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From stem cells and cadaveric matrix to engineered organs

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The definitive treatment for end-stage heart failure, organ transplant, is limited by the supply of donor organs. Successful allograft recipients suffer significant adverse effects from chronic antirejection medications. Positive clinical treatment of injured myocardium with stem/progenitor cells has led to hope that one day autologous stem-cell-derived whole or partial donor organs can be generated. Advances in the ability to isolate (or generate) stem or progenitor cells that can give rise to beating cardiocyte-like cells and vascular components, and the advent of human iPS cell technology when combined with recent advances in the generation of perfusable complex tissue scaffolds has moved the field closer to creation of a transplantable heart. As cardiac tissue engineering matures, several other simpler cardiac tissues, such as patches for focal use and human cell test beds for drug screening and drug discovery, are emerging.

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Introduction

Cardiovascular disease (CVD) exceeds infection and cancer as the leading cause of death throughout much of the world [13]. Not only is a rise in the prevalence of hypertension, obesity, and type 2 diabetes [14–16] driving the onset of CVD at a younger age [17], but also the increasing number of patients surviving acute myocardial infarction (AMI) [18] is elevating the number of individuals developing heart failure (HF). In fact, up to 50% of post-AMI patients (about half a million annually) manifest symptomatic HF in the United States alone. Because HF is in part due to significant fibrosis and loss of contractile cells throughout myocardium and an inability of remaining cells to keep pace with the demand for contraction, replacing lost or dying cells is a therapeutic option that makes sense to both physicians and patients. This is the genesis for cardiovascular cell therapy. Yet, stem or progenitor cell trials for HF lag behind those for AMI because the therapeutic burden is greater. Preinfarction vascular repair is the target; postinfarction both injured myocardial muscle and vasculature become the focus. With the onset of failure, whole or partial organs must be rebuilt and either integrated with terminally scarred myocardium or transplanted as its replacement [19].

A short summary of cardiovascular cell therapy

Typically CV cell therapy is performed with a population of stem or progenitor cells that can differentiate down multiple lineages giving rise to different cell types depending on the need. Bone marrow (BM), skeletal muscle, fat, cardiac muscle, and umbilical cord blood have all been sources of uncommitted adult stem or progenitor cells for cardiovascular cell therapy [19]. Ideally, such a population of cells would survive in injured myocardium, give rise to new mature cardiocytes and vasculature and integrate with surrounding host tissue. Data from several laboratories suggest this is feasible, although it appears to be a rare event [20]. Data further suggest [21], that when injected into injured or infarcted heart, stem cells (SCs) can develop into fibroblasts, cartilage, and fat, or in the case of embryonic [22,23] (or presumably inducible pluripotent) SCs, even into benign tumors or teratomas. Understanding this and controlling it are active areas of investigation that must progress before undifferentiated pluripotent cells will be a viable target for clinical myocardial repair or for engineering of an organ.

BM is the most commonly used cell source for cardiac repair. In fact, more than 1000 patients have been treated in phase I and II clinical studies with BM aspirate containing a 'cocktail' of stem or progenitor cells — usually after AMI. These trials are reviewed elsewhere [24]; however, recent meta-analyses suggest that BM-derived cells provide significant benefit when administered post-AMI [25–27]. Beyond BM and peripheral blood (PB), skeletal muscle, adipose tissue, and of course heart have more recently been used as a source of SCs for cardiac repair and regeneration. The most promising and challenging of these sources is likely adult heart.

Cardiac stem cells

Currently, at least four undifferentiated cell populations (expressing c-kit, MRD-1, isl-1, or sca-1, and lacking expression of hematopoietic markers) have been isolated from neonatal or adult hearts [28–32]. Interestingly, the number of some of these cells increases after AMI, but is

very low in failing hearts suggesting a role of these cells in ongoing repair that becomes insufficient in HF. We recently isolated an SSEA+ population of *u*ncommitted cardiac *p*rogenitor *c*ells (UPCs) in both neonatal and adult rat hearts and showed that these cells can be expanded *in vitro*, give rise to many of the SC populations described previously, and be differentiated down cardiac myocyte, smooth muscle, and endothelial cell pathways [23]. Cell population with similar potential has recently been isolated from epicardium [31,32]. Data like these begin to suggest that adult myocardium contains a dynamic population of progenitors likely left from development that contributes to low level muscle and vascular repair and speaks to the therapeutic potential of these cells even though an ability to harvest and expand CSCs remains limited. Recently, a breakthrough occurred when Messina and colleagues [33] developed methods to derive myogenic cells from adult cardiac biopsies. This emerging ability to derive, isolate, and expand CSCs, and their capacity for cardiac muscle and vascular maturation suggest a potent role for these cells in the future of regenerative medicine.

