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Review

고분자 생체재료와 줄기세포를 이용한 조직공학과 재생의학의 최신 동향

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Recent Applications of Polymeric Biomaterials and Stem Cells in Tissue Engineering and Regenerative Medicine

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Abstract: Tissue engineering and regenerative medicine strategies could offer new hope for patients with serious tissue injuries or end-stage organ failure. Scientists are now applying the principles of cell transplantation, material science, and engineering to create biological substitutes that can restore and maintain normal function in diseased or injured tissues/organs. Specifically, creation of engineered tissue construct requires a polymeric biomaterial scaffold that serves as a cell carrier, which would provide structural support until native tissue forms *in vivo*. Even though the requirements for scaffolds may be different depending on the target applications, a general function of scaffolds that need to be fulfilled is biodegradability, biological and mechanical properties, and temporal structural integrity. The scaffold's internal architecture should also enhance the permeability of nutrients and neovascularization. In addition, the stem cell field is advancing, and new discoveries in tissue engineering and regenerative medicine will lead to new therapeutic strategies. Although use of stem cells is still in the research phase, some therapies arising from tissue engineering endeavors that make use of autologous adult cells have already entered the clinic. This review discusses these tissue engineering and regenerative medicine strategies for various tissues and organs.

Keywords: biomaterials, polymers, extracellular matrix, stem cells, tissue engineering, regenerative medicine.

Introduction

Tissue engineering, a major component of regenerative medicine, encompasses the principles of cell transplantation, materials science, and engineering to create living tissue constructs that can implant to the patients to restore and maintain normal function of diseased or injured tissues/organs.[1-4] Tissue engineering strategies generally fall into two categories: the use of cell-free scaffolds, which depend on the body's natural ability to regenerate for proper orientation and direction of new tissue growth, and the use of cell-seeded scaffolds, which mimic the composition of the native tissues. These tissue-engineered scaffolds can be prepared by manufacturing artificial templates derived from natural materials or synthetic polymers or by removing cellular components from tissues using mechanical and chemical manipulation to produce collagen-based tissue matrices.[5-9] These tissue-engineered scaffolds slowly degrade following implantation and are subsequently replaced by the extracellular matrix (ECM) proteins secreted by the seeded cells or the in-growing host cells.

A small piece of donor tissue is dissociated into individual cells when cells are used for tissue engineering. Donor tissue can be heterologous (such as bovine), allogeneic (same species, different individual), or autologous (from the host). Complications are greatly minimized when autologous cells are selected to produce structural and functional tissue replacements as the biopsy tissue is obtained from the host. While an immune response is possible, the use of autologous cells could avoid rejection and eliminates the need for immunosuppressive medications, which have deleterious side effects. Therefore, most tissue engineering strategies are dependent upon obtain-

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ing autologous cells from the diseased organ of the host. Patients with extensive end-stage organ failure, however, are poor candidates for obtaining autologous cells since a tissue biopsy may not yield adequate numbers of healthy cells for expansion and transplantation. Moreover, primary autologous human cells cannot be expanded from certain organs, such as the pancreas. Alternative sources of viable stem cells include discarded human embryos, fetal-related tissue (amniotic fluid or placenta), or from adult sources (bone marrow, fat, or skin).[10] Therapeutic cloning has also played significant a role in the development of the field of regenerative medicine.

This review describes cells and biomaterials used in tissue engineering, including regeneration of specific tissue structures in the body. Therapies at the cellular, tissue, and organ levels are stated, as well as the specific challenges and applications in tissue engineering and regenerative medicine research.

Polymeric Biomaterials

General Consideration. Biomaterials, which replicate the biological and mechanical functions of the native ECM found in tissues in the body, should be biodegradable and bioresorbable and serve as an artificial ECM. Incompatible materials produce an inflammatory or foreign-body response that eventually leads to rejection and/or necrosis. Degradation products, if produced, should be removed from the body via metabolic pathways at an adequate rate to limit the concentration of these degradation products in the tissues to a tolerable level.[11] Selected biomaterials should also provide an environment in which appropriate regulation of cell behavior (adhesion, proliferation, migration, and differentiation) can occur to facilitate the formation of functional tissue. Multiple interactions of cells within their microenvironment will impact cell behavior in the newly formed tissue, including interactions with cell-adhesion ligands[12] and with soluble growth factors.[13] Choice of appropriate biomaterials should provide temporary mechanical support and allow the engineered tissue to maintain sufficient mechanical integrity to support itself in early development. In late development, the biomaterials begin degradation, hence tissue growth is unhindered.[14]

Generally, three classes of biomaterials have been utilized for engineering tissues: naturally derived materials, decellularized tissue matrices, and synthetic biodegradable polymers. These classes of biomaterials have been tested with respect to their biological properties.[15] While naturally derived materials and decellularized tissue matrices have the potential advantage of biological recognition, synthetic polymers can be produced on a large scale with controlled properties of their strength, degradation rate, and microstructure and with a high degree of reproducibility.

Naturally Derived Biopolymers. Naturally derived materials are defined as those that are produced by the cells of a living organism. In particular, structural proteins such as collagen, elastin, and fibronectin have been used for tissue engineering. Collagen has found widespread use as a scaffold and carrier for cells in various tissue engineering applications, specifically, soft tissue applications such as skin[16,17] and cartilage.[18,19]

Natural carbohydrates have been utilized as hydrogels not only for drug delivery, but also for tissue engineering.[20] Among these, the linear glycosaminoglycan hyaluronic acid (HA), which is composed of repeating disaccharide units of glucuronic acid and *N*-acetylglucosamine, is widely distributed in the ECM and critical to vertebrate tissue morphogenesis.[21] HA has been approved for use in clinic both as viscous fluid and sheet formulations, and HA is indicated for knee pain and surgical adhesions, respectively. Many large clinical trials have confirmed the effectiveness of HA for these applications.[22-26] The activity of HA, like that of other relatively simple carbohydrate matrix components, may be enhanced by physical and/or chemical modification to promote cell migration, spreading, and multiplication.

