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Recent Advances in Image-Based Stem-Cell Labeling and Tracking, and Scaffold-Based Organ Development in Cardiovascular Disease

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Abstract : Myocardial infarction (MI) and heart failure (HF) are leading causes of mortality and morbidity in the Western World. Therapeutic approaches using interventional cardiology and bioengineering techniques have thus far focused on either salvaging viable tissue post-infarction or preserving cardiac function in the failing myocardium. Regenerative medicine on the other hand, attempts to renew damaged tissue and enhance cardiac functional performance. Tremendous advances have been made in this field since the introduction and ethical approval for use of stem-cells (SC) and relevant technologies in pre-clinical and clinical practice. While study outcomes are still ambivalent on the potential translational impact of SCs, renewed hope has arisen since the introduction of induced pluripotent stem-cells (iPS) and the prospect of intact organ development and transplantation. The aim of this work is to review recent discoveries and the patent landscape employing stem-cell engineering, labeling and image-based monitoring strategies, their use in bioreactors and constructions of enriched bio-artificial membranes, as well as the potential role in artificial organ development and transplantation, with relevance to anticipated impact in pre-clinical screening and widespread clinical use.

Keywords: 3D printing, myocardial infarction, organ development, scaffolds, scar regeneration, stem cell, translational research.

INTRODUCTION

Human SC Technologies: State-of-the-Art

Cardiovascular disease [CVD] (coronary heart disease, hypertension, and congestive heart failure) is still the primary cause of mortality and morbidity in the Western World. Since the early 1900's, it has constituted the primary etiology of morbidity, with more than 4.3 million deaths being reported in Europe every year. In addition to cardiac pathology and its complex time-dependent evolution (Fig. **1**), the inherent inability of native cardiomyocytes to regenerate leads to a progressive cumulative cardiac degeneration, which is exacerbated with increasing age, eventually causing heart failure (HF). Interventional, surgical, or pharmacological treatment of CVD in Europe exceeds €192 billion in yearly costs.

Over the past 16 years, since the discovery of embryonic stem cells (SC) by Gearhart's team [2] and Thomson *et al.* [3], implantation of SC's has provided a methodological pathway that promises tissue regeneration, aiming to improve global and local cardiac function following ischemic injury [4] (Fig. **2**). In 2001, Orlic *et al.* [5] suggested the *de novo* regeneration of the infarcted myocardium in animals using locally derived bone-marrow stem cells. Spurred upon Orlic's findings, similar applications emerged employing cardiac progenitor cells (CPCs) [6, 7]. Reinforcement of such work on both such cell types was the subsequent migration of their use to clinical trials.

Evidence of the limited cardiomyocyte regeneration [8, 9] has stimulated a plethora of approaches to repair myocardial injuries by injecting myogenic cells into the scarred myocardium [5, 10-13], or replacing scar tissue with engineered grafts [14, 15] composed of collagen gels [16] and fibers [17]. More recently, the de-novo construction of intact organs using de-cellularization-re-cellularization techniques [18], and the introduction of three-dimensional (3D) SC printing technologies [19], has generated increased excitement within the scientific community as alternative pathways for transplantation and CVD therapy.

While the feasibility of SC technologies has been proven, efficacy is still in question (Fig. **3**), with contradictory results documented in humans [20]. Several studies demonstrate improvements of cardiac function post-injection of SCs in the coronary circulation [21], or directly into the myocardium [22], however, the multiple types of SCs used, the numerous methodologies and protocols employed for SC extractions, culturing, purification, implantation, and long-term fate, resulted in a wide variety of outcomes in other studies, both in humans and animals. Nevertheless, renewed hope has arisen since the introduction of induced pluripotent

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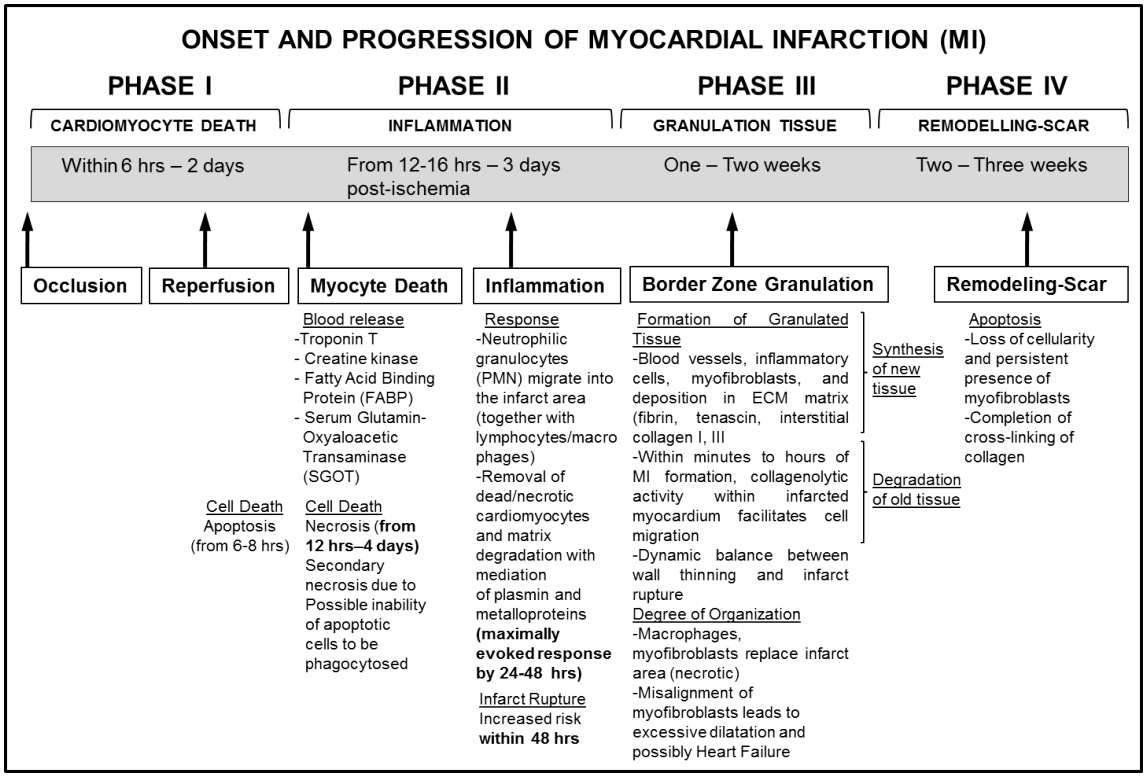

Fig. (1). Major myocardial tissue responses (Phases I-IV) during and following the onset and progression of MI. Proposed objectives argue in favor of secondary interventions (such as additional, direct, peri-infarct injections of SCs and transforming growth factor (TGF) up/down-regulation) during Phase III (granulation tissue phase) of the evolution of MI and associated remodeling changes. The diagram is based on the work of W. M. Blankesteijn *et al.* [1].

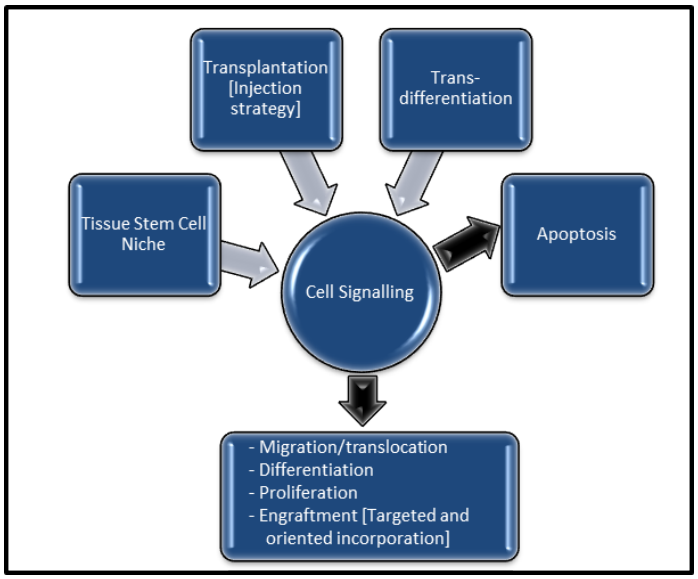

Fig. (2). Diagrammatical representation of cellular transplantation, innate stem-cell niche recruitment, and cellular fate in regenerative therapy.

stem-cells (iPS) [23] and intact organ development and possible transplantation [18].

The basic approach of SC therapy involves the direct transplantation of cells (Fig. **2**), followed by translocation, migration, differentiation and proliferation, ultimately attaining homing and engraftment. Furthermore, stimulation of the endogenous pool of adult SCs (from the SC niche) may result in similar patterns of trans-differentiation, apoptosis and necrosis, as a result of intra- and inter-cellular cell signaling cascades. In addition to improvements in global and regional cardiac function – one of the main aims of the SC therapy – long-term remodeling can be assessed using markers including those for apoptosis, fibrosis, collagen deposition and cross-linkers, and scar formation (Fig. **3**) [10].

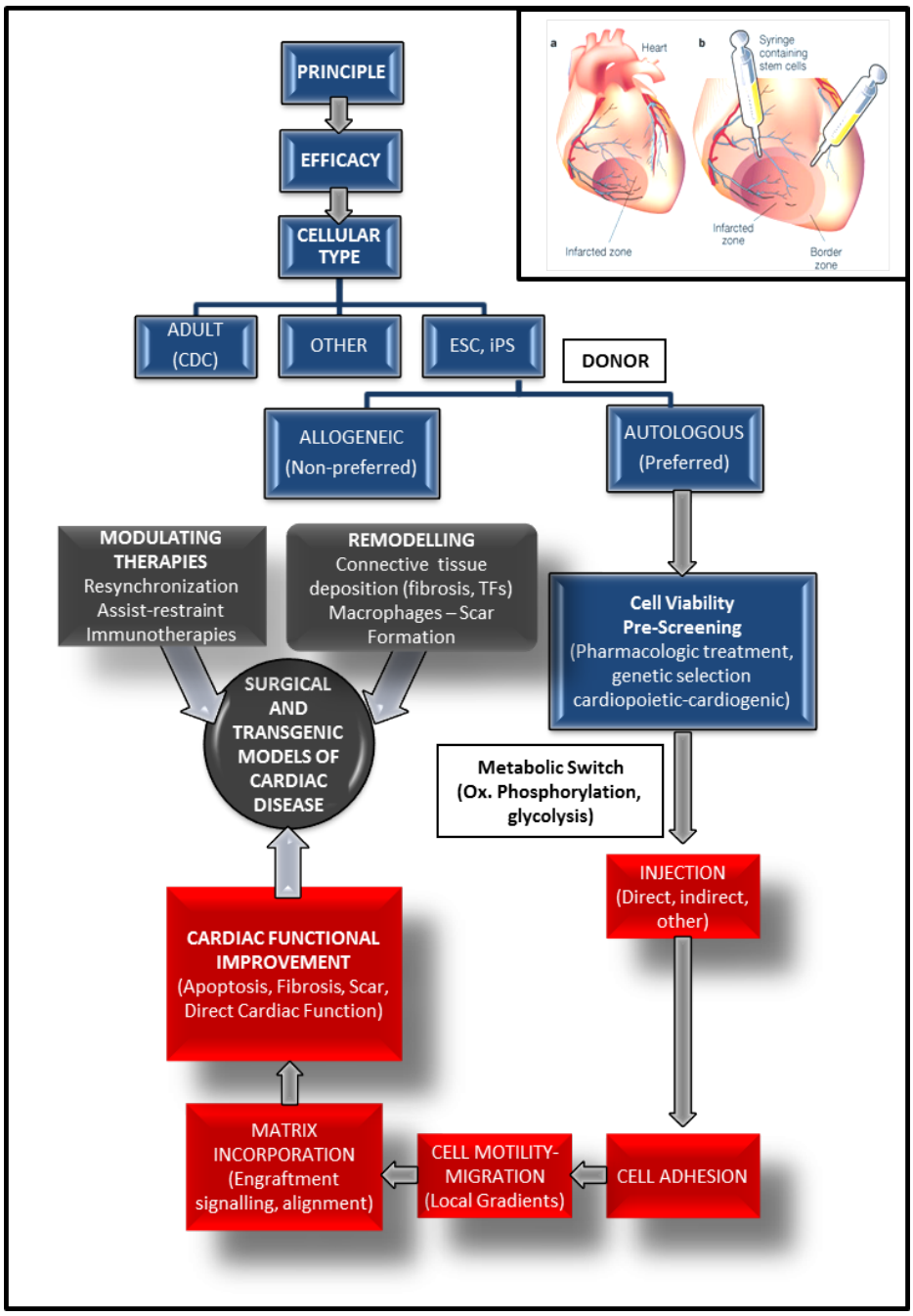

Fig. (3). Summary of state-of-the-art stem cell technology and methodologies for SC therapy. Shown on the top right are cartoon representations of the (**a**) infarcted zone location (**b**) and the peri-infarct (border zone) stem cell injection methodology.

Preclinical Advances

Myocardial tissue characterization pre- and post-implantation therapy has thus become an active area in clinical and basic science research. Endogenous cardiac SCs, which can be isolated and expanded *in vitro* have been shown to improve cardiac function in rodent models [6, 10, 24]. The mechanisms of action of administered SCs have been informed by detailed studies of cardiogenesis during development, including *ex vivo* and *in vivo* studies of cellular motility, migration [25], cellular signaling [26], differentiation, metabolism [26], engraftment [27], and matrix-alignment and tethering [28, 29].

Cellular Labeling and Tracking

The need to visualize the fate of implanted cells to define optimal strategies for SC therapy has become a subject of continuing research [30, 31]. Relevant studies focused on imaging the tissue distribution of administered cells, after pre-labeling with a fluorescent probe, transduced expression of a fluorescent protein [32] or labelling with iron oxide particles (MPIOs) [30]. MPIO-labeled cells allowed for non-invasive tracking and identification of the hypo-intense cells with MRI, at cellular spatial scales, facilitating studies of their migratory patterns *in vivo*. More recent findings at the cellular signaling level, point to the importance of the tissue

matrix stiffness as a contributory factor to SC migration patterns, and to the active and passive matrix properties, promoting cellular differentiation and proliferation [33].

This article provides a review of the historical evolution of SCs and the current European and American patent landscape, extending focus on recent advances and discoveries in image-based SC labeling and tracking in CVD, enriched bio-artificial membranes and direct (decellularization-recellularization), or indirect (3D printing), intact organ development.

STEM-CELLS IN CARDIOVASCULAR REGENERATIVE MEDICINE: HISTORICAL EVOLUTION, CELL TYPES, AND PATENT LANDSCAPE

Historical Overview

The first SC isolation reports date back to 1981 [34, 35]. However, the first breakthrough in successful identification and derivation of embryonic SCs (ESCs) is attributed to John Gearhart's team at Johns Hopkins on primordial germ cells and blastocysts taken from human fetal tissue (early stage embryo) [2]. Almost concurrently, Thomson's publication emerged from the University of Wisconsin [3] on cells extracted from human embryos created *in vitro*. Although Gearhart's research was never based on United States (US) federal funding, Thomson's work was banned (under the Dickey-Wicker Congressional amendment and a subsequent executive order) from being eligible for federal funding. Based on the ban's terms, ESC research was permitted only for available lines on existing cell cultures; extraction of cells from discarded embryos was prohibited. Despite the lifting of the ban by the successor to the US executive office, a federal court has reinstated the ban in 2011. As such, regulations on SC research in the USA vary from state to state.

In comparison to the current state of affairs in the USA, the European landscape differs. Regenerative medicine research and innovation is governed by the European Union (EU) Biopatent and Human Tissues and Cells Directives [36, 36]. While both the European Science Foundation (ESF) and the European Medical Research Councils (EMRC) have long supported human SC research, the Biopatent Directive excludes human embryo uses from patentable outcomes [38]. Following a recent ruling by the European Court of Justice (ECJ) (based on the Brustle case) [39], legally binding for all EU Member-States (MS), procedures involving human ECSs (hESCs) are not allowed to be patented. Fewer ethical and legal constraints exist for other (non-hESC) SC types.

Nevertheless, the legal definition of the term 'embryo' is lacking and interpretation of the term has become ambiguous. Overall, European Member-State positions on SC research can be classified as very permissive, permissive with restrictions, restrictive by default, very restrictive, and unlegislated [38]. Correspondingly, in some European countries SC research is completely forbidden, while in others it has been progressing for several years.

Stem Cell Types

Stem cells are classified according to their a) origin (from fetal or adult tissue), b) organ or tissue from which they were derived, c) differentiation fate, and d) expression of surface markers. The existing types of SCs [40] and their current and envisaged uses [succinctly summarized by the EuroStemCell initiative (www.eurostemcell.org)] include a) human ESCs (hESC), b) iPS, c) umbilical cord SCs (USC), d) tissue somatic SCs (hSSC), e) adult, f) mesenchymal SCs (MSC), g) bone marrow derived mononuclear (BMMNC), h) hematopoietic (HSC), i) endothelial progenitor cells (EPC), j) endogenous cardiac stem cells (CSC) and k) skeletal myoblasts (SKM).

ESCs are obtained from the inner cell mass (ICM) of the blastocyst and are pluripotent. They can differentiate into different types of cells and self-renew, possessing unrestricted plasticity. Nevertheless, they are associated with ethical and religious concerns. USCs are extracted from the umbilical cord and their multipotency refers to their ability to differentiate into different types of blood cells. To this date, they have been used to treat children with blood disorders, although limited applicability in adults has been reported, primarily reflecting their limited abundance [41, 42].

Tissue SCs, or alternatively, human somatic SCs (hSSCs)[1] are obtained directly from specific body tissues and are multipotent. However, hSSCs are associated with limited expansion ability and failing plasticity [41, 42].

Bone marrow is a source for MSCs and BMMNCs. Such cells can differentiate into skeletal, fat, bone cartilage, or blood cells. They are readily available from the donor's bone marrow and differentiate into skeletal tissue and vessels. It is yet uncertain whether their direct administration through the blood circulation can lead to beneficial effects or proper homing/engraftment in body tissues, however they are postulated to have anti-inflammatory-immune properties, making them a possible cell source for allogeneic therapy [43].

The major discovery of Takahashi in 2006 led to the development of inducible pluripotent SCs (iPS) [23] (for which Prof. Yamanaka was jointly awarded the 2012 Nobel Prize in Medicine and Physiology), through retroviral overexpression of the OCT4, SOX2, c-Myc, and KLF4 transcription factors in mouse fibroblasts, and subsequent relevant work on human skin fibroblasts [44, 45]. These cells overcame in various respects the ethical dilemmas and controversies associated with the use of ESCs, providing renewed alternative opportunities for research worldwide. Inducible iPS cells can be reprogrammed in the lab from adult cells (often skin cells), to a phenotype comparable to that of ESCs. Their major advantage includes their ability to provide patient-specific treatment, avoiding immune rejection. However, the iPS technique is time-consuming, and it has generated concerns about its efficiency to revert to a fully-embryonic state. It is also associated with an accelerated senescence, and long-term stability [41]. Moreover, the cells are tumorigenic and recent evidence [46] supports the need to ensure sufficient differentiation.

[1]The terms somatic (hSSC) and adult SC are used interchangeably; somatic SCs refers specifically to cells from the body (Greek, soma), that is, not germ cells, sperm or eggs.

Patent Landscape

Major trends on SC patenting presented herein are based on recent scientific reports [41, 47] and the UK Intellectual Property (Patent) Office (UKIPO) report (for the period of 2008-11), summarizing the UK National Stem Cell Network (UKNSCN) report on published and granted patents on SCs [48]. Specific patent referencing, demarcating recent scientific advances on image-based labeling, cellular tracking, and artificial organ and explant construction relevant to CVD is addressed in subsequent sections.

The UKNSCN and the UKIPO reports [48] indicated that recently published patents focus on research areas utilizing MSCs, ESCs and iPSCs. Complementarily, granted patents exhibit intense focus on ESCs, hematopoietic SCs and neural progenitor SCs. Prominent international role in granted and published patents is associated with both the corporate and academic worlds (sectors that account for almost 85% of the total awarded patents) [48]. Overall, the number of published patents has grown exponentially since the early emergence of SCs, dating back to 1991. Collectively, dominance in invention origin resides with US patents (and the USPTO), followed by World patents (WO), the Japanese patent office, the EPO, Korea, and the UK. The Wisconsin Alumni Research Foundation (WARF) and Kyoto University rank top internationally, whereas the University of Edinburgh has amassed the most published and granted patents in the UK. The major technology areas relevant to recent patents include ophthalmic (30% of total granted patents), neurological (24%), cardiovascular (18%), and antineoplastic (14%). For published cardiovascular patents, the major market share (43%) is held by academia, whereas corporations hold the majority sectoral share (60%) for granted patents [48].

To date, more than 92 hESC-related patents have been granted by the USPTO, referring to the processes of cellular isolation, culturing, purification, manipulation, or differentiation [41]. As a result, several patents have been claimed and granted by the USPTO on the derivation of pluripotent hESCs [49, 50-53] and the improvement of methods for culturing and monitoring hESCs, including cryopreservation of human cells, elimination of cells that spontaneously differentiate, and cell-subtrate imaging [55-57] (Table **1**).

While increased interest has been documented as a result of prior patent filings, controversy has not been lacking. Correspondingly, the EPO has refused (since the Brustle ruling in 2011) to grant hESC patents. Despite the controversies, ethical and legal issues, there is a noted pattern of growing number of awarded patents (by non-EPO offices) on hESC during the past 5 years [41].

On the forefront of iPSCs, recent US landscape analyses [47] indicate geographical clustering in patent activity, with Boston institutions exhibiting leading roles (benefitting from elite universities and two private corporations), followed by Japan (primarily Kyoto University), WARF and Cellular Dynamics at Wisconsin, and others. Not-surprising is the fact that, in a similar fashion to the global landscape, there is a growing trend in US patenting, exemplified by the numbers of filed patents [47].

Use of SCs in Cardiac Regenerative Medicine

Given the limited intrinsic ability of myocardial tissue to self-renew following injury, and the complex pathophysiological pattern of post-ischemic remodeling (Fig. **1**), much interest and excitement has been attributed to cellular regenerative therapies in ischemic heart disease (IHD) and HF [63, 64]. To this date, numerous cell types have been considered for therapies [40, 65, 66], including SKMs [67-69], BMMCs and BM-HSCs [5], MSCs [70-73], CSCs [6, 7, 24, 74], cardiosphere-derived cells (CDCs) [10, 58, 60, 65, 75, 76], and ESCs [78-81].

Preclinical (phase 1), phase 2 and phase 3 clinical trials are designed to evaluate the safety and efficacy of SCs. Early pre-clinical trials provided adequate proof-of-principle, justified by stable grafts and retention of the cardiac phenotype post-SC implantation. However, while clinical trials have indicated adequate safety profiles, efficacy has been inconsistent, with low cellular retention in tissue. For example, both BM-HSCs and SKMs have undergone extensive testing, in both preclinical and clinical studies, however, in addition to raised doubts regarding their cardiomyogenic potential [65, 66], there have

Table 1. Recent patents relevant to major breakthroughs with SC technologies.

Patent Description	Stem Cell Type	Host Species	Reference
Derivation of pluripotent hESCs from frozen—thawed embryo	hESCs	Human	[50]
Isolation, expansion and preservation of CDCs for cell transplantation and myocardial repair	CDC	Human or animal	[58]
Method for cultivation, propagation and production of differentiated cells and ESCs	Fibroblasts	Mouse or human	[59]
Isolation of inner cell mass for production of hESC	hESCs	Human	[52]
Identification of secreted proteins relevant to paracrine factors from CSC and CDCs targeted to therapeutic use	Cardiac, myocytes	Human	[60]
Establishment of hESCs stem cell line using mammalian cells, exhibiting population-specific characteristics (expression of pluripotent cell surface markers)	hESCs	Human	[51]
Methodologies for expansion of undifferentiated stem cells in culture systems	hESCs	Human	[53]
Microfluidic device for SC rapid prototyping			[61]
Bioartificial organ synthesis from organ scaffolds through cell seeding	Endothelial cells or cell progenitors	Human or animal	[62]

been significant concerns about arrhythmogenic potential, as exemplified by the early termination of the MAGIC human trial study [82]. Additionally, ESC and iPS cells (beyond the presented ethical issues) have exhibited significant risk of teratoma formation and immune rejection, thereby shifting interest for human therapy to adult cells (despite their poor survival post-transplantation [66]). On the other hand, BMMC-derived cells (MSCs and BMMNCs) are the most frequently used types to treat myocardial infarction (MI) clinically, exhibiting excellent safety and feasibility, positive clinical [83] but limited functional outcomes characterized by improvements in ejection fraction (EF) ranging from 2-5% in early non-randomized pilot studies [40, 84, 85].

Cardiac stem cells (CSCs), a term originally introduced by Deischer [48], relate to a niche pool of endogenous SCs (with an estimated number of 1 cell in 10000 cardiomyocytes and a turnover of 1% per year [65]), which received increased attention following patented results and publications by Messina *et al.* on cardiospheres [58, 77]. The expansion of cardiospheres as adherent monolayers to give cardiosphere-derived cells (administered in humans *via* intracoronary injections) led to improved function in rodents [10, 86] and other animals, and to reduced scar size in the clinical trial [87]. However, CDCs are associated with a lack of definitive and specific identification cellular markers [40].

Certainly, numerous literature reports exist on cellular therapies, pre-clinical and clinical studies and relevant outcomes. However, it is beyond the scope of this article to summarize all such prior efforts. Instead, the interested reader is referred to the excellent reviews on the topic by Malliaras *et al.* [65], Buyn *et al.* [40] and Zhu *et al.* [66]. Some of the major pre-clinical and clinical trials are summarized on Table **2** below, compiled from data in such publications and reviews.

Overall, SC cardiac cellular therapy has opened new therapeutic treatment avenues but is still associated with limitations, controversies, and variable functional improvements. Beneficial outcomes seem to be increasingly associated with induced (secondary-in-nature) paracrine effects (favorable for ventricular remodeling, collateral formation, limited inflammation, and host cell survival), instead of enhanced primary homing and engraftment as the primary reason for functional changes. Some of the prominent limitations of such approaches include poor electromechanical integration and increased arhythmogenesis, immune rejection, low retention, technologically cumbersome and surgical administration methodologies, early apoptotic responses, and complex cellular preparation and prescreening [40, 66].

Table 2. Examples of clinical trials of SC therapy in human cardiovascular diseases [40, 65, 66]. Delivery methods included epicardial, intracoronary, trans-endocardial injections or administrations.

Trial-Study	Cell Type	Host Species	Major Findings	Reference
MAGIC	SKM	Human	Early termination-no effect (ICM)	[82]
Erbs *et al.*	PC	Human	Improved EF and perfusion, reduced infarct size (ICM)	[88]
Hendrikx *et al.*	BMMNC	Human	(ICM)	[89]
TOPCARE-CHD	BMMNC *vs* PC	Human	Improved EF and regional contractility (ICM)	[90]
FOCUS-CCTRN	BMMNC	Human	Improved EF but no change in defined endpoints (LVESV, MVO_2)	[91]
TOPCARE	BMMNC *vs* PC	Human	Improved EF and perfusion, reduced infarct size (Acute MI)	[83]
Chen *et al.*	MSC	Human	Improved EF, viability (Acute MI)	[92]
BOOST	BMMNC	Human	Transient EF improvement and diastolic function (Acute MI)	[85]
REVIVAL-2	BMSC	Human	No EF change (Acute MI)	[93]
MAGIC cell-3-DES	BMSC	Human	Improved EF (Acute MI)	[94]
REPAIR-AMI	BMMNC	Human	Improved EF (Acute MI)	[83]
FINCELL	BMMNC	Human	Marginally beneficial (Acute MI)	[21]
REGENT	BMMNC	Human	Marginally beneficial (Acute MI)	[95]
Janssens *et al.*	BMMNC	Human	No EF change, decrease in scar size (Acute MI)	[96]
BONAMI	BMMNC	Human	Increase in viability (Acute MI)	[97]
HEBE	BMMNC	Human	Negative functional effect (Acute MI)	[98]
SCAMI	BMMNC	Human	Negative functional effect (Acute MI)	[99]
CADUCEUS	CDC	Human	Ongoing (Acute MI)	[100]
NCT00474461	CDC	Human	Ongoing (HF)	[101]
POSEIDON	BMMNC	Human	Ongoing (ICM)	[102]
PROMETHEUS	MSC	Human	Ongoing (Chronic ICM)	[103]

Table 3. Summary of promising clinical trials conducted in Europe utilizing various forms and types of SCs. Adapted from information in ESF's report (p. 12) on Human Stem Research and Regenerative Medicine, October 2013 [38].

Target Tissue-Organ	Synopsis of Clinical Trial Activities and Outcomes	Reference
Eyes	Transplantation of adult human retinal SCs and photoreceptor neural cells targeting human degenerative diseases that lead to blindness	[108, 109]
Liver	hESC-derived hepatocytes targeting liver damage. Mechanistic evaluations of parenchyma development targeting/exploring therapeutic options.	[110-112]
Brain	hESC-derived neurons targeting stroke, traumatic brain injury and dementia. Exploration of use of neural crest cells and MSCs targeting therapies in neurological diseases.	[113-115]
Oncology	MSC-derived from fat and bone marrow targeting tumor behavioral responses	[116-118]
Neurology	hiPS-derived neural progenitors targeting therapy for amyotrophic lateral sclerosis	[119]
Dentistry	SCs derived from human dental pulp targeting the osteogenetic potential	[120]
SC-based Tissue-Engineered Organs	Pediatric trachea transplant in congenital tracheal stenosis.	[121]
Ears	hESC-derived and differentiated into auditory neurons targeting deafness	[122, 123]
Endocrinology	hESC-derived pancreatic progenitors targeting diabetes. Additionally, ESC-derived thyroid cells targeting hypothyroidism	[124]
Dermatology	SC-derived skin cells targeting skin cancers	[125-127]
Trauma and Orthopedic Surgery	MSC-derived and hematopoietic cells targeting therapies of the osteoarticular system	

Non-Cardiac Human Trials – The Current State of Affairs

Within Europe, clinical trials using SCs (totaling 514 in accordance to the EU Clinical Trials registry in October 2013) conducted by academic and industrial sites, have generated promising results [38]. Studies have focused primarily on MSCs, hESCs, and iPS and have engaged to this date individuals from MS [104], industries [105, 106], and consortia coordinated by a network of countries [107].

Examples of promising scientific results reported by the ESF [38] listing potential clinical applications of SCs in Europe are classified into those relevant to eyes, liver, brain, oncology, neurology, dentistry, SC-based-tissue-engineered-organs, ears, endocrinology, dermatology, and trauma and orthopedic surgery, and are summarized in Table **3** below [38]. Noteworthy are also the advanced stage of Phase 3 clinical trials on congestive heart failure and Crohn's disease [38, 105, 106] by two Belgian companies (engaging US investigators from the Mayo Clinic).

IMAGE-BASED LABELING AND TRACKING OF STEM-CELLS AND STEM-CELL ENRICHED BIO-ARTIFICIAL MEMBRANES IN ACUTE REPERFUSED MYOCARDIAL INFARCTION

Within the realm of SC therapies (including SC-enriched scaffolds, passive 2D-explant membranes [30], 3D extracellular-matrix [ECM] or other biomimetic materials [19, 128-131], and artificial organs [18]), non-invasive imaging and tracking of labelled cells and their functional impact have taken leading and prominent roles in recent years (Fig. **4**). SC therapy limitations, relevant to therapeutic dose, timing of cellular delivery, methodology of SC administration, and homing and engraftment, have, and will continue to benefit from image-based cellular labeling, tracking, and serial monitoring [132].

Despite the recent advancements in cellular and molecular imaging using numerous non-invasive imaging modalities (including computer tomography [CT], positron emission tomography [PET] and X-ray imaging), this article primarily focuses on MRI-relevant methodologies and breakthroughs, as such may complement progress in optical imaging (fluorescence or bioluminescence) or imaging of nano-compounds.

The emergence of the field of nanotechnology and its recent scientific progress has led to a multitude of new applications comprising novel nanoparticles (including, but not limited to magnetic nanoparticles, quantum dots, and carbon nanotubes). Such applications can be classified under the categories of a) cellular labeling, b) tracking, c) delivery, and d) scaffolds and platforms. The newly developing field of organ synthesis is envisaged to also benefit tremendously from imaging advances in the near future.

This section succinctly reviews the recent progress and granted patents in such areas. The fourth category of scaffolds and platforms is discussed in sections below.

Cellular Labeling

Biocompatibility, target specificity and cellular sensitivity are the three most critical properties an ideal cellular label must possess [132, 133]. Label classification-schemes include: a) receptor-based, b) reporter gene-based and c) direct labeling. As Fu *et al.* [132] argue the first two categories are inappropriate for cardiac SC therapy; both schemes suffer from the lack of expression of specific markers on the SC surface and are associated with cellular changes upon differentiation into the cardiac phenotype. Consequently, direct cellular labeling approaches have gained preference and increased interest. Label synthesis and label-dependent drug-delivery are topics beyond the scope of this work and the reader is referred to recent reviews by Bertorelle *et al.* [134] and Wadajkar *et al.* [133].

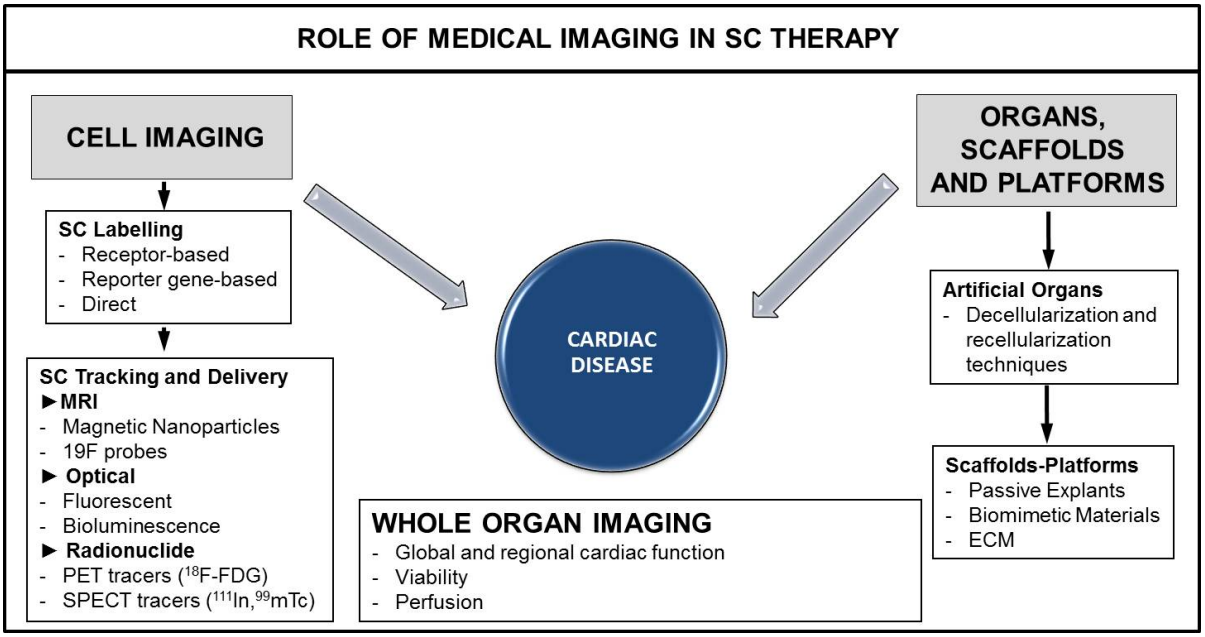

Fig. (4). Diagrammatical representation emphasizing the role of medical imaging in SC therapy of CVD. Target areas include SC labeling, tracking and delivery, artificial organ and scaffold development.

Despite the increased sensitivity of (single photon, multiphoton or confocal) fluorescence (ranging between 10^{-9} and 10^{-12} mol/l) and bioluminescence (ranging between 10^{-15} and 10^{-17} mol/l) [135] such techniques exhibit poor resolution characteristics and low imaging penetration [13]. *In vivo* applications thus become extremely challenging. Contrary to such techniques, MRI exhibits a significantly lower sensitivity (of the order of 10^{-3} and 10^{-5} mol/l), yet has exquisite spatial resolution, soft tissue contrast and depth of penetration, and provides added advantages of real time cardiac imaging, and interventional monitoring of cellular injections.

Generally, direct labeling is achieved by label introduction into the cell or attachment to the cellular membrane. Simple incubation of SCs with markers achieves adsorption, often followed by cell-mediated endocytosis or phagocytosis and endosomal packaging [134]. Although, traditionally, fluorescent probes have been associated with use in optical imaging, and super-paramagnetic nanoparticles (SPIO) with MRI [137], smart, efficient and biodegradable MR-nanoparticles (NP) continue to be synthesized, that have complementarily contributed to intracellular miRNA delivery, enhancing SC stability [134].

Fluoroscent Probes

Fluorescent probes refer to dyes or nanoparticles which label cells by direct attachment to cellular nuclei or the cytoplasmic membrane. Migration to SC applications has reflected the increased stability of such probes (e.g. CellTracker™) and their non-toxic properties [132]. High resolution optical imaging [138] can allow visualization [136], often combined with post-mortem histological assessment for validation.

Quantum Dots

Quantum dots (Qdots) are nano-crystals structures that emit light. They often incorporate Cadmium (or other atoms from groups II-IV of the periodic table such as Zn or Pb). They are particularly attractive because of their photostability, longevity and ability to track cellular dynamic processes. They can also provide multicolor optical imaging, and they are readily incorporated *via* incubation or peptide-mediated uptake [139-141]. Their size (typically of the order of tens of nm, precludes their entry into adjacent cardiomyocytes through the syncytium of interconnections *via* gap junctions [132, 141]. Their *in vivo* sensitivity was assessed in mice to range at approximately 10^5 cells [141]. Their toxic nature (Cadmium base) and the necessity for dedicated equipment for imaging have precluded their widespread clinical use so far.

Magnetic Labels

Paramagnetic Contrast Agents

Chelated gadolinium-based (PEG-functionalized Gd_2O_3, Gd-DTPA, Gd-DOTA, Gadofluorine-M-Cy3 [GdFM-M-Cy3]) agents [132, 133, 142] or Manganese ($MnCl_2$, MnO) T_1-contrast agents have been extensively used for intracellular labeling [132]. Limited access of these agents to free intracellular water leads to fair efficiencies in modulating image contrast [143]. Furthermore, toxicity effects (due to possible un-chelation upon failure of engraftment of labelled cells) led to limited widespread clinical use.

Paramagnetic Iron Oxides

Following the initial major discovery by Weissleder's group on intravascular dextran monocrystalline iron oxide nanocompounds (MION) [144, 145] and their subsequent ^1H and multinuclear applications as contrast agents [146], their clinical ferumoxide formulation emerged (Feridex – Advanced Magnetics, Endorem or Berlex). However, the inability of such particles (having sizes larger than 10-100

nm) to traverse vessel fenestrations, subsequently stimulated the synthesis of superparamagnetic (or ultrasmall superparamagnetic) iron oxide nanoparticles (SPIOs or USPIOs and their clinical formulation Combidex), that also exhibited potential for SC labeling and tracking. Incorporation of iron oxide particles (consisting of either magnetite Fe_3O_4 or magnemite Fe_2O_3), using conventional magnetofection [147], or magnetoporation techniques [148] for nanoparticles or endocytosis for larger particles [30], showed no effects on cellular viability, or proliferation [132]. Initial cardiovascular studies on large animals [149] were followed by SC labeling in rodents [150] leading to negative [due to susceptibility induced losses, behaving primarily as T_2- or T_2^*-contrast agents] or positive [151] cellular image contrast and discriminatory tracking power. An added advantage of such particles in tracking inflammation includes their incorporation into macrophages in infarct zones and the visualization capacity of the cardiomyocyte phenotype shift to a less-inflammatory state [152]. Larger, micron-sized iron oxide particles (MPIOs) have also been used to track SCs in rodents [10, 30] but have not been used clinically. Limitations associated with SPIOs include their detection limits (of the order of 10^4-10^5 cells) [153] in cardiac imaging, label dilution effects (as a result of the proliferative, differentiation and viability capacity of SCs), and inability of *in vivo* localization (intracellular or extracellular spaces, or within macrophages) for quantitative purposes. A recent FDA decision banned further Feridex, Combitex and Sineren use in clinical practice.

Fluorinated Labels

The ^{19}F MR-visibility and lack of its *in vivo* tissue/organ abundance provides fluorinated labels an added advantage as tracking agents. Prior [154-156] and newer [157] fluorinated SC labelling and MR imaging efforts have been documented. In contrast to the paramagnetic contrast agents listed above, ^{19}F-MRI aims to the direct detection of the label rather than its effect on the abundant water signal, despite the potential sensitivity limitations of this technique. Furthermore, fluorine imaging, necessitates use of specially designed hardware and dedicated pulse sequences that are often limiting for widespread clinical use.

Radionuclide Labels

Particularly beneficial to radionuclide labels is their endogeneously unique cellular phenotype upon injection, given the lack of any background radioactivity from target organs and tissues. With much increased imaging sensitivity (compared to MRI), ^{111}In oxyquonoline, ^{99}mTechnicium (Tc) and ^{18}F fluor-deoxy-glucose (FDG) are widely used labels for pre-clinical and clinical positron emission tomography (PET) and single photon emission tomography (SPECT) work. Their advantages reflect their long half-lives (up to several days); although, at the same time, they are associated with increased cytotoxicity and radioactivity persistence (from cellular lysis to scavenging of cellular debris, imposing effects on surrounding healthy tissue and cells). Specifically, Chin *et al.* [158] showed feasibility with SPECT in ^{111}In oxine (commercially available, long half-life of approx. 67 hours suitable for serial, dynamic imaging) radiolabelled MSCs in swine in MI, tracked with imaging in a semi-quantitative manner, over a period of 2 weeks. While feasibility was proven, cardiac localization was poor. The study provided, however, important insights for the choice of proper SC administration routes. In a follow-up study, Kraitchman [159] combined use of ^{111}In oxine with SPECT and X-ray CT in a canine MI model for MSC tracking [co-labelled with ferumoxides-poly-L-lysine (PLL)], demonstrating high sensitivity of cellular detection (even compared to PCR or immunofluorescent techniques), allowing tracking of 10^5 cells, for up to 7 days post-injection (facilitating serial studies), superior attributes compared to MRI. This study showed minimal localization of SCs in the heart. ^{99m}Tc labelled BMMNC SPECT imaging was also employed in rats post-MI to track and monitor their bio-distribution post-injection and ascertain optimal SC administration strategies (e.g. systemic intravenous *vs* direct ventricular cavity injections) [160]. Noteworthy are also the PET studies by Hofmann *et al.* [161] and Wu *et al.* [162] targeting human and rat myocardium, respectively. Wu *et al.* demonstrate for the first time the feasibility of monitoring transplanted cells (cardiomyoblasts) onto animal myocardium using reporter gene imaging (with microPET and optical bioilluminescence), noting drastic signal reductions within 4 days post-transplantation, in an effort to determine cell delivery, route and optimal injection number. Hofmann studied intracoronary-administered BMMNC homing and bio-distribution in the infarcted myocardium in patients post-stent insertion, using ^{18}F-FDG PET.

Cellular Tracking and Delivery

The ability to quantitatively assess the efficacy of SC delivery and viability is fundamentally critical and guarantees adoption of cellular tracking techniques in clinical trials. Although little prior documentation exists on non-invasive cellular viability assessment *in vivo* (post SC injections), cellular tracking has been repetitively studied in CVD [31, 143, 163]. Acute MI studies in large animals [149, 153] have thus far documented successful tracking and localization to peri-infarct borders, often in association with delayed contrast enhanced MRI [149, 153]. Novel ^{19}F tracking has been recently documented on the rat hindlimb [157] and cardiac and cerebral ischemia [164].

Patent Landscape and Recent Imaging Breakthroughs

While the target of some of the recent patents may relate to brain imaging, applicability of such breakthroughs can be readily extended to cardiovascular imaging. Classification of recent efforts relates to the a) introduction of novel methods and systems for tissue and cellular imaging, b) imaging of labelled cells, and c) targeted NP delivery and therapy, as summarized in Table **4**.

In accordance to these categories, Ragan *et al.* [172] has recently presented a multi-photon optical imaging technique to image tissue samples (prior and after sectioning) in 3D, ameliorating prior shortcomings of existing imaging techniques relevant to resolution, image penetration and ability to visualize in all spatial dimensions.

Table 4. Examples of major breakthroughs relevant to imaging modalities and labels recently patented.

Patent Description	Cell Label	Imaging Technique	Reference
Biocompounds targeted to specific ligands for thermotherapeutic applications	Bioprobes	MRI, PET, SPECT, Bioimpedance	[165]
Methodologies for *in vivo* quantification of labelled cells with perfluorocarbons using MRI	PFC	^{19}F MRI	[166]
Ex vivo cellular labelling using perfluorocarbons and their *in vivo* detection using MRI	PFC	^{19}F MRI	[167]
Use of labelled nanoparticles to enhance image quality properties of biological tissue	Polymeric iron oxide nanoparticles	MRI	[137]
Use of magnetic fields to transfer energy to magnetically labelled cells, causing them to function as positive contrast agents	Magnetically labelled molecules or cells	Radiofrequency detection apparatus and MRI	[168, 169]
Methodology to quantify labelled cells *in vivo*	Fluorocarbon label	^{19}F MRI/MRS	[170]
Use of a novel fluorescence imaging system for tissue imaging *in vivo* and *in situ*	Fluoroscent labels	Optical imaging	[136]
Synthesis of high sensitivity labels for SC tracking	Long hydrocarbon chain	Near Infrared Optical Imaging, PET, SPECT	[171]
Use of a multi-photon system to image tissue samples (prior and after sectioning) in 3D		Optical imaging	[172]
Detection and tracking of labelled cells in inflammation	Fluorinated labels	^{19}F MRI/MRS	[173]

On the forefront of imaging labelled cells, prominent methodological strides are attributed to Gray [171] and Wang [168, 169]. While Gray's invention relates to novel probes for multi-modality imaging (Near Infrared, PET, and SPECT) for SC tracking, Wang's inventions are MR-relevant. In the two awarded patents, both the apparatus and the imaging methodology is described to allow localized application of a magnetic field for attraction of magnetically-labeled cells in the human or animal body, followed by regional transfer of energy (*via* a second magnetic field) to labeled cells. Heating the labeled cells leads to positive cellular contrast during MR imaging.

However, noteworthy have been the five patents awarded to Eric Ahrens on *in vitro* and *in vivo* NMR and MRI of fluorocarbon synthesis and imaging [166], novel ferritin gene reporter [171], cellular labeling and quantification [167, 170], detection of inflammation and tracking [173], spanning the past 9 years. Scientifically, the most prominent achievement was the viral cellular transfection of the metalloprotein ferritin [171] that led to local intracellular iron accumulation, converting the labeled cells to nanomagnets. Local field susceptibility allowed their tracking and imaging using MRI.

A magnificent example of targeted NP delivery and therapy is also described by Ahrens [173] using fluorocarbon imaging for inflammation detection and image-guided treatment of MI *in vivo*. Complementary to such efforts are also catheter-based therapeutic approaches *via* NP targeted delivery [165, 175] and donor cell tracking using fluorinated nanoparticles [176].

BUILDING FROM THE BOTTOM-UP: SCAFFOLD-BASED CUSTOMIZED ORGAN DEVELOPMENT

End-stage organ failure is the terminal clinical outcome following IHD. More than six million American citizens suffer from HF, with half a million new cases reported annually [177, 178]. Organ transplantation remains the only treatment of organ-failure, nevertheless, and waiting lists of about 3500 listed recipients anticipating heart donation (third in demand after the kidney and liver) are reported in the USA alone [179], with the United Network for Organ Sharing indicating that over 100000 patients are anticipating a transplantable organ graft [178, 180]. Even still, multiple organ-failure, immunosuppression and clinical complications associated with transplanted organ rejection, often pose additional challenges towards this therapeutic pathway.

Pioneering work by D. Taylor's group on decellularization/recellularization strategies on rat hearts [181] has successfully led to bioartificially engineered organs [62, 182]. The approach is simple, yet its fundamental basis relies on knowledge of complex molecular biology signaling, spatial developmental cues, physical cellular environmental stimuli and cell regulation. In this approach, cellular material is removed from organs from cadavers or animals (using detergent chemicals and well-defined protocols *via* the innate vasculature [178-180]) leaving the protein scaffold intact, thereby preserving the organ's ability to maintain the structure and biomechanics of native tissue [131, 183]. The effort concludes with cellular repopulation and vascular re-endotheliation using dedicated SCs, often exposed to appropriate preconditioning [178]. The ingenious approach bypasses and eliminates any issues associated with immunogenic organ rejection. Nevertheless, functional discrepancies still exist in synthesized organs that require substantial additional work before the technique finds its way to large animal transplantations and routine clinical use. Moreover, from the engineering standpoint, and despite early and latest mechanical tests of both the synthesized left [178] and right ventricle [184], indicative of tensile muscular response, stiffness and anisotropy, much work still remains, to allow complete functional characterization of synthesized hearts, and optimization of decellularization/recellularization protocols. Evidently, the role and contribution of image-

based techniques, including non-invasive MR imaging is expected to be integral and most prominent.

THREE-DIMENSIONAL (3D) PRINTED STEM CELL TECHNOLOGIES

While Taylor's and Ott's work provides a remarkable strategy to synthesize 3D organs [181, 182], alternative strategies have been explored. The team led by W. Shu [61] has successfully managed to use 3D printing techniques to arrange hESCs, aiming to the creation of three-dimensional tissues and structures [19]. Such groundbreaking work allowed printed cells to be driven by pneumatically controlled processes, through open/control control microvalve stages, ensuring SC viability and differentiation. In this way, the technology can be directly applicable for use with natural ECM scaffold substrates (rather than hydrogels or other polymers) to design optimal structures for cell seeding [185].

MR IMAGE-BASED HOMOGENEOUS MEMBRANE AND ORGAN DEVELOPMENT USING TISSUE-MIMICKING BIOMATERIALS

An alternative regenerative approach to intact organ synthesis is the development of scaffolds or membranes that anatomically and functionally mimic the ECM and local cardiac geometry [129, 131] for repair or replacement of lost tissue. SC-enriched biodegradable scaffolds, often constructed from native extracellular matrix of host (or allogeneic) decellularized organs, allowed for tremendous progress in the field of tissue engineering [18]. This section focuses on recent imaging advances of tissue-mimicking (biomimetic) materials; for the topics of biomimetic platforms (bioartificial tissues, decellularized matrix scaffolds, scaffold-bioreactor systems) the reader is referred to excellent reviews and articles by Vunjak-Novakovic *et al.* [131], Ott [18], Song *et al.* [178], and Park *et al.* [180].

In the pursuit of these approaches, increased interest has been documented for identification of biomimetic materials that match local tissue morphology, and mechanical [13, 186] and imaging properties [13, 187]. As reported by Kossivas *et al.* [130], polymeric or elastomeric materials have traditionally been locally injected or attached and have been found to possess biocompatibility and bioactivity properties that facilitate good adhesion with the surrounding environment, ensuring mechanical stability and proper structural moduli [13], withstanding static and dynamic loading [188]. Recent studies have investigated different classes of scaffold biopolymers as passive epicardial restraint bio-artificial membranes in IHD [13, 189] or for controlled delivery of SCs, with and without pre-conditioning, such as electromechanically synchronous stimulation [187, 190].

Of these, Poly(glycerol sebacate) [PGS] [13, 130, 191], hydrogels [192], and polyethylene terephthalate [187] have proven to possess excellent biocompatibility, biodegradability, and good stiffness and strength suitable for biological applications [32, 193-195], and for use in drug carrier-release applications, both *in vitro* and *in vivo* [196].

Consequently, recent efforts have focused on the production of engineered tissue mimicking materials that match the morphology and emulate the *in vivo* murine and human cardiac tissue mechanical and imaging characteristics [130, 191]. Such efforts [130] therefore argue in favor of use of PGS elastomers in conjunction with image-based modelling and rapid prototyping manufacturing approaches, for accurate 2D/3D model construction and tissue membrane synthesis emulating biological anatomy, of ultimate value and importance to cardiac transplantation. Results from a recent implementation of such an approach with elastomeric materials possessing well-known mechanical constitutive behaviors (depicted in Fig. **5**) shows the MR-based reconstructions of cardiac anatomy that drive the finite element modeling and subsequent manufacturing (at any desired scale), using stereo-lithographic (or other) processes.

Integral to such efforts has been the ability to synthesize elastomers (in solid or semi-solid form) with proper imaging properties, carefully controlled for relaxivity and contrast in MR imaging. Relaxivity adjustment was achieved by doping (i.e. adding an MR contrast agent, in this case gadoteric acid – *Dotarem*) at two different concentrations (D1 and D2), as shown in Fig. (**6**). Future MR-specific applications of un-doped and PGS doped materials (possessing negative or positive contrast) is expected to facilitate their *in vivo* use and monitoring as synthesized patches or implants with ischemic heart disease severity and progression [102].

Capitalizing on recent advancements in electrospinning, Dzenis *et al.* [197] have reported polyacrylonitrile nanofiber structures possessing increasing toughness with stretch, rendering them stronger than any other commercially available commercial fibers. Kouwer *et al.* [198] also reported synthetic polyisocyanopeptide hydrogels that mimic gels prepared from intermediate filaments. Presented results indicate not only the synthetic ability to match mechanical stiffness (constitutive stress-strain relationships) but also the degree of gel bundling. Given that electrospinning technologies have advanced to allow for material construction of any complexity, including deposition of directional fiber deposition, the combination of this technique with MRI, will facilitate empirical optimization of image-properties, myocardial fiber directionality, and local material properties. Such engineering and synthetic approaches are thus envisaged to become fully capable to reproduce myocardial (and any other) biological tissue complexity in the near future.

CURRENT AND FUTURE DEVELOPMENTS

While currently used SC methodologies and their applicability to cardiac disease have been described in prior sections, envisaged future developments and applications of medical imaging are expected to continue to focus on cardiac functional characterization, viability and perfusion, following SC treatments. Specifically, future work is anticipated to include studies focusing on recellularized hearts, post-transplantation follow-up and tissue characterization methodologies, 3D SC printing of membranes, scaffolds or entire organs, and SC therapies employing bio-artificially synthesized scaffolds (attempting

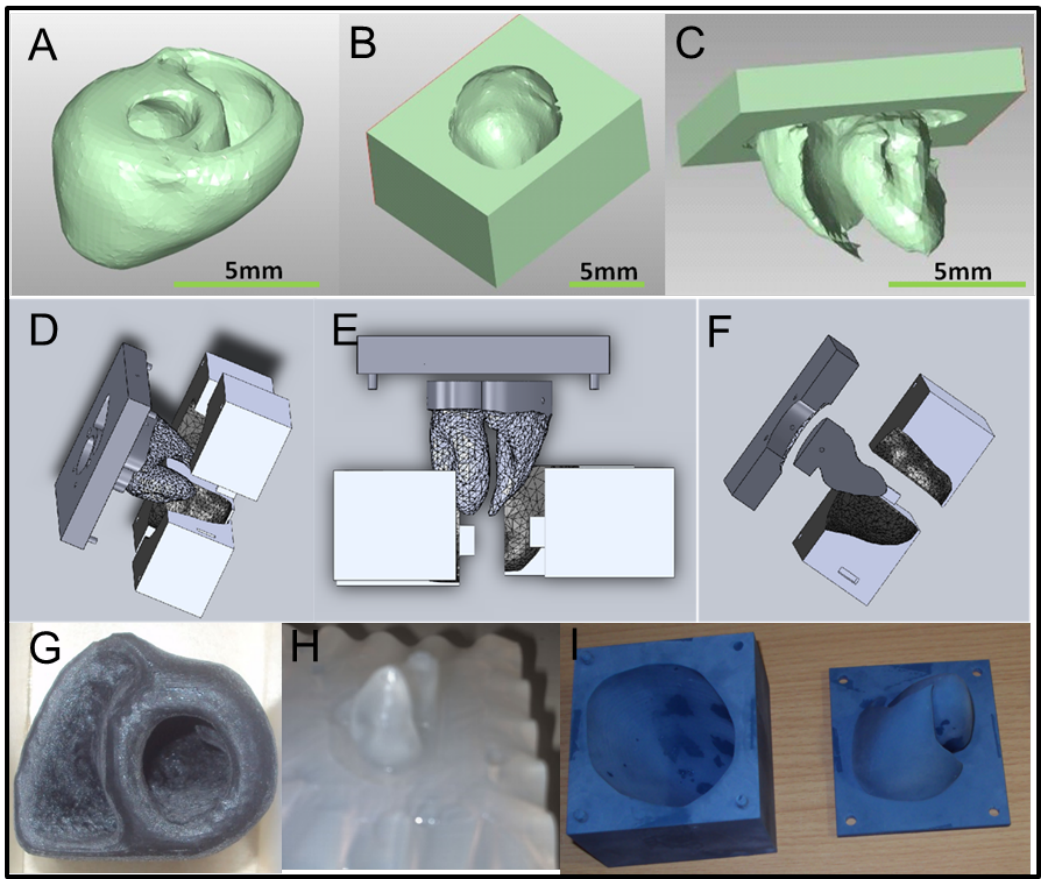

Fig. (5). (**A**) 3D reconstructed surface models of the murine heart based on MRI and (**B**, **C**) negative molds designs. (**D-F**) Cardiac finite element computational model renditions of the designed mold prototype. The design can easily accommodate aided-supports and the synthesized material filling. (**G**) Construction of the elastomeric heart showing accurate representations of the left and right ventricular heart geometry and (**H**) negative elastomeric mold at the scale of the rat (Materialize, Belgium); (**I**) a second type of solid negative mold showing left and right ventricular cavities and ventricular geometries, appropriate for fabrication using elastomeric filling (in liquid form). [Part of this figure is reproduced from figure 4 in Kossivas F, Angeli S, Kafouris D, Patrickios C, Constantinides C. MRI Based Morphological Modeling, Synthesis, and Characterization of Cardiac Tissue Mimicking Materials. IOP Biomedical Materials 7(3), 2012 [130], with permission].

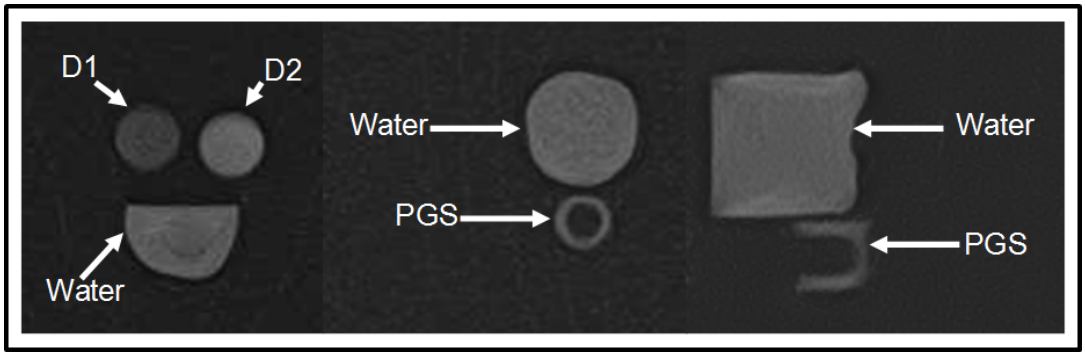

Fig. (6). (Left) Axial MRI images of the two synthesized and doped cylindrical PGS2:2 elastomers (D1, D2) at 1.5T demonstrating that controlled MR contrast can be achieved by appropriate doping of the elastomer; (Middle, right) Typical axial and sagittal images of synthesized polymer in a glass negative mold of annular shape, exemplifying the ability to shape the MR-visible material into any desired geometry. The elastomer imaged existed in semi-solid form (polymerization reaction incomplete). [This figure is reproduced from Kossivas F, Angeli S, Kafouris D, Patrickios C, Constantinides C. MRI Based Morphological Modeling, Synthesis, and Characterization of Cardiac Tissue Mimicking Materials. IOP Biomedical Materials 7(3), 2012 [130], with permission].

to increase SC proliferation rates, facilitate directional injection schemes, or incorporate alternative pre-injection treatments).

On the forefront of cellular imaging, image-based labelling and tracking techniques will continue to develop, facilitating temporal-dependent monitoring of homing,

engraftment and viability of implanted cells. Novel markers are also expected to emerge to allow imaging of the remodeling processes in the interstitial versus the intracellular spaces, while cardiac tractographic studies will contribute towards the understanding of the complex myocardial structure and its laminar composition, the identification of dominant and non-dominant layers, the existence of possible inter-species variations, and the structural alterations in myocardial remodeling.

CONCLUSION

Remarkable breakthroughs and advancements in SC technology, bioartificially synthesized organs, biomimetic scaffolds and tissue-mimicking materials have been evidenced during the past decade. Imaging advancements have concurrently allowed scaffold, cellular labeling, quantitative *in vivo* tracking, and image-based therapy. The role of medical imaging is foreseen to be integral and prominent in the next decade, when advancements in bioartificial organ synthesis, 3D tissue, scaffold and explant printing are anticipated to progress towards routine clinical use.

The direct applicability of any SC technique or methodology to human therapy has, and will continue to hold great potential for establishing efficient SC therapies in human cardiovascular disease.

ABBREVIATIONS

BMMNC = Bone marrow derived mononuclear
CSC = Cardiac stem cells
CT = Computer tomography
CVD = Cardiovascular disease
DOTA = 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid
DTPA = Diethylene triamine pentaacetic acid
ECM = Extracellular matrix
EF = Ejection Fraction
EMRC = European Medical Research Councils
EPO = European Patent Office
EPC = Endothelial progenitor cells
ESC = Embryonic stem cells
ESF = European Science Foundation
EU = European Union
FAK = Focal Adhesion Kinase
GFP = Green fluorescent protein
hESC = Human embryonic stem cells
hSSC = Human somatic stem cells
HSC = Hematopoietic stem cells
HF = Heart failure
IHD = Ischemic heart disease
iPS = Induced pluripotent stem-cells
MI = Myocardial infarction
MPIO = Metal paramagnetic iron oxide
MS = Member States
MSC = Mesenchymal stem cells
NIR = Near Infrared
NP = Nanoparticle
PEG = Polyethylene Glycol
PET = Positron emission tomography
PFC = Perfluorocarbons
PGS = Poly(glycerol sebacate)
SC = Stem-cells
SKM = Skeletal myoblasts
SPECT = Single Photon Emission Computer Tomography
SPIO = Super-paramagnetic iron oxide
TGF = Transforming Growth Factor
UKIPO = UK Intellectual Property Office
USC = Umbilical cord stem cell
US = United States
USPTO = US Patent Office

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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