Is the future of cardiac regenerative medicine bioartificial organs?

Creation of an autologous-cell-derived bioartificial heart could theoretically solve the major problems associated with heart transplantation — a shortage of donor organs, and side effects [34] of life-long immunosuppression. But until recently, engineering whole heart constructs was not deemed feasible. In fact, creation of any functional, complex, vascularized, 3D tissue or organ has been viewed as an overarching unmet need in tissue engineering in large part because organ ultrastructure could not be readily recreated *in vitro* and the complex vascular networks necessary for the maintenance of tissues of any complexity have proven difficult to engineer. Building a heart, or portion thereof, poses additional unique challenges because myocardium is a contractile tissue with specific structural and energetic specifications. For example, cardiac muscle must integrate with the cardiac valves appropriately to allow blood to flow in and out of each cardiac chamber at the appropriate time, which in turn demands specific spatial orientation as well as functional coupling of cells and matrix both electrically and mechanically. Furthermore, cardiac muscle is exquisitely hypoxia-sensitive such that cardiocytes must be intricately coupled with patent vasculature to survive and contract appropriately. Finally, cardiac muscle contains few stem or progenitor cells with the capacity to generate mature cardiac muscle (including atrium, ventricle, and nodal tissue) and thus finding appropriate building blocks has posed a specific challenge to cardiac repair.

Bioengineering advances

Numerous approaches have been taken to engineer simpler cardiac tissues such as acellular or simple cell-based patches. But, even the creation of cardiac patches beyond a few hundred microns in depth, has been limited by an inability to support the high oxygen and energy demands of cardiomyocytes at a greater depth from the surface [35,36]. In an attempt to overcome this, investigators have proposed combining channeled synthetic extracellular matrix (ECM) constructs with oxygen carriers, or stacking single layer cardiac cell sheets [37–39] (Figure 1), which have reinforced the direct relationship between perfusion and graft thickness or cell density [36,40]. Two promising approaches, engineered contractile rings and cardiomyocyte cell sheets, have been used additively (Figure 1b) to generate more complex versions of simple structures transplanted in small animals to improve ventricular function [41–43].

Despite these incremental improvements, engineering cardiac tissues with a thickness on the order of human heart remains a challenge and is unlikely to occur in the absence of concomitantly engineered vascular beds. To meet this high demand for the perfusion of cardiac cells — recognizing that cardiac ECM contains a complex vascular network capable of meeting the demands of working heart — we developed a perfusion method to remove all cellular constituents from cadaveric tissue while retaining acellular vascular networks [8] throughout the remaining ECM (Figure 1c) that can be re-lined with functional endothelial cells. If this ability to recreate vasculature in the depth of cardiac ECM can be sustained, it begins to suggest the feasibility of engineering complex cardiac structures.

