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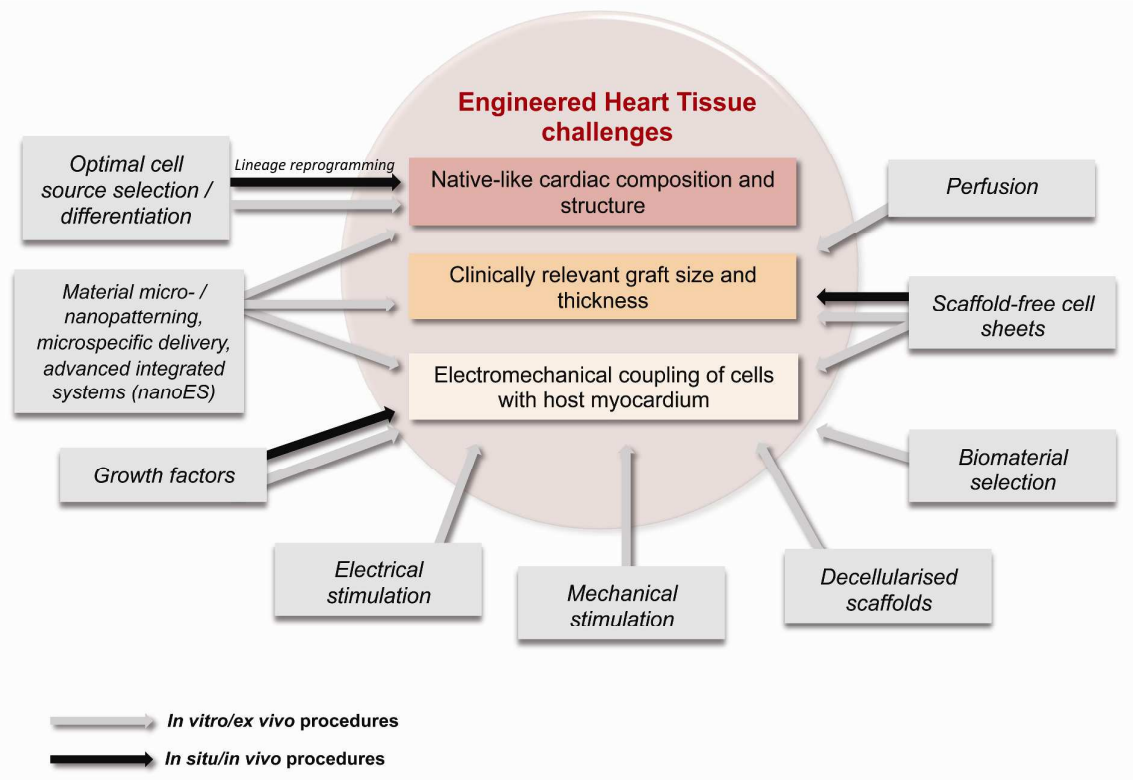

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CRITICAL REVIEW

Cardiac tissue engineering: renewing the arsenal for the battle against heart disease.

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The development of therapies that lead to the regeneration or functional repair of compromised cardiac tissue is the most important challenge facing translational cardiovascular research today. During the last 25 years huge efforts have been made towards restoring the physiologic functions of the heart by means of delivering cell implants into the insulted heart, initially through 'naked cell' injections and more recently through the principle of cardiac tissue engineering and the use of elaborate delivery systems and priming mechanisms that include scaffolds, bioreactors or ex vivo manipulations of cells and support structures. In this review we summarise various approaches towards cardiac repair and highlight advances in the field of tissue engineering, ranging from a review of cell types used, to advances that attempt to address mechanistic and functional elements that are critical for successful restoration of the heart, including the maintenance of the extracellular matrix through scaffoldless cardiac sheets, strategies that promote neovascularisation and the precise micro-delivery of cell populations to form three-dimensional structures through bioengineering methods such as microfabrication.

Insight, Innovation, integration

The complex anatomical characteristics, functional mechanisms and the almost complete inability of cardiac tissue to regenerate have rendered traditional therapeutic approaches to cardiac disease ineffective or incomplete. During the last 25 years the advent of cardiac tissue engineering has brought together experts from diverse fields, such as molecular and cell biology, engineering, medicine and computer science, in order to develop translational platforms that consider the different mechanistic qualities and tissue requirements of the heart milieu. This review gives an overview of the different interdisciplinary approaches and presents the seminal advances that attempt to address the main challenges in cardiac tissue engineering research.

Introduction

Cardiovascular disease and consequent heart failure remains one of the major causes of death in the world [1]. Acute or chronic pathophysiological events such as myocardial infarction (MI), ischaemic or idiopathic cardiomyopathy and other systemic conditions lead to the progressive functional compromise of the heart, manifested by the replacement of healthy, functioning myocardium by non-contractile scar tissue [2]. Hundreds of thousands of patients per year survive an acute heart attack while others succumb due to the primary event. Those who survive the acute infarct are likely to become progressively incapacitated and require expensive medical and/or surgical support [3].

The human heart was until recently considered to be a postmitotic, terminally differentiated organ but recent evidence suggests that it does possess a small regenerative capacity both physiologically (ranging from 1% in early adulthood to 0.45% in septuagenarians) [4] as well as following myocardial injury due to cardiovascular disease [5]. This innate ability for regeneration, however, is far from sufficient to replenish the failing organ without clinical or pharmacological intervention, and as a result the progressive loss of heart muscle

contractility, due to the replacement of functioning myocardium by non-contractile scar tissue, leads to congestive heart failure. The lack of significant endogenous regenerative capacity in the heart therefore necessitates the development of therapeutic interventions that will replace or regenerate the injured tissue and restore the functional properties of the myocardium. The current status of interventional therapies for heart disease includes several mechanistic procedures such as insertion of a 'stent' (coronary catheter) [6] and use of a pacemaker for cardiac synchronisation in the left ventricle (LV) (left ventricular assist device or LVAD) [7] as well as surgical procedures such as LV reconstructive surgery [8]. However, none of these clinical therapeutic strategies, invasive or pharmacological, have been able to efficiently regenerate the diseased heart, instead offering a temporary and unsustainable form of palliative care. To date, whole heart transplantation remains the most efficient form of therapy, particularly in cases of end-stage heart disease. The disproportionately small ratio of donors to recipients, however, as well as the fact that recipients have to rely on a constant immunosuppressive regime post-operatively, suggest that heart transplantation cannot constitute a pragmatic therapy for heart disease.

From naked cells to 'matrix'-based delivery systems

Naked-cell injection (cardiomyoplasty): overview and advances

Considering the great number of cells that are lost following an MI episode (up to 25% of LV mass within a few hours post-MI) [9], there is a clear need for the development of therapies that can lead to the efficient and stable regeneration of the compromised organ. Towards this goal, the direct injection of cells into the diseased myocardium (known as 'cellular cardiomyoplasty' or 'cardiac cell therapy') was proposed as a potential form of cardiac repair more than 15 years ago. Since its inception, several cell types have been employed as an injectable source of cells toward *in situ* cardiac regeneration/repair, including: adult stem cells, embryonic stem cells (ESCs) and, more recently, induced pluripotent stem cells (iPSCs). Furthemore, the recent advent of direct lineage reprogramming of non-cardiac cells into cardiomyocytes (CMs) promises to bypass some of the caveats presented by the delivery of unprogrammed somatic cells.

All types of adult stem cells have undergone preclinical studies and, in most cases, large-scale clinical trials have been carried out to test the efficacy and safety of these. With regard to adult stem cells, initial work focused on myogenic cell sources, namely either that of skeletal myoblasts (SMs) as an autologous source [10,9,11,12] or fetal and neonatal cardiomyocytes, as an allogeneic source [13,14,15,16], drawing on the similar, already established, protocols for cell therapies of muscular dystrophies and in particular myoblast transfer therapy (MTT) [17,18]. Subsequent injection of SMs into human myocardium led to development of arrhythmias [19]. The MAGIC clinical trial, a double-blind randomised phase-I clinical trial using autologous myoblasts injected into ischaemic hearts of patients, found little evidence of improved LV function but at the same confirmed the higher rate of arrhythmias in the myoblast recipients [20], thus ending the possibility of SMs being used as a cell source of *in vivo* cardiac restoration.

