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Formation of Stable Vascular Networks in Engineered Tissues

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1. Introduction

Proper vascular network structure is essential for normal tissue function. The vasculature provides oxygen, nutrients and immune cells, as well as removes tissue waste and by-products. (Jain 2003) In tissue engineering and regenerative medicine, the existence of functional blood vessels is critical to the survival of the newly generated tissue. Current strategies for promoting new blood vessel formation, or neovascularization, have mostly focused on angiogenesis, which is the formation of new capillaries from pre-existing vessels by sprouting of endothelial cells (ECs). (Carmeliet 2003) Another mechanism of neovascularization, vasculogenesis, which is the *in situ* assembly of endothelial progenitors into capillaries, has also been explored as a method for stimulating new vessel formation. Genes or proteins of angiogenic factors have been delivered systematically or directly to a target tissue to promote neovascularization via either angiogenesis or vasculogenesis. For example, proteins from the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families delivered by various methods have been investigated extensively for enhancing neovascularization. (Brey *et al.*, 2005; Brey & McIntire, 2008)

While the delivery of angiogenic factors has been successful in promoting new capillary formation, the structure of the newly formed vessels is often non-ideal. The vessels can be immature, with small diameters and lack of some essential cellular and extracellular components required for proper function. Immature vascular structure may result in poor blood perfusion and possibly vessel regression upon a decrease in the vascular stimulus. (Benjamin *et al.*, 1999) In addition, the formation of small capillaries alone is not likely to be sufficient for vascularization of large, complex tissues, which are often needed for tissue engineering. Therefore, the goal of neovascularization in engineered tissue should be focused not only on the initial assembly of capillaries via angiogenesis or vasculogenesis, but also the expansion and stabilization of new vessels, which means the formation of mature, long-lasting vessels with dimensions that meet the requirement of high conductance blood flow. (Carmeliet & Conway, 2001) In this chapter, we will discuss current status in the generation of stable, long-lasting vascular networks in tissue engineering and regenerative medicine, identifying recent advances and limitations yet to be overcome.

2. Angiogenesis stabilization: basic mechanisms

The process of angiogenesis includes four stages: (a) initiation, in which a certain angiogenic stimulus increases vessel permeability and protein leakage; (b) progression, characterized

by the production of proteolytic enzymes that degrade the basement membrane (BM) lining endothelial cells (ECs) and surrounding extracellular matrix (ECM) to allow EC invasion and migration; (c) differentiation, which involves the assembly of ECs into a network with a luminal structure; and (d) stabilization and maturation, where mural cells (pericytes and vascular smooth muscle cells) are recruited and ECs deposit new BM. (Bussolino *et al.*, 1997) It is important to note that the MCs may be recruited after initial network function or simultaneous with vessel assembly. (Brey *et al.*, 2004) Numerous cellular and molecular factors are involved in the regulation of this process. Failure in this regulation could result in excessive vessel generation or premature regression, which is seen in many pathological conditions. In the following sections we will describe the basic components of the vessel and their role in vessel stability.

2.1 Cells

The walls of stable blood vessels are composed of two distinct cell types: ECs and mural cells (MCs). (Figure 1) While ECs form the inner lining of vascular tubes, MCs associate with and coat the outside of the endothelial tube. (Gaengel *et al.*, 2008) In the case of capillaries the MCs, or pericytes, are intermittently present on the surface. For larger vessels the MCs, or vascular smooth muscle cells (VSMCs), coat the entire vessel surface often in multiple layers. Interactions between the ECs and MCs play a critical role in the regulation of vascular formation, stabilization, remodeling, and function. Abnormal interactions between the two cell types are implicated in a number of pathological conditions, including tumor angiogenesis, diabetic microangiopathy, ectopic tissue calcification, and stroke and dementia syndrome. (Armulik *et al.*, 2005; Carmeliet 2003)

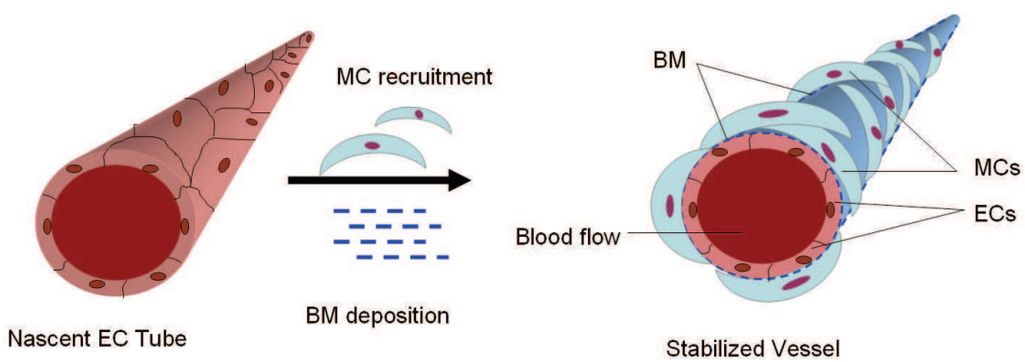


Fig. 1. Process of blood vessel stabilization. Endothelial cells (EC) in immature vessels secrete signals that stimulate the migration and proliferation of mural cells (MC). ECs also secrete a thin layer of matrix, known as the basement membrane (BM) that lines the vessels

Junctional structures, including adherent junctions and tight junctions, are present between ECs and provide the endothelium with integrity, mechanical strength and tightness, and a barrier to molecular transport. (Carmeliet 2003) During vascular sprouting, junctions are partially disorganized to allow cell migration and proliferation, and to increase vessel permeability. During vessel stabilization, junctional integrity is re-established and permeability is tightly controlled. (Dejana, 2004) As vessels sprout and migrate, they will

form branches. These sprouts then inosculate with other vessels to form complex networks. Typically, a high vessel density is formed in response to the initial vascular stimuli. This vasculature must be remodeled for tissue-specific transport and function. Apoptosis of ECs are involved at this later stage to remove excessive vessels. (Darland & D'Amore, 2001) However, excessive apoptosis may result in premature vessel regression. Growth factors, which are essential for angiogenesis, not only stimulate EC proliferation and migration but also inhibit EC apoptosis. (Dimmeler & Zeiher, 2000) Thus, the prevention of EC apoptosis may improve the survival and durability of blood vessels formed in engineered tissues.