Other carbohydrate polymers such as chitosan and alginate, which are derived from the exoskeleton of shellfish and brown algae, respectively, have been used in several applications. Chitosan is a polycationic material produced by the deacetylation of chitin. It readily forms hydrogels that have been used in a number of gene and drug delivery and tissue engineering.[27,28] Alginate has been also used extensively in hydrogel form for cell encapsulation and drug delivery,[29] as well as in tissue engineering.[30] Although many advances in alginate-based materials have been reported, translation to clinical applications will require several improvements. The purity of the input algal source is critical, along with the development of validation strategies for alginate extraction and purification processes. Maintenance of an open exchange of nutrients, oxygen, and therapeutic factors released by the encapsulated cells while simultaneously avoiding swelling and subsequent rupture of the microcapsules remains a key engineering challenge in designing immunoisolating alginate-based microcapsules for use in cell encapsulation.[31]

Decellularized Tissue Matrices. The normal biological ECM, which contributes to mechanical integrity, has important

signaling and regulatory functions in the development, maintenance, and regeneration of tissues. ECM components combined with soluble signals provided by growth factors and hormones direct a variety of transduction mechanisms to elicit tissue-specific control of gene expression.[32-34] Furthermore, the ECM is itself a dynamic structure that is actively remodeled by the cells within the microenvironment.[35] Improving development of scaffolds that more closely recapitulate the biological and mechanical properties of native ECM remains an important area of tissue engineering.[36] Deconstructing mature ECM and understanding its complex functions in mature or regenerating tissues continues to be a challenging task. In the current absence of methods for *de novo* construction of a true ECM mimic from purified components, decellularized tissue matrices are considered as an ideal scaffolding system due to their structural and mechanical similarity to native tissues and their possession of tissue-specific ECM proteins which remain after decellularization.[37] As a result, these tissue matrices have been used for a number of tissue engineering applications and have yielded an understanding of the physicochemical properties of ECM, as well as a tissue-specific scaffold for engineering functional tissues. For example, by harnessing the cell-matrix interactions that are crucial for cell adhesion, proliferation, and differentiation,[33] several studies have promoted tissue formation by combining cells with tissue-specific ECM from decellularized heart,[38] urinary bladder,[39] liver,[40] lung,[41] kidney,[42] and small intestinal submucosa (SIS).[43] Each of these decellularized tissue matrices maintained the mechanical and structural integrity of the original tissue with minimal disruption to the ECM. More importantly, these ECM proteins play a vital role in tissue maintenance and regeneration of a specific tissue type; furthermore, they can modulate cell adhesion and migration, growth factor storage and release, and progenitor cell activation and differentiation.[44,45]

The use of xenogeneic materials is possible due to the relatively high degree of evolutionary conservation of many ECM components. Various decellularized tissue matrices have been utilized successfully for tissue engineering in animal models, and a limited number of xenogeneic products have received regulatory approval for clinical use.[46] Indeed, several decellularized xenogeneic medical products are being introduced into the market. Despite many advantages, there are concerns surrounding decellularized tissue matrices, including the potential for immunogenicity, the possible presence of infectious agents, variability among preparations, and the inability to completely specify and characterize the bioactive components of the material. Many of the soluble bioactive molecules in the tissue matrices can be lost during the decellularization process.

Synthetic Polymers. Biodegradable synthetic polymers offer a number of advantages for applications in tissue engineering. These polymers can be easily synthesized with reproducible quality and purity and fabricated into various shapes with desired bulk and surface properties. Specific advantages include the ability to tailor the mechanical properties and degradation kinetics of these materials to suit various applications. Poly(α-hydroxy acid)s, such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymer poly(glycolide-*co*-lactide) (PLGA), are the most widely used biodegradable polymers for tissue engineering applications. These polymers have gained popularity due to their processing consistency, adequate mechanical properties, and biodegradability, and they are FDA-approved for clinical use in a variety of applications, including suture material and drug delivery systems. The ester bonds in these polymers degrade by non-enzymatic hydrolysis, and their nontoxic degradation products are eliminated from the body in the form of carbon dioxide and water. The degradation rate of these polymers can be controlled by alteration of their crystallinity, initial molecular weight, and the copolymer ratio of lactide and glycolide. Moreover, the degradation rates that can be achieved range from several weeks to several months. Because these polymers are thermoplastics, they can be configured into a 3-D structure with a desired microarchitecture, shape, and dimension. In addition, poly(ε-caprolactone) (PCL) has been widely used as an ideal material for 3-D bioprinting process due to its structural integrity, biodegradability, and relatively low melting point.[47] However, these polymers generally lack intrinsic biological activity, and their degradation products may cause adverse effects or alter local microenvironment *in vivo*. In addition, the surface hydrophobicity of synthetic polymers may mediate protein denaturation in the vicinity of the implant and induce fibrous encapsulation.[14,48]

A number of research groups are exploring the synthesis of biomaterials that unite the advantages of "smart" synthetic polymers with the biological activities of proteins at the chemical level. The concept of smart polymers was initially derived from the development of materials that show large conformational changes in response to environmental stimuli such as temperature, ionic strength, pH, or light.[49] The responses of the polymer may include precipitation or gelation, reversible adsorption on a surface, collapse of a hydrogel or surface graft,

and alternation between hydrophilic and hydrophobic states.[50] In many cases, the change in the state of the polymer is reversible. Smart synthetic polymers may revolutionize improvements in tissue engineering scaffolds. Beyond the physical properties of these polymers, imparting smart biomaterials with the specific properties of signaling proteins, such as ECM components and growth factors, remains a major goal.

Cell Sources for Use in Tissue Engineering

Primary Tissue-specific Cells. Cell-based approaches to organ replacement are limited by the inability to grow tissue-specific cell types in large quantities. By studying the privileged sites for committed precursor cells in specific organs, as well as exploring the conditions that promote differentiation, the obstacles that limit cell expansion *in vitro* might be cleared. Over the past decades, several protocols have been developed that identified the undifferentiated cells and led to the ability to maintain this cell population in an undifferentiated state during the growth phase.[51-53] Theoretically, these methods of cell culture enabled expansion from a single specimen that initially covered a surface area of 1 cm^2 to a surface area of 4202 m^2 (the equivalent of one football field) within 8 weeks.[51] These studies illustrate the possibility of collecting autologous cells from human patients, expanding them in culture, and returning them to the donor in sufficient quantities for reconstructive purposes.[51,52,54] Within the past few decades, major advances in cell culture techniques have led to the expansion of a variety of primary human tissue-specific cell types. Furthermore, development of specific techniques has demonstrated that use of autologous cells may now be feasible for clinical application.

Embryonic Stem Cells. Human embryonic stem (ES) cells exhibit two remarkable properties: the ability to proliferate in an undifferentiated but pluripotent state (self-renewal), and the ability to differentiate into many specialized cell types.[55] ES cells can be isolated by immunosurgery from the inner cell mass of the embryo during the blastocyst stage (5 days post-fertilization) and are usually grown on feeder layers consisting of mouse embryonic fibroblasts or human feeder cells.[56] Several reports have shown that these cells can be grown without the use of a feeder layer,[57,58] and thus, avoid the exposure of these human cells to mouse viruses and proteins. ES cells have demonstrated longevity in culture by maintaining their undifferentiated state for at least 80 passages when grown using the established protocols.[59]

Human ES cells have been shown to differentiate into cells from all three embryonic germ layers *in vitro*. Skin and neurons have been formed, indicating ectodermal differentiation.[60,61] Blood cells, muscle cells,[62] chondrocytes, and vascular cells[63] have been formed, indicating mesodermal differentiation.[64-66] And, liver cells and pancreatic cells have been formed, indicating endodermal differentiation.[67,68] As further evidence of their pluripotency, ES cells can form embryoid bodies, which are cell aggregations comprising all three embryonic germ layers. The tendency to form teratomas *in vivo*, however, is a limitation to widespread usage of human ES cells in cell therapy and tissue engineering due to difficulty, if not impossibility, in controlling the tumorigenicity of these cells *in vivo*, which leads to a number of safety concerns surrounding the usage of these cells for therapeutic benefit.[69] Additionally, the ethical considerations surrounding the destruction of human embryos to obtain ES cells are yet to be resolved. Finally, the use of human ES cells is banned in some countries, including the US; therefore, their use in tissue engineering is infrequent in those countries at this time.