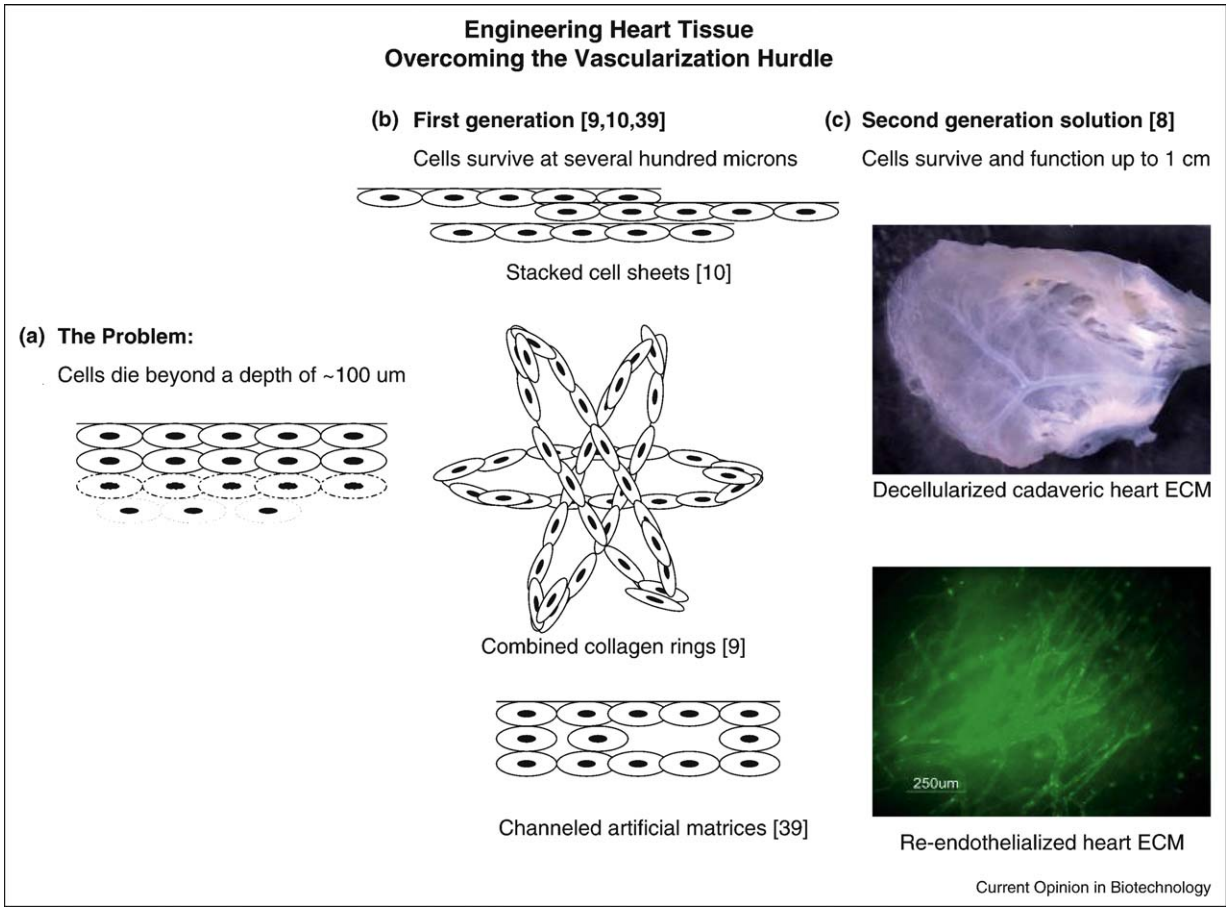
Stem cell biology advances

Two recent advances in SC biology have further increased the likelihood that personalized cardiac tissue can be generated in the laboratory. First is the isolation of SCs from adult heart that can give rise to beating cardiocyte-like tissues, and vascular progenitors *in vitro* [3,23,32]. Second is the ability to generate adult-derived pluripotent SCs that can be driven down by a cardiac lineage [7]. These when combined with the generation of complex scaffolds [8], open a door toward engineering of 'personalized' autologous tissues and organs for both *in vitro* drug testing and for therapeutic use. Similar models of (albeit nonpersonalized) human stem embryonic SC-derived test beds are already being proposed for drug development and drug [44].

Building cardiac patches for focal repair

The idea of cardiac bioengineering over the past 10 years has primarily involved seeding an artificial scaffold (e.g. hydroxyapatite, collagen, and fibrin) with cells and cultivating it in the laboratory to be used as a cardiac patch. In principle, such a patch could be an off-the-shelf product applied to scar with the goal of lessening scar expansion and progression to HF. The most well-known approach to engineering cardiac patches is the application of neonatal

