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Reclaiming a natural beauty: whole-organ engineering with natural extracellular materials

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Abstract

The ability to engineer whole organs as replacements for allografts and xenografts is an ongoing pursuit in regenerative medicine. While challenges remain, including systemic tissue integration with angiogenesis, lymphangiogenesis and neurogenesis, ongoing efforts are working to develop novel technologies to produce implantable engineered scaffolds and potentially engineered whole organs. Natural extracellular matrix materials, commonly utilized *in vitro*, are now being used as effective, natural, acellular allografts, and are being integrated into nanoscale scaffolds and matrices with programmable responsiveness. Based on the significant use of natural scaffolds for tissue regeneration and bioengineering strategies, this review focuses on recent and ongoing efforts to engineer whole organs, such as the tooth, featuring natural extracellular matrix molecules.

Keywords

decellularization; electrospinning; functional scaffolds; natural extracellular matrices; smart materials; tissue and organ engineering

Since the advent of the auricular implant grown on a mouse's back [1,2], whole-organ engineering has been proof-of-concept and a growing pursuit in the field of regenerative medicine. Whole-organ engineering endeavors to create functional organs, in addition to furthering our understanding of the composition of individual tissues, and the interfaces between tissue types. The extracellular matrix (ECM) offers not only a physical structure to house the cells within tissue, but also supplies directions for the morphogenetic process integrated into the tissue. Proliferation, differentiation, adhesion, migration and survival are all intrinsically linked to cell-surface receptor binding sites found on ECM molecules.

Natural ECMs consist of proteins and polysaccharides secreted by cells. Many are 'multimodal' molecules with areas for both cell and ECM protein binding sites [3]. Others have written comprehensively on specific natural ECM molecules' and respective functions [4–8]. The two major components to the ECM in organ systems include the basal lamina and the stromal matrix. While the ECM composition can vary from tissue to tissue, the basal lamina, found bordering epithelial sheets, primarily consists of collagen IV and laminins; stromal or interstitial matrices include diverse compositions such as fibrillar collagen I or hyaluronic acid [3].

There are many benefits to incorporating natural materials into engineered tissues and organs. In addition to functional cell and ECM adhesion sites, natural ECM molecules offer improved biocompatibility over synthetic alternatives as well as genetic conservation in terms of use with xenogenic products [8,9]. Over the years, biomaterials have advanced from incorporating inert materials into the body, to creating bioactive and bioresponsive material implantations, in the pursuit of repairing organ functionality or regenerating tissue. The use of natural ECM materials spans these efforts. The main focus of this review is to showcase the latest developments in whole-organ engineering, which reclaims natural materials and natural/synthetic blends in innovative ways (Table 1). In this article, the tooth is an illustrative example of a developing organ, and efforts toward whole-organ tooth engineering with current and future applications using natural ECM molecules.

The current state of the art for natural scaffolds

While still in its infancy, current advances in engineering whole organs with natural materials include decellularizing cadaveric or animal tissue for scaffold use, electrospinning nanofiber scaffolds or functionalizing environmentally responsive matrices. Whereas decellularization attempts to reuse, maintain and characterize the 3D tissue architecture in place, electrospun fibers and responsive materials attempt to recreate the niche for cells within a 3D-fabricated environment. These technologies vary along a continuum from prototypical *in vitro* models to human clinical studies. However, much can be learned from the incorporation of natural ECM molecules in all of these efforts as we progress towards engineered whole organs.

Decellularization of tissue

As reconstructing the entire structure of an ECM remains an elusive task, decellularization, a process of cell and associated nucleic acid debris removal, has been used to create acellular scaffolds for tissue and organ engineering. These scaffolds retain the original architecture of organs and tissue interfaces, giving rise to a source of allogenic and xenogenic whole-organ grafts. Decellularization has been employed on a variety of tissues of various organs such as the urinary bladder, small intestinal submucosa, blood vessels, heart valves, pericardium, trachea and esophagus, as well as musculoskeletal regions such as the temporomandibular joint [9–22]. This method of creating acellular scaffolds has resulted in commercial products and tissue substitutes [23–27].