MCs are commonly subdivided into VSMCs, which are associated with arteries and veins forming multiple concentric layers; and pericytes, which are associated with small diameter capillaries. (Gaengel *et al.*, 2009) Intermediate-size vessels, arterioles and venules, have MCs with properties between those of typical VSMCs and pericytes. Since VSMCs and pericytes share some common cellular markers, such as α-smooth muscle actin (α-SMA) and desmin, they are believed to represent phenotypic variants of the same lineage, and may function as progenitors for each other depending on external stimuli. (Gerhardt & Betsholtz, 2003)

The presence of MCs on the surface of ECs plays a critical role in vessel stability. Intercellular adhesion between MCs and ECs is mediated by integrin $\alpha_4\beta_1$, which is expressed by proliferating ECs, and its ligand VCAM-1, which is expressed by proliferating MCs. (Garmy-Susini *et al.*, 2005) Studies have shown that the association of MCs to the newly formed endothelial plexus stabilizes the vasculature, while the disruption of EC-MC associations results in excessive regression of vasculature. (Benjamin *et al.*, 1998) Co-culture of EC with SMC in a 3D *in vitro* model results in a mature, quiescent EC phenotype with increased number junctional structures and increased resistance to apoptosis. (Korff *et al.*, 2001) However, the presence of MCs in the microvasculature does not always guarantee vessel stability. Vessels with extensive MC can still regress following loss of neovascular stimuli. (Brey *et al.*, 2004) Therefore, some additional signals, such as ECM and soluble growth factors, are also required for vessel stabilization.

2.2 Extracellular components

Extracellular matrices (ECM), including BM (primarily collagen IV and various laminin isoforms) and interstitial matrices (typically collagen I, elastin and fibronectin but depends on the particularly tissue), play important roles in angiogenesis. (Francis *et al.*, 2008) Firstly, the ECM scaffold provides mechanical support for ECs to form channels, preventing vessels from collapsing. In addition, EC adhesion to ECM regulates EC proliferation, migration, and survival, mediated by different signaling pathways. (Davis & Senger, 2005) And finally, the deposition of ECM, particularly BM, which occurs after MC recruitment stabilizes the vessel by decreasing permeability and preventing vessel regression. (Stratman *et al.*, 2009)

The ECM is dynamically regulated during angiogenesis. In the initiation of angiogenesis, ECM is degraded by proteinases, such as matrix metalloproteinases (MMPs). The degradation of ECM not only allows EC migration and proliferation, but also releases soluble growth factors trapped in the matrix. (Ghajar *et al.*, 2008) After lumen formation, MCs are recruited to EC tubes and induce the deposition of new BM as well as the secretion of tissue inhibitor of metalloproteinases (TIMPs), which inhibit MMPs proteolysis. (Stratman

et al., 2009) Dysregulation of ECM, such as glycation of collagen due to diabetes and aging, can slow ECM vessel assembly. (Francis-Sedlak *et al.*, 2010)

2.3 Signaling pathways

The formation of stable vasculature is tightly regulated by the spatial and temporal kinetics of numerous signaling pathways. A stable, operational vascular network results from a balance between signals that favor angiogenesis and vascular stabilization, and those that promote vascular regression. (Bussolino *et al.*, 1997) Signaling pathways affect vascular stabilization presumably by regulating proliferation and apoptosis of ECs and MCs, as well as the deposition and degradation of ECM. (Jain 2003) In this section, we will discuss some of the signaling pathways and their roles in vascular stabilization.

2.3.1 Platelet derived growth factor (PDGF) family

The PDGF family is composed of disulfide-bonded homodimers of four subunits, PDGF-A, PDGF-B, PDGF-C and PDGF-D, as well as the heterodimer PDGF-AB. PDGF isoforms exert their biological effects through the activation of two tyrosine kinase receptors, PDGFR-α and PDGF-β, which are present on MCs and messenchymal cells. (Hellberg *et al.*, 2010)

Specifically, PDGF-BB, secreted by proliferating ECs during angiogenic sprouting, stimulates the migration and proliferation of MCs, which express PDGFR-β. In addition, PDGF-BB induces the differentiation of messenchymal cells toward a MC lineage.(Hirschi *et al.*, 1999) Genetic knockout of *pdgf-b* or *pdgfr-β* in mice results in MC deficiency, vascular leakage and embryonic lethality (Hellstrom *et al.*, 1999; Lindahl *et al.*, 1997) The more recently described PDGF-C and PDGF-D isoforms are also involved in vessel maturation and stabilization. PDGF-C drives the recruitment and differentiation of MCs resulting in improved BM integrity and a more mature, stable vascular wall with a lower permeability. (di Tomaso *et al.*, 2009) PDGF-D induces macrophage recruitment and vessel stabilization by improving the SMC coating of angiogenic blood vessels and decreases their permeability. (Uutela *et al.*, 2004)

2.3.2 Angiopoietins

The angiopoietin (Ang) family consists of Ang-1, Ang-2, Ang-3 and Ang-4. The angiopoietins are ligands for the Ties, a family of receptors that are expressed within the vascular endothelium. (Yancopoulos *et al.*, 2000) Ang-1 is predominantly expressed by MCs, suggesting a paracrine mode of action; whereas Ang-2 is mostly expressed by ECs, suggesting an autocrine function, although expression in MCs has also been reported. (Gaengel *et al.*, 2009) Ang-3 and Ang-4 are less well understood at this time.

Ang-1 stabilizes nascent vessels and make them leak-resistant, presumably by facilitating interactions between ECs and MCs. Genetic deletion of Ang-1 or its receptor Tie2 in mice resulted in defects in vessel remodeling with poor association of ECs with BM, and failure to recruit MCs. (Sato *et al.*, 1995; Suri *et al.*, 1996) Transgenic overexpression of Ang-1 in mice lead to a significant increase in vessel diameter and increased resistance to vessel leakage. The resistance is presumably due to the ability of Ang-1 to maximize interactions between ECs, MCs and BM. (Thurston *et al.*, 1999, 2000) Ang-2, on the other hand, acts as an antagonist of Ang-1, destabilizes vessels and contributes to vessel regression. Transgenic overexpression of Ang-2 disrupts blood vessel formation in the mouse embryo, resulting in

similar effects to Ang-1 or Tie 2 knockouts. (Maisonpierre *et al.*, 1997) Thus, angiopoietins positively or negatively regulate vessel stabilization, however, the detailed mechanism of the signaling pathways are not yet well-described.

