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Review

Decellularization and Recellularization Technologies in Tissue Engineering

Ru-Huei Fu,*†[1] Yu-Chi Wang,‡[1] Shih-Ping Liu,†§ Ton-Ru Shih,* Hsin-Lien Lin,* Yue-Mi Chen,* Jiun-Huei Sung,* Chia-Hui Lu,* Jing-Rong Wei,* Zih-Wan Wang,* Shyh-Jer Huang,¶ Chang-Hai Tsai,#** Woei-Cherng Shyu,*† and Shinn-Zong Lin*†††‡‡

*Graduate Institute of Immunology, China Medical University, Taichung, Taiwan
†Center for Neuropsychiatry, China Medical University Hospital, Taichung, Taiwan
‡Biomedical Technology and Device Research Laboratories, Industrial Technology Research Institute, Hsinchu, Taiwan
§Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan
¶Chinese Medicine Research and Development Center, China Medical University Hospital, Taichung, Taiwan
#Department of Pediatrics, China Medical University, Taichung, Taiwan
**Department of Healthcare Administration, Asia University, Taichung, Taiwan
††Department of Neurosurgery, China Medical University Beigang Hospital, Yunlin, Taiwan
‡‡Department of Neurosurgery, Tainan Municipal An-Nan Hospital, China Medical University, Tainan, Taiwan

Decellularization is the process by which cells are discharged from tissues/organs, but all of the essential cues for cell preservation and homeostasis are retained in a three-dimensional structure of the organ and its extracellular matrix components. During tissue decellularization, maintenance of the native ultrastructure and composition of the extracellular matrix (ECM) is extremely acceptable. For recellularization, the scaffold/matrix is seeded with cells, the final goal being to form a practical organ. In this review, we focus on the biological properties of the ECM that remains when a variety of decellularization methods are used, comparing recellularization technologies, including bioreactor expansion for perfusion-based bioartificial organs, and we discuss cell sources. In the future, decellularization–recellularization procedures may solve the problem of organ assembly on demand.

Key words: Biomaterial; Decellularization; Extracellular matrix (ECM); Organ bioengineering and regeneration; Recellularization

INTRODUCTION

Tissue engineering studies have until now been able to partially increase cell engraftment by reinforcing cells and cell contact in animal models and by providing non-immunogenic matrices before transplantation (45). However, these methods are limited to transplantation into a smaller amount of vascularized areas and two-dimensional structures (58,61,95). Promising clinical tests involve the use of two main scaffolds: synthesized material and a natural matrix (6,59). Artificial scaffolds are made of polymers such as polypropylene or polyglycolic acid. Decellularized natural matrices are another source of material for the scaffold. The use of decellularized scaffolds is an exquisite technique because the resulting material is likely to maintain the original architecture of the tissue or organ, including the function of primitive microvascular systems (4).

A review of tissue decellularization techniques and developments was published in 2006 (28), but improvements in these techniques using three-dimensional whole organ decellularization have since appeared (17). The purpose of the present review is to focus on the biological characteristics of the extracellular matrix (ECM) that remains when various decellularization methods are used,

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[1]These authors provided equal contribution to this work.
Address correspondence to Shinn-Zong Lin, M.D., Ph.D., Center for Neuropsychiatry, China Medical University Hospital, No. 2, Yude Road, Taichung 40447, Taiwan, ROC. Tel: +886-4-22052121, ext. 6034; Fax: +886-4-22080666; E-mail: shinnzong@yahoo.com.tw *or* Ru-Huei Fu, Ph.D., Graduate Institute of Immunology, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan, ROC. Tel: +886-4-22052121, ext. 7826; Fax: +886-4-22052121, ext. 7810; E-mail: rhfu@mail.cmu.edu.tw

after which stem or progenitor cells can be seeded on the biological scaffold with ECM proteins, and the cells are able to proliferate and differentiate, as well as to compare recellularization technologies.

