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Forum Review Article

Remuscularizing Failing Hearts with Tissue Engineered Myocardium

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Abstract

Supporting or even replacing diseased myocardium with *in vitro* engineered heart muscle may become a viable option for patients with heart failure. The key to success will be to (1) generate human heart muscle equivalents *in vitro*, (2) integrate the latter into a failing heart, (3) ensure long-term functional competence of the grafts, and (4) prevent unwanted effects including arrhythmias, inflammation/rejection, and tumor formation. Several promising tissue engineering technologies have already been developed and are presently being tested in animal models. The rapidly evolving field of human stem cell biology has in parallel identified unique cell sources of potential clinical relevance. Somatic cell reprogramming and nontransduced, nonembryonic pluripotent stem cells may be of particular interest to eventually provide patient-specific cells and tissues. Yet, limited cardiac differentiation and cell immaturity still restrict a broad application of any stem cell type in cardiac muscle engineering. Bioreactor technologies, transgenic "optimization," and growth factor, as well as physical conditioning, have been used to address these caveats. This review summarizes different tissue engineering modalities, speculates on potential clinical uses, provides an overview on cell sources that may ultimately facilitate a patient-specific application, and discusses limitations of tissue engineering-based myocardial repair. *Antioxid. Redox Signal.* 11, 2011–2023.

Introduction

Heart failure is one of the leading causes of death in industrialized countries and is expected to become a global epidemic within the 21st century. It does consequently not come as a surprise that novel therapies are being rapidly developed to address this public health and socioeconomic threat. One of the most promising, but at the same time highly controversially debated, novel treatment concepts is cell-based cardiac repair. It "simply" aims at adding fresh contractile elements to a failing heart. First proof-of-concept for this approach has been provided by a seminal study by Soonpaa *et al*. in 1994 (91). Subsequently, different cell types, including skeletal myoblasts (60, 62, 96) and bone marrow-derived stem cells (3, 5, 38, 59, 76, 78, 85, 106) amongst others (reviewed in refs. 22 and 68), have been implicated as "magic bullets" for heart failure treatment. However, cell implantation studies are generally hampered by low cell retention and survival (52, 66), as well as a restricted cardiomyogenic potential in most of the applied cells (6, 31, 70).

Tissue engineering follows a different rational. Here, cells are assembled into functional units already *in vitro*; the resulting tissues may subsequently be subjected to a quality control step that should involve an assessment of contractile performance *ex vivo*, ideally directly before implantation to support or replace a significant portion of a failing heart *in vivo* (114). Up to date, several groups have been able to provide proof-of-principle for a tissue engineering-based myocardial repair approach in animal models (63, 119), and only recently one year follow-up data from a first human tissue engineering trial (MAGNUM-trial) was published (13). Yet, the latter study did not involve contractile tissue constructs, but focused primarily on "passive" physical support of failing hearts with bone marrow cell-modulated collagen sponges. This approach may indeed be feasible to restrain dilating myocardium and control aneurysmatic defects; it will, however, not reintroduce

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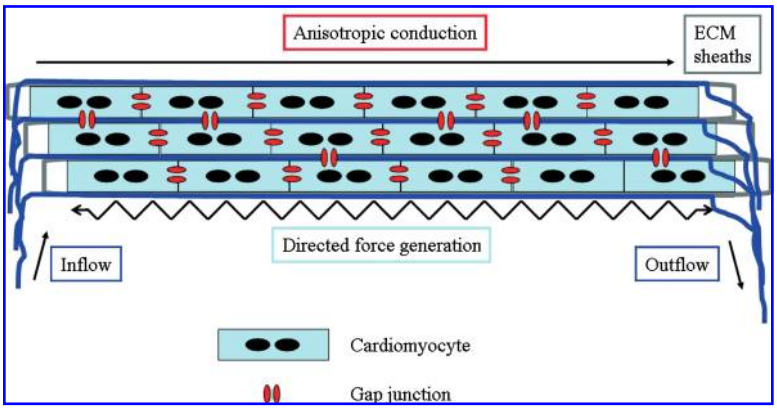

FIG. 1. Schematic view of an ideal assembly of tissue engineered myocardium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

contractile elements and is therefore unlikely to reach the ultimate goal of cell-based cardiac repair (*i.e.*, to "remuscularize" a heart).

One of the key deliverables in the myocardial tissue engineering field is to develop large, force-generating human heart muscle equivalents (Fig. 1). The principle technologies to create such tissues *in vitro* appear to be available (114). The lack of a scalable human cardiomyocyte source has, however, been a major drawback for further development. The availability of human stem cells, mainly human embryonic stem cells (ESCs), has somewhat alleviated the problem (41, 97). Yet, ESCs are essentially allogeneic and may ultimately not be an ideal cell source for myocardial repair applications. This caveat has been addressed by the recent advent of alternative stem cell technologies, enabling the derivation of pluri-/multipotent stem cells from nonembryonic tissue, either by gene transduction-mediated reprogramming (94, 105), by selecting germline stem cells (GSC) from adult testes (32, 39), or by inducing parthenogenetic development of unfertilized oocytes (21, 30, 45, 95). Either of the resulting stem cells is apparently pluripotent or at least multipotent and can give rise to *bona fide* cardiomyocytes. Yet, quantity and quality remain to be optimized to enable the allocation of sufficient numbers of terminally differentiated cardiomyocytes to large-scale clinical applications. Availability of cardiomyocytes will, however, be only one side of the coin; providing cardiac growth and function supporting non-myocytes, including endothelial cells, smooth muscle cell, fibroblasts, and potentially other cells, will likely be of equal importance to reconstitute heart muscle equivalents (72).

