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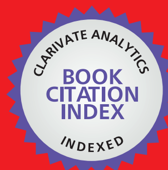

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Angiogenesis and Vascularity for Tissue Engineering Applications

Remo A. Largo[1], Venkat M. Ramakrishnan[1], Martin Ehrbar[2], Algirdas Ziogas[2], Jan A. Plock[3] and Daniel Eberli[1]
[1]Division of Urology, University Hospital Zurich
[2]Division of Obstetrics, University Hospital Zurich
[3]Division of Plastic and Hand Surgery, University Hospital Zurich
Switzerland

1. Introduction

Tissue engineering is a field of medicine that has experienced significant growth in prominence over the past three decades. Though traditional interventions exist for many medical maladies, tissue engineering aims to combat such disorders through the synthesis of body tissues and organs, resulting in functional implants. Tissue engineering takes an innovative approach, often utilizing autologous stem cells for tissue construction or materials that are biocompatible while avoiding immune rejection (Nomi *et al.*, 2002).
Despite its immense successes, a major hurdle still faces tissue engineering. Large volumes of implanted tissue are unable to stimulate the formation of necessary blood vessels required for their survival. In the body, naturally occurring, equivalent vascular networks serve vital functions in gas and nutrient exchange, metabolic processes, and waste expulsion. Though individual, large vessels have been successfully engineered for implant, it is still exceptionally difficult to fashion a stable and sustainable network of vessels for large volumes of tissue (Nomi *et al.*, 2002). Neovascularization after tissue damage requires a level of positive and negative control that has not been successfully replicated in a laboratory environment to date. As such, rapid *de novo* synthesis of a controlled, established vascular network remains a challenge today.
Angiogenesis is the morphogenic process of forming new blood vessels from pre-existing ones (Laschke *et al.*, 2006; Dai and Rabie, 2007; Li and Rabie, 2007). This event plays an important, normal physiological role in wound healing, tissue repair, pregnancy, and exercise (Ferrara and Davis-Smyth, 1997), and exists in contrast to vasculogenesis (the formation of the every first blood vessels in the body, and especially predominant in embryological development). Yet, the abuse of angiogenesis, leading to an uncontrolled vascular formation as a consequence of epigenetic influence, nucleotide polymorphisms, or endocrine irregularities can also result in tumor formation (Verbridge *et al.*, 2010). However, angiogenesis is clearly an activity that is central to development and tissue maintenance. Successful modulation of angiogenesis can have profound therapeutic outcomes for organs and tissues deprived of an adequate, stable vasculature. Studies from the last two decades have shown that the manipulation of various factors directly influences angiogenic outcome.

Angiogenesis requires the activity of soluble factors such as Vascular Endothelial Growth Factor (VEGF; outlined in the next section and in Tables 2 & 3), basic Fibroblast Growth Factor (bFGF), Platelet-derived Growth Factor (PDGF), Transforming Growth Factor-β (TGF- β) (Nomi *et al.*, 2002; Dai and Rabie, 2007; Li and Rabie, 2007; Kanczler and Oreffo, 2008; Bates, 2010), Keratinocyte Growth Factor (KGF) (Elia *et al.*, 2010), Hepatocyte Growth Factor (HGF) (Hoot *et al.*, 2010), Ephrin-B2 (Herbert *et al.*, 2009), and Angiopoietin(Han *et al.*, 2010) (Table 1). Morphologically, angiogenesis can be attributed to endothelial cell migration and proliferation as well as pericyte recruitment, migration and differentiation (Egginton, 2010).

