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REVIEW

ESC Working Group on Cellular Biology of the Heart: position paper for Cardiovascular Research: tissue engineering strategies combined with cell therapies for cardiac repair in ischaemic heart disease and heart failure

Rosalinda Madonna[1,2†], Linda W. Van Laake[3†], Hans Erik Botker[4], Sean M. Davidson[5], Raffaele De Caterina[1,2,6], Felix B. Engel[7], Thomas Eschenhagen[8,9], Francesco Fernandez-Aviles[10,11], Derek J. Hausenloy[12,13,14,15,16,17], Jean-Sebastien Hulot[18,19,20], Sandrine Lecour[21], Jonathan Leor[22], Philippe Menasché[23,24,25], Maurizio Pesce[26], Cinzia Perrino[27], Fabrice Prunier[28], Sophie Van Linthout[29,30,31], Kirsti Ytrehus[32], Wolfram-Hubertus Zimmermann[33,34], Peter Ferdinandy[35,36*†], and Joost P.G. Sluijter[37*†]

[1]Institute of Cardiology and Center of Excellence on Aging, "G. d'Annunzio" University—Chieti, Italy; [2]University of Texas Medical School in Houston, USA; [3]Cardiology and UMC Utrecht Regenerative Medicine Center, University Medical Center Utrecht, The Netherlands; [4]Department of Cardiology, Aarhus University Hospital, Aarhus N, Denmark; [5]The Hatter Cardiovascular Institute, University College London, London, UK; [6]University of Pisa, Pisa University Hospital, Pisa, Italy; [7]Experimental Renal and Cardiovascular Research, Department of Nephropathology, Institute of Pathology, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany; Muscle Research Center Erlangen, MURCE; [8]Institute of Experimental Pharmacology and Toxicology, University Medical Center Hamburg Eppendorf, Germany; [9]DZHK (German Centre for Cardiovascular Research), Partner Site Hamburg/Kiel/Lübeck, Hamburg, Germany; [10]Department of Cardiology, Hospital General Universitario Gregorio Marañón, Instituto de Investigación Sanitaria Gregorio Marañón, Universidad Complutense, Madrid, Spain; [11]CIBERCV, ISCIII, Madrid, Spain; [12]Cardiovascular & Metabolic Disorders Program, Duke-National University of Singapore Medical School, Singapore; [13]National Heart Research Institute Singapore, National Heart Centre, Singapore; [14]Yong Loo Lin School of Medicine, National University Singapore, Singapore; [15]The Hatter Cardiovascular Institute, University College London, London, UK; [16]The National Institute of Health Research University College London Hospitals Biomedical Research Centre, Research & Development, London, UK; [17]Tecnologico de Monterrey, Centro de Biotecnologia-FEMSA, Nuevo Leon, Mexico; [18]Université Paris-Descartes, Sorbonne Paris Cité, Paris, France; [19]Paris Cardiovascular Research Center (PARCC), INSERM UMRS 970, Paris, France; [20]Hôpital Européen Georges Pompidou, AP-HP, Paris, France; [21]Hatter Cardiovascular Research Institute, University of Cape Town, South Africa; [22]Tamman and Neufeld Cardiovascular Research Institutes, Sackler Faculty of Medicine, Tel-Aviv University and Sheba Medical Center, Tel-Hashomer, Israel; [23]Department of Cardiovascular Surgery, Hôpital Européen Georges Pompidou, Paris, France; [24]Université Paris-Descartes, Sorbonne Paris Cité, Paris, France; [25]INSERM UMRS 970, Paris, France; [26]Unità di Ingegneria Tissutale Cardiovascolare, Centro Cardiologico Monzino, IRCCS, Milan, Italy; [27]Department of Advanced Biomedical Sciences, Federico II University, Naples, Italy; [28]Institut Mitovasc, INSERM, CNRS, Université d'Angers, Service de Cardiologie, CHU Angers, Angers, France; [29]Berlin-Brandenburg Center for Regenerative Therapies, Charité, University Medicine Berlin, Campus Virchow Klinikum, Berlin, Germany; [30]Department of Cardiology, Charité, University Medicine Berlin, Campus Virchow Klinikum, Berlin, Germany; [31]DZHK (German Centre for Cardiovascular Research), Partner Site Berlin, Berlin, Germany; [32]Department of Medical Biology, UiT, The Arctic University of Norway, Norway; [33]Institute of Pharmacology and Toxicology, University Medical Center Göttingen, Göttingen, Germany; [34]DZHK (German Centre for Cardiovascular Research), Partner Site Göttingen, Göttingen, Germany; [35]Department of Pharmacology and Pharmacotherapy, Semmelweis University, Nagyvárad tér 4, III-V Floor, H-1089 Budapest, Hungary; [36]Pharmahungary Group, Szeged, Hungary; and [37]Department of Cardiology, Experimental Cardiology Laboratory, Regenerative Medicine Center, University Medical Center Utrecht, Utrecht University, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands

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Abstract

Morbidity and mortality from ischaemic heart disease (IHD) and heart failure (HF) remain significant in Europe and are increasing worldwide. Patients with IHD or HF might benefit from novel therapeutic strategies, such as cell-based therapies. We recently discussed the therapeutic potential of cell-based therapies and provided recommendations on how to improve the therapeutic translation of these novel strategies for effective cardiac regeneration and repair. Despite major advances in optimizing these strategies with respect to cell source and delivery method, the clinical outcome of cell-based therapy remains unsatisfactory. Major obstacles are the low engraftment and survival rate of transplanted cells in the harmful microenvironment of the host tissue, and the paucity or even lack of

* Corresponding authors. Tel: +31 88 7555555, E-mail: j.sluijter@umcutrecht.nl (J.P.G.S.); Tel: +36 1 210 4416, E-mail: peter.ferdinandy@pharmahungary.com (P.F.)

† These authors contributed equally to this work.

endogenous cells with repair capacity. Therefore, new ways of delivering cells and their derivatives are required in order to empower cell-based cardiac repair and regeneration in patients with IHD or HF. Strategies using tissue engineering (TE) combine cells with matrix materials to enhance cell retention or cell delivery in the transplanted area, and have recently received much attention for this purpose. Here, we summarize knowledge on novel approaches emerging from the TE scenario. In particular, we will discuss how combinations of cell/bio-materials (e.g. hydrogels, cell sheets, prefabricated matrices, microspheres, and injectable matrices) combinations might enhance cell retention or cell delivery in the transplantation areas, thereby increase the success rate of cell therapies for IHD and HF. We will not focus on the use of classical engineering approaches, employing fully synthetic materials, because of their unsatisfactory material properties which render them not clinically applicable. The overall aim of this Position Paper from the ESC Working Group Cellular Biology of the Heart is to provide recommendations on how to proceed in research with these novel TE strategies combined with cell-based therapies to boost cardiac repair in the clinical settings of IHD and HF.