Induced Pluripotent Stem Cells. A recent study reports the successful transformation of adult cells into pluripotent stem cells through a type of genetic "reprogramming".[70] The reprogramming technique involves de-differentiation of adult somatic cells to produce patient-specific pluripotent stem cells without the use of embryos. Cells generated by reprogramming would be genetically identical to the somatic cells (and thus the patient who donated these cells) and would not be rejected. Takahashi and Yamanaka were the first to discover that mouse embryonic fibroblasts (MEFs) and adult mouse fibroblasts could be reprogrammed into an "induced pluripotent state (iPS)".[70] Their group used MEFs engineered to express a neomycin resistance gene from the Fbx15 locus, a gene expressed only in ES cells. They identified four key genes (Oct3/4, Sox2, c-Myc, and Klf4) that, when introduced into the reporter fibroblasts, resulted in drug-resistant cells. The resultant iPS cells possessed the immortal growth characteristics of self-renewing ES cells, including expressed genes specific for ES cells, and generated embryoid bodies *in vitro* and teratomas *in vivo*. In addition, this initial study was significantly improved by infecting fibroblasts with retroviral vectors and selecting the activation of endogenous Oct4 or Nanog genes.[71] DNA methylation, gene expression profiles, and the chromatin state of the reprogrammed cells were found to be similar to those of ES cells. In addition, teratomas induced by the reprogrammed

cells contained differentiated cell types representing each of the three embryonic germ layers. Most significantly, the reprogrammed cells from this experiment shared a similarity with ES cells in their ability to form viable chimeras and contribute to the germ line and suggests that these iPS cells were completely reprogrammed.

Recent studies have shown that reprogramming of human cells is possible. Yakahashi *et al*. showed that retrovirus-mediated transfection of Oct3/4, Sox2, Klf4, and c-Myc generated human iPS cells that were similar to human ES cells in terms of morphology, proliferation, gene expression, surface markers, and teratoma formation.[72] Yu *et al*. showed that retroviral transduction of Oct4, Sox2, Nanog, and Lin28 could generate pluripotent stem cells without introducing any oncogenes (c-Myc). Both studies showed that human iPS were similar but not identical to human ES cells.[73] Of concern is the increased risk of tumorigenesis due to the presence of three to six retroviral integrations (one for each factor) in iPS cells. Okita *et al*. studied the tumor formation in chimeric mice generated from Nanog-iPS cells and found that 20% of the offspring developed tumors due to the retroviral expression of c-Myc.[74] An alternative approach would be the use of a transient expression method, such as adenovirus-mediated system, as demonstrated by both Okita *et al*.[74] and Meissner *et al*.[75] who showed strong silencing of the viral-controlled transcripts in iPS cells. This indicates that these viral genes are required only for the induction, not the maintenance, of pluripotency. Furthermore, developing non-viral induction methods to produce clinically relevant cell sources is essential.

Adult Stem Cells and Amniotic Fluid-derived Stem Cells. Adult stem cells (derived from bone marrow, fat, skin, etc.) have been used as an important cell source because terminally differentiated cells produce tissue-specific phenotypes in damaged tissue.[76-79] Among somatic stem cells, bone marrow-derived mesenchymal stem cells (MSCs) have been used for autologous cell transplantation in patients. MSCs can be differentiated into multi-lineage cell types, including bone, cartilage, tendon, ligament, and muscle.[80] Moreover, the ability of MSCs to evade immunosurveillance after transplantation and to suppress the immune response has made MSCs attractive candidates for clinical use. MSCs are relatively difficult to expand *in vitro*, however, and they lose regenerative potential with serial passages in culture.

An alternate source of stem cells is the amniotic fluid, which is known to contain multiple partially differentiated cell types derived from the developing fetus. Our research group has isolated stem cell populations from this source, termed amniotic fluid-derived stem cells (AFSCs), and which express embryonic and adult stem cell markers.[81] The undifferentiated stem cells expand extensively without a feeder layer, and the population doubles every 36 h. Lines maintained for over 250 population doublings retained long telomeres and a normal karyotype. The AFSCs are broadly multipotent. Cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal, and hepatic lineages, can be induced to differentiate from clonal human lines verified by retroviral marking.[81] Therefore, these cell lines may be classified as pluripotent stem cells as they fit the criteria, but no implication that they can generate every adult tissue. Unlike ES cells, AFSCs do not form tumors *in vivo*, which would be advantageous in clinical applications.[10] Moreover, 90% of AFSCs express the transcription factor OCT4, which is related to the maintenance of the undifferentiated state and pluripotency in ES cells.[82] More interestingly, AFSCs are also able to suppress inflammatory responses *in vitro*.[83] Possession of extensive self-renewal capacity and the possibility for banking indicates AFSCs are an attractive source of stable, well characterized ‘‘off the shelf’’ immunomodulatory cells for clinical use.

Tissue Engineering Applications

Skeletal Muscle and Innervation. Skeletal muscle injuries due to trauma or tumor ablation usually require reconstructive procedures in order to restore normal tissue function. Currently, muscle pedicle flap from adjacent regions is the primary method practiced. This option is challenged by host muscle tissue availability and donor site morbidity such as functional loss and volume deficiency.[84,85] Recent advances in cell therapy using myoblasts have provided an alternate therapeutic opportunity to regenerate muscle tissue for functional augmentation.[86,87] Injection of cultured myoblasts has shown some efficacy;[88-90] however, this approach may not be suitable for treating large muscle injuries.[89] Creation of an implantable functional muscle tissue that could restore muscle tissue defects may be a possible solution to treating larger wound areas.[91,92]

An essential step in engineering a functional skeletal muscle construct is to mimic the structure of native muscle which is comprised of highly oriented myofibers formed from numerous fused mononucleated muscle cells.[93] Because structure and organization of myofibers dictate tissue function, muscle cell

alignment that permits organized myotube formation is a crucial step in the musculoskeletal myogenesis.[94] Very recently, we developed an aligned electrospun PCL/collagen hybrid scaffold for diaphragmatic muscle reconstruction.[95] The hybrid scaffolds were implanted into a central left hemi-diaphragmatic defect (approximately 70% of the diaphragmatic tissue on the left side) in rats. After 6 months of implantation, histological and immunohistochemical evaluations revealed ingrowth of muscle tissue into the scaffolds. The mechanical properties of the retrieved diaphragmatic scaffolds were similar to those of normal diaphragm at the designated time points. Our results show that the aligned electrospun hybrid scaffolds allowed muscle cell migration and tissue formation. Therefore, aligned scaffolds may provide implantable functional muscle tissues for patients with diaphragmatic muscle defects.

