

Notch1 activation favors the commitment of Cardiac Progenitor Cells to the myocyte lineage

PhD in Systems Physiopathology (XIX cycle: 2003-2007)

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INTRODUCTION.

1 The heart is a self-renewing organ.

1.1 Cardiac primitive cells. Till few years ago the scientific community considered the heart as a terminally differentiated post mitotic organ in which the cell number is defined soon after birth. According to this view the post-natal heart would depend uniquely on the hypertrophic growth of preexisting cells in order to reach the adult dimension. This would imply that cardiac cells have to maintain their viability throughout the life of the organism. Moreover, if this were the case, cell death had to be a very rare event to avoid the progressive loss of the cardiac mass over time. It is somehow difficult to imagine a human cardiomyocyte contracting on average 70 times per minute 24 hours a day for 80 years or more without considering the hypothesis of a cell turnover dedicated to the preservation of tissue homeostasis. Conversely, it is reasonable to envision that in the heart, like in several other organs and tissues, a limited number of primitive cells with self-renewing properties is constantly maintained in an undifferentiated-low proliferative state and that a fraction of these cells undergoes differentiation following physiological or pathological stimuli. Newly formed differentiated cells may preserve cardiac homeostasis and replace damaged tissue after injury.

This scenario is anything but uncommon in other organs in which cells with these properties have been described. All organs of the organism contain two categories of cells: non-dividing cells and dividing cells. However non-dividing cells could be temporarily arrested in G_0 in a dormant condition and be capable of reentering the cell cycle following growth activation and becoming terminally differentiated losing permanently their ability to divide (Zhu and Skoultchi, 2001). This phenomenon has been described in the small intestine at the tip of the villi (Mariadason et al., 2005), in the auditory cells of the ear (Chen et al., 2003) and in the upper layer of the epidermis (Hotchin et al., 1995). In the liver, hepatocytes are arrested in G_0 and it is well known that, after injury, the hepatic tissue is able to regenerate thanks to the proliferation of several cell types including hepatocytes, biliary epithelial cells and fenestrated endothelial cells (Taub, 2004). The liver contains also undifferentiated cells, the oval cells, which could contribute to the regeneration process of the hepatic tissue after expansion and maturation into fully mature cells (Vessey and de la Hall, 2001). Although the possible mechanism underlying liver regeneration is still debated, the existence of primitive cells playing a major role in tissue homeostasis and regeneration has been proven and accepted in the bone marrow (hematopoietic stem cells) (Uchida et al., 1998), in the skin (skin stem cells) (Blanpain et al., 2004), in the central nervous system (neural stem cells) (Eriksson et al., 1998; Lie et al., 2005; Rakic, 2004; Sanai et al., 2004) and in the skeletal muscle (satellite cells) (Dhawan and Rando, 2005) (Fig. 1).

In our laboratory it has been recently demonstrated that the heart contains its own stem cell compartment since putative primitive cells have been characterized in the heart of small and large mammals including humans (Beltrami et al., 2003; Linke et al., 2005; Martin et al., 2004; Matsuura et al., 2004; Oh et al., 2003). Resident **cardiac stem cells (CSCs)** have been isolated from the myocardium and described as self-renewing, multipotent and clonogenic (Bearzi et al., 2007; Beltrami et al., 2003; Linke et al., 2005).

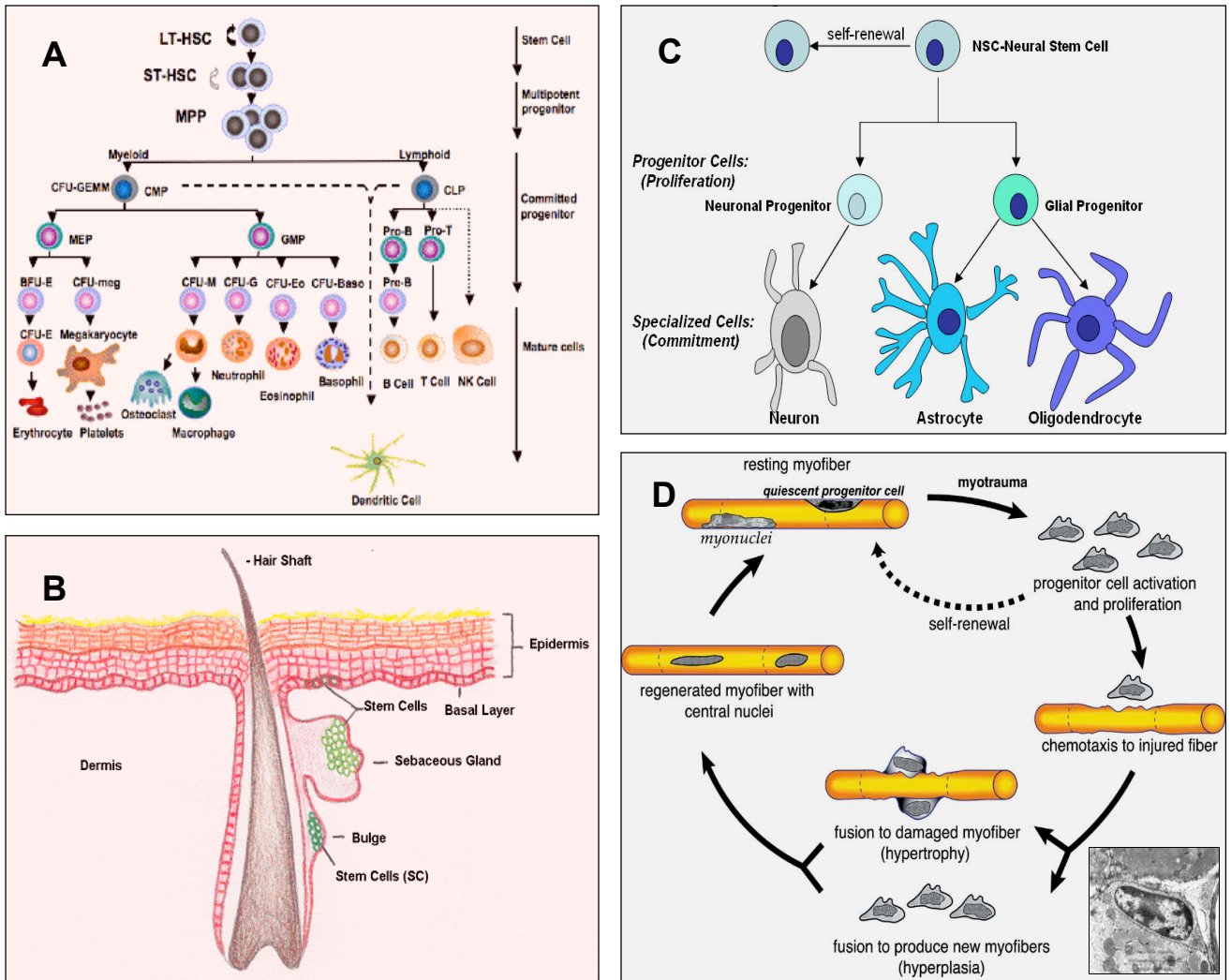


Figure 1: Primitive cells in different tissue. (A) Hematopoietic stem cells (HSCs), their commitment to the myeloid and lymphoid progenitors and their differentiation into blood mature cells. (B) Skin stem cells localized in the bulge and in the sebaceous gland of the dermis and in the basal layer of the epidermis. (C) Neural stem cells (NSCs) and their commitment to neuronal and glial progenitors and their differentiation into mature cells. (D) Skeletal muscle stem cells (satellite cells) and their involvement in the repair of damaged myofibers and in the generation of new myofibers.

Such properties are essential for the maintenance of the stem cell pool and, at the same time, for the replacement of the cardiac cell lineages (cardiomyocyte, smooth muscle cells, endothelial cells and fibroblasts).

The immunophenotypic features of CSCs are still under further investigation due to the technical difficulties encountered in their isolation and in the distinction between the true stem cells and their early committed progeny. In this regard, FACS analysis has been utilized for the characterization and enrichment of the different categories of cardiac primitive cells. However, this approach implies cell membrane permeabilization for the detection of cytoplasmic antigens precluding the possibility to utilize viable cells for further experiments.

So far, the main marker that has been utilized in our laboratory for the identification and the isolation of putative CSCs is the stem cell antigen **c-kit** that is the receptor for the stem cell factor (SCF). Its presence at the level of the cell membrane may be coupled with the presence of other two molecules that are expressed, although not uniquely, in undifferentiated cells: Sca-1 (stem cell antigen-1) considered the predominant stem cell marker in the mouse bone marrow (Leri et al., 2005; Nadin et al., 2003; Shizuru et al., 2005), and MDR1 (multi drug resistance-1) that belongs to the ABC family of transporters that mediate the efflux of the Hoechst dye out of the cell in the side population of bone marrow (Bunting, 2002; Bunting et al., 2000) and skeletal muscle (Goodell et al., 2005; Leri et al., 2005; Majka et al., 2003). CSCs are classically characterized by the presence of the stem cell-related markers c-kit, Sca-1 and MDR-1 in variable combinations. Quantitative data in the mouse, rat, dog and human heart demonstrated that there is one CSC per 30,000-40,000 myocardial cells and that ~65% of them express the three stem cell antigens c-kit, Sca-1 and MDR1 together, ~20% express two stem cell antigens and ~15% express only one of them (Leri et al., 2005; Linke et al., 2005; Urbanek et al., 2005).

CSCs do not express transcription factors and membrane or cytoplasmic proteins of bone marrow cells, neural cells, skeletal cells and cardiac cells and, therefore, they are considered lineage negative. True CSCs divide rarely and remain in an undifferentiated dormant status till their activation. Additionally, they are consistently small in size (~7 μm of diameter) and they have a high nucleus-to-cytoplasm ratio (Fig. 2). As mentioned before, CSCs are typically self-renewing (able to give rise to a cardiac stem cell progeny), clonogenic (able to form clones in culture) and multipotent (able to differentiate into cardiomyocytes, smooth muscle and endothelial cells).

CSCs have to undergo molecular and morphological modifications in order to become fully differentiated. The hallmark of premature commitment of a CSC is the nuclear expression of cardiac transcription factors (GATA4, GATA6, Mef2C, Nkx2.5, and Ets1 among others) that define their lineage destiny. At this stage of maturation, the cells usually retain the stem cell marker c-kit (Sca-1 and/or MDR1) and are classified as **cardiac progenitor cells**.

When progenitor cells start to express cardiac transcription factors together with membrane or cytoplasmic proteins (i.e. contractile proteins: α -sarcomeric actin, smooth muscle actin) they enter a more mature state and they are classified as **cardiac precursor cells**. These cells can be negative for the stem cell marker c-kit (Sca-1 and/or MDR1) and appear larger in size being the cytoplasmic component more present (Fig. 3). Considering the expression of cardiac transcription factors and cytoplasmic proteins, it has been documented by our laboratory that clonogenic CSCs (positive for c-kit and/or Sca-1 and/or MDR1 in different combinations) preferentially differentiate into myocytes (~30-32%), to a less extent into smooth muscle (~22-29%) and endothelial (~6-12%) cells and fibroblasts (~12-18%) in culture. Importantly, c-kit positive CSCs showed a greater clonogenic capacity than that of Sca-1-like positive, MDR1 positive and c-kit-Sca-1-like-MDR1 positive CSCs.

Cardiac progenitor and precursor cells (CPCs) can actively divide giving rise to a pool of **transient amplifying committed cells** identified by the expression of cardiac transcription factors together with myofibrillar proteins and cell cycle markers such as Ki67 and MCM5 (Urbanek et al., 2003). This fraction of cells represents a valuable resource for the replacement of old and damaged cells.

Following a proliferation phase and the accomplishment of the differentiation program, young myocytes undergo progressive hypertrophy and, in the end, they permanently exit the cell cycle becoming positive for p16^{INK4a} (inhibitor of cell cycle and marker of cell senescence). Interestingly, the lifespan of myocytes seems to correlate with their volume; in this regard, young, amplifying myocytes are less than 180 μm^2 in cross-sectional area, adult non dividing cells are 200-250 μm^2 , old non dividing cells are 300-500 μm^2 and senescent non dividing cell are 600-900 μm^2 (Rota et al., 2007; Urbanek et al., 2003).

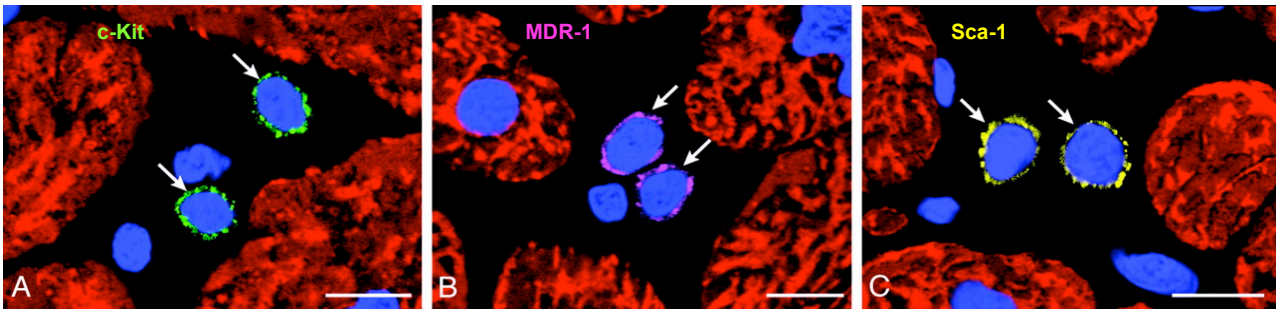


Figure 2: Putative cardiac stem cells. Shown are the detection of c-kit (A, green), MDR1 (B, purple), and Sca-1-reactive protein (C, yellow) in primitive cells (arrows) of hypertrophied hearts. Nuclei are stained by propidium iodide (PI; blue) and myocytes by cardiac myosin (red). (Bars = 10 μ m). From: (Urbanek et al., 2003).

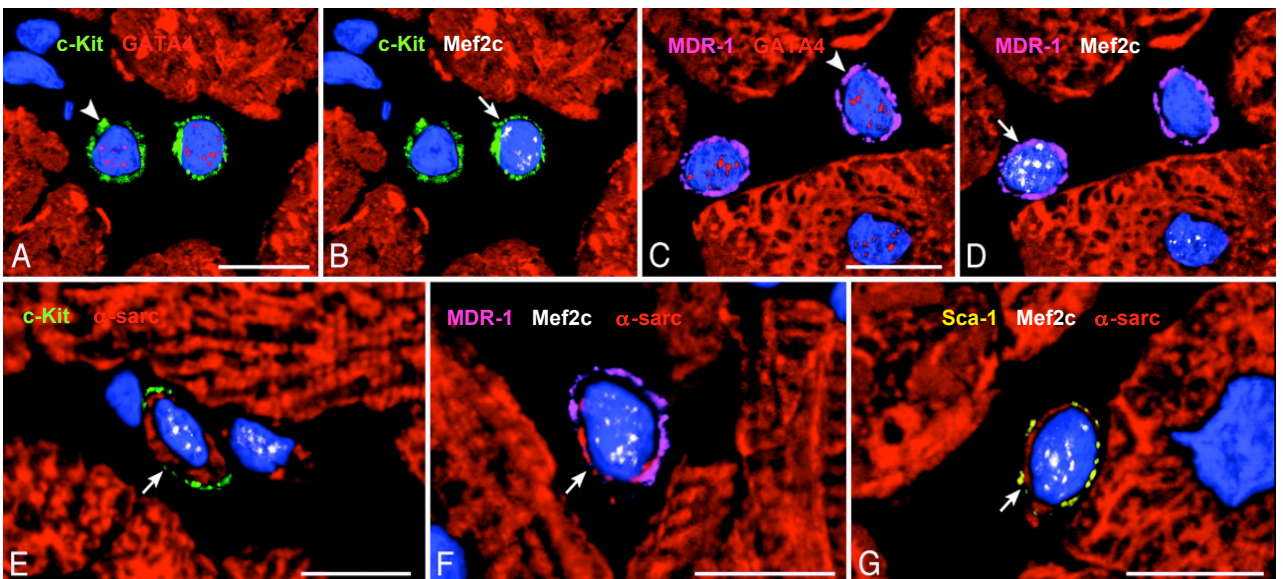


Figure 3: Commitment of primitive cells with cardiac hypertrophy. (A and B; the same field) A cardiac progenitor (A, arrowhead) shows c-kit on the surface membrane (green) and GATA-4 (A, red) in the nucleus, and the myocyte progenitor (B, arrow) exhibits in its nucleus GATA-4 (A, red) and MEF2 (B, white). (C and D; the same field) A cardiac progenitor (C, arrowhead) shows MDR-1 on the surface membrane (purple) and GATA-4 (C, red) in the nucleus, and a myocyte progenitor (D, arrow) exhibits in its nucleus GATA-4 (C, red) and MEF2 (D, white). (E, F, and G) Myocyte precursors (arrows) show on the surface membrane c-kit (E, green), MDR-1 (F, purple), or Sca-1-like (G, yellow). A thin cytoplasmic layer is positive for cardiac myosin (E-G, red) and nuclei express MEF2 (E-G, white). (Bars = 10 μ m). From: (Urbanek et al., 2003).