DECELLULARIZATION AND RECELLULARIZATION TECHNOLOGIES

The objective of the decellularization protocol is to minimize any adverse influence on the composition, biological activity, and mechanical incorruptness of the remaining ECM after efficiently removing all cellular and nuclear materials (Fig. 1). Any processing step intended to remove cells during decellularization will alter the native three-dimensional architecture of the ECM. The challenge in decellularization is to find the optimal protocols that will reserve the native ECM for clinical use, depending on its intended purpose.

The most commonly used modus for the decellularization of tissues comprises a combination of chemical, enzymatic, and physical approaches. Various procedures have been developed to remove the cellular component of both native and engineered tissues while minimally disrupting the ECM. Decellularization protocols are typically initiated with lysing the cell membrane by usage of physical treatments or ionic solutions, and then dissociating the cellular components from the ECM by using enzymatic treatments and detergents for the solubilization of cytoplasmic and nuclear cellular elements, and finally removing cellular debris from the tissue. These steps can be done with mechanical agitation to enhance their utility. To prevent an adverse host–tissue response to the chemicals, all residual chemicals must be removed after decellularization. According to the report of Crapo et al., DNA content needs to be less than 50 ng/mg tissue for adequate decellularization (17).

The detailed methods depicted in the literature combine several of these principal techniques in order to enhance the efficiency of decellularization and, at the same time, to reduce the potential damage to the ECM by exposing it to less detrimental conditions.

Chemical Compounds

Acids and bases (69) work to denature proteins, solubilize cell elements, and alter nucleic acids, thus rupturing