Bone marrow (BM)-derived cells (BMCs) have also been used as a source for direct injection into the myocardium, providing a stable, autologous source for cardiomyoplasty. These included c-kit+ BM-derived progenitor [21] and stem cells [22] as well as BM-derived cardiomyocytes [23]. A subtype of BM-derived cells, mesenchymal stem cells (MSCs), have also been used a cell source [24,25,26,27,28,29] in cardiomyoplasty studies and are complemented by their localised immunosuppressive properties which render them a good candidate as an allogeneic source. A recent study found that addition of MSCs into partially CM-depleted cardiac constructs led to improved contraction [30]. Once again, however, any positive effects of BMCs/MSCs proved to be transient due to limited *in situ* differentiation, while the concomitant improvement in cardiac function following injection of such cells has been suggested to be a result of their paracrine effects conferring neovascularisation and cytoprotection to resident cell populations [31,32]. Furthermore, it was observed that what was originally thought to be transdifferentiation of BM-derived cells in the host myocardium was in fact the result of cell fusion between donor and host cells [33] and this functional misnomer also applied to the use of adipose-derived cells (ADCs) as a cell source [34]. Therefore, in the case of SM-type and BM-derived cells (including MSCs), although initial preclinical studies showed positive results with regard to incorporation and mechanical integration, clinical trials and additional preclinical studies indicated that a) the statistically significant improvement in myocardial function was in most cases transient and not clinically relevant and b) in many cases the assimilation of the donor cells by the host tissue led to arrhythmogenic events that could increase morbidity and mortality in recipient subjects. These observations are in contrast to the principal aim of restoring cellular electrical conductance and contractility in the host tissue. Furthermore, the finding that injection of non-cardiac cells into the diseased cardiac tissue can facilitate some degree of improvement, albeit transient, indicates that remodelling/regeneration is driven, to some degree, by paracrine factors secreted by those non-cardiac cells [35]. To date, the identity of these specific factors remains unknown, although clearly the characterisation of these factors is crucial to the improvement of cardiomyoplasty whatever the cell source. Other noteworthy sources of stem cells used in cardiac regeneration studies include endothelial progenitor cells (EPCs) [36,37] and smooth muscle cells [38] (for a review see [39]).

Resident cardiac progenitor cells (CPCs), a number of which express either c-kit [40,41,42] or Sca-1 [43], have also been put forward as candidates for cardiomyoplasty. A mixed population of c-kit+ and Sca-1+ CPCs that are able to form spheroid cultures in suspension, termed 'cardiospheres', have also been a promising source for myocardial restoration. Two recent clinical trials published the first results

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of c-kit+ cells (SCIPIO) [44] and cardiospheres (CADUCEUS) [45] being injected into human patients' hearts. Although a decrease of fibrotic scar tissue was observed, no significant functional improvement (in terms of ejection fraction) was observed and further studies with larger patient samples and placebo controls are required for the complete clinical evaluation of CPCs. Aside from the results of the latter phase I clinical trials, the limited proliferation of CPCs, their apparent absent role in cardiac response and their potential senescent molecular and cellular status, due to their patient-specific endomyocardial-derived source, pose limitations to their use as an injectable cell source.

With relation to the abovementioned cell types and their use in clinical studies, low donor cell retention/engraftment post-implantation is a considerable drawback. Excluding factors such as tissue homology (delivery of autologous or heterologous donor cells) and the choice of cell injection route (intramyocardial, intravascular or intracoronary), it appears that the state of the host cardiac tissue that is to receive the donor cells (i.e. whether the donor site is physiologic, ischaemic, inflamed or scarred) may influence donor cell survival and uptake [46]. For example, an animal study by Terrovitis and colleagues showed that approximately 17% of CPCs survived following intramyocardial delivery, whereas this percentage increased to 75% when cells were injected within 1 hour from the induction of a myocardiac infarction [47]. The latter pathophysiological milieu however is not clinically relevant since patients will receive grafts within days, at best, of an insult. As already mentioned, some of the positive effects of cardiac cell therapy regimes are attributed to paracrine effects; these paracrine effects occur prior to the formation of gap junctions (a process that is necessary for electrical integration between donor and host cells) and therefore can elicit their beneficial action without the need of cellular electrical connectivity.

Embryonic stem cell (ESC)-derived cardiomyocytes are another potential cell source due to their pluripotent nature and their ability to remain in culture for unlimited periods of time. Although animal studies have demonstrated some restoration of heart function following ESC injection through electromechanical coupling of the ESC-derived CMs with the native myocardium, there are significant disadvantages accompanying their use, including teratoma formation (in the case of undifferentiated ESCs being used), immunological rejection by the host tissue and, of course, ethical considerations [48,49,50,51,52]. For those reasons no ESC clinical trials have been carried out to date. A recent study showed that human ESC-derived CMs, when transplanted into a guinea pig MI model, lead to contractions that are synchronous to the host tissue, improve mechanical function of the host heart and reduce the risk of arrhythmia and tachycardia [53].

iPSCs are a promising cell source for cardiac regeneration since, like ESCs, they are pluripotent and can give rise to all three germ layers. The use of iPSC-derived CMs as an injectable cell source for cardiac therapy can potentially bypass the immunogenic rejection elicited by orthotopic injection of ESCs, although the potential of teratoma formation cannot be excluded [39]. CMs have been obtained through iPSC differentiation and, upon transplantation into rodent MI models, have shown functional integration with the host tissue but as yet no clinical trials have been carried out [54,55]. Furthermore, iPSCs-derived CMs isolated from long QT syndrome (LQTS) mice showed *in vitro* all the pathophysiological phenotypic characteristics of cells isolated directly from affected subjects, thus suggesting that patient-specific iPSC-derived CMs could offer clues for pharmacological patient-specific design that is more relevant to the advent of pharmacogenomics [56].

During the last three years major advances have been made towards efficient histological and, most importantly, functional restoration of the insulted myocardial tissue through a novel conversion strategy termed 'lineage reprogramming' and virtually parallel studies have shown that it is possible to induce the reprogramming of fibroblast cells (usually dermal fibroblasts) into functional cardiomyocytes. Stemming from the seminal work of Takahashi and Yamanaka [57] whereby somatic cells (embryonic or tail-tip fibroblasts) were induced to adopt a pluripotent (iPSC) state by the retroviral introduction of four transcription factors (TFs), work by Deepak Srivastava and colleagues initially showed, through single factor elimination, that the ectopic expression of three TFs (Mef2c, Gata4 and Tbx5, collectively known as 'GMT') could facilitate the direct *in vitro* lineage reprogramming of cardiac and tail-tip fibroblasts into CMs without an intermediate transition into a pluripotent state [58]. The implications of that study were significant with regard to the clinical manifestation of scar (fibrotic) tissue formation through fibroblast proliferation: scar tissue formation leads to a decrease in cardiac pump ability and subsequent manifestation of arrhythmias. However, the *in vitro* conversion rate of fibroblasts to CMs was extremely low (20% conversion based on transgenic reporter expression and 1% conversion into beating CMs), suggesting that the absence of a 'native' environment (such as that found *in vivo*) was required for the efficient cardiac reprogramming of the fibroblasts into functional, contracting CMs [59]. Recent studies utilising the GMT TF combination [60], or with the addition of the Hand2 TF to the GMT set (GHMT) [61] have taken lineage reprogramming a step further by achieving *in vivo* reprogramming of cardiac fibroblasts (CFs) into CMs via direct retroviral injection of the respective TF combination into mouse myocardium: both studies reported low percentage conversion of CFs into CMs following experimental infarction but, most importantly, improvement in cardiac function and reduction in fibrous tissue formation. More recent studies have questioned the complete reprogramming of the starting CFs into CMs, arguing that a global epigenetic rearrangement, which is required for complete reprogramming, has not been shown [62] or does not seem feasible [63]. Certainly more work is needed towards assessing the full extent of lineage reprogramming in the heart. Another noteworthy study showed that direct lineage reprogramming of CFs into CMs can be achieved both *in vitro* and *in vivo* by transient expression of a single microRNA, miR-1 [64], providing further evidence for the possibility of reprogramming fibroblasts by canonical modifications. For a more detailed

review of the various cell types used to date in cardiac cardiomyoplasty, including clinical trials, see [39,65,66,67,68].