1.2 The cardiac niche. In several organs the stem cells are stored in organized microenvironments, called niches, which protect them from adverse stimuli. By definition, a niche is characterized by two components: the stem cells, which are located in the inner portion of the niche, and the surrounding supporting cells (Fig. 4).

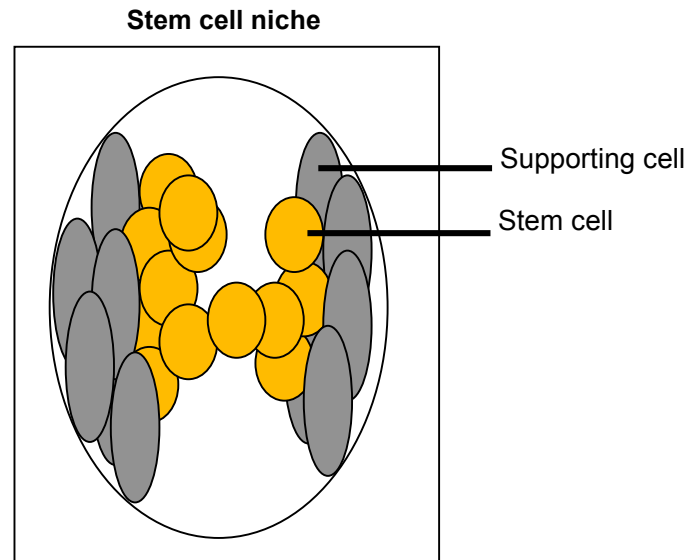


Figure 4: Schematic representation of a stem cell niche.

A considerable amount of information regarding the structure and the signaling regulatory mechanisms of the niche has been collected in *D. melanogaster* (Spradling et al., 2001). In specific organs of simple organisms, such as the *Drosophila* gonad, the architectural organization of the tissue gives reliable reference points that make the identification of the niches relatively easy (Lin, 2002). Conversely, in mammals, the localization of a stem cell niche presents several obstacles related to the more complex architecture of the tissue.

However, this task is simplified in organs with an epithelial lining that offers a useful reference point for the identification of the region in which the supporting cells reside. This is particularly apparent in the skin (Tumbar et al., 2004) in the bulge of the hair follicles (Cotsarelis, 2006) and in the intestine (Kim and Shibata, 2002).

The stem cells stored within a niche have the capability to perpetuate themselves following (a) symmetric division through which two self-renewing daughter stem cells are formed and/or (b) asymmetric division resulting in the generation of one daughter stem cell and one committed amplifying cell which give rise to a progeny of newly differentiated cells. Additionally, symmetric division can produce two daughter-committed cells (c) and this event ends up with a progressive depletion of the stem cell pool in the niche over time (Watt and Hogan, 2000) (Fig. 5).

Similarities in the spatial arrangement of putative stem cells and committed cells can be found in the mouse bone marrow (Yoshimoto et al., 2003) and in the rat brain (Doetsch, 2003; Palmer, 2002), providing important elements of analogy between these organs and the heart. It has been recently demonstrated that CSCs and CPCs are stored in niches in the adult heart (Leri et al., 2005; Urbanek et al., 2006), (Appendix). Lineage negative cells expressing a stem cell marker c-kit, Sca-1 or MDR1, separately or in combination, have been detected clustered together with progenitor and precursors cells. In such clusters it is possible to recognize also small myocytes and other cells in early phases of differentiation (Anversa et al., 2003).

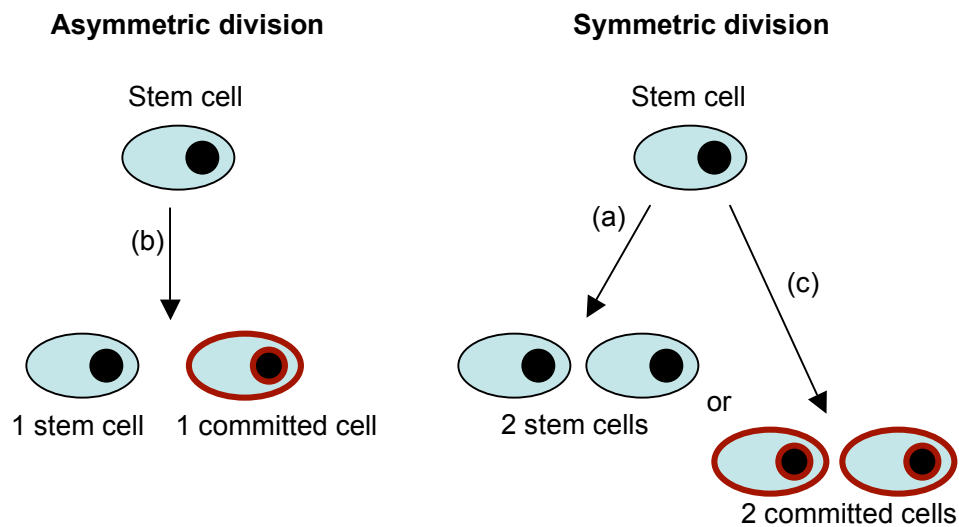


Figure 5: Scheme of asymmetric and symmetric cell division in which different proportions of undifferentiated and committed cells are formed. One committed cell and one stem cell are formed after asymmetric cell division (b), two stem cells (a) or two committed cells (c) are formed after symmetric cell division.

A **cardiac niche** is defined by CSCs and their committed progeny (CPCs) surrounded by terminally differentiated cardiomyocytes and fibroblasts that function as supporting cells. The cells forming a niche are structurally and functionally interconnected through adherent junctions (N, E-cadherins and β -catenin) and gap junctions (connexins 40, 43 and 45) that guarantee mechanical and functional coupling, respectively. These connections are necessary to physically keep the stem cells within the niche and to preserve stem cell characteristics (Fuchs et al., 2004; Urbanek et al., 2006). Additionally, different classes of integrins expressed on the stem cell surface mediate the interaction of stem cells with the intercellular matrix and regulate stem cell migration (Urbanek et al., 2006) (see Appendix). Although small clusters of CSCs are scattered throughout the entire myocardium, true cardiac niches are preferentially located in regions with low exposure to mechanical stress such as the atria and the apex. Cardiac niches

are more rarely found in regions of the heart exposed to a high hemodynamic load such as the base and the mid portion of the left ventricle (Fig. 6).

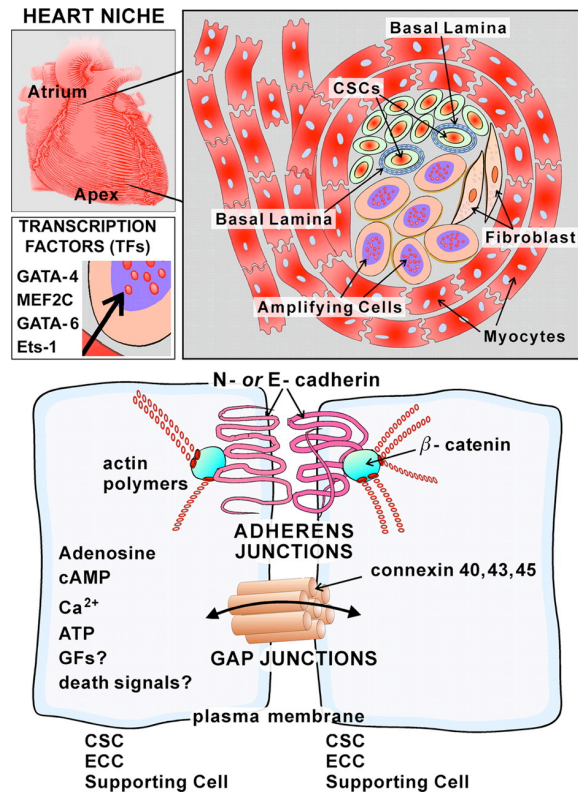


Figure 6: Schematic representation of CSC niches. Cardiac niches are prevalently located in the atria and apex and consist of differentiated myocytes that surround clusters of CSCs and highly dividing amplifying cells. The amplifying cells are committed cells and express transcription factors of the cardiac (GATA-4), myocyte (MEF2C), smooth muscle cell (GATA-6), and endothelial cell (Ets1) lineages. The interaction among CSCs, early committed cells (ECCs), and supporting cells occurs via junctional proteins (cadherins and connexins). From: (Leri et al., 2005).

Within a niche, a stem cell maintains a quiescent state by its anchorage to a neighboring supporting cell; when the stem cell starts to proliferate and differentiate this connection is lost. Committed cells are able to change location within the niche and, eventually, to leave the niche environment in response to specific stimuli migrating into areas of the tissue where new parenchymal cells and new vessels have to be formed. In this regard, mechanical deformation and high wall stress represent physical stimuli able to trigger cell migration, proliferation, differentiation and death (Estes et al., 2004; Leri et al., 2005). The combination of these events dictates the homeostasis of the cardiac niche.

The balance between proliferation and apoptotic death depends not only on the cell itself but also on the signals coming from the surrounding environment that reflect the needs of the organ. This phenomenon has been studied and well characterized during embryonic and fetal development. If the same equilibrium is operative in the adult heart and if the same mechanisms may be impaired in pathological condition, it is still under investigation.

Importantly, apoptosis is a phenomenon occurring not only in differentiated, progenitors and precursor cells, but also in stem cells, which were considered until recently an example of immortal cells. The role of apoptosis in the definition of the stem cell number in a niche and throughout the parenchyma has been recently demonstrated in the bone marrow (Domen et al., 2000), the intestine (Renehan et al., 2001), the brain (Lindsten et al., 2003) and the heart (Anversa et al., 2006; Chimenti et al., 2003; Urbanek et al., 2005).

1.3 CSCs and the biology of myocardial regeneration. The old paradigm that the heart is a post mitotic organ is slowly shifting to the new exciting notion that views the heart as a self renewing organ in which tissue homeostasis is maintained by a delicate balance of three different, although related processes: cell death, cell proliferation and cell differentiation.

Experimental observations on the human, murine and dog heart have demonstrated two important phenomena. First, myocyte can divide as documented by the activation of the cell cycle machinery coupled with BrdU incorporation, histone-H3 phosphorylation and Ki67, MCM5, Cdc6 and cyclin B1 expression in myocyte nuclei (Leri et al., 2005) indicating that the heart is not a post mitotic organ. Second, myocardial regeneration spontaneously occurs in humans following ischemic (Beltrami et al., 2001; Leri et al., 2005; Urbanek et al., 2005) and non-ischemic injury (Leri et al., 2005; Urbanek et al., 2003), confirming that the heart is an organ with self-renewing properties (Fig. 7). The documentation of mitotic figures in myocytes forced the researchers to answer the fundamental question concerning the origin of this subpopulation of replicating myocytes. Essentially there are two possibilities: a dedifferentiation process involving mature myocytes or a generation of newly formed dividing myocytes by the activation and differentiation of primitive cells. The former possibility implies that a fully mature cell could go backwards the differentiation program, disassembling the myofibrillar apparatus and reentering the cell cycle. This represents a quite complex and biologically challenging event. However, the documentation of the existence of resident CSCs, originally in the rat heart and subsequently in dogs, pigs, rats, mice and humans, gave the opportunity to see the process of myocyte proliferation under a new light (Anversa et al., 2006).

We now know that the mammalian heart contains self-renewing, multipotent and clonogenic primitive cells able to differentiate into the main cardiac cell lineages (cardiomyocytes, smooth muscle cells, endothelial cells and fibroblasts). These properties are fundamental for the

maintenance of tissue homeostasis and, importantly, represent a prerequisite for **myocardial regeneration**.

This dynamic view of the heart has been reinforced in the last few years by several laboratories that have documented the presence of a stem cell compartment in the heart (Anversa et al., 2006). The activation of such cells and the consequent continuous turnover of myocytes result in a heterogeneous cell population consisting of young, adult, old and senescent myocytes.

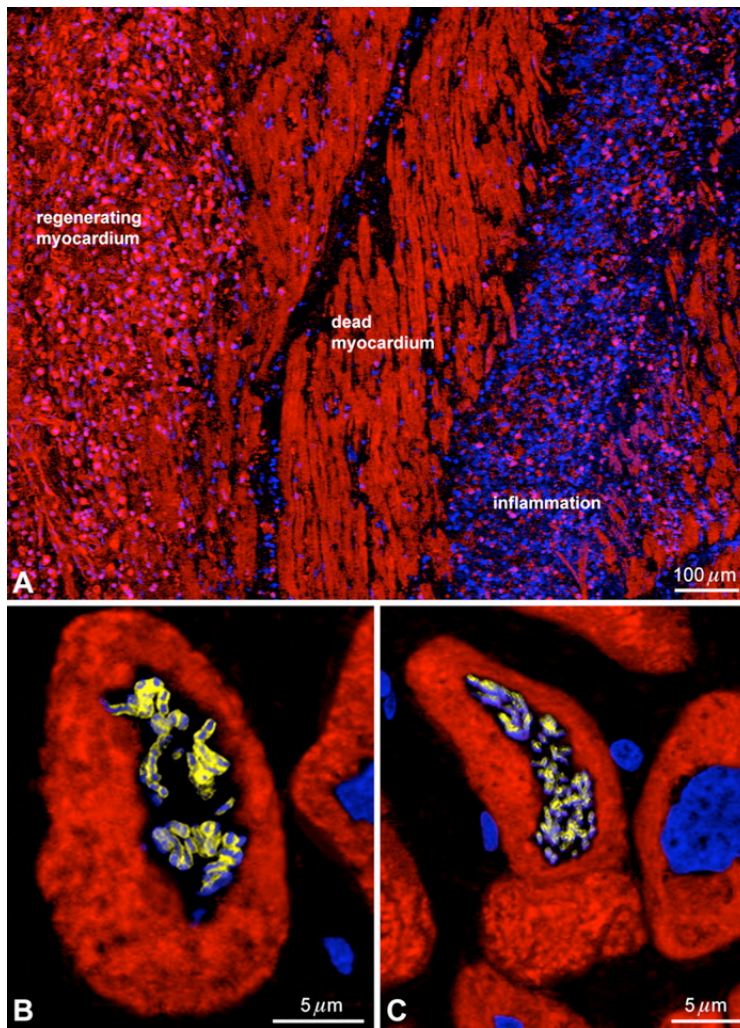


Figure 7: Myocardial regeneration in the human heart. *A:* an area of spontaneous formation of myocytes (-sarcomeric actin, red) is present in proximity of dead, infarcted myocardium and a region of inflammatory reaction. *B* and *C:* metaphase chromosomes (PI, blue and phospho-histone H3, yellow) are apparent in two small dividing myocytes (cardiac myosin heavy chain, red) in the heart of patients affected by chronic aortic stenosis. Modified from: (Urbanek et al., 2003).

Myocyte proliferation and activation of resident primitive cells both take place adjacent to (border zone) and far from the infarcted region. However, in mammals, (at variance with amphibians) this population of cells fails to colonize the dead tissue and differentiating in mature myocytes to fully restore the myocardium. The limited ability of mammalian tissues to repair the damage still remains without a reasonable explanation. In this regard, the physiological and

molecular events that underlie myocardial regeneration are still poorly understood. However, some factors have been identified as possible candidates. For example, the insulin-like growth factor-1 (IGF-1) and the hepatocyte growth factor (HGF) have been proposed as important molecules able to trigger cell proliferation and survival and cell migration, respectively (Linke et al., 2005). In this regard, CSCs and early committed cells express the HGF receptor (c-Met) and the insulin-like growth factor-1 (IGF-1) receptor and can synthesize and secrete the corresponding ligands HGF and IGF-1. Activated CSCs injected in the border zone of the infarct and stimulated with a cocktail of IGF-1 and HGF reconstituted dead myocardium and recovered myocardial functions in infarcted mice (Urbanek et al., 2005).