Keywords Cardiac tissue engineering • Ischaemic heart disease • Heart failure • Biomaterials • Cells

1. Tissue engineering and cell therapy: are they useful approaches?

Most complex organisms have lost the ability to fully regenerate the heart. However, stimuli to reactivate regenerative processes mammalian cells have been identified.[1] Despite this knowledge, we have learned over the years that reactivation of heart muscle regeneration is much more difficult than suggested by earlier studies.[1] Nature Biotechnology published an editorial in 2017, referring to 'A futile cycle in cell therapy'[2] because of the none-to-marginal benefits of cardiac cell therapy. The discredit on the entire research area has increased following the emergence of unreliable publications, especially in the infancy of the field, by unreliable unscrupulous scientists simply willing to ride a fashionable horse, recapitulating the story of gene therapy 20 years before. All that has prompted individual scientists declared 'the death of this research field'. Yet, it is not a novel observation that after an unreasonable hype and a phase of frustration that the true value of scientific discoveries unveil. And most importantly, in light of the huge clinical need it is in our view fully warranted to further scrutinize the possibility to regenerate the failing heart by remuscularization. Obvious roadblocks (e.g. poor cell retention and lack of proper integration) must be overcome to unfold the true potential of novel regenerative treatments. For this is important to ask: What have we learned so far and what needs to be achieved to obtain better result? In recent years, a consensus has been reached on several aspects that still require attention for the successful implementation of myocardial cell therapy.[3,4] To this aim, different cell types endowed with various regenerative activities have been used, with various outcomes. Currently, we consider first-generation clinical cell therapy candidates, i.e. cell types that can be relatively easy prepared for clinical applications, but exhibit limited regenerative potential, including bone marrow-derived mononuclear cells or mesenchymal stromal cells (MSCs) with a focus on stimulation of endogenous regenerative responses.[5] Second-generation clinical cell therapy candidates need more refined isolation and *ex vivo* amplification procedures, but have a higher regenerative potential, such as several cardiac derived progenitor cells in the form of cardiospheres and pluripotent stem cell-cardiac derivatives, including cardiac progenitor cells and cardiomyocytes and are considered more like an exogenous regenerative approach to replace lost myocardial cells. However, and irrespective of the cell source, a major problem for cell therapy is the low level of retention of infused or injected cell products. Indeed, although encouraging results have been reported, most studies concur that only few of the transplanted cells survive in the hostile environment of the host tissue, such as that occurring after an infarction, and even fewer integrate and are retained in the host myocardium/myocardial scar. Transplanted cells quickly disappear from the injection site because they simply die in the disease struck and thus typically hostile environment or are 'washed out' into the circulation.[6] The poor cell retention in the receiving tissue is primarily related to typically used delivery methods, such as intramyocardial (IM) injection, anterograde intracoronary perfusion, or retrograde delivery via the coronary venous (RV) delivery with short-term engraftment of approximately 10–15% can be detected, regardless of the dose of injected cells,[7] long-term engraftment (>1 month) is reported to be less than 1%,[6] questioning their direct contribution to myocardial remuscularization. Irrespective of the cell type, a significant fraction of cells (~35%) localizes to the lungs after IM delivery apparently due to clearance through venous myocardial drainage.[8] MSCs applied attached to small gelatinous carriers resulted in reduced drainage from the myocardium compared with freely suspended MSC controls.[8] Although such approaches are promising, initial high cell retentions may be lost when cells detach in time in the myocardium, subsequently causing a significant drop in cell numbers.[9] More advanced tissue engineering (TE) approaches have led to long-term cell retentions of more than 80%, and therefore have gained much attention in recent years.[10]

The success of TE in the treatment of other medical conditions[11] should motivate the continuation of work in the cardiovascular field. In this position paper, we therefore discuss how new technologies, such as TE/biomaterials tools, can be used to promote the success rate of cell therapies for ischaemic heart disease (IHD) and heart failure (HF). In this context, some semantic considerations in terms of TE and regenerative medicine must be made to better understand how the two fields intersect and synergize each other.

TE aims at assembling functional constructs that restore, maintain, or improve damaged tissues or entire organs, through the combined use of scaffolds, cells, and biologically active molecules. Regenerative medicine includes TE, but, in addition, also includes research on self-healing—where the body uses endogenous mechanisms, sometimes with the help of foreign biological materials—to recreate cells and rebuild tissues and organs. TE emphasizes the starting materials and scaffolds used to create *de novo* tissue implants, while regenerative medicine encompasses the formation of new tissue induced by tissue-engineered materials. The Committee on the Biological and Biomedical Applications of Stem Cell

Research (https://www.ncbi.nlm.nih.gov/books/NBK223688/) stated that in the new era of TE combined with regenerative medicine, 'regenerative medicine seeks to understand how and why stem cells, whether derived from human embryos or adult tissues, are able to develop into specialized tissues, and seeks to find new ways of applying cells and their derivatives in order to empower cell based repair and regeneration that will restore lost function in damaged organs'. Therefore, for the purpose of this Position paper, we will discuss on TE as a strategy that can help the regenerative process initiated by the cellular component, providing a biomolecular and spatial environment conductive to cell survival, proliferation and vascularization, aimed at supporting cell and tissue growth. We will focus on how biomaterials, in combination with a cellular component (culture systems or cell products) can be used to increase the success rate of cell therapies for IHD and HF by inducing reparative processes.