Figure 1

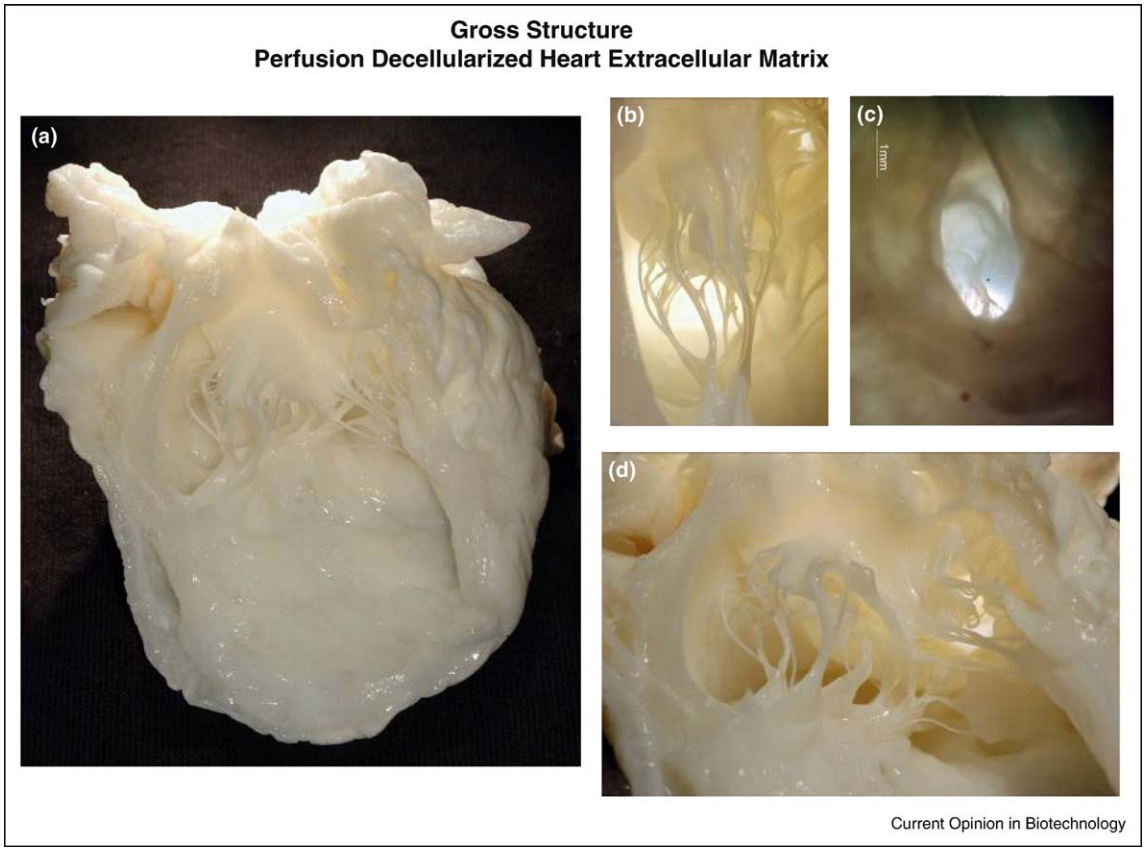


(a) Engineering heart tissue: overcoming the vascularization hurdle. Currently cardiac muscle cannot be engineered at thicknesses beyond several hundred microns because cardiocytes die because of lack of oxygen. (b) Several first generation attempts to overcome this limitation include stacking of cardiac cell sheets grown in a single monolayer on temperature-sensitive artificial matrix [10]; building channels into artificially constructed matrices [36], and creating simple collagen rings with minimal cell thickness that can be combined to give a multi-arm construct [9]. (c) A second generation solution [8] is to use perfusion-decellularized cardiac matrix containing acellular vascular conduits that can conduct medium and oxygen throughout the tissue construct and to rebuild a re-endothelialized vascular network and endocardium. Green staining represents live CMFDA+ endothelial cells throughout large and small diameter vessels in the arterial and venous tree and lining the endocardial surface.

cardiomyocytes to a collagen gel that is then subjected to cyclic mechanical stretch to induce maturation [9,45]. Recently, we undertook a different approach. Because nature had already engineered a vascularized scaffold that serves as the basis of heart, we reasoned that it would serve as the ideal patch substrate. Thus we proposed to remove the cellular components of the myocardium presuming it was possible to obtain a 3D scaffold comprising native cardiac ECM in the original four-chambered geometry and architecture of the native heart. As shown in Figure 2 we have been able to do so. Perhaps the most surprising finding was the retention of fine architecture and cardiac geometry within the acellular matrix (Figure 2). Briefly, after completely removing cellular structures [8] (<3% of deoxyribonucleic acid remaining) from a rat or pig heart using detergent-based perfusion decellularization, both gross structural and biochemical properties of the native heart were retained. As shown in Figure 2, cardiac wall structure, valves, papillary muscles, and trabeculae could all be seen after cell removal. Decellularization did not decrease glycosaminoglycan content in the myocardial matrix. Nor did it alter passive mechanical properties of the matrix. The perfusability of the matrix was proven either by infusing resin to show structural integrity of the vascular tree from the major coronary vessels to fourth/fifth order vessels or by heterotopically transplanting the decellularized construct and obtaining angiograms showing essentially normal blood inflow.