Cardiac Tissue Engineering: overview and advances

The concept of 'naked', cellular cardiomyoplasty (notwithstanding the recent aforementioned lineage reprogramming studies which cannot be explicitly classified as such) therefore proved to be one that offers low cell retention by the host tissue and a transient form of incorporation which, most importantly, does not lead to a stable restoration of the electromechanical properties of the host heart organ. It has become apparent that the lack of a sustainable therapeutic effect does not lie solely on the choice of cell source but also on the delivery mode and the auxiliary constituents that form the 'implant niche'. Cardiac tissue engineering (CTE) as a discipline addresses those issues: CTE is the therapeutic strategy that aims to mimic the native environment of the host cardiac tissue by constructing new tissue *in vitro*, *in situ* or *in vivo* to facilitate the assimilation of the bioengineered, 'donor' tissue by the host milieu.

In CTE, aside from the cell source, the underlying extracellular matrix (ECM) (either in its native form or in the form of a biomaterial substitute) is incorporated, as well as, in some cases, the supply or presence of bioactive molecules (such as growth factors) and oxygen supply. Some researchers incorporate the advent of cardiomyoplasty into CTE [69], and indeed in some cases the former constitutes a section of the latter (and this notion extends to the principle of tissue engineering at large). CTE is, however, traditionally defined as the strategy that employs a cell source along with a native or experimentally-made incorporated matrix/scaffold, with any one of various mechanical, stimulatory (e.g. electrostimulation) or chemical implementations forming a 'bioreactor' system.

Considering that the cell source in CTE can, theoretically, be any cell type from those described above, the next consideration is the scaffold used for the delivery of cells as well as the presence (if any) of a bioreactor. It is important to note that certain experimental designs in CTE may omit a cell source altogether, instead attempting to stimulate *in situ* cardiac repair through the direct injection/deposition of acellular materials with or without the addition of bioactive molecules; in this way, cardiac remodelling that leads to scar tissue formation can be decreased through an increase in infarct thickness which will in turn result in decreased wall stress on the healthy portion of the myocardium [70].

In order to understand the principles of CTE, one must consider its goal which is to re-establish physiological function as well as cellular structure at different hierarchical levels [71]. As such, an ideal CTE construct has to fulfil the following requirements:

a) Be biocompatible with the host tissue and therefore not be immunologically rejected or elicit an inflammatory response *in vivo* that would lead to significant cell loss of the construct and/or deterioration of the host milieu.
b) Have morphological properties that are similar and in accordance to those of the native myocardium (this element includes selection of an appropriate cell source). Specifically in the case of CMs being used in the construct, the biomaterial used should promote CM alignment and differentiation *in vitro* as well as *in vivo*, therefore further improving the contractile properties of the graft.
c) Remain viable prior to and following delivery/implantation to the host tissue. This is achieved via culture medium perfusion *in vitro* (through the use of bioreactors) or via blood perfusion *in vivo* (through efficient vascularisation of the construct via assimilation by the host tissue).
d) Allow sufficient oxygen diffusion through itself. This requirement directly affects the thickness of viable constructs by limiting it to 200μm in the absence of experimentally-applied perfusion [72].
e) The biomaterial used has to be biodegradable at a rate that facilitates assimilation of the donor cells and at the same time is coordinated with the native tissue rate of repair/regeneration.
f) Be biomimetic by 'mirroring' the extracellular matrix (ECM) of the host tissue.
g) Facilitate cell-cell adhesion via the formation of gap junctions and membrane channels.
h) Generate force during contraction that is in the range of the native tissue and also conduct electrical signals [73]. The biomaterial used must therefore facilitate the electrical integration of the CTE construct with the host tissue to allow synchronous contraction of the two by matched excitability of host and graft, and support of electrical propagation [74]. An appropriate cell source used in the CTE construct is one that will not provoke arrhythmias following integration to the native myocardium.
i) Possess as well as confer mechanical integrity that will allow *in vitro* manipulation of the biomaterial during transplantation as well as mechanical support during *in vivo* regeneration/repair. The overall physical characteristics of the construct should include mechanical 'stiffness' that can even be slightly in excess of that of the fibrotic tissue in order to prevent scar expansion.

Overall, a clinically relevant functional cardiac tissue should possess the following characteristics: a tissue thickness of ~0.5cm, a high cell density of ~10^8 cells/ml, the ability to generate a 2-4mN/mm^2 force during contraction and to facilitate electrical signal propagation at ~25cm/sec, and a vasculature with intercapillary distances of ~20μm [75,76].

Towards reaching the above requirements, six different CTE approaches can be identified [71].

1) Mechanical stimulation of cells in hydrogels.
2) Electrical stimulation of cells contained in porous scaffolds.
3) Culturing of cells on perfused channelled scaffolds.
4) Decellularisation of the native heart and repopulation with donor cells.
5) *In situ* cell delivery via injectable hydrogels.

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6) Culturing of scaffold-free cell sheets.

In terms of the use of scaffolds used in cardiac constructs, three possibilities exist: a) the use of a specific scaffold type (see below), b) the use of a decellularised heart as a scaffold which is seeded with a combination of cell types and c) the use of scaffoldless cellular sheets. Another classification highlights the cell delivery system as an identifying factor, thus separating CTE intra- or epicardial administration of donor cells in: a) naked cell injection, b) hydrogel administration and c) cardiac patch deposition [71].

Although the complete listing of different CTE scaffolds is beyond the scope of this review, we briefly list below the main categories of scaffold materials and arrangements that are employed in CTE. Several categorisations exist according to materials, structure and method-of-use and we herein attempt to give all major categories along with the most prominent members of each category but we only focus on the advances in mechanistic combinations of scaffolds rather than those pertaining to material. For a more detailed review of biomaterial type and scaffold structure, including use in pre-clinical and clinical studies, see [77,78].

Scaffold biomaterials:

a) *Natural polymers*: Collagen I (alone or mixed with Matrigel), fibrin, ECM, Matrigel, glycosaminoglycans (GAGs) (hyaluronic acid (HA)), alginate, gelatin, chitosan.
b) *Synthetic polymers*: Poly(lactic-co-glycolic acid) (PLGA), poly(glycerol sebacate), poly(*N*-isopropylacrylamide) (PNIPAAm), polycaprolactone, PTMCLA, polyurethane, polyethylene glycol (PEG).
c) Certain combinations of natural and synthetic polymers (e.g. polyurethane + collagen I).

Scaffold structures:

a) *Preformed*: Sponges (PLGA or collagen I). Decellularised organ (decellularised ECM).
b) *Hydrogel*: fibrin, collagen, chitosan (Natural). PLGA, PEG (Synthetic).
c) *Scaffoldless*: e.g. (PIPAAm) thermo-responsive surfaces.

Bioreactor types:

a) *Static or mixed flask*: constructs are suspended in culture medium.
b) *Rotating vessel*: constructs are suspended in medium with constant rotational flow.
c) *Perfusion cartridge*: constructs are perfused at interstitial velocities that are similar to native tissue blood flow [79,74].

Natural polymer scaffolds are highly biodegradable and biocompatible and can be a source of chemical signals by virtue of direct contact/assimilation or following enzymatic degradation. At the same time natural materials have weak mechanical properties and native enzymes can lead to excessive degradation of such scaffolds; their manipulation can lead to the disruption of their three-dimensional architecture while on some occasions they can elicit an immunological response. Polymeric scaffolds can be designed to have a range of mechanical and chemical properties but their lack of innate chemical signals usually necessitates their combination with various cytokines [66,74].

Some recent cardiac therapy studies that utilised fibrin scaffolds reported significant cardiac functional improvement: Xiong and colleagues delivered hESC-derived endothelial cells (ECs) and smooth muscle cells (SMCs) along with a fibrin porous scaffold into ischaemia/reperfusion (I/R)-subjected mouse and pig hearts and observed an improvement in ejection fraction (mouse) as well as improved contractility, recruitment of endogenous cardiac progenitors, decreased apoptosis, LV wall stress and cardiac metabolism [80,81]. Those findings were attributed to an increased amount of neovascularisation achieved by the implanted cells and similar observations were made following implantation of hiPSC-ECs and hiPSC-CMs into infacted pig hearts [82]. Another study found that rat MSCs seeded onto a collagen-I patch led to improvement in contractility and perfusion of the infarcted segment, along with a decrease in infarct size and improved neovascularisation [83]. In a study that used a combination of synthetic and natural polymers, in this case a PEGylated fibrin biomatrix, mouse BMCs were injected along with the biomatrix which was complemented with hepatocyte growth factor (HGF) into infarcted murine hearts, leading to an improvement in LV dilation and fibrosis size as well as a decrease in apoptosis and neovascularisation [84]. The use of other biomaterials in CTE strategies are discussed in the sections below.