Importantly, mechanical deformation and high wall stress have been proven to mimic the effects triggered by chemokine and growth factors in terms of cell migration, proliferation, differentiation and death (Estes et al., 2004; Sadoshima and Izumo, 1997).

An important aspect addressing the issue of myocardial regeneration is represented by the extracellular matrix. Myocardial infarction has two effects: cell death and disassembly of the tissue scaffold. As in every organ, the parenchymal cells and the extracellular network constitute two interdependent components of the heart. The experimental observations of the regenerated heart have shown that the structural organization of the newly formed myocardium is characterized by myocytes oriented longitudinally to the tangential stress. The absence of the more complex tissue organization (cardiomyocyte bundles) typical of the ventricular wall may depend, in part, on the disruption of the extracellular scaffold. The usage of biomaterials for the synthesis of self-assembling scaffolds has become recently a novel and common strategy (Davis et al., 2005; Eschenhagen and Zimmermann, 2005). These scaffolds can drive an orderly growth of myocytes and coronary vessels and can be loaded with peptides able to stimulate the differentiation and the mobilization of resident progenitor cells (Koh and Atala, 2004; Narmoneva et al., 2004). Additionally, the combination of **tissue engineering** with self-assembling biomaterials and **cell therapy** with CSCs/CPCs may offer more powerful tools to overcome the injured heart.

However, a better understanding of the cardiac niche and a deep knowledge of the molecular mechanisms of tissue homeostasis would allow the stimulation of resident CSCs to become functionally competent, the induction of their migration and translocation from the niches to reach and repair the damaged tissue. This approach may provide new hints to face tissue injury offering new tools to induce myocardial regeneration and counteract tissue deterioration in the pathologic and aged heart.

2. Notch pathway overview.

In recent years a substantial body of evidence supported the notion that molecular pathways classically involved in tissue specification during embryonic development (Shh, Wnt and Notch) may be also operative in adult self-renewing organs (Wilson and Radtke, 2006).

The evolutionary conserved Notch pathway is one of the most important mechanisms that controls binary cell fate decisions during embryonic development and tissue homeostasis in adult life (Bray, 2006). The Notch gene was named for the phenotype of a mutation in *D. melanogaster* causing characteristic indentations (notches) in the rear portion of the fruit fly's wing (Morgan, 1917) (Fig. 8). The identification of new alleles allowed the studies on the recessive neurogenic phenotype (Metz and Bridges, 1917; Poulson, 1939) and, over the years, the analysis of these phenotypes eventually led to the discovery of different functions of Notch.

Most importantly, in the last few years, three different Notch activities during development have been uncovered: lateral inhibition, boundary formation and cell fate assignment (Bray, 1998). The first insights into the function and mode of action of Notch signaling came from studies in *D. melanogaster* and *C. elegans* (Fehon et al., 1990; Greenwald, 1985; Wharton et al., 1985; Yochem et al., 1988) which allowed the discovery of a core set of molecules involved in Notch signaling and led to the understanding of how they organize into a signaling pathway.

To date, four Notch isoforms have been described in mammals: Notch1 to Notch4 (Artavanis-Tsakonas et al., 1999). The Notch receptors are large single-pass type I transmembrane proteins and exist at the cell surface as a proteolytically cleaved heterodimer comprising a large ectodomain (Notch extracellular domain, NECD) and a membrane-tethered intracellular domain (Notch intracellular domain, NICD). Notch receptors interact with single-pass type I transmembrane ligands expressed on neighboring cells and this restricts the Notch pathway to regulating short-range intercellular interactions through lateral inhibition or inductive interaction (Bray, 2006).

In mammals, there are five genes encoding the Notch ligands: three Delta-like (*DLL1*, *DLL3* and *DLL4*) (Bettenhausen et al., 1995; Dunwoodie et al., 1997; Shutter et al., 2000) and two Jagged (*JAG1* and *JAG2*) (Lindsell et al., 1995; Shawber et al., 1996a) (Fig. 9).

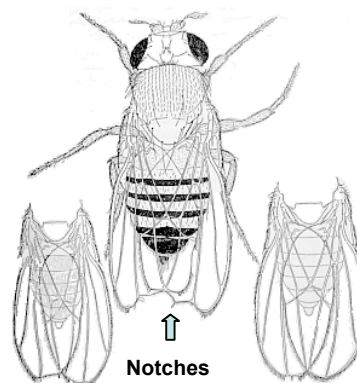


Figure 8: Notch wings phenotype. (left to right) average, extreme condition, nearly normal.

From: (T.H. Morgan, "The physical basis of heredity", 1919).

In *Drosophila*, there is only one Notch-encoding gene, one Delta and one Jagged homologues (Lissemore and Starmer, 1999; Maine et al., 1995). In *C. elegans*, there are two genes encoding for Notch (*lin-12* and *glp-1*) and several Delta/Serrate/Lag-2 (DSL) homologues (Greenwald, 1994; Lissemore and Starmer, 1999; Maine et al., 1995). The study of the genetics of simpler organisms such as *C. elegans* and *D. melanogaster* has provided enormous insights into the mechanics of Notch signaling and has paved the way for a better understanding of how Notch acts in higher eukaryotes.

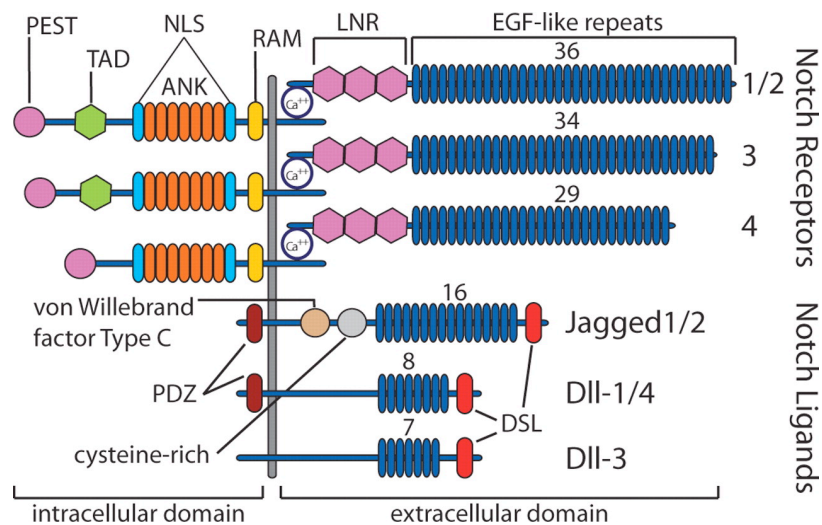


Figure 9: Structure of mammalian Notch receptors and their ligands. Mammals possess 4 Notch receptors (Notch1-4) and five ligands (Jagged1 and 2, and Delta-like 1, 3 and 4). Notch receptors are expressed on the cell surface as heterodimeric proteins. They are composed of an extracellular domain (NECD) and an intracellular domain (NICD). NECD contains up to 36 EGF-like repeats that mediate the interaction between Notch and its ligands, a cysteine-rich region known as Lin-12 repeats (LNR) and heterodimerization domains that bind non-covalently to the membrane-tethered intracellular Notch. NICD contains a RAM domain (RBPjk Associate Molecule), repeated structural motifs named Ankyrin repeats that mediate the interaction between Notch and CBF1/Su(H), two nuclear localization sequences (NLS), a transactivation domain (TAD) and a PEST domain that is involved in the degradation of Notch. Notch ligands Delta (DII1, 3 and 4) and Jagged (Jagged1 and 2) are also expressed on the cell surface. The extracellular domains contain a Delta/Serrate/Lag2 (DSL) domain unique to Notch ligands and also contain multiple EGF repeats. Jagged1/2 also contains a cysteine-rich domain and a von Willebrand factor type C domain. The intracellular domains of Jagged1 and DII1 have been shown to contain PDZ domains, which may interact with downstream signaling components to activate transcription. From:(Niessen and Karsan, 2007).

Notch receptor activation. The Notch receptor mediates molecular signaling between two neighboring cells. Upon binding with its ligands, the Notch receptor undergoes conformational changes that allow a proteolytic cleavage at the level of the Notch ectodomain. This event is catalyzed by metalloproteinases of the ADAM family and determines the release of the NECD from the cell membrane. Following a second proteolytic cleavage mediated by the γ -secretase complex NICD is released from the inner surface of the cell membrane. The presence of nuclear localization sequences (NLSs) targets the NICD to the nucleus (Fig. 10). Once in the nucleus, the NICD forms a complex with the sequence-specific DNA-binding protein of the CSL family RBP-jk (recombination signal binding protein for immunoglobulin kappa J region). In the absence of NICD, the RBP-jk protein binds to specific DNA sequences in the regulatory elements of various target genes and represses their transcription by recruiting histone deacetylases and other components to form a co-repressor complex. The nuclear translocation of NICD displaces the histone deacetylase-co-repressor complex from the RBP-jk protein. The NICD-RBP-jk complex recruits other proteins, such as the mastermind-like 1 (MAML1) protein and histone acetyltransferases, leading to the transcriptional activation of Notch target genes (Tanigaki and Honjo, 2007).

Among the most commonly induced Notch target genes are the basic helix-loop-helix (bHLH) transcriptional repressors of the hairy and enhancer of split/hairy and enhancer of split related with YRPW motif (Hes/Hey) family (Iso et al., 2003b; Kageyama et al., 2007).

Functions of the Notch pathway. The Notch signaling results in the up-regulation or in the down-regulation of transcription in several cell systems under physiologic and pathologic conditions. For this reason the Notch pathway has been described as a mechanism with a typical pleiotropic effect (Fig. 11).

The Notch pathway is a major determinant of binary cell fate decision that is achieved by two processes: lateral inhibition and asymmetric cell division.

Lateral inhibition is a fundamental mechanism dedicated to the definition of cell fate and cell spatial patterning during development and is probably the best characterized function of the Notch pathway (Heitzler and Simpson, 1993; Le Borgne and Schweisguth, 2003). Although lateral inhibition is a phenomenon mainly observed in insect neurogenesis (Niessen and Karsan, 2007) (Gibert and Simpson, 2003; Martin-Bermudo et al., 1995; Parks et al., 1997), there are data documenting that this process takes place in mice during hair cell development in the inner ear (Kiernan et al., 2005). Asymmetric cell division relies on cell polarization and is characterized by an asymmetric distribution of cell fate determinants before cell division that induces the different cell fate identity of the daughter cells.

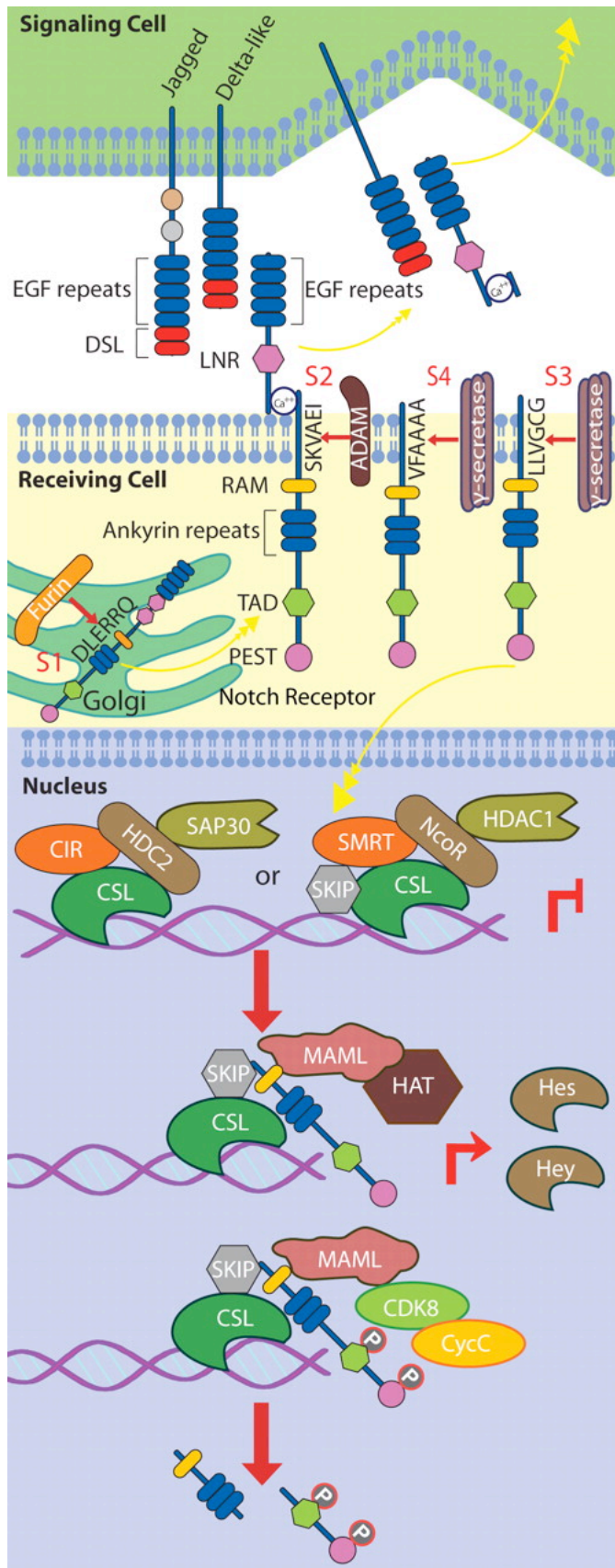


Figure 10: The Notch signaling pathway. Notch receptors undergo processing in the trans-Golgi network by Furin and are expressed on the cell surface as a non-covalently linked heterodimer stabilized by a calcium ion. Receptor-ligand interaction results in 3 additional cleavage events that result in the release of the ectodomain (S2) and the intracellular region of the Notch receptor (S3, S4). The ectodomain of the Notch receptor and the ligand are thought to be endocytosed by the signaling cell. NICD then translocates to the nucleus, where it binds and converts CSL from a transcriptional repressor to a transcriptional activator of the Hes and Hey family of genes. Notch signaling is negatively regulated by hyperphosphorylation of nuclear NICD by the nuclear kinase CycC:CDK8, which is recruited to NICD by the Notch pathway coactivator MAML. From: (Niessen and Karsan, 2007).

The best example of asymmetric cell division is in *D. melanogaster* neurogenesis. The asymmetric distribution of regulators of Notch signaling activity (Bazooka, Numb and Neuralized among others) determines the identity of the daughter cells as signal-sending or signal-receiving cells. Once Notch signaling is elicited, the cells differentiate according to binary cell fate decision mediated by Notch (Frise et al., 1996; Hutterer and Knoblich, 2005; Le Borgne and Schweisguth, 2003). Asymmetric cell division is a mechanism occurring also in vertebrate species as suggested by recent data (Knoblich, 2001).

Another important example of binary cell fate choices is the role of Notch in the maintenance of stem cell populations. Notch mediates the decision whether a cell should differentiate or remain in an undifferentiated state both in embryonic and in post-natal stem cell systems (Chiba, 2006).