Molecule	Known Properties	Citations
Basic Fibroblast Growth Factor (bFGF)	Stimulates activity of fibroblasts, neurons, smooth muscle cells, and endothelial cells; acts via tyrosine kinase receptors; induces production of VEGF during angiogenic stimulation.	(Pepper *et al.*, 1992; Lee *et al.*, 2003; Arkudas *et al.*, 2007; Jung *et al.*, 2010; Wu *et al.*, 2010)
Platelet-derived Growth Factor (PDGF)	Certain isoforms possess survival and mitogenic functions; implicated in tumor angiogenesis; compared to VEGF, PDGF: (a) has comparable angiogenic activity, (b) produces blood vessels with decreased permeability and leakage, and (c) is thought to produce functionally different blood vessels.	(Li *et al.*, 2010; Wu *et al.*, 2010)
Transforming Growth Factor β (TGF-β)	Promotes and inhibits angiogenesis and tumor invasion via stimulation of Hepatocyte Growth Factor (HGF) expression.	(Hoot *et al.*, 2010)
Keratinocyte Growth Factor (KGF)	Member of FGF family; certain isoforms have been implicated in wound healing as well as the inhibition of neovascularization.	(Wang *et al.*, 2010)
Hepatocyte Growth Factor (HGF)	Stimulates endothelial cell growth, migration, scatter, and elongation independently of VEGF.	(Hoot *et al.*, 2010)
Ephrin-B2	Implicated in arterial/venous differentiation (see section: Modulation of Notch Signaling).	(Herbert *et al.*, 2009)
Angiopoietin	Signals through Tie receptors; certain isoforms are angiogenic inhibitors, while others promote inhibition of apoptosis; Stabilize blood vessels and reduce leakage.	(Han *et al.*, 2010)

Table 1. Notable Ancillary Growth Factors and Their Properties

2. VEGF and its receptors: a brief overview

In studying the development of cells, tissues, and organs, Vascular Endothelial Growth Factor (VEGF) has been identified as a key, though not sole, proponent of angiogenesis (Takahashi and Shibuya, 2005; Arkudas *et al.*, 2007; Dai and Rabie, 2007; Yla-Herttuala, 2009;

Bates, 2010; Elia *et al.*, 2010). VEGF exists in six classes (Table 2), lettered A through F, though the vast amount of literature concerning VEGF-A indicates that it is by far the most widely understood of all. VEGF-A exists in at least eight homodimeric isoforms, all conceived by alternative mRNA splicing(Ferrara, 2009) and differing by their amino acid number. Of these, $VEGF_{121}$ and $VEGF_{165}$ are predominant and show great promise in tissue engineering applications.

VEGF Class	Known Properties	Citations
A	Induces proliferation of arterial, venous, and lymphatic vascular endothelial cells; stimulates monocyte chemotaxis; hematopoetic effects; eight isoforms in humans.	(Ferrara, 2009)
B	May play role in atrial conduction, but not required for cardiovascular development.	(Olofsson *et al.*, 1996a; Olofsson *et al.*, 1996b; Aase *et al.*, 2001)
C	Involved in embryonic angiogenesis, lymphangiogenesis, & lymphatic vessel maintenance; mitogenic for cultured endothelial cells.	(Kukk *et al.*, 1996; Orlandini *et al.*, 1996; Jeltsch *et al.*, 1997; Yamada *et al.*, 1997; Dumont *et al.*, 1998)
D	Thought to be involved in pulmonary development, endothelial cell mitogen; Function(s) still generally unclear.	(Orlandini *et al.*, 1996; Yamada *et al.*, 1997; Achen *et al.*, 1998; Farnebo *et al.*, 1999)
E	Proteins are encoded by Orf-viruses and predominantly expressed in sheep, goats, and rarely in humans.	(Lyttle *et al.*, 1994; Meyer *et al.*, 1999)
F	Derived from snake venom; enhances the formation of vascular fenestrations in guinea pigs.	(Klein and Catargi, 2007; Matsunaga *et al.*, 2009; Yamazaki *et al.*, 2009)
Placental Growth Factor (PlGF)	Induces vascular permeability; supplements VEGF activity during wound-healing; may enhance VEGF-driven angiogenesis.	(Carmeliet *et al.*, 2001; Adini *et al.*, 2002; Hattori *et al.*, 2002; Luttun *et al.*, 2002; Odorisio *et al.*, 2002)