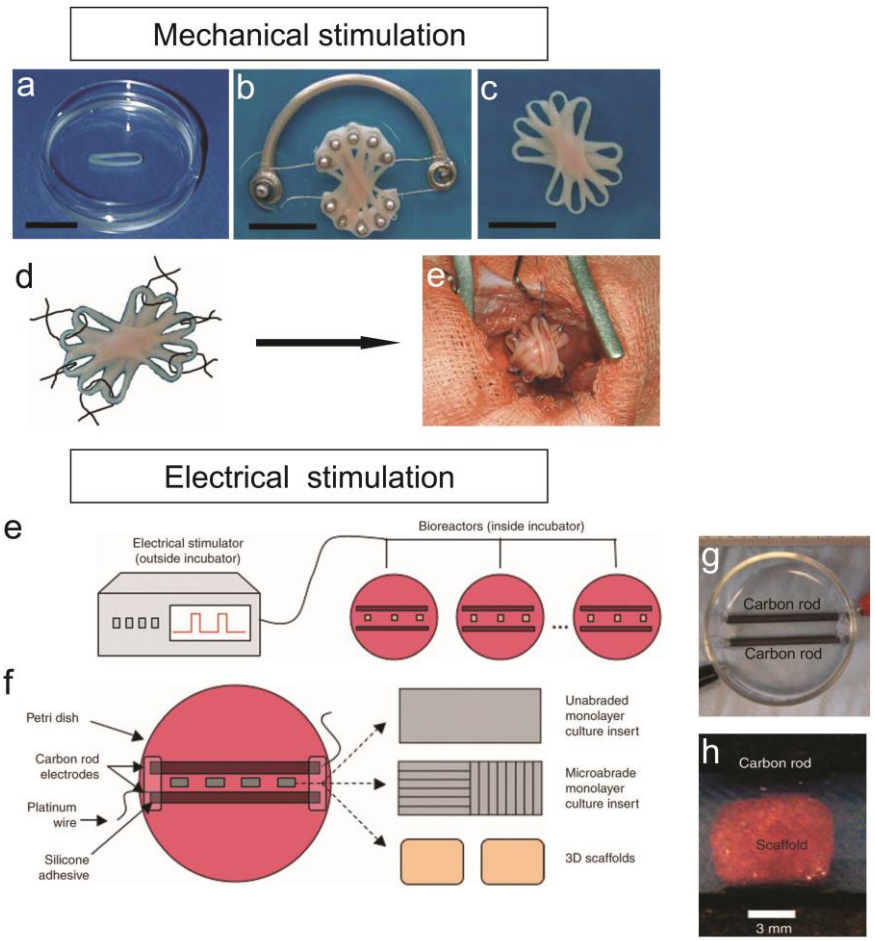
The application of a 'bioreactor system' is required for, at least, three of the CTE tissue engineering approaches: mechanical or electrical stimulation of donor cells and perfusion of cells [75]. A bioreactor is an apparatus or experimental setup that aims to enhance tissue regeneration and can provide to the CTE constructs the necessary mechanical and physiological properties required to maintain or enhance native-like cardiac function, including constant physiological temperature, oxygen and CO_2 gradients [85]. Bioreactors were first used in cardiac studies in 1997 [86] and their abovementioned variations have since targeted the optimisation of cell homeostasis and electromechanical properties.

The various CTE approaches, scaffold materials and structures and bioreactor systems are exemplified in some of the recent advances in the field and are presented below:

Mechanical stimulation: The production of the first engineered heart tissue (EHT) in 1997 was a result of the mixing of primary CMs with collagen gel which, following gelling of the cells with the collagen matrix, led to spontaneously contracting tissue *in vitro* [87]. Recent modifications of that original protocol led to the generation of custom 'multiform loop' EHTs, a prime example of the 'mechanical stimulation' CTE approach [88,89]: neonatal rat CMs were cast into a mixture of collagen I and Matrigel and were used to construct circular EHTs ('loops') following stretching on a cycling stretching device (auxotonic stimulation). These multiform EHTs were implanted in infarcted rat hearts and initially showed a great degree of vascularisation, and 4 weeks post-implantation showed electrical integration with the host myocardium and, most importantly, evidence, for the first time, of restoration of systolic function in the host heart [89]. It is important to note that immunosuppression was required for the *in vivo* survival of the EHTs but nevertheless these studies provided the first evidence that cardiac cell constructs could functionally improve injured cardiac tissue. A very recent study by the same group that designed the aforementioned EHT's used nonembryonic parthenogenetic stem cells (PSCs) in order to derive cardiomyocytes (PSC-derived CMs) and directly compared, favourably, their pluripotency and cardiogenicity with those of ESCs. The ability to match the major histocompatibility complex (MHC) haplotype of PSC-derived CMs with the recipient haplotype, due to the PSCs' MHC haploidentity, makes them an ideal allogeneic cell source candidate for CTE strategies. The authors demonstrated that the use of PSCs in EHTs led to a functional improvement, when compared to 'sham' EHTs, namely thicker anterior wall and enhanced systolic anterior wall thickening [90].

Electrical stimulation: Given the importance of synchronous contraction of bioengineered cardiac constructs and their ability to respond to electrical pacing by proper excitation-contraction coupling, the application of external field stimulation through *in vitro* bioreactor systems has been explored. Using an electrical 'stimulation chamber', the Vunjak-Novakovic group provided the first evidence that physiologically relevant electrical stimulation of cardiac cells (CMs, CFs and ECs) on a collagen porous scaffold induces the formation of parallel, elongated cardiac muscle fibres, with an improvement in cardiac cell marker expression, tissue architecture and morphology and contraction amplitude [91,92]. Refinement of the bioreactor system showed that the use of symmetric biphasic square pulses, when compared to analogous monophasic pulses, leads to improved gap junction formation and contractility [93].

Perfusion: In order to allow for the high oxygen demand of CMs, cells have been perfused *in vitro* with culture medium containing oxygen carriers (such as perfluorocarbon) and grown on channelled synthetic polymer scaffolds [94,72,95]. This approach leads to the



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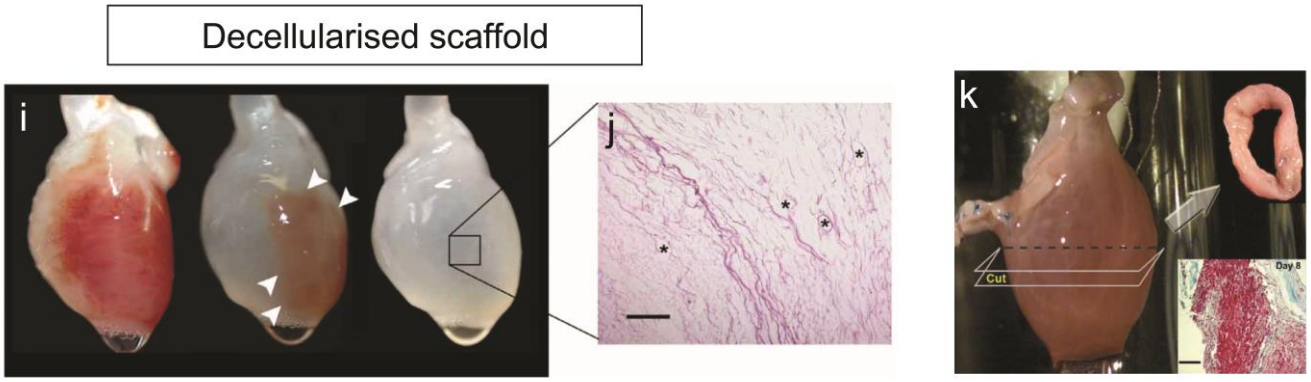