2.3.3 Sphingosine-1-Phosphate (S1P)

S1P is a bioactive lipid mediator that is primarily secreted by platelets. It activates a family of G-protein coupled receptors ($S1P_{1\ to\ 5}$), formerly known as endothelial differentiation gene (Edg)-receptors. (Gaengel *et al.*, 2009) Mice deficient in S1P have severe vascular defects resulting in embryonic lethality, with incomplete coverage of MCs. Mice deficient in $S1P_1$, its receptor, also showed severe defect in vascular maturation, suggesting the role of $S1P/S1P_1$ signaling pathway in promoting vascular stabilization. (Mizugishi *et al.*, 2005) Moreover, knockout of the EC-specific $S1P_1$ in mice embryos resulted in a similar phenotype as obtained for the $S1P_1$ full knockout, suggesting that $S1P_1$ functions mainly through ECs instead of other cell types. (Allende *et al.*, 2003) It was found that $S1P/S1P_1$ signaling pathway promotes vascular stabilization by activation of N-Cadherin, which is a cell adhesion molecule connecting ECs and MCs. (Paik *et al.*, 2004)

2.3.4 Transforming growth factor-β (TGF-β)

TGF- β, a growth factor family with three different isoforms TGF-β1, TGF-β2 and TGF-β3, is expressed by a number of cell types, including ECs and MCs. Depending on its receptor TGF-β mediates distinct signaling pathways. TGF-β can be both pro- and anti-angiogenic. (Pepper 1997) The major receptors for TGF-β in regulating vascular stabilization include activin receptor-like kinase (Alk)-1, Alk-5, and endoglin. (Gaengel *et al.*, 2009)

Activation of Alk-1 triggers EC migration, proliferation, and inhibits vessel maturation and MC differentiation; whereas activation of Alk-5 instead mediates inhibition of EC migration, reduced proliferation, increased vessel maturation and MC differentiation, suggesting that TGF-β regulates vessel maturation and stabilization via a balance between Alk-1 and Alk-5. (Goumans *et al.*, 2002) The determination of which signaling pathway TGF-β activates appears to depend on its concentration. At low doses, TGF-β promotes EC proliferation by activating TGF-β /Alk-1 pathway; while at high doses, Alk-5 is activated instead. (van den Driesche *et al.*, 2003) Endoglin, a TGF-β co-receptor expressed specifically in ECs, modulates Alk-1 and Alk-5 mediated pathways, possibly by favoring the TGF-β/Alk-1 pathway. (Letamendia *et al.*, 1998)

2.3.5 Vascular endothelial growth factor (VEGF) family

VEGF is a family of angiogenic growth factors expressed by a variety of cells, including keratinocytes, macrophages, mast cells, vSMCS, and ECs. Members of the VEGF family include VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). VEGF is a major mitogen for ECs, mediated via VEGF tyrosine kinase receptors (VEGFRs) expressed on ECs. (Bussolino *et al.*, 1997; Yancopoulos *et al.*, 2000) It has been shown that VEGF plays a central role in neovascularization by stimulating ECs proliferation, migration and increasing vascular permeability in a number of physiological and pathological processes. (Nor *et al.*, 1999)

However, the role of VEGF in regulating vascular stabilization can be both positive and negative, depending on its spatial and temporal kinetics. On the one hand, VEGF enhances

EC survival and protects EC from apoptosis by up-regulating the expression of Bcl-2 (B-cell lymphoma 2), an apoptosis regulator protein. (Nor *et al.*, 1999) Withdrawal of VEGF prior to stabilization and remodeling may result in excessive vessel regression by EC apoptosis. (Benjamin *et al.*, 1999; Jain, *et al.*, 1998) On the other hand, VEGF is also considered as a negative regulator for vessel maturation by disrupting VSMC function and pericyte coverage of nascent vascular sprouts, leading to vessel destabilization. (Greenberg *et al.*,2008). The overexpression of VEGF results in leaky, immature and unstable vessels, which contribute to a number of pathological conditions, such as tumors, diabetic retinopathy, and age-related macular degeneration (AMD). Therefore, the dosage of VEGF must be tightly regulated in a spatial, temporal and quantitative manner in therapeutic applications to avoid abnormal or aberrant vascular structure. (Yancopoulos *et al.*, 2000)

2.3.6 Ephrins and notch

Ephrins and Notch are two signals that are generally involved in the differentiation of many cell types. In angiogenesis, the two signaling pathways are involved in the determination of arterio-venous vessel fate. (Jain 2003) Thus, the ephrins and Notch signaling pathways are considered novel targets for therapeutic angiogenesis. (Sullivan & Bicknell, 2003; Cristofaro & Emanueli, 2009) The ephrin ligands and Eph receptors are both transmembrane molecules expressed on ECs and other cell types.(Yancopoulos *et al.*, 2000; Cristofaro & Emanueli, 2009) The ligand ephrin-B2 is expressed specifically in the arterial endothelium, while the receptor EphB4 expresses in the venous endothelium. The distribution suggest a role in defining boundaries between arterial and venous domains. (Adam *et al.*, 1999; Wang *et al.*, 1999). Notch is a family of transmembrane protein receptors that bind to ligands Delta/Serrate. The Notch pathway, in angiogenesis, is also required for arterial-venous differentiation. (Sullivan & Bicknell, 2003; Gaengel *et al.*, 2009) Activation of Notch signaling leads to repression of venous cell fate in EC, while ablation of Notch function resulted in defects in blood vessel formation similar to those associated with improper arterial-venous specification. (Lawson *et al.*, 2001; Liu *et al.*, 2003)

2.3.7 Others

There are a number of other signaling pathways involved in the formation of stable vasculature. For example, brain derived neurotrophic factor (BDNF) is a neurotrophin for neuron survival and differentiation. It is also an EC survival factor involved in intramyocardial vessel stabilization, by activation of trk B receptors expressed on cardiac microvascular ECs.(Donovan *et al.*, 2000) Monocyte chemotatic protein-1 (MCP-1) is a small cytokine best known for its ability to recruit monocytes after injury. However, recent studies have shown that MCP-1 improves vessel stabilization during angiogenesis by promoting MC recruitment and inducing VSMC proliferation. (Aplin *et al.*, 2010; Selzman *et al.*, 2002) Nitric oxide (NO), a multifunctional gaseous molecule that regulates various physiological functions is also involved in vascular stabilization. An *in vivo* tissue-engineered blood vessel model revealed that NO mediates EC-MC interaction and induces MC recruitment, vessel branching, and longitudinal extension and subsequent stabilization of the vessels. (Kashiwagi *et al.*, 2005) A summary of the of numerous signalling pathways is provided in Table 1, with ligand/receptor pairs, cells that express these molecules, and their roles in vessel maturation and stabilization.