Table 2. Six General VEGF Classes

The binding of VEGF to any of its many receptors, such as VEGF-R1 (Flt-1) (Shibuya *et al.*, 1990; de Vries *et al.*, 1992), VEGF-R2 (KDR) (Terman *et al.*, 1991), VEGF-R3 (Flt-4) (Fitz *et al.*, 1997), and Neuropilin (NRP-1 and -2) (Soker *et al.*, 1996; Soker *et al.*, 1998; Dallas *et al.*, 2008), has been shown to trigger a signaling cascade that results in the activation of angiogenesis (Stefanini *et al.*, 2009). Not surprisingly, VEGF receptor and ligand placement and density have been implicated in the successful transmission of the signaling cascades required for angiogenesis (Stefanini *et al.*, 2009). Modulating receptors and their densities appear to be a logical avenue for further efforts in modulating neovascularization. Further, Heparin Sulfate Proteoglycan (HSPG) has been implicated in the binding of VEGF to their receptors, but its exact function remains unclear (Ferrara *et al.*, 2003; Lee *et al.*, 2010). Preliminary findings suggest that HSPGs inhibit VEGF binding to its receptors, and consequently, angiogenic activity (Lee *et al.*, 2010).

3. Modulations to VEGF and its delivery

Though VEGF has a significant influence on cell migration, proliferation, and vasodilation, the uncontrolled or sole use of VEGF *in vivo* has been shown to result in the disordered growth of blood vessels into a dense mass (hemangioma), malignant tumor angiogenesis, and the assembly of leaky vessels (Takahashi and Shibuya, 2005; Bates, 2010). As such, the fundamental function of VEGF in physiological development and maintenance, contrasted by its role in tumorigenesis and vessel instability, is paradoxical. In instances where VEGF is heterogeneously present in a microenvironment, areas of high VEGF expression resulted in abnormal angiogenesis. However, implanting VEGF-transfected myoblasts, with each cell equally producing VEGF over time, led to the formation of a stable, normal vascular network (Misteli *et al.*, 2010). The latter provides a compelling case for carefully-controlled VEGF release, distribution, and kinetics in tissue engineering applications. As discussed below, several current tissue-engineering efforts are positioned to resolve this dilemma.

It should not be assumed that blind administration of VEGF is solely responsible for a successful angiogenic effort. Many studies have illustrated that the promotion of successful angiogenesis depends on a prolonged exposure to a low dose of VEGF (Wernike *et al.*, 2010), while other studies claim that micro-environmental conditions must be taken into account (Ferrara and Davis-Smyth, 1997; Misteli *et al.*, 2010). With regards to the latter, techniques like FACS purification (Misteli *et al.*, 2010) and microdialysis (Hoier *et al.*, 2010; Marcus *et al.*, 2010) are becoming increasingly prevalent in closely monitoring VEGF expression in the microenvironment. Further, many attempts at eliciting controlled angiogenesis also focus on coupling the properties of VEGF with certain other growth factors – most notably bFGF (Arkudas *et al.*, 2007) or KGF (Elia *et al.*, 2010) (see Table 1); stable vessels were formed when VEGF was combined with either of these two factors.

There exist contradictions regarding the parameters of tissue exposure to VEGF. In one comparison, a study highlighting growth factor implementation in orthopedic applications suggested that VEGF delivery for over 14 days may have interfered in the vascularization during bone healing and restoration (Wernike *et al.*, 2010). This suggestion challenges an argument that a longer VEGF exposure (of approximately one month) was necessary for the production of stable, but leaky, vessels as brief exposure to VEGF (less than 15 days) resulted in the formation of unstable vessels. Further, these vessels actually degenerated after VEGF delivery cessation (Tafuro *et al.*, 2009). Disparities such as this are common in the

literature and demonstrate the sheer complexity of VEGF activity in various tissue environments.

The view that successful VEGF delivery and angiogenic response are strictly dose-dependent is challenged by factors such as delivery kinetics, which appear to be critical for proper vasculature (Borselli *et al.*, 2010; Wernike *et al.*, 2010). For instance, a characteristic problem with the bolus delivery of VEGF is the outcome of variable systemic effects (Matoka and Cheng, 2009) such as haemorrhage, hypotension, or flu-like symptoms (Benjamin *et al.*, 1999). This method of delivery also fails to achieve the prolonged supply of physiological low dose of VEGF necessary to produce a mature, lasting vascular network. The lack of lasting vessel formation observed in many therapeutic trials is probably due to difficulties in the delivery of VEGF, a growth factor with an apparently narrow therapeutic window (Hariawala *et al.*, 1996; Lee *et al.*, 2000; Dor *et al.*, 2003; Ozawa *et al.*, 2004), in the optimal time and dose for maintaining sufficient vascularity.