Fig.1 Main CTE approaches and methods. Mechanical stimulation: Five EHTs ((a) shows a single EHT), each consisting of rat cardiomyocytes, collagen I and basement membrane-like matrix (BME/Matrigel), are subjected to auxotonic stimulation on a custom-built device (b) that leads to the EHTs fusing and contracting synchronously as a multiform loop (c) which can then be engrafted *in vivo* through suturing onto recipient hearts (d, e) leading to mechanical and electrical integration of the EHT with the host cardiac tissue. Scale bars d-f: 10µm ((a)-(e) reprinted with permission from [89]). Electrical stimulation (modifiable setup according to Vunjak-Novakovic's group): electrical pulses are generated by an electrical stimulator and are transmitted into bioreactors that are incubated at physiologic conditions (e). The electrical stimulation chamber consists of a Petri dish with two inserted carbon rods, into which different cell-containing inserts or CTE constructs/scaffolds can be placed (f-h). Application of electrical impulses has been shown to lead to elongated cardiac muscle fibres with an improved tissue architecture and contractile properties ((e)-(h) adapted with permission from [92]). Decellularisation of cardiac matrix: Perfusion of whole rat hearts with 1% SDS for 12hrs (i) leads to complete decelullarisation (as shown through H+E staining in (j)) (scale bar in j: 50µm). Following electrical stimulation and under physiological load, the decellularised scaffold is then reseeded with cardiac or endothelial cells through coronary perfusion culture in a bioreactor (k, left panel shows recellularised heart at 4d into perfusion). The upper right insert in (k) shows a cross-sectional ring harvested at 8d of perfusion culture, while the lower right insert in (k) shows a ring thin section of stained with Masson's trichrome, indicating the presence of cells throughout the thickness of the wall (scale bar in k: 250µm). Force generation in recellularised rings is comparable to the maximal force generated by rings created by artificial ECM ((i)-(k) adapted with permission from [103]). (continued overleaf)

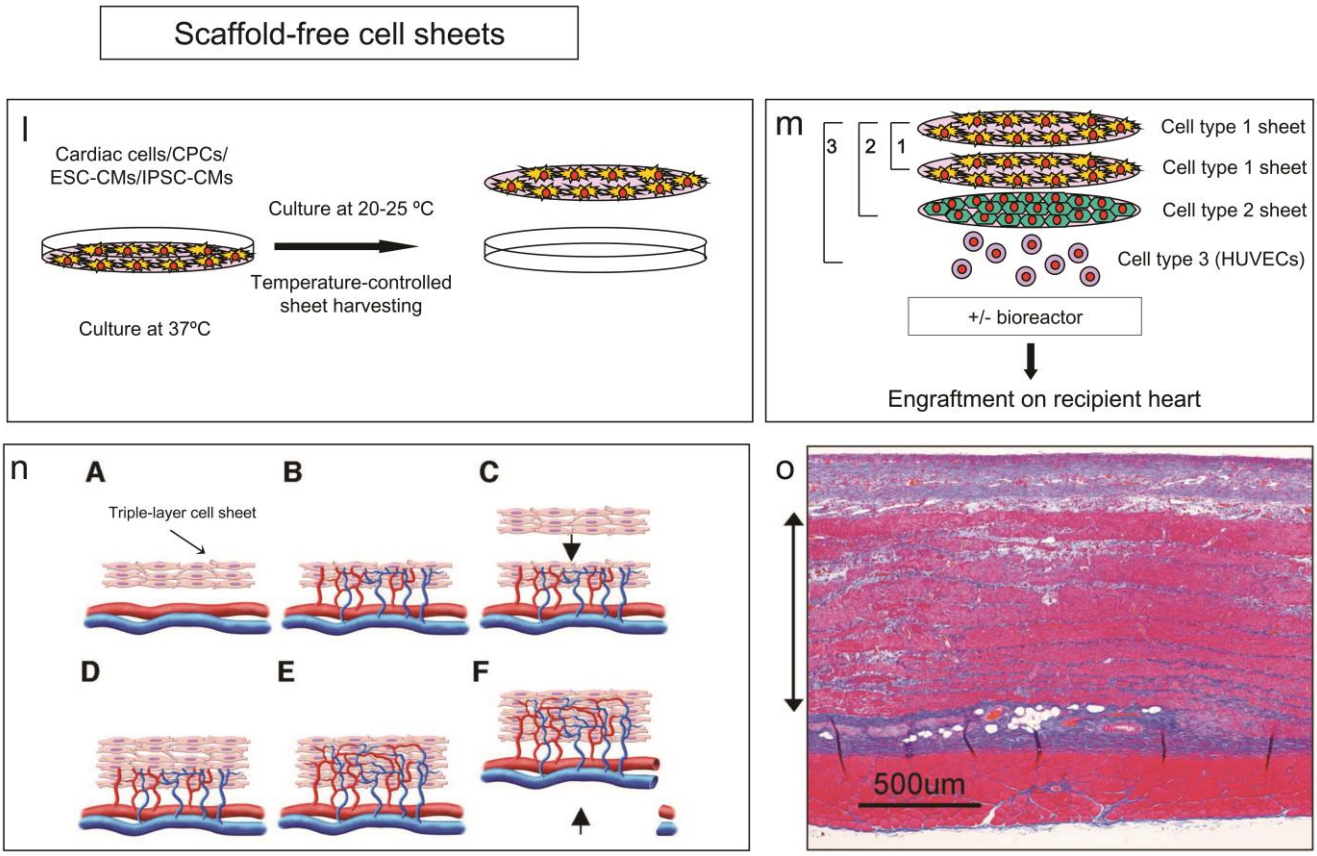

Fig. 1 (continued) Scaffold-free cell sheets: Cells that have been grown at 37 ºC on poly (*N*-isopropylacrylamide) (PIPAAm) plates are harvested as intact sheets, along with their ECM, following incubation at 20-25 ºC. Various cell sources such as cardiac cells, CPCs, ESC-derived cardiomyocytes (ESC-CMs) and IPSC-derived cardiomyocytes (IPSC-CM) have been used as a cell source (l). Stacking of homotypic cell sheets (connection 1, m), heterotypic cell sheets (connection 2, m) or cell sheets with cell suspension (e.g. human umbilical vein endothelial cells (HUVECs)) (connection 3, m) can be carried out, leading to the fabrication of multilayered sheets that can be further conditioned in bioreactors, following which they are engrafted onto the recipient heart (m). In the 'polysurgery cell sheet approach', multi-layered (triple) cardiac sheets can be vascularised *in vivo* if they are transplanted over a surgically accessible host vein and artery (panel A, n), resulting to the graft being vascularised through the host vessels (panel B, n). Following vascularisation of the initial graft, a second triple sheet is transplanted over the first graft (panels C and D, n) and allowed to be vascularised also by the host vasculature perfusing the initial graft (panel E, n). The resulting vascularised construct is surgically resected along with part of the host vasculature (panel F, n) and can then be transplanted ectopically. 10-times polysurgery with 1-day intervals leads to the creation of a multilayer myocardial tissue (indicated by the bidirectional arrow) that contains organised microvessels (o). ((n)-(o) are adapted with permission from [117]).

creation of compact cardiac tissue and has been combined with mechanical [96] and electrical stimulation [97].

Hydrogels (in situ injection): Apart from serving as a matrix for *ex vivo* processing/expansion of cells, hydrogels were recently also used for *in situ* injection of cells into the host heart. This strategy aims to bypass the caveat of cell loss and limited engraftment of naked-cell suspensions. The use of injectable materials has the advantage of not presenting the host tissue with an immunogenic reaction (due to the cells being encapsulated in the hydrogel material). The first such study showed that when myoblasts were injected along with fibrin glue into infarcted rat hearts, infarct size was decreased when compared to naked cell injection or fibrin glue alone [98]. A modified protocol of the injectable scaffold strategy utilised decellularised porcine myocardial tissue as an injectable hydrogel which, when injected into rat hearts, led to increased EC and smooth muscle cell migration and arteriole formation *in vivo* [99]. For a review of injectable hydrogel applications in CTE see [100].

Decellularised scaffolds: The presence of the ECM is instrumental in the attachment and orientation of cells, as well as the expansion of tissue, either *in vitro* or *in vivo* [101,102]. The Taylor group decellularised whole rat hearts by means of detergent perfusion and used the decellularised organ as a scaffold onto which cardiac cells were seeded [103]. This process results in an acellular skeleton of the whole organ on which cells can be seeded and left to orientate using the topographical cues of the organ's preserved ECM. Following *in* vitro medium perfusion of the recellularised 'construct', as well as electrical stimulation, a pumping function corresponding to 2% of that observed in the native adult rat heart was observed. Through this approach the ECM constituents of the acellular scaffold are retained while the scaffold preserves the mechanical properties of the native organ. Recent studies have shown that use of decellularised hydrogels leads to an improved maturation of ESC-derived CMs [104]. These studies highlighted the importance of a biomimetic approach to the delivery of cardiac constructs to the host tissue, whereby the structure and composition of the native ECM is recapitulated as faithfully as possible and recently the decellularisation procedure was extended to human hearts [105].