We reasoned that perfusion-decellularized ECM should provide a perfusable scaffold on which we could reapply

Figure 2



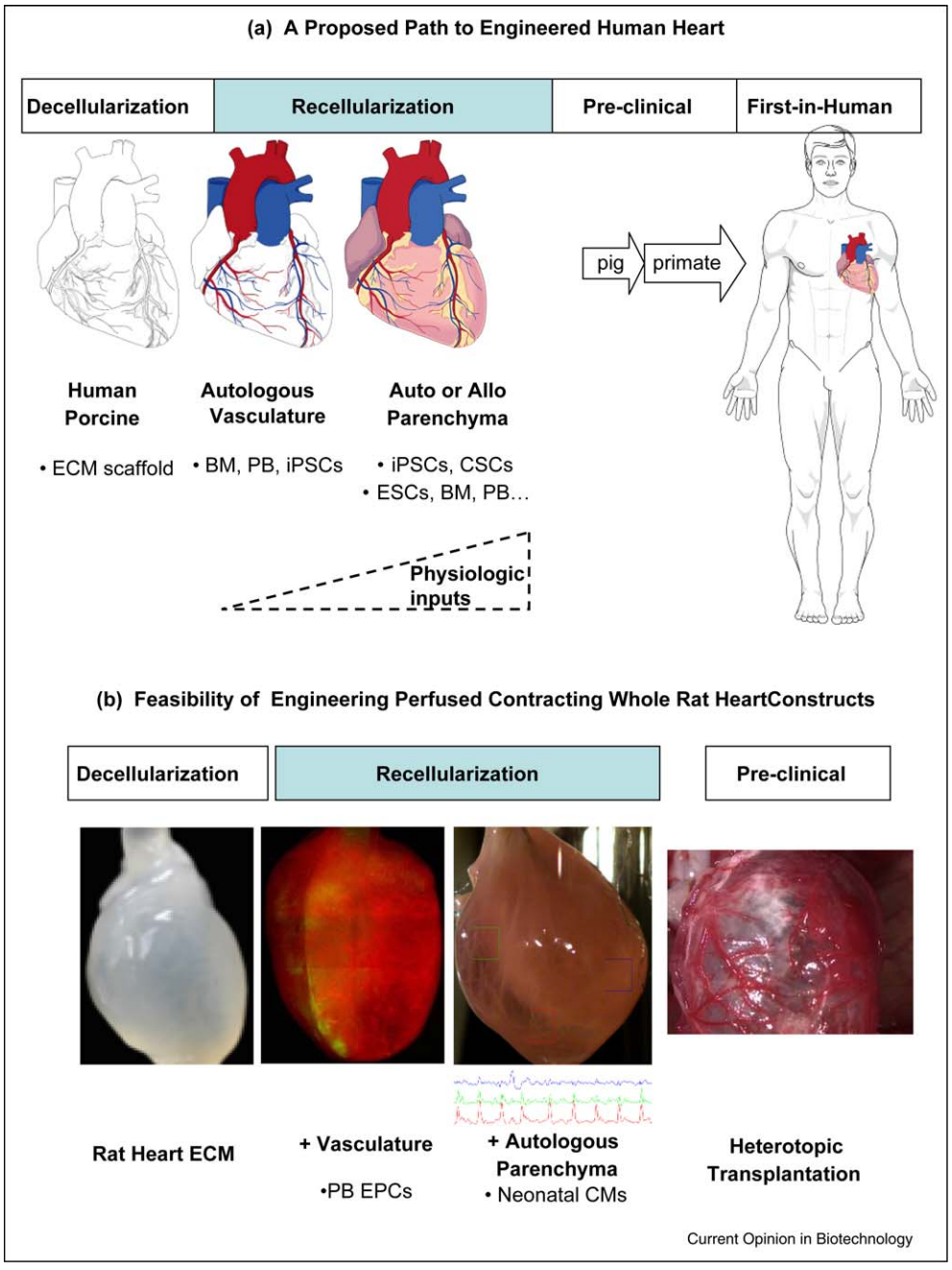
Gross structure of decellularized cadaveric heart ECM showing maintenance of fine structure in both large (porcine) and small (rat) animal hearts. **(a)** Decellularized porcine heart sliced in half to show the retention of whole organ architecture, intricate intrachamber geometry, and the presence of vascular conduits after removal of cells. **(b)** and **(d)** Valve chordae and leaflets after perfusion decellularization of pig heart. **(c)** Transparent aortic valve leaflets demonstrating valve patency after perfusion decellularization of rat heart.