Ligand (Cells)	Receptor (Cells)	Roles in vessel stabilization
Ang-1 (MCs)	Tie2 (ECs, MSCs)	Stabilizes vessels by mediating EC-MC and EC-ECM adhesion; Induces production of factors such as HB-EGF, HGF and MCP-1.
Ang-2 (ECs)	Tie2 (ECs; MSCs)	Destabilizes vessels by binding to Tie2 to attenuate the effect of Ang-1.
Bcl-2 (ECs)	ERKs (ECs)	Prevents EC apoptosis and enhances vessel durability.
BDNF (neurons; ECs)	Trk-B (neurons; ECs; MCs)	Promotes intramyocardial arteries and capillaries stabilization by enhancing EC survival during embryogenesis.
Delta/Serrate (ECs)	Notch (ECs)	Determines arterial-venous fate of ECs by repressing venous fate in arterial ECs.
Ephrin-B2 (Arterial ECs)	Eph-B4 (Venous ECs)	Determines arterial and venous EC specialization.
HB-EGF (ECs)	ErbB1, ErbB2 (MCs)	Promotes MCs proliferation and protects from apoptosis, involved in Ang(s) regulation.
HGF (ECs, MSCs)	C-met (ECs, SMCs)	Induces SMC migration, mediating angiopoietins regulated vessel stabilization.
MCP-1 (ECs, MCs)	CCR-2 (MCs)	Mediates spontaneous and Ang-1 induced MC recruitment.
PDGF-BB (ECs)	PDGFR-β (MCs; MSCs)	Promotes MC migration and proliferation; Induces MSCs differentiation to MCs.
S1P (Platelets)	S1P1 (MCs)	Activates adhesion molecule connecting ECs and MCs; directs MC recruitment and association to vessels.
TGF-β (ECs; MCs)	Alk1 (ECs; MSCs)	Promotes EC migration and proliferation; Inhibits vessel maturation and MC differentiation.
	Alk5 (ECs; MSCs)	Induces MC differentiation and ECM production; Inhibits EC migration and proliferation.
	Endoglin (ECs)	Modulates Alk-1 and Alk-5 mediated pathway by favoring Alk-1.
VEGF (Macrophages; MCs; fibroblasts)	VEGFRs (ECs)	Enhances EC survival by inducing Bcl-2 expression; Inhibits MC association and increases vessel permeability.

Table 1. Signaling Pathways Regulating Vessel Stabilization

Many of the signaling pathways mentioned above work in a coordinated fashion to regulate vessel stabilization. For example, MCP-1 was found to be one of the many cytokines released during angiopoietin induced MC recruitment.(Aplin *et al.*, 2010) Other cytokines involved in angiopoietin function include EC-derived heparin binding-epidermal growth factor (HB-EGF) (Iivanainen *et al.*, 2009; Stratman *et al.*, 2010) and hepatocyte growth factor (HGF) (Kobayashi *et al.*, 2006). PDGF-BB was found to promote MC proliferation partially by inducing the release of fibroblast growth factor-2 (FGF-2), which in turn transactivates FGFR-1. (Millette *et al.*, 2006) PDGF-B stimulation also induces an acute production of TGF-β, which in turn, negatively regulates Ang-1 expression induced by the PDGF-B stimulation. (Nishishita & Lin, 2004) Thus, a complex interaction of multiple signaling pathways is required to maintain vascular homeostasis and vascular stabilization.

3. Tissue engineering strategies

Extensive attention has been given to tissue engineering for its potential to restore, maintain or improve tissue functions. (Langer & Vacanti, 1993) However, the clinical application of engineered tissues requires the ability to form extensive, stable microvascular networks within engineered tissues. (Brey & McIntire, 2008) While strategies for neovascularization, including angiogenesis, vasculogenesis and arteriogenesis, have been extensively reviewed elsewhere (Brey *et al.*, 2005; Ennett & Mooney, 2002; Sneider *et al.*, 2009), increased attention is being given to the later stage of neovascularization, which is the maturation and stabilization of newly formed vessels. Different tissue engineering strategies, including protein delivery, gene therapy and cell therapy (Table 2) have been developed and tested in both *in vitro* and *in vivo* models.

	Advantages	**Disadvantages**
Protein Delivery	Better dose control than gene therapies; Temporal control over release can be achieved with certain delivery systems.	Expensive to produce and isolate proteins; Short protein half-lives *in vivo*;
Gene Therapy	Long-term active growth factor expression; Delivery restricted to area of modified cells	Safety issues based on delivery methods; Difficult to control the level and duration of gene expression; Limited transfection efficiency.
Cell Therapy	Introducing cell population that is sensitive to stimuli unlike many cells in the disease/damaged tissue Cells can stimulate neovascularization by incorporation into new vessels or release of soluble factors	Costly and time consuming for cell isolation and expansion; Limited cell sources for autologous transplantation; Immunosuppresive medications needed for allogeneic or exnogeneic transplantation.