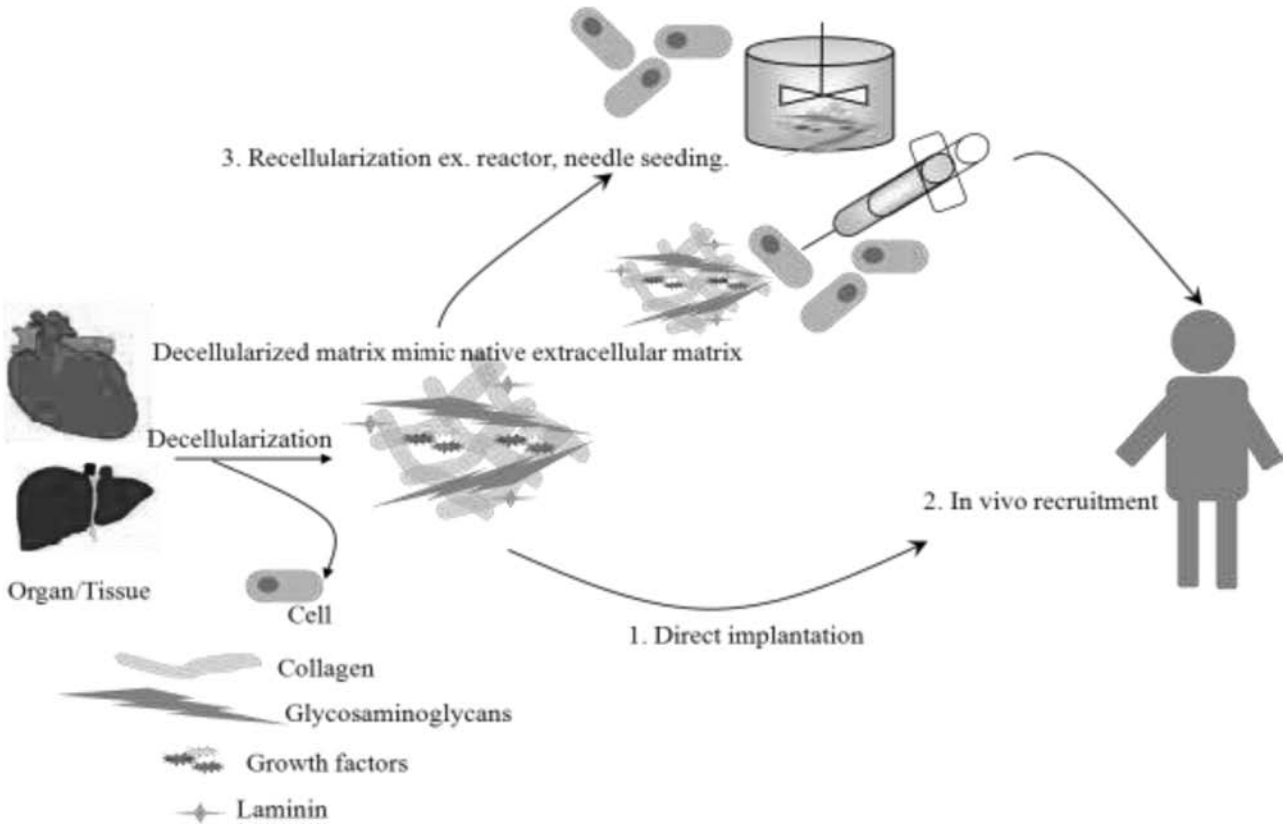

Figure 1. Rationale of tissue decellularization. The cell-free scaffold can originate from the decellularization of native material or from synthesized material. The resulting scaffold can be directly transplanted into a patient (1), relying on its capacity to stimulate cells toward endogenous tissue repair (2). (3) In addition, prior to implantation, the extracellular matrix (ECM) can be seeded with cells that guide the material (to enhance remodeling or vascularization) or have a specific function (proliferation or differentiation).

the cells. However, these compounds are not selective, and so they can adjust the ECM elements, particularly collagen, glycosaminoglycan (GAG), and growth factors.

Hypertonic and hypotonic solutions (99) disrupt cells through osmotic shock and also interfere with the interaction between DNA and proteins. Although being relatively efficient at disrupting cells, they do a disappointing job of removing cellular residues.

Ionic or nonionic detergents, or a combination of both (79), destroy DNA–protein interactions, lipids, and lipoproteins and may denature proteins. As a result of their action on cells, they also damage the ECM ultrastructure, leading to wear and tear of collagens, growth factors, and GAG.

Other solvents, such as alcohol and glycerol, aid in dehydrating and lysing cells during tissue decellularization (67). Acetone can also be applied to remove lipids during decellularization (49,54). However, like alcohols, acetone is a tissue fixative (40) and damages the ECM ultrastructure (15), guaranteeing conservative use, especially for biological scaffolds used in load-bearing clinical applications (30,31). Tributyl phosphate (TBP) is an organic solvent with viricidal characteristics. For decellularization of dense tissues such as tendons, TBP is more effective than detergents such as Triton X-100 and sodium dodecyl sulfate (SDS), with varying effects on the preservation of ECM constituents and its native mechanical properties (13,20).

Biologic Solutions

Enzymes (66) can be used to target the vestiges of nucleic acids after cell rupture or to target the peptide bonds that anchor the cells to the ECM. They tend to remain in the tissue in significant amounts and may lead to higher levels of immune response. Long-term exposure can also result in the removal of collagens, laminin, fibronectin, elastin, and GAG, as well as damage to the ECM ultrastructure.

Nonenzymatic agents—chelating agents such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid—have a particular way of congregating with ions and metal ions that advances cells to dissociate from ECM proteins by sequestering metal ions (27,43).

Other Agents: Physical and Miscellaneous

Pressure, temperature, force, and nonthermal permanent electroporation (NTIRE) can also be used in decellularization protocols. Multiple freeze–thaw cycles may be used (16) that do not significantly enlarge the loss of ECM proteins from tissue (65). Cells on the surface of a tissue or organ such as urinary bladder, small intestine, skin, and amnion can be efficiently removed by mechanical abrasion in combination with enzymes (36), hypertonic saline, or chelating agents, all of which facilitate dissociation of cells from their subjacent basement membrane. NTIRE has also been investigated as an approach to tissue decellularization (74).