Effective innervation is another critical requirement to successfully engineer a functional muscle tissue *in vivo*.[96] The established contacts of engineered muscle constructs with host nervous tissue is vital following implantation, as improper or incomplete innervation leads to atrophy of muscle tissue and loss of contractile function.[97] When muscle cells/fibers are implanted in the body, host nerves contact with the muscle fibers to form neuromuscular junctions (NMJs). The process of innervations into denervated muscle is slow, however, and substantial time is required before muscle tissue is functional. Therefore, methods to accelerate innervations are needed. Previous reports indicate that the expression of acetylcholine receptors (AChRs) and their clustering on muscle fibers are necessary to induce neural contacts on newly formed muscle fibers in a natural biological system.[98-100] For this reason, we investigated whether pre-induction of AChR clusters on engineered muscle fibers using agrin, a neural-released trophic factor, would accelerate innervation when implanted *in vivo*. Agrin has a critical role in inducing AChR clustering on the differentiated muscle cells.[101-104] Neural-agrin stimulates the rapid phosphorylation of a muscle-specific kinase (MuSK), which has been shown to be necessary for the formation of the NMJs and leads to the redistribution of previously unlocalized AChR-proteins to synaptic sites.[105] Thus, we demonstrated that agrin molecules could facilitate the formation of AChR clusters on the engineered muscle constructs.[106] Our results indicate that agrin treatment increased AChR cluster formation on the engineered muscle fibers and these pre-inducing AChR clusters facilitated accelerated contacts with dorsal root ganglion (DRG) nerve *in vitro*, or with the host nerve in engineered muscle constructs treated with agrin *in vivo* (Figure 1).[106] Moreover, the implanted agrin-treated muscle constructs displayed a mature myofiber structure with myosin heavy chain (MHC) expression in the vicinity of the host nerve, indi-

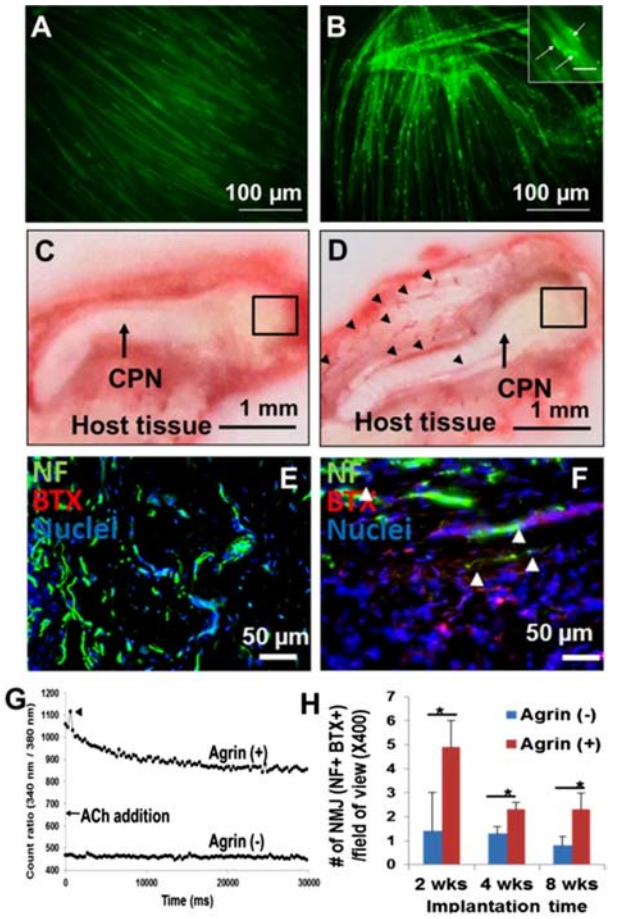

Figure 1. Pre-induction of AChR by agrin treatment accelerates innervations *in vitro* and *in vivo*. *In vitro* myotube formation (A) without and (B) with agrin treatment; agrin treatment induced enhanced AChR expression on myotubes [α-BTX$^+$ indicated by arrows on the surface of myotubes (box in B)] (C,D) Pre-fabricated C2C12 myotubes in fibrin gel was implanted subcutaneously embedded with common peroneal nerve (CPN) at 2 weeks; numerous large blood vessels were observed in the implant treated with agrin (D), whereas there was no remarkable blood vessels surrounding the implant without agrin treatment (C). (E,F) Immunofluorescence of the harvested C2C12/fibrin gels; higher numbers of innervated structures [neurofilament (NF)$^+$/α-BTX$^+$ double staining (arrowheads)] were observed in agrin treated group (F) than in no treated group (E) and quantification of their number (H). Student *t*-test, **p*<0.05, n=3. (G) *In vitro* functional properties of AChR expression on myotubes by calcium imaging; agrin-treated myotubes displayed depolarization-induced increases in steady-state intracellular calcium levels in the kinetic curve with the sharp increase (arrowhead) in agrin-treated myotubes. Reproduced with permission from Ko *et al*., *Biomaterials*, 34(13), pp. 3246-55 © Elsevier.[106]

cating enhanced interaction with the host nerve. It is our belief that the effective use of a NMJ-inducing factor, agrin, could induce proper maturation of engineered muscle constructs for accelerating further host nerve integration (innervation).

Vascular Grafts. Cardiovascular diseases, including coronary artery and peripheral vascular diseases, are the leading cause of mortality in the US.[107] There remains a significant clinical demand to improve technology for vascular grafts. Segments of autologous vessels continue to be the standard for many revascularization procedures. Unfortunately, autografts are limited in supply and dimensions, while allografts and xenografts are limited by strong immunogenic response.[108-110] Synthetic vascular grafts have been explored for over half a century. Substances such as expanded polytetrafluoroethylene (ePTFE) or Dacron (polyethylene terephthalate fiber) work well as large diameter (> 5 mm) bypass conduits, but such synthetics have not proven generally satisfactory for small diameter grafts, due to the high frequency of thrombosis, stenosis, occlusion, and infection.[111,112]

Tissue engineering offers an attractive approach to vascular grafting, particularly for small diameter (< 5 mm) vessels. The basic approach is to create vessels by combining autologous cells with a natural and/or synthetic scaffold under suitable culture conditions, resulting in a tubular construct[113] that can be implanted *in vivo*. Coating the vascular lumen of synthetic grafts with endothelial cells (ECs) has reduced acute thrombosis.[109,110] The presence of a permanent synthetic graft remains an issue, as it may lead to chronic inflammatory responses and other problems. Preferably, biodegradable scaffolds could be replaced gradually by cells to generate an essentially normal engineered blood vessel. Indeed, vascular scaffolds combined with viable cells have been shown to allow grafts to remodel when introduced *in vivo*.