Table 2. Advantages and Disadvantages of General Strategies for Promoting Stable Neovascularization in Engineered Tissues

3.1 Protein delivery

3.1.1 Single factor delivery

The formation of a stable vascular network during normal healing involves a complex spatial and temporal regulation of a large number of angiogenic stimulators and inhibitors. (Jain, 2003) While it may seem logical that all of these factors would be required in order to generate vessels in engineered tissues, researchers have attempted to initiate the entire process through the delivery of a single factor. Members of the VEGF and FGF families were some of the first identified angiogenic growth factors and have been used in clinical trials for the treatment of limb ischemia. (Cao *et al.*, 2005) However, the short term delivery of basic FGF (FGF-2) or VEGF may result in immature vessels, stable for only a short time prior to regression. (Dellian *et al.*, 1996) Based on these issues others have chosen to use factors with a known role in angiogenesis stabilization. For example, PDGF-BB, Ang-1, HGF, and ephrin-B2 are some of the factors currently under investigation in animal and *in vitro* models. (Brey & McIntire, 2008)

It is possible that the short lifetime and immaturity exhibited by vessels stimulated in these cases is not the result of using only a single factor, but instead due to the transient nature of their presence. The short lifetime of proteins and their rapid transport out of tissues can results in only a short-term stimulus. A single protein delivered in a controlled manner may be able to provide enough signal to prolong the vascularization response and, possibly, increase vessel maturation. A number of delivery systems, including natural and synthetic biomaterials, have been developed for growth factor delivery. Commonly used materials include naturally occurring materials, such as alginate, gelatin and fibrin, as well as synthetic materials, such as poly (lactic-co-glycolic acid) (PLGA), and modified polyethylene glycol (PEG). Sustained growth factor delivery can be achieved and controlled using those materials in the form of hydrogels, micro/nano particles, fibers, porous matrix, or their combinations. (Chen & Mooney, 2003; Zisch *et al.*, 2003a) The materials can be used to modulate delivery via diffusive resistance or through direct interactions between the materials and the growth factors. For example, the release of FGF-1 encapsulated in alginate microbeads can be modulated based on alginate composition. The release of FGF-1 from this relatively simple biomaterial can result in significantly prolonged increase in vascular density compared to a bolus injection of FGF-1. The enhancement in neovascularization can persist for greater than 6 weeks. In addition, release of FGF-1 from alginate can lead to a greater number of capillaries interacting with MCs but the fraction of MCs was not changed. (Moya *et al.*, 2009, 2010)

In addition to diffusion-controlled release, molecules can also be covalently immobilized to a polymeric surface or matrix for growth factor delivery and presentation. For example, VEGF has been covalently bound to fibrin gels resulting in cell demanded release. The immobilized VEGF cannot diffuse out and is only released as the fibrin is degraded. Compared to VEGF that diffuses from the fibrin gels, which formed leaky and chaotic vessels, the VEGF immobilized fibrin gels were able to stimulate the formation of nonleaky, organized vasculature with MC coverage and BM deposition. (Ehrbar *et al.*, 2004) Synthetic polymer matrixes have also been used for immobilized growth factor delivery. PEG hydrogels modified with MMP sensitive degradation sequences and cell adhesion sequences have been used in combination with a number of growth factors, including VEGF (Leslie-Barbick *et al.*, 2011; Moon *et al.*, 2010; Zisch *et al.*, 2003b), Ephrin-A1 (Moon *et al.*, 2007), and PDGF-BB (Saik *et al.*, 2011). Capillary-like structures were formed only in the contact area with the immobilized VEGF *in vitro*, while after *in vivo* implantation, functional vessels were

formed with MC coverage. (Moon *et al.*, 2010) Compared to single dose delivery, the sustained delivery of growth factors allows a more precise spatial control over the formation of vascular networks and may be able to lead to a more persistent vascular response and mature vessel formation even in the absence of additional maturation factors.

3.1.2 Multiple factors delivery

Since the formation of a stable vascular network appears to require the collaboration of a number of growth factors, the delivery of a combination (cocktail) of angiogenic molecules has been investigated as a method for improved vascular maturation. For example, co-delivery of both FGF-2 and VEGF from an accellular collagen-heparin scaffold resulted in higher vessel density and more MC covered mature vessels, compared to single factor delivery. (Nillesen *et al.*, 2007) In addition, some of the molecules involved in the angiogenesis stabilization signaling pathways (Table 1) have been delivered to the target tissue together with stimuli involved in the initiation step. PDGF-BB, a signaling protein stimulating MC proliferation and recruitment, has been used in combined delivery with VEGF (Chen *et al.*, 2007; Richardson *et al.*, 2001) and FGF-2 (Cao *et al.*, 2003; Lu *et al.*, 2007). Tested in many animal models, the dual delivery of PDGF-BB, a vessel maturation factor, with VEGF or FGF-2 resulted in not only an increase in vessel quantity, but also improved vessel quality. In a mouse corneal micropocket model, the dual delivery of PDGF-BB with FGF-2 led to stable vessels lasting for more than a year, while vessels formed by single delivery of PDGF-BB or FGF-2 regressed within 70 days (Cao *et al.*, 2003). Other vessel maturation factors, such as Ang-1 (Peirce *et al.*, 2003) and S1P (Tengood *et al.*, 2010), have also been investigated in combined delivery systems with VEGF resulting in stable, long-lasting vessels compared to VEGF delivery alone.

Moreover, it appears that in dual factor delivery, the sequence of the molecules administrated matters. Angiogenic factors, such as VEGF and FGF, are involved in the initial stages of angiogenesis primarily acting on ECs, while vessel maturation factors, such as PDGF-BB, Ang-1 and S1P, are involved in the later stage of vessel stabilization, mostly targeting MCs. Therefore, a temporal separation for distinct kinetics is likely preferred with initial administration of angiogenic factor(s) followed by a vessel maturation factor(s). The sequential delivery of VEGF followed by S1P (Tengood *et al.*, 2010) and FGF-2 followed by PDGF (Tengood *et al.*, 2011) resulted in increased vessel density and stability compared to simultaneous dual delivery. A reverse in the sequential delivery results in decreased vessel density and stability, presumably due the factors working against each other. It was proposed that early delivery of vessel maturation factors restricts EC tube formation and expansion, resulting in decreased vessel density and vessel diameter. The late delivery of angiogenic factors was thought to then inhibit MC recruitment, resulting in excessive formation of immature vessels susceptible to regression. (Tengood *et al.*, 2010, 2011)

With certain modifications, sequential delivery of multiple growth factors can be achieved with delivery materials. For example, alginate can be made into multilayer microbeads with an internal region for slow protein release and external layer for faster protein release. (Khanna *et al.*, 2010) PLGA, a biodegradable copolymer, can be made into microspheres encapsulated porous matrix, in which the microspheres are loaded with factor required for later stage of angiogenesis (PDGF-BB) with slow release kinetics, while the porous matrix is loaded with factor required for initial stage of angiogenesis (VEGF) with fast release rate.