The optimal conditions for decellularization agents are reliant on tissue characteristics such as thickness and density, the agents being used, and the predictable clinical application of the decellularized tissue. Before decellularization agents are applied, any undesirable overdose tissue may be removed to simplify the cell removal process (26). Still, some problems remain common to all agents. First, all existing techniques rely on cell lysis. The resulting cell debris can thus generously adsorb to the remaining matrix, consequently raising immunogenicity, leading to contradictory results (8). Second, existing techniques have been proven to degrade some components of the ECM (17). On the other hand, pathogenic viruses that place the recipient at extensive risk are thought to be eliminated by endonuclease treatment, as integrated viral DNA/RNA is likewise fragmented. Maintenance of the native tissue architecture, ECM components, and growth factors for proper cellular homing and differentiation are the significant properties of these scaffolds. The autologous or allogeneic cells are seeded back into the scaffolds to repopulate the matrix and bring back function to the organ. Numerous methods are used to reseed scaffolds, with dynamic cell-seeding methods tending to be more effective than static methods (92,96). To date, static seeding is the most frequent method in the construction of tissue, in which a dense cell suspension is passively introduced to a scaffold. This seeding technique yields seeding efficiencies of approximately 10–25% (3). This technique has several limits, however, such as low seeding competence and minimal cell penetration of scaffold walls. To conquer some of these drawbacks, alternating seeding techniques have been developed, including magnetic, dynamic, electrostatic, vacuum, and centrifugal seeding (92). The most common of the dynamic methods for reseeding is to straightly add cells at elevated concentration into the vascular perfusion line just upstream of the organ, allowing cells to travel directly through the vascular tree into the scaffold and parenchyma. This method is extensively used in recellularization (64) of the blood vessels of the heart, lungs (63), and liver (7,85). Many researchers have delivered cells via the scaffold vasculature to an organ parenchyma, and the cells are thought to traverse the vascular lining through holes or pores created by the decellularization procedure. During this reseeding process, low flow rates are used to decrease the shear stress on the cells. For the liver, a high seeding efficiency (86–96%) has been reported by directly injecting into the portal vein via the bioreactor inlet port; dividing the cells into multiple injections is superior to a single infusion with the identical total number of cells (85,91).

Portal vein-seeded endothelial cells were indicated to be primarily deposited in the periportal regions of the liver lobule, while the vena cava-seeded endothelial cells were primarily concentrated in the regions of the central veins and in infertile branches and vessels (11). The second way to recellularize the liver is to inoculate cells into the bulk media and permit them to recycle through the circuit to reseed the organ; however, this achieved a lower seeding effectiveness (69%) than did the method described previously (85).

The organ parenchyma cells might be seeded together in a mixture (7,63) with endothelial cells or via separate inoculations of unmixed cell populations (85,91). Using a small-gauge needle, direct inoculation of cells into numerous locations of organ parenchyma is a substitute method of recellularization (64,78). However, this method was less efficient than delivering hepatocytes through the vasculature (85). Although the vascular system is most commonly used to present cells into a scaffold, other routes may be needed to populate the diverse cell types that make up an organ. The lung is the most developed model for alternative delivery of customized cells, with the tracheobronchial tree primarily used to deliver pneumocytes or mesenchymal stem cells (10). Delivery of specialized cells such as urothelium to growing kidneys and cholangiocytes to the liver will likely necessitate direct seeding through the ureter or bile duct, respectively.

A cylindrical configuration and spinner flask are accessible in bioreactors used for cell seeding. Bioreactors have both advantages and disadvantages. The cylindrical configuration limits dead zones and promotes mixing, as determined by mathematical modeling and experimentation (46). An often used bioreactor device is a spinner flask with a stir bar on the bottom, which is specifically useful when seeding cells into bulk media. The continuous flow stirred tank reactor keeps cells in suspension that may have failed to lodge in the scaffold, allowing for multiple passes through the scaffold. However, the cells used in recellularizing an organ are adhesion dependent, and so anoikis may happen if cells recirculate for extended periods. The organ is typically suspended on the inlet line, and stirring the bulk fluid may lead to adverse rotational shear on the organ. Pausing the stir bar will minimize lysis of cells caught between the vessel and the stir bar (71). During organ decellularization, flow resistance is reduced since the scaffold is inversely proportional to cell density and porosity (55). But as reseeded cells flow into the vascular tree, pores will fill with cells, and the pressure drop will enlarge as the porosity reduces (46). During reseeding, increasing the cell seeding density increases the probability of cell aggregates occluding vessels and forming thick polylayers. This can result in oxygen and nutrients becoming mass transfer limited, leading to hypoxia and the promotion of a necrotic core (39,42,52,98). Experimentation to determine the optimal media content is critical. The addition of extra growth factors may be necessary if sufficient levels are not retained in the ECM. However, it is important to maintain a suitable balance. For example, vascular endothelial growth factor (VEGF) is necessary for endothelial cells to seed the scaffold vasculature and to constitute new vessels. But when high levels of VEGF and fibroblast growth factor (FGF) are added, giant cells and aggregates form (93). At times, modification of the oxygen level is demanded.