A need exists for biomaterials or stable scaffolds that enable slow, sustained VEGF release(Rocha *et al.*, 2008) for a predictable and functional outcome. Studies in different laboratories have indicated that incorporation of growth factor into slow-release polymer formulations could present a means for better control of dose, location, and duration of active signals in tissue (Edelman *et al.*, 1991; Lee *et al.*, 2000; Sheridan *et al.*, 2000; Ehrbar *et al.*, 2004). The kinetics of VEGF delivery today depends on the physical properties (such as cross-linking and porosity, for instance) of biocompatible conduits such as fibrin-gels (Ehrbar *et al.*, 2004; Arkudas *et al.*, 2007), gelatin microparticles (Patel *et al.*, 2008), collagen / fibronectin hydrogels (Glotzbach *et al.*, 2010), and PLG(A) scaffolds (Murphy *et al.*, 2000)(Rocha *et al.*, 2008; Matoka and Cheng, 2009; Borselli *et al.*, 2010; Golub *et al.*, 2010). Moreover, transfecting or transducing developing cells (muscle precursor cells or myoblasts, for instance) with VEGF and subsequently injecting them into a site can also provide a steady, longer-term delivery of the growth factor (Misteli *et al.*, 2010).

Once VEGF is coupled with a delivery conduit, a more intricate approach to controlling VEGF delivery involves modifying the factor itself. Extensive delivery-mechanics research was conducted with the isoform $VEGF_{121}$. Though $VEGF_{121}$ is initially confined within a biomaterial (such as a fibrin-gel matrix) upon implant, plasmin and metalloproteinase degradation of the implant over time allows for the rapid, free diffusion of $VEGF_{121}$ into the whole body environment (Ehrbar *et al.*, 2004), potentially resulting in angiogenesis that may not be localized. Additionally, the short biological half-life of VEGF impedes its use in long-term applications. A synthetic variant of $VEGF_{121}$, known as TG-$VEGF_{121}$, cross-links to fibrinogen by the transglutaminating activity of factor XIII during fibrin-gel polymerization. This covalently tethered TG-$VEGF_{121}$ is protected from rapid diffusion. Gradual degradation of the fibrin-gel by local fibrinolytic activities results in a local liberation of low levels of TG-$VEGF_{121}$ into tissue. Experimental animal models have shown that fibrin-conjugated TG-$VEGF_{121}$ produced more structurally stable vessels than $VEGF_{121}$ while avoiding vascular leakage (Ehrbar *et al.*, 2004; Ehrbar *et al.*, 2008).

4. Hypoxia-mediated control and modulation of notch signaling

Hypoxia presents another means of employing direct control on VEGF and ancillary angiogenic factors. In the low-oxygen environments of normal muscle, VEGF mRNA experiences decreased degradation and increased expression (Ikeda *et al.*, 1995; Levy *et al.*, 1995; Levy *et al.*, 1996; Tang *et al.*, 2004), though severely hypoxic settings will actually

impede VEGF up-regulation (Milkiewicz *et al.*, 2004). VEGF mRNA stability is made possible by its interaction with HuR, a complex that binds and stabilizes RNAs, consequently regulating gene expression (Levy *et al.*, 1998). The increase in VEGF production can be attributed to the Internal Ribosome Entry Site (IRES), which is accountable for efficient factor synthesis under hypoxic conditions (Stein *et al.*, 1998). Other proteins can also impact VEGF efficiency and activity. Control of expressed VEGF lies with ORP150 (oxygen regulated protein), a chaperone that transports VEGF from the cell's endoplasmic reticulum to the Golgi apparatus (Kuwabara *et al.*, 1996; Ozawa *et al.*, 2001). An increase in ORP150 levels correlates with an increased production of VEGF during hypoxia (Ozawa *et al.*, 2001). Further, Hypoxia-inducing factor (HIF) is responsible for activating the transcription of genes associated with neovascularization (Covello and Simon, 2004; Ramirez-Bergeron *et al.*, 2006). These proteins represent points of control for VEGF modulation.