As studies advance towards more complex geometries, the decellularization process has evolved to meet stricter standards needed for allograft studies. As Gilbert *et al.* suggested, there is variability amongst tissue types in terms of effective decellularization methods [24]. While a decellularization treatment may efficiently remove cellular material in some tissues, denser, thicker tissues and organs may need improved penetration into the tissue. The effectiveness of cell removal and tissue damage varies based upon the method of decellularization (chemical, enzymatic or physical) [13,14,24,28]. After determining methods of decellularization, optimization of decellularization reagent incubation time, temperature, concentration or number of solvent cycles has been reported with varying degrees of success based upon histology, scanning-electron microscopy and mechanical testing [12,19,20,28].

In human allograft studies of tracheal tissue, penetration was achieved by circulating cycles of detergents to remove nucleic materials [19,20]. While verification of cell removal is typically quantified by DNA content postdecellularization by means of spectrophotometry [29–32], further characterization of the remaining MHCs is essential in clinical human allograft studies. For the decellularized human whole-trachea graft, MHC class I and II removal was monitored. To decellularize the tissue, multiple washes in 4% sodium

deoxycholate and 2000 kU deoxyribonuclease I were alternated with water rinses. After 25 cycles of detergent washes, immunohistochemistry demonstrated removal of HLA-A, HLA-B and HLA-C antigens, with minimal expression of MHC II antigens. Likewise, serology screens of HLA recipient antidonor antibody production verified complete absence of foreign proteins for as long as 2 months postsurgery [20].

Excessive decellularization can degrade the ECM, resulting in collagen damage, glycosaminoglycan depletion, as well as elastin cleavage [24,33]. Accordingly, protocols should be designed to detect and minimize destruction, while preserving the mechanical integrity of the tissue. Often, retention of ECM architecture is validated in comparison to untreated cadaveric tissue with microscopy to ensure tissue integrity is not compromised [12,13,17,22,28,32,33]. In histology, the extent of ECM damage in collagen-dominated tissue has been quantified by collagen crimping amplitude and periodicity, resulting from exposure to detergents such as sodium dodecyl sulfate [17,33]. Other imaging techniques visualize nanostructures (e.g., the weave, coil and strut fibers in the decellularized heart study) using scanning-electron microscopy comparing decellularized tissue with cadaveric tissue [32]. However, with optimal treatment, decellularized tissues can maintain ECM antibody epitope expression in such molecules as collagens, laminin and fibronectin in immunohistochemistry and immunofluorescence studies [13,15,18,21,30,34]. Table 2 summarizes the various ECM molecules characterized in select decellularized organ and tissue studies.

Just as the chemical composition of the ECM affects organ functionality, mechanical integrity must also be preserved in decellularized tissues. Mechanical testing quantitatively evaluates the effects of decellularization. As an example, to accommodate ventricular load and associated cardiac stresses, decellularized rat heart cross-sections were able to match the ventricular tissue anisotropy to cadaveric tissue with respect to stiffness and tangential modulus.

Recent developments in decellularization focus on retaining whole-organ functionality in animal models [32] and allogenic clinical studies [20]. As mentioned previously, Ott *et al.* has characterized the mechanical properties and ECM composition in an animal model, whereas Macchiani *et al.* characterized clinical factors for allograft implantation of tracheal tissue. Yet, both decellularization groups utilized functional studies to recapture the necessary physiologic functions of the recellularized organ.

The measure of restored function has been quantified by physiological parameters. For example, 24 h after recellularization in a coronary perfusion bioreactor, electrical stimulation was introduced *in vitro* to the whole rat heart. The heart, repopulated with a mixed population of cardiac cells, regained approximately 2% pump function of an adult rat heart, and 25% pump function of a 16-week fetal human heart as evaluated by its performance under physiologic preload, afterload, intraventricular pressure and electrical stimulation. Furthermore, doses of phenylephrine administered to the recellularized whole heart were also able to stimulate contractility similar to extrinsic control mechanisms that exist physiologically [32].

Similarly, in the decellularized human whole-trachea graft study, the restored physiological function of airway clearance in the allogenic, trachea graft was evaluated. The ratio of forced expiratory volume in 1 s to forced vital capacity (FEV₁:FVC) was taken before and 3 months after surgery, and determined that the graft resulted in a reversal of airway obstruction [20]. As additional clinical studies are completed, we will continue to learn more about the instructive nature of decellularized grafts such as the preservation of angiogenic

cytokines [20], as well as other chemokines preserved from previous niches that may affect morphogenesis [4,14,35,36].