(Carmeliet & Conway 2001; Richardson *et al.*, 2001) Although the temporal and spatial kinetics of the growth factor(s) delivered to the target tissues can be controlled using certain delivery systems, there are still limitations existing with this strategy. The production and purification of massive amounts of active protein factors can be very expensive. And since most growth factors have relatively short half-lives, their bioactivity can be compromised due to storage, delivery routes, and metabolism *in vivo*. Moreover, the optimal combination and concentration of growth factors for different engineered tissues and the any side effect after long-term administration still need to be identified.

3.2 Gene delivery

3.2.1 Genes

While the use of protein delivery offers improved control of the dose for the growth factors, there are also some disadvantages for those strategies, including expensive mass production of the proteins, and short protein half lives *in vivo*. Those limitations can potentially be overcome by means of gene therapy. (Rissanen *et al.*, 2001) Gene therapy allows prolonged overexpression of proteins, theoretically leading to sustained therapeutic effects after a single application, with minor change in systemic growth factor concentrations. (Gosh *et al.*, 2008) Therefore, DNA sequences encoding for growth factors involved in angiogenesis can be used to improve neovascularization. In this section, we will only review genes used directly to the testing model or patients, either by systemic or local injection, or release from polymeric system. Genetic modification to cells for neovascularization will be introduced in the following section, cell therapies.

Transient *vegf* gene expression *in vivo* results in potent angiogenic sprouting, however, the resulting vasculature appears to be immature and leaky, similar to the results of VEGF protein delivery. (Rissanen *et al.*, 2003) When *vegf* gene is co-administrated with genes encoding angiogenic maturation factors, such as *pdgf-b* (Hao *et al.*, 2004a; Korpisalo *et al.*, 2008; Kupatt *et al.*, 2010) and *ang-1* (Chen *et al.*, 2007; Siddiqui *et al.*, 2003; Su *et al.*, 2009), the vasculature is longer-lasting and less permeable,with increased perfusion, which is also similar to the results of dual protein growth factor delivery. Other genes delivered for stable vascular networks formation includes *ang-1* (Shyu *et al.*, 1998), *pdgf-b* (Shea *et al.*, 1999), *fgf-2* with *pdgf-b* (de Paula *et al.*, 2009; Hao *et al.*, 2004b), *inf-β* (Dickson *et al.*, 2007), and *neutrophin-3* (Cristofaro *et al.*, 2010).

3.2.2 Vectors

Vectors are needed in order for the genetic material to be transferred into cells and expressed. Commonly used vectors for gene delivery are plasmids and viral vectors. (Rissanen *et al.*, 2001) Plasmids are DNA sequences that can automatically repeat and express in a host cells. They are easy to manufacture in a large quantity, and have low toxicity with a low immune response. However, naked plasmid DNA usually has very low transfection efficiency except in muscle tissue, therefore biomaterials have been used to increase plasmid-based gene transfer efficiency. (Yia-Herttuala & Alitalo, 2003) For example, PLGA, a degradable copolymer that has been widely used as a tissue engineering scaffold and drug delivery vehicle, has been applied as a carrier for plasmid. PLGA can be made into nanoparticles with the modification of cationic polymer chitosan to increase plasmid transfer efficiency. (Tahara *et al.*, 2008) It can also be prepared as a matrix for sustained plasmid delivery, which in turn, results in longer term growth factor expression. (Nie *et al.*,

2008) Viral vectors, on the other hand, have much higher transfection efficiency compared to naked plasmids, but have significant safety concerns. Adenoviruses (Levanon *et al.* 2006) and adeno-associated viruses (AAV) (Arsic *et al.*, 2003) are the most commonly used viral vectors for growth factor gene delivery. While adenoviral vectors may cause inflammatory reactions and lack sustained expression, AAV only causes a very mild immune response and has shown long-term transgene expression, which makes it more attractive in gene therapy. (Dickson *et al.*, 2007)

Despite the success of gene therapies for the formation of stable vasculature in some animal models, clinical efficacy has yet to be observed. The pharmacokinetics and pharmacodynamics of gene vectors and products remain largely unknown and difficult to control. In addition, data from long-term follow up of clinical studies is lacking. (Yia-Herttuala & Alitalo, 2003) Moreover, the method of gene delivery must be carefully selected to balance the need for maximum transfer efficiency with minimum risk of vector related safety issues. (Brey & McIntire, 2008)

3.3 Cell therapies

Although the delivery of growth factors or their genes has shown great potential to activate the host angiogenic response, certain pathological conditions may also require the transplantation of appropriate blood vessel forming cells, due to loss of, or damage to, the host vascular cells. In addition, these cells may rapidly assemble into vessels which can inosculate with the host, decreasing the time required for tissue perfusion relative to protein/gene approaches. In cell therapies, the cells would have to be isolated from donor or host sources, expanded and implanted into the target tissue, where the cells proliferate and incorporate into functional vessels. (Ennett & Mooney, 2002) ECs are of great interest in this strategy for their potentials to mimic vasculogenesis and assemble into capillary structures, as well as to release multiple angiogenic factors following implantation. (Brey *et al.*, 2005, 2008) Transplantation of mature ECs have been shown to increase neovascularization in engineered tissues. (Nor *et al.*, 2001) However, the limited availability and proliferation capability of mature ECs limtis this approach. (Kim & von Recum, 2008) Therefore, a number of other cell types and modifications may be needed in addition to the used of mature ECs for stable vasculature formation.