2. Major properties and requirements of cell types

Cardiac cell-based TE aims to remuscularize post-infarct myocardial scars, provide paracrine support for activation of endogenous repair mechanisms, and add substitute tissue for failing hearts using a three-dimensional approach. Depending on the specific therapeutic goal, the cell requirements vary and different cell types are needed. If the primary objective is remuscularization, a large number of bona fide cardiomyocytes are required, which eventually couple with endogenous cardiomyocytes. The general feasibility of cardiomyocytes engraftment with electromechanical integration was demonstrated earlier for foetal[12] and pluripotent stem cell derived[13] cardiomyocytes as well as engineered heart muscle (EHM)[14] allografts, using either voltage indicator dyes and two-photon imaging or multi-array electrode recordings of the epicardial spread of electrical excitation. Murry *et al.*[15] pioneered the use of genetically encoded calcium indictors to demonstrate electrical coupling of human ES-cell-derived cardiomyocyte grafts in injured guinea-pig and non-human primate.[16] Whilst the original studies in only mildly injured non-human primate demonstrated ventricular arrhythmias in all investigated animals,[16] recently published data suggest a lower risk of ventricular arrhythmias and ectopy rather than re-entry as the underlying mechanism.[17] In case of activation of host-associated repair pathways via paracrine signalling, the flexibility of cell type is greater. TE is here mainly directed at optimizing the early cell retention, which allows the release of paracrine factors, a strategy which might later be replaced by the administration of their secretome only.[18] Independent of the objective, the cells used for TE should be compliant with the 'Guideline on human cell-based medicinal products' from the European Medicines Agency, related to the identity of the cell population, potency, purity, viability, safety, efficacy, and suitability for intended use. For more details see ref.4

2.1 Cells and their requirements for TE-directed remuscularization

With the advent of human pluripotent stem cells (hPSC), including human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC), the availability of large-scale production in bioreactors and differentiation/purification protocols, sufficient quantities of essentially pure human cardiomyocytes can be produced according to GMP requirements and applied in the engineering of heart muscle.[19]

Human EHM rings and patches[19–21] as well as human engineered heart tissue strips[22] from human embryonic (hESC)- and hiPSC-derived cardiomyocytes could be implanted and used to partially repair large muscle defects in rat and guinea-pig hearts. These studies collectively demonstrated long-term cardiomyocyte retention (>200 days), maturation, graft vascularization, and a variable degree of proliferation; further electrical coupling to the host myocardium was observed in some, but not all investigated guinea-pigs,[22] in line with earlier studies demonstrating the isolation of human engineered muscle xenograft grafts by scar formation in athymic rats.[23] Three-dimensional culture and maturation of hiPSC-cardiomyocytes can be stimulated in clinical scalable 3D cultures, displaying robust electromechanical coupling, consistent H-zones, I-bands, and evidence for T-tubules and M-bands, and there is a general agreement that engineered three-dimensional heart muscle cultures achieve a greater resemblance to bona fide human myocardium in comparison to standard 2D monolayer cultures.[21,22,24–33] Several specific interventions have been identified to improve the maturation of hPSC-derived cardiomyocytes including (i) addition of thyroid hormone,[34] (ii) application of electrical stimulation,[33,35,36], (iii) mechanical loading[21,27,37–39], or (iv) co-culture with cardiac-specific cell types including endothelial cells[22] and cardiac fibroblasts[21,31,40–42] or a mixture of fibroblast-like stromal cells.[37,43] A good understanding of the optimal cellular make up as well as reliable quality markers for cardiomyocytes and non-myocyte maturation should be instrumental to further improve the sophisticated architecture and function engineered myocardium.

Current protocols mainly give rise to ventricular-like cardiomyocytes. Going forward, the field would benefit from the development of precise, directed standardized protocols for cardiomyocytes subtype specification and functional maturation. Accordingly, several steps have been taken, including the formation of atrial myocytes specification,[44] epicardial progenitor cells,[45,46] and sinoatrial node cells.[47] Recently, protocols for the engineering of EHM with distinct atrial and ventricular properties have been reported.[48] Non-cardiomyocytes are included in most TE studies—either by their addition or by a contamination of the cardiomyocyte pool. Whether and in particular how they contribute to tissue assembly, maturation, and survival after implantation needs further mechanistic studies. It seems clear that endothelial cells can support angiogenesis[49] and fibroblasts contribute to extracellular matrix (ECM) homeostasis as well as its viscoelastic properties.[21] In addition, there is certainly paracrine cross-talk which may be instrumental in the heart muscle tissue formation process.

Additional open questions include: Are there any circumstances in which non-cardiomyocytes can be damaging to graft or host? Is there an ideal mixture of cardiomyocytes with the multiple stroma cell species found in the healthy heart? Recently Gao *et al.*[50] reported the use of human induced PS derived cardiomyocytes (hiPSC), smooth muscle cells (hiPS-SMC), and endothelial cells (hiPS-EC) that were mixed into a fibrin scaffold for 7 days to create a cardiac muscle patch. The patch was used to cover an infarct area in a pig heart. Beneficial effects, likely mediated by paracrine cross-talk, were observed after 4 weeks.[50] It is clear that the proximity between capillary endothelial cells and cardiomyocytes in the healthy adult heart has a functional role beyond lining of the capillary,[51] which includes pro-angiogenic paracrine signalling.[52]

2.2 Cells and their requirements for TE-directed endogenous regeneration

Cells committed to a cardiac lineage such as right ventricle-derived cardiosphere-derived cells,[53] bone marrow-derived MSC engineered to

express cardiac transcription factors,[54] and ESC-derived cardiac progenitor cells[55] may be used in TE to stimulate endogenous regeneration by the release of paracrine factors. ESC/iPSC-derived cells carry the risk of teratoma formation due to contamination with residual pluripotent cells that have not responded to lineage-specific instructive cues, and therefore have retained their state of uncontrolled proliferative potential. To avoid this contamination, a selection step based on surface antigens is mandatory for obvious safety reasons.[56] From a translational point of view a more realistic approach is to control the differentiation and proliferation processes properly to exclude the presence of teratogenic cells. With regards to the prediction of the efficacy of cells to be used for TE, a cardiopoietic index was recently established.[57] This biomarker-based index relies on canonical cardiac transcription factors employing gene expression profiles as a means to assess the regenerative quotient of patient-derived cells.

2.3 Allogeneic vs. autologous cells

The use of allogeneic cells is preferential over autologous cells since they are not exposed to patient risk factors (i.e. age, diabetes mellitus, and smoking), known to impair the potential of autologous stem cells[58] and allow off-the-shelf use: streamlined logistics, consistency and immediate availability of the product and guaranteed dosage. However, allogeneic cells will always induce an immune response. Alloreactivity depends on foreign peptide presentation by major histocompatibility complex (MHC) on antigen presenting cells and detection by T cells[59] and can be modulated by T cell suppressors, including calcineurin inhibitors (cyclosporine and tacrolimus are commonly used) and corticosteroids. Immunosuppressive treatment is associated with a number of unwanted effects, including hypertension due to kidney damage, transaminitis due to liver damage, an increased risk for infections, and if used long term with malignancies). These are the same risks heart transplant patients are exposed to and have been proven to be manageable. An alternative strategy to avoid the need for long-term immunosuppression is modulation of the innate immune system. A very recent paper from Braza *et al.*[60] demonstrated the feasibility of promoting long-term organ transplant acceptance by inhibiting macrophage activation with nano therapeutics.

Another strategy to prevent allogeneic cell transplantation-related immune rejection is the use of MHC-matched donor cells or to engineer grafts with immune tolerant properties such as recently demonstrated by a functional HLA knock-out via beta-2-microgbulin (B2M) deletion with subsequent insertion of a B2M-HLA-E fusion to overcome T- and NK-cell mediated killing.[61] Although similarly appealing as autologous cells from the immunology point of view, these cells will be more difficult to eradicate if unwanted side effects occur, because they will not be detected as foreign by the endogenous immune system. A potential strategy for eradication of immune tolerant cells is the use of suicide genes, as pioneered by Malcom Brenner and Helen Heslop for treatment of graft vs. host disease following adoptive cell therapy.[62]

Systematic review and meta-analysis of cell therapy in large animal studies concluded effects of autologous and allogeneic cell therapy for IHD were similar, irrespective of immunosuppressive therapy[63] which can be taken as an additional indirect evidence for a similar cell loss with predominantly paracrine mechanisms of action in the reported studies.

In the context of TE, strict immunologic monitoring as well as for example monitoring circulating cell-free allograft DNA[64] will be helpful to if needed adapt immune suppression protocols for enhanced graft survival. It appears likely that a differentiated use and need for immunosuppressive therapy and MHC matching will depend also on the cell type and TE approach used.

For a TE strategy directed at stimulating cardiac endogenous regeneration via paracrine effects, a short-term immunosuppressive treatment would be recommended with the premise that the transplanted cells would be likely short-lived and act via paracrine signalling.[18] This short-term immunosuppressive regime is underscored by the finding that although allogeneic cells are expected to be eliminated more rapidly than autologous cells, their transient presence shortly after transplantation is sufficient to yield equivalent long-term benefits.[65] In the case of TE-directed remuscularization with iPS- or ESC-derived cardiomyocytes, durable engraftment of the cells/graft is required along with long-term state-of-the-art immunosuppressive therapies. MHC matching of iPS-derived cardiomyocytes has been shown to enhance cell engraftment in non-human primates.[66] Translated to patients, a large repository with a variety of MHC-homozygous stem cell lines, collected from donors or engineered, may solve this issue.[67]

3. Methods for engineering myocardial tissue

As indicated, major hurdles to therapeutic applications have been observed, including low cellular survivals, and poor localization to the target area. To further facilitate integration and prolonged activities, several approaches are under development that each have various properties and advantages. These approaches include (i) seeding of cells on preformed scaffolds, (ii) self-assembly of cells in hydrogels, and (iii) cell sheet engineering (reviewed in ref.[68,69]).

3.1 Hydrogels

Hydrogel-based injection and TE are promising techniques in which hydrophilic structures are used, made of either synthetic or natural polymers, which can assemble into a three-dimensional polymeric network.[70] Hydrogels have a high number of essential scaffold requirements, including the exchange of oxygen, nutrients and metabolites due to their porosity and the possibility of including growth factors and other molecules to mediate cross talk between cells.[71] However, in general the hydrogel structures lack structural supportive characteristics and the correct stiffness of cardiac materials.[72]

3.2 Engineered myocardial tissue

Engineered myocardial tissue can be used to aim at the restoration and improvement of cardiac function in terms of tissue regeneration, as indicated above, however, they can also be used to develop three-dimensional *in vitro* models able to mimic the native heart muscle. Construction of engineered heart tissue/muscle (EHT/M) was pioneered and continuously developed by the laboratories of Eschenhagen and Zimmermann[21,73–76] and became widely used recently.[49,77–79] To this end, a range of innovative three-dimensional culture *in vitro* systems for cardiac disease modelling has been developed. In both approaches overlapping characterizations are needed, including the presence of (i) native-like biochemical, electrophysiological, and mechanical cell-ECM and cell–cell interactions, (ii) dynamic *in vivo* like conditions such as fluid flow and shear stress, and (iii) correct cell characteristics and morphologies and structural micro-architectures.

3.3 Extracellular matrix

In addition to the cellular components of myocardial tissue, the ECM is a major player in normal cardiac functioning and homeostasis and cellular behaviour. Ideally, a hydrogel or engineered construct should perfectly mimic native cardiac ECM and provide a physiological microenvironment for cells. The cardiac ECM consists of a complex network of structural and non-structural (matricellular) proteins, of which three-dimensional hydrogel scaffolds have been generated from decellularized cardiac ECM[80] and used as injectable biomaterial for myocardial repair,[81] currently under clinical investigation (NCT02305602).

In addition to the native cardiac ECM, naturally occurring ECM proteins are suitable materials to be used as a hydrogel-based scaffold in cardiac TE due to their bio-mimicking and bioactive properties.[82] Some of the frequently used hydrogels from natural sources include collagen,[21] fibrin,[35] gelatin,[83] hyaluronic acid,[84] and alginate.[10] Matrigel was originally introduced as being supportive in the engineering of rat EHT[76] with also subsequent application in human TE, but will not be applicable in clinical applications.

While the primary advantage of these materials is to maintain transplanted cells alive in the myocardium and retain them *in situ* following transplantation, a potential shortcoming is the possibility that even small modifications of local mechanical compliance could activate fibrosis due to the emerging stiffness-sensitivity of myocardial resident stromal cells.[85] This problem may be overcome by finely controlling the viscoelastic properties of the materials employed to encapsulate cells, or by providing them with sufficiently controlled level of degradation to avoid changes in myocardium compliance. In this respect, employment of 'bio-ink' materials, such as gelatin methacryloyl (GelMA) hydrogels, whose stiffness may be easily adjusted by, e.g. pre-injection photo-polymerization[86] offer a new opportunity for advanced myocardial TE applications.

3.4 Cell sheets

Alternatively, non-cardiomyocytes can be employed for endogenous ECM production, resulting in a replacement of exogenous scaffold material.[87] Similarly, in cell sheet engineering[88] it is well established that cell-secreted ECM plays a key role in sheet assembly and epicardial connection after implantation.[89] Cell sheet engineering makes use of temperature-responsive polymer surfaces to enable the controlled release of cell monolayers; free-floating sheet of cohesive cells that can, for example, be placed onto the epicardium with or without stiches.[89] Using this approach 3–4 monolayers can be fused without a palpable core necrosis. The cell sheet approach can be applied to all cell types, which are capable of forming a biomechanically interconnected monolayer, such as cardiomyocytes for contractile support and non-myocytes for the delivery of secreted factors.[90–92] This technology has been recently tested clinically for the delivery of skeletal myoblasts to the failing human heart[93] and is presently further exploited for the delivery of allogeneic iPSC-derived cardiomyocytes.[94] Issues with this approach are the frailty of these sheets which may cause their folding or tearing during manipulations and the limited number of sheets which can be stacked on each other without cell death.[95]

3.5 Biofabrication

The major concern with classical TE, which entails the seeding of prefabricated scaffolds with cells, is that only inhomogeneous cell densities can be achieved, because of the cells propensity to remain at the scaffolds surface, and thus only weakly contracting cardiac tissues can be fabricated. This caveat may not apply to the delivery of non-cardiomyocytes with a primary mode of action related to their paracrine activity. In hydrogel approaches, cell content is typically more homogeneous, and anisotropic growth is typically guided by mechanical stimuli. A better understanding of how to achieve dense anisotropic muscle structures with capillarization would be important for the development of the next generation of tissue-engineered products. Accordingly, tremendous effort is invested into improved biofabrication technologies combining additive manufacturing techniques with cell printing to create hierarchical tissue-like structures; commonly known as three-dimensional printing.[96,97] In principle, biofabrication allows the production of cardiac tissues layer by layer utilizing multiple print heads and inks (e.g. shear thinning gels) containing distinct cell-types. In this way, various parameters can be controlled such as correct cellular composition, the positioning of various cell-types and materials, vascularization, and the incorporation of bioactive substances.[98] Currently, the field focusses on improving the hardware, to provide more additional gels for printing, and to include materials that can change their shape after printing upon defined stimuli (4D biofabrication) to generate for example vascular-like networks.[99,100] Although promising and potentially very versatile in its applicability, it remains a key challenge to printing tissues at meaningful dimensions for heart repair applications.

4. How biomaterials and myocardial engineering can influence cell therapy in the repair and regeneration of the heart

Two substantially different strategies can be endeavoured in myocardial TE: (i) cell-based methods in which differentiated myocardium tissue patches, or progenitor cells 'niches', can be implanted directly in areas lacking contractile tissue; or (ii) *in situ* strategies, in which composite biomaterials (cells + ECM) may be designed to support the regeneration capacity of endogenous myocardial cells.

4.1 Cell-based methods

For clinical translation, the first discussed strategy requires not only fine-tuning of materials/cells combinations to recapitulate the physiology of the normal tissue environment, but also an enhancement of the propensity for electromechanical and vascular coupling with the recipient myocardial tissue. The latter aspect is crucial to promote stable patch engraftment and coordinated action potential propagation from pre-existing myocardium (reviewed in ref.[80]). A second problem inherent to the generation of myocardial patches concerns the status of cell maturity inside the engineered grafts. Commonly used stem or progenitor cell derivatives have a variable level of proliferation and phenotypic immaturity, which results in the formation of rather immature muscle *in vitro*. Maturation occurs after implantation *in vivo*, rendering the cells indistinguishable from native myocardium in an allograft setting.[13] Whether similar factors would drive human cardiomyocyte maturation in the diseased heart remains an open question. Data on advanced maturation after implantation even in a xenograft setting suggest that similar maturation capacities exist in human PSC-derived cardiomyocytes.[19,69] The underlying process of *in vivo* maturation are however not well understood and further optimization of engineering paradigms may benefit from further mechanistic insight. A possible implementation of engineering systems to overcome these

shortcomings may consist in using biomaterial fabrication criteria supporting coordinated multi-layered vascular and myocardial cell growth,[100] systems favouring electro-mechanical coupling of cells in the patch[101,102] and, finally, materials with defined biophysical characteristics (e.g. stiffness) promoting maturation of myocyte action potential propagation.[103]

An emerging area of interest in myocardial engineering is the modelling of the so-called cardiac niches, which can be defined as individual 'functional units' that include spatially prearranged biochemical/biophysical information. These functional units can instruct the coordinated differentiation of stem/progenitor cells into terminally differentiated cardiomyocytes, while maintaining stable amounts of self-renewing cells in its core.[104] By exploiting the natural stem/progenitor cardiogenic differentiation process, this strategy may lead to a new 'organoid'-based approach to cardiac (re)generation.[105] In case standardization and scalability will be demonstrated, this method could be employed to produce large amounts of allogenic or even personalized tissue 'building blocks', that could be transplanted into the recipient myocardium using appropriate delivery systems. *Trans*-endocardial injection guided by real-time electrophysiology myocardial mapping (e.g. NOGA®) is an example of delivery systems, with the caveat that endocardial deliveries of a bulk material need a careful safety assessment because of the potential of systemic embolization of the material end-products. An alternative without this limitation is the percutaneous delivery of tissue-engineered products with a shape-memory onto the epicardium.[106]

4.2 *In situ* strategies

A systematic investigation of the conditions promoting stem/progenitor cells cardiogenic differentiation could either lead to improved existing regenerative approaches by 'combining' materials/cells, or even cell-free *in situ* strategies. A first possibility is offered by embedding therapeutic cells into unstructured biomaterials (e.g. hydrogels) and combining them with survival/cardiogenic factors which may preserve their vitality, proliferation and/or enhance differentiation capacity in the potentially hostile environment.[107] In a second formulation, the injection of 'smart' materials that instruct *in situ* cellular specifications has been demonstrated, at least at a proof of concept level.[108] In this formulation, the therapeutic approach could consist of hydrogels containing instructing signals for cardiac cell reprogramming (e.g. slowly releasing miRNAs[109]), or stem cell-derived secretome survival factors.[110] In the case where the combination of materials/bioactive factors includes formulations with stiffness characteristics consistent with myocardium elasticity,[111] materials making them electrically conductive[102,112] or topologically arranged substrates,[113] there will be valuable options to operate cell-free cardiac regeneration that could lead to the delivery of clinically effective therapies. In fact, these applications would not only boost the inefficient myocardium regeneration process,[114] but would also permit a coordinated and spatially-organized regeneration of myocardial contractile tissue in areas where cardiomyocyte death and myofibroblast-dependent fibrosis typically limit the replacement with new contractile cells. In this regard, the inclusion of materials (e.g. silk-derived proteins[115]) or drugs (e.g. anti-inflammatory molecules[116]) capable of suppressing pathways mediating the early inflammatory response leading to myofibroblast activation in response to myocardium damage, or of stimulating cardiomyocyte cell-cycle re-entry in the myocardium,[116,117] could be effective enhancement strategies limiting the extent of post-ischaemic damage and, at the same time, promoting cardiac regeneration.

4.3 How to prevent inflammatory/immune reactions in the host environment

One of the most important challenges in the field of cardiac TE is to avoid adverse foreign-body host immune response to implanted scaffolds. Novel biocompatible, immunomodulatory biomaterials can reduce the foreign-body response and improve engraftment.[118] Furthermore, because inflammation is a critical component influencing cardiac regeneration,[118,119] immunomodulation by smart biomaterials is a potential strategy to overcome this major challenge in cardiovascular regenerative medicine. The modification of the physicochemical properties of biomaterials can modify the host inflammatory response, which can improve biomaterial integration and their interaction with immune cells including the reparative cells such as macrophages and MSC, as well as the controlled delivery of anti-inflammatory small molecules and cytokines.[118]

5. Cell-free (secretome) approaches

5.1 Acellular biomaterials

These cell-free materials include the use of material itself for directing cardiac responses or as delivery vehicles for biologics. The material is typically natural (e.g.: alginate, collagen, hyaluronic acid, chitosan, decellularized ECM), or less commonly, a synthetic polymer (reviewed in ref.120). The proposed mechanisms of benefit include reduction of wall stress by mechanical support, and modulation of host cellular responses including inflammation, neovascularization, fibroblast activity, stem cell recruitment, and protection against cell death. The potential advantages of this approach are that it is cost-effective, localized (vs. systemic), and avoids the need to consider cell survival in the injected biomaterial. The method of delivery is an important factor, with injectable methods that have the advantage of a minimally invasive delivery route, though the need for injection confers several design limitations on the gels.[120] To date, results of the few clinical trials of acellular biomaterials have been mixed.[121–123] Another promising approach is the injection of VentriGel™ (Ventrix), an injectable hydrogel derived from porcine myocardial ECM, which showed benefits in pigs[81] and is currently in a Phase I clinical trial in patients with HF post-myocardial infarction (MI) (NCT02305602).

Biomaterials can be used to deliver biologics such as growth or survival factors. This places further limitations on the biomaterial, necessitating carefully controlled rates of degradation and release. In a successful example, an epicardial collagen patch was used to deliver recombinant follistatin-like 1 (Fstl1), stimulating cardiomyocyte proliferation, and improving cardiac function and survival in mouse and swine models of myocardial infarction.[124] To improve the localization and retention in the heart once the factors are released, the biomaterial can be modified to bind the factors. This approach has seen some success in small and large animals,[120] but clinical translation may be limited by the expense of synthesizing and incorporating peptide growth factors.

5.2 Exosomes and microvesicles with biomaterials

The secretome of stem cells contains a rich cocktail of growth factors and other pro-regenerative molecules including extracellular vesicles (EVs) such as exosomes and microvesicles.[125] In fact, the secretome may mediate much of the benefit in cardiac function that has been observed after the injection of stem cells into the heart, via mechanisms that include paracrine stimulation of pro-angiogenesis pathways, alterations of

macrophage phenotype and anti-fibrotic effects.[3,126] In a recent head-to-head comparison, EVs from human cardiovascular progenitors outperformed the cells themselves at improving cardiac function in mice with HF post-MI.[127] Although some long-lasting benefits have been observed after a single injection of EVs, it may be presumed that controlled release of the secretome from an injected biomaterial would allow for maximum and/or prolonged benefit.[128] Although there are few studies using this approach to date, its feasibility has been demonstrated by the use of a hydrogel based on polyethylene glycol (PEG), end-modified with ureidopyrimidinone moieties to deliver growth factors in the soluble phase with no exosome delivery to pig myocardium.[129,130] A gelatin and laponite nanocomposite hydrogel has been used to deliver the complete secretome of human adipose-derived stem cells (hASC) to the peri-infarct myocardium of rats, and was found to reduce relative infarct size after 21 days recovery, and significantly improve EF.[110] Finally, in a small feasibility study in patients, hESC-derived cardiovascular progenitors were encapsulated in a fibrin patch which was then sewn to the epicardial surface of the hearts of six patients with HF. Although this was a safety study, an improvement in cardiac regional systolic contractile function was seen, which was most likely mediated via secreted paracrine factors.[55]

6. Modes of cell applications and retention with biomaterial carriers

6.1 *In vitro* and *in vivo* engineering and the three routes of cell delivery

The ideal strategy to deliver cells in biomaterial carriers depends on multiple factors including features of the diseased heart (type of cardiomyopathy, location of scarring), the clinical setting [e.g. whether or not it is combined with coronary artery bypass grafting (CABG)], biomaterial characteristics, and to a lesser extent cell characteristics. The actual TE process can take place *in vitro* where the cells are seeded or grown into a pre-formed structure, or *in vivo* after injection of cells in conjunction with a biomaterial. The stimulation of endogenous repair by transplanted cells, or the direct reprogramming of other cell types into cardiomyocytes, is sometimes also referred to as *in vivo* TE.

IM injection can be performed via the endocardial or epicardial route. The epicardial route is the only one that allows direct visualization, and therefore, usually used in preclinical (small animal) research or in combination with open chest surgery, most often CABG for ischaemic cardiomyopathy. The endocardial application, via a percutaneous approach, is less invasive and allows for precisely targeted therapy if a special electromechanical mapping system (e.g. NOGA) is used, although these mappings have their limitations in their anatomical visualizations. The transvenous approach represents a combination of the two techniques, as cells are injected through a coronary vein puncture[131] under IntraVascular UltraSound guidance. IM injection thus has the advantage of delivery to a specific location but the risk of rapid wash-out of cells via venous drainage is high[8] and should be overcome with suitable biomaterials.

The epicardial application can also be in the form of an engineered tissue patch that is sutured on top of the heart. This ensures an excellent cell retention mechanically, but electromechanical integration via this route may be more challenging and the technique currently depends on open chest surgery. Future technical developments should allow for less invasive epicardial (and potentially endocardial) application, parallel with developments in minimal invasive valve surgery. Intracoronary infusion is minimally invasive and easy to perform with standard catheterization lab equipment, but caution should be taken not to induce embolization, especially when thicker biomaterials are used. In addition, the coronary arteries may be inaccessible due to the nature of the disease. The interstitial retrograde coronary venous infusion may then serve as an alternative. It is performed by placing a balloon-catheter in the coronary sinus or one of the coronary veins and occluding the distal side temporarily to allow the cells to disseminate into the heart.[132] In each case, the cells need to be able to migrate across the microvascular endothelium to reach their planned site of action. Finally, because repeated dosing may be required to achieve a maximal therapeutic benefit,[133] less invasive approaches likely need to be considered and in this setting it might be worth further investigating the intravenous route.

6.2 Injectable hydrogels

Non-gelated, switchable hydrogels can be administered using a syringe and incorporated into any catheter-based application. Cells, and additives such as growth factors, are suspended in or co-injected with the liquid material provided it features visco-elastic properties allowing it to take a liquid form as the shear stress increases. The solution should be designed to polymerize/crosslink quickly after arrival *in vivo* in the heart, usually due to pH or temperature change, and remain stable afterwards to prevent embolization. Degradation should occur without toxic byproducts.[131] This has been reported to enhance cell retention significantly and possibly improve differentiation and functional effects in preclinical models.[107]

6.3 Porous scaffolds

Therapeutic cells can be grown into a three-dimensional construct on a porous or fibrous scaffold, which usually needs additional treatment to maximize cell attachment.[69] In cell sheets, usually cardiomyocytes or progenitor cells are combined with support cells which enhance tissue organization and robustness.[134–136] Until now, patches have been placed on the epicardial side of the myocardium via open chest surgery but the development of less invasive techniques may change this requirement in the future.

6.4 Microcapsules

Sized in the order of hundreds of microns or less, microcapsules consist of cells encapsulated with nanoporous materials.[137] The capsules protect the cells from recognition by the immune system and may enhance the long-term paracrine action of the transplanted cells. Although intracoronary infusion of encapsulated glucagon-like peptide-1-eluting mesenchymal stem cells preserved left ventricular function in a porcine model of acute MI, the suggestion of coronary occlusion halted these preclinical studies. For cardiac applications, biodegradable hydrogels such as gelatin seem most appropriate.[9] So far, application has been via IM injection due to the size of the cell-laden capsules and the necessity to pass the endothelial barrier in intravascular routes. On top of microcapsules or carriers, also (mixed) aggregates[138] of iPSC-cardiomyocytes in capsules is a potential route to improve cellular retention and thereby increase myocardial function.[139]

7. Towards clinical applications

The clinical use of tissue-engineered constructs in myocardial regeneration is still at an early phase. Although the regulatory framework needed

to achieve authorization of a tissue engineering product is similar to that of cell-based therapy, the combination of cells and materials in an advanced therapy medicinal products will require more stringent quality criteria, and therefore, more advanced preclinical testing before it can be approved. This includes a careful assessment of potential benefits and obvious risks in a well-defined patient population and ultimately also economic considerations.[136,140]

7.1 Factors influencing results/limitations

In fact, although effective carrier materials and/or engineering approaches may enhance cell or tissue retention, the problems with sources of autologous cell and survival in the host tissue remains problematic, similarly to any cell-based therapies. The quality and number of cells may diminish in patients who are older or have comorbidities (together with specific medications for the comorbidity) or genetic defects. Moreover, cell/tissue survival is also effected by the host tissue environment with comorbidities, aging, gender, etc. (for review, see ref.[3]). Unfortunately, little is known about the effect of major comorbidities and risk factors on quality of cell source and cell survival in the host tissue.[141,142]

Other limitations are the logistics and high costs of such advanced therapy medicinal products as well as the failure of many biomaterials to meet translationally relevant requirements. The first in man phase one trials using products of TE will require dedicated GMP-production and clinical infrastructure for the implantation procedure and follow-up. GMP-production according to the relevant regulatory demands would for preclinical trials ideally be at one site to ensure quality and comparability of a TEP. This will be particularly important in case of multicentre studies. This requires qualified protocols and carriers for TEP delivery to the point-of-care. In this context, recent data on transatlantic shipping of EHM without an apparent loss in potency and quality is encouraging.[19] Early clinical trials would also in light of the high costs associated with the set-up of GMP-production clearly be facilitated by single production units. If later phase clinical testing and market authorization is considered based on compelling clinical data, production logistics will have to be reconsidered. A general concern is related to the high cost of advanced therapy medicinal products in general, leading to their withdrawal from the market despite approval by the European Medical Agency (EMA) and no safety or efficacy concerns.[143] Thus, setting up cost effective production processes will be at least as important as compelling data from clinical trials.

7.2 Potential risks: arrhythmias, tumour development, rejection, calcification

Since myocardial tissue is subject to the coordinated propagation of electromechanical stimuli, the development of arrhythmias is a key risk for all cell therapeutics and especially so for conductive myocyte-containing therapeutics. This caveat has been clearly recognized as clinically relevant in the first clinical trials on skeletal muscle cells for heart remuscularization.[144] A number of important preclinical large animal studies have further underscored this risk in case of the application of cardiomyocytes.[16,145,146] Conversely, a recent study on fibrin-patch mediated delivery of ESC-derived cardiac progenitors did not provide evidence for arrhythmia,[18] which is in line with the primarily secretome associated mechanism of action but also suggests that avoidance of IM punctures may mitigate the risk of uneven integration of the grafted cells and the attendant predisposition of inhomogeneous coupling at the graft-host interface to trigger arrhythmias.

Potential risks that could constitute a serious obstacle to the clinical translation of tissue-engineered myocardium could arise from uncontrolled proliferation and unwanted differentiation of cells in the constructs that may lead to, e.g., tumour formation in case of iPSC/ESC,[147,148] or to calcification of the host myocardium by MSC.[148] These issues remain essentially the same as those of classical cell-based cardiomyoplasty approaches, with the caveat that an increased efficiency of cell survival and engraftment, resulting from embedding cells into hydrogels or structured tissue patches, could enhance the risks of side effects. Another relevant issue that may need to be solved is the problem of immune-rejection against cells/materials combinations. In this regard, the materials causing no inflammatory or foreign body reactions would be ideal (reviewed in ref.[149]). This class of materials includes, (i) naturally derived polymers with an innate anti-inflammatory activity (e.g. silk), (ii) ECM matrix components deriving from decellularization or lyophilization procedures, or finally, (iii) materials with controlled release of anti-inflammatory/immune-suppressive molecules. A final strategy, which can be used for cardiac TE with allogenic cells could derive from the exploitation of cell combinations that maximize the suppression of immune responses *in vivo*.[150]

7.3 Safety, regulatory and ethical frameworks related to the use of tissue engineering products in myocardial repair

According to the EMA classification (EC No. 1394/2007), tissue-engineered constructs and combinations of cells/biomaterials fall in the definition of advanced therapy medicinal product. A conceptually similar definition (Human Cells, Tissues, or Cellular and Tissue-Based Products HCT/Ps) is adopted by the Food and Drug Administration in United States.[151] In either cases, the adoption of stringent quality criteria complying with the Good Manufacturing Practice (GMP) production under a manufacturing authorization by the local competent authority is the basis to ensure standardization, safety, traceability, and potency of the final product. Recognizing the diverse nature of advanced therapy medicinal products, including the tissue-engineered products (TEP), EU guidelines (e.g. Guidelines on Safety and Efficacy Follow-up: Risk Management of Advanced Therapy Medicinal Products—EMEA/149995/2008) provide guidance as to how to (i) ensure quality of the production process, (ii) evaluate potential risks, and (iii) demonstrate potency and efficacy of the final product with *in vitro/in vivo* tests. Issues that are particularly recognized include: (i) '*transmission of infectious agents to the patient and to close contacts*', (ii) '*graft dysfunction and/or rejection*', (iii) '*induction of autoimmunity or immunogenic reactions*', (iv) '*induction of malignancies*', and (v) '*impossibility of discontinuing or removal of the product*'. In case of cell/materials combinations, the same guideline additionally recommends specific testing of biodegradation and mechanical factors, thus asking for an evaluation of long-term patient/graft interactions possibly affecting the graft performance at long term. These latter recommendations may be particularly important for setting myocardial therapy using, for example, cellularized patches. These patches should, alternatively, be produced with fully bioabsorbable materials, able to release therapeutic cells without eliciting inflammatory responses secondary to material degradation, or be designed to resist to biodegradation, thereby prompting a full functional integration in the host's myocardium. Finally, each TEP has to be evaluated by the responsible regulatory authority to specify the required manufacturing and preclinical strategy before embarking on first-in-patient trial.

At the time of the preparation of our current paper, the available EMA 'Scientific Recommendations' lists (www.ema.europa.eu/ema/in

Table 1 Ongoing human clinical trials for cardiac tissue engineering

Clinical trial name	Material/cells/tissue	Trial identifier www.clinicaltrials.gov	Phase (enrolled patients)—(publication)
Epicardial infarct repair using CorMatrix®-ECM: clinical feasibility study (EIR)	CorMatrix-extracellular matrix	NCT02887768	Phase 1[119]
Transplantation of human embryonic stem cell-derived progenitors in severe heart failure (ESCORT)	Human ESC-derived progenitors embedded into a fibrin patch	NCT02057900	Phase 1[57,116]
A study of VentriGel in post-MI patients	VentriGel extracellular matrix hydrogel	NCT02305602	Phase 1 (recruiting)
Myocardial assistance by grafting a new bioartificial upgraded myocardium (MAGNUM trial)	Collagen matrix seeded with bone marrow cells	NCT01429415	Completed[140]

dex.jsp?curl=pages/regulation/general/general_content_000301.jsp&mid=WC0b01ac05800862c0) the following advanced therapy medicinal products for myocardial regeneration, such as e.g. autologous/allogenic hematopoietic stem cells ($CD34^+/CD133^+$), adult bone marrow/adipose tissue-, or placental (Wharton Jelly)-derived mesenchymal stem cells, none of which is based on material/cells combinations.

7.4 Ongoing clinical trials

We are aware of six clinical trials (according to www.clinicaltrials.gov), which have been organized up to date with a TE approach in myocardial repair. In *Table 1*, we report the five clinical trials according to www.clinicaltrials.gov. In an actively recruiting trial, a CorMatrix™ ECM sheet is applied on the epicardium of patients undergoing bypass implantation after an acute ischaemic event. This study was corroborated by a preclinical investigation performed in pigs supporting the cardioprotective and *pro*-vasculogenic effect of the matrix. In another recently completed trial, a combination of allogeneic embryonic stem cell-derived cardiac progenitor cells and fibrin was employed to create an engineered tissue patch that was sutured to the necrotic portion of the ventricle cover by a pericardial flap. Results of this study in six patients were recently published,[18] and showed a promising outcome as to safety (the primary endpoint of the trial) and hints supporting the concept of functional recovery of the heart. In a different approach, therapeutic material (a myocardium specific ECM matrix) is delivered inside the myocardium using a minimally invasive procedure. In this case, the choice of the regions of the myocardium to be treated is guided by the mapping of myocardial viability, available by electric voltage mapping (e.g. NOGA system). The clinical trial approved in Japan and previously mentioned (see Section 4.3) is registered in the Japanese clinical trial registry. It will for the first time test human iPSC-derived cardiomyocytes implanted epicardially as cell sheets in patients with HF. As such it is a follow-up study to the completed skeletal myoblast cell sheet trial by the same group of investigators.[93] The propensity for arrhythmia induction and tumour formation as well as immune responses to the allograft will have to be monitored carefully. Following the fast track developmental strategy of the Japanese government, the study is planned to be performed in three patients under a conditional approval of the cardiomyocyte cell sheet for application in HF repair.[94]

8. Recommendations

- The application of non-myocytes appears to be safe in patients with IHD and HF, but so far largely ineffective. Low cell retention at the site of injury and false expectations as to the outcome of the mostly small clinical trials, which were naturally designed to test for safety and feasibility, but often misinterpreted as efficacy trials, contributed to the perception of futile outcome. These underpowered trials were successful in all cases, but not designed with sufficient power to test efficacy. Sufficiently long retention of the biologically active constituent, i.e. either cardiomyocytes or cells with paracrine activity, must be ensured to render tissue-engineered products effective in the remuscularization/regeneration of the failing heart;
- TE strategies should be further explored and optimized as they offer means to significantly enhance cell retention at the site of injury.
- Advanced preclinical development including manufacturing of tissue-engineered products should be in close interaction with the relevant regulatory authorities to be in-line with regulatory demands. In addition to an agreement on the GMP-manufacturing process this also includes a detailed discussion of typically rodent and large animal experiments.
- Further refinement of TE strategies, including the testing of three-dimensional printing, are attractive to optimize the biological activity of tissue-engineered products, i.e. either contractile performance or secretome activity or both, to continuously develop the next generation tissue-engineered products as well as strategies for their optimal administration.

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