The bioreactor may need to supply the growing organ with physical, electrical, or chemical advance, or a combination of these, depending on the situation. For example, the heart tissue requires itself to be mechanically stretched and electrically stimulated. Mechanically stretching tissue facilitates cell alignment, elongation, and expression of connexin-43, a cardiac marker (51). Electrical stimulation can also be used in tandem with mechanical stretching (51,88). The lung requires particular consideration for the bioreactor design. As the lung is inflated and deflated, the ECM must retain appropriate mechanical compliance. In order to accomplish this, the common method used in a bioreactor is to suspend the lung scaffold in a container of media and to connect the lungs to a ventilator that matches the volume and respiratory rate of the animal (53,68,81,82). In a lung transplanted from a rat, with the bioreactor design, it is promising to recellularize the cadaveric lung to such an extent that improvement in oxygen exchange can be demonstrated with the recellularized lung at 7 days (81,82). The kidney does not necessitate mechanical stimulation, but benefits from chemical stimulation. Humes and Cieslinski found that when kidneys were cultured with convert growth factor β1 and *trans*-retinoic acid, renal proximal tubule cells grew as a monolayer and produced lumens with polarized epithelial layers, microvilli, and tight junction complexes; this did not occur without these factors (37). After culturing, the cells were incorporated into hollow fibers and coupled through an extracorporeal circuit to dogs with renal failure. The hollow fibers containing proximal tubule cells enlarged the level of activated vitamin D (1,25-dihydroxy: vitamin D_3) by 5.8 pmol/ml from the uremic dog's pretreatment baseline in excess of the course of 3 days, whereas activated vitamin D levels in dogs with sham-control hollow fibers decreased by 4.0 pmol/ml from their pretreatment level (24).

Tissue decellularization might be applicable to any organ. The construction of perfusion-decellularized native ECM scaffolds that match organs in size and structure is the first step in the direction of generating functional tissue that can be transplanted. However, they necessitate enhancement and customization in size, category, species, and cell sources. More important, the resulting

scaffolds need to be reproducible, sterile, and preservable for future procedures.

THE ECM

For each tissue and organ in the body, the ECM is a mixture of structural and practical proteins, lipids, proteoglycans, and crystals that has a distinct composition and physical characteristics. The ECM has been shown to, in particular, promote cell aggregation, adhesion, migration, proliferation, and differentiation in a way that reflects the functional necessities and biological uniqueness of tissues (17). Such guiding elements may be retained, at least in part, even in the lack of living cellular components. On the basis of this reason, the decellularized ECM has received more and more attention in the field of regenerative medicine as an off-the-shelf and immune-compatible alternative to living grafts for tissue and organ restoration (Fig. 1). Decellularized ECM is expected to stimulate regenerative processes, not only through specific "organomorphic" structures (89) but also by its physiological introduction. The ECM is involved in cell communication, as well as in defining the shape and stability of tissues (83). The instructive scaffold materials derived from decellularized ECM could be activated by living cells prior to implantation, with the hypothesis that the ECM is capable of directing the differentiation fate of the seeded cells (19,22,41,73). During the process of tissue decellularization, preservation of the native ultrastructure and composition of the ECM is highly desirable (2,5,60,64,66,80,91). The effectiveness of decellularization and the preservation of the ECM can be evaluated through a variety of methods. Some commonly used agents (e.g., chemical, enzymatic, and physical) and their effects on cellular and extracellular tissue constituents are reviewed in the following sections (17).