Previous work by our group demonstrated that a decellularized porcine vessel scaffold coated with autologous ECs could replace a small diameter blood vessel in a sheep carotid arterial interposition model.[114,115] The implanted decellularized vascular grafts could remain patent for at least 130 days. While the use of cells bound to a naturally derived vascular tissue matrix demonstrates the principle of vascular tissue engineering, this approach has several drawbacks that would be expected to hinder clinical translation. These include the limited supply of vessels of the required dimensions, inconsistency in donor tissue composition, and potential contamination by pathogens. Therefore, it is essential to develop a synthetic vascular scaffolding system that would allow for the consistent production of small diameter vessel substitutes of any desired dimensions.

A number of approaches have been attempted with the goal of producing an ideal vascular scaffold. Among these, electrospinning technology, which uses high-voltage electrostatic fields to generate nanofibers, appears particularly attractive because it provides a biomimetic environment that may be designed to resemble that of ECM architecture of native vasculature. Electrospinning permits fabrication of fibrous matrices of nano- to micro-scale. In addition, it is possible to control the composition, structure, dimension, and biomechanical properties of fabricated scaffolds. Numerous reports have documented that synthetic biodegradable polymer-based materials can be electrospun to generate candidate vascular scaffolds with *in vitro* characteristics of strength and biological properties that appear consistent with clinical requirements.[113,116-119] Many efforts attempted to develop a durable biomaterial capable of resisting physiological forces and maintaining structural integrity until mature vascular tissue forms *in vivo*. It is also important that engineered vessels should be compliant, resistant to kinking and compression, and possess sufficient tensile and shear strength to resist fraying at cut edges and tearing out of sutures.[120]

With these considerations in mind, we have developed a vascular scaffold that is a composite of poly(ε-caprolactone) (PCL) and type I collagen.[113,117] We observed that this scaffold can withstand physiologically relevant vascular conditions over a period of 1 month *in vitro* and after implantation *in vivo* in a rabbit aorto-iliac bypass model.[121] The introduction of collagen to PCL appeared to increase the structural stability under high physiological pressure as well as cell-compatibility. Interestingly, the hydrated PCL/collagen scaffolds showed tensile properties similar to those of native blood vessels due to the stiffness of collagen. Most recently, we have developed a bilayered scaffolding system that provides different pore sizes to facilitate adequate cellular interactions. Thus, our system allows for EC adhesion onto the luminal surface and homogenous infiltration of smooth muscle cells (SMCs) into the outer layer (Figure 2). Moreover, this scaffold provides sufficient mechanical properties that withstand physiologically relevant vascular conditions. Our findings suggest that bilayered scaffolds may facilitate endothelialization and smooth muscle maturation for improved vessel tissue function. A preclinical large animal study for the application of this fully cellularized vascular scaffold is currently being conducted. These studies will evaluate the utility of vascular tissue engineering to provide

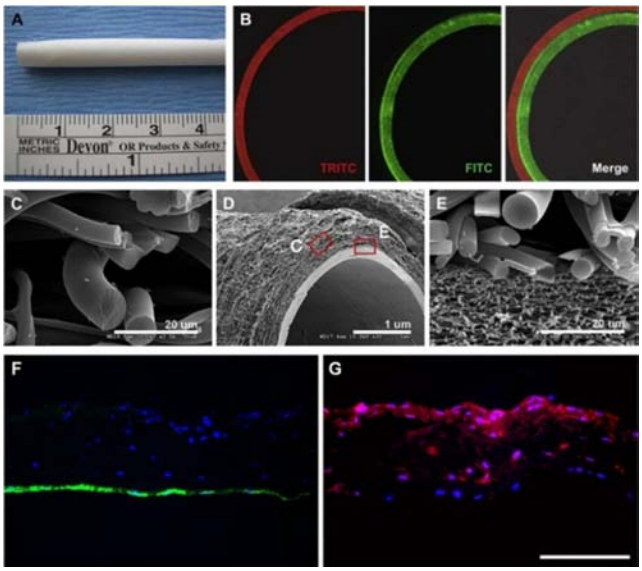

Figure 2. Bilayered electrospun PCL/collagen vascular scaffolds: (A) Gross appearance, (B) fluorescent images of bilayered scaffold s incorporated fluorescent dyes. SEM images of cross-sectional (C) outer layer, (D) entire, and (E) interface between outer and inner layers of bilayered scaffolds. Immunofluorescent images of EC and SMC seeded bilayered electrospun PCL/collagen vascular scaffolds; (F) EC seeded luminal layer (CD31 expression, green) and (G) SMC seeded outer layer (α-SMA expression, red). Scale bar indicates 500 μm (magnification, ×100). ECs formed a monolayer on the surface of the inner layer (F) and SMCs infiltrated into the outer layer (G) of the bilayered scaffolds. Reproduced with permission from Ju *et al*., *Biomaterials*, 31(15), pp. 4313-21 © Elsevier.[113]

platform technologies for rehabilitation of patients recovering from severe and devastating cardiovascular diseases. The long-term goals are to provide alternatives to vascular grafting using tissue-engineered blood vessels derived from the patient's own cells. We believe that completion of this study has the potential to lead directly to clinical translation and address an important unmet medical need.

Bone. Large bone defects in humans still remains a significant clinical problem, as they often result in a reduced quality of life for many patients, as well as a significant socioeconomic cost.[122] Despite some reports of successful bone healing with autografts and allografts, which are the most common clinical treatments for large non-union defects, these techniques have not yet been able to meet all clinical needs.

Current bone tissue engineering strategies consist of three major components: (1) osteogenic cells, including primary osteoblasts or stem cells, (2) an osteoconductive and/or osteoinductive biomaterial scaffold that closely mimics natural bone tissue biochemically and mechanically, and (3) signals that support to direct the cells to the phenotypically desirable type. Current approaches require *in vitro* expansion of stem cells with high proliferative and osteogenic potential as well as a suitable scaffold that provides cell support for new bone tissue formation. One type of cell that is commonly used in bone tissue engineering is the bone marrow-derived MSCs.[123] MSCs are relatively difficult to expand *in vitro*, however, and they lose their regenerative potential during serial passaging. As mentioned above, AFSCs have recently become an interesting source of cells for cell-based tissue engineering. AFSCs have a high proliferation rate and have a remarkable ability to differentiate into multiple cell types.[81] As a result, it appears that AFSCs have several advantages over other stem cell populations. Previous studies have also shown that human AFSCs can give rise to osteogenic lineages.[124-126]

Scaffolds play a critical role by defining the 3-D template for tissue regeneration and by serving as a synthetic ECM environment for tissue regeneration. Thus, various natural and/or synthetic biomaterials have been developed.[117] Supporting cell growth and differentiation, controlling interactions of cells within the biological milieu, and maintenance of the mechanical and physical properties required for the selected application is the role of the scaffold. Bone tissue engineering scaffolds should be biocompatible, osteoconductive and/or osteoinductive and possess structural stability, a proper degradation rate over time, and high porosity with large interconnected pores to enable mass transport and infiltration of cells.