This review will provide an introduction into available tissue engineering modalities (see refs. 24, 114, 116, 118 for more comprehensive reviews on tissue engineering techniques) and discuss the potential of tissue engineered myocardium in heart muscle repair. A major part of this review will be devoted to stem cell technologies and the perspective to derive patient-specific cells for an autologous application at a clinical scale as well as to caveats associated with this approach.

Tissue-Engineering Modalities

Several tissue engineering technologies have been developed and refined throughout the past decade (reviewed in ref. 114). These include (Fig. 2): (a) the classical biomaterial approach to create a scaffold and "vitalize" the latter by seeding cells onto the scaffold (10, 11, 56, 57, 79); (b) a cell entrapment approach, aiming at concentrating cells at a high density within a defined three-dimensional environment to facilitate spontaneous cell-aggregation (23, 117, 120); (c) the cell sheet engineering approach, aiming at stacking cell monolayers to generate tissue "sandwiches" (90); (d) the micro-tissue technology, being essentially a modification of the commonly used hanging-drop cell culture system (44); (e) the organ decellularization–recellularization approach (77).

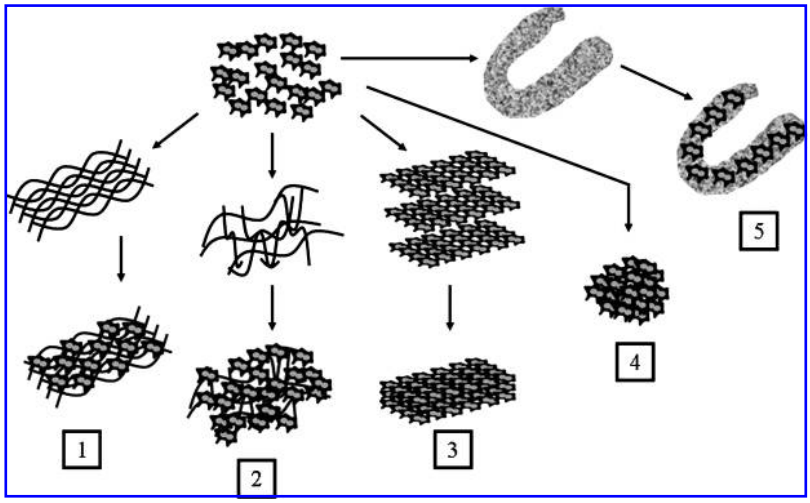

FIG. 2. Cardiac tissue engineering technologies: (1) classical biomaterial approach; (2) cell entrapment approach; (3) cell sheet engineering; (4) myocardial micro-tissue; (5) organ decellularization–recellularization.

Classical biomaterial approach

Biomaterial scientists have been for many years at the forefront of the tissue engineering field (54). They naturally focus on chemical engineering of biocompatible materials and utilization of the latter as a growth substrate for cells. The underlying hypothesis of this approach is that cells from a suitable source would be able to generate a tissue if seeded on an appropriate blueprint. The latter would ideally be designed to offer initial structural support, but be degraded over time. To this end, several materials have been used, including polylactic acid (PLA) and polyglycolic acid (PGA) and mixtures thereof (10, 11). Alternatively, alginates (56), polysaccharides derived from seaweed, and gelatin/collagen sponges (57, 79) have been employed. Clear advantages of the biomaterial approach are the possibility to generate any given geometry and to create fully chemically defined matrices that would be easy to produce under good manufacturing practice (GMP) regulations. Clear disadvantages, at least for cardiac muscle engineering, are the apparent lack of cardiomyocyte differentiation if grown on or in most of the so far used matrices and the unphysiological mechanical properties of most matrices.

Cell entrapment approach

Spontaneous assembly of mostly spherical cell aggregates occurs if cardiomyocytes are cultured at high densities in bioreactor suspension cultures (1, 65). This apparently random process can be spatially controlled by entrapping cells within a hydrogel [*e.g.*, collagen from rat tails (23, 117, 120)]. During the naturally occurring condensation process of hydrogel/cell mixtures, muscle aggregates fuse to form a functional syncytium. This process can be optimized by imposing mechanical strain onto the resulting tissue constructs (25, 117, 120). Importantly, hydrogel-based engineered heart tissues (EHTs) display several structural and functional properties of native myocardium and can further be optimized by growth factor conditioning, electrical stimulation, and addition of nonmyocytes to the initial cell mixture (72, 120). The EHT approach has the clear advantage of supporting differentiation of cardiomyocytes and enabling the generation of strongly contracting tissue constructs with various geometries (72, 111). A disadvantage of the EHT-approach is the necessity to use several animal products, including collagen from rat tails, exudate from mouse Engelbreth–Holm–Swarm tumors (*i.e.*, Matrigel®), chick embryo extract, and horse serum (117, 119, 120), as well as devices to impose strain on and support ideally auxotonic contractions of EHTs (25, 119). Although the aforementioned means are useful to generate EHT with an optimal tissue structure and contractile performance, we have recently been able to developed partially serum-free and Matrigel®-free EHT reconstitution and culture protocols (72). Further studies are ongoing to engineer and culture EHTs eventually under fully defined conditions.

Cell sheet engineering

The development of culture surface coatings containing poly(N-isopropylacrylamide), being either hydrophobic or hydrophilic, depending on the environmental temperature to facilitate cell attachment and detachment at high (37°C) and low (20°C) temperatures, respectively, has enabled the controlled release of monolayer cell cultures from its underlying substrate (75). Stacking cell layers can eventually be utilized to generate complex three-dimensional tissue with a specific organ function (109). The absence of vascularization does, however, restrict the size of individual cell sheet stacks to three to four layers (90). This limitation may be overcome *in vivo* by a so-called "polysurgery approach" that would, however, necessitate repeated access to the implantation site to sequentially stack cell sheet sandwiches *in vivo* after previous grafts have become properly vascularized (89). In a modification of the original cell sheet engineering approach, spontaneous detachment of cell monolayers from uncoated or laminin coated culture dishes has been exploited to generate cardiac muscle constructs (4). The advantage of cell sheet engineering is clearly the lack of exogenous matrix material and good contractile performance. A main disadvantage is high fragility of the layered tissue constructs.