Chemical Compounds

Alkaline and acid treatments are used to solubilize the cellular components dissolved in the cytoplasm, as well as to remove nucleic acids such as RNA and DNA. For example, acetic acid, peracetic acid (PAA), hydrochloric acid, sulfuric acid, and ammonium hydroxide can effectively disrupt cell membranes and intracellular organelles in decellularization protocols (21,23,25). Unfortunately, these chemicals also dissociate important molecules such as GAGs from collagenous tissues. In porcine urinary bladder, for example, the concentration of PAA at about 0.10–0.15 (w/v) is highly efficient in removing cellular material (47), and the effect of PAA treatment on ECM components has been studied extensively. However, the microstructure of the collagen fibers is not the same as before the treatment (12). Like hyaluronic acid, heparin, heparin sulfate, chondroitin sulfate A, and dermatan sulfate preserve many of the native GAGs following PAA treatment (34). PAA treatment retains the structure and function of numerous growth factors that are resident in the ECM, including transforming growth factor-β, essential FGF, and VEGF (35,94).

Nonionic detergents destroy lipid–lipid and lipid–protein interactions, but leave protein–protein interactions intact; thus, proteins within a tissue or organ should be left in a functional conformation below nonionic detergent management (77). Triton X-100 is the most widely studied nonionic detergent for decellularization protocols. In valve tissue, Triton X-100 led to almost complete loss of GAGs and reduced the laminin and fibronectin content (32). The most commonly used ionic detergent is SDS (70). Compared with other detergents, SDS yields more complete resection of nuclear remnants and cytoplasmic proteins, such as vimentin. SDS tends to sabotage the native tissue structure, however, and to result in reduced concentrations of GAG and loss of collagen integrity (97).

TBP did not have an effect on the tensile strength of collagen fibers isolated from rat tail tendon, compared with the native control, but it did on the anterior cruciate ligament, resulting in a reduction in collagen content (97) after TBP treatment. TBP appears to be a promising chaotropic agent that has minimal impact on the mechanical manners of the ECM and so is worthy of advanced study (20).

Biologic Solutions

Trypsin is one of the most frequently used proteolytic enzymes in subculture for passaging cells. However, enzymatic methods of decellularization are not without adverse effects on the extracellular components of tissues and organs. Long-term treatment with trypsin/EDTA has been shown to result in disruption of the normal pulmonary valve ECM structure, but did not influence the amount of collagen in the tissue (76). Extended exposure to trypsin/EDTA significantly decreased the elastin content and GAGs over time, with *o*-sulfated GAGs (chondroitin sulfates, keratin sulfates, and dermatan sulfates) showing the greatest decrease. Such treatments can decrease tensile strength by up to 50%.

The ECM that remains after such enzymatic decellularization protocols still supports endothelial cell growth in vitro despite the removal of ECM components (32,76). It is nonetheless acceptable to limit exposure to trypsin/EDTA treatment to minimize the disruptive effects on the ultrastructure and composition of the ECM.

Other Agents: Physical and Miscellaneous

The temperature must be carefully controlled to avoid the formation of ice, as well as to interrupt the rate of alteration of the ECM. Although freezing can be an effective method of cell lysis, it must be followed by processes to remove the cellular material from the tissue (48). Even when the number of cycles was enlarged to

10, freeze–thaw cycling alone could not remove all of the cell nuclei (48). However, freeze–thaw cycling combined with other methods can be used to achieve more satisfactory decellularization. According to some reports, freeze–thaw cycling can be combined with detergent and nucleases to accomplish optimal decellularization of menisci (86). It has also been indicated that cellular components can be removed by freeze–thaw cycling and ammonium hydroxide, while retaining the ECM in two-dimensional culture systems (33).

Cells can be lysed by applying straight pressure to tissue, but this method is effective only for tissues or organs that are not characterized by densely organized ECM (e.g., liver, lung). Organs such as the small intestine (62) and bladder (72) are characterized by natural planes of dissection in which mechanical force can be used to delaminate the tissue layers from these organs.