cells to build a 3D cardiac construct or even a whole heart (Figure 3). By reapplying cells that could give rise to mature cardiac muscle and vasculature (Figure 3b), and providing appropriate physiologic parameters, we should be able to generate a myocardial construct that could synchronously contract, respond to drugs and pump against an afterload. Proof-of-this-concept was performed by the injection of neonatal cardiac-derived cells into the scaffold followed by maturation in a bioreactor under pacing and mechanical load. By 8–10 days, contractions of the recellularized LV segments were recorded (Figure 3b) and drug responsivity was seen [8]. More recently we have generated decellularized matrix from uninjured, infarcted, or failing heart (Figure 4) from small and large animals of multiple ages or developmental stages, and of both sexes. This ability to generate matrix of virtually any genotype or phenotype provides a powerful tool for testing the potency and capacity of SCs to be retained, survive, migrate, differentiate, and function in a controlled but physiologically relevant setting. Studies are currently underway to evaluate the capacity of several adult and embryonic SC populations to form cardiac muscle and vessels in this novel controlled milieu. Recently we placed pluripotent human SCs on decellularized cardiac matrix and showed contraction of the resulting cell/matrix patch. These cumulative data suggest we have moved one step closer to a creation of autologous organ and constructed a tool to test hypotheses relevant to developmental biology, disease pathophysiology and cell therapy. In doing so, we have, to a reasonable degree, overcome several major limitations of cardiac tissue engineering. Obviously, time will determine which of the current engineering approaches will translate into a clinical product.

Building test beds for drug discovery and drug screening

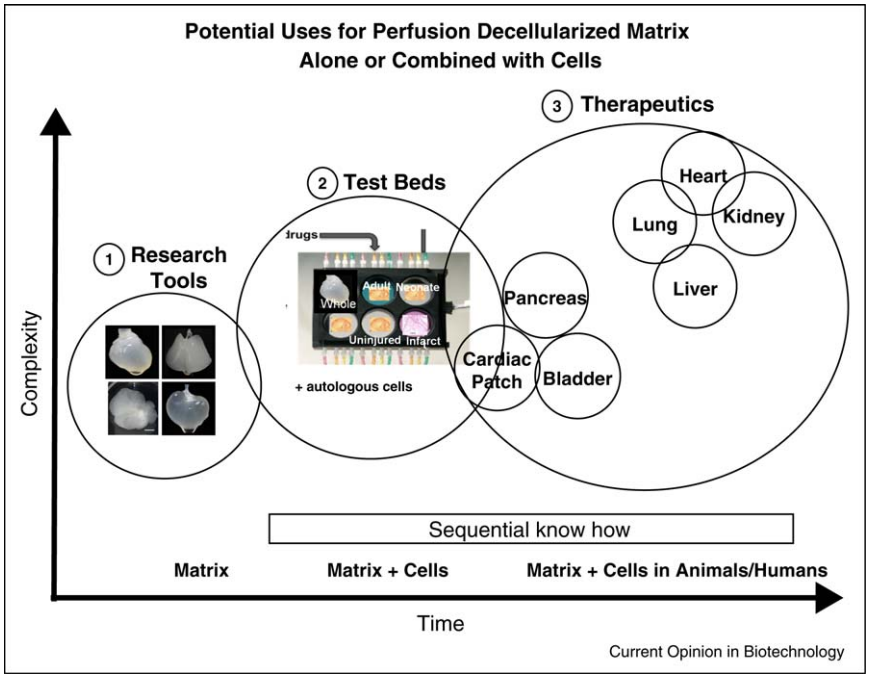
Each year promising pharmaceutical compounds move into costly clinical trials (or beyond), only to be halted by safety problems that were not detected in existing 2D or 3D human tissue models. Cardiotoxicity is a