Recent preclinical studies have shown that stem and progenitor cells derived from embryos or adult bone marrows have the potential to restore tissue vascularization after ischemic events. (Rafi & Lyden, 2003) Unlike mature cells, stem and progenitor cells have the potential to self-renew and differentiate into multiple cell types. While mature ECs seeded into collagen-fibronectin gels and implanted into different animal models suffered from poor perfusion and fast regression (Au *et al.*, 2008b; Koike *et al.*, 2004), ECs derived from human embryonic stem (hES) cells formed durable and functional blood vessels *in vivo*, supporting perfusion for over 150 days. (Wang *et al.*, 2007) Co-delivery of ECs with mesenchymal stem cells (MSC) can also result in stable and functional vessels, lasting for more than 130 days, which was not achieved when either of the cell types were used alone. (Au *et al.*, 2008a)

Endothelial progenitor cells (EPCs) derived from circulating blood or bone marrow have also been used to introduce neovascularization *in vivo*. (Park *et al.*, 2004) However, in order to form stable and durable vascular network, EPCs are often co-implanted with other cell types, such as VSMCs (Melero-Martin *et al.*, 2007) or mesenchymal progenitor cells (MPCs)

(Melero-Martin *et al.*, 2008). EPCs are expected to form the lumen of new vessels and VSMCs/MPCs serve as perivascular cells adjacent to the lumen providing survival and stability signals. EPCs derived from adult peripheral blood (PB) as well as umbilical cord blood (CB) have been compared for their ability to form functional long-lasting vessels *in vivo*. When co-implanted with 10T1/2 cells, a type of mouse MPCs, PB-EPCs and CB-EPCs both formed new vessels. While PB-EPCs formed vessels were unstable and regressed in 3 weeks, CB-EPCs formed normal-functioning vessels that lasted for more than 4 months, possibly due to a greater proliferative capacity of these cells. (Au *et al.*, 2008b) Therefore, CB-EPCs hold great potential for their ability to form stable vasculature in engineered and regenerated tissues. Adipose-derived stromal cells (ASCs) have also been investigated as another potent candidate for neovascularization cell therapy, due to their ability to stimulate angiogenesis as well as vessel maturation *in vivo*. (Rubina *et al.*, 2009)

Cell therapies can also be used in combination with single or multiple growth factors or their genes to improve the function of the transplanted cells. Stromal cell derived factor-1 (SDF-1), a small chemokine involved in recruiting EPCs, was delivered in combination with EPCs to promote EPC engraftement in ischemic muscle. (Kuliszewski *et al.*, 2011; Yu *et al.*, 2009) Dual delivery of VEGF and MCP-1 from alginate microbeads has been shown to support EC transplantation, with VEGF improving survival of transplanted ECs and MCP-1 inducing MCs recruitment. (Jay *et al.*, 2010) A similar effect was observed when co-delivering FGF-2 and granulocyte-colony stimulating factor (G-CSF) with bone marrow cells transplanted in a rodent model of critical limb ischemia. While FGF-2 directs EC migration and proliferation, G-CSF promotes the homing of bone marrow stem cells (in particular EPCs) to the ischemic site. (Layman *et al.*, 2011) PLGA microparticles loaded with VEGF and hepatocyte growth factor (HGF), as well as Ang-1, were delivered in combination with cord blood derived vasculogenic progenitor cells. The delivery of triple growth factors significantly enhanced the effect of cell therapy in multiple animal models, with increased progenitor cell incorporation, improved vessel function, and stabilization. (Saif *et al.*, 2010)

In addition to combined gene or protein delivery with cells, ECs and EPCs can also be genetically modified *ex vivo* prior to implantation to enhance vascular stabilization. For example, Bcl-2 is an anti-apoptosis protein that is upregulated during angiogenesis. ECs transfected with the *bcl-2* gene to inhibit EC apoptosis have shown increased vascular density and stability. Vessels formed with *bcl-2* transduced ECs connect with host circulation and last for at least 2 months. (Enis *et al.*, 2005; Schechner *et al.*, 2000; Shepherd *et al.*, 2009) EPCs with *vegf* gene overexpression have been shown to increase EPC migration and reduce serum starvation-induced apoptosis *in vitro*. (Yu *et al.*, 2009) When implanted *in vivo*, the *vegf* transfected EPCs stimulated greater blood flow and angiogenesis in animal models of ischemia than EPCs alone. (Ikeda *et al.*, 2004)

In spite of the promising results seen with cell therapies, certain limitations still exist. First, the sources of cells for transplantation, either mature cells or progenitor cells, can be problematic. If autologous cells have to be used, there are usually limited number of cells available, not to mention that their viability and activity are compromised due to disease or aging of the patient. (Brey & McIntire, 2008) If allogeneic and xenogeneic cells can be used, they would initiate a host immune response, which requires immunosupressive medication, making the patient vulnerable to infection and other diseases. Secondly, the expansion of isolated cells *ex vivo* can be costly, time-consuming and technically challenging. If mature

ECs and VSMCs are used for transplantation, it often requires longer times to reach a sufficient number, due to their low self-renewal capability. Stem and progenitor cells, on the other hand, have much higher proliferation rate. However, the expansion of these cells while maintaining their multipotency may be a technical challenge. (Ennett & Mooney, 2002) In addition, determination of the optimal cell types and concentration, delivery route, as well as identifying growth factors or genes to couple with cell transplantation requires further research. And lastly, the exact roles and ultimate fates of the transplanted cells remain largely unknown. Whether or not the transplanted cells would cause some other adverse complications, such as tumor angiogenesis, atheroma formation and retinopathies, is still unknown. (Rafii & Lyden, 2003) Those questions need to be answered with preclinical studies before proceeding to extensive clinical applications.

3.4 Analytical methods

3.4.1 *In vitro* models

In order to investigate the efficacy of different tissue engineering strategies for neovascularization, a serial of preclinical tests should be done with different analytical models before jumping to any conclusion and proceeding with patients. *In vitro* models are usually used to allow the evaluation of a single or a combination of factors (protein, gene or cells) in a relatively simple, robust, and controlled system. Most early *in vitro* models of neovascularization only used ECs to spontaneous form tubes in a 2D or 3D environment to mimic angiogenesis or vasculogenesis. (Francis *et al.*, 2008) Those models have been used to investigate the effect of soluble or insoluble factors, as well as genetic manipulation to the formation of new vessels *in vitro*. However, since the stabilization and remodeling stage of angiogenesis involves the interaction between ECs and MCs, many later studies have incorporated SMCs co-cultured with ECs. ECs and SMCs with a defined number can be co-cultured as a spheroid and put into a 3D scaffold. By fluorescently labeling the two cell types with different dyes, the cells can be visualized and distinguished under fluorescence microscope. The coculture spheroid can spontaneously organize into a core of SMCs and a surface layer of ECs, which is a spherical mimic of cylindrical blood vessels where there is a surface of ECs and coating MCs. (Korff *et al.*, 2001) This model allows study of the interactions between ECs and SMCs and their roles in angiogenesis and vessel stabilization. (Brey *et al.*, 2005)