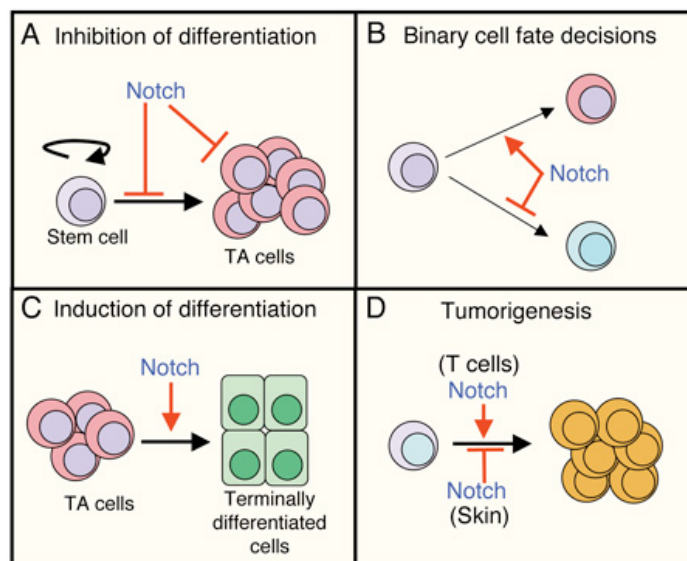


Figure 11: Pleiotropic effect of the Notch pathway. The four major roles of the Notch cascade that are relevant within self-renewing tissues or during tumorigenesis are schematically illustrated. A. Gate-keeper function: Notch maintains stem and/or transient amplifying cells (TA) in an undifferentiated state. In the intestine for example, Notch prevents crypt progenitor cells (TA) from differentiating. B. Binary cell fate decisions: In the lymphoid system Notch specifies the T cell lineage at the expense of the B cell lineage from an (at least) bi-potent early thymocyte progenitor. C. Induction of differentiation: In the skin, Notch induces terminal differentiation events of TA cells, and during thymocyte differentiation Notch1 promotes differentiation of pro-T cells into pre-T cells. D. Tumorigenesis: Overexpression of Notch within hematopoietic bone marrow cells or in T cell progenitors results in T cell leukemias, and as such, Notch functions as an oncogene. However in the skin, Notch functions as a tumor suppressor since loss of Notch signaling results in the development of basal cell carcinoma-like tumors. From: (Radtke F. web site).

The effects of Notch on the maintenance of stemness or initiation of the differentiation process are dose-, context- and time-dependent and, therefore, Notch modulates multiple phases of cell growth and turnover (Bray, 2006; Tanigaki and Honjo, 2007). Although Notch signaling ensures a proper balance between stem cells and their progeny, Notch may interfere or favor cell commitment. This lineage switch is apparent in the skin where Notch inhibits the formation of epidermal keratinocytes and promotes the generation of hair follicles (Estrach et al., 2006). Similarly the effects of Notch on bone marrow cells vary according to the state of maturation of the stimulated cells (Tanigaki and Honjo, 2007). The synthesis of the Notch ligand Jagged1 by osteoblasts increases the number of hematopoietic stem cells in the bone marrow niches (Calvi et al., 2003). In contrast, Notch activation in hematopoietic progenitors induces the generation of T lymphocytes (Radtke et al., 1999; Wilson et al., 2001). In the brain, Notch signaling expands the pool of immature precursors and enhances glial formation (Androutsellis-Theotokis et al., 2006).

For completeness it is important to underline that increasing evidence has been collected in support of the notion that Notch can effect multiple cellular processes independently from its CBF/Su(H) activity. For example, in vertebrates, Notch has been shown to inhibit skeletal muscle cell differentiation in a CSL-independent manner (Shawber et al., 1996b).

The Notch pathway plays also a role in boundaries formation during somitogenesis in vertebrates or in invertebrates during the establishment of the D/V boundary in the wing imaginal disc (de Celis and Bray, 1997; Major and Irvine, 2005; Micchelli et al., 1997).

Lastly, given the importance of Notch signaling in development it is not surprising that several human diseases are linked to defects of genes involved in Notch signaling (see Table 1). Mutations of the *Jagged1* gene are responsible for the Alagille syndrome, an autosomal dominant mutation that causes liver, kidney, eye, heart and skeleton developmental abnormalities (Artavanis-Tsakonas, 1997; Li et al., 1997; Oda et al., 1997). Mutations of the human *Notch1* and *Notch3* are responsible for the cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy syndrome (CADASIL) (Gridley, 2003; Harper et al., 2003). Deregulation of Notch signalling activity is at the basis of T-cell acute lymphoblastic leukaemia (Sjolund et al., 2005; Weng et al., 2004). Finally, a family of diseases, spondylocostal dysostosis, is caused by mutations of *Dll3* and is characterized by vertebral defects (Gridley, 2003).

Notch pathway defects and human disease

Disase	Cause
Alagille syndrome	mutations on the Jagged1 gene.
CADASIL syndrome	mutations on Notch1 and Notch3.
T cell acute lymphoblastic leukemia	mutation involving either the Notch heterodimerization domain or the PEST domain. Translocation of a truncated form of Notch resulting in signaling Hyperactivation.
Spondylocostal dysostosis (SD)	mutations in Dll3. Epigenetic results suggest Lunatic Fringe mutations could also cause SD.

Table 1: Notch and diseases.

3. The Notch pathway in the cardiovascular system.

3.1 Notch and cardiac development. The heart is the first functional organ that is formed during vertebrate development (Fig. 12). In mice at embryonic day 7 (E7.0) cardiac markers start to be expressed in the primitive streak (one of the first sign of gastrulation). At this stage cardiac precursors expressing *CITED2* (considered the earliest transcriptional activator expressed during heart development) undergo epithelial-to-mesenchymal transformation (EMT) and migrate bilaterally from the primitive streak forming the **primary heart field** (left and right) (Schlange et al., 2000). This event corresponds to the expression of the transcription factors *Mesp1* and *Mesp2* (Kitajima et al., 2000). The endoderm underlying the primary heart field plays an important role in the definition of the cardiac phenotype due to the secretion of several factors including the bone morphogenetic protein (BMP) family, sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF-8). Additionally the ectoderm and the endoderm secrete Wnt inhibitors required for the induction of the cardiogenic fate. Even at this early stage of development, the compartmentalization of heart chambers occurs (atrial precursors in the posterior region and ventricular precursors in the anterior region of the heart field) and the main markers of the primary heart field are apparent (*Nkx2.5*, *BMP2*, *GATA4*, *GATA5*, *GATA6* and *Tbx20*) (Redkar et al., 2001).

During development the right and left heart fields undergo anterior-medial migration and subsequently fuse at the anterior giving rise to the **cardiac crescent**. The cardiac crescent is quickly divided into a ventral myogenic domain (future myocardium of the heart tube) and a dorsolateral non-myogenic domain (future mesocardial and pericardial roof cells) (Raffin et al., 2000).

As in several other organs, the Notch pathway plays an important role during heart development. Evidence has been accumulated that the Notch signaling controls cardiac cell fate downstream of heart field specification. In this regard, it has been documented in *X. laevis* that the activation of the Notch pathway by the ligand *Serrate1* suppresses the myogenic potential of the cells residing in the dorsolateral domain; however, the expression of the primary heart field markers *Nkx2.5* and *GATA4* are unaffected (Rones et al., 2000). Additionally, in CSL-deficient mouse embryos the primitive heart tube and heart field specification appear normal (Oka et al., 1995; Souilhoul et al., 2006).

During subsequent folding of the embryo the cardiac crescent fuses into a tube-like structure that starts to beat at E8.0 in the mouse and after about 3 weeks of gestation in humans.

A secondary heart field located in the splanchnic mesoderm and expressing common cardiac markers such as *Nkx2.5* and *GATA4* together with peculiar markers such as *Nkx3.1* and FGF-

10, has been identified in the chick and mouse embryos, but its contribution to the adult heart remains unclear (Kelly and Buckingham, 2002; Schneider et al., 2000).

The primitive heart tube undergoes conformational changes (heart tube looping) that align and fuse the heart chambers. Data support a function of the Notch pathway in heart tube looping. At this stage Notch pathway inhibition (null mouse embryos for CSL or Notch and embryos deficient for Dll1) produces randomized heart looping and axial rotation (Krebs et al., 2003; Przemeck et al., 2003; Raya et al., 2003). However, these effects are not reproduced in Notch1-deficient and Notch2-deficient embryos (Hamada et al., 1999; Krebs et al., 2003).

At E9.5 the mouse cells of the atrio-ventricular (AV) canal and the outflow tract (OFT) are activated by signals originated in the myocardium and by inter-endocardial signaling pathways to undergo endothelial-to-mesenchymal transition (EMT). This process is necessary for the formation of mesenchymal cells from the endocardial cell during cardiac cushion development. As development progresses mesenchymal cells proliferate determining the fusion of the cardiac cushions within the heart tube lumen forming the initial septa. The cardiac cushions undergo further remodeling producing protruding leaflets composed by endocardial cell and extracellular matrix that concur to the formation of the cardiac valves. The Notch pathway is involved in the onset of EMT during heart development. Loss and gain-of-function studies have revealed that Notch1, Notch4 and Dll4 are expressed at the beginning of the EMT in the AV canal and in the OFT. Both Notch1-deficient and CSL-deficient mouse embryos show a significant reduction in the EMT at the level of the AV canal (Timmerman et al., 2004). Additionally, zebrafish injected with constitutively active Notch1 resulted in hypercellularized AV tract and enlarged valves as a result of increased EMT (Timmerman et al., 2004). Another indication that Notch signaling is clearly involved in EMT is provided by *in vitro* experiments showing that the activation of the Notch pathway in endothelial cells resulted in EMT (Nosedá et al., 2004).

The role played by the Notch pathway in EMT seems to be restricted only to the initial phases of this process and does not appear to be required for the maintenance of the mesenchymal phenotype as indicated by the high expression of Hey2 (target of Notch) at the onset of EMT and its absence in the mesenchymal cells.

Notch is important for the proper trabeculation of the ventricle since Notch-deficient embryos show a decrease in myocardial proliferation early in development. Ventricular chamber morphogenesis, first manifested by trabeculae formation, is crucial for cardiac function and embryonic viability. In this regard, Notch1 expression is found to be highest at the presumptive trabecular endocardium and, importantly, RBP-jk and Notch1 mutants show impaired trabeculation (Grego-Bessa et al., 2007). Additionally, Notch signaling plays an important role in the differentiation of cardiac conduction cell lineage in the ventricles. Notch1 expression coincides with the expression of a conduction marker, HNK-1, at early stages. The expression

of constitutively active Notch1 (NICD) in early heart tubes in chick produced a significant increase in the expression of conduction cell markers HNK-1 and SNAP-25 (Chau et al., 2006). However, this study reported a significant decrease in the expression of cardiac muscle markers in cell positive for NICD.

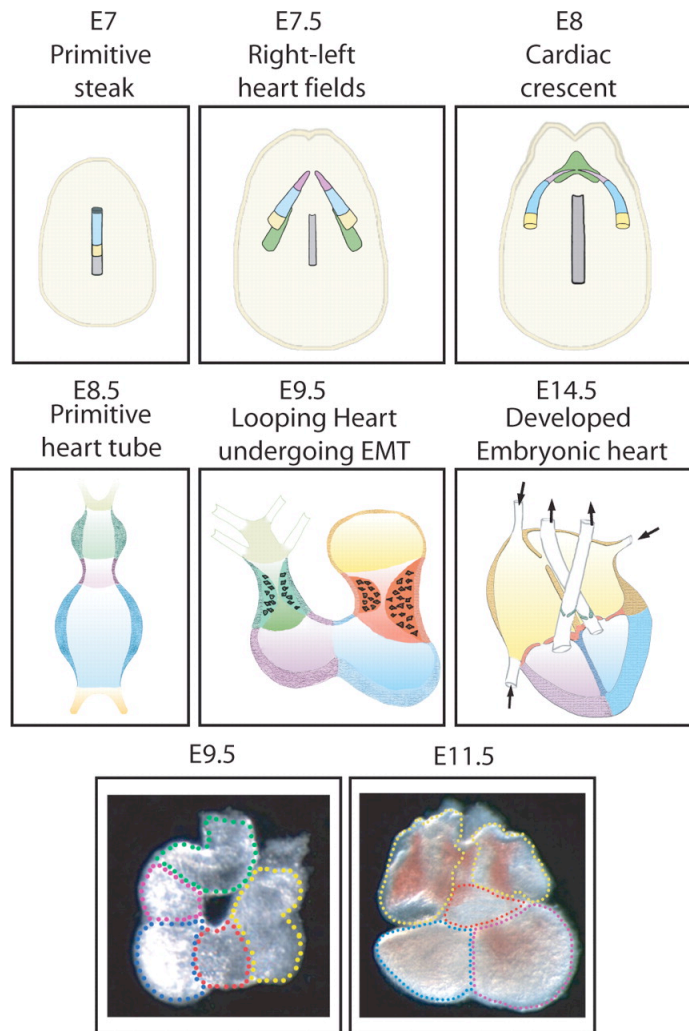


Figure 12: Cardiac Development. Cardiac precursors are identifiable in the anterior third of the primitive streak (blue and yellow), but not in the node (dark grey). As embryonic development progresses the heart precursors undergo EMT and migrate bilaterally from the primitive streak forming the left and the right heart fields (E7.5). The primary and secondary (green) heart fields undergo anterior-medial migration and fusion forming the cardiac crescent (E8.0). The primitive heart tube is formed by the fusion of the cardia crescent at embryonic midline at E8.5. The primary heart field gives rise to the left ventricle (blue), right ventricle (purple) and atria (yellow) while the secondary heart field gives rise to the cells of the outflow tract (green). The heart tube undergoes elongation at the atrial and ventricular ends and looping forming a C-shaped structure is initiated shortly after the formation of the heart tube (E8.5). At E9.5 EMT is initiated in the AV canal (red) and OFT (green) and the cardiac cushions become cellularized by EMT. EMT-derived cells from the AV contribute to the membranous atrial septum and mitral and tricuspid valves, while OFT EMT-derived cells combined with the neural crest-derived cells contribute to the aortic and pulmonary valves. Panels at E9.5 and E11.5 represent images of mouse embryonic hearts at the respective stages. From (Niessen and Karsan, 2007).

3.2 Notch and vascular development. During development the endothelial cells originates from the embryonic mesoderm and in particular from mesodermal progenitors constituting the **angioblast**. The angioblast initially differentiates into a primary vascular plexus that following extensive remodeling gives rise to arteries, veins and capillaries.

Although the expression of the components of the Notch pathway is dynamic during development rendering complicated their detection, three Notch receptors (Notch1, 3 and 4), three Notch ligands (Dll4, Jagged1 and 2) and three Notch effectors (HERP1, 2 and 3) seem to be involved in the vascular system (Iso et al., 2003a).

The Notch pathway is important for arterial specification and this is in line with the fact that even though the Notch pathway components are initially expressed throughout the developing cardiovascular system, they are typically restricted to cells that will acquire an arterial fate later in development. In this regard, the primary vascular plexus (E9.5) is characterized by the expression of Notch1, Notch4 and Dll4, but later (E13.5) the receptors Notch1, Notch2 and Notch4 and the Notch ligands Dll4, Jagged1 and Jagged2 result mainly expressed in the arterial endothelium (Villa et al., 2001). In mice, Notch1/Notch4-double deficient and Dll4-deficient embryos display defects in arterial specification (Gale et al., 2004; Krebs et al., 2004; Krebs et al., 2000; Xue et al., 1999).

The capillary endothelium is also characterized by the expression of Notch1, Notch4 and Dll4. Smooth muscle cells express high levels of Notch3 and Jagged1, but low level of Notch1 (Villa et al., 2001). Interestingly, it has been reported that, after injury, arteries express several Notch receptors and Notch ligands (Lindner et al., 2001).

Concerning the molecular patterning in arterial vein specification, recent studies have produced data supporting a sequential activation of Shh, vascular-endothelial growth factor (VEGF) and Notch in a signaling cascade in which Shh is upstream of VEGF and VEGF is upstream of Notch (Niessen and Karsan, 2007). The connection between these molecules has been demonstrated in zebrafish in which Shh deficiency resulted in the decrease of VEGF expression and loss of arterial specification (Lawson et al., 2002).

Evidence exists that Notch target genes play a role in the arterial-venous specification. In this regard, mouse embryos double deficient for Hey1 and Hey2 display defects in arterial specification (Fischer et al., 2004). Additionally, experiments conducted in zebrafish highlighted the function of gridlock (Hey homolog in *D. rerio*) in promoting arterial specification and inhibiting vein specification (Zhong et al., 2001; Zhong et al., 2000). However, opposite results have been obtained (Lawson et al., 2001).

Preliminary remarks:

Although the role of the Notch pathway has been described in the developing heart, the contribution of this molecular signaling to tissue homeostasis in the adult heart remains largely unknown.

The goal of the present study was to uncover possible implications of the Notch pathway during myocyte formation in the adult mouse heart. In particular my interest has been focused on a possible connection between the activation of the Notch1 receptor and the up-regulation of the cardiomyocyte transcription factor Nkx2.5 that is one of the earliest marker of cardiomyocyte lineage specification.

This study has been developed at the Cardiovascular Research Institute,
New York Medical College, Valhalla, NY in the laboratory led by Prof. Piero Anversa.