Figure 3



(a) A proposed path to engineered human heart. Engineering human heart requires a scaffold, vascular, and parenchymal cells, and a method for promoting cell survival maturation and ultimately function. Choices exist for each. Cadaveric human or porcine tissue could be perfusion-decellularized to generate a four-chambered cardiac scaffold with acellular valves and vascular conduits [8]. This is the decellularization phase. Recellularization includes rebuilding of autologous vasculature and auto or allogeneic cardiac muscle — depending on the cells available. Cells that can give rise to vasculature would likely be derived from autologous bone marrow (BM) or peripheral blood (PB) mononuclear cells, which have been shown to participate in angiogenesis *in vivo*; or alternatively from inducible pluripotent SCs derived from skin. Building cardiac muscle and valve components would more likely rely on the use of cardiac-derived stem cells (CSCs), or human iPS cells. However, recently BM and PB mesenchymal cells have been shown to differentiate down cardiac cell lineages and could provide an alternate cell source. Although decellularized matrix is capable of driving some degree of cell differentiation and maturation, it is very likely that electrical and mechanical inputs to the nascent tissue will be required to coordinate synchronous productive organ contraction and promote cell–cell coupling that appears to be required for organ maturation. Before any human safety studies, rigorous preclinical large animal testing would have to occur. To mimic the human clinical scenario it is likely that autologous cells would be used whenever possible. **(b)** Demonstration of feasibility: engineering perfused contracting whole rat heart constructs. Pictorially illustrates the feasibility of the steps described in (a). First is decellularization of whole rat heart followed by the recellularization of the vascular conduits via arterial and venous perfusions with labeled rat aortic endothelial cells. Addition of approximately 120 million neonatal cardiac-derived myocytes, fibroblasts, and stem or progenitor cells into the left ventricle was followed by electrical and mechanical pacing for four days resulting in a visibly contractile construct by day 4. Movement can be seen in three colored tracings below the image corresponding to the colored squares on the image. To demonstrate biologic compatibility and perfusion-decellularized cardiac matrix was transplanted into the abdomen of nonsyngeneic rats and allowed to persist *in vivo* for an average seven days. A heart scaffold immediately after connection to the recipient aorta is shown as evidence of this preclinical step.

Figure 4



Potential uses for perfusion-decellularized matrix. Perfusion-decellularized matrix can be used alone and with cells *in vitro* or *in vivo* to build research tools, personalized test beds, and/or therapeutics. Knowledge gained with each component of the system builds upon the prior. Perfusion-decellularized matrix can be generated from any organ or tissue including rat heart, liver, lung, and kidney as depicted. These matrices can provide research tools for evaluating stem cell capacity for organ-specific differentiation, engraftment, repair, etc. depending on the type of matrix used for testing. Examples of the kinds of tools or test beds that can be generated include ECM from organs of any age, developmental stage, disease state, or sex. Understanding the capacity for SCs to incorporate into these environments could provide significant insights into the potential for that cell type in a cell therapy or regenerative medicine role. Importantly the knowledge gained building test beds should translate directly into transplantation of cells for whole organ bioengineering.

common reason for nonapproval or drug withdrawal by the FDA; yet, there are few if any human *in vitro* heart models that provide safety data in a 3D renewable, versatile system. *Thus the challenge:* developing bona fide living complex vascularized human organ surrogates that recapitulate 3D structure/function (i.e. anatomy and physiology). Generating such a 3D tissue requires: firstly, a geometrically and spatially appropriate scaffold — which now exists; secondly, vascularization for tissue perfusion — which appears feasible (Figure 1); thirdly, the availability of cells that can give rise to parenchymal and vascular components, which likewise has emerged in the past several years; fourthly, an ability to tune the microenvironment to alter cell physiology and function; and fifthly, a capacity for driving tissue/organ maturation *in vitro*. We have created a proof-of-principle beating rat heart construct from decellularized rat heart ECM and neonatal rat cardiac cells, but as of yet, have not attempted a human organ. The next five years will likely bring: firstly, off-the-shelf 3D perfusable whole organ scaffolds that can be populated with mixed populations of human stem/progenitor cells to develop personalized testing tools; secondly, an understanding of molecular and/or physiologic inputs required to promote proliferation, differentiation, and maturation of human SCs in a 3D cardiac environment; thirdly, a human assay system for drug testing comprising decellularized organ scaffold plus human cells subjected to cardiac appropriate inputs and functional outputs.

Conclusion

Along with cell therapy, cardiovascular tissue engineering is a new frontier in the treatment of CVD. Although the successes are very preliminary, cardiac patches and bioartificial organ constructs could offer a wide range of solutions in the future. This will be bolstered by recent SC advances, such as iPS cell technology and could well culminate in the creation of personalized organs, eliminating both the donor shortage and the need for harsh immunosuppressive regimes. This, combined with a capacity for creating human organ surrogates to provide more accurate drug screening and toxicity testing, could potentially save billions of dollars in health care costs annually.

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