REVIEW ARTICLE

Beyond cardiomyocyte loss: Role of Notch in cardiac aging†

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Abstract

The knowledge of the cellular events occurring in the aging heart has dramatically expanded in the last decade and is expected to further grow in years to come. It is now clear that impaired function and loss of cardiomyocytes are major features of cardiac aging, but other events are likewise important. In particular, accumulating experimental evidence highlights the importance of fibroblast and cardiac progenitor cell (CPC) dysfunction. The Notch pathway regulates cardiomyocyte, fibroblast and CPC activity and, thus, may be critically involved in heart disease associated with advanced age, especially heart failure. In a translational perspective, thorough investigation of the Notch system in the aging myocardium may lead to the identification of molecular targets for novel therapies for age-related cardiac disease.

Keywords: cardiac; aging; fibroblast; fibrosis; cardiac progenitor cells; regeneration; Notch.

Introduction

Aging profoundly affects cardiac structure and function (Chen and Frangogiannis, 2010). Age-related remodelling of the heart contributes to the limitation in exercise capacity typical of the elderly. Furthermore, it often prepares the ground for the development of cardiac disease, especially heart failure (HF), but also arrhythmia and ischemic heart disease. Cardiac deterioration during the late phase of life may be in part ascribed to cardiomyocyte impairment and death, which have been the focus of research for many years in the field of cardiac aging (Sheydina et al, 2011). More recently, two major conceptual breakthroughs have opened new perspectives in the study of the mechanisms that make the heart old and predispose to HF and other types of cardiac disease: i) fibrosis is as important for cardiac pathology as cardiomyocyte dysfunction or loss, and ii) the evidence that the heart can, in principle, regenerate.

The first two paragraphs of this review will address these two aspects with respect to cardiac aging. As a consequence of understanding the importance of fibrosis and heart regeneration, interest has arisen in the identification of signaling pathways affecting cells responsible for these processes, i.e. fibroblasts and cardiac progenitor cells (CPC), respectively. This appears to be the case with the Notch system, which is discussed in the third paragraph. This review is a result of a systematic literature search by Pubmed (simple subjects search) using the following key concepts: Notch receptors, Notch ligands, heart, aged endothelium, aged heart, cardiac progenitor cells, aging, fibrosis, myofibroblast.

1. Cardiac Fibrosis and Aging

The complexity of heart function is accounted for by a complicated tissue organization, where cardiomyocytes are bundled together in close spatial relation with several other cell types, among which a major role is played by fibroblasts and the intramural coronary vasculature. This cytoarchitecture is ensured by the extracellular matrix (ECM), which enwraps cardiac cells and vessels, connecting one to another, and continues with the *chordae tendineae, valve annuli* and leaflets (Bowers et al, 2010;Rienks et al, 2014;Li et al, 2014). It is now established that ECM does not simply provide physical support to cardiomyocytes, but also exerts functional roles. Indeed, it keeps myofibers aligned and prevents their overstretching or slippage, integrates the contraction of individual cardiomyocytes into coordinated apical-to-basal cardiac shortening, and avoids chamber deformation when blood pressure or volume increase (Bowers et al, 2010;Rienks et al, 2014;Li et al, 2014). Furthermore, binding of components of the ECM to membrane receptors and the cytoskeleton of parenchymal cells converts mechanical stimuli into biochemical signals. The ECM is made of proteins and other macromolecules, such as glycoproteins and proteoglycans (Bowers et al, 2010). Collagen is the most abundant protein within the ECM; the enzyme lysil oxidase crosslinks extracellular collagen to form collagen fibrils, which then further assemble in very resistant fibres. The amount of ECM is the result of the balance between new synthesis and degradation. If ECM deposition outweighs breakdown, fibrosis ensues (Li et al, 2014;Rienks et al, 2014). Traditionally, fibrosis that develops following cardiomyocyte necrosis is referred to as “reparative”, while the term “reactive” is used to indicate fibrosis involving the perivascular spaces and interstitium and not initiated by cardiomyocyte death. Activated fibroblasts, namely myofibroblasts, are primarily implicated in either form of fibrosis (Goldsmith et al, 2014). In physiological conditions, the presence of

myofibroblasts in the adult heart is limited to valvular leaflets. Following injury or disease, the number of myofibroblasts dramatically increases mainly because of the activation and differentiation of resident fibroblasts. Myofibroblasts produce ECM by default, as a protective reaction aiming at maintaining tissue homeostasis (Goldsmith et al, 2014;Li et al, 2014;Rienks et al, 2014). In case of myocardial infarct (MI), this process leads to the formation of a scar that protects against ventricular wall rupture: if the heart is exposed to pressure or volume overload, myofibroblast-driven fibrosis cooperates with cardiomyocyte hypertrophy in diminishing parietal stress. Nevertheless, fibrosis becomes detrimental in the long term (Burchfield et al, 2013). Indeed, at the cellular level, it entraps cardiomyocytes, hindering oxygen and nutrient supply and causing atrophy, while predisposing to arrhythmia by interfering with the transmission of electrical impulses. In addition, myofibroblasts release factors that alter cardiomyocyte function via paracrine modulation or through heterocellular myofibroblast-cardiomyocyte gap junctions. As far as cardiac mechanics is concerned, accumulation of ECM may eventually stiffen the heart or impair the generation of an integrated contractile force, leading to diastolic or systolic dysfunction, respectively.

Current evidence indicates that aging is characterized by enhanced cardiac fibrogenesis (Chen and Frangogiannis, 2010). The old heart contains more collagen than the younger one and experimental data suggest that ECM degradation decreases with aging. Furthermore, it has been reported that collagen cross-linking is increased (de Souza, 2002). These abnormalities in ECM turnover are paralleled by recruitment and activation of fibroblasts. Several signals may lead to this fibroblast dysregulation, among which transforming growth factor (TGF)-β and angiotensin II have long been known (Brooks and Conrad, 2000;Billet et al, 2007). As new factors stimulating myofibroblast generation and activity are identified, it becomes evident that the

signaling networks responsible for age-related fibrosis are extremely complex and, at least in part, specific to the fibroblast lineage. For instance, chronic low-grade inflammation has been shown to be a key player in inducing myofibroblast differentiation from myeloid precursors (Cieslik et al, 2011). On the other hand, the low-density lipoprotein receptor, LOX-1, has been inversely correlated with cytoskeletal disorganization, reduced proliferation, and increased collagen secretion in aged cardiac fibroblasts (Wang et al, 2013), despite this receptor being classically induced by pro-inflammatory cytokines. While fibrogenesis is basally enhanced, the fibrotic response to MI in preclinical murine models appears to be paradoxically impaired with aging (Bujak et al, 2008;Cieslik et al, 2013). Fibroblasts infiltration of the infarcted area is significantly lower in old than young animals and, consistently, collagen deposition is decreased. As a result, the scar that forms is defective and maladaptive dilative cardiac remodeling occurs. Efforts have been put to solve this apparent conundrum, i.e. that baseline fibrosis is augmented in the aging heart, while the one induced by MI is blunted. A possible explanation resides in the fact that myofibroblasts derive from different cell populations. Specifically, it has been proposed that a population of non-myeloid mesenchymal stem cells (MSCs) within the myocardium becomes unresponsive to TGF-β with age, because of TGF-β receptor downregulation. On one hand, this causes these MSCs to lose their ability to differentiate into myofibroblasts, giving rise to fibroblasts which retain TGF-β resistance and thus express higher levels of monocyte chemoattractant protein-1 (MCP-1). In turn, MCP1 promotes the migration of leukocytes into the cardiac tissue and their differentiation into myeloid myofibroblasts; as a consequence, fibroblast population substantially expands. On the other side, non-myeloid MSC-derived fibroblasts are dysfunctional and synthetize less collagen upon stimulation by TGF-β. As a result, fibrotic reaction to MI is hindered (Cieslik et al, 2014), (Figure 1). As further discussed below,

attenuation of Notch1 signaling may contribute to the increased fibrogenesis and blunted regenerative response observed in the aged heart. One mechanism by which Notch1 may ameliorate cardiac fibrosis is inhibition of TGF-β signaling, which was shown to prevent fibroblast-myofibroblast transition (Sassoli et al, 2013). However, because resistance to TGF-β stimulation leads to MCP-1 production by aged MSCs (Cieslik et al, 2014), it cannot be excluded that also Notch1-induced suppression of TGF-β signaling favors MCP-1 release by these cells, consequently promoting leukocyte recruitment and differentiation into myeloid myofibroblasts.

2. Cardiac Progenitor Cells and Aging

Historically, the central dogma of cardiac biology has been based on the assumption that the heart is a terminally differentiated organ without regenerative potential, with cardiac hypertrophy being only secondary to enlargement and hypertrophic growth of pre-existing resident cardiomyocytes (Karsner et al, 1925;Leri et al, 2014). Cardiovascular disease is still a major socio-economic burden in Western countries, with MI the most common cause of cardiac injury. Although MI mortality rate has significantly decreased in the last years, thanks to significant progress of interventional cardiology, a growing number of MI survivors are at high risk of developing HF, especially as they become older. Unfortunately, in this scenario the ultimate cure is still represented by heart transplantation, which is limited by shortage of donors, side complications and is not a feasible option for elderly patients (Braunwald, 2013).

Nevertheless, the identification in 2003 of a population of endogenous CPC residing within the rat heart and endowed with stem-like properties and the potential of supporting myocardial regeneration following MI (Beltrami et al, 2003), has revolutionized cardiac medicine. This finding challenged the idea of the adult heart as a terminally differentiated organ without

restoration potential, introducing the novel concept of multipotent CPC residing in niches scattered within the myocardium. Such hypothesis opened up to the fast development of CPC biology, by identifying their phenotype upon the expression of specific stem cell-related (c-kit, Sca-1) and/or early cardiac developmental (Isl1, Nkx2.5) markers, their *in vitro* culture properties (cardiospheres and cardiosphere-derived cells), or their tissue origin (epicardium-derived progenitor cells, EPDC), as extensively reviewed elsewhere (Bollini et al, 2011). CPC represent a small, yet promising, reservoir of endogenous immature progenitors within the adult myocardium which, upon injury or appropriate stimulation, can either differentiate into the three main cardiovascular lineages to replace damaged cells, or can exert beneficial paracrine effects by releasing soluble molecules, overall resulting in a significant improvement of cardiac function (Bollini et al, 2011;Feng et al, 2012). So far, CPC have mainly been exploited for cell therapy approach, as they can be easily isolated from endocardial/myocardial biopsy obtained during cardiac surgery interventions and expanded *in vitro* prior to being transplanted back in the injured heart, exerting beneficial effects on the whole organ function (Barile et al, 2007;Messina et al, 2004;Bolli et al, 2013;Matsuda et al, 2013). Indeed, clinical trials have already been carried out using either c-kit+ (SCIPIO trial) and cardiosphere-derived (CADUCEUS trial) CPC in patients with ischemic HF or left ventricular dysfunction, showing safety and feasibility of autologous human CPC injection with reduction of the infarct size, increase of viable mass, and improvement of left ventricular function (Malliaras et al, 2014;Chugh et al, 2012). Alternatively, the endogenous regenerative programme of resident CPC can also be activated by appropriate stimulation with soluble factors by paracrine therapy, thus avoiding *in vitro* manipulation and *in vivo* transplantation (Urbanek et al, 2005;Rota et al, 2008;Aghila Rani and Kartha, 2010;Croquelois et al, 2008;Smart et al, 2011;Limana et al, 2005). Hence, CPC represent a

regenerative reservoir that may be activated for therapeutic purposes. Notably, CPC activation is widely active in both lower vertebrates (such as the zebrafish) and in neonatal mammals. Indeed, activation and cardiovascular differentiation of CPC following injury has been demonstrated to be much stronger and responsive in the neonatal mouse heart compared to the adult (Jesty et al, 2012) and some specific subpopulations, such as the EPDC, become completely quiescent and unresponsive soon after birth (Smart et al, 2007), unless specifically stimulated via cardioactive paracrine effectors (Smart et al, 2011) this suggesting that age can significantly affect CPC biology.

Since resident CPC are physiologically retained in low amount and with limited injury response in pathological situations, different strategies have been investigated in order to enhance their regenerative potential - either by their direct transplantation into the myocardium or via paracrine stimulation - as summarized in Table 1.

By means of both in vitro and in vivo methods, several key aspects of human, rodent and swine CPC biology have been addressed, including the improvement of their survival and proliferative capacity, their differentiation potential and paracrine influence, with the latter mediating relevant cardioprotective and healing effects. Indeed, preconditioning strategy by using specific substances or stimuli, such as hypoxia (Yan et al, 2012;Cai et al, 2012), or by over-expressing the apurinic/apyrimidinic endonuclease/redox factor 1 (APE1, (Aonuma et al, 2016), have shown to promote CPC survival via expression of pathways involving phospho-Akt, Bcl2, TAK1 and NF-kB activation, while enhancing the transplanted cell therapeutic effect and increasing cardiac function through the activation of the SDF-1a/CXCR4 axis and downstream pro-survival pathways (Yan et al, 2012). Likewise, in vitro stimulation by SCF, the TGFβRI inhibitor A83-01 along with ALK5 silencing, and microRNA-21 transfection have also been applied to enhance

CPC proliferative potential, resulting in the activation of PTEN/PI3K/AKT, MAPK and MERK/ERK signaling (Ho et al, 2016;Shi et al, 2017;Vajravelu et al, 2015). Distinctive paracrine effects relevant to cardiac repair mechanisms have also been reported following in vivo transplantation of human and rodent cardiosphere-derived CPC into the injured murine cardiac tissue, including: i) significant inhibition of cardiomyocyte apoptosis; ii) improvement of local angiogenesis and iii) of cardiac function by TGF-β1/Smad signaling blocking, along with iv) the holding back of pathological ventricular remodeling and v) the local secretion of growth factors such as VEGF, IGF-1 and HGF, (de Couto et al, 2015;Malliaras et al, 2012;Tseliou et al, 2014). As well, growing interest has been dedicated towards the immunomodulatory competence of CPC in both xenogeneic and allogeneic transplantation preclinical models of rat myocardial infarction in order to define safety and efficacy of putative future cell therapy with mismatched progenitors. While rat allogeneic CPC showed to elicit negligible lymphocyte proliferation in vitro, xenogeneic human progenitors induced a strong response from the immune cells. This trend was further confirmed in vivo when showing acute rejection of human CPC within one week from injection and limited survival of allogeneic ones as well. Nevertheless, the treated rat cardiac tissue presented increased secretion of trophic factors, smaller scar size and recruitment of endogenous c-kit^{+} cells, respectively, overall suggesting a more specific paracrine role for the transplanted CPC, as supported by rare cardiomyogenic and angiogeneic differentiation activity (Malliaras et al, 2012). Indeed, more recent results further emphasized the low retention of transplanted rat CPC, yet showing macrophage polarization to cardioprotective phenotype away from M1 lineage (de Couto et al, 2015). Remarkably, transplanted CPC have been reported to trigger relevant regenerative modulatory influence in stimulating resident cardiomyocyte proliferation and cell cycle progression via the expression of Cyclin D1, CdK4, Cyclin E, CdK2,

Cyclin A1-2 and E2F1 (Malliaras et al, 2012;Malliaras et al, 2013a;Malliaras et al, 2013b;de Couto et al, 2015) .

Restoration of the embryonic programme of murine resident epicardial CPC has also been suggested by paracrine stimulation with the small peptide thymosin beta 4 in a mouse myocardial infarct model, as a proof of principle of reactivation of endogenous mechanism of regeneration, overall resulting in cardiomyocyte, endothelial and smooth muscle differentiation, up-regulation of phospho-SMAD1/5/8, phospho-SMAD2, SNAIL, SLUG and reactivation of the Wt1 and Raldh2 embryonic genes via C/EBP proteins, combined with CPC increased paracrine potential (Huang et al, 2012;Smart et al, 2007;Smart et al, 2011;Zhou et al, 2011).

Notably, more recent studies have revealed that extracellular vesicles released by CPC, including exosomes, can recapitulate most of the significant paracrine effects exerted by these cells (including reprogramming of fibroblasts into a less fibrotic phenotype by suppression of phosphorylated Smad 2/3,4, Snail1 and increased secretion of SDF-1 and VEGF), possibly via direct transfer of their RNA content (i.e. microRNAs like miR-210 and miR-132, or Y RNA fragment), thus suggesting a new cell-free approach to still obtained progenitor-mediated beneficial and regenerative effects (Barile et al, 2014;Cambier et al, 2017;Ibrahim et al, 2014;Tseliou et al, 2015).

Noteworthy, HF and cardiovascular disease represent the most common cause of hospitalization for patients over 65 years. Aging is associated with a progressively increased risk of ischemic coronary disease and MI (Thomas and Rich, 2007). Hence, the impact of aging on CPC biology and on their regenerative potential has to be evaluated in details in order to identify a working strategy. Several preclinical and clinical evidences support the hypothesis that CPC residing in the old heart might be affected by aging, showing impaired cell function and becoming less

responsive to external stimulation, thus penalizing any possible regenerative strategy in the event of injury (Hu et al, 2014). In particular, nucleostamin and Pin1 have been lately identified as playing a key role in maintaining CPC function, as their silencing has revealed to significantly induce premature cell senescence and to affect their proliferative potential (Table 1)(Hariharan et al, 2015;Toko et al, 2014) .

While the number of human CPC seems to increase in the old myocardium, especially in women (Kajstura et al, 2010), a recent study showed that the percentage of human c-kit+ CPC can be negatively correlated with aging, especially when associated with age-related disease, such as diabetes mellitus and coronary heart disease, resulting in the depletion of the progenitor pool, overall affecting the endogenous heart potential (Hu et al, 2014). Therefore, a growing body of evidence suggests that the aged heart can be compromised from chronological CPC aging. Indeed, within the old myocardium the migratory capacity of human c-kit+ CPCs declines with a putative mechanism described in the alterations of signaling regulated by the receptor EphA2 controlling human CPC motility, which, in turn, are caused by age-associated accumulation of reactive oxygen species and a significantly higher oxidative stress. Besides, old human CPC with altered trafficking are unable to translocate within the myocardial tissue, with important consequences for cardiac repair (Goichberg et al, 2013). Likewise, senescent CPC, which have lost their telomerase activity because of aging, may give rise to structurally old cardiomyocytes destined to become less functional, with severely depressed mechanical performance and a marked tendency to apoptosis, along with the manifestation of an aging cardiac phenotype (Kajstura et al, 2010). Cardiosphere-derived cells (CDC) isolated from aged mice showed a decline in the expression of CDC stem markers, such as c-kit and Sca-1, together with reduction

of their clonogenic potential, suggesting failure of regenerative potential with age (Hsiao et al, 2014).

Thus, there is mounting claim for finding novel approaches besides current conventional medical care. Indeed, to be clinically feasible, CPC must be isolated and expanded from aged and/or diseased tissue. In such scenario, CPC functional lifespan might be ensured via the appropriate stimulation by protective soluble molecules (such as insulin-like growth factor-1) preserving telomere length and the regenerative potential (Siddiqi and Sussman, 2013); pharmacological approaches based on ACE-inhibitors blocking the angiotensin II signaling and oxidative stress affecting CPC, have also been suggested (Cesselli et al, 2013). Moreover, novel strategies aiming at preserving the pool of competent CPC might be based on defining the specific environmental clues and molecules orchestrating and supporting the transient regenerative potential of the early/neonate heart, a process that has been showed to temporally correlate with the existence of functional CPC. This might represent an ideal strategy to maintain and restore the progenitor potential while correcting their impairment due to the aging process and temporal limitations (Jesty et al, 2012;Zaruba et al, 2010;Beltrami et al, 2012).

3. Notch Signaling in the Heart

The Notch pathway is an ancient system of communication between adjacent cells. In mammals there are four Notch receptors (Notch 1-4) and five ligands (Delta-like ligand (Dll)1, 3, 4 and Jagged1 and -2) located on cell surface and characterized by the presence of a DSL (Delta, Serrate, and Lag2) domain required to interact with Notch (D'Souza et al, 2010). Binding of a ligand to the receptor triggers two proteolytic cleavages releasing the active form of Notch (NIC), which binds to the transcription factor recombination signal binding protein for

immunoglobulin kappa J region (RBP-Jk) and regulates the transcription of genes related to cell proliferation, survival, and cell-type specification. The most studied Notch target genes belong to Hes and Hey families, which are negative regulator of transcription (Espinoza and Miele, 2013), (Figure 2) and (Table 2). Recent studies have shed further light on the complexity of Notch signaling and have shown that the pool of genes modulated by Notch is larger than previously thought, greatly differs among cell types, and is context-dependent in the same cell (Andersson et al, 2011). Furthermore, non-canonical ligands have been identified lacking the DSL domain and comprising a group of structurally diverse proteins that include integral and glycosylphosphatidylinositol (GPI)-linked membrane as well as secreted proteins (D'Souza et al, 2010).

Notch plays a major role during heart development, patterning the embryonic endocardium, enabling region-specific differentiation and critical interactions of the endocardium (or its derived mesenchyme) with other cardiac tissues (like cardiac neural crest, myocardium), so that specialized structures (as cardiac valves and chambers) are generated (D'Amato et al, 2016b;MacGrogan et al, 2016;Luxan et al, 2016;D'Amato et al, 2016a), (Figure 2) and (Table 2). A large body of evidence suggests that Notch exerts a critical function in the adult overloaded or damaged myocardium as well, even though less is known about Notch role within the heart during post-natal life compared to embryonic development (Ferrari and Rizzo, 2014), (Figure 2) and (Table 2). Neonatal cardiomyocytes rapidly proliferate within the very early post-natal stages and express high levels of Notch1. Conversely, in the adult myocardium these cells lose the ability to proliferate and down-regulate Notch signaling. Notch1 is reactivated in cardiomyocytes located in the MI border zone or in the overloaded myocardium, where it

counteracts cardiomyocytes apoptosis and hypertrophy (Ferrari and Rizzo, 2014);Nistri et al, 2017). Consistently, the expression of components belonging to its signaling pathway has also been observed in myocardial biopsies from HF patients (Ferrari and Rizzo, 2014). However, it is possible that Notch activation following cardiac injury is protective only if temporary, since Campa et al. have shown that prolonged activation of Notch1 in cardiomyocytes is detrimental and induces apoptosis (Campa et al, 2008). In agreement with the hypothesis suggesting that prolonged and/or dysregulated Notch signaling could be detrimental for heart function, elevated levels of non-canonical Notch ligand periostin were found to be associated to myocardium fibrosis (Zhao et al, 2014) and to symptoms severity in dilated cardiomyopathy (Norum et al, 2017) and, similarly, elevated levels of canonical Notch ligand Dll1 have been linked to worse prognosis of dilated cardiomyopathy (Norum et al, 2016) , HF (Norum et al, 2017) and symptomatic aortic stenosis (Abraityte et al, 2015).

Notch activation in the damaged myocardium has been linked to CPC and MSC regulation (Ferrari and Rizzo, 2014). Indeed, Notch1 is present on the membrane of CPC in its inactive form and, following MI, it gets activated by Jagged1 exposed on the surface of adjacent cardiomyocytes. Notch activation, in turn, induces the transcription factor Nkx2.5, which is involved in the expression of early cardiomyogenic transcripts and in the inhibition of vascular cells markers (Boni et al, 2008); it also stimulates CPC growth, survival and differentiation via mTORC1, while enhancing their lineage commitment and protective signaling (Gude et al, 2015). Furthermore, in a mouse model of pressure-overloaded myocardium, overexpression of Jagged1 by cardiomyocytes favoured the CPC differentiation into Nkx2.5-cardiac precursor cells, while inhibiting myofibroblast proliferation and cardiac fibrosis mediated by TGF-

β/connective tissue growth factor (Nemir et al, 2012). Notably, while inhibition of fibrosis by Notch has also been observed *in vitro*, where activation of Notch interfered with TGFβ-induced cardiac fibroblast-into-myofibroblast transition (Fan et al, 2011;Sassoli et al, 2013), a population of GFP-genetically labelled epicardial CPC from transgenic Notch reporter (TNR) mice showed a dynamic Notch-dependent activation following myocardial injury - obtained as either by myocardial infarction or by thoracic aorta banding – with modest cardiogenic potential and a more pronounced commitment to the fibroblast lineage (Russell et al, 2011).

Notch plays a major role also in the regulation of MSC, as suggested by a study showing that deletion of Notch1 in these cells impairs their recruitment, proliferation, and survival leading to decreased ability to repair the myocardium damage compared to MSC with functional Notch1 signaling (Ferrari and Rizzo, 2014). Consistently, pathological conditions such as HF affect MSC functions and deregulates Notch pathway (Ferrari and Rizzo, 2014). The influence of Notch on triggering the MSC cardioprotective potential could be synergic to CPC activation, as suggested by an *in vitro* study showing enhanced proliferation of immature cardiomyocytes and Notch1 activation by co-culture with Jagged-1 expressing MSC (Sassoli et al, 2011). The critical role of Notch in stem cell-mediated cardiac repair has also been investigated in a preclinical mouse model of MI following doxorubicin treatment (DOX-MI mice) (Merino and Singla, 2014). The study showed the pivotal role of Notch in mediating the protective effect obtained following transplantation of embryonic stem (ES) and induced pluripotent stem (iPS) cells, overall improving heart function and reducing adverse cardiac remodelling.(Merino and Singla, 2014).

In addition to fibrosis, down-regulation of calcium-handling proteins with decreased intracellular Ca^{2+} decay and altered oxidative balance represent hallmarks of age-related cardiac diastolic

dysfunction (Loffredo et al, 2014). Crosstalks between the Notch and Ca^{2+} signaling networks have been observed in cardiomyocytes (Kasahara et al, 2013) and, in leukemia cells, inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) impairs the maturation and activity of Notch1 (Roti et al, 2013). Similarly, there is evidence of reactive oxygen species (ROS) modulation by Notch in the myocardium. *In vitro* studies have shown that moderate oxidative stress induces the expression of cardiac markers in MSC through the activation of Notch signaling (Boopathy et al, 2013) and that Notch deficiency aggravates postburn myocardial injury through increased ROS levels (Cai et al, 2016).

Notch signaling is active in the endothelial (EC) and vascular smooth muscle cells (VSMC) of cardiac vessels. The Notch pathway controls angiogenesis and protects the endothelium from dysfunction caused by inflammatory cytokines, ischemia, and turbulent blood flow (Rizzo et al, 2012); moreover, it controls proliferation, survival, and function of VSMC (Ferrari and Rizzo, 2014). Studies in mouse model of MI have shown that activation of Notch1 signaling improves cardiac function by promoting myocardial angiogenesis (Ferrari and Rizzo, 2014;Lassaletta et al, 2014).

Notch in the Aged Heart

Whether and how Notch signaling is affected in the aging heart has not been extensively investigated yet (Figure 2) and (Table 2). Lower expression of Notch1, Jagged1, and Delta-like ligand 1 has been observed in skeletal muscle biopsies from older men compared with muscle from younger men (Carey et al, 2007). The reduced activation of Notch in the skeletal muscle impairs its regeneration and exposure of satellite cells from old mice to serum of young ones

enhances the expression of the Notch ligand (Delta), increases Notch activation, and enhances their proliferation *in vitro* (Conboy et al, 2003;Conboy et al, 2005).

Hastening of cardiomyocyte apoptosis and senescence have been proposed as mechanisms underlying decreased hemodynamic performance and increased risk of HF in the elderly (Sussman and Anversa, 2004). Despite Notch signaling components were not found among the age-regulated genes in mouse ventricular cardiac muscle cells (Bodyak et al, 2002), the hypothesis that aging impairs Notch activation in cardiomyocytes under ischemic or overloaded conditions or in other myocardial cell types, cannot be excluded. Consistently, microarray studies have shown that ischemic stress generates a much greater degree of contractile impairment and cellular damage in aged versus young hearts and these changes are associated with selective changes in transcription levels of Ca^{2+}, Wnt, Notch, and G-protein coupled receptor signaling pathways in aged versus young hearts (Ashton et al, 2006). In aging mice, the impairment in MSC function has been linked to Notch inhibition (Mutyaba et al, 2014). Old mice exhibit a decrease in MSC number, with limited proliferation, adipogenesis, and inconsistent osteogenesis associated to decreased basal Notch signaling activity, even though these cells are fully responsive to Jagged1 stimulation (Mutyaba et al, 2014). Taken together, these studies suggest that aging could interfere with the activation of Notch required to reduce pathological remodeling in the damaged myocardium.

The effects of aging on Notch within the vasculature have been investigated in a rat model of thoracic aorta injury by balloon catheter, in which aging-exaggerated proliferation of VSMCs has been linked to the attenuation of Jagged1 expression in EC (Wu et al, 2008). In this artery injury model, the interaction of Jagged1 on EC with Notch3 on VSMC in the intima prevented VSMC proliferation and vessel stiffening (Wu et al, 2011). In addition to excessive proliferation,

reduced apoptosis of VSMC plays a key role in aging-associated enhanced response to vascular injury. VSMC co-cultured with senescent EC, expressing reduced levels of Jagged1 compared with young EC, exhibited decreased susceptibility to H_2O_2-induced apoptosis compared with those co-cultured with young EC (Qian et al, 2011). It is unknown whether Jagged1 downregulation is involved in the exaggerated neointimal hyperplasia after percutaneous coronary intervention in the elderly as well as in aging-related vascular remodeling and stiffening . Dysregulation of Notch signaling could play a role in the progressive calcification of the aortic valve, which affects a large number of people over 65-years old, since elevated Notch1 levels and enhanced Notch1 activation were found to play a major role in augmentation of the pro-osteogenic response of interstitial cells of stenotic valves (Zeng et al, 2013).

Aging is associated with impaired vascular endothelial function (Donato et al, 2007), which is implicated in the HF pathophysiology, particularly of HF with preserved ejection fraction (Lam and Brutsaert, 2012). As previously discussed, the Notch pathway has a protective role in the endothelium (Rizzo et al, 2012) and there is evidence of an age-related endothelial Notch dysregulation. During vein graft adaptation to the arterial environment, both Dll 4 and Notch4 expression were found to be downregulated in an endothelial aged background, compared to a young one, and loss of Notch4 was linked to loss of attenuation of *neointima* (Kondo et al, 2011). Currently, it is not known whether aging leads to downregulation of endothelial Notch1, which is required to prevent the expression of calcification in the endothelium of aortic valve (White et al, 2015). If this were the case, age-related calcific aortic stenosis could be linked to dysregulation of Notch in both VSMC and endothelial cells.

Gender is associated to differences in symptoms and susceptibility to specific cardiovascular diseases which have been ascribed to gonadal hormones (Arnold et al, 2017). Specifically pre-menopausal women are protected against ischemic heart disease compared to age-matched men and this protection disappears after menopause due, as suggested by many studies, to the dropping levels of estrogens (Hayward et al, 2000). The Notch signaling is modulated by estrogens in estrogen receptor positive- breast cancer cell lines (Rizzo et al, 2012), in neuronal (Ruiz-Palmero et al, 2011) and in endothelial cells (Caliceti et al, 2013). In endothelial cells, treatment with 17β-estradiol increases the levels of active Notch1 (Caliceti et al, 2013) which is required to protect the endothelium against TNF α-induced apoptosis (Fortini et al, 2017). Considered the protective role of Notch in the endothelium (Theodoris et al, 2015;Pannella M et al, 2014;Briot et al, 2015), the increased risk of ischemic heart disease in post-menopausal women (Hayward et al, 2000) and in breast cancer women undergoing aromatase inhibitor treatment (Seruga et al, 2014;Abdel-Qadir et al, 2016), could be related to a decrease of endothelial Notch1 signaling caused by low levels of estrogens. Similarly, since estrogens controls the expression of periostin in breast cancer (Ratajczak-Wielgomas et al, 2017), the lack of estrogens in post-menopausal women could lead to elevated levels of periostin, thus affecting cardiac function (Norum et al. 2016). Studies in patient are needed to assess the involvement of Notch in the observed differences in cardiovascular risk or symptoms among genders.

4. Conclusions

The knowledge of cellular events occurring with age in the heart has dramatically expanded in the last decade and is expected to further grow in years to come. It is now clear that impaired activity and apoptosis or necrosis of cardiomyocytes are major features of cardiac aging, but

other events are likewise important. In particular, accumulating experimental evidence highlights the importance of basal activation of fibroblasts with fibrosis in spite of a defective response to injury, as well as of depletion and dysfunction of CPC. Detailed investigation of the signaling pathways involved in all these cellular abnormalities, such as the Notch one, may allow the discovery of novel therapies for age-related cardiac disease. As discussed in the previous paragraphs, the activation of myocardial Notch i) prevents the transformation of cardiac fibroblasts in myoblasts, ii) promotes the proliferation of CPC, and iii) favours their differentiation into cardiomyocytes, rather than myofibroblasts. In resemblance with what observed in the vascular endothelium, in which aging attenuates Jagged1 expression, it would be of great interest to determine whether fibrotic and reduced regenerative responses in the aging heart are caused by the attenuation of Notch signaling due to reduced levels of Jagged1. The weak responsiveness to TGF-β in the aging heart would be consistent with this hypothesis, since it has been observed that, at least in MSC, TGFβ induces Jagged1 (Kurpinski et al, 2010). Aging-related and attenuated expression of endothelial Jagged1 could potentially affect also angiogenesis and endothelium functions, thus promoting the pathological remodeling of the aged heart. In this scenario, reinstating the pre-existing levels of Jagged1 could help preventing altered fibrotic and regenerative response in the aged myocardium (Figure 3).

Conflict of interest

None of the authors has conflicts of interest to declare.

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Figure Legend

Figure 1. Effects of aging on cardiac fibroblasts. Downregulation of the oxidized low density lipoprotein receptor-1 (LOX-1) in aged fibroblasts has been associated with reduced proliferation, cytoskeletal disorganization and increased collagen secretion. Differentiation of precursors into myofibroblasts can be induced by a variety of stimuli (grey boxes). Cardiac mesenchymal stem cells (MSC) of non-myeloid origin become unresponsive to transforming growth factor (TGF)-β with aging, and thus they loose their ability to differentiate into myofibroblasts. Instead, they give rise to mesenchymal myofibroblasts which produce less collagen in response to TGF-β and release large amounts of monocyte chemoattractant protein-1 (MCP-1). MCP-1 promotes the migration of leukocytes in the cardiac tissue and their differentiation into myeloid myofibroblasts. Dysfunction of mesenchymal fibroblasts and expansion of the myeloid myofibroblasts pool may account for the impairment in scar formation and the enhancement in interstitial collagen deposition observed in the aged heart. Finally, cardiac injury was shown to induce epicardial cardiac progenitor cells (CPC) to commit to the fibroblast lineage via Notch1 activation

Figure 2. Expression of isoforms of Notch receptors , ligands, effector genes and proteins involved in the modulation of this pathway in fetal, adult and aged heart. The names of the enzymes involved in Notch processing are shown in red. The question mark indicates lack of data on that particular protein/gene. The maturation process of the Notch receptor involves cleavage in the Golgi complex by Furin. The activation of Notch signaling pathway is mediated by a direct contact between ligand and the extracellular domain of the receptor. This interaction

triggers two proteolytic cleavages by ADAM and the γ-secretase releasing the intracellular domain of the Notch receptor (NIC), NIC translocates into the nucleus and interacts with the transcription factor RBP-JK, and converts it into a potent transcriptional activator of downstream target genes Abbreviations: ADAM, A Disintegrin And Metalloprotease; CM, cardiomyocytes; CSC, cardiac stem cell; Dll1-4, Delta-like ligands 1,4; DLK1, Delta-like 1 homologue; DLX2, Deltex E3 Ubiquitin Ligase 2; EC, endothelial cells; ER, endoplasmic reticulum; Hey, hes related family bHLH transcription factor with YRPW motif; Hes, hes family bHLH transcription factor; MAM, transcriptional coactivator Mastermind; MF, myofibroblasts; MSC, mesenchymal stem cells; NFL, full lenght Notch; NIC, intracellular active Notch; POFUT1, protein O-fucosyltransferase 1; RBP-Jk, transcription factor recombination signal binding protein for immunoglobulin kappa J region; S, serum; VSM, Vascular Smooth Muscle Cells; WH, whole heart.

Figure 3. Effects of aging on cardiac Notch pathway. The aged heart is characterized by increased fibrosis and reduced regenerative response affecting cardiac function and setting the stage for pathological remodelling. These processes are regulated by Jagged1-activated Notch signaling. Thus, Jagged1 could represent a new therapeutic target to reduce fibrotic response and replenish the CPC pools within the aging myocardium (CPC, green; myofibroblasts, red; cardiomyocytes, pink; collagen fibers, red/green rods).

Table 1. Summary of experimental and preclinical studies showing CPC regenerative potential.

CPC	*In Vitro* Model	*In Vitro* Results	*In Vivo* Model	*In Vivo* Results	Ref.
Human c-kit$^+$ CPC	Preconditioning with CoPP; H_2O_2 oxidative stress	Improved survival, Cox2 up-regulation, increased expression of pNFR2 and pERK1/2, BCL2, BCL-XL and MCL-1; paracrine release of EGF and FGF			**(Cai et al, 2012)**
	Stimulation of c-kit activation by SCF	Increased proliferative and chemotactic response via PI3K-AKT and MAPK			**(Vajravelu et al, 2015)**
Mouse c-kit$^+$ CPC	Hypoxia preconditioning	Improved survival by pAkt and Bcl2 expression	i.m. injection into MI mouse model	Lower cardiac cell death; increased cardiac function via SDF-1/CXCR4 axis	**(Yan et al, 2012)**
	Nucleostamin silencing	Increased cell senescence; lower expression of stemness markers, up-regulation of p53 and p16	Nucleostamin knock-out mouse model	Early cardiac aging; decreased cardiac function; CPC depletion	**(Hariharan et al, 2015)**
	Pin1 silencing	Cell arrest in G1 phase; lower expression of Cyclin D and B with increased expression p53 and Rb	Pin1 knock-out mouse MI model	Reduced proliferating CPC	**(Toko et al, 2014)**
Rat c-kit$^+$ CPC	miR-21 transfection	Increased proliferation via PTEN/PI3K/Akt pathway			**(Shi et al, 2017)**

Mouse Sca-1$^+$ CPC	APE1 overexpression; oxidative stress in co-culture with rat NVCM	Inhibition of apoptosis via TAK1 and NF-kB activation;	i.m. injection into mouse model of MI	Improved survival of APE1-CPC graft; restoration of cardiac function; reduced inflammation and fibrosis	**(Aonuma et al, 2016)**
	Stimulation by the TGFβRI inhibitor A83-01 and ALK5 silencing	Increased proliferation via MERK/ERK-pathway by Birc5 up-regulation			**(Ho et al, 2016)**
Rat CS			i.m. injection into MI mouse model after 1 month	Increased cardiac function, reduced fibrosis and sustained angiogenesis with inhibition of TGF-β1/Smad signaling by paracrine effect	**(Tseliou et al, 2014)**
Human CDC	MLR with rat cells with inflammatory cytokines analysis	Strong proliferative response, activation of responder lymphocytes with secretion of inflammatory cytokines	i.m. xenogeneic transplantation into MI rat model	Acute rejection within 1 week post transplantation; increased paracrine secretion of VEGF, IGF-1 and HGF in the first 24h post MI	**(Malliaras et al, 2012)**
Rat CDC	MLR on allogeneic rat cells with measurement of inflammatory cytokines	Negligible proliferative effect	i.m. sex-mismatched syngeneic or allogeneic transplantation into MI rat model	Limited survival of transplanted cells with allogeneic CDC cleared more quickly post MI; smaller scar size; rare events of cardiomyogeneic and angiogeneic differentiation; resident cardiomyocyte proliferation; recruitment of endogenous c-kit$^+$ cells; improved paracrine secretion of VEGF, IGF-1 and HGF in the first	**(Malliaras et al, 2012)**

				week.	
	Indirect co-culture with peritoneal and bone-marrow rat macrophages	Reduced M1 gene expression in primed macrophages	i.c. allogeneic infusion into rat I/R injury model	Low retention of transplanted cells; sustained cardioprotection for 2 weeks; reduced number of CD68+ cells; macrophage polarization to cardioprotective phenotype away from M1 lineage.	**(de Couto et al, 2015)**
Mouse CDC			i.m. transplantation into MI mouse model	Up-regulation of resident cardiomyocyte proliferation with expression of Cyclin D1, CdK4, Cyclin E, CdK2, Cyclin A1-2, E2F1; recruitment of endogenous progenitors; improved heart function and increased viable myocardium.	**(Malliaras et al, 2013b)**
Swine CDC			i.c. allogeneic infusion into minipig model of MI with MRI monitoring	No sign of systemic immunogeneicity; paracrine stimulation of resident cardiomyocyte proliferation, upregulation of endogenous progenitors and local angiogenesis; improved cardiac function with increased viable myocardium.	**(Malliaras et al, 2013a)**
Human CS-EV	Human dermal fibroblast (hDF) priming	Dose-dependent decrease of pro-fibrotic phenotype by phosphorylated Smad 2/3,4, Snail1			**(Tseliou et al, 2015)**

	Matrigel angiogeneic assay and rat NVCM survival assay under H_2O_2 oxidative injury with conditioned media from CS-EV primed hDF	suppression; increased secretion of SDF-1 and VEGF Increased angiogenesis; cardiomyocyte protection against stress-induced apoptosis.			
Rat CS-EV			i.c. injection of primed fibroblasts 4 weeks after injury into rat MI model.	Increased cardiac function; improved angiogenesis; reduced scarring.	**(Tseliou et al, 2015)**
Human CDC-Ex	HUVEC angiogeneic assay; rat NVCM proliferation and survival assay under H_2O_2 oxidative injury.	Enhanced angiogenesis and proliferation; increased survival	i.m. injection into acute and chronic mouse model of MI	Improvement in cardiac function, increased myocardial viable mass and angiogenesis via miR-146a exosomal transfer	**(Ibrahim et al, 2014)**
Human CDC-EV	Co-culture of neonatal rat cardiomyocyte exposed to H_2O_2 oxidative injury with CDC-EV primed rat bone marrow-macrophages.	Reduction of cardiomyocyte apoptosis by enhanced secretion of IL-10 from macrophages primed with CDC-EV via direct transfer of Y RNA fragment.	i.c. injection into I/R myocardial injury rat model	Reduced infarct size, decreased $CD68^+$ macrophages, reduced cardiomyocyte apoptosis and increased levels of IL-10 due to Y RNA fragment enrichment	**(Cambier et al, 2017)**
Human atrial appendage CPC-EV	HL-1 cardiomyocyte apoptosis assay by starvation; HUVEC angiogeneic assay	Inhibition of starvation-induced apoptosis and stimulation of angiogenesis via miR-210 and miR-132 transfer and down-regulation of ephrin A3, PTP1b and RasGAP-p120	i.m. injection into mouse model of MI	Improvement of cardiac function; inhibition of cardiomyocyte apoptosis; decreased fibrosis; promotion of angiogenesis.	**(Barile et al, 2014)**

Mouse EPDC	Paracrine activation by Tβ4	Reactivation of quiescent cells; fibroblast, endothelial and smooth muscle differentiation	Paracrine therapy by i.p. injection of Tβ4 in a MI mouse model	Increased cardiac function; cardiomyocyte, endothelial and smooth muscle differentiation; upregulation of pSMAD1/5/8, pSMAD2, SNAIL, SLUG and secretion of angiogenic factors; reactivation of the Wt1 and Raldh2 embryonic genes via C/EBP proteins.	**(Huang et al, 2012;Smart et al, 2007;Smart et al, 2011;Zhou et al, 2011)**
Mouse Notch-activated Epicardial Cells			MI and thoracic aorta banding mouse models	Fibroblast differentiation with modest cardiomyogenic potential	**(Russell et al, 2011)**

APE1: APurinic/apyrimidinic Endonuclease/redox factor 1; BCL2: B-cell lymphoma 2; BCL-XL: B-cell lymphoma-extra large; C/EBP: CCAAT/enhancer binding protein; CDC: Cardiosphere-Derived Cells; CdK: Cyclin-Dependent Kinase; CoPP: Cobalt Protoporphyrin; Cox2: Cyclooxygenase 2; CPC: Cardiac Progenitor Cells; CS: CardioSphere cells; EGF: Epidermal Growth Factor; EPDC: Epicardium-Derived progenitor Cells; EV: Extracellular Vesicles; Ex: Exosomes; FGF: Fibroblast Growth Factor; HGF: Hepatocyte Growth Factor; HUVEC: Human Umbilical Vein Endothelial Cells; i.c.: intra-coronary infusion; i.m.: intra-myocardial injection; i.p.: intra-peritoneal; I/R: Ischemia/Reperfusion; IGF-1: Insulin Like Growth factor-1;IL-10: InterLeukin-10; LV: Left Ventricle; MAPK: Mitogen-Activated Protein Kinase; MCL-1: Induced myeloid leukemia cell differentiation protein; MI: Myocardial Infarction; miR-21: microRNA 21; MLR: Mixed Lymphocyte Reaction; MRI: Magnetic Resonance Imaging; NVCM: Neonatal Ventricular CardioMyocytes; pAkt: phosphorylated Akt; pERK1/2: phosphorylated Extracellular signal–Regulated Kinases 1/2; PI3K: PhosphoInositide 3 Kinase; Pin1: Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; pNFR2: phosphorylated Nuclear Factor (erythroid-derived 2)-like 2; PTEN: tensin homolog deleted on

chromosome ten; Raldh2: Retinaldehyde dehydrogenase 2; Rb: Retinoblastoma; Ref.: Reference; Sca-1:Stem Cell Antigen-1; SCF: Stem Cell Factor; SDF-1: Stromal Derived Factor-1; TAK1: β-Activated Kinase 1; TGF-β-1: Transforming Growth Factor Beta-1; TGFβRI: TGF-β type I receptor; TLR: Toll Like Receptors; Tβ4; Thymosin Beta 4; VEGF: Vascular Endothelial Growth Factor; Wt1: Wilms tumor 1.

Table 2. Expression of proteins and target genes of the Notch pathway in the heart at different stages of life

Stage	*Ligands*	*Receptors*	*Processing and Regulatory enzymes*	*Nuclear targets*	Ref.
Fetal	Jagged1, Jagged2 Dll1, Dll4 (whole heart)	Notch1-4 (whole heart)	Furin, ADAM17, Presenilin1, 2 (whole heart)	Hey1, Hey2 (whole heart)	**(Susan-Resiga et al, 2011;High and Epstein, 2008;de la Pompa, 2009;Albrecht et al, 2006)(Shi et al, 2003)**
Adult	Jagged1 (myofibroblasts, vascular smooth muscle cells)	Notch1 (cardiomyocytes, cardiac stem cells, myofibroblasts, vascular smooth muscle cells, endothelial cells)	Furin, ADAM17, Presenilin1, 2 (cardiomyocytes, cardiac stem cells, myofibroblasts, vascular smooth muscle cells, endothelial cells)	Hey1, Hey2, Hes1 (cardiomyocytes, cardiac stem cells, myofibroblasts, vascular smooth muscle cells, endothelial cells)	**Wang et al, 2009;Boni et al, 2008;Croquelois et al, 2008;Kratsios et al, 2010;Nemir et al, 2012)**
	Dll1, Dll4 (endothelial cells, serum)	Notch2, Notch4 (endothelial cells)			**(Briot et al, 2015;Norum et al, 2016;Shutter et al, 2000)**
	DLK1, periostin, (whole heart, serum)	Notch3 (myofibroblasts, vascular smooth muscle cells)			**(Abraityte et al, 2015;Norum et al, 2017;Ragot et al, 2016;Zhang et al, 2016;Zhao et al, 2014)**
Aged	Decreased (?) Jagged1, Dll1, Dll4	Decreased (?) Notch1-4?	Decreased DTX2, POFUT1 (whole heart)	Decreased Hey1,Hey2, HeyL (mesenchymal stem cells)	**(Ashton et al, 2006;Mutyaba et al, 2014)**

Abbreviations: ADAM, A Disintegrin And Metalloprotease; Dll1-4, Delta-like ligands 1,4; DLK1, Delta-like 1 homologue; DTX2, Deltex E3 Ubiquitin Ligase 2;Hey, hes related family bHLH transcription factor with YRPW motif; Hes, hes family bHLH transcription factor; POFUT1, protein O-fucosyltransferase 1.

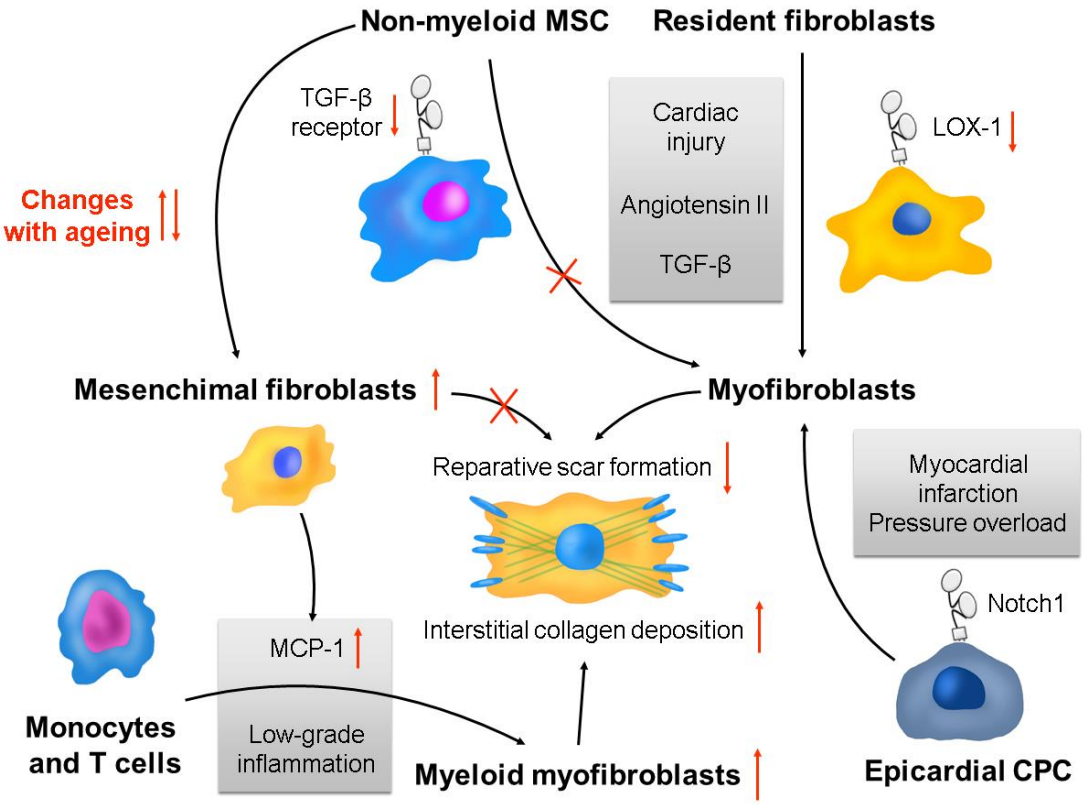

Figure 1

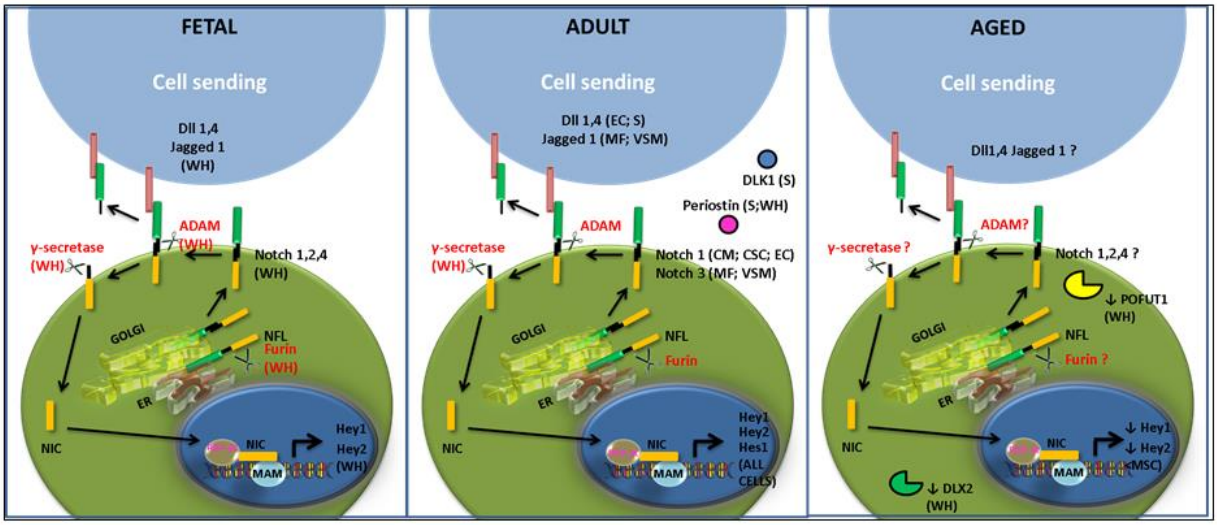

Figure 2

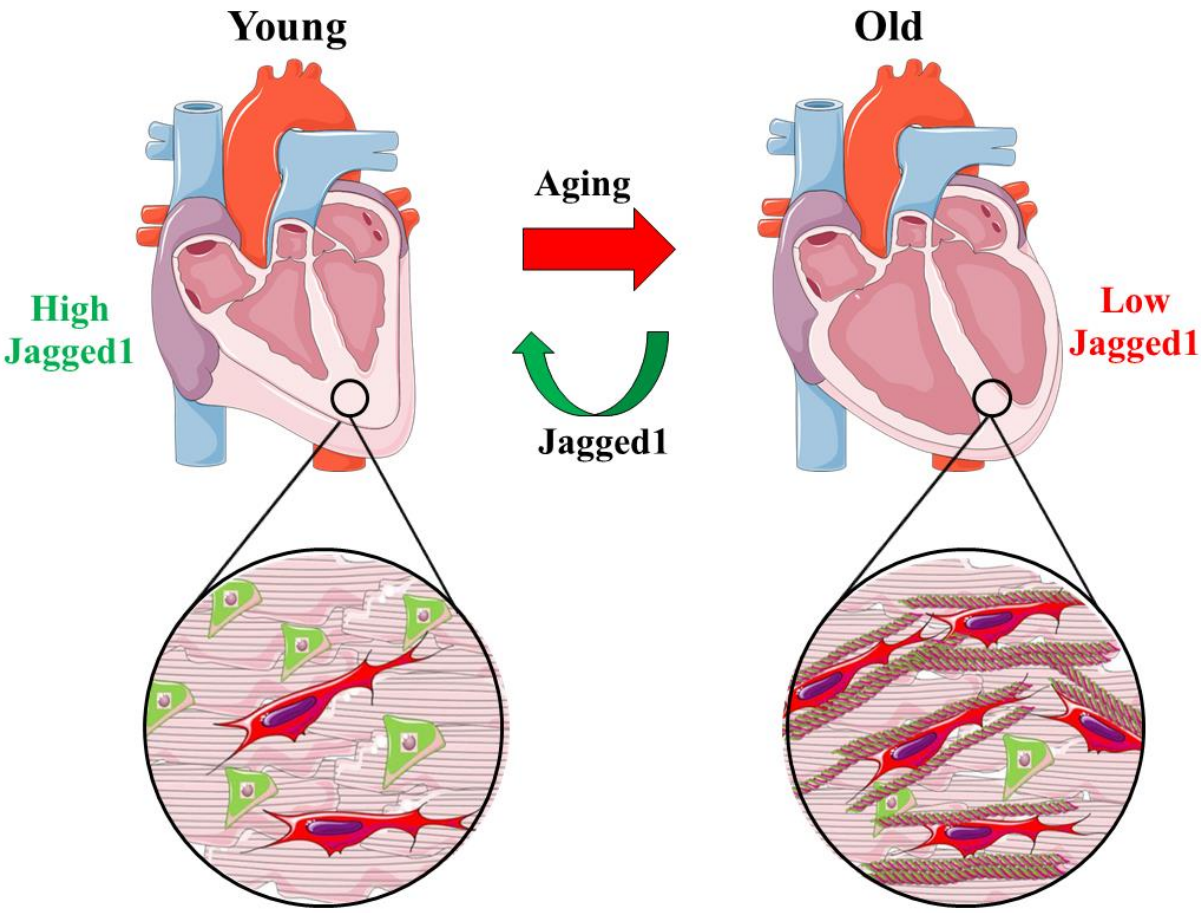

Figure 3