Scaffold-free cell sheets: One of the most exciting novel approaches in CTE has been the use of scaffold-free cell sheets, a process pioneered by the group of Teruo Okano. In order to produce sheets of various cell types, temperature-responsive dishes are grafted with the polymer poly(*N*-isopropylacrylamide) (PIPAAm) [106]. The grafting process is achieved by means of electron beam irradiation and produces PIPAAm-coated dishes that are slightly hydrophobic and cell adhesive at 37°C. Below 32°C the dish surface becomes hydrophilic and non-cell-adhesive. This particular technology allows for cells grown on those PIPAAm-coated dishes to be harvested as sheets along with their ECM and consequently be laid on top of other cell sheets for the creation of contracting multi-sheets [107]. When such CM-consisting cell sheets are placed on top of each other, they form gap junctions, establish electrical coupling and are able to contract *in vitro* [107,108]. Further studies using rat CM-consisting sheets indicated that donor cells survived more than 1 year post-implantation [109] and led to an improvement in cardiac performance and exhibited angiogenesis [110] and lack of arrhythmias [111]. In terms of autologous SM sheets, rat studies observed that there was an improvement in LV contraction and a reduction in fibrosis following autologous implantation of SM sheets into infarcted rat hearts [112], while similar results were achieved following the implantation of SM sheets into a canine heart failure model [113] and porcine MI model [114]. A very recent study found that transplantation of rat SM sheets into infarcted rat hearts led to a significant decrease in ventricular arrhythmias, compared to intramyocardial injection, and confirmed the almost 10-fold difference in survival of donor cells [115]. It is noteworthy that implantation of autologous SM sheets in a patient with dilated cardiomyopathy (DCM) (five four-layered sheets implanted in different sites of the dilated heart) led to an improvement in LV function, with no occurrence of arrhythmias one year after the implantation and allowed for the removal of the LVAD that was being used pre-operatively [116]. In an effort to address the issue of insufficient vascularisation of grafts following transplantation into the host myocardium, the Okano team sequentially grafted triple-layered cardiac sheets onto the myocardium of host rats and demonstrated electrical coupling between the implanted sheets when the surgical interval was one or two days. They then performed serial implantation of ten such cardiac sheets onto an area of host rat hearts overlying a connectable vein and artery of the host myocardium. This process resulted in *in situ* vascularisation of the scaffoldless constructs and allowed for their resection (along with parts of the perfusing vasculature) and further re-implantation [117]. In essence, this study demonstrated the exploitation of the *in vivo* milieu for the proper vascularisation and expansion of the implanted construct, whereby the host microenvironment serves as the 'bioreactor system'. Further studies tested combinations of mixing cell types (CMs and ECs) prior to sheet formation, which led to improved cardiac function and angiogenesis and a reduction in fibrosis, [118] as well as combinations of different types of formed sheets for multistacking of 3D sheet constructs (dermal fibroblast sheets stacked with EC sheets) [119] and showed that specific combinations led to improved neovascularisation in infarcted rat models [119,120]. More recent cardiac cell sheet studies have focused on the selection of an optimal cell source, including CPCs [121], mouse ESCs [122] and hiPSCs [123], with CPC sheets having been tested in an MI model and having led to a functional improvement in cardiac function. Implantation of MSC sheets into infarcted rat hearts showed an improvement in cell retention number, LV function and contractility [124]. Transplantation of hiPSC-CM sheets into a rat MI model led to an improvement in cardiac performance, an increase of neovascularisation and an attenuation in ventricular remodelling [125]. A very recent study by the same group found that when hiPSC-CM sheets were transplanted into pig hearts along with omentum, there was increased vascular density compared to donors where similar sheets were transplanted without the omentum [126]. This was attributed to the rich vasculature of the omentum and highlighted further the importance of perivascularisation (also see next section). A notable variation in the strategy for the production of cardiac cell sheets is by cell aggregation following incubation of ESC-derived CMs (hESC-CMs) in an orbital shaker [127]. This process, which does not use thermo-sensitive surfaces, led to cardiac sheets that showed synchronous contraction *in vitro*. An *in vivo* study using such 'cell aggreggation' sheets found that tri-culture sheets containing hESC-derived CMs, HUVECs and mouse embryonic fibroblasts (MEFs) led to improved vascularisation of the patches and improved engraftment of those patches into rat hearts [128]. For a review of sheet studies using various cell sources and animals models see [129,130].

The use of cardiac sheets bypasses obstacles presented by the use of biodegradeable scaffolds: i) Cardiac sheets can be deposited directly onto the host tissue without the use of any mediating scaffolds, therefore avoiding the replacement of the three-dimensional space occupied by the scaffolds (following deposition onto/into the host tissue) by ECM that can lead to erroneous sparse cell-cell connectivity [131]. ii) By avoiding proteolytic harvest of the cells, cell surface proteins that are critical for the formation of gap junctions and electrical coupling of myocytes, such as connexins, are maintained. The three-dimensional stacking of cardiac sheets facilitates the 3D formation of diffuse gap junctions and at the same time favours the dense cytoarchitecture of cardiac tissue. This is of particular importance in the case of myocardial tissue, wherein cells are dense and compact, as opposed to cell-sparse tissues such as bone and cartilage that can be repaired more efficiently through the use of biodegradable scaffolds [132]. iii) The absence of scaffolds also alleviates the potential problem of a host immunogenic response linked to synthetic polymer scaffolds [132]. iv) Considerations about the scaffold's porous structure with regard to vascularisation are also dispensable due to the direct contact of the cardiac sheets with the host tissue and the superior proangiogenic property of the cell sheets is demonstrated in their pre-clinical results. v) Finally, limited oxygen diffusion is one of the key limiting factors of scaffold-based CTE approaches. The sequential deposition of cell sheets, as exemplified by the *in vivo* polysurgery study [117] limits the hypoxic effects and allows for both more efficient nutrient diffusion as well as vascularisation of the implants. Fig. 1 presents an outline of four of the main CTE approaches.

Vascularisation-specific considerations

Sufficient vascularisation and oxygen diffusion in cardiac constructs of any type is the most important challenge facing CTE research (the other one being the functional electromechanical integration of donor and host tissues). As presented above, different experimental

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setups have addressed oxygen diffusion *in vitro* through the use of bioreactors although more work is needed in order to establish CTE protocols that will facilitate the neovascularisation of the implant and of the nascent, repaired tissue *in vivo*. As already mentioned, oxygen diffusion limits the thickness of standalone engineered constructs to approximately 200μm since CMs lying in non-perfused scaffolds can only survive if the distance from the construct surface is <100μm, which is oxygen's perfusion depth threshold [133] and this diffusion limit cannot be overcome by any of the bioreactor convective system types [71].

In order to specifically address construct vascularisation, the following CTE approaches exist: a) Cell tri-cultures b) addition of proangiogenic factors, and c) fabrication (and microfabrication) of proangiogenic scaffolds. Several of the abovementioned studies attempted to address the issue of oxygen diffusion in cardiac constructs or observed proangiogeneic events. Zimmermann's EHTs showed unexpected formation of primitive capillaries emanating from and forming into the EHTs, indicating *de novo* vascularisation possibly due to paracrine action by the host tissue, as well an innate ability of the constructs to promote angiogenesis [88,89]. Radisic's channelled perfusion bioreactor promoted oxygen diffusion by the addition of oxygen carriers that led to the efficient oxygenation of thick constructs while improving their cellularity and contractile properties [72]. Ott's decellularised heart scaffold preserved its vascular structure network thus allowing for myogenic or endothelial cells to be seeded onto the acellular organ skeleton [103]. And Shimizu's polysurgery strategy succeeded in the *in vivo* vascularisation of the sheet implants by establishing minimal time intervals necessary for the perfusion of each sheet by the host vasculature system [117].

Co-culturing studies, which aim to exploit paracrine effects between allotypic cells, have shown that the defined addition of non-myogenic cells improves the vascularisation of constructs whether the three cell types are grown simultaneously [134,135] or CMs are seeded onto co-cultured fibroblasts and ECs [136,137]. Variations either in the monolayer seeding of ECs and CMs prior to scaffoldless sheet formation or in the stacking of formed EC and CF sheets have produced positive results in the *in vivo* vascularisation in rat MI models [118,119,120].