Myocardial micro-tissue

Micro-tissues can be generated by aggregating cardiomyocytes in hanging-drop cultures (43, 44). They demonstrate several features of a functional myocardial syncytium. Whether they can serve as optimized and possibly more stable grafting material as compared to cell suspensions remains to be demonstrated. Disadvantages of micro-tissues are their small size and apparently random organization.

Organ decellularization–recellularization

Recently, organ harvest from mammals followed by a decellularization step to dispose of all potentially immunogeneic structures and subsequent reseeding the remaining extracellular matrix (ECM) by "intramural" cell injection has attracted considerable attention in the scientific community and in the lay press (77). Although fascinating, ultimate proof for complete reseeding and full functional competence of a recellularized heart has not been provided, so far. It appears in fact unlikely that intramural injections would suffice to completely refurbish the "naked" ECM skeleton of a heart. Reseeding through cell-free vascular structures could be an option, but would similarly require the cells to migrate into the retained ECM, identify the right "spot" to differentiate, and connect with equally "intelligent" cells. Whether stem cells can cope with these exceptional challenges remains to be demonstrated.

Potential Application of Tissue-Engineered Myocardium

Several treatment modalities and combinations thereof can be envisioned once clinically applicable surrogate myocardium would become available (Fig. 3), that is, (a) partial myocardial reconstruction, (b) transmural wall repair, (c) reconstruction of myocardial structures, (d) global heart support, (e) paracrine support, and (f) cardiac restraint.

Partial myocardial reconstruction

An average myocardial infarction causes a loss of ~1 billion myocytes (28). Subsequently, scar tissue forms and the remaining myocardium responds to the loss of contractile tissue by cardiomyocyte hypertrophy. The latter may transiently suffice to compensate for the loss in myocardial performance but will in the long-run perpetuate the myocardial

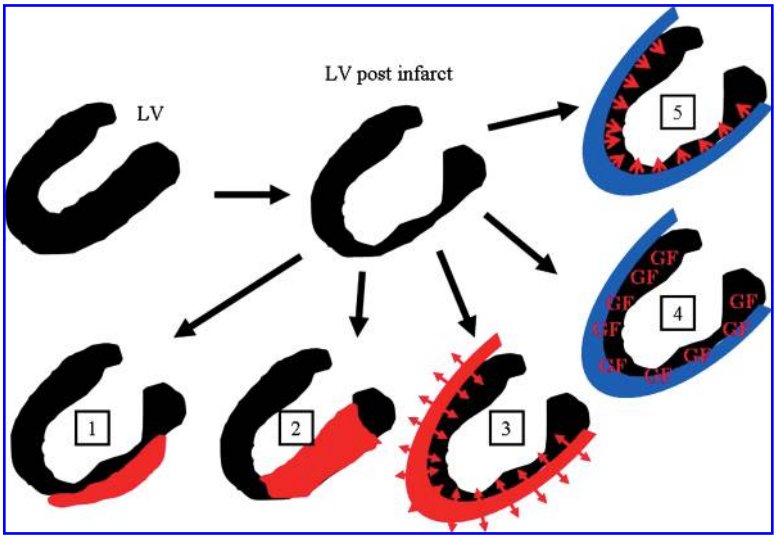

FIG. 3. Applications of tissue engineered myocardium: (1) partial myocardial reconstruction; (2) transmural myocardial repair/reconstruction of myocardial structures; (3) active global heart support (*arrows* indicate contraction and relaxation of a biological assist device); (4) paracrine support (GF symbolizes secreted growth factors); and (5) cardiac restraint (*arrows* indicate unidirectional tension imposed on the myocardium to prevent dilation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

damage, eventually leading to heart failure. If a defined defect can be clearly localized after a myocardial insult, engineered heart muscle may be placed on top of it, to locally support the failing heart and inhibit further deterioration. Thickening the ventricle by adding a patch may by itself have a beneficial effect on myocardial performance, as it may attenuate wall stress and consequently oxygen consumption (LaPlace law). Computational modeling of heart function after injection of cell-free biomaterials, as well as a recent study in pigs on the effects of intramyocardial alginate injections, have in fact confirmed this notion (53, 101). Yet, to achieve long-term improvement of myocardial contractility, addition of muscle is likely a prerequisite (119).

Transmural wall repair

In an alternative approach, localized defects (*e.g.*, aneurysms and transmural scars) may be surgically excised, using for example a Dor-procedure (20), and replaced with tissue-engineered myocardium (8, 51, 61). This approach is expected to be superior to an epicardial application of tissue-engineered myocardium, because it avoids potentially interfering effects of otherwise underlying noncontracting scar tissue. It may, however, especially if applied in the left ventricle, be complicated by (a) high systolic left ventricular pressures (requiring bioartificial grafts with high burst strength), (b) thrombus formation (requiring nonadhesive/nonthrombogenic tissue surfaces), and (c) arrhythmias (requiring seamlessly integrated and physiologically conducting grafts bridging the transmural defect).

Reconstruction of myocardial structures

Tissue engineering may also be exploited to reconstruct other myocardial structures besides defined areas within the left ventricular free wall (*e.g.*, in hearts with congenital malformations). Potential applications may be to generate muscle to be used in ventricular augmentation in children with hypoplastic ventricles or severe cardiac septation defects (51). In addition, cardiac muscle engineering may also be used to provide conducting elements to establish, for example, electrical circuits between atria and ventricles in patients with permanent AV block (16).