Another *in vitro* model commonly used in studying angiogenesis and vessel stabilization is called aortic ring assay. (Nicosia & Ottinetti, 1990) The model results from the isolation of a 1-2 mm cross-section of aorta from rats or mice which is then embedded in a 3D biomaterial. Under certain stimuli, new vessels form with an inner core of ECs and a outer layer of MCs, which can then be visualized by immunofluorescent staining for different cellular markers. By treating the culture with vessel maturation factors, such as Ang-1 and MCP-1, increased MC recruitment was observed in this model. (Aplin *et al.*, 2010; Iurlaro *et al.*, 2003) However, since the model uses an organ part with multiple cell types, many other undefined cell types can also contribute to the network formation. It is difficult to isolate the contribution of each cell type in this model. (Brey *et al.*, 2005)

3.4.2 *In vivo* models

While the *in vitro* models provide convenient evaluation methods for the study of vessel assembly, the controlled environment differs dramatically from the actual *in vivo*

environment where other factors, such as blood flow, inflammatory response, endocrine regulation, etc. contribute to the process. Therefore, studies with *in vitro* models are often followed by *in vivo* studies. A number of *in vivo* models have been developed to isolate the process of neovascularization, with different aspects of focus in different organ systems. Commonly evaluated parameters in most *in vivo* models include: vessel density, SMC coverage rate, vessel lasting or regression time, vessel size distribution, blood perfusion rate, vessel permeability, etc.

Subcutaneous implantation of scaffold materials is a commonly used method to evaluate protein, gene or cell therapies. (Jay *et al.*, 2010; Shea *et al.*, 1999; Tengood *et al.*, 2010) Growth factors, genes or cells can be embedded a matrix material, which will then be implanted subcutaneously to animals as a "plug". The presence of therapeutic agents will affect local neovascularization and tissue invasion into the material. At different time points after the implantation, local tissues will be harvested for evaluation. Typically histologic analysis of vessel density, diameter, and SMC coverage are performed, but more sophisticated techniques such as confocal microscopy, vascular casts, and microCT following contrast enhancement can be used to provide a better understanding of vascular structure. This model is relatively easy to use, and can be applied to evaluate different therapeutic strategies. However, all animals need to be sacrificed in order for the neovascularization assessmment, and it only provides the evaluation of some basic parameters. Therefore, other animal models are often used to provide greater detail into the process.

The mouse corneal micropocket assay allows for monitoring neovascularization without sacrificing animals. The method has been used by Cao *et al.* for the evaluation of vascular stability in response to dual growth factor delivery. Briefly, growth factor-loaded micropellets are implanted into a mouse corneal micropocket. Vessel lengths and vascularization areas of the eyes can be measured at various time points. The method allows the observation of neovascularization frequently without invasive surgery, thus limiting the number of animals used. Moreover, it allows long-term observation of vessel persistence. This method has only been used for evaluating growth factors or gene delivery, either as soluble factors (Cao *et al.*, 2003; Lai *et al.*, 2001; Lu *et al.*, 2007) or immobilized in a hydrogel (Moon *et al.*, 2010; Saik *et al.*, 2010). Use of this model to evaluate cell therapies is still rare to our knowledge. On the other hand, cranial windows model of severe combined immunodeficient (SCID) mouse has been used to evaluate cell therapies. (Au *et al.*, 2008a,b; Wang *et al.*, 2007) Similar to the corneal model, the cranial windows model also allows observation of neovascularization for several months without sacrificing the animals.

Mice, rats or rabbits hindlimb ischemia models have been used to evaluate the efficacy of protein delivery, gene therapy and cell therapy in neovascularization. (Cao *et al.*, 2003; de Paula *et al.*, 2009; Laymen *et al.*, 2011) The model applies ligation to the femoral artery and its branches to create ischemia in one limb. This model attempts to mimic peripheral arterial occlusive disease (PAOD), however this model only recreates the ischemic event and not the chronic vascular disease that lead to the condition in humans. After a certain treatment strategy, a number of neovascularizaiton parameters can be assessed, including flow recovery rate. The intensity of blood perfusion in the created ischemic area can be measured with different imaging techniques include laser Doppler or after systemically injection of fluorescent- or radio-labeled particles. Another ischemic model is the myocardial infraction heart model. Instead of femoral artery, the left anterior descend

artery (LAD) is ligated in the animal to create ischmia. (Hao *et al.*, 2004) Besides common neovascularation parameters, cardiac morphology and function can also be evaluated using this model.

There are also a number of other animal models used in evaluating angiogenesis and vascular stability. The chorioallantoic membrane (CAM) model with chicken or quail embryos is a simple method to evaluate vascularization in response to different therapies. (Saif *et al.*, 2010; Stratman *et al.*, 2010) After therapeutic agents injection or implantation, the angiogenic effect can be easily assessed by imaging vascular regions of the CAM. This model is cost efficient and easy to perform compared to other mammalian animal models. Suitable animal models should be selected based on the target function of the therapeutic strategy. In most cases, one single animal model may not be sufficient to testing all the aspects of interest in neovascularization, therefore multiple *in vivo* models may be required to provide extensive understanding of the potential strategy. In addition, if the technique is ultimately prepared for a specific tissue engineering application then the appropriate pre-clinical model of the diseased or damaged tissue should be used.

4. Conclusions

The formation of stable vascular networks is critical for the survival of regenerated tissue in tissue engineering applications. In many cases, current strategies can result in immature vessel formation, providing poor perfusion and eventually leading to vessel regression. Recent studies have focused more on the later stage of neovascularization, which is the stabilization and remodeling of new vasculature. The process is highly regulated by both soluble and insoluble factors. Studies in the cellular and molecular mechanisms involved in vascular maturation have provided insight towards the development of strategies for the formation of stable vascular networks in engineered tissues. Successes have seen in many preclinical studies with these strategies; however, future success requires collaboration from interdisciplinary fields, including molecular and cellular biology, engineering and medicine.

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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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