The addition of proangiogenic growth factors and peptides can be carried out either by direct addition as soluble factors, by microparticle delivery or by immobilisation of the factors onto the scaffold biomaterial. Vascular endothelial growth factor (VEGF) has been delivered in constructs via microparticles either along with hepatocyte growth factor (HGF) and angiopoietin 1 (Ang-1), without the addition of cells [138], or along with monocyte chemotactic protein 1 (MCP-1) during EC implantation [139]. In both cases there was improved vessel formation and vascular stability while increased EC survival was observed in the case of EC incorporation. Furthermore, induction of VEGF in CMs through erythropoietin led to increased proliferation of ECs and recruitment of EC progenitors in the injured rat myocardium [140]. Immobilisation of VEGF with Ang-1 [141,142] or of a VEGF fusion protein with a collagen-binding domain (CBD) [143] onto collagen scaffolds led to increased EC proliferation and neovascularisation in infarcted rat hearts [141,142] as well as a decrease in scar tissue formation [143]. In an experimental protocol echoing the prevascularisation work of the sheet polysurgery study [117], Dvir and colleagues subjected alginate scaffolds to *in vivo* prevascularisation by implantation into the rat omental cavity (a vessel-rich subperitoneal membrane) following incubation with soluble VEGF, stromal cell-derived factor 1 (SDF-1) and insulin-like growth factor 1 (IGF-1) [144]. Following reimplantation into infarcted rat myocardium, the prevascularised constructs integrated mechanically and electrically with the host tissue and led to improved scar thickness, contractility and angiogenesis and decreased chamber dilation [144]. A recent study found that the addition of VEGF in iPSC cultures leads to increased transdifferentiation of iPSCs to induced CMs, compared to non-VEFG assays [145]. Finally, thymosin β4 (Tβ4), a small peptide that has been shown to promote vasculogenesis and angiogenesis [146] was found to induce neovascularisation and stabilise the vascular network of ischaemic rat hearts [147].

With regard to proangiogenic scaffolds, the generation of size-specific pores and channels through micropatterning of scaffolds has resulted in improved capillary formation and angiogenesis *in vitro* and in *in vivo* rat MI models [148,149,150]. Pertaining further to *in vivo* vascularisation, but without the addition of growth factors, others have seeded rat CMs along with Matrigel and an arteriovenous vessel loop into a synthetic chamber that was initially implanted into the groin of animals that led to extensive vascularisation of the construct within weeks [151]. Addition of ADCs into the construct or addition of CMs secondary (day 7) to the presence of the loop led to improved CM survival, indicating that the formation of a microvascular network could be instrumental for the survival of seeded cells [152,153]. For a detailed review of proangiogenic molecules and scaffolds used in CTE see [154].

Micro- and nanofabrication approaches to CTE

The complex and compact ultrastructural organization of the myocardium necessitates an accurate recapitulation of its components from the centimetre to the nanometer level. We have already presented studies that consider the topographical guidance of cardiomyocytes and cardiac fibres with regards to the mechanical functionality and the pre- or post-implantation vascularisation of tissue by patterning of scaffold materials, as well as the electromechanical integration of the engineered constructs with the host tissue through the application of bioreactor systems that aim to induce the formation of gap junctions between cells. We list below some examples of novel approaches that specifically address the requirements of the cardiac microenvironment and in particular cell alignment, cell-ECM interactions and

ECM mechanical properties, and microvascularisation. For a more detailed review of micro- and nanofabrication strategies employed in CTE, see [75,155,156,157].

Topographical cues can influence CM attachment, hypertrophy, ion channel activation, cytokine release, mechanical stress and structural remodeling of CMs [75,158]. Given that the parallel alignment of CMs and cardiac fibres is essential for maximum force contraction and impulse propagation along the fibre axis, efforts have been made to recapitulate this natural alignment in engineered constructs. Nanopatterning of hydrogels [159], 3D-map guided microcontact printing of fibronectin surfaces for CM alignment [160,161], rotary spinning of polymer nanofibres [162] and a combination of micropatterning with electrical stimulation [163] are strategies that have led to improved CM anisotropy and electrical conductance. Cell alignment and nutrient diffusion of CMs was considered in another study where cells were seeded onto 'mesoscopic posts' contained in PDMS matrix, thus controlling the spatial pattern of the scaffold tension[164].

Electrospinning, a method whereby natural or synthetic fibres (or a combination of both) are deposited by application of an electric field that leads to the production of an electrically charged jet that carries the biomaterial solution, has also been used for the production of geometrically-controlled, nanoscale-sized scaffolds. The high surface-to-volume ratio of electrospun fibres/scaffolds can inherently enhance properties such as cell attachment and mass transfer, while loading of macromolecules and pharmacological agents can be incorporated into the electrospun scaffold and, as such, electrospinning has also been used for seeding of living cells, including cardiac cells [165,166]. In a recent study, CMs were cultured on electrospun fibres and deposited in a 'collector' with an insulating gap that had been modified to act as a capacitor that separates charge. The electrostatic forces present in the collector drove the anisotropic alignment of CMs, promoting cell elongation and formation of native-like tissue [167]. A very recent study showed that cells that were electrospun along with modified Matrigel exhibited similar amounts of cell death to non-spun cells and could be traced following subcutaneous injection into mice, thus paving the way for concurrent cell electrospinning of biomaterial and cells, including applications that use cardiac cell population [168]. Bio-electrospraying (BES) is a technique similar to electrospinning where the application of an electrical field leads to the generation of micro- to nano- sized droplets that can be deposited in specified distances from each other on an underlying substrate/scaffold [169]. The application of an electrical field to the cell-bearing

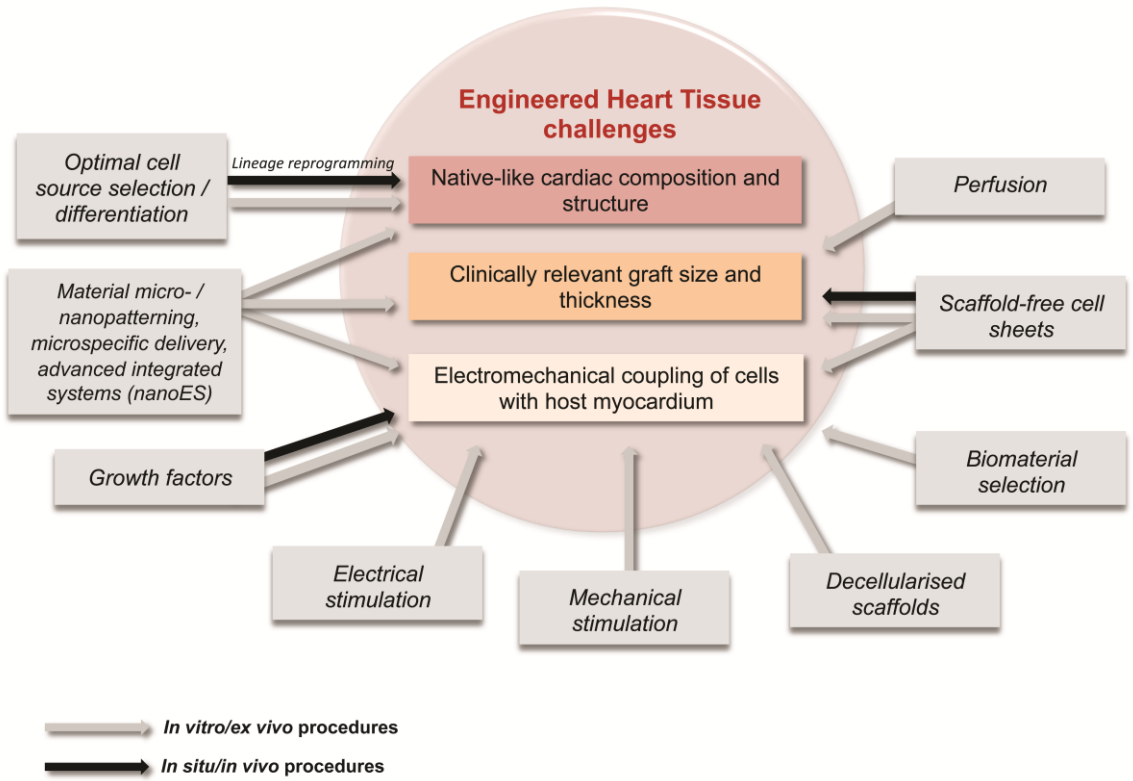

Fig. 2 Integrative approach of different CTE strategies and technologies in addressing the main CTE challenges. The three main challenges in the development/fabrication of engineered heart tissue are the maintenance of a native-like composition and structure (which is primarily addressed by the choice of donor cell sources as well as the development of advanced spatial geometries), the clinically relevant size and thickness of the grafted construct (which is mainly addressed by the perfusion of constructs) and the electromechanical coupling of the construct's cells with the host tissue (which is addressed by various strategies). Grey arrows indicate procedures/events that take place *in vitro/ex vivo* and black arrows indicate procedures/events that take place *in situ/in vivo* (i.e. where the host microenvironment is exploited in order to confer native-tissue like properties to the delivered cells/construct). The above schematic is not absolute but serves to indicate the multidisciplinary and integrative approaches and

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techniques employed for the development of optimal cardiac tissue constructs, as well as the multitude of qualitative 'checkpoints' encountered by researchers in CTE studies. CE: cell electrospinning. BES: bio-electrospraying. nanoES: nanoelectronic hybrid scaffolds.

droplets does not have an adverse effect on the survival or gene expression profile of BES-processed CMs [170,171] and offers the opportunity for defined spatial construction of cardiac tri-cultures on various scaffold or scaffoldless surfaces which can then be implanted into the host myocardium [172], while both CE and BES can be combined with viral-mediated gene replacement therapy [173].