Global heart support

Congestive heart failure is generally the consequence of chronic myocardial damage, stemming mostly from myocardial infarctions, hypertension, myocarditis, genetic predisposition, or drug toxicity (*e.g.*, in patients with anthracycline treatment). In contrast to a localized defect, globally failing hearts require full left ventricular support. This may be achieved by myocardial tissue constructs that can be wrapped around the heart. To this end, we have recently developed a "Biological Ventricular Assist Device (BioVAD)" and could demonstrate its principle applicability as heart embracing biological grafts in healthy rats (111). Future studies will have to scrutinize whether the BioVAD concept can be used in a model of congestive heart failure. Alternatively, tissue-engineered muscle tubes may be applied to perform as extra-myocardial assist devices, supporting arterial blood flow from the left ventricle into the systemic circulation, thereby reducing afterload (88), similarly as mechanical left ventricular assist devices, but without their typical complications such as device failure, thrombus formation, foreign body reactions, and infections.

Paracrine support

Cell implantation into the heart has resulted in an improved myocardial performance in some clinical trials (3, 85, 106), whereas it has not in others (38, 59). In addition, several animal studies have provided evidence for a therapeutic effect of cell grafts (reviewed in ref. 22) that may be beyond a passive wall strengthening effect. A potential mechanism is paracrine support of the surrounding viable myocardium through cytokines and/or growth factors released from engrafted cells (29). Consequently, tissue-engineered muscle or in this case even non-muscle structures may be used as paracrine support devices to, for example, provide angiogenic (VEGF-A, SDF-1), anti-apoptotic (IGF-1, PDGF-BB), and muscle growth supporting (IGF-1) factors.

Restraint devices

Myocardial dilation generally develops during advanced stages of heart failure. Offering restraint to dilating hearts has been the rational for several surgical interventions, such as the

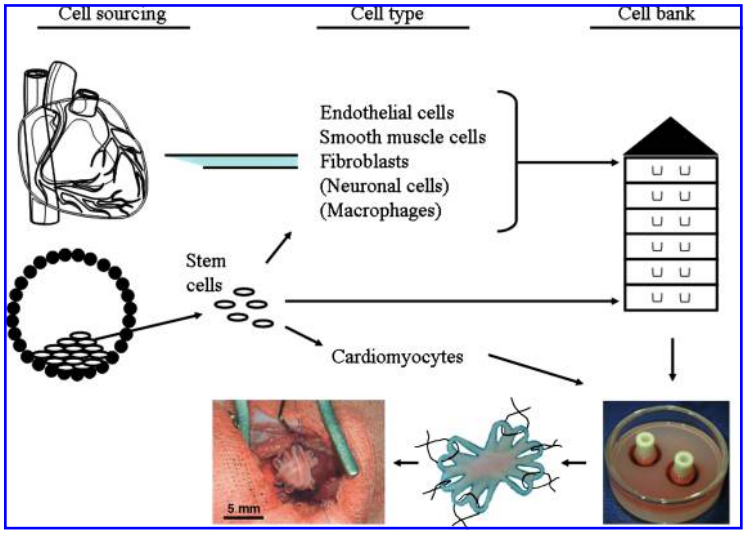

FIG. 4. Utilization of different cell types to generate distinct myocardial structures from biopsy material or from banked cells. Endothelial cells, smooth muscle cells, fibroblasts, neuronal cells, and macrophages may be isolated from biopsy material (*e.g.*, from heart or other suitable tissue), subsequently propagated, and stored in a tissue bank until needed to generate a cardiac tissue construct. Cardiac myocytes and also nonmyocytes may be derived from stem cells. Undifferentiated stem cells can be banked until further use. Stem cell-derived cardiomyocytes cannot be stored and would have to be used immediately for cardiac tissue engineering. *Bottom panels* show circular EHTs in a casting mold (*right*), an assembled star-shaped EHT before (*middle*) and after implantation (*left*; adapted from refs. 120 and 119). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

implantation of Acorn CorCap Cardiac Support Devices (102) or myocardial splints (34). Similarly, tissue-engineered biological restraint devices may be engineered to embrace the heart and thereby prevent its further enlargement (111). In contrast to the Acorn and Myosplint approach, tissue-engineered restraint devices would be purely biological and may ideally not cause foreign body reactions or other inflammatory responses. Yet, the concept of biological restraint remains to be tested in a relevant animal model.

Cell Sourcing for Tissue Engineering Applications

Assembling functional myocardium will require all cell types that naturally reside within the native myocardium (72). These include myocytes and nonmyocytes. The former represent 30% of the total heart cell number, but account for ~70% of the normal heart mass (71). Myocytes within tissue engineered myocardium would have to form an anisotropically organized myocyte network; nonmyocytes should ideally generate blood vessels and ECM (Fig. 1). In addition, cells would preferable be derived from the respective patient in need for a therapeutic intervention or alternatively from a stem cell bank, containing all relevant immunotypes (Fig. 4). Ultimately, mixtures from banked and biopsied cells may turn out to be useful to generate optimal tissue engineered heart muscle rapidly.

