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Pericytes and cardiac stem cells; common features and peculiarities.

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Abstract

Clinical data and basic research indicate that the structural and functional alterations that characterize the evolution of cardiac disease towards heart failure may be, at least in part, reversed. This paradigm shift is due to the accumulation of evidence indicating that, in peculiar settings, cardiomyocytes may be replenished. Moving from the consideration that cardiomyocytes are rapidly withdrawn from the cell cycle after birth, independent laboratories have tested the hypothesis that cardiac resident stem/progenitor cells resided in mammalian hearts and were important for myocardial repair. After almost two decades of intensive investigation, several (but partially overlapping) cardiac resident stem/progenitor cell populations have been identified. These primitive cells are characterized by mesenchymal features, unique properties that distinguish them from mesodermal progenitors residing in other tissues, and heterogeneous embryological origins (that include the neural crest and the epicardium). A further layer of complexity is related to the nature, *in vivo* localization and properties of mesodermal progenitors residing in adult tissues. Intriguingly, these latter, whose possible perivascular pericyte/mural cell origin has been shown, have been identified in human hearts too. However, their exact anatomical localization, pathophysiological role, and their relationship with cardiac stem/progenitor cells is emerging only recently. Therefore, aim of this review is to discuss the different origin, the distinct nature, and the complementary effect of cardiac stem cells and pericytes supporting regenerative strategies based on the combined use of both myogenic and angiogenic factors.

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1)   Introduction

The concept of the heart as a terminally differentiated organ was extensively debated between the end of 1800 and the beginning of the last century, until the seminal work of Karsner, Saphir and Todd strongly supported this view, by showing, with quantitative pathology approaches on histological sections, that the adult heart increases its size by hypertrophy only[1]. One of the strongest arguments supporting this thesis was the difficulty of pathologists to find mitotic figures in adult human hearts[1]. However, in subsequent years, this static notion was challenged, employing a plethora of different techniques, such as stereology[2], ^{3}H-thymidine labeling[3], bromodeoxyuridine labeling[4], immunofluorescence and confocal microscopy[5], and, most recently, mass spectrometry of ^{14}C incorporation into cardiomyocyte DNA[6]. As a result, nowadays the possibility that cardiomyocytes can divide is not anymore regarded to as a heresy. However, both the magnitude of this phenomenon and the origin of the proliferating cardiomyocytes are at the center of a heated debate, where evidence in support of their generation from either adult cardiomyocytes or cardiac stem cells have been reported in the literature (reviewed Cesselli et al. in this isssue).

In nature, the clearest examples of myocardial regeneration, that yield a complete *restitutio ad integrum* of the injured organ, occur in teleost fishes[7] and urodele amphibians[8]. Additionally, recent reports have shown that neonatal mice too have a transient potential to regenerate cardiac injury that vanishes 1 week after birth[9]. Although this result has the potential to reveal the mechanisms that distinguish myocardial scarring from regeneration, other reports have either refuted this finding[10] or demonstrated that the extent of regeneration depends upon the type and size of damage[11]. At first, the main mechanism of myocardial regeneration was considered to be, in these models, the activation of cardiomyocyte proliferation[12, 13]. However, in recent years, a more complex picture has emerged, that involves: a cardiomyocyte de-differentiation program, mediated by miR99/100 and Let-7a/c[14] or stimulated by the neuregulin/ErbB2 axis[15], the infiltration of macrophages with a unique polarization phenotype[16], epicardium-derived factors[17], a specific extracellular matrix[18], and, possibly, stem cells[17, 19, 20].

The natural history of cardiac pathology is characterized by a series of molecular, cellular, histologic, conformational, metabolic and functional changes, which have been collectively named

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cardiac remodeling. This latter term, indeed, describes a large spectrum of pathologic modifications (including: cardiomyocyte hypertrophy[21], cardiomyocyte death[22], cardiomyocyte de-differentiation[23], alterations of both calcium dynamics[24] and myofilament calcium sensitivity[25], profound modifications of myocardial metabolism[26], and cardiac fibrosis[27]), that constitute the common pathophysiologic mechanism leading a wide range of cardiac diseases of different etiology towards overt heart failure.