Nanocomposite materials can be used not only as fabrication tools for CTE scaffolds but also as complementing elements that accentuate the electrical conductance and cardiac tissue formation [157]. Gold nanowires incorporated into alginate scaffolds led to thicker and more anisotropically aligned cardiac tissue constructs that contracted spontaneously [174]. Culturing of CMs on a polymer hydrogel scaffold seeded with gold particles led to increased expression of connexin-43 (Cx43) and cardiac markers such as cardiac troponin T (cTnT) [175]. Finally, a very recent study incorporated nanoelectronic arrays into synthetic 3D matrices and the resulting nanoelectronic hybrid scaffolds (nanoES) were used to culture, among others, CMs and smooth muscle cells [176]. Intriguingly, the integrated capability of the nanoelectronic arrays into the nanoES allowed for real-time monitoring of the electrical activity of the nanoES-based CMs, as well as the response of those CMs to drug dosage, creating a nanoscale-specific system for cardiac activity monitoring [176].

Conclusion

During the last few years there has been great progress in the field of CTE and the studies presented in this review highlight examples of the culmination of collaborative research between different scientific disciplines. In the sections above, we have described in detail the starting cell types and the methods of preparation and engraftment and we have mentioned, in some cases, the functional effects of current CTE therapy strategies. However, it is probably fair comment that at this stage of method development and irrespective of donor cell source, scaffold design or precise methodology, retention of engrafted cells is poor and clinically-relevant functional improvement is disappointing. It is widely acknowledged that the translational context of cell sources and scaffold/bioreactor design used in rodent models do not necessarily mirror the clinical situation due to the difference in organ size, variation in cell population ratios and subsequent effect of the latter two in cell dynamics. Although several preclinical studies have shown promising results, no clinical studies have presented a safe and efficient protocol for the restoration of cardiac function. Accordingly, it has not been possible to identify specific cell types or auxiliary elements (scaffold, bioreactor setup, cytokine) which offer a clear clinical advantage over other relevant candidates: The advent of lineage reprogramming offers the possibility of a robust cell source that can be differentiated to the desired lineage without the caveats presented by the use of ESCs and iPSCs, however there is promise in the plasticity and relative safety of iPSC-derived donor cells. The development of increasingly elaborate and effective bioreactor systems that enhance the mechanical and electrical integration of engineered cardiac constructs holds great promise for the near-physiological priming of the constructs. The need of proper vascularisation of constructs, which is the most limiting factor in successful cardiac repair by CTE approaches, has been met to a considerable degree, particularly through prevascularisation protocols and, more recently, by the development of scaffoldless constructs that facilitate superior oxygen and nutrient diffusion as well as *in vivo* vascularisation. The application of defined spatiotemporal patterning and cell delivery through microfabrication and controlled cell/tissue delivery has enhanced the survival and expansion of donor tissue in relevant CTE approaches. Table 1 collectively presents various important studies, in terms of functional observations, that have used either cardiomyoplasty or tissue engineering approaches, along with the functional observations.

In conclusion, various strategies are employed at different stages of the development of engineered heart tissue and, in terms of their aim and design, converge at the main 'checkpoints' that define an optimal construct/implant (Fig. 2). Within 20 years from the establishment of tissue engineering as a novel approach to tissue repair [177], not only has the field of cardiac tissue engineering developed new strategies that ameliorate the outcome of cardiac disease but it has been at the forefront of interdisciplinary research that brings together molecular, cell and chemical biologists, mechanical and electrical engineers, material scientists and clinicians. The general consensus in acknowledging the consideration and manipulation of the donor tissue, scaffold and host micro- and nano-enviroment has led to the development of protocols that have taken cardiac therapeutics closer to its aim, which is the complete regeneration of the diseased heart.

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CRITICAL REVIEW

Referenced study	Donor cell source / *recipient model*	Scaffold (+ bioreactor / stimulation)	Time post-ischemia for cell implantation	Evaluation time (max)	LV functional effects: LV Volume and Dimensions, Effect on infarct wall thickness	Other observations
Jain et al 2001[10]	Rat SMs / *rat*	-	7 days	6weeks	↑	Regeneration ↑ Exercise capacity
Orlic et al 2001 [22]	Mouse BMCs / *mouse*	-	3-5 hrs	9 days	↑	-
Christman et al 2004 [98]	Rat SMs / *rat*	Fibrin glue (injectable)	7 days	6 weeks	↑	↑ Neovascularisation
Zimmermann et al 2006 [89]	Rat CMs / *rat*	Collagen I + Matrigel (+ mechanical loading)	14 days	4 weeks	↑	Neovascularisation of EHTs
Sekine et al 2008 [118]	Rat CMs + EC sheets / *rat*	(cell sheet)	14 days	4 weeks	↑	↑ Angiogenesis
Dvir 2009 [143]	Rat CMs / *rat*	Alginate (+ Matrigel + SDF-1 + IGF-1 + VEFG) + 7d omental prevascularisation	7 days	4 weeks	↑	↑ Angiogenesis
Maureira 2012 [83]	Rat MSCs / *rat*	Collagen (patch)	28 days	4 weeks	↑	↑ Perfusion
Qian et al 2012 [60]	Mouse iCMs (*in vivo* lineage reprogramming) / *mice*	(+/- thymosin β4)	Immediate	12 weeks	↑	(Further cardiac functional improvement with the addition of thymosin β4)
Song et al 2012 [61]	Mouse iCLMs (*in vivo* lineage reprogramming) / *mice*	-	Immediate	12 weeks	↑	Attenuation of fibrosis
Kawamura et al 2012 [125]	hiPSC-CMs sheets / *swine*	(cell sheet)	4 weeks	8 weeks	↑	↑Neovascularisation
Xiong et al 2013 [82]	hiPSC-ECs + hiPSC-SMCs / *swine*	Fibrin (patch)	Immediate	4 weeks	↑	↑ Perfusion and neovascularisation ↑ Bioenergetics Endogenous cardiac progenitor recruitment Attenuation of hypertrophy Attenuation of apoptosis
Narita et al 2013 [115]	Rat SM sheets / *rat*	(cell sheet)	ND	4 weeks	↑	↑ Neovascularisation Attenuation of arrhythmias Attenuation of hypertrophy

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CRITICAL REVIEW

Table 1: Summary of selected CTE studies. Note: All functional parameters listed are those found statistically significant when compared to sham operation or control conditions. ↑symbol indicates an improved clinical trait rather than an absolute increase or decrease. BMCs: bone marrow cells. CMs: cardiomyocytes (neonatal in all cases). ECs: endothelial cells. EHTs: engineered heart tissues. hESC-CMs: human embryonic stem cell-derived endothelial cells. hESC-SMCs: human embryonic stem cell-derived smooth muscle cells. hiPSC-ECs: human induced pluripotent cell-derived endothelial cells. hiPSC-SMCs: human induced pluripotent cell-derived smooth muscle cells. iCMs: induced cardiomyocytes. iCMLs: induced cardiac-like myocytes. IGF-1: insulin-like growth factor 1. LV: left ventricular. MSCs: mesenchymal stem cells. ND: not disclosed. SDF-1: stromal cell-derived factor 1. SM: skeletal myoblasts. VEGF: vascular endothelial growth factor.

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