These methods are efficient, causing minimal disruption to the three-dimensional architecture of the ECM within these tissues. In the current review, we have evaluated the outcome of the most commonly used chemical and physical decellularization methods on the ECM in order to optimize a decellularization method for future studies. Further studies are necessary to get better preparation processes and to optimize the methods of applying ECM scaffolds for tissue regeneration.

CELLS FOR RECELLULARIZATION

The clinical quality and scale of the scaffolds is barely the first step of many toward the regeneration of viable and functional tissues. At the current stage of technology, an attempt to recapitulate the entire procedures of embryogenesis from the single-cell stage to organogenesis in the laboratory setting seems unrealistic. The ideal clinically viable progenitor population as a source of cells has not yet been identified. Embryonic stem cells (ESCs) take along ethical and supply issues and are immunogenic, thus affecting their clinical value. Prompted pluripotent stem cells derived from adult cells are an alternative, although disease-related mutations might need to be corrected [e.g., bone morphogenetic protein receptor type II (*BMPR2*) mutations in pulmonary hypertension], and concerns surrounding genetic alterations may demand to be resolved. Creating differentiated cell numbers essential for the regeneration of human-sized organs exceeds current progenitor cell technology and poses significant challenges in phenotypic control at a great scale (56).

Distinct cell sources, including pig cells, fetal cells, induced pluripotent stem cells (87), and ESCs (44), are currently accessible or being investigated as a new paradigm for future treatments of various intractable diseases. The use of stem cells in several clinical trials has begun, although the consequences are controversial (1,9,90). Cells are engineered and multiplied in culture before transplantation, or after use with minimal manipulation after harvesting or isolation, and then delivered directly or by use of bioengineering strategies. The transplanted cells are expected to home to the site of interest (e.g., the liver) or to reserve at the injection site, depending on the particular application. The main challenge in this area is to transition rapidly from the identification of candidate cell populations to the development of valid delivery approaches.

Xenogeneic cells may also be a potential source of cells that could resolve many of the challenges in curing organ failure. The xenotransplantation of cells is not limited by the availability of donors, could be performed repeatedly if needed, and may be effective for the therapy of viral disease (63,100). The most arduous obstacle to xenotransplantation is the immunological rejection of organ transplantation, but major efforts are ongoing to elucidate the exact mechanism involved in cell rejection. Previous studies displayed that porcine hepatocytes survive immune inhibition for up to 3 months after injection in monkey spleen, thus demonstrating that xenotransplantation of pig hepatocytes into patients with severe liver failure (35,57) may become an option in the future.

These studies show that the usefulness of decellularization and the alterations to the ECM vary, depending on the source of the tissue, its composition, its density, and other factors (28). Recently, as a result of the progress in guiding cell differentiation toward specific lineages, in vitro engineered tissues are also being considered as a substrate for decellularization. This approach opens the possibility of producing large quantities of standardized, customized grafts.

The potential applications of decellularized matrix in tissue engineering have been illustrated for a number of tissues, including the liver (11,91), kidneys (60), pancreas (29), bladder (99), artery, esophagus (18), skin (75), and trachea (50). More recently, another group reported the decellularization of a whole heart through perfusion, preserving the original architecture and original microvascular network and allowing for extensive recellularization (64).

FUTURE PROSPECTS

We have reviewed the latest technology in regenerative therapies necessitating entire organ decellularization and the effectiveness of the application. Many of the present efforts aimed at progress strategies for expansion and stemness [e.g., fibroblast reprogramming that skips pluripotent progenitor stages (38) and ESC-derived protein-induced pluripotency (14)] might deliver original solutions applicable to organ engineering in the foreseeable future. Compared with the successful clinical application of this technology in skin or vessels, which was

established early on, there is a huge need to overcome many functional and structural issues before it can be used for organ substitution. This method has presently achieved only short-term functionality in vivo and then only in rodent models. Work in tissue engineering in recent decades, and currently work in the field of stem cell research, is producing encouraging results, which some day might answer the problem of organ assembly on demand (84). Solid organ regeneration based on perfusion-decellularized native ECM scaffolds has an enormous potential in patients with organ failure, one that is apparently still an ambitious goal.

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