Electrospun nanofibers

Electrospinning is a nanofabrication technique that generates nonwoven fibers of uniform, reproducible composition on a physical scale that is topographically compatible with the cells that reside upon them. Electrospun natural materials offer the benefits of incorporated peptide motifs that synthetic materials lack, although natural materials can be too weak to form reproducible nanofibers, and are therefore often blended with biocompatible, synthetic polymers to create composite fibers with added strength [37]. Furthermore, the addition of natural polymers to synthetic fibers has improved the adhesion, viability and proliferation over synthetic material alone [38,39].

Besides having the ability to form nanoscale fibers, 3D assemblies of natural or natural/synthetic blend fibers can also be used to create composite structures, allowing for a closer replication of natural nanoscale ECM fiber composition within an organ. For instance, the basement membrane bordering epithelial sheets consists primarily of fibrillar proteins such as collagen IV and laminin [3]. In terms of structure, electrospun laminin I meshes have recently been fabricated to resemble basement membrane [40]. Bead-like structures, usually indicative of spinning limitations in terms of thinness, were preserved in the laminin spun fibers due to the replication of 'matrisome' structures found in the natural basement membrane. Laminin meshes, in comparison to laminin films, induced more adherent, elongated morphologies commonly associated with neuronal-like cells, as well as $\beta 3$ tubulin expression in serum-free media, without the use of chemical additives. The addition of exogenous NGF did not increase the frequency of neurite extensions per cell, in comparison to the frequency of laminin nanofibers without NGF. Neal *et al.* postulated that the topography and the associated high surface area of electrospun fibers were attributable to the extensions [40].

Layer-by-layer depositions, multilayer mixing, dual-mandrel electrospinning and magnetic electrospinning have also been used to manufacture multidimensional scaffolds [41–44]. Elastomeric vascular graft-fabrication methods have been investigated for over 30 years. Recent efforts have incorporated electrospinning to create a composite graft from poly(ethylene oxide), segmented polyurethane and UV-crosslinked collagen I by rotating a traversing movable mandrel [41]. The use of cytotoxic crosslinkers was avoided for the bioadhesive, collagen component of the scaffold. Instead, UV crosslinking was used to render the collagen insoluble, but still capable of swelling.

Since Kidoaki *et al.* demonstrated multidimensional construction of an electrospun vascular graft, other groups have proceeded to implant and characterize 3D electrospun scaffolds *in vivo*. Using sheep and rabbit models [45], a poly(ϵ -caprolactone)/collagen vascular graft was evaluated based upon functional and physiologic parameters such as patency, platelet adhesion resistance, which was based on previous studies that had established upper limits for burst pressure (4912 ± 155 mmHg), tensile strength (4.0 ± 0.4 MPa) and adequate elasticity (2.7 ± 1.2 MPa) [46].

The challenge now is to reduce the cytotoxic solvent and/or crosslinker levels currently used to produce natural and natural/synthetic blended fibers [47]. The process of electrospinning often includes the addition of organic solvents to dissolve polymers prior to spinning [48]. Crosslinkers are used to strengthen spun fibers and render natural fibers more water resistant [40]. Some natural-spun fibers have circumscribed or reduced cytotoxic solvent or crosslinker usage by using techniques such as melt electrospinning, or by replacing organic solvents and crosslinkers with less toxic alternatives [37,40,49,50].

Electrospun fibers are increasingly becoming incorporated into many different applications. 3D tissues are also being formed with spun nanofibers incorporated into the composite design [45,51–54], and hydrogel 3D composites with stitched fibers have been tested in animal models [51,52]. Thus, as biomimetic studies reduce the amount of cytotoxic crosslinkers and solvents used in electrospun scaffolds, continued efforts to improve biocompatibility may increase the frequency of electrospun scaffold use in clinical trials for whole-organ engineering applications.

Responsive materials, remodeling & engineered gradients

The term ‘smart material’ encompasses many materials that recapitulate the dynamic nature of ECM remodeling in an engineered whole organ. Traditionally, ‘smart materials’ include materials such as piezoelectric sensors, shape memory alloys and polymer-responsive gels, which can respond to environmental fluctuations through physical or chemical changes. Smart polymers respond to changes in pH, ionic strength, chemical species, enzyme–substrate interactions, magnetic fields, temperature, electric fields, as well as ultrasound irradiation [55,56]. These materials exploit responsiveness for a triggered release. In response to the changing environment, smart polymer hydrogels swell, thus facilitating an intended deployment of the triggered response, such as drug release.