RESULTS.

1. Abstract

The Notch pathway mediates cell fate decision in multiple organs by regulating the expression of transcription factors that control cell differentiation. In the current work we tested the hypothesis that Nkx2.5 is a target gene of Notch1 and thereby regulates myocyte commitment in the adult mouse heart. Cardiac progenitor cells (CPCs) in the Niche express the Notch1 receptor and the supporting cells exhibit the Notch ligand Jagged1. In CPCs stimulated with Jagged1, the nuclear translocation of Notch1 intracellular domain (NICD) was coupled with the up-regulation of Nkx2.5 and the formation of cycling myocytes in vitro. NICD and RBP-jk form a protein complex in CPCs that, in turn, binds to the promoter of the Nkx2.5 gene initiating transcription. In contrast, transcription factors of vascular cell lineages are down regulated following Jagged1 stimulation and Notch1 activation. Consistent with these in vitro observations, inhibition of Notch1 signaling in infarcted mice impaired cardiomyogenesis. Conversely, the commitment of resident CPCs to myocytes that retained a high proliferative state was preserved in control animals. These results indicate that Notch1 activation favors the early commitment of CPCs to the myocyte lineage, but the expression of Nkx2.5 does not abrogate their ability to divide. Cycling Nkx2.5 positive myocytes correspond to a transient amplifying cells which condition the replicative capacity of the heart in physiologic and pathologic states.

2. Notch1 activation favors cardiomyocyte differentiation through the expression of the cardiac transcription factor Nkx2.5.

2.1. Cardiac niches contain Notch1-positive CPCs. Notch signaling controls the size and the composition of stem cell niches in several organs (Androutsellis-Theotokis et al., 2006; Calvi et al., 2003; Song et al., 2007). *c-kit*-positive CPCs are organized in niches that are located preferentially in the atria and in the apex of the adult heart (Urbanek et al., 2006). To establish whether the Notch pathway was involved in the maintenance of mouse CPCs homeostasis, the first step was to demonstrate the presence of the components of the Notch pathway (Notch receptors and Notch ligands) in CPCs within a niche. Most cardiac niches in the mouse heart contain Notch1 positive CPCs that are nested in the myocardial interstitial space and are connected to the surrounding myocytes by the junctional proteins connexins 43 and N-cadherin (Fig. 13).

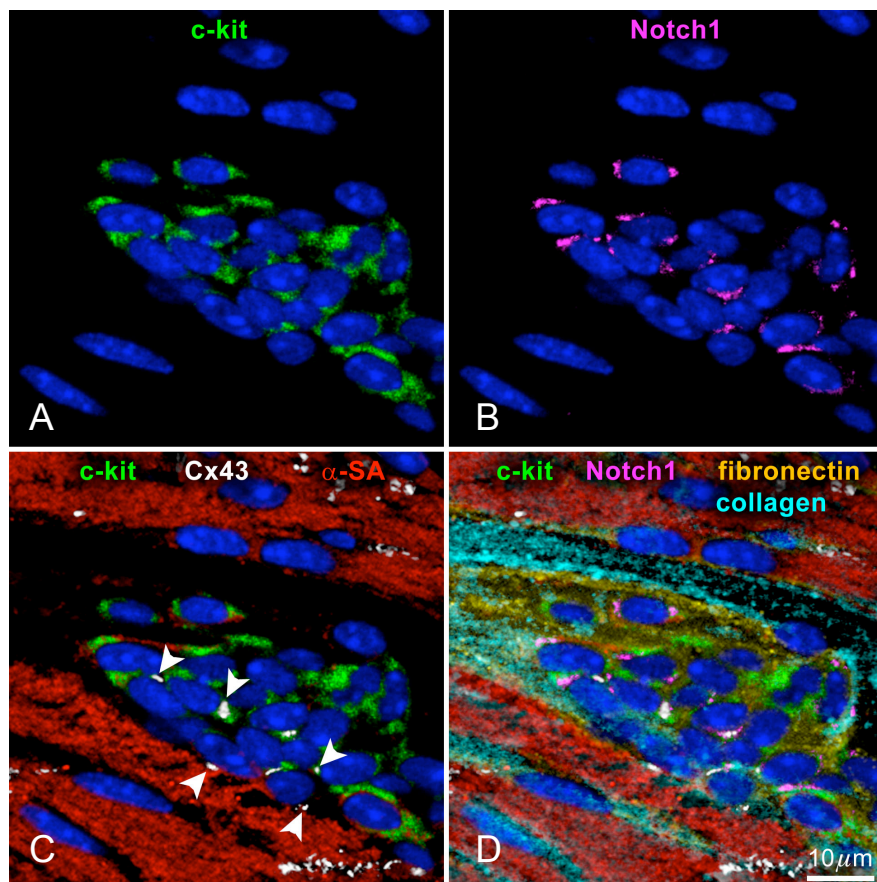


Figure 13. Cardiac progenitors, *c-kit* and *Notch1* receptor. (A-D) Cardiac niche in normal mouse myocardium. Majority of *c-kit*-positive cells (green) express *Notch1* receptor (B, magenta). Connexin 43 (C, white dots, arrowheads) is present between *c-kit*-positive cells and between *c-kit*-positive cells and myocytes (α -sarcomeric actin, α -SA, red); (D) merge and distribution of fibronectin (yellow) and collagen (light blue).

The presence of such connections demonstrates that Notch1 positive CPCs are functionally and mechanically connected to the surrounding myocardium. Importantly, the Notch ligand *Jagged1* is expressed in proximity of *c-kit* positive CPCs and is distributed at the interface between CPCs and between CPCs and myocytes (Fig. 14). The Notch ligand Delta-like-4 does not show a similar distribution being present predominantly in coronary vessels (Fig. 15).

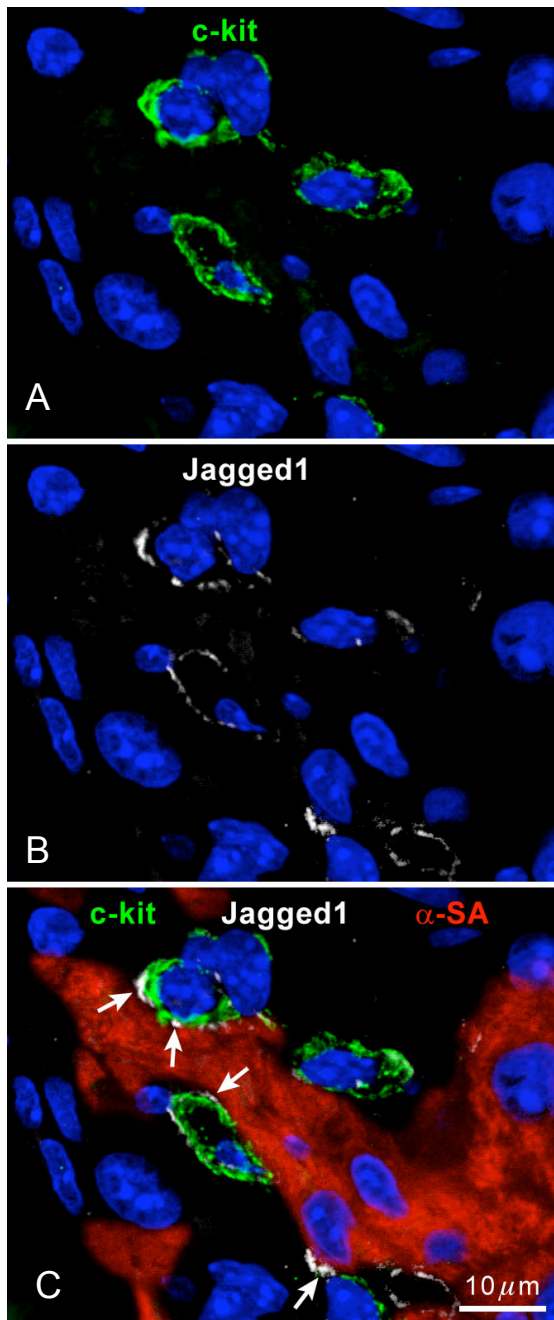


Figure 14. (A-C) *c-kit*-positive cells (green) located in the atrial myocardium; (B and C) distribution of *Jagged1* (white, arrows) between *c-kit*-positive cells and myocyte.

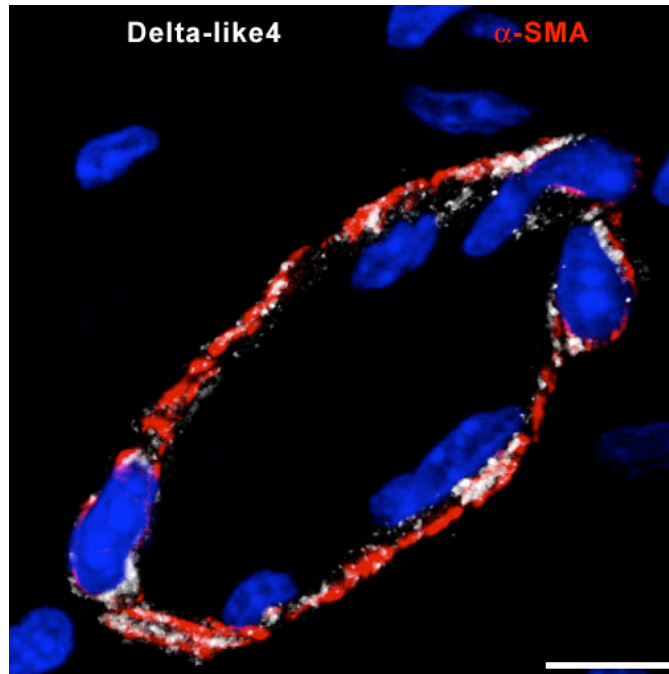
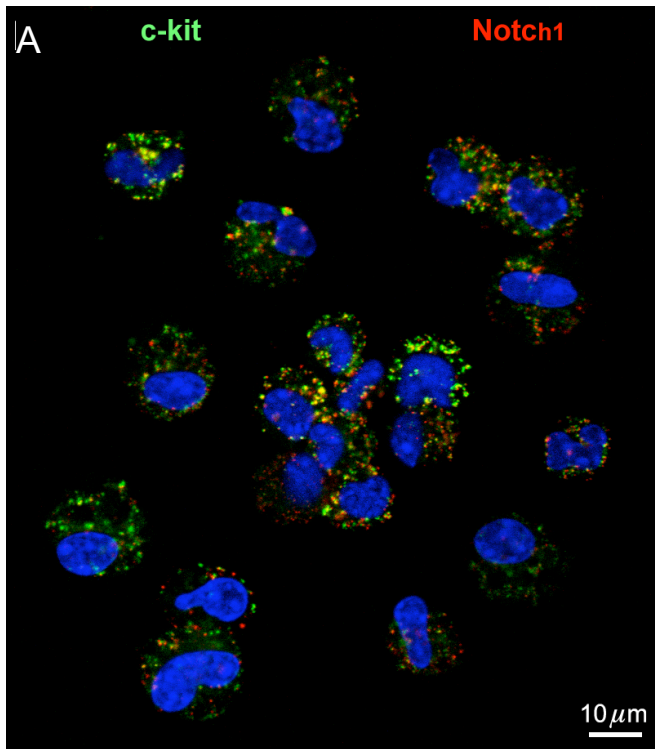


Figure 15: Small vessel in normal mouse myocardium. Smooth muscle cells (α -smooth muscle actin, α -SMA, red) express the *Notch* ligand *Delta-like-4* (white). Bar=10 μ m.

2.2. *Notch1* regulates CPCs commitment. Given the existence of Notch1-positive CPCs in the mouse heart and because of the role that Notch plays in stem cell fate, we assessed first the degree of expression of this receptor in freshly isolated mouse CPCs. By immunocytochemistry, an average of 60% of CPCs were Notch1 positive. Notch1 was restricted to CPCs lacking the expression of the transcription factor GATA4, employed as a marker of cell commitment (Urbanek et al., 2006). This suggested that the presence of the intact form of the Notch1 receptor identified lineage negative CPCs. Only 20% of Notch1 negative CPCs expressed GATA4 (Fig). Importantly, c-kit and the other Notch receptor isoforms Notch2, Notch3 and Notch4 were found in ~10% of cells, pointing to Notch1 as the major component of the Notch pathway in CPCs (Fig. 16).

Because the number of progenitor cells, ~250, in the adult mouse heart is small (Urbanek et al., 2006), CPCs were expanded in vitro to characterize the effect of Notch1 on cell differentiation. Therefore, freshly isolated CPCs at late passages (P8-11) were employed for these studies. The properties of CPCs at baseline were assessed by immunocytochemistry: 60 \pm 11% and 35 \pm 2% of cultured CPCs was c-kit positive and expressed Notch1, respectively.



C

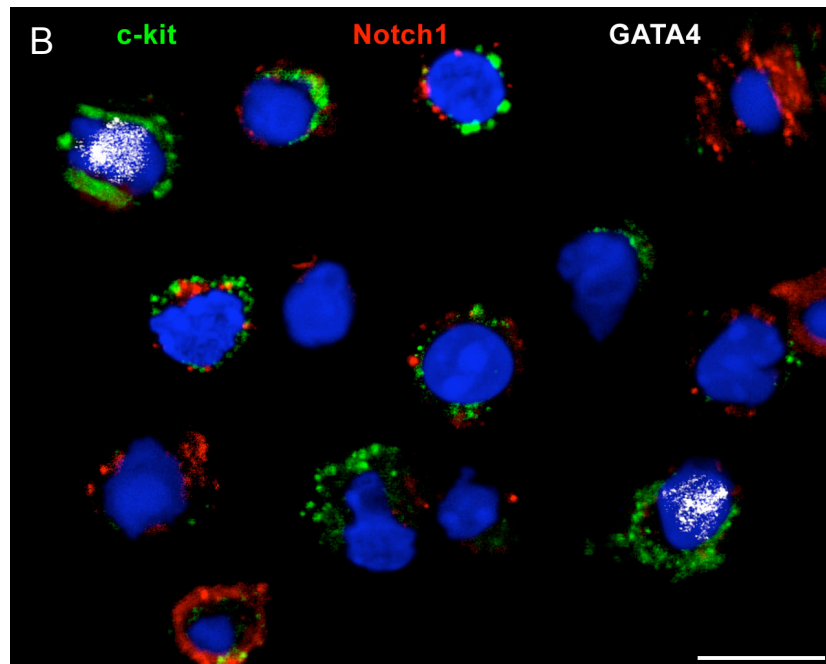
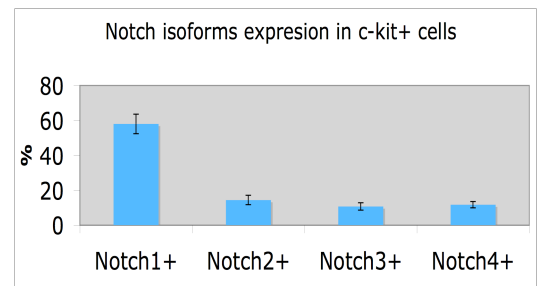


Figure 16. (A) Cytospin preparation of *c-kit*-positive CPCs: *c-kit*-positive cells (green) co-express *Notch1* receptor. (B) *c-kit*-positive (green) *Notch1*-positive (red) cells (arrows) are negative for *GATA4* (white dots). (C) Notch1, 2 and 3 isoforms expression in *c-kit* positive cells.

Importantly, $63\pm 11\%$ of c-kit positive cells expressed Notch1 and $96\pm 2\%$ of Notch1 positive cells were c-kit positive. Cultures of CPCs were then exposed to the immobilized Jagged1 ligand in the presence or absence of γ -secretase inhibition (Kornilova, AY JBC 2003); the γ -secretase cleaves the Notch1 receptor and, thereby, releases NICD activating the transcription of Notch1 dependent genes (Fortini, 2002). Conversely γ -secretase blockers interfere with Notch1 function.

Since Hes1 is a well-established target gene of Notch, its expression was employed to document the state of activation and inhibition of the Notch1 pathway in CPCs. Jagged1 resulted in a time dependent increase in the quantity of Hes1 transcript while γ -secretase inhibition markedly attenuated, although did not abrogate, Hes1 transcription (Fig. 17). The short half-life of the γ -secretase inhibitor may oppose complete repression of the Notch pathway.

Based on these results, the consequences of Jagged1 stimulation together with γ -secretase inhibition on the localization of NICD in CPCs were measured at 2, 5 and 8 days after plating. Similarly, the expression of Nkx2.5 and GATA4 was determined to identify committed CPCs. For this purpose, CPCs were stained with an antibody that recognized exclusively the active cleaved form of Notch1, NICD. The loss of the extracellular domain of Notch1 was accompanied by the translocation of NICD to the nucleus. In contrast, NICD was absent in cells that displayed high Notch1 expression on the plasma membrane. Intermediate stages of co-localization were also present (Fig. 18).

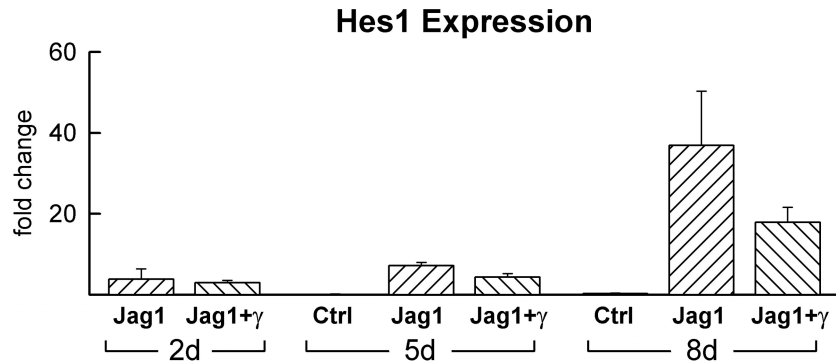


Figure 17: Notch pathway activation with *Jagged1* in the absence (*Jag1*) and presence of gamma-secretase inhibitor (*Jag1+g*). *Hes1* mRNA levels measured by real time RT-PCR at 2 (2d), 5 (5d) and 8 (8d) days in culture. Fold change vs. Ctrl at 2 days.

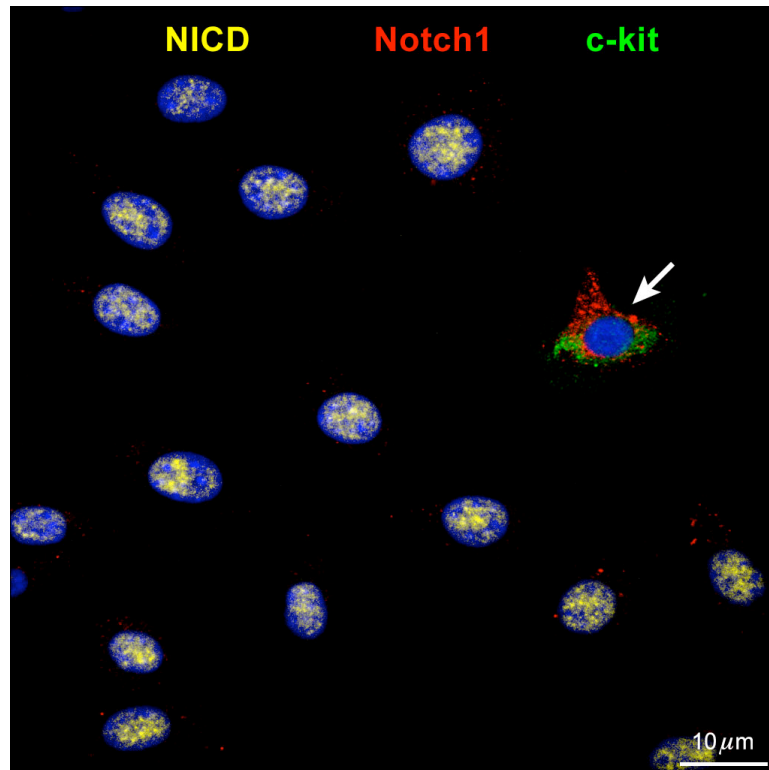


Figure 18. *Notch1* stimulation and nuclear localization of its active form (*Notch* intracellular domain, NICD). Majority of stimulated CPCs express NICD in their nuclei (yellow) and are negative for *c-kit*. CPC in which *Notch* pathway remains not active (arrow) continue to express *Notch1* (red) and *c-kit* (green) on the cell surface.

Following Jagged1 stimulation, the percentage of CPCs positive for Notch extracellular domain decreased dramatically from 2 to 5 days and remained low at 8 days (Fig. 19).

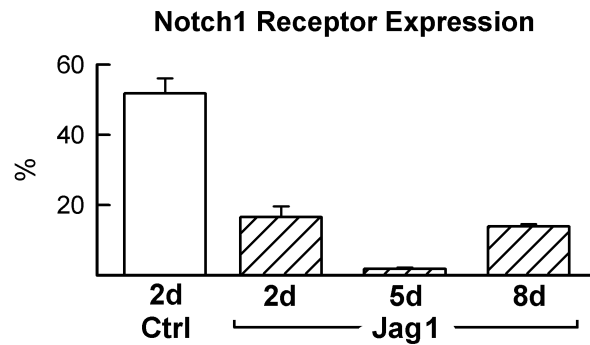


Figure 19. *Jagged1* stimulation and *Notch1* receptor activation. *Notch1* activation in stimulated cells (*Jag1*) at 2, 5 and 8 days in culture results in a significant loss of the membrane-bound *Notch1* receptor compared to non-stimulated cells (*Ctrl*).

To identify the function of Notch1 in the acquisition of the myocyte lineage by CPCs, the expression of NICD, Nkx2.5 and GATA4 was established. Nkx2.5 was consistently co-expressed with NICD and Jagged1 markedly enhanced this association and myocyte formation (Fig. 20). In the presence of Jagged1, the fraction of CPCs positive for NICD and Nkx2.5 increased sharply at 5 days after stimulation and remained elevated at 8 days (Fig. 21). The up-regulation of NICD promoted by Jagged1 was not characterized by a comparable response of Nkx2.5 in CPCs at 2 days. However the induction of these two proteins was essentially identical at 5 and 8 days, suggesting that the transcriptional regulation of Nkx2.5 in CPCs may depend partly on Notch1 activation. Importantly blockade of Notch1 signaling abrogated Jagged1 function. These histochemical results were confirmed by real time RT-PCR (Fig. 22). Conversely, Notch1 activation had a negative impact on GATA4; the number of GATA4 positive CPCs increased with γ -secretase inhibition pointing to a repressive role of the Notch1 pathway in the transcription of GATA4 (Fig. 23).

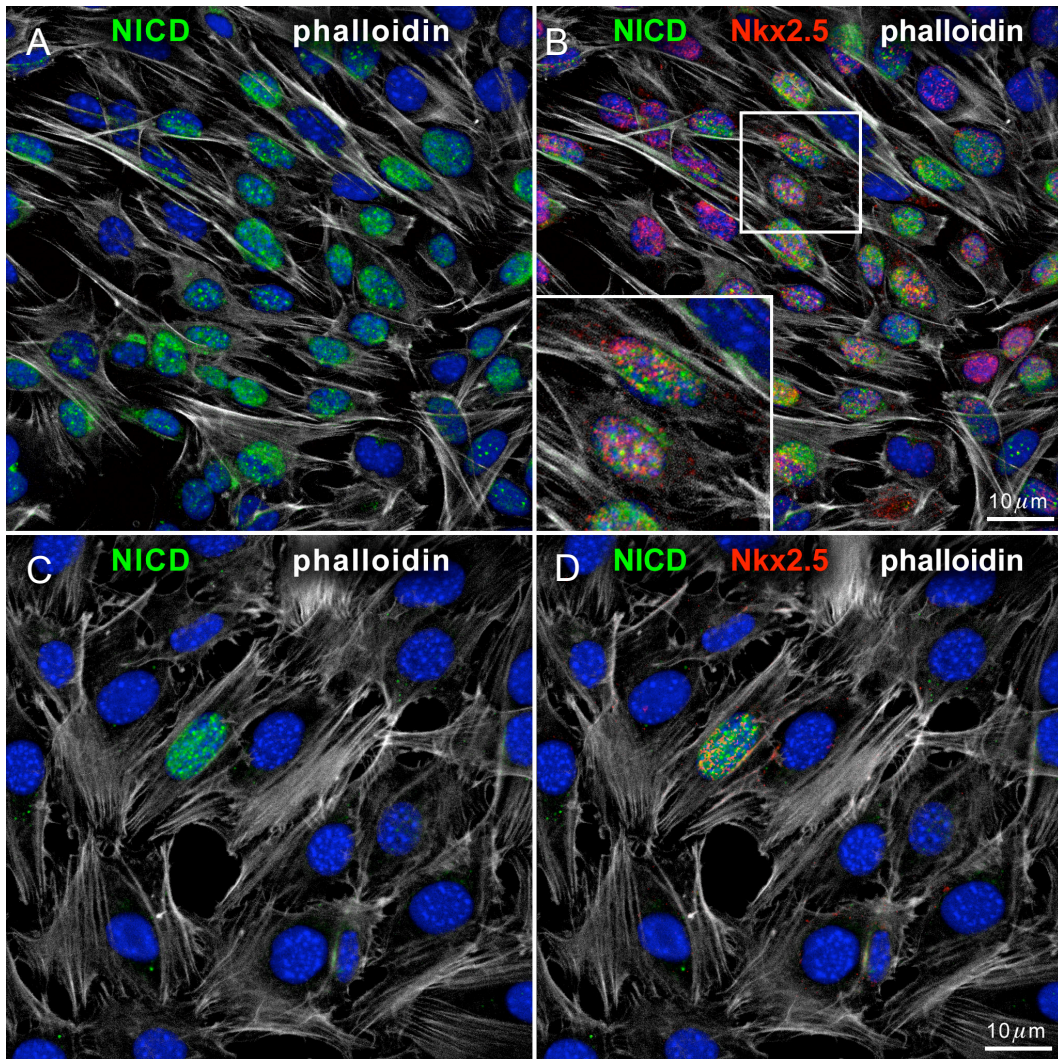


Figure 20. (A-D) *Notch1* stimulation and inhibition affect cardiomyocyte commitment of CPCs. (A and B) *Jagged1*-stimulated CPCs express NICD (green) together with *Nkx2.5* (magenta) in their nuclei. Area included in the square is shown at higher magnification in the inset. (C and D) Majority of *Jagged1*-stimulated CPCs in presence of γ -secretase inhibitor are negative for NICD and *Nkx2.5*. Phalloidin staining (white) illustrates cell bodies.

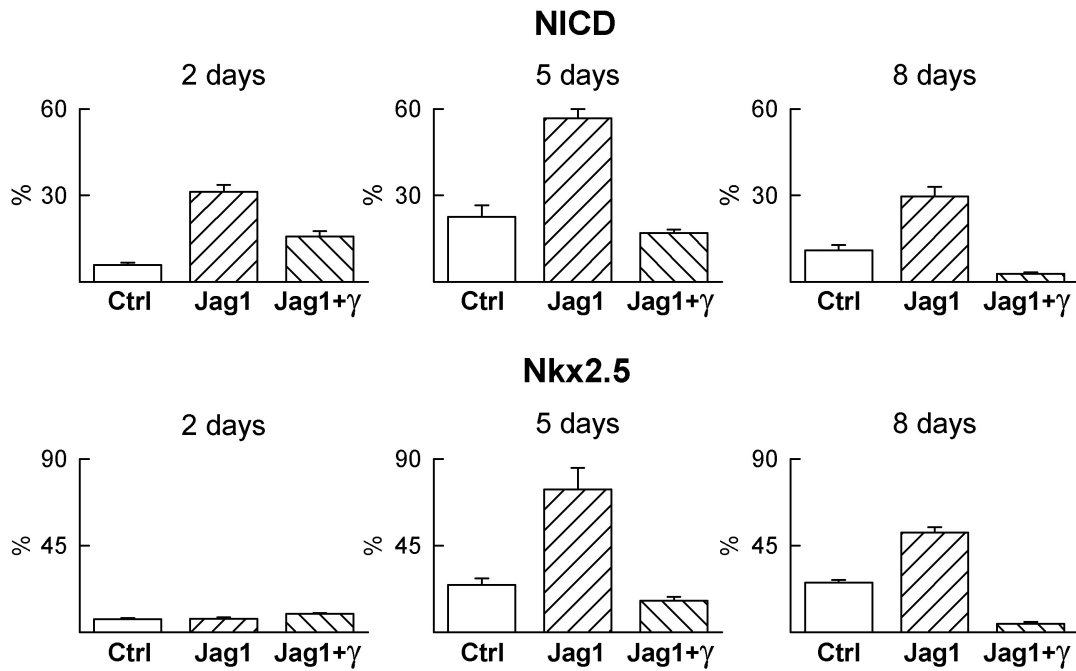


Figure 21. Fraction of CPCs positive for *NICD* and *Nkx2.5* after 2, 5 and 8 days of culture under different conditions: uncoated (Ctrl), *Jagged1*-coated (*Jag1*) and *Jagged1*-coated treated with γ -secretase inhibitor (*Jag1+ γ*).

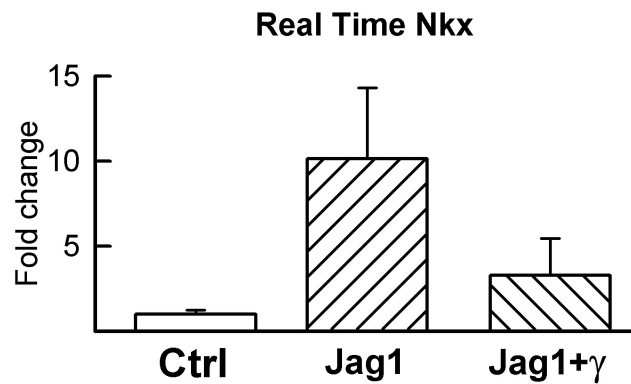


Figure 22. *Notch* pathway activation (*Jag1*) and inhibition (*Jag1+ γ*) and *Nkx2.5* mRNA levels measured by real time RT-PCR.

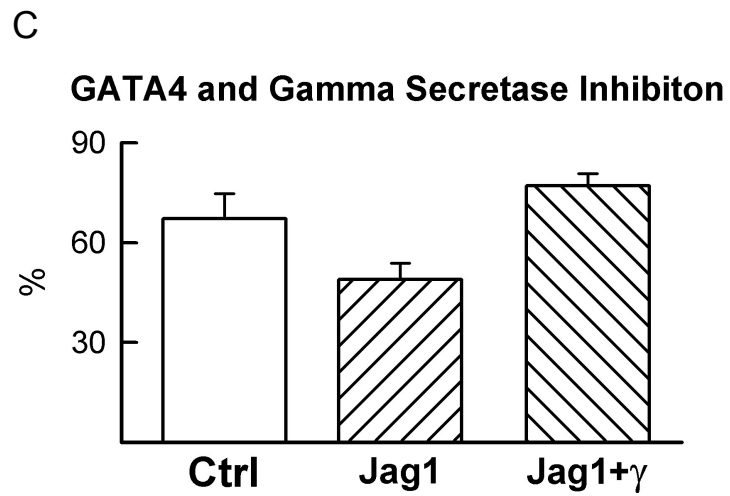
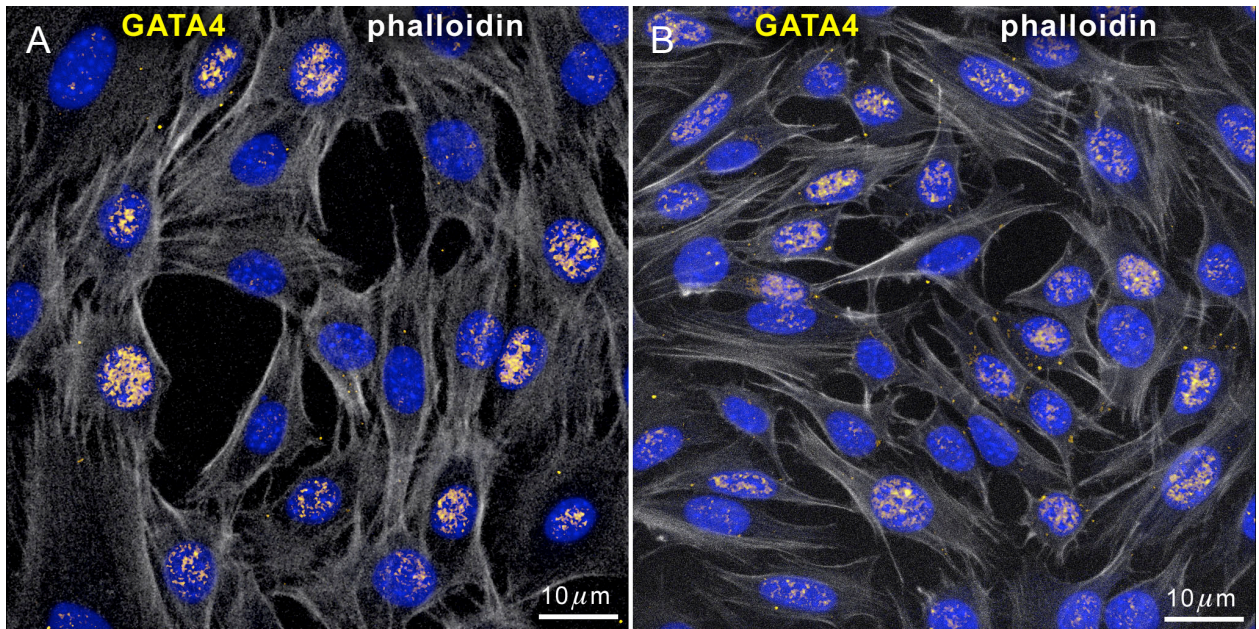


Figure 23. (A-B) *GATA4* expression (yellow) in CPCs stimulated by *Jagged1* in the absence (A) and presence (B) of γ -secretase inhibitor. (C) Fraction of CPCs positive for *GATA4* under different culture conditions.

2.3 Notch1 and cardiac cell differentiation. The recognition that Notch1 activation is linked to the expression of Nkx2.5 and myocyte commitment raised the question whether Notch1 signaling represses or promotes the acquisition of endothelial cell (EC) and smooth muscle cell (SMC) lineages by CPCs. Therefore the mRNA quantity for the EC transcription factor *VeZF1* and the SMC transcription factor *GATA6* was measured by real time RT-PCR in CPCs treated in a manner identical to that discussed above. In the presence of Jagged1, *VeZF1* and *GATA6* transcripts decreased consistently over time. Following 8 days of stimulation, a nearly 60% reduction in the expression of these transcription factors was detected. This negative response was partially reversed by γ -secretase inhibition (Fig. 24). Importantly, the effects of Jagged1 on *Nkx2.5* and *GATA4* mRNA were similar to those shown above at the protein level by immunocytochemistry.

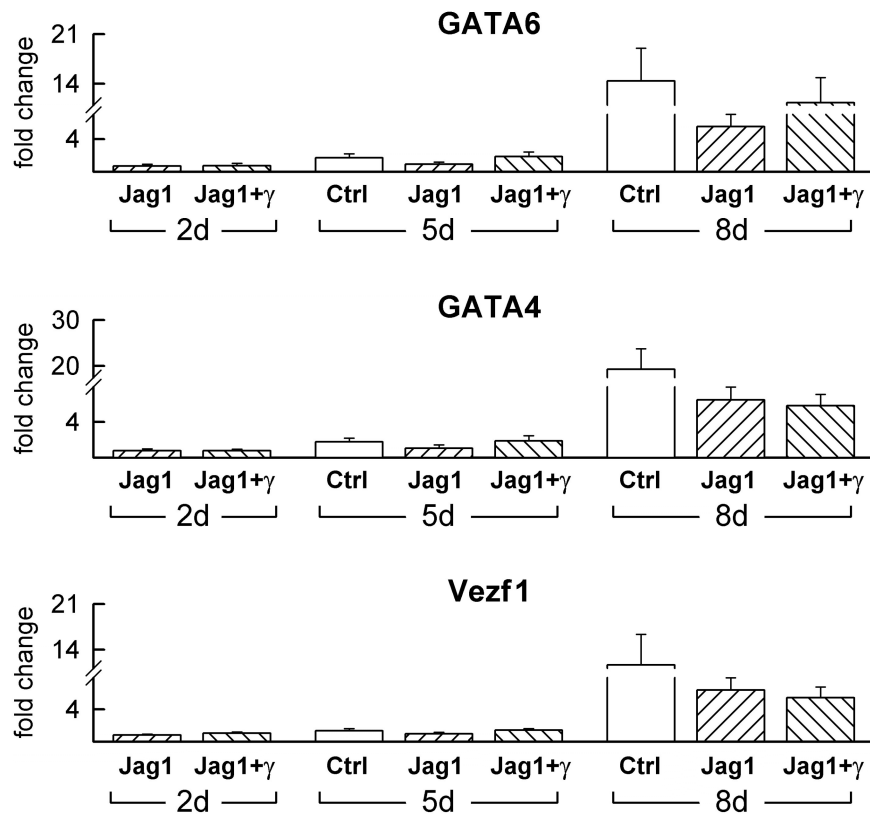


Figure 24. Notch1 and CPCs differentiation. *Notch* pathway activation (*Jag1*) and inhibition (*Jag1+ γ*) and mRNA levels of *GATA6*, *GATA4* and *VeZF1* measured by real time RT-PCR at 2, 5 and 8 days in culture. Fold change vs. Ctrl at 2 days..

Although Notch1 activation appeared to be selectively coupled with the acquisition of the myocyte lineage, these data failed to document whether this cell commitment was accompanied by the loss of the replicative potential of newly formed myocyte progenitors. The ability of Jagged1 to enhance or inhibit CPCs division was evaluated by the expression of the cell cycle protein Ki67 at 5 days. This time point was selected because the number of Nkx2.5 positive CPCs reached its maximum at this interval (see above). The up-regulation of the Notch pathway resulted in a 2.4 fold increased in CPCs proliferation and nearly 65% of cycling cells was Nkx2.5 positive (Fig. 25).

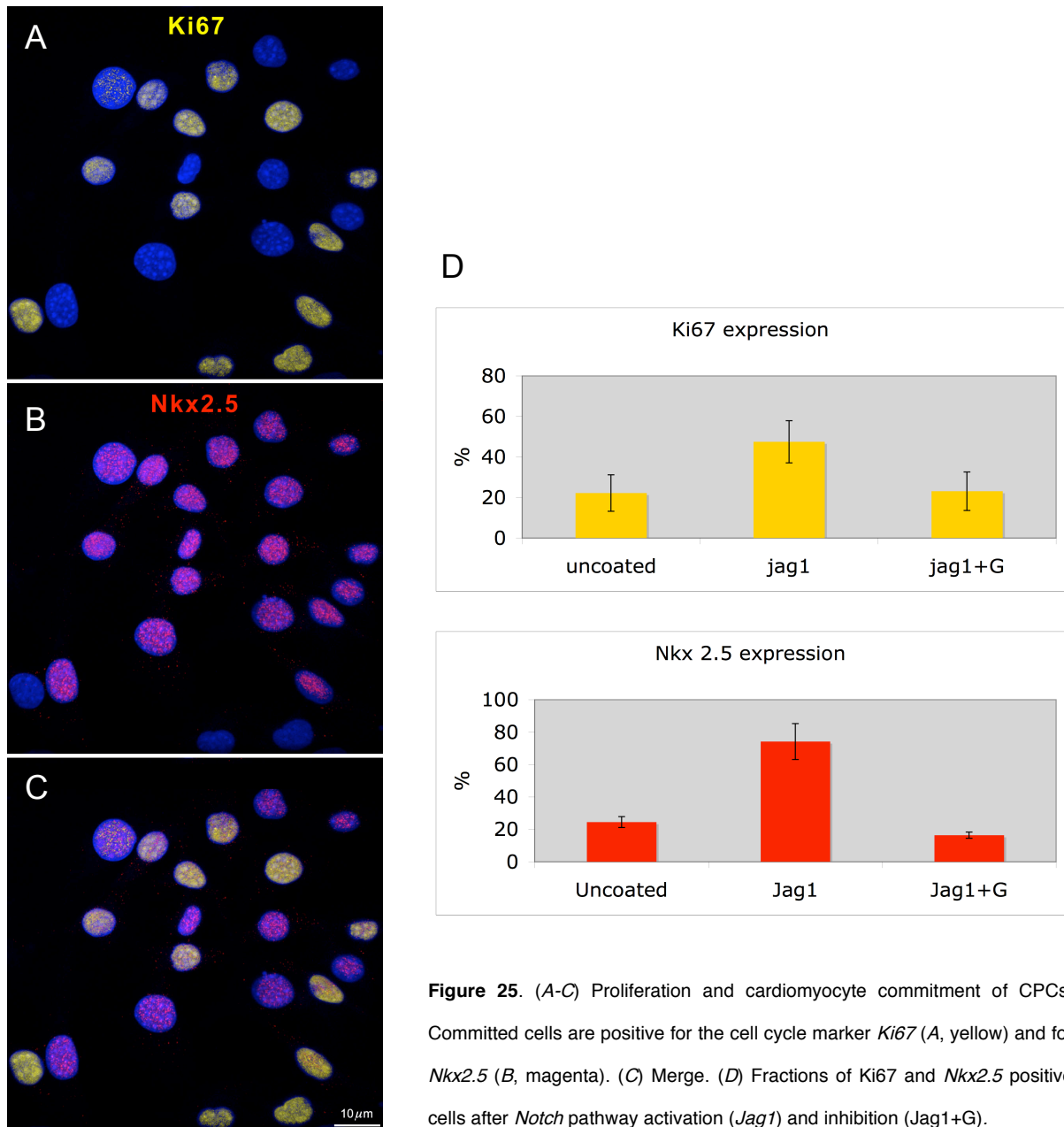


Figure 25. (A-C) Proliferation and cardiomyocyte commitment of CPCs. Committed cells are positive for the cell cycle marker *Ki67* (A, yellow) and for *Nkx2.5* (B, magenta). (C) Merge. (D) Fractions of *Ki67* and *Nkx2.5* positive cells after *Notch* pathway activation (*Jag1*) and inhibition (*Jag1+G*).

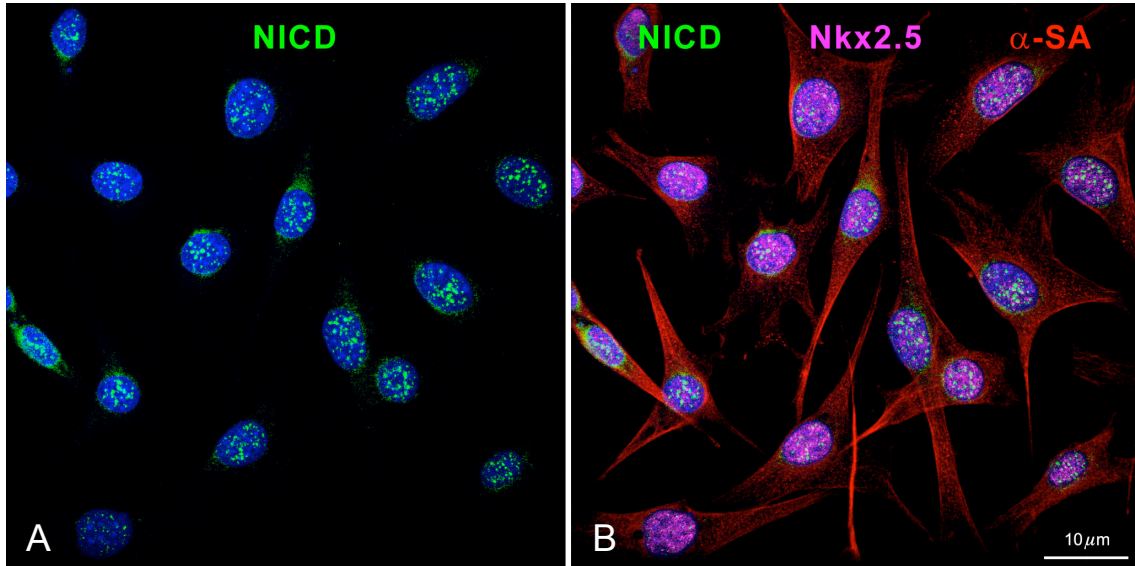


Figure 26. (A and B) *Notch* pathway activation and co-localization of *NICD* (A, green) and *Nkx2.5* (B, magenta) in cells positive for α -sarcomeric actin (B, red).

Collectively our observations support the notion that Notch1 regulates the transition of CPCs from the primitive immature phenotype to the compartment of amplifying myocytes (Fig. 26). This cell category has the ability to divide and simultaneously to differentiate (Watt, 1998). In stem cell regulated tissues, the pool size of transient amplifying cells defines the growth reserve of the organ and its ability to maintain homeostasis and adapt to injury.

3. Molecular mechanism of Nkx2.5 activation.

3.1. RBP-jk binds to the Nkx2.5 promoter. The molecular mechanism by which Notch1 regulates CPCs differentiation into myocytes is unclear. Notch1 activation may lead to the formation of protein complex between RBP-jk and NICD that, in turn, binds to the Nkx2.5 promoter initiating transcription. To test this hypothesis, lysates of freshly isolated CPCs were immunoprecipitated with an antibody against RBP-jk; Western blotting with a NICD-antibody was then performed (Fig. 27). This pull-down assay documented that NICD and RBP-jk formed a complex in CPCs raising the possibility that RBP-jk is converted by NICD into a transcriptional activator of the Nkx2.5 gene. However, the limited number of CPCs in the mouse heart and the necessity to implement complex protocols of co-transfection precluded to performing such studies in this cell population. We employed the embryonic cell line P19CL6 that upon stimulation generates clusters of spontaneously beating myocytes and vascular cells. P19CL6 cells have been used previously for the analysis of gene expression and myocyte differentiation (Brown et al., 2004).

The RBP-jk transcription factor preferentially binds to DNA regions containing the 5'-GTGGGAA-3' sequence (Tun et al., 1994). A perfect consensus site for RBP-jk in the Nkx2.5 promoter was identified at position -2209 -2202 from the transcription starting point. To establish whether RBP-jk and the promoter of Nkx2.5 form a protein-DNA complex, P19CL6 cells were transfected with a plasmid expressing RBP-jk. Nuclear extracts from non-transfected and transfected P19CL6 cells were then utilized in an electrophoretic mobility shift assay. An oligonucleotide of 17bp including the binding site for RBP-jk was used as a probe and a shifted RBP-jk band was found. Exposure to excess unlabeled self-oligonucleotide opposed the appearance of the RBP-jk shifted band while pre-incubation with an RBP-jk antibody super-shifted the protein-DNA complex (Fig. 27).

To confirm the role of Notch1 in the regulation of Nkx2.5 transcription, the association between RBP-jk and CPC genomic DNA was analyzed by chromatin immunoprecipitation (ChIP). ChIP recognizes the physical interaction of transcription factors with endogenous DNA regulatory elements. Formaldehyde cross-linked chromatin was immunoprecipitated with an antibody against RBP-jk and the purified DNA fragments were amplified and sequenced. RBP-jk occupied the region of the Nkx2.5 promoter containing the -2209/-2202 RBP-jk consensus site (Fig. 28).

The relevance of the data obtained with P19CL6 cells was reinforced by similar studies conducted utilizing CPCs obtained from the mouse heart, which were subsequently expanded in vitro. CPCs at P8-11 were utilized and ChIP assay was performed. As expected, RBP-jk was found to be linked to the Nkx2.5 promoter that comprised the RBP-jk consensus site. Thus a

binding site for RBP-jk is located in the promoter region of *Nkx2.5*, suggesting that *Nkx2.5* is a novel target gene of Notch1.

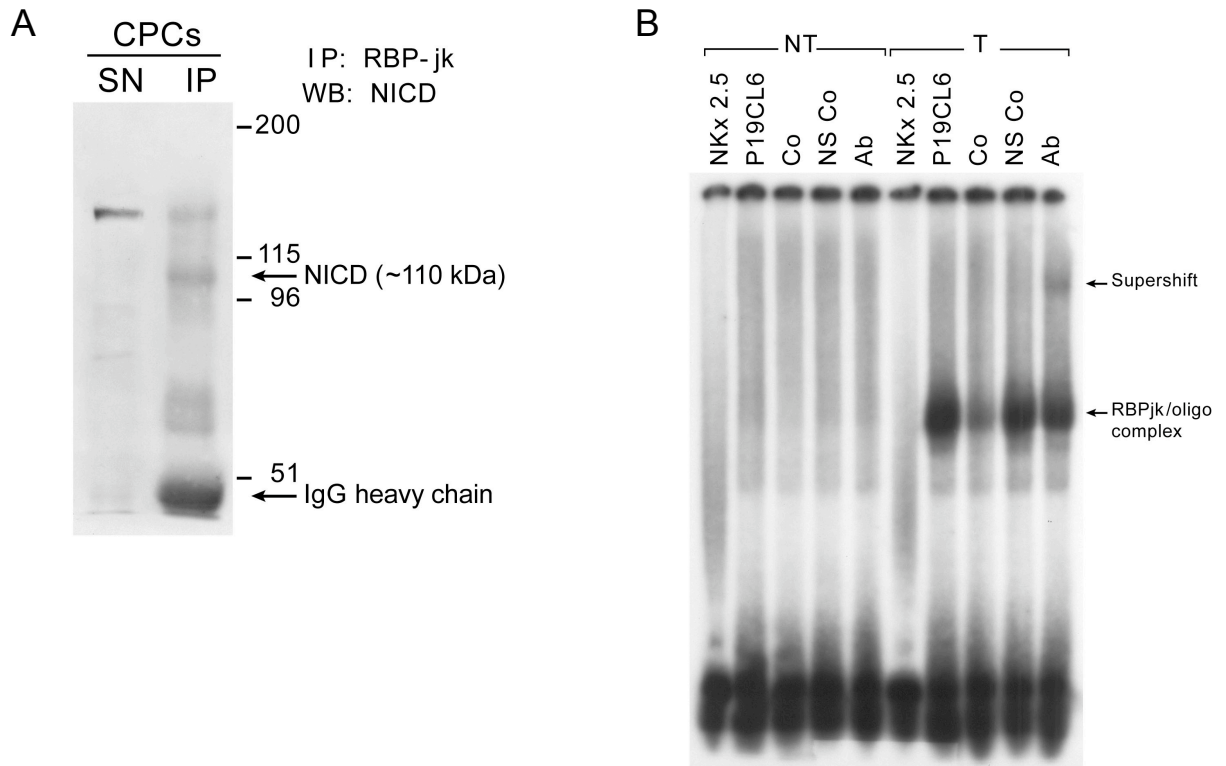


Figure 27. (A) Formation of a protein complex between *RBP-jk* and *NICD* in CPCs; immunoprecipitation (IP)/Western blotting (WB) of whole protein extract with an antibody anti-*RBP-jk* and an antibody anti-*NICD*, respectively. (B) *RBP-jk* binding to its consensus sequence is shown by electrophoretic mobility shift assay in *P16CL6* cells transfected (T) with an expression vector for *RBP-jk*. RBP-jk/oligo complex formation is documented by the shifted bands and the specificity of the assay is documented by the supershifted band (Co: specific competitor, NS Co: non-specific competitor, Ab: antibody anti-*RBP-jk*).

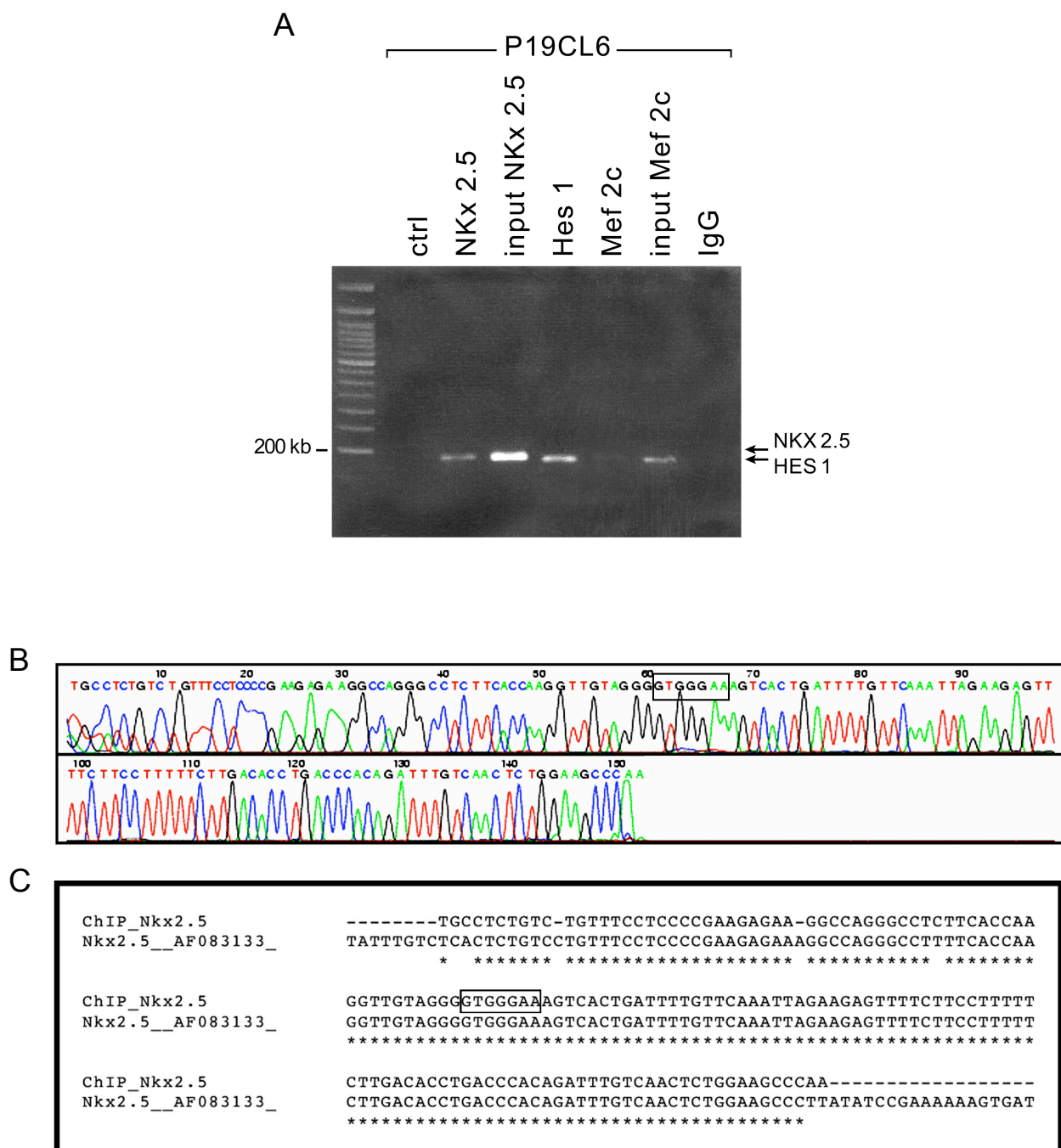


Figure 28. (A) *RBP-jk* binding to the genomic *Nkx2.5* promoter is shown by chromatin immunoprecipitation assay in CPCs using an antibody anti-*RBP-jk*. *Hes1*: positive control, *Mef2c*: negative control, IgG: isotype control. (B) Sequence of immunoprecipitated DNA fragments amplified with promoter-specific *Nkx2.5* primers and (C) sequences alignment.

3.2. Notch1 enhances Nkx2.5 promoter activity. To establish the function of NICD/RBP-jk complex on Nkx2.5 transactivation, we performed a gene reporter assay with an artificial Nkx2.5 promoter containing 10 Kb of the 5' flanking region of the gene. P19CL6 were transiently transfected with the β -galactosidase reporter construct controlled by the Nkx2.5 promoter. Cells were also co-transfected with expression plasmids carrying constitutively active NICD. The expression of exogenous NICD was assessed by Western blotting (not shown). In spite of the high level of over-expression of NICD, no β -gal activity was detected, suggesting that the NICD/RBP-jk complex alone was unable to promote transcription of Nkx2.5.

The Nkx2.5 promoter/enhancer region is long, complex and contains several binding sites for multiple transcription factors. The regulation of the Nkx2.5 gene requires the cooperation of several proteins (Brown et al., 2004; Lien et al., 2002). In this regard, the RBP-jk binding sequence was found to be located in proximity of one Smad4 and two GATA4 consensus sites in the downstream portion of the Nkx2.5 promoter. Of relevance, the transcription of Nkx2.5 requires the physical interaction of the Nkx2.5 promoter with GATA4 and Smad4 (Brown et al., 2004). Therefore, P19CL6 cells were co-transfected with NICD, GATA4 and Smad4 expression plasmids in various combinations. Maximal Nkx2.5 promoter activity was reached by transfection with the three expression plasmids together: GATA4, Smad4 and NICD. With respect to baseline, there was a three-fold increase in β -gal enzymatic activity. Importantly, NICD increased by nearly 2-fold the activity of the reporter gene induced by GATA4 and Smad4 combined (Fig. 29). Thus, Notch1 forms a complex with RBP-jk, which in turn enhances transcription of the Nkx2.5 gene.

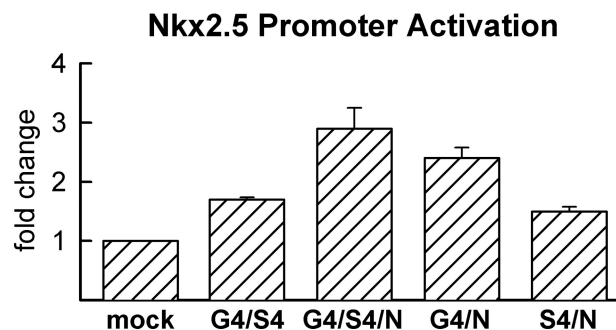


Figure 29. *Nkx2.5* promoter activation measured by β -galactosidase activity assay after transfection with the indicated combination of expression vectors (G4: *GATA4*, S4: *Smad4*, N: *NICD*). Fold changes vs. mock-transfected cells.

4. Function of Notch1 in vivo

Our in vitro results are consistent with in vivo observations suggesting that in self-renewing organs the Notch pathway may regulate the pool size of transient amplifying cells (Blanpain et al., 2006; Lowell et al., 2000). If this possibility would apply to the heart, Notch1 signaling could have important implication in the myocardial response to infarction and the ability of the heart to sustain extensive myocardial damage. The experimental protocol involved the intraperitoneal administration of the γ -secretase inhibitor to mice for three days prior to coronary artery ligation and myocardial infarction. At the time of surgery, the γ -secretase inhibitor was injected intramyocardially and animals were continuously treated intraperitoneally for additional 9 days up to the time of sacrifice. Control infarcted mice were injected with the vehicle only. Moreover, infarcted mice received two injections of BrdU/day for 9 days. This protocol was utilized because myocardial infarction in humans is associated with activation of resident CPCs and formation of new myocytes in the region bordering the infarct (Beltrami et al., 2001).

Doublets, triplets and small clusters of CPCs were detected in the viable myocardium adjacent to the infarct in both animal groups. Jagged1 was also distributed in the border zone together with c-kit positive cells. In the absence of γ -secretase inhibition, the localization of NICD to the nucleus of CPCs was accompanied by the expression of Nkx25 (Fig. 30). This adaptation was markedly attenuated in mice exposed to the γ -secretase inhibition.

Quantitatively, blockade of the Notch pathway resulted in a 70% reduction in the pool of CPCs distributed in the border zone and in an 80% decrease in the fraction of CPCs expressing NICD (Fig. 31). Similarly, the number of CPCs positive for NICD and Nkx2.5 was 84% lower in animals treated with the γ -secretase inhibitor. Therefore, the commitment of resident CPCs to the myocyte lineage appeared to be impaired by Notch pathway inhibition.

To test this possibility we measured the extent of cardiomyogenesis at the level of the border zone. New myocytes corresponded to small cells that were BrdU and α -sarcomeric actin positive. In infarcted mice not exposed to the γ -secretase inhibitor, several regenerated myocytes were identified (Fig. 32). In contrast, newly formed myocytes were rarely found in animals with blockade of the Notch pathway. Quantitatively, the number of BrdU positive myocytes in the border zone was ~50% lower in animals treated with the γ -secretase inhibitor (Fig. 32 B).

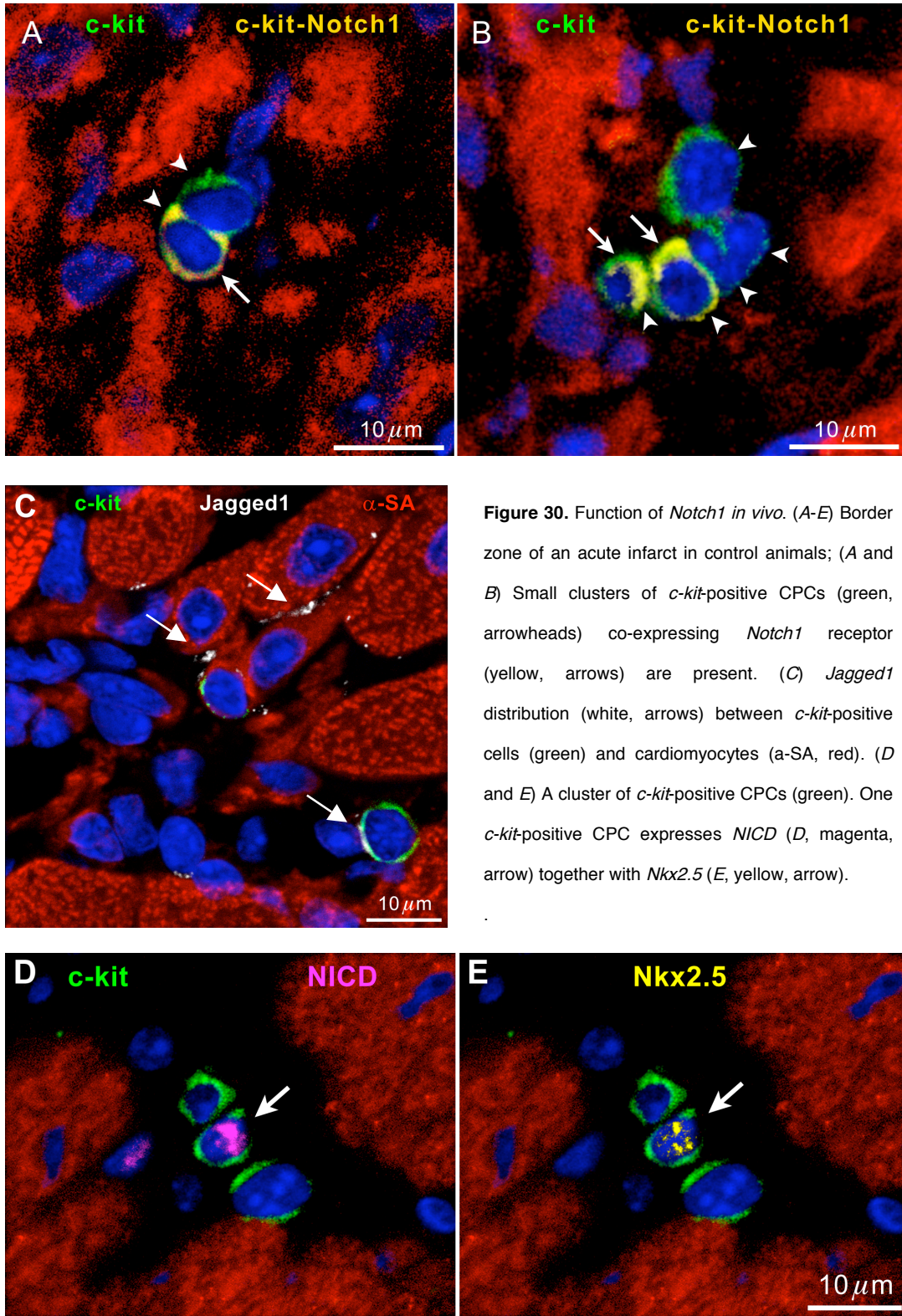


Figure 30. Function of *Notch1* in vivo. (A-E) Border zone of an acute infarct in control animals; (A and B) Small clusters of *c-kit*-positive CPCs (green, arrowheads) co-expressing *Notch1* receptor (yellow, arrows) are present. (C) *Jagged1* distribution (white, arrows) between *c-kit*-positive cells (green) and cardiomyocytes (α -SA, red). (D and E) A cluster of *c-kit*-positive CPCs (green). One *c-kit*-positive CPC expresses *NICD* (D, magenta, arrow) together with *Nkx2.5* (E, yellow, arrow).