The following paragraphs will give an overview on cells with a restricted differentiation potential to provide nonmyocytes and pluripotent cell species to provide cardiac myocytes and/or nonmyocytes for cardiac tissue engineering applications. Finally, up-scaling of the cell allocation process, being essentially equally important as identifying the "right" cell source, will be discussed:

Cells with a restricted potency

The myocyte fraction within the adult heart is naturally postmitotic and essentially "unipotent". Regeneration of myocytes depends ultimately on their inherent ability to constantly renew or repair cellular components (especially sarcomeres, but also mitochondrial proteins, cell membrane and associated proteins, as well as DNA). Loss of this activity results in rapid myocyte death, which cannot be compensated by myocyte proliferation. Similarly, meaningful proliferation of adult primary myocytes cannot be observed *in vitro*, precluding an application of adult myocytes in cardiac muscle engineering. Other specialized mesodermal cells, including endothelial cells, smooth muscle cells, and fibroblasts, can proliferate under certain physiologic and pathologic *in vivo* conditions und maintain this activity also *in vitro*. Consequently, myocardial biopsies may be instrumental to yield essential nonmyocyte species for cardiac muscle engineering (Fig. 4). While the heart may indeed be the ideal source for cardiac nonmyocytes, it would be preferable to harvest nonmyocytes from tissues that are easier to access. In fact, cells from the dermis (42) and bone marrow (7, 13, 58, 84) have already been applied to generate noncontractile cardiac patches and offer paracrine, angiogenic, and/or passive mechanic (*i.e.*, restraint) support to failing hearts.

Pluripotent cells

Pluripotent stem cells can give rise to all somatic cells. They exist naturally within the inner cell mass of a blastocyst stage embryo and can be harvested from the latter (97). Embryos and ESCs can also be generated by nuclear transfer technologies (100). Alternative, embryo-sparing technologies involve chemical activation of unfertilized oocytes to generate pluripotent parthenogenetic stem cells (PSCs) (15, 21, 99), selection of multipotent GSCs from pluripotent spermatogonial stem cells (SSCs) (18, 32), and genetic reprogramming of somatic cells (93, 94) (Fig. 5). The latter technologies are ethically less controversial because they do not require the destruction of potentially viable embryos and in addition may be applicable to generate autologous therapeutic cells.

Since cells with a cardiac differentiation capacity are principally available for cardiac tissue engineering, it becomes essential to scale-up the cell allocation procedure and separate wanted from unwanted cell species. Genetic engineering, facilitating expression of selectable reporter genes such as antibiotic resistances and fluorescing proteins under the control of cell-type restricted promoters, can be exploited to purify specifically differentiated cell populations from naturally heterogeneous embryoid body cultures (47, 67). Using similar

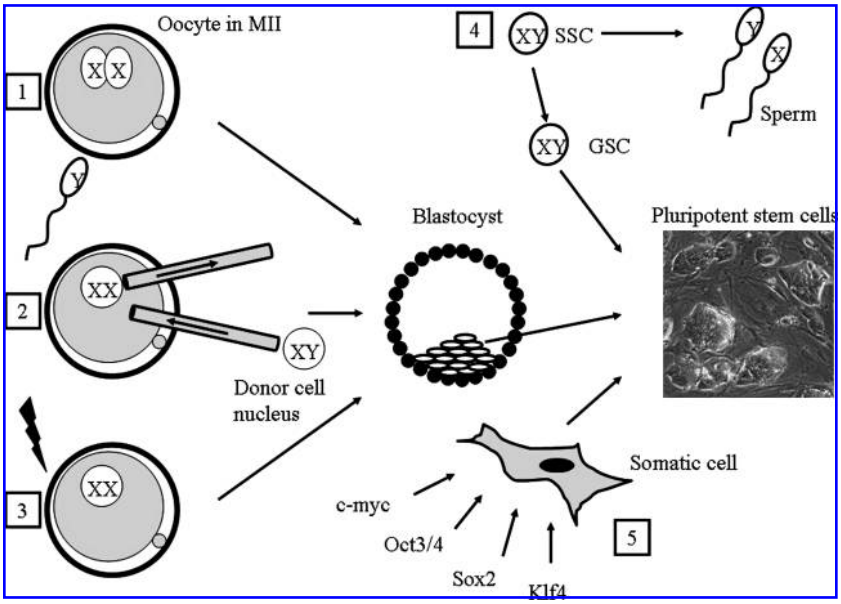

FIG. 5. Exploitable stem cell technologies: (1) ESCs from the inner cell mass of fertilized oocyte-derived blastocysts; (2) stem cells derived from SCNT-blastocysts; (3) PSCs derived from chemically activated oocytes; (4) testis-derived GSCs; (5) iPS-cells derived from reprogrammed somatic cells. MII: meiosis II.

technologies, a putative "master cardiac progenitor" may also be isolated (Fig. 6). To this end, several markers of mesodermal development [including Brachyury T (37, 50), MesP1 (19), Isl1 (55, 64), Nkx2.5 (64), and Flk1/KDR (40)] have been identified. A master stem cell would ideally be used to allocate all cardiac mesoderm derivatives (cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts) to cardiac tissue engineering from a single cell source. Alternatively, cytokine induction protocols (110) may turn out to be instrumental to generate ideal heart cell mixtures for cardiac tissue engineering without the need for genetic cell manipulations or sophisticated cell sorting (see below).

The following paragraphs identify cell species that may give rise to a master progenitor and discuss alternative strategies to provide an "ideal" cardiac mesodermal cell mixture from pluripotent stem cell cultures for cardiac tissue engineering:

Pluripotent embryonic stem cells. ESCs are generally derived from the inner cell mass of blastocyst stage embryos (9, 97). They can differentiate into any cell type of the body. Controlling ESC differentiation, especially into a mesodermal and eventually cardiac lineage, remains difficult, because their default differentiation program appears to direct them into ectodermal lineages (69). Genetic selection strategies are, however, useful to purify "rare" cell populations from differentiating embryoid body cultures (47, 67), but will, for example, not increase the total number of resulting cardiomyocytes because of their very early withdrawal from the cell cycle after cardiomyocytotic specification (46). To get a better grip on very early and potentially more "plastic" and "proliferating" cardiac mesoderm progenitors, several groups have identified novel early [*e.g.*, Brachyury T (40), Mesp1 (19)] or later [*e.g.*, flk1, Isl1, Nkx2.5 (40, 55, 64)] cardiac lineage markers. Yet, none of these markers is essentially unambiguous and even more so, most of these markers are not suited for classical and clinically feasible cell purification using either fluorescence-activated cell sorting (FACS) or magnetic cell sorting (MACS).