Because angiogenesis is the synthesis of vasculature from existing vessels, constant remodeling and modification of vessels takes place *in vivo*. Existing endothelial cells, such as stalk and tip cells, have receptors that respond to environmental conditions like hypoxia, in which Notch signaling (driven by VEGF-A presence) is most prevalent. The presence of VEGF-A leads to an increase in the presence of Delta-like Ligand 4 (DLL4), a major Notch ligand. It is believed that Notch signaling modulates the ratio of VEGF to its receptors through the inhibition of VEGF-R2.

Manipulation of the Notch signaling pathway presents another means of controlling angiogenesis. Cao, et al., discussed the role of Notch signaling in modulating VEGF activity, resulting in effective pruning and branching of vascular vessels (Cao *et al.*, 2009; Cao *et al.*, 2010). Activation of the Notch signaling pathway inhibits VEGF signaling by down-regulating the VEGF receptor synthesis. With fewer available receptors for free VEGF to bind, endothelial cell proliferation is effectually curbed. This process occurs at the cellular level of the lining endothelium, a major component of vascular vessels, and vascular growth in any particular direction is controlled.

5. Applications in regenerative medicine: notable case studies

Recent advances in understanding the angiogenic process and isolating potent and specific angiogenic growth factors prompted their therapeutic usage. Evidence that VEGF is a specific endothelial cell growth factor suggested its potential in therapeutic angiogenesis. Injection of the $VEGF_{165}$ protein enhanced revascularization of rabbit ischemic hindlimbs (Takeshita *et al.*, 1994). VEGF treatment induced collateral vessel formation, endothelium-dependent blood flow and tissue perfusion. VEGF has been tested for potential beneficial effects on wound repair in diabetic animal models (Greenhalgh *et al.*, 1990; Tsuboi and Rifkin, 1990; Frank *et al.*, 1995). The growth factor treatment regimens accelerated granulation, tissue formation, and wound closure. However, due to the high clearance and/or degradation of the proteins from the administration site, topically administered growth factors would require high dosages and frequent delivery.

An alternative strategy for therapeutic angiogenesis is gene therapy using recombinant angiogenic growth factors. The first study using $VEGF_{165}$ cDNA was performed by gene transfer into the iliac artery of an ischemic hindlimb of a rabbit (Bauters *et al.*, 1994; Takeshita *et al.*, 1996). VEGF protein was expressed at the site of injection, augmenting the formation of collateral vessels. Subsequently, intramuscular gene transfer of VEGF cDNA

was used in a similar model with similar results (Asahara *et al.*, 1996). This technique was further employed in patients with peripheral vascular disease and critical limb ischemia (Isner *et al.*, 1996). Clinical trials showed significant improvement in collateral blood flow, healed ischemic ulcers, and most importantly, salvage of limbs in patients in whom amputation was imminent (Isner, 1998; Isner and Takayuki, 1998). VEGF protein and cDNA have been used for coronary revascularization, resulting in improved myocardial perfusion and increased collateral density (Isner and Losordo, 1999).

As previously discussed, a successful angiogenic outcome is not solely attributed to VEGF implementation alone (discussed in Section 3 and Table 1). Wilcke, et al., showcased the coupling of VEGF with bFGF in a fibrin dermal substitute (Wilcke *et al.*, 2007). This led to a marked improvement in factor delivery, with a notable, prolonged release and resulted in a higher density of newly developed vessels in *in vivo* murine models. Concurrently, an *in vivo* experiment performed by Zacchigna, et al., studied the effect of a VEGF and Angiopoietin 1 (Ang 1) combination (delivered via an adeno-associated viral vector, for long-term protein production and release) on the muscle blood flow (MBF) and vascular permeability of rat skeletal muscle (Zacchigna *et al.*, 2007). The grouping elicited a marked increase in both resting MBF and perfusion post exercise stimulation. To contrast, VEGF expression alone did not enhance the resting MBF and actually reduced tissue perfusion after exercise.