Various corneal tissue-engineering groups have harvested *ex vivo* cellular sources found in short supply, and expanded and implanted the cells using tissue sheets of a noninvasively thermoreversible polymer [57,58]. While poly(*N*-*N'*-(dimethylacrylamide)) (PIPAA-m)-implanted cells restored function to the affected corneas, PIPAA-m sheets restrict applications to 2D and cannot fully replicate cell-matrix remodeling dynamics [55]. Replacement of PIPAA-m and other synthetic scaffold materials with natural ECM polymers is more desirable for potential 3D activities.

Natural matrix remodeling has been well documented in organs such as bone. In orthopedics, guided bone regeneration has been pursued with natural materials fabricated into scaffolds, such as demineralized bone, which possesses osteo-inductive properties [59]. Recently, demineralized bone has been manufactured as a nanoscale bone matrix (NBM) powder and incorporated within poly(L-lactide) (PLA) electrospun fibers [60]. Human mesenchymal stem cells osteodifferentiated on both PLA and PLA–NBM scaffolds *in vitro*, and accelerated mineralization on PLA–NBM fibers occurred *in vivo* [61]. PLA–NBM also provided a scaffold with mechanical properties higher in average tensile strength and Young’s modulus than PLA alone, but provided lower values than cortical bone [60]. As a result, PLA–NBM constructs can provide osteoinductive cues and may approximate the mechanical properties of immature bone undergoing remodeling with minimal loading [60]. After 12 weeks *in vivo*, the defect was almost 90% smaller than its original size, demonstrating the responsive remodeling potential of PLA–NBM [61].

In addition, in soft-tissue remodeling, the fabrication of heart muscle using natural ECM matrices is being claimed as a new type of natural smart material [62]. Blan *et al.* manufactured a composite scaffold of chitosan, a natural biomaterial that was solubilized with acetic acid, and later pH neutralized after freezing and lyophilization. Fibrinogen was added for scaffold gelation followed by a thrombin coating, the incorporation of cardio myocytes and an added layer of fibrin. In terms of organ function, this group monitored contractility as well as ECM architecture. For a responsive scaffold, degradation time was manipulated based upon lysozyme concentrations from 2 h to 2 weeks. One advantage of using a responsive enzyme-substrate scaffold is the ability to match changes in matrix metalloprotease activity to that of cell-secreted matrix remodeling [62,63]. With controlled

degradation, cell migration can also be manipulated, although it is important to consider and control for potential unbalanced remodeling and tumorigenic consequences [64–66].

Scaffold degradation can also dynamically change cellular cues such as migration. As proteolytic activity degrades the ECM, cryptic binding sites are unveiled, and matrix-bound growth factors become untethered [64,67]. Cell migration occurring along a gradient, such as that which occurs during chemotaxis, haptotaxis and durotaxis, can therefore be manipulated based upon the design of the ECM [36,68]. Further studies in 3D have been able to demonstrate more physiologically relevant applications by creating matrices with natural materials that take advantage of durotaxis [69,70].

Although advances in whole-organ engineering have origins in 2D *in vitro* models, current methods for bioengineering functional tissues, such as tissue decellularization and use of electrospun nanofibers and smart materials, are progressing to 3D scaffolds. It has been shown to be an oversimplification that cells behave the same in 3D as they behave in 2D [70–74]. Cell-matrix adhesions to the ECM are exaggerated in 2D cell cultures – the adhesions are stronger than those of cells in 3D models and of the cell *in vivo* [72]. Likewise, 3D matrix adhesions are not recreated in 2D [72]. Still, 3D models mimic static, short-term conditions and may oversimplify parameters such as oxygen diffusion [71,75]. Therefore, it is important to verify *in vitro* results with parallel *in vivo* model investigations.

Whole-tooth engineering

The tooth is a prime example of a developing organ with interactions between mesenchymal and epithelial tissues over time. Bioengineering strategies have targeted tooth regeneration with various scaffolds, growth factors and cells [76–79]. Tissue recombination and pelleting cells in a scaffold-free environment are also popular strategies in whole-tooth engineering [80,81]. In addition, groups have investigated the location of the implantation site for engineered implants as a morphogenetic means of developmental signaling. The diastema, or toothless region within the rodent jaw, has been shown to provide a site for whole-tooth formation, thus demonstrating the potential for teeth to regenerate and erupt in a native environment [82,83].

While cell pellets have been used in other studies to replace the need for scaffolds [84,85], Nakao *et al.* included a collagen droplet method to contain pelleted E14.5 epithelial and mesenchymal cells within a collagen gel matrix [77]. The dissociated cell pellets from molars as well as from incisors were able to form predentin, dentin and enamel-like tissue, with specificity confirmed by *in situ* hybridization studies. These E14.5 droplet constructs were able to reassemble into proper tissue layers, whereas older E16.5 cells could not self-assemble [77]. As the effects of collagen were not explored, more can be learned about spatial and temporal tooth development within an ECM.

Ongoing studies in the Yelick laboratory have worked towards engineering a whole tooth, as well as hybrid tooth–bone constructs [86–92]. What remains to be understood is how to maintain spatial organization of the tissues, not only of the shape and size of a tooth, and surrounding alveolar bone, but also of the compartmentalization of individual tissue types [87]. For instance, studies have observed ectopic alveolar bone growth within dental tissues [76,91]. Likewise, the ECM components associated with functional tooth-root formation and for functional tooth eruption are critical to our understanding. Preliminary immunohistology and histology analyses in a 5.5-month-old porcine molar model have demonstrated some ECM fiber organization within the tooth-bud and surrounding alveolar bone. Areas of collagen, keratin and fibronectin co-localize within vascular regions of the dental pulp as well as in enamel epithelium (Figure 1). Future studies will elucidate further ECM organization and composition.

With respect to human tooth re-engineering efforts, further characterization of the ECM molecules involved during critical stages of tooth development are imperative. Others have applied the roles of biglycan, and subsequent amelogenin expression, to nanofiber scaffolding to study enamel maturation [93–95]. Mouse knockout models have also elucidated the role of Tbx1 and enamel formation [96]. Still, further exploration of additional ECM molecules and corresponding spatial organization within the tooth are needed for future scaffold designs. ECM molecules such as collagen I, osteocalcin, amelogenin, dentin sialoprotein and bone sialoprotein are present in the bioengineered tooth-bud and tooth–bone hybrid constructs made of silk and poly(glycolic acid) (PGA)/poly(lactide-co-glycolide) (PLGA), respectively [87,90,91]. As we continue to characterize the locations and 3D organizations of additional fibrillar proteins and proteoglycans in native tissue and natural ECM scaffolds, we will gain an understanding of the spatial organization and function of the natural tissues within the tooth and bone, which guide and maintain final form and functions.

Ongoing challenges in whole-organ engineering

Thus far, many of the clinically relevant grafts have been constructed for hollow organs [52]. Implicit in the creation of solid organs, including teeth, is the proper integration of functional angiogenesis, lymphangiogenesis and neurogenesis within an organ or organ system [97,98]. An ongoing obstacle in angiogenesis is defining a targeted approach to invade and provide oxygen to tissues with volumes greater than 2–3 mm³. As host-vessel ingrowth takes a finite time to penetrate into the depth of the implanted tissue, necrosis can occur prior to vascularization. Many studies have explored means to facilitate bioengineered angiogenesis [99,100] and ongoing *in vitro* attempts to prevascularize engineered tissue may have a future in *in vivo* applications [97,101–103]. Notably, one study has already demonstrated evidence of angiogenesis *in vivo* attributed to multicell type coculture [104]. Others have incorporated mesenchymal cells with endothelial cells in a 3D scaffold in a mouse model to successfully create network formation [105].

Despite these advances, the extent of penetration of vessels into bioengineered tissues and organs has yet to be determined. Additional efforts have considered designing vascular network-based physiological models that consider length, diameter, pressure and flow rate as vessels anastomose into vascular beds as well as recon-verge; these networks are especially applicable in branched, ordered tissues such as the lung or liver [98]. Furthermore, control of vessel architecture is relevant not only in terms of geometric function, but also in terms of maintaining vessel growth [66,99,106,107]. Specific to matrix-induced angiogenic efforts, groups are beginning to identify pro- and anti-angiogenic motifs on natural ECM peptides such as collagen IV [106,108,109]. From this information, gradients to drive angiogenic potential within a construct have been manufactured [110].

Another relatively new subset of angiogenic tissue engineering involves integrating lymphatic system formation in bioengineered tissues and organs. Similar concerns that exist in promoting angiogenesis persist with lymphatogenesis, in terms of controlling tissue growth and preventing metastatic behavior [111,112]. Animal models are currently being explored to investigate balancing pathologies in the lymphatic system [113–115]. However, it was found that lymphatic endothelial cells do not sprout to form a vessel unit, but rather the cells migrate to a site individually and allow unidirectional flow [116]. Perlecan, an ECM molecule involved in vascular homeostasis, was produced in a tissue-engineered model of skin regeneration within a rat model, although interestingly the ECM molecule was only formed after the construction of the lymph vessel and was expressed in the direction of flow [116]. Therefore, integration of a lymphatic system to prevent edema from developing in an engineered organ may also be affected by changes in the ECM environment. As

engineered organs become more prevalent, systemic integration with the lymphatic system will provide an immune response ‘watchdog’ to maintain homeostasis via lymphatic vessels, and to sustain hydrostatic and osmotic pressures at vessel–tissue interfaces [115].

Although tissue-engineering platforms exist to guide axonal growth [117], more cohesive strategies are needed to innervate engineered constructs in a controlled manner. Currently, autologous epineurium [118], or better still an autologous sural graft [119], serve as gold standards for peripheral nerve regeneration. However, autografts have caveats, from supply shortages to the fact that they may not be made-to-order in terms of nerve length, may create wound pain and often have a motor function recovery rate of less than 40% [118,119]. To innervate an organ, the graft should match the mechanical properties of surrounding areas, allow for diffusion, provide the appropriate degradation when a nerve regenerates and provide migratory guidance cues to recruit nearby axons from a neural growth cone or other means of taxis [117,119]. Current neural-tube materials include, but are not limited to, PGA–collagen, PLGA, poly-L-lactic acid, silicon and hydroxyapatite (HA)–collagen hydrogels [118,119]. While synthetic neural tubes may offer improved stiffness, the same synthetic materials have caused complications such as inflammatory responses and scarring [118,119].

Directing neurogenesis in a precise manner to integrate the implanted tissue within the host, and to test for restored function of the nerve, are ongoing struggles. A recent rabbit facial nerve defect model, which tested a HA–collagen hydrogel construct with NT-3 growth factor and xenogenic neural stem cells, found minimal signs of immune response and migration of donor cells to affected areas [118]. However, Zhang *et al.* were not able to demonstrate improved neuromuscular function using HA–collagen implants, based on ethology and electromyography results. Functional innervation studies are still difficult to prove in animal models, although various tests have been established for peripheral nerve recovery [119,120]. As Li *et al.* have previously surmized that axonal guidance needs multiple cues for nerve regeneration that could include integrating physical stimulation [117]. For example, 780 nm laser phototherapy, when applied transcutaneously to peripheral nerve-damaged patients, significantly improved motor function as well as voluntary muscle activity in partially paralyzed limbs [121]. These studies demonstrate that cell growth and specific antibody expression should be coupled to a test of restored function, and may need to include multiple cues for guided regeneration.

Unmet needs

Efforts to engineer organs, including teeth, are still struggling to identify available and reliable cell sources, and to optimize efficient incorporation of cells into scaffolds. While the multipotency of adult stem cells is being realized as an autologous source for various tissues, limitations remain for re-engineering immature tissue [122]. For instance, since enamel-forming tissues have undergone apoptosis prior to tooth eruption, it is necessary to identify a dental epithelial cell source for bioengineered enamel production. Although current studies use murine, rat and porcine tooth germ tissues, animal-derived cells are not suitable for clinical applications in humans. Current attempts to reprogram cell sources include the development of spontaneously derived immortalized murine dental epithelial cell lines, which when grown in low calcium-supplemented media, can express enamel markers such as amelogenin [123,124], and the use of single-cell suspensions generated from tooth germ tissue [125]. Lessons from these studies should assist efforts to generate suitable human dental epithelial cell lines from available tissues such as Hertwig’s epithelial root sheath or epithelial cell rests of Malassez. Chimeric teeth, generated from autologous dental mesenchyme combined with xenogenic dental epithelium, may also eventually be used as a clinically relevant alternative approach [81,85].

Incorporating cells into decellularized and/or fabricated scaffolds is also a parameter in need of improvement. While many studies use bioreactors to facilitate cell seeding, reports of uneven adhesion and proliferation persist [11,32]. Direct injection of cells into scaffolds may also create cell clusters rather than even cell distributions, depending on cell migration and cell adhesion receptor binding sites. For example, although Blan *et al.* directly injected cardiomyocytes into the smart chitosan material, this approach did not produce the necessary contractile responses owing to low cell retention. However, injection of cells mixed with fibrin gel allowed for cell adhesion to the scaffold [62]. Methods for cell penetration into, retention on and proliferation within scaffolds are anticipated to improve as strategies to promote angiogenesis throughout bioengineered organs continue to advance, and allow for continuous recruitment of host progenitor cells to the implant site.

As supporting scaffolds degrade over time, questions remain, such as: will the regenerating tissue unit be able to grow and remodel? Will engineered organs possess the appropriate ECM signals for proper growth and remodeling, based upon the proposed regression, and pruning models of blood vessel and nerve formation [67,106,119]? Although defined methods to properly manipulate and monitor angiogenesis, lymphangiogenesis and neurogenesis in bioengineered organs remain unclear, they should become clearer as additional functional, long-term studies using whole-engineered organs are completed.

Future perspective

Whole-organ engineering is a new field, with plenty of room to grow and a multitude of inter-disciplinary talents involved in the pursuit. As we continue to learn more about natural ECM materials, fabrication technologies will also advance. Over the next few years, we will need to learn more about the composition, degradation and remodeling rates of decellularized cadaveric allografts as the demand for clinical studies and the number of potential commercial products increases, and methods for improved removal and detection of MHC antigens continue to improve. As microarray techniques continue to improve high-throughput screening, we will be able to better characterize serology screens for foreign antigens in decellularized cadaveric tissues.

Likewise, the generation of thin electrospun fibers with less toxic solvents and crosslinkers will improve current tissue-engineering efforts. Natural crosslinkers, such as genipin, have already shown improved biocompatibility over glutaraldehyde in tissue fixation [126–128]. Other groups have demonstrated incorporation of genipin into natural ECM hydrogels [69,129] and natural electrospun nanofibers [50]. With improved nanofiber production methods, more complex 3D electrospinning techniques can be used to find better ways to integrate a variety of fabricated scaffold layers on the mesoscopic scale.

Smart polymer technologies should increase in clinical relevance as the demonstrated use of composite corneal implants becomes more prevalent. As 3D synthetic scaffolds develop or as natural ECM analogs are better characterized based on remodeling characteristics, implants will become more dynamic, thus reducing the number of surgeries needed for a procedure. Similarly, more long-term studies will assist in our understanding of the degradation processes of scaffolds over time, along with the associated cascade of matrix metalloprotease activities and soluble cytokines, which redefine and remodel the ECM.

Looking beyond the next few years, pluripotent reprogramming technologies such as induced pluripotent stem cells and piggyBac will continue to improve [130–132], and new and useful human embryonic cell lines will be created. Thus, these cells will provide new sources for organ engineering to study ECM synthesis and turnover. As instructive scaffold technologies continue to improve, we anticipate being able to directly control and monitor

cell behavior, migration and differentiation throughout the morphogenetic process of any organ formation.

Executive summary

The current state of the art for natural scaffolds

- The goal of decellularized tissue is to retain the mechanical, functional and compositional properties of the intended graft, while demonstrating nucleic acid removal.
- Electrospun fibers attempt to recreate the properties of extracellular matrix (ECM) nanofibers. Fibers can be constructed in 3D for biomimetic applications. There is also a trend to reduce or replace cytotoxic solvents and crosslinkers.
- Responsive scaffolds try to recreate niche responses through chemical and physical changes such as matrix metalloprotease secretion. Such degradation provides the necessary cues for ECM remodeling. 3D scaffolds allow for cell adhesion behavior to mimic that which is experienced *in vivo*.

Whole-tooth engineering

- The tooth is a prime example of organ development and consequential attempts at organ engineering.
- Multiple groups are trying to incorporate ECM molecules within their whole-tooth engineered organ.