Auricular Cartilage. Varying degrees of outer auricular defects can result due to congenital or accidental reasons.[127-129] Since the auricle is a primary identification feature on the face, reconstruction of a defective ear is aesthetically critical for the patient to regain self-confidence. Currently, use of autologous costal cartilage is a standard treatment method for auricular reconstruction.[130-132] The main advantages include low incidence of rejection, minimal treatment or care during the lifetime of the patient, and improved self-image and self-esteem experienced by the patient following reconstruction of a prominent facial feature.[133] The use of costal cartilage is a complex procedure, however, and it is difficult to configure the shape that matches the contralateral auricle.[131,132,134] As such, complications, including inadequate shape, progressive dimensional change, and donor site morbidity, continue to be problematic.[133,134] Alternative approaches using alloplastic implants, such as silicon or high-density polyethylene (HDPE), have been introduced and approved by the Food and Drug Administration (FDA) for clinical applications.[135,136] Although

alloplastic implants are biologically stable and provide several advantages, including no donor site morbidity and long-term structural integrity when compared to autologous costal cartilage tissue, problems with vascularization and tissue integration at the interface between the alloplastic implant and the overlying skin often lead to infection, inflammation, and overlying skin necrosis, resulting in implant failure.[137]

Tissue engineering strategy has been considered as an alternative means to reconstruct auricular defects.[138] Creation of an auricle built with patient's own cells is attractive as this approach eliminates the concerns associated with immunological rejection or foreign body reaction. Engineering of cartilage tissue *de novo* for total auricular reconstruction does require an extensive cell expansion process and lengthy wait time while the new cartilage tissue forms. More importantly, the current limitations of tissue-engineered cartilage tissue constructs for clinical application are primarily due to the challenges in maintaining tissue shape resulting from poor mechanical strength and long-term stability *in vivo*.[138]

To overcome these limitations, we previously have introduced an approach that utilizes alloplastic implants coated with autologous chondrocytes.[139] The chondrocytes coated on the surface of implant material are able to form a cartilage tissue layer covering the entire ear implant. These results suggest that tissue-engineered cartilage covered alloplastic implants provide a more natural interface between the implant and recipient's tissue.[139] Therefore, the objective of this study was to demonstrate the clinical feasibility of placing engineered cartilage-covered human ear-shaped alloplastic implants in the subcutaneous tissue under the skin. The overlying skin tension and pressure loaded over the ear implants might be critical factors for determining long-term success of the ear implants for auricular reconstruction. We also examined the morphological stability and phenotypic characteristics of the engineered cartilage-covered ear-shaped implants in a mouse subcutaneous implantation model.

Kidney. The kidney is a complex organ with multiple cell types and a complex functional anatomy that renders it one of the most difficult organs to reconstruct.[140] Previous efforts in tissue engineering of the kidney have been directed toward the development of extracorporeal renal support systems made of biological and synthetic components,[141-145] and *ex vivo* renal replacement devices are known to be life-sustaining. Patients with end-stage kidney disease (ESRD) would benefit tremendously if these devices could be implanted long-term without the need for an extracorporeal perfusion circuit or immunosuppressive drugs.

We previously applied the principles of both tissue engineering and therapeutic cloning in an effort to produce genetically identical renal tissue in a large animal model, the cow (Bos taurus).[146] Bovine skin fibroblasts from adult Holstein steers were obtained by ear notch, and single donor cells were isolated and microinjected into the perivitelline space of donor-enucleated oocytes (nuclear transfer). The resulting blastocysts were implanted into progestin-synchronized recipients to allow for further *in vivo* growth. After 12 weeks, cloned renal cells were harvested, expanded *in vitro*, then seeded onto scaffolds consisting of three collagen-coated cylindrical polycarbonate membranes. This created a renal neo-organ with a mechanism for collecting the excreted urinary fluid. The scaffolds with the collecting devices were transplanted subcutaneously into the same steer from which the genetic material originated. Chemical analysis of the collected urine-like fluid, including urea nitrogen and creatinine levels, electrolyte levels, specific gravity, and glucose concentration, revealed that the implanted renal cells possessed filtration, reabsorption and secretory capabilities. Histological examination of the retrieved implants revealed extensive vascularization and self-organization of the cells into glomeruli and tubule-like structures.

Combining functional renal cells with a decellularized porcine kidney scaffold is an effective approach to whole organ engineering.[42] The complexity of the kidney structure with over thirty different cell types, an intricate vascular network, and array of functional structures, makes the use of the underlying ECM comprising the unique microenvironment a logical starting point for the engineering of a transplantable whole kidney. The efficacy of cellular removal and biological properties of the preserved tissue matrices, coupled with scaffold reproducibility, are critical to the success of this approach. To perform the decellularization, we have designed and constructed high-throughput system for decellularization process. This system showed significant cellular removal (<50 ng DNA/mg dry tissue). And, decellularized kidneys retained intact microarchitecture including the renal vasculature and essential ECM components (Figure 3). Our method ensures clearance of porcine cellular material, which directly impacts immunoreactivity during transplantation, and preserves the ECM and cellular compatibility of these renal scaffolds. Thus, we have developed a rapid decellularization method that can be scaled up for use in other large organs. Our results represent a step toward development of a transplantable organ using tissue engineering techniques. A preclinical large animal study for the application

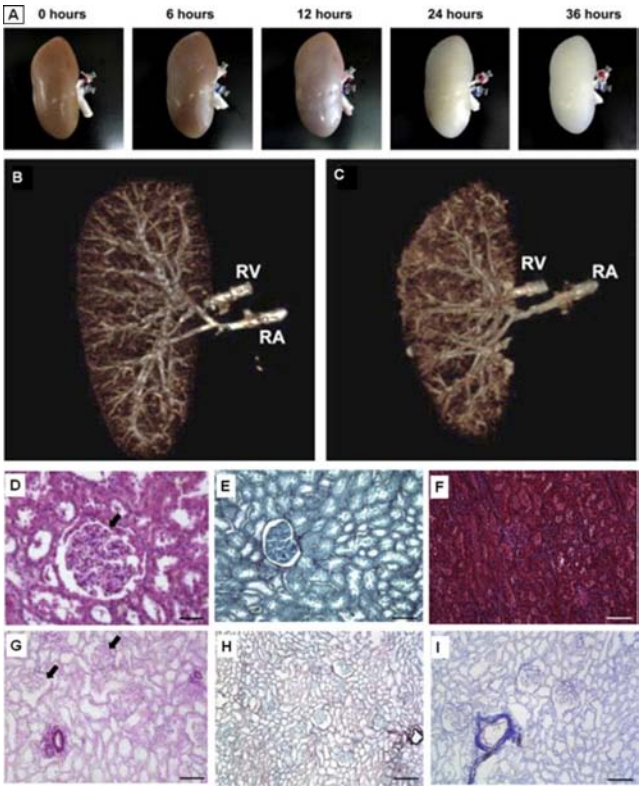

Figure 3. Decellularization of porcine kidney: (A) Representative time lapse of decellularization process of porcine kidney. (B,C) Comparison of whole organ vasculature system between (B) native and (C) decellularized porcine kidney illustrating the preservation of the arterial–venous renal system by continuation of the renal artery (RA) to the renal vein (RV). Histological analyses of (D-F) native and (G-I) decellularized porcine kidney; (D,G) H&E, (E,H) Alcian Blue/Sirius Red, and (F,I) Masson's Trichrome. Reproduced with permission from Sullivan *et al*., *Biomaterials*, 33(31), pp. 7756-64 © Elsevier.[42]

of this technology is currently being conducted.

Urethra. Various strategies have been proposed over the years for the engineering of urethral tissue. Woven PGA meshes without cells[147,148] or with cells[149] were used to engineer urethral tissue constructs in various animal models. Decellularized bladder submucosa (5) and urethral submucosa[150] have also been tested in animal models for urethral reconstruction. Bladder submucosa matrix (BSM) proved to be a suitable graft for repair of urethral defects in rabbits (5). These results were confirmed clinically in a series of patients with a history of failed hypospadias reconstruction. The urethral defects in these patients were repaired with human BSM.[151] The neo-urethral tissue was created by anastomosing the matrix to the urethral plate in an onlay fashion.

These techniques, which employed decellularized tissue matrices that had not been reseeded with cells, were applied experimentally and clinically in a successful manner for onlay urethral repairs. Further study, however, indicated that when tubularized urethral repairs with unseeded tissue matrices were attempted experimentally, adequate urethral tissue regeneration was not achieved, and complications, such as graft contracture and stricture formation, occurred.[152] To determine if seeding the scaffold with cells from the urinary tract could improve the results of tubularized urethral repairs, autologous rabbit bladder epithelial cells and SMCs were grown and seeded onto preconfigured tubular scaffolds. Entire urethra segments were then resected and urethroplasties were performed with tubularized scaffolds either seeded with cells or without cells. The tubularized scaffolds seeded with autologous cells formed new tissue which was histologically similar to native urethra. The tubularized scaffolds without cells lead to poor tissue development, fibrosis, and stricture formation. These findings were confirmed clinically when a trial using tubularized non-seeded scaffolds for urethral stricture repair was performed.[153] Most recently, we were able to show that synthetic biomaterials can also be used in urethral reconstruction when they are tubularized and seeded with autologous cells.[154] We used PLGA-coated PGA nonwoven scaffolds seeded with autologous cells derived from bladder biopsies taken from each patient. These cell-seeded scaffolds were then used to repair urethral defects in five boys. Upon follow-up evaluation, it was found that most of the boys had excellent urinary flow rates postoperatively, and voiding cystourethrograms indicated that these patients maintained wide urethral calibers. Urethral biopsies revealed that the grafts had developed a normal appearing architecture consisting of urothelial and muscular tissues.

Bladder. The current methods of bladder replacement or repair have been limited. Gastrointestinal segments are commonly used for this purpose, but they are designed to absorb specific solutes, whereas bladder tissue is designed for the excretion of solutes. Numerous investigators have attempted to use alternative materials and tissues for bladder replacement or repair due to the problems encountered with the use of gastrointestinal segments. The ability to use donor tissue efficiently and to provide the right conditions for long-term survival, differentiation, and growth determine the success of cell transplantation strategies for bladder reconstruction. These principles were applied in the creation of tissue-engineered bladders in an animal model that required a subtotal cystectomy with subsequent replacement with a tissue-engineered organ in beagle

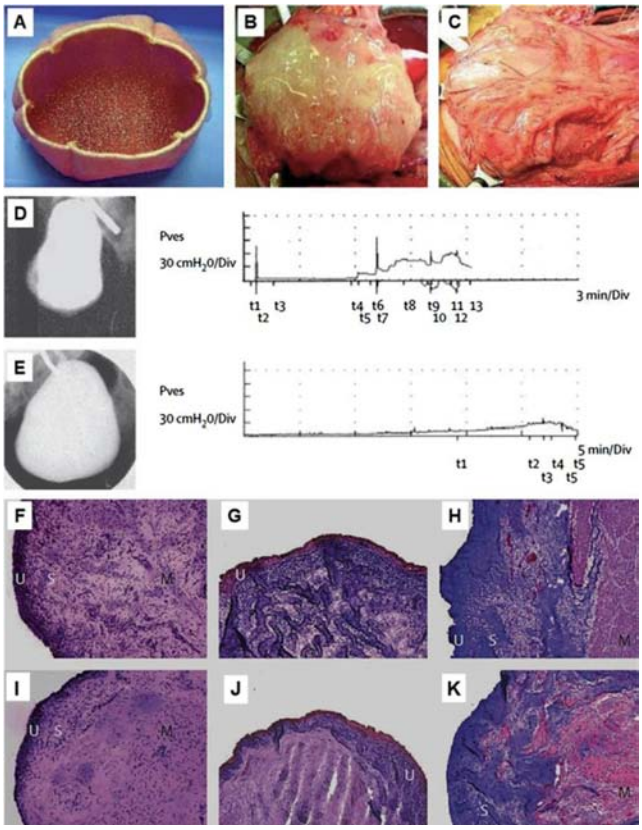

Figure 4. (A) Scaffold seeded with cells, (B) engineered bladder anastomosed to native bladder with running 4-0 PGA sutures, and (C) implant covered with fibrin glue and omentum. (D) Preoperative and (E) 10-month postoperative cystograms and urodynamic findings in patient with a collagen-PGA scaffold engineered bladder. (F), (G), and (H): cystoscopic biopsies of implanted engineered bladders 31 months after augmentation shows extent of regeneration. Engineered bladder tissue showed tri-layered structure, consisting of lumen lined with urothelial cells, U, surrounded by submucosa, S, and muscle, M. (F) H&E, immunocytochemical analysis with (G) anti-pancytokeratin AE1/AE3 antibodies and (H) anti-α smooth muscle actin antibodies showed presence phenotypically normal urothelium and smooth muscle. (I), (J), (K): native bladder tissue. Original magnification: ×100. Reproduced with permission from Atala *et al*., *Lancet*, 367(9518), pp. 1241-6 © Elsevier.[156]

dogs.[155] Bladder urothelial cells and SMCs were separately expanded from an autologous bladder biopsy and seeded onto a bladder-shaped biodegradable polymer scaffold. The results from this study revealed the possibility of engineering bladders that are anatomically and functionally normal.

A clinical trial involving engineered bladder tissue for cystoplasty reconstruction was conducted starting in 1999. A small pilot study of seven patients was reported, using a collagen scaffold seeded with cells either with or without omentum coverage, or a combined PGA/collagen scaffold seeded with cells and omental coverage. The patients reconstructed with the engineered bladder tissue created with the cell-seeded PGA/collagen scaffolds showed increased compliance, decreased end-filling pressures, increased capacities and longer dry periods (Figure 4).[156] Although the results of this clinical trial are show promise toward safe implantation of engineered tissues, the goal of engineering fully functional bladders has yet to be achieved.

Vascularization of Engineered Tissues

Delivery of Angiogenic Factors. A major challenge in tissue engineering is the lack of proper vascularization in the engineered tissue constructs when implanted. To develop complex tissues or organs, fully vascularized tissue constructs should be provided to attain long-term cell survival and function of cell-constructs not only at the margin, but also at the center of the tissue grafts.[157] In fact, the growth of a new microvascular system persists as one of the major limitations to the successful introduction of tissue engineering products to clinical practice.[158] Accordingly, the focus of much research in tissue engineering has evolved to include a better understanding of angiogenesis. Considerable effort has been made to overcome this limitation, and enhancement of angiogenesis within the host tissue has been addressed using several approaches.

Delivery of growth factors and cytokines that play central regulatory roles in the process of angiogenesis, which is thought to induce ingrowth of capillaries and blood vessels into an engineered tissue constructs has demonstrated diminished hypoxia-related cell damage. The delivery of such angiogenic factors has been achieved either by incorporating the desired bioactive molecules into the scaffold material to be used or by genetic modification of the cells to be used in the engineering process by inducing the cells to express factors such as vascular endothelial growth factor (VEGF), one of the most potent angiogenic factors.[159,160]

A study evaluated controlled release of VEGF by incorporating VEGF directly into PLGA scaffolds or by incorporating VEGF encapsulated in PLGA microspheres into scaffolds.[161] VEGF incorporated into scaffolds resulted in rapid release of the cytokine, whereas the pre-encapsulated group showed a delayed release. In addition, both systems showed negligible release of VEGF into the systemic circulation, yet their use led to enhanced local angiogenesis *in vivo* for up to 21 days. These studies demonstrated the delivery of VEGF in

a controlled and localized fashion *in vivo*. This angiogenic factor delivery system was applied to bone regeneration, and its potent ability to enhance angiogenesis within implanted scaffolds was followed by enhanced bone regeneration and outlines a novel approach for engineering tissues in hypovascular environments.[162]

As another example, Borselli *et al*. developed a dual delivery system combining VEGF and insulin-like growth factor 1 (IGF-1) to enhance transplantation and dispersion of cultured myogenic cells.[163] Localized delivery of VEGF and IGF-1 from alginate scaffolds into injured muscle enhanced local angiogenesis, altered muscle fiber type, and enhanced muscle regeneration in a model of severe muscle damage. Transplanting cultured myoblasts on scaffolds that delivered VEGF/IGF-1 dramatically enhanced their direct participation in muscle regeneration, and enhanced angiogenesis and the return to normal tissue perfusion levels compared to growth factor delivery alone. This approach holds promise in the transplantation of many cell types used to promote the regenerative response of multiple tissues.

Transplanted Endothelial Cells. Our group used human vascular ECs and skeletal myoblasts transfected with adenovirus encoding the gene for VEGF to regenerate a vascularized engineering muscle construct.[159] The transfected cells were injected subcutaneously in athymic mice where muscle tissue formed with neovascularization with maintenance of their muscle volume as evidenced by histological and immunohistochemical analyses. Engineered muscle of non-transfected cells had a significantly smaller mass of cells with loss of muscle volume over time, less neovascularization, and no surviving ECs. Our results indicate that a combination of VEGF and ECs may be useful for inducing neovascularization and volume preservation in engineered tissue.

The development of a method of formation and stabilization of endothelial vessel networks *in vitro* in engineered skeletal muscle tissue[164] has shown promising results. A 3-D multicultural system consisting of myoblasts, embryonic fibroblasts, and ECs co-seeded on highly porous, biodegradable polymer scaffolds. These results showed that pre-vascularization of the implants improved angiogenesis and cell survival within the scaffolds. Moreover, this research group emphasized that co-cultures with ECs and SMCs may also be important for inducing differentiation of engineered tissues.

Recently, Moon *et al*. developed synthetic, biomimetic hydrogels that allow the rapid formation of a stable and mature vascular network.[165] Hydrogels were fabricated with integrin binding sites and protease-sensitive substrates to mimic the natural provisional ECM, and ECs cultured in these hydrogels organized into stable, intricate networks of capillary-like structures. The resulting structures were further stabilized by recruitment of mesenchymal progenitor cells that differentiated to a SMC lineage and deposited collagen IV and laminin.

Conclusions and Future Outlook

Engineered tissues of many varieties are at different levels of development, with some in current clinical use. The diversity in stem cells and biomaterials used for tissue engineering applications is immense, and it is imperative that research continues to increase our understanding of the impact of these important technologies to interact in biological systems. Achieving regulatory approval for clinical trials using novel tissue engineering and regenerative medicine technologies will rely on our understanding the fundamental principles of biological interactions between cells and biomaterials.

Principally, biomaterials provide mechanical support, shape, and 3-D architecture for neo-tissue regeneration *in vitro* or *in vivo* as seeded cells expand and organize. Most biodegradable polymeric biomaterials used to date comprise a class of synthetic polyesters and/or naturally derived materials such as collagen. A multitude of fabrication techniques have been devised and afford an abundance of potential shapes, sizes, porosities, and architectures. The blending of cell and molecular biology with materials science and biomedical engineering will provide new applications in regenerative medicine in the form of interactive biomaterials that serve to orchestrate cell attachment and growth, as well as tissue morphogenesis.

Future advances in tissue engineering and regenerative medicine will rely on the development of ‘smart’ polymeric biomaterial scaffolding systems that actively participate in recovering tissue function. Desirable smart biomaterials could be designed as scaffolds that mimic the natural biological system and integrate the necessary structural and biological properties. Hence, solid understanding of materials science combined with extensive knowledge of the clinical challenges and cell biology is vital for development of clinically applicable biomaterials to be used in tissue engineering. Interdisciplinary collaboration between materials scientists, engineers, cell biologists, physiologists, and clinicians will aid the development of novel biomaterials for use in tissue engineering applications that might enhance or improve current regenerative medicine therapies.

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