Elcin, et al., researched *in vitro* release kinetics and *in vivo* angiogenic effects of human VEGF-loaded PLGA sponges in rats (Elcin and Elcin, 2006). When compared with control sponges (containing no factor) and bolus injections of VEGF, the use of VEGF-loaded PLGA sponges led to the establishment of neovascularized sites suitable for tissue engineering purposes. Patel, et al., who utilized VEGF-loaded gelatin microparticles infused in biodegradable composite scaffolds, outlined another notable attempt in VEGF delivery modulation in the field of orthopedics (Patel *et al.*, 2008). Findings suggested that modulating the degree of gelatin cross-linking could affect VEGF release into the microenvironment and, consequently, angiogenic outcome. Ennett, et al., studied the temporally regulated delivery of VEGF *in vivo*, hypothesizing that the means of VEGF-loading into a delivery scaffold would have an impact on the factor release kinetics (Ennett *et al.*, 2006). They compared (a) VEGF loaded directly into a PLG scaffold with (b) VEGF pre-encapsulated in PLG microspheres that were later used to fabricate a PLG scaffold. Though pre-encapsulated VEGF microspheres further delayed the factor's release into the surrounding environment, this approach produced a desirable angiogenic outcome, with significant local angiogenesis and negligible systemic effects.

Pre-encapsulation of VEGF by nanoparticles or microspheres has also been investigated in conjunction with Matrigel hydrogels, PLGA-, and collagen-scaffolds. While it is clear that encapsulation protects the factor(s) within the nanoparticles and microspheres, it also offers a mechanism of controlled release – especially ideal for a potent angiogenic factor like VEGF. Even though pre-encapsulated VEGF-loaded PLGA scaffolds performed better than similarly loaded Matrigel hydrogels (with respect to release profiles in a saline solution; also in comparison with free VEGF) *in vitro*, both of the loaded delivery conduits improved angiogenesis *in vivo*. An increase in both endothelial cell counts and red blood cells was noted at the sites of implantation (des Rieux *et al.*, 2010). Further, VEGF-loaded PLGA microspheres combined with collagen elicited strong enhancements to vascular sprouting and activation of endothelial cells *in vivo* and *in vitro*, respectively (des Rieux *et al.*, 2010).

The idea of optimizing delivery agents combined with the notion that co-factor usage is ideal for neovascularization resulted in an experiment that analyzed the co-implementation of VEGF with FGF-2 via an acellular collagen scaffold implant (Nillesen *et al.*, 2007). An implant containing both factors resulted in the highest vessel density and the most mature blood vessels (characteristics of an enhanced, stable vasculature) in a rat model when compared with scaffolds containing either of the factors alone or no factor at all.

6. Conclusion

A significant challenge facing tissue engineering lies in eliciting controlled neovascularization. Controlled neovascularization can have a profound impact in vessel and organ synthesis, as well as in the treatment of damaged tissues. Many current attempts for such control entail the use of VEGF. Attempts at fabricating an ordered vascular network include changes to VEGF through structural modification, the employment of strategies controlling its release, and coupling VEGF to other growth factors.

Yet, despite the promises shown by VEGF, significant obstacles remain. Many researchers propose that use of growth factors alone will not ensure stable angiogenesis. Rather, a combination of growth factors (VEGF and bFGF, for example), delivery methods, and modulation of inflammatory responses (via fibroblast, macrophage, cell-adhesion molecule and cytokine manipulation, for instance) and pathways (such as hypoxia and Notch) are thought to facilitate adequate vessel fabrication and stability.

Additionally, investigation into the mechanisms of cellular crosstalk is necessary to better understand angiogenesis in general. Moreover, the refinement and implementation of microenvironment monitoring technologies are vital in ensuring proper vascular development. Such technologies would enable researchers to closely study the impact of gross- or modulated-release of growth factors on the delicate balance required for vascular formation and branching.

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8. References

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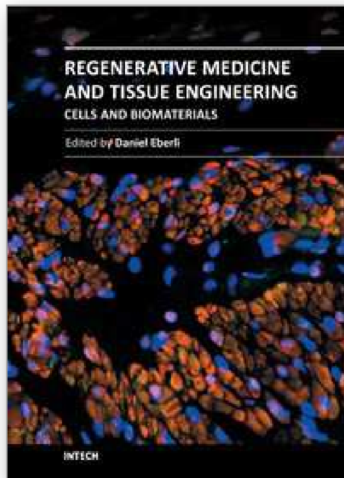

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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage 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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51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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