Pluripotent somatic cell nuclear transfer-derived cells. The enucleated oocyte contains factors that can reprogram nuclei from differentiated cells into an embryo-like stage (100). The identity of these reprogramming factors still remains elusive. A potential advantage of SCNT is that derivatives could be generated from any patient. Yet, SCNT is associated with enormous technical (mainly low efficiency) and ethical hurdles (large numbers of donor oocytes would be required to generate a single pluripotent stem cell line). Moreover, it should be noted that human SCNT-derived stem cell lines have not been generated up until now and it is unlikely that this will be done for a tissue engineering application, given the availability of apparently better "autologous" stem cells.

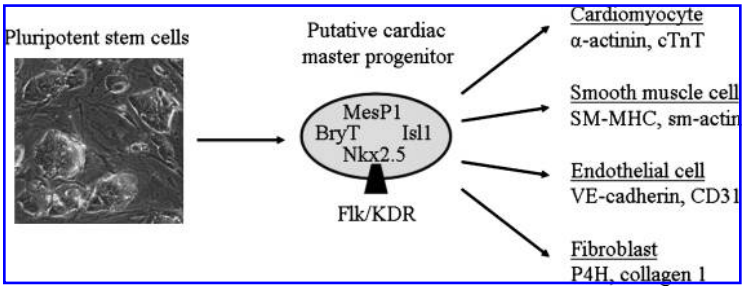

FIG. 6. Identification and utilization of a master cardiac progenitor. BryT, MesP1, Isl1, and Flk1/KDR have been identified as markers of early mesoderm formation. Genetic selection approaches (*e.g.* fluorescence-activated cell sorting [FACS] for GFP positive cells in BryT-GFP transgenic stem cell cultures (40)) or FACS-isolation of Flk1/KDR positive cells may be used to purify a common mesoderm progenitor. The latter may subsequently be induced to generate cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts for applications in myocardial tissue engineering (40).

Pluripotent parthenogenetic stem cells. Asexual reproduction, namely parthenogenesis, is a common trait of plants and nonmammalian animal species including amphibians, fish, and some birds (14). In contrast, mammalian parthenotes cannot develop to term as a consequence of defective extraembryonic tissue development. However, parthenogenesis can be induced in mammalian oocytes by chemical stimuli (17, 26, 81). Resulting parthenogenetic blastocysts develop an inner cell mass containing pluripotent cells. This cell source appears especially attractive for tissue engineering applications given the high efficiency of PSC derivation from unfertilized oocytes (50% in ref. 45), its potentially autologous applicability in the respective oocyte donors, and the possibility to induce PSC development by defined pharmacological stimuli.

Pluripotent spermatogonial stem cells. SSCs can be derived from adult testes and include a subpopulation of multipotent GSCs (32). The latter have the capacity to give rise to *bona fide* cardiomyocytes (33). The recent identification of similar cells in testes biopsies from patients of various age groups [17–81 years; (18)] may render these cells highly attractive for autologous cell-based therapy in male recipients. Yet, for pre-clinical testing and eventually clinical scale applications, stable cell lines still need to be established,

Induced pluripotent stem cells. The advent of a somatic cell reprogramming technology using retroviral transfer of four stemness factors/oncogenes (c-myc, Klf4, Sox2, Oct3) has raised enormous attention (94, 105). The original technology developed by Yamanaka and colleagues has already been modified extensively in terms of identification of alternative reprogramming factors (112), the omission of one (c-myc) and most recently two (c-myc and Klf4) of the four factors (36, 104), drug-induced expression, as well as control of reprogramming factors (103), and the use of apparently nonintegrating adenovirus (92). Recent studies moreover demonstrate that iPS cells can give rise to cardiomyocytes, yet at an apparently lower rate than ESCs (86). Nevertheless, iPS cells appear to be a highly attractive cell source for cardiac repair applications and the variations in differentiation capacity might just be the consequence of using a "suboptimal" iPS line. Despite the enthusiasm and enormous efforts to advance the iPS technolgoy, it is important to note that the exact mechanism of reprogramming still remains unclear. Naturally, studies have focused on the above mentioned "magnificent four" reprogramming factors, which appear to be at least partially exchangeable (94, 112). Another, at the moment purely speculative mechanism, could be that "lucky" random integration of transgenes or DNA fragments caused a relaxation of chromatin structure and thereby enabled transcription of essential endogenous reprogramming factors. This possibility has to be considered especially in light of very low reprogramming efficiencies [$<0.01\%$; (93)].

Up-scaling of the cell allocation process for cardiac muscle engineering applications

While several technologies and cell sources for human myocardial tissue engineering appear to be in place, there is a substantial limitation with regard to allocation of sufficient cell numbers and cell purity. It is important to emphasize that only $\sim 1(-10)\%$ of the ESC-derivatives develop into *bona fide* cardiomyocytes in standard embryoid body cultures (47). Similar cardiac differentiation capacity can be observed in other pluripotent stem cells species. In contrast, cardiac differentiation of "adult stem cells", including resident, circulating, and bone marrow stem cells, is at least *in vitro* negligible.

Although various protocols have been proposed to increase cardiomyocyte number in ESC cultures, none has so far been widely accepted. To this end, new data employing a sequential cytokine induction protocol appears most promising (110). Here knowledge from cardiac tissue development in the embryo was conferred to ESC cultures (Fig. 7). This resulted in the derivation of ESC-derived cell mixtures being composed of $\sim 50\%$ cardiomyocytes and 50% other mostly mesenchymal cells. High myocyte numbers can principally also be achieved by manual enrichment (41), gradient centrifugation (52, 107, 108), and genetic selection (47, 67). Yet, as pointed out before, pure myocyte populations are unlikely to be optimal for cardiac tissue engineering applications. Cytokine induction would overcome this limitation and could likely also be applied in large-scale bioreactor cultures (73, 87) to yield large "cardiospecific" progeny. Ultimately, cell quantity does not equate to cell quality and it will be essential to further identify factors that will advance stem cell-derived myocytes and nonmyocytes into terminal differentiation. These factors would likely not only include cytokines but also physical triggers [*e.g.*, shear stress, mechanical strain, and electrical stimulation (25, 79)].

Tissue Engineering-Related Caveats

A reparative myocardial tissue engineering approach will require full electrical and structural integration of the respective tissue construct into the architecture of a failing heart. To this end, terminal differentiation of the surrogate myocardial tissue *in vivo* will likely be essential. Tissue engineered myocardium must also not be encased in scar tissue once implanted and should ideally not cause an immune or inflammatory response. Moreover, long-term survival is a prerequisite to provide lasting support to a failing heart. This will not only require structural integration of myocytes but also vascularization to meet the necessary oxygen and nutrient demands of "physiological" heart tissue *in vivo*. In addition, arrhythmias should not originate from or be provoked by the

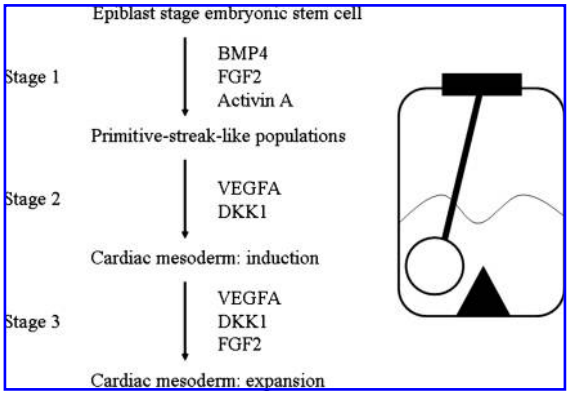

FIG. 7. Cytokine induction, as developed by Yang *et al.* (110), would ideally be combined with bioreactor technologies (87, 113) to allow large scale production of cardiac mesoderm derivatives for clinical scale cardiac muscle engineering.

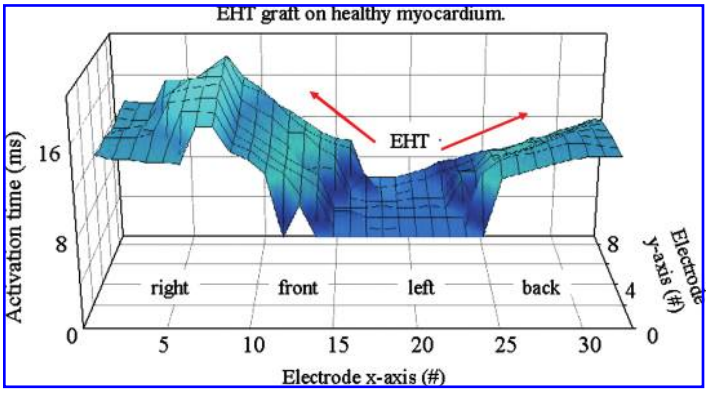

FIG. 8. Extra beat originating from an EHT implant on a healthy rat heart. Spread of excitation was recorded in a Langendorff-perfused heart by high resolution epicardial mapping (collaboration with S. Dhein, Clinic for Cardiac Surgery, Heart Centre Leipzig, University of Leipzig). These data provide additional evidence for electrical coupling of EHT grafts to recipient myocardium (119). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

biological graft material. Finally, it appears as if stem cells would be the only realistic cardiomyocyte source for clinical scale tissue engineering. This would naturally go along with the risk of malignant growth (48, 74). The following paragraphs will address each of the aforementioned caveats:

Cell integration

Myocytes within the heart are organized as a highly aniostropic functional syncytium (98). Gap junctions at the end-to-end connections of individual cardiomyocytes ensure undelayed impulse propagation within this structure. Exogeneous myocytes will have to be seamlessly integrated into this delicate electrical network and should essentially display similar electrophysiological and calcium-handling properties as native myocytes to ensure anisotropic impulse propagation and contraction.

Several studies have provided unequivocal evidence that myocyte "transplants" cannot only establish connecting gap junctions to neighboring myocytes, but also display similar calcium-handling properties as the latter (80, 83, 91). Similarly, tissue engineering studies have provided evidence for electrical integration *in vivo* (27, 115, 119). Yet, establishing new electrical contacts may also cause irregular electrical activity. For example, engrafted spontaneously active EHTs can principally activate a recipient heart by retrograde impulse propagation, that is, spread of excitation from the EHT-graft to the native myocardium (Fig. 8). In addition, integration of new electrical entities may go along with the risk of creating reentry circuits that may lead to fatal arrhythmias.

Most myocardial tissue engineering studies have been performed in fast heart rate rodents. This model may in fact underestimate the risk of arrhythmia induction, given the situation that all so far developed myocardial tissue constructs had a spontaneous beating rate far below the natural heart rate of the respective recipient (*e.g.*, beating rate of rat EHT *vs.* rat heart rate: ~120 *vs.* 360). Once coupled to the host myocardium, this would result in a permanent overstimulation situation with hardly any chance for retrograde activation. Moreover, there is a constant mismatch of current load, originating from a rather small graft, and the current sink, being essentially the remote myocardium (82). This situation will also make regular retrograde activation unlikely and may explain why we did not observe graft-derived arrhythmias in "EHT-treated" rats with severe myocardial infarctions (119).

It is, however, likely that spontaneously active tissue engineered myocardium would elicit severe arrhythmias in slow heart rate animals and essentially humans. In these models, lack of electrophysiological abnormalities may at least at early time-points after implantation argue against relevant functional integration of new electrical elements into the heart. It is therefore likely that any relevant cardiac remuscularization procedure would have to go along with pharmacological and/or electrophysiological rate and rhythm control.

Cell differentiation in vitro *and* in vivo

Adult cardiomyocytes are highly specialized and essentially postmitotic. Once isolated from native myocardium, there is a rapid decay in function and structure leading to cell death within hours or at best a few days. Consequently, myocardial tissue engineering relies on the allocation of immature but developmentally fully competent myocytes. Most studies so far have employed primary heart cells from neonatal rats or embryonic stem cells (10–12, 35, 56, 57, 117, 120). Ideally, these cells would already *in vitro* reach terminal differentiation and, in case of working myocardium, be quiescent. The latter would obviously also be beneficial with respect to the anticipated arrhythmogenic risk. Yet, reaching terminal differentiation would require either the already committed (immature myocyte) and even more so the not committed cells (*e.g.*, ESCs) to be exposed to "physiological" growth promoting triggers. To achieve a perfect match of the *in vivo* situation appears in this respect overly optimistic. It would, however, be expected that myocardial tissue grafts have the capacity to reach a state of terminal differentiation once exposed to the host's *in vivo* milieu. This appears to be the case after implantation of neonatal heart cell-derived EHT (115). Whether this also holds true for stem cell-derived myocytes remains to be demonstrated.

Scar formation, immune response, and inflammation

Engrafted cells and scaffold materials may cause immune or inflammatory responses and are consequently often encased in scar tissue. Scar tissue formation may even increase with time and eventually cause a structural and subsequently electrical demarcation of engrafted cells (80). This appears to be a major caveat for any nonautologous tissue engineering application. Consequently, cells and materials should be utilized that are either autologous or naturally biocompatible. On the other hand, transient inflammatory responses or foreign body reactions may be reasonably controlled pharma-

cologically (*e.g.*, by applying steroids) at least transiently until engrafted cells and scaffolds have properly integrated or are degraded.

Vascularization

Myocyte survival depends crucially on the allocation of oxygen and nutrients. This is physiologically ensured by a dense network of capillaries penetrating the normal myocardial syncytium (49). All so far applied tissue engineered muscle constructs do, however, rely mainly on diffusion, at least during the first days after an *in vivo* application. Here vascularization was reported to occur within 1–3 days after implantation (56, 89, 115, 119). This may at least in part be facilitated by the presence of preformed primitive capillary networks and angiogenic cells in tissue engineered myocardium (119, 120) and is apparently sufficient to enable cell survival in the respective animal models.. "Slow" vascularization is, however, unlikely to sufficiently support the survival of thick "human scale" muscle implants *in vivo*. Consequently, tissue engineered myocardium would ideally contain a functional micro- and macrovasculature that may be readily connected to the recipient's circulation by the respective transplant surgeon.

Tumor formation

It is likely that any clinically relevant tissue engineering approach would have to rely on the use of stem cells. Given the lack of cardiac differentiation in adult stem cells, most of the research efforts in cardiac tissue engineering have concentrated on pluripotent stem cells. The latter are capable of unlimited self-renewal if maintained in an undifferentiated state. All pluripotent stem cells have consequently the capacity to grow also *in vivo* and subsequently form teratomas (48, 74). Thus, any application of stem cells and their derivatives has to be controlled to stop unwanted growth. This may, for example, be achieved by integrating pharmacologically activatable suicide genes, such as herpes simplex virus thymidine kinase (2), into the respective cells or pharmacological induction of terminal differentiation in all transplanted stem cell progeny.

Conclusions

Cardiac tissue engineering offers an exciting perspective for the treatment of severe myocardial defects. To this end, several tissue engineering technologies and human cell sources are already available and may eventually be exploited to generate large autologous tissue patches for clinically relevant cardiac repair applications.

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Abbreviations

AV-block, atrioventricular block; BioVAD, biological ventricular assist device; BMP4, bone morphogenic protein 4; BryT, brachyury T; c-myc, cellular myelocytomatosis oncogene; CD31, cluster of differentiation 31; cTnT, cardiac troponin T; DKK1, dickkopf homolog 1; DNA, deoxynucleic acid; ECM, extracellular matrix; ESC, embryonic stem cells; EHT, engineered heart tissue; FACS, fluorescence-activated cell sorting; FGF-2 (basic), fibroblast growth factor-2; Flk1/KDR, fetal liver kinase 1/kinase insert domain protein receptor; GF, growth factor; GFP, green fluorescing protein; GMP, good manufacturing practice; GSC, germline stem cell; IGF-1, insulin-like growth factor-1; iPS, induced pluripotent stem cells; Isl1, islet-1; Klf4, Krueppel-like factor 4; LV, left ventricle; MII, meiosis II; MACS, magnetic cell sorting; MesP1, mesoderm posterior 1; ms, millisecond; Nkx2.5, NK2 transcription factor related locus 5; Oct 3/4, octamer 3/4; P4H, prolyl-4-hydroxylase; PDGF-BB, platelet-derived growth factor-BB; PECAM, platelet/endothelial cell adhesion molecule; PSC, parthenogenetic stem cells; PGL, poly-glycolic acid; PLA, poly-lactic acid; SCNT, somatic cell nuclear transfer; SSC, spermatogonial stem cells; SDF-1, stromal cell-derived factor-1; sm-actin, smooth muscle actin; SM-MHC, smooth muscle myosin heavy chain; sox2, SRY (sex determining region Y)-box 2; VE-cadherin, vascular/endothelial cadherin; VEGF-A, vascular endothelial growth factor-A.

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