td>
<td>9.82</td>
<td>0.568</td>
</tr>
<tr>
<td>d</td>
<td>7</td>
<td>736</td>
<td>-94.8</td>
<td>9.03</td>
<td>-0.222</td>
</tr>
<tr>
<td>d</td>
<td>7</td>
<td>374</td>
<td>-456.8</td>
<td>7.21</td>
<td>-2.042</td>
</tr>
<tr>
<td>d</td>
<td>7</td>
<td>817</td>
<td>-13.8</td>
<td>9.35</td>
<td>0.098</td>
</tr>
<tr>
<td>d</td>
<td>7</td>
<td>1279</td>
<td>448.2</td>
<td>10.85</td>
<td>1.598</td>
</tr>
<tr>
<td>c</td>
<td>21</td>
<td>0</td>
<td>-5.33333</td>
<td>0.35</td>
<td>-1.01667</td>
</tr>
<tr>
<td>c</td>
<td>21</td>
<td>16</td>
<td>10.66667</td>
<td>2.52</td>
<td>1.153333</td>
</tr>
<tr>
<td>c</td>
<td>21</td>
<td>4</td>
<td>-1.33333</td>
<td>1.55</td>
<td>0.183333</td>
</tr>
<tr>
<td>c</td>
<td>21</td>
<td>12</td>
<td>6.666667</td>
<td>2.31</td>
<td>0.943333</td>
</tr>
<tr>
<td>c</td>
<td>21</td>
<td>0</td>
<td>-5.33333</td>
<td>0.76</td>
<td>-0.60667</td>
</tr>
<tr>
<td>c</td>
<td>21</td>
<td>0</td>
<td>-5.33333</td>
<td>0.71</td>
<td>-0.65667</td>
</tr>
<tr>
<td>d</td>
<td>21</td>
<td>0</td>
<td>-5.83333</td>
<td>0.42</td>
<td>-1.04167</td>
</tr>
<tr>
<td>d</td>
<td>21</td>
<td>17</td>
<td>11.16667</td>
<td>2.56</td>
<td>1.098333</td>
</tr>
<tr>
<td>d</td>
<td>21</td>
<td>4</td>
<td>-1.83333</td>
<td>1.6</td>
<td>0.138333</td>
</tr>
<tr>
<td>d</td>
<td>21</td>
<td>11</td>
<td>5.166667</td>
<td>2.25</td>
<td>0.788333</td>
</tr>
<tr>
<td>d</td>
<td>21</td>
<td>0</td>
<td>-5.83333</td>
<td>0.46</td>
<td>-1.00167</td>
</tr>
<tr>
<td>d</td>
<td>21</td>
<td>3</td>
<td>-2.83333</td>
<td>1.48</td>
<td>0.018333</td>
</tr>
</tbody>
</table>
According to the goodness-of-fit test, there is insufficient evidence to reject the null hypothesis that the residuals are normally distributed (p>0.05). We proceed to perform two-way ANOVA on the cube root-transformed data. Results from the effects test indicates that there are significant effects of time and interaction between time and treatment:

Subsequently, we perform Tukey’s Honest Significance test on all pairwise interactions. The following results table summarizes all statistically significant interactions: