Beyond cardiomyocyte loss: role of Notch in cardiac aging:

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Abstract

The knowledge of the 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 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. In a translational perspective, detailed investigation of the signaling pathways that regulate both fibroblasts and CPC activity, such as the Notch system, may lead to the identification of molecular targets for novel therapies for age-related cardiac disease.

Aging profoundly affects cardiac structure and function (Chen and Frangogiannis 415-22). At best, age-related remodeling of the heart chiefly contributes to the limitation in exercise capacity typical of the elderly. Furthermore, it often prepares the ground for the development of all the major types of cardiac disease, including heart failure (HF), 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 in fact been the focus of research on cardiac aging for many years (Sheydina, Riordon, and Boheler 315-29). More recently, two major conceptual breakthroughs have opened new perspectives in the study of the mechanisms that make the heart old: that fibrosis is as important for cardiac pathology as cardiomyocyte dysfunction or loss and that the heart can, in principle, regenerate. The first two paragraphs of this brief review address these two aspects with respect to cardiac aging. As a consequence of the understanding of the importance of fibrosis and heart regeneration, interest has arisen in the identification of signaling pathways that affect the cells responsible for these processes, i.e. fibroblasts and cardiac progenitor cells (CPCs), respectively. This appears to be the case with the Notch system, which is discussed in the third paragraph of this review.

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 there are in particular 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 and valve annuli and leaflets (Bowers, Banerjee, and Baudino 474-82;Rienks et al. 872-88;Li et al. 916-27). It is now

established that the ECM does not simply provides physical support to cardiomiocytes, but also has functional roles. It keeps the 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, Banerjee, and Baudino 474-82;Rienks et al. 872-88;Li et al. 916-27). 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, Banerjee, and Baudino 474-82). Collagen is the most abundant protein; 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. 916-27;Rienks et al. 872-88). Traditionally, fibrosis that develops following cardiomyocyte necrosis is referred to as reparative, while the term reactive is used to indicate fibrosis that involves the perivascular spaces and interstitium and is not initiated by cardiomyocyte death. Activated fibroblasts, or myofibroblasts, are primarily implicated in either form of fibrosis (Goldsmith et al. 92-99). In normal conditions, the presence of myofibroblasts in the adult heart is limited to the valvular leaflets. In disease, the number of myofibroblasts dramatically increases because of the differentiation of resident fibroblasts, as well as of cardiac mesenchymal cells and bone marrow-derived precursors. Myofibroblasts produce ECM as a default, protective reaction aiming at maintaining tissue homeostasis (Goldsmith et al. 92-99;Li et al. 916-27;Rienks et al. 872-88). In the case of 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. On the long term, however, fibrosis becomes detrimental (Burchfield, Xie, and Hill 388-400). At the cellular level, it entraps cardiomyocytes, hindering oxygen and nutrient supply and causing atrophy, and predisposes to arrhythmia by interfering with the transmission of electrical impulses. In addition, myofibroblasts release factors that alter cardiomyocyte function in a paracrine way 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 415-22). 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 325-35). These abnormalities in ECM turnover are paralleled by recruitment and activation of fibroblasts. Several signals may lead to such fibroblast dysregulation, among which transforming growth factor (TGF)-β and angiotensin II have long been known (Brooks and Conrad 187-95;Billet et al. 1914-25). 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, a role for chronic low-grade inflammation inducing myofibroblast differentiation from myeloid precursors has been shown(Cieslik et al. 248-56). 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. 184-90), despite this receptor being classically induced by pro-inflammatory cytokines. While fibrogenesis is basally enhanced, the fibrotic response to MI in

mouse models appears to be paradoxically impaired with aging (Bujak et al. 1384-92;Cieslik et al. 26-36). 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, but the one induced by MI is blunted. A possible explanation resides in the fact that myofibroblasts derive from different cell populations, mesenchymal stem cells (MSCs) and leukocytes Specifically, it has been proposed that MSCs become unresponsive to TGF-β with age, because of the downregulation of TGF-β receptor. On the one hand, this causes MSCs to mature into fibroblasts and express higher levels of monocyte chemoattractant protein-1 (MCP-1), which in turn promotes the migration of leukocytes into the cardiac tissue and their differentiation into myeloid myofibroblasts. Therefore, the fibroblast population substantially expands. On the other side, MSC-derived fibroblasts are dysfunctional and synthetize less collagen upon stimulation by TGF-β. As a result, the fibrotic reaction to MI, which chiefly depends on the contribution of MSCs, is hindered (Cieslik et al. 56-63).

Cardiac progenitor cells and aging

Historically, the central dogma of cardiac medicine has been based on the assumption that the heart is a terminally differentiated organ without regenerative potential, cardiac hypertrophy being only secondary to enlargement and hypertrophic growth of pre-existing resident cardiomyocytes(Karsner, Saphir, and Todd 351-72;Leri et al. 631-46). Cardiovascular disease is still a major socio-economic burden in western countries, with myocardial infarction (MI) the most common cause of cardiac injury. Although the mortality rate of MI has significantly decreased in

the last years with fewer people dying thanks to significant progress of interventional cardiology, MI survivors are at high risk of developing HF when they become older, with the ultimate cure still represented by heart transplantation which is limited by shortage of donors and side complications and is not an option for elderly patients(Braunwald 430-32).

In this scenario, the identification in 2003 of a population of endogenous CPCs in the rat heart with stem-like properties and the potential of supporting myocardial regeneration following MI (Beltrami et al. 763-76), 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 a multipotent CPCs residing in niches scattered within the myocardium. Such hypothesis opened up to the characterisation of CPCs biology, by identifying their phenotype on the expression of specific stem cell-related (c-kit, Sca-1) and/or early cardiac developmental markers (Isl1), or upon their in vitro culture properties (cardiospheres and cardiosphere-derived cells), or on their tissue origin (epicardium-derived progenitor cells, EPDCs), as extensively reviewed elsewhere(Bollini, Smart, and Riley 296-303). CPCs represent a small, still receptive, reservoir of endogenous immature progenitors within the adult myocardium which, upon injury or appropriate stimulation, can either differentiate into the three main cardiovascular lineages providing new cells to replace damaged ones or can exert beneficial paracrine effects by releasing soluble molecules, overall resulting in a significant improvement of cardiac function (Bollini, Smart, and Riley 296-303;Feng et al. 65-77). So far, CPCs have mainly been exploited for cell therapy approach, as they can be easily isolated from endocardial/myocardial biopsy and expanded *in vitro* prior to being transplanted back in the injured heart with beneficial effects on the whole organ function (Barile et al. S9-S14;Messina et al. 911-21;Bolli et al. 122-31;Matsuda et al. 222-31). Indeed, clinical trials have already been carried out using c-kit+ (SCIPIO trial) and

cardiosphere-derived (CADUCEUS trial) CPC in patients with ischemic HF or left ventricular dysfunction, showing the safety and feasibility of autologous human CPC injection with reduction of the infarct size, increase of viable mass, and improvement of left ventricular function [32,33]. Alternatively, endogenous resident CPC can also be activated by the stimulation with soluble factors by paracrine therapy, avoiding *in vitro* manipulation and *in vivo* transplantation(Urbanek et al. 663-73;Rota et al. 107-16;Aghila Rani and Kartha 157-65;Croquelois et al. 3173-85;Smart et al. 640-44;Limana et al. e73-e83) .

Notably, 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 381-87), thus the impact of aging on CPCs biology and on their regenerative potential has to be evaluated in details. Several preclinical and clinical evidences support the hypothesis that CPCs in the old cardiac tissue might be affected by aging, showing impaired cell function and becoming less responsive to any external stimulation, hence penalizing any possible regenerative strategy in the event of injury (Hu et al. 582-90).

While the number of human CPCs seems to increase in the old myocardium, especially in women (Kajstura et al. 1374-86) , a recent study showed that the percentage of human c-kit+ CPCs 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, thus affecting the endogenous heart repair process (Hu et al. 582-90). Hence, a growing body of evidence suggest that the aged heart can be compromised from chronological CPCs aging. Indeed, within the old myocardium the migratory capacity of human c-kit+ CPCs declines with a putative mechanism described in the alterations of the signalling regulated by the receptor EphA2 controlling human CPCs motility, which, in turn, are caused by age-associated accumulation of

reactive oxygen species and a significantly higher oxidative stress. Besides, old human CPCs with altered trafficking are unable to translocate within the myocardial tissue, with important consequences for cardiac repair (Goichberg et al. 2211-23) . Moreover, senescent CPCs, which have lost their telomerase activity because of ageing, 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. 1374-86) . Similarly, cardiosphere-derived cells (CDCs) isolated from aged mice showed a decline in the expression of CDCs stem markers, such as c-kit and Sca-1, together with reduction of their clonogenic potential, suggesting the failure of regenerative potential with age (Hsiao et al. 1027-36).

Therefore, there is mounting claim for finding novel approaches besides current conventional medical care. Indeed, to be clinically feasible, CPCs must be isolated and expanded from aged and/or diseased tissue. In such scenario, CPCs functional lifespan might be ensured via stimulation by protective soluble molecules (such as insulin-like growth factor-1) preserving telomere length and their regenerative potential (Siddiqi and Sussman); pharmacological approaches based on ACE-inhibitors blocking angiotensin II signalling and oxidative stress affecting CPCs have also been suggested (Cesselli et al. 81-97). Moreover, novel strategies aiming at preserving a pool of competent CPCs 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. 13380-85;Zaruba et al. 1992-2000;Beltrami, Cesselli, and Beltrami 21-29).

Notch signaling in cardiac aging

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-1, 3, 4 and Jagged1 and -2) located on the cell surface. 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 the Hes and Hey families, which are negative regulator of transcription (Espinoza and Miele 95-110). Recent studies have shed further light on the complexity of the Notch signaling and have shown that the pool of genes modulated by Notch is large, greatly differs among cell types, and in the same cell is context-dependent (Andersson, Sandberg, and Lendahl 3593-612).

Notch plays a major role during heart development, in which it patterns the embryonic endocardium, enabling region-specific differentiation and critical interactions of the endocardium (or its derived mesenchyme) with other cardiac tissues (cardiac neural crest, myocardium), so that specialized structures (cardiac valves and chambers) are generated (de la Pompa and Epstein 244-54).

Compared to its role during development, less is known about Notch in the heart during post-natal life even accumulating evidence show that Notch activation plays a pivotal role in the overloaded or damaged myocardium (Ferrari and Rizzo 2140-45). Neonatal cardiomyocytes are rapidly proliferating and express high levels of Notch1. Conversely, in the adult myocardium these cells lose the ability to proliferate and down-regulate Notch signalling. Notch1 is reactivated in

cardiomyocytes located in the MI border zone or in the overloaded myocardium to counteract cardiomyocytes apoptosis and hypertrophy (Ferrari and Rizzo 2140-45). Consistently with the in vivo data showing reactivation of Notch under pathological conditions, expression of Notch signalling components has also been observed in myocardium biopsies from HF patients (Ferrari and Rizzo 2140-45). It is possible that Notch activation in the damaged heart is a temporary event, since Campa et al. have shown that prolonged activation of Notch1 in cardiomyocytes is detrimental and causes apoptosis (Campa et al. 129-41).

Notch activation in the damaged myocardium has also been linked to the regulation of CPCs (Ferrari and Rizzo 2140-45). Notch1 is present on the membrane of CPCs in its inactive form and it is activated following a MI by Jagged1 exposed on the surface of adjacent cardiomyocytes. Notch activation induces the transcription factor Nkx2.5 which is involved in the expression of cardiomyogenic transcripts and in the inhibition of vascular cells markers. Remarkably, in a mouse model of pressure overloaded myocardium, the overexpression of Jagged1 on cardiomyocytes favoured the differentiation of CPCs into Nkl2.5-positive cells while inhibiting myofibroblasts proliferation and TGF-β/connective tissue growth factor-mediated cardiac fibrosis (Nemir et al.). The MSCs compartment is also modulated by Notch, as deletion of Notch1 in these cells impairs their recruitment, proliferation, and survival leading to decreased ability to repair the myocardium damage compared to MSCs with a functional Notch1 signalling (Ferrari and Rizzo 2140-45). In aging mice, the impairment of MSCs function has been linked to Notch inhibition (Mutyaba et al. S20-S23). Geriatric mice exhibit reduced MSCs number, proliferation, adipogenesis, and inconsistent osteogenesis associated to decreased basal Notch signaling activity, even though these cells were fully responsive to Jagged1 stimulation (Mutyaba et al. S20-S23).

In addition to fibrosis, altered oxidative balance and down-regulation of calcium-handling proteins with decreased intracellular Ca2+ decay are hallmarks of age-related cardiac diastolic dysfunction (Loffredo et al. 97-107). A crosstalk between the Notch and Ca2+ signaling networks have been described in cardiomyocytes (Kasahara et al. 734-37) and, in leukemia cells, inhibition of sarcoplasmic/endoplasmic reticulum Ca2+ ATPase (SERCA) impairs the maturation and activity of Notch1 (Roti et al. 390-405). Studies *in vitro* have shown that Notch signalling controls both oxidative balance and metalloproteases production (Delbosc et al. 1430-40;Boopathy et al. 43) and negatively regulate TGFβ-induced cardiac fibroblast-myofibroblast transformation (Fan et al. 739-48).

Notch signalling is also active in the endothelial (EC) and vascular smooth muscle (VSMC) cells of cardiac vessels. The Notch pathway controls angiogenesis and protects the endothelium from dysfunction caused by the inflammatory cytokines, ischemia, and turbulent blood flow (Rizzo, Miele, and Ferrari); moreover, it controls proliferation, survival, and function of VSMCs (Ferrari and Rizzo 2140-45). Data from a mouse model of MI show that the activation of Notch1 signalling improves cardiac function by promoting myocardial angiogenesis (Ferrari and Rizzo 2140-45;Lassaletta et al. 743-51). For more details on the cited literature on the role of Notch in pathological cardiac remodeling the reader is referred to Ferrari et al. (Ferrari and Rizzo 2140-45).

Whether and how Notch signaling is affected in the aging heart has not been extensively investigated. 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. 9-

17). The reduced activation of Notch in the skeletal muscle impairs its regeneration and the exposure of satellite cells from old mice to young serum enhances the expression of the Notch ligand (Delta), increases Notch activation, and enhances proliferation *in vitro* (Conboy et al. 1575-77;Conboy et al. 760-64).

Acceleration 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 29-48). Notch signalling components were not found among the age-regulated genes in mouse ventricular cardiac muscle cells (Bodyak et al. 3788-94), but the possibility 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 that this is associated with selective changes in transcription levels of Ca2+, Wnt, Notch, and G-protein coupled receptor signaling pathways in aged versus young hearts (Ashton et al. 189-204). Additionally, geriatric mice exhibit reduced MSCs number, 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. S20-S23). Taken together, these studies suggest that aging could interfere with the activation of Notch required to reduce pathological remodeling in the damaged myocardium.

Neointimal hyperplasia after percutaneous coronary intervention is exaggerated in the elderly, but the role of Notch in this context is not known. The effects of aging on Notch in the vasculature have been characterized 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. 800-08). In this model of artery injury the interaction of Jagged1 on EC with Notch3 on VSMCs in the intima prevented VSMC proliferation and vessel stiffening (Wu et al. 2000-06). In addition to excessive proliferation, reduced apoptosis of VSMCs plays a key role in aging-associated enhanced response to vascular injury. VSMCs co-cultured with senescent ECs, expressing reduced levels of Jagged1 compared with young ECs, exhibited decreased susceptibility to H_2O_2-induced apoptosis compared with those co-cultured with young ECs (Qian et al. 207-13). It is unknown whether Jagged1 dowregulation is also involved in aging-related vascular remodeling and stiffening. Similarly, dysregulation of Notch signalling could play a role in the progressive calcification of the aortic valve, which affects a large number of people over age 65. 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. 1580-90).

Aging is associated with impaired vascular endothelial function (Donato et al. 1659-66)which is particularly implicated in the pathophysiology of HF with preserved ejection fraction (Lam and Brutsaert 1787-89). As previously discussed, the Notch pathway has a protective role in the endothelium (Rizzo, Miele, and Ferrari). During vein graft adaptation to the arterial environment, both Delta-like ligand 4 and Notch4 expression were found to be down-regulated in an aged, but not a young, background and loss of Notch4 is associated with loss of attenuation of neointima (Kondo et al. e149-e160). It is not known whether the Notch signalling is down-regulated in the endothelium of the vasculature of aging myocardium predisposing to endothelial dysfunction.

Conclusions

The knowledge of the 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 CPCs. 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 Notch signaling prevents the transformation of cardiac fibroblasts in myoblast and Jagged1 promotes the differentiation of immature cardiac cell into cardiomyocytes and the proliferation of CPCs. In analogy with the endothelium, in which ageing attenuates Jagged1 expression (Wu et al. 800-08) , it would be of interest to determine whether inhibition of Notch signalling caused by reduced levels of this ligand is linked to the fibrotic and reduced regenerative responses in the aging heart. Because TGFβ induces Jagged1 in MSCs (Kurpinski et al. 734-42), the weak responsiveness to TGFβ observed in aging heart could also lead to low levels of Jagged1. In this scenario, reastablishing the preexisiting levels of Jagged1 in the aged myocardium could help preventing altered fibrotic and regenerative response (Figure 1).

Conflict of interest

None of the authors has conflicts of interest to declare.

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