In addition, vascular, perivascular and stromal cells participate in cardiac remodeling and repair. During the development of hypertrophy, capillary endothelial cells also dynamically undergo a phenotypic change to support contractile function of the myocardium[28, 29]. Morphometry analyses of hypertrophied hearts in animal models showed that capillary microvasculature and myocytes grow proportionally to the increase of heart mass[30, 31], suggesting that an insufficient growth of capillaries could cause myocardial ischemia in pathologically hypertrophied hearts[32, 33].

Perivascular mesenchymal cells, alias pericytes, are abundant in the cardiac muscle but their function in cardiac remodeling has not yet been well documented. Recent evidence suggests that cardiac pericytes may contribute to cardiomyogenesis[34]. Clonal analysis, lineage tracing studies and *in vitro* differentiation experiments showed that coronary smooth muscle cells derive from pericytes in the adult heart[35, 36]. However, whether pericytes represent a homogeneous population remains a matter of debate. A recent study reported that immunosorted $CD146^+/CD34^-$ perivascular cells from fetal and adult myocardium share some phenotypic and developmental similarities with skeletal muscle pericytes, yet exhibit different antigenic, myogenic, and angiogenic properties, thus suggesting the heterogeneity of pericytes derived from diverse anatomical locations *(vide infra)*[34].

Although for many years the remodeling process was thought to be irreversible[37], an increasing body of literature is indicating that a fraction of patients in optimal medical and device treatment undergo profound modifications of the ventricular shape, shifting the pressure-volume relationships of their ventricle to the left. Intriguingly, reverse remodeling predicts prognosis in patients affected by idiopathic dilated cardiomyopathy and is related to the duration of heart failure[38]; consistently, reverse remodeling is very pronounced in infants[39]. In some instances,

these macroscopic functional benefits have even been coupled with the reversal of more subtle, cellular and molecular alterations, such as cardiomyocyte hypertrophy or the re-expression of fetal cardiac genes[40]. Importantly, according to different investigators, cell therapy can promote reverse remodeling[41, 42]. Furthermore, for specific pathologic conditions (e.g. acute lymphocytic myocarditis, peripartum cardiomyopathy[40] and Takotsubo cardiomyopathy[43]), the spontaneous recovery from heart failure symptoms and a nearly complete normalization of left ventricular function and structure were described, suggesting that, under favorable circumstances (especially after acute, not chronic injury) myocardial recovery can occur[40]. Intrigued by this observation, some investigators verified if, when cardiac injury spares cardiac resident stem cells and (at least in part) tissue architecture, these cells are both necessary and sufficient to promote myocardial regeneration[44].

In line, by extensively investigating, since the early 2000s, the possible ability of stem cell therapy to promote myocardial repair, together with the mechanism of action of donated cells, a novel field of studies, aimed at promoting cardiac regeneration, was created[45]. Indeed, the observed ability of primitive cells to stimulate cardiomyocyte proliferation started a new paradigm shift, which now considers feasible to unlock the proliferative potential of the heart. Prompted by these pioneering studies, several new approaches are now under investigation, that include: the combined use of progenitors cells of different origin and complementary mechanisms of action[46], the use of exogenous microRNA[47], the use of these latter within exosomes[48], the direct reprogramming of fibroblasts to cardiomyocytes[49] or the identification of factors, such as thymosin β4[50], the neuregulin Erbb2 axis[15] or the Hippo/Yap pathway[51] able to stimulate myocyte proliferation.

Given these premises, aim of this review is to discuss the different origin, the distinct nature, and the complementary effect of cardiac stem cells and pericytes supporting regenerative strategies based on the combined use of both myogenic and angiogenic factors

2)   The elusive nature of cardiac stem/progenitor cells.

Stimulated by evidence in support of cardiac myocyte proliferation following acute myocardial infarction[5], we and other authors started to chase after primitive cells in adult mammalian hearts. The rationale for this was that mature cardiomyocytes are withdrawn from the

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cell cycle few days after birth[52], while primitive, motile cells, able to differentiate in cardiomyocytes could also account for the observed chimerism of cardiac myocytes in male recipient of donated female hearts[53, 54]. By pursuing this task, Hierlihy and colleagues first described the existence of a cardiac side population (SP), able to differentiate both into hematopoietic cells and cardiomyocytes[55]. Immediately thereafter, the presence of primitive, cardiac resident cells was given employing a single or few antigenic markers. Specifically, cells with stem/progenitor cell characteristics that expressed either the stem cell factor receptor c-Kit[56] or the stem cell antigen-1 (Sca-1)[57] were initially identified. Subsequently, independent investigators confirmed the existence of cells with stem/progenitor properties in the postnatal mammalian heart, by evaluating cells expressing the developmental lineage marker Islet-1[58], employing a selective culture system that promotes the expansion of cells as self-adhesive, floating, clusters (i.e. cardiospheres)[59] or culturing plastic adherent cardiac cells with mesenchymal stem cell features (MSC, see discussion later)[60]. Although it was shown that, within the cardiac SP, Sca-1^+CD31^- cells were the most cardiomyogenic ones[61], that both c-Kit$^+$ and Sca1$^+$ cells were present within mouse cardiospheres[59], that Sca1 was expressed by most cardiac MSC, while cKit was expressed only by a minor fraction of the same cells[60], that human cKit$^+$ cells coexpressed MSC antigens[62], and that Sca1 expression may be required for c-Kit$^+$ cell function[63], conflicting reports on the relationship between the different primitive cell populations can be found in the literature.

To clarify the relationship between the different cell types, gene expression analyses were conducted by independent investigators. By comparing freshly isolated, cardiac derived, c-Kit$^+$, Sca1$^+$ cells, and SP cells with cardiomyocytes of mouse origin employing cDNA microarrays, Dey and collaborators observed that Sca1$^+$ cells and SP cells displayed the highest similarity in gene expression with cardiomyocytes, while c-Kit$^+$ cells showed the lowest correlation with all the other cell types[64]. The latter population appeared to be a distinct, more primitive cell type, which was, however, distinct from bone marrow (BM) derived c-Kit$^+$ cells and BM-MSC[64]. Additionally, by comparing c-Kit$^+$ cells of cardiac and hematopoietic origin, it was shown that cardiac cKit$^+$ cells are enriched in endothelial cell specific and angiogenesis related genes, including *Wt1*[64]. Investigating the differences among MSC derived from heart, BM and kidney, Pelekanos and collaborators showed that, despite a high degree of correlation, strong biases in gene and protein expression were observed (e.g. increased expression of *Mef2C, Tbx4, Fgf10*, and, to a less extent, *Isl1* in cardiac MSC), that could account for organ specific functions[60]. Similar results were obtained by

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Rossini and collaborators, who showed that MSC obtained from the heart and the bone marrow differed in their gene, miRNA, and protein expression profiles, where *c-Kit*, *GATA4*, *GATA6*, *KLF5*, and *myosin light chain-2a* were more expressed in cardiac MSC[65]. Importantly, a common feature of MSC cells obtained from the different tissues is the expression of ≈66% of the genes comprised within the pluripotency network (plurinet)[60]. Analyzing SP and Sca1 expressing cells by single cell quantitative RT-PCR, Noseda and collaborators showed that *Pdgfra* coexpression identifies cells enriched for the cardiogenic factors *Gata4/6*, *Mef2a/c*, *Tbx5/20* and *Hand2*, but mostly negative for *Hand1*, *Isl1*, and *Nkx2-5*, suggesting a similarity between these cells and the developing cardiac mesoderm[66]. Notably, these features were maintained even in clonal cultures of cardiac SP cells[66]. Intriguingly, when Gaetani and collaborators compared the gene expression profile of cultured human cardiac progenitor cells that were isolated employing either cKit or Sca1 and propagated either as monolayer cultures or as spheroid suspension cultures, they observed a very high degree of similarity among the different cell types. Notably, gene expression variability was mostly related to individual donor differences and to culture conditions (i.e. adhesion versus suspension culture). Last, we compared the gene expression profile of human multipotent cells with MSC features isolated from different tissues, including the heart[67]. Importantly, these latter were additionally characterized by cKit positivity[68]. Although in this early report we showed a high degree of transcriptional similarity among cells obtained from heart, liver and bone marrow[67], in a subsequent work that employed the cap analysis of gene expression (CAGE) technique, we showed that the expression profile of cardiac cells could be clearly distinguished from that of bone marrow or adipose tissue derived cells[69].

Despite a general agreement on the notion that adult cardiac resident primitive cells have a transcriptional identity that is distinct from mesenchymal cells residing in other adult tissues, less clear is the embryonic origin and the developmental potential of cardiac stem/progenitor cells residing in adult organs. Isl1^{+} cells may be originated from the cardiac progenitor fields during embryogenesis. However, postnatal Isl1 expressing cells were found to be mainly differentiated cells in parasympathetic ganglions, in the great arteries, in the outflow tract and in the sinoatrial pacemaker[70]. More complex is the origin of cKit expressing cells, which, could be remnants of the cardiogenic fields[71, 72] or, according to Hatzistergos and collaborators, would derive from the cardiac neural crest[73]. A third possible source of cKit expressing cells is the proepicardium (*vide infra*). Some lines of evidence support this possibility, such as: the localization of cKit^{+} cells in

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epicardial regions, both in mice[74] and in normal human hearts[75], and the reported co-expression of cKit with both Wt1 and Tbx18[76]. Last, *cKit* gene expression may be also induced by the Oncostatin M mediated de-differentiation of cardiomyocytes[23]. Lineage negative SP Sca1$^+$ cells, instead, do not derive from either neural crest, hematopoietic cells or cardiomyocytes, but rather from *Nkx2-5* or *Isl1* expressing cells. However, they may have a proepicardial origin as well, since fate mapping studies suggested their derivation from cells expressing the embryonic epicardial genes *cGATA5* and *Wt1*[66]. A proepicardial origin has been also claimed for cardiac MSC, following lineage tracing analysis experiments in mice[77]. Similar results were also obtained when we analyzed the transcriptome of human cardiac progenitor cells, comparing it with the most comprehensive transcription start site-based atlas obtained from the majority of mammalian cells and tissues. With this approach, we observed that cardiac progenitors show a high degree of similarity to mesothelial cells[69].

In line, epithelial to mesenchymal transition (EMT) of epicardial cells is a potential mechanism able to generate cells with progenitor features from the mesothelium. EMT describes the conversion of fixed cells, firmly anchored to neighboring cells (i.e. epithelial cells) into motile, matrix-degrading, mesenchymal cells [78]. This process, that plays a central role in embryogenesis, wound healing, stem cell homeostasis, and cancerogenesis[79], is driven by the expression of master regulators, including SNAIL, TWIST, and zinc-finger E-box-binding (ZEB) transcription factors, that inhibit the expression of epithelial genes (e.g. E-Cadherin) and promote the expression of mesenchymal genes (e.g. N-Cadherin)[80]. EMT is controlled by signaling pathways that are triggered by extracellular stimuli, such as TGFβ family members, which represent one of the strongest inductors of this process[80]. In development, EMT and its opposite mesenchymal to epithelial transition -MET- play a central role in the formation of the epicardium. Specifically, epicardial progenitors would arise from the splanchnic mesoderm likely via MET[81]. Conversely, concomitantly with the coverage of myocardial surface by proepicardial cells to form the epicardium, subsets of these cells acquire migratory and invasive properties via EMT and invade the myocardium as epicardial derived cells (EPDC). These latter are heterogeneous and comprise distinct subpopulations expressing semaphorin 3D (Sema3D), scleraxis (SCX), WT1 and Tbx18. WT1 and Tbx18 expressing cells contribute to smooth smooth muscle cells, pericytes, and fibroblasts, but rarely give rise to endothelial cells, while SCX and Sema3D expressing cells also contribute to the endothelial and endocardial lineage[82, 83]. Importantly, Wt1 and Tbx18 would exert opposite

roles, where Wt1 inhibits and Tbx18 promotes EMT[84]. In the human heart, TGFβ signaling and EMT are required for the formation of cardiospheres from cell monolayers[85], moreover epicardial cells exposed *in vitro* to TGFβ can undergo EMT and upregulate the expression of cKit[86]. Intriguingly, following acute myocardial infarction the epicardium becomes activated, and promotes myocardial healing[50, 87], however, in humans, chronic ischemic heart disease is associated with structural and molecular modifications of the epicardial layer[86].

Altogether these results indicate that the adult mammalian heart hosts one or few primitive cell populations, with overlapping mesenchymal characteristics, which are clearly distinguished from stem/progenitor cells residing in other tissues.

3)   Pericytes, mesenchymal stromal cells and cardiac stem cells

Studies conducted in the late '60s and in the mid '90s showed that osteogenic, multipotent, fibroblast colony forming units (CFU-F) can be found both in the bone marrow and in the connective tissue of many organs, including the heart[88, 89]. Following these early studies, different authors had the ambition to pin down the *in vivo* identity of these mesodermal progenitors. Bianco and Cossu were among the first ones to hypothesize a perivascular/mural origin for these cells, considering the localization of alkaline phosphatase positive stromal cells in the bone marrow and the osteo-chondrogenic potential of perivascular cells isolated from both the retina and postnatal arterial walls[90]. Intriguingly, clonogenic assays and cell fate tracking experiments suggested the existence of a close relationship between angiogenic cells and mesodermal progenitors, supporting their possible origin from the mesoangioblast, a cell type that was initially identified in the embryonic dorsal aorta, and whose origin has been recognized in a subpopulation of the hemogenic endothelium[91, 92]. Moving from this hypothesis, it was first described that bone marrow osteoprogenitor cells had a perivascular localization and could be prospectively enriched employing CD146, an antigen shared with pericytes/mural cells[93]. In analogy, a myogenic population, distinct from satellite cells, was identified that expressed pericyte markers (including NG2, alkaline phosphatase and PDGFRβ) and resided in the skeletal muscle[94]. These results were later generalized by identifying *in situ* and prospectively sorting cells with a pericyte phenotype (i.e. CD146^{high} CD34^{-} CD45^{-}), demonstrating their clonogenicity, multipotency, and the coexpression of MSC markers such as NG2 and PDGFRβ[95]. To corroborate this finding, vascular/perivascular cells expressing the MSC markers CD146, CD73, CD90, CD105, CD271, and

NG2 have been identified in several tissues *in vivo*[96]. However, both the possibilities that pericyte subsets act as *bona fide* MSC and that pericytes are a uniform population of cells that can be cultured as MSC have been questioned. Specifically, in line with results obtained by us[69] and others[65, 77] on mesodermal progenitors isolated from different sources, recent data indicate that CD146 expressing cells isolated from different human tissues are clearly distinct from the bone marrow derived ones, both from a potency and transcriptional standpoint[97, 98]. As a further piece of evidence supporting the heterogeneity of the pericytes, it has been shown that CD146 cannot be considered a universal marker of this cell population, since it is expressed by endothelial cells too, but it is absent on CD34$^+$ adventitial pericytes. These latter are localized in the external layer of large vessels nearby the *vasa vasorum*, express some MSC markers (i.e. CD44, CD73, CD90, and CD105), and generate clonogenic multipotent progenitors, but are phenotypically distinct from microvascular pericytes (i.e. adventitial pericytes do not express CD146, NG2, and PDGFRβ)[99, 100]. Moreover, in the spinal cord and skeletal muscle, different pericyte subtypes have been described[101, 102]. Specifically, in the skeletal muscle type 1 pericytes are Nestin$^-$NG2$^+$, while type 2 pericytes are Nestin$^+$NG2$^+$. Both type 1 and type 2 pericytes express PDGFRβ and CD146, but only type1 pericytes express PDGFRα[102]. In young mice, muscular type 2 pericytes have myogenic potential while type 1 pericytes appear quiescent. In aged animals, however, type 2 pericytes show diminished myogenic capacity while type 1 pericytes produce collagen[102]. For these reasons, the term MSC does not identify a cell type with identical differentiation capacities, therefore it should be abandoned when referring to extramedullary mesodermal progenitors. Intriguingly, according to Sacchetti and collaborators[97], tissue-specific mesodermal progenitors could be recruited to a mural fate, suggesting that pericytes would serve as local stem/progenitor cells. Importantly, mechanical cues can instruct mesodermal progenitors *in vitro* to differentiate towards neural, muscle or osteogenic fates, therefore it is tempting to speculate that physical forces may modulate the differentiation of these cells *in vivo* too[103]. However, this hypothesis was recently challenged, employing lineage tracing experiments of cells expressing the transcription factor *Tbx18*, that, according to the authors, would label most pericytes more specifically than PDGFRβ[104]. Although cells that express Tbx18 (albeit at extremely low levels) are multipotent *in vitro*, those that derive from Tbx18 expressing cells *in vivo* maintain their cell identity during aging and do not contribute, for example, to cardiomyocyte turnover in the heart[104]. Moreover, in pressure overloaded hearts, only a minority of pericytes derived from *Tbx18* expressing cells would contribute to fibrosis[104]. However, PDGFRα has been associated with widespread organ

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fibrosis, suggesting that PDGFRα^{+} cells may also have a role in skeletal muscle and cardiac fibrosis [105]. Type 1 pericytes and fibro-adipogenic progenitors express this receptor, and like pericytes, fibro-adipogenic progenitors line the skeletal muscle vasculature, suggesting that their roles might overlap[98]. The contribution of perivascular PDGFRα^{+} cells to cardiac fibrosis has not been determined yet.

Pericyte anatomy and biology have been extensively studied over the years. These cells, that were first described to be anatomically localized within the vascular basement membrane of microvessels, have now been identified in the adventitial region of large vessels too (reviewed in [106, 107]). Pericytes contribute to basement membrane secretion, possibly as a consequence of their interaction with endothelial cells[106]. Furthermore, pericyte contractility regulates endothelial cell proliferation, via contact inhibition of endothelial cell growth and activating chemomechanical signaling[108], while pericyte coverage promotes vessel stabilization, preventing oedema or hemorrhagic complications[109]. Adventitial pericytes have been associated with vascular remodelling and neointima formation[110] and more recently recognized for their healing potential in preclinical studies of peripheral and myocardial ischemia [99, 111]. Last, pericytes would originate cardiac smooth muscle cells in a Notch3 dependent fashion[36].

An intriguing and still open question regards the relationship between cardiac pericytes and cardiac progenitor cells. Several lines of evidence indicate, at least, a close resemblance between the two cell types. Specifically, a common epicardial origin with pericytes has been described for a subset of Sca1 positive cells[66], cardiac MSC[60], and, possibly, human cardiac progenitors[67]. Moreover, both cardiac MSC[60] and human cardiac progenitors[46] coexpress some pericyte markers (e.g. NG2 and PDGFRβ) and share with fetal cardiac pericytes the expression of pluripotency genes[35]. Last, although cardiac pericytes expanded *in vitro* from fetal, neonatal and adult hearts have been reported not to express the stem cell marker cKit[34, 35], a vascular and perivascular localization has also been described for subsets of cKit[20, 112] and Sca1[57, 113] expressing cells. These differences could reflect the existence of distinct cell types *in vivo* that are possibly recruited to a perivascular localization, as suggested by [97]. However, a modification of the properties of the cells could have been also induced by protocols for their *in vitro* expansion.

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Therefore, since pericytes may regulate crucial processes required for the preservation of a normal cardiac structure and function (e.g. angiogenesis, blood flow, vessel stability, maturation, and vascular permeability, as well as production of trophic factors), we recently experimented their possible use for the treatment of acute myocardial infarction[111]. Specifically, pericytes derived from a large vessel (e.g. saphenous vein leftovers collected at time of coronary artery bypass grafting surgery) were implanted in infarcted mouse hearts.

4)   Cardiac regeneration from a clinical perspective.

The notion that the heart possesses some degree of renewal potential has opened the avenue to restorative therapies especially for the increasing number of patients with heart failure (HF). In the US, adults with the disease could reach 8 million by 2030, up from 5.7 million in 2012, while the cost of treatment could more than double, from $31 billion to $70 billion. The mainstays of novel regenerative treatment of ischemic HF consists of boosting cardiopoiesis and angiogenesis.

Cardiopoietic cell therapy has been introduced by the two seminal trials, the Scipio and the Caduceus. The Scipio reported initial encouraging results in humans, but several reporting bias have reduced the validity of the study[114]. The randomized CADUCEUS clinical trial showed a single administration of cardiosphere-derived cells significantly reduces the size of the scar that had resulted from a large heart attack, and concomitantly increases the muscle mass at the affected area[115, 116].

Several companies have now engaged into the cardiopoiesis arena. Capricor, a biotechnology company focused on the discovery, development, and commercialization of biological cardiovascular therapeutics, has conducted a Phase I/II HOPE clinical study based on CAP-1002 (allogeneic cardiosphere-derived cells, CDCs), whose results are expected to be released during 2017. The same company has developed CAP-2003 exosomes derived from CAP-1002 CDCs. The exosome technology is being developed under a license agreement with the Cedars-Sinai Medical Center. In October 2016, Capricor was launched a new research program on CAP-2003 exosome therapy for treating patients with hypoplastic left heart syndrome.

The CHART-1 trial from Celyad[117], a Belgian biotechnology company, represents the world's first Phase III trial for a pre-programmed cellular therapy targeting HF. It involves taking cells from a patient's bone marrow and through a proprietary process re-programming those cells so that

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they become heart precursor cells with the aim of replicating the normal process of cardiac development in the embryo and healing the failing heart. The cells are then injected back into the patient's heart through a minimally invasive procedure, with the goal of repairing damaged tissue and improving heart function, clinical outcomes and quality of life. This Phase III trial follows the successful outcome of the Phase II trial[118]. A report presented at the European Society of Cardiology Congress in 2016 showed there was no difference in deaths or worsening of heart failure between patients given Celyad's C-Cure and those given a sham procedure. The product did, however, benefit a subset of patients — those with severely enlarged hearts — so Celyad is refocusing on this subpopulation. But designing such a new trial requires a large investment from potential partners, which may be dissuaded to join the research due to the mixed signals about other cardiac stem cell trials.

Mesoblast has recently launched two trials based on allogeneic precursor cells. NCT02032004 (DREAM HF-1) is Double-blind, Randomized, Sham-procedure-controlled, Parallel-Group Efficacy and Safety Study of Allogeneic Mesenchymal Precursor Cells (Rexlemestrocel-L) in chronic HF due to LV Systolic Dysfunction (Ischemic or Nonischemic). In the first quarter of 2017, Mesoblast will release interim results on about 300 patients from this phase 3 study of 600 people. The Mesoblast NCT01781390 is Safety Study of Allogeneic Mesenchymal Precursor Cell Infusion in MyoCardial Infarction (AMICI) currently ongoing but not recluting patients.

Proangiogenic gene and cell therapies aim to stabilize the infarct scar and reduce its extension, by modulating the extracellular matrix remodeling, promoting cardiomyocyte salvage in the area at risk, and awakening the hibernated myocardium. Furthermore, the improvement in myocardial perfusion is expected to treat angina and improve exercise tolerance. The majority of studies used angiogenic -endothelial and hematopoietic- progenitor cells as an adjuvant therapy to optimally-treated reperfused myocardial infarction (MI) or chronic coronary artery disease (CAD), reporting mixed results[119-122]. Only a few studies have focused on developing a cell treatment for patients with non-revascularizable CAD. Seminal trials using hematopoietic CD133^{+} and CD34^{+} cells in refractory angina have provided initial evidence of feasibility with reported attenuation of angina[123-125] and improvement of SPECT perfusion score[126]. However, one study showed that cell mobilization caused cardiac enzyme elevations, suggestive of non-ST segment MI, in ~5% of patients, which instills safety concerns[123]. Angiogenesis gene therapy has been

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revitalized by the advent of significant improvements in vector technology[127]. Nevertheless, the incapacity of single growth factors to promote mature vessels represents a persistent limitation.

The combination of cardiopoietic and angiogenic progenitor cells could additively implement current regenerative strategies. However, there are few studies investigating combinatory cell therapy approaches. A recent report by Williams et al [128] combining human cardiac stem/progenitor cells (CSC) and bone marrow MSC in a swine model of MI showed that each cell therapy reduces MI size relative to placebo, with the MI size reduction being 2-fold greater in combination versus either cell therapy alone. These results are similar to those published by us using CSC and adventitial pericytes. In a study conducted in mice with non-reperfused MI, we showed that human adventitial pericytes and cKit+ CSCs in combination work better to reduce infarct size and collateralization. Moreover, while pericytes acted mainly by promoting angiogenesis, CSCs were superior in stimulating cardiomyocyte proliferation [46]. However, while Williams and colleagues showed that dual cell therapy substantially improved LV chamber compliance and contractility over the single cell treatments, we did not observe further improvements in cardiac function over single cell therapy. Moreover, as opposed to Williams' report (that identified a large effect on cell engraftment with the combined cell therapy), we were not able to demonstrate any improvement on this parameter. A possible difference between the two studies, that may account for the observed discrepancies, is the timing of cell administration. In fact, while we injected cells immediately after the coronary artery occlusion, Williams performed cell implantation 14 days after infarction. Despite these differences, we are confident that combination cell therapy approaches that employ stem cells obtained from surgical samples that are discarded during the procedures may represent one of the best opportunities for cardiac repair. Regarding the mechanism of action of the combined cell therapy, a cross-talk between pericytes and cardiac progenitors could also be documented, since implanted cells were able to promote the recruitment of both cKit+ cells *in vivo* and Sca1+ cells *in vitro*[46]. Furthermore, an interaction between saphenous vein-derived pericytes and cultured human cardiac progenitors was shown *in vitro*, being the crosstalk between the two cell types able to modify their secretome and promote the synergistic release of SDF-1α[46]. However, the molecular mechanism that is responsible for progenitor cell recruitment is under investigation. Concerning cardiopoiesis, CSC were able to increase the frequency of cardiomyocytes incorporating the thymidine analogue EdU and reduced cardiomyocyte hypertrophy. The mechanisms promoting this beneficial effect are still

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partially unknown. In fact, although, as anticipated, we observed low levels of cell engraftment and direct differentiation of the implanted cells towards the cardiomyocyte lineage, the secretome of both cell types has powerful biologic effects and may hide important and novel therapeutic factors.

5)   Conclusion

Optimized medical treatment and novel interventions have shown that cardiac remodeling may be, at least partially, reversed, suggesting that the heart has an inherent capacity for restoring its structure and function. Moving from these premises and supported by experimental results achieved both with cell therapy and in regenerating animal models, an entire field of investigation developed, that aims at identifying the best modalities to unlock the potential for myocardial regeneration. Almost two decades of investigation on cardiac resident primitive cells have delineated a complex picture, where the heart hosts one or few primitive cell populations, with overlapping mesenchymal characteristics, which are clearly distinguished from stem/progenitor cells residing in other tissues, whose embryological origin includes the cardiac neural crest and the epicardium. Mesodermal progenitors have been identified in several adult tissues and have, at least, a common perivascular localization with pericytes (and could be associated both with capillaries or with the adventitia of large vessels). Recent data indicate that tissue resident mesodermal progenitors/pericytes have distinguishing, organ specific, and possibly complementary properties. Preclinical data and clinical experimentation have demonstrated not only the feasibility, but also the potential benefit of cell therapy. Most importantly, the combination of cardiopoietic and angiogenic progenitor cells has been shown to promote the recruitment of cardiac resident progenitors, an increase in collateralization, and cardiomyocyte proliferation. The mechanisms responsible for this positive effect have not been completely elucidated, but their investigation has the potential to indicate novel methods to promote cardiac regeneration.

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Figure Legend

Figure 1. Localization of cells expressing putative stem/progenitor markers in adult mammalian hearts. Cartoon depicting the localization of putative stem cells or their derivative in the adults. Insets show a schematic view of the tissue histology of the relative dashed squares (A - C) superimposed to the picture. **A**: examples of the two subsets of Isl1 expressing cells that were identified in the adult heart[70, 129]. The left panel shows a cluster of Isl1 positive cells that is negative for cardiomyocyte markers, but may express neuronal ones. The rightmost panel shows a cluster of Isl1 expressing cells positive to cardiomyocyte markers. Isl1 is depicted in green, nuclei are shown in blue, cardiomyocyte cytoplasm is shown in pink with red striations, extracellular matrix is shown in cyan. **B**: cartoon showing an activated epicardium re-expressing the epicardial developmental markers Wt1 and Tbx18, as well as cKit[76]. WT1 and TBX18 are shown as pink and green dots in epicardial cell nuclei, respectively. cKit is shown as orange labeling of two epicardial cells. Nuclei are shown in blue, cardiomyocyte cytoplasm is shown in pink, extracellular matrix is shown in cyan. **C**: scheme showing the localization of cKit and Sca1 expressing cells in the myocardium. cKit expressing cells (labeled in orange) are shown to be localized in the endothelium, within the vessel wall, and in the intersitium. Sca1 positive endothelial and interstitial cells are shown in green. Adventitial and microvascular pericytes are shown in cyan and indicated by arrows. A close relationship between microvascular and advential pericytes and cardiac resident stem/progenitors is shown.

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ISL1

ISL1

WT1
TBX18
cKit

Activated
Epicardium

Sca-1
cKit
Pericyte

A

B

C