Ongoing challenges

- Beyond depths associated with hollow or thin organs, engineered tissues need to include angiogenesis, lymphangiogenesis and neurogenesis.

Unmet needs

- Alternative cell sources to adult stem cells need to be pursued to create a sustainable source of immature cells such as human dental epithelium.
- Cell-seeding protocols should improve to evenly distribute cells throughout the construct as angioneogenesis is better incorporated into engineered tissues and organs.
- Long-term studies need to describe the balance between degradation and remodeling processes.

Future perspective

- More will be discovered in terms of composition, degradation and remodeling rates of decellularized cadaveric allografts as the demand for clinical studies and the number of potential commercial products increases, and methods for improved removal and detection of MHC antigens continue to improve.
- Electrospun scaffolds and smart polymers will also improve over time as more biocompatible materials are fabricated based on knowledge gleaned from previous scaffold efforts and from the composition of natural ECM molecules within organs.
- Whole-organ engineering will advance through the use of natural materials, particularly when incorporated with new sources of reprogrammed progenitor cells and embryonic stem cells.

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Figure 1. Extracellular organization of the porcine molar tooth-bud and surrounding bone

Masson's trichrome histological analyses (A–C) show positive staining for collagen (blue) and keratin (red). Immunohistochemical analyses (E–G) display positive fibronectin expression (green–brown). Higher magnification images of the boxes in (A) are found in (B) and (C). Likewise, higher magnification images of the boxes in (E) are found in (F) and (G). Arrows denote fibronectin expression in the stellate reticulum (F). Composite collagen, keratin and fibronectin organization are depicted schematically (D), with emphasis on the enamel epithelium (B & F), vasculature and dental papilla (C & G). Scale bars = 1000 μ m in (A & E) and 100 μ m in (B, C, F & G).

ab: Alveolar bone; am: Ameloblast; bv: Blood vessel; d: Dentin; e: Enamel; oe: Outer enamel epithelium; p: Pulp; sr: Stellate reticulum.

Table 1

Natural extracellular matrix molecules incorporated into engineered organs and tissues.

Natural ECM components	Engineered organ/tissue	Ref.
Collagens	Cartilage	[2,39]
	Blood vessels	[41,45,46,110]
	Nerves	[42,118]
	Ligament	[51]
	Bladder	[52]
	Teeth	[77,89–92,125]
	Lymphoids	[114]
Gelatin	Skin	[48,49,128]
	Blood vessels	[41]
	Cartilage	[127]
Hyaluronic acid/hyaluronan	Blood vessels	[39,110]
	Nerves	[118]
Laminin	Peripheral nerves	[40]
Genipin	Skin	[50,128]
	Nerves	[69]
	Cartilage	[127]
Silk/fibroin	Skin	[50]
	Ligament	[51]
	Mineralized osteodentin	[87]
Chitin/chitosan	Cardiac muscle	[62]
	Bone	[63]
Fibrin	Cardiac muscle	[62]
	Blood vessels	[67]
Syndecan-1	Breast	[65]
Perlecan	Lymph vessel	[116]

ECM: Extracellular matrix.

Table 2

Extracellular matrix molecules characterized in decellularized organ and tissue studies.

Natural ECM components	Decellularized organ/tissue	Ref.
Collagens	Bladder	[9]
	Blood vessels	[11]
	Heart valve	[12,13,15,17]
	Pericardium	[18]
	Trachea	[20]
	Esophagus	[21]
	Cornea	[28]
	Pulmonary roots	[29]
	Meniscus	[30]
	Ligament	[31,33,34]
	Heart	[32]
<hr/>		
Laminin	Bladder	[9]
	Heart valve	[13]
	Esophagus	[21]
<hr/>		
Elastin	Blood vessels	[11]
	Heart valve	[12,13,15,17]
	Pericardium	[18]
	Esophagus	[21]
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Cytokeratins	Trachea	[20]
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Fibronectin	Esophagus	[21]
	Heart	[32]
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Chondroitin sulfate	Meniscus	[30]
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Glycosaminoglycans	Bladder	[9]
	Pericardium	[18]
	Heart valve	[12,13,17]
	Meniscus	[30]
	Ligament	[31,33,34]

ECM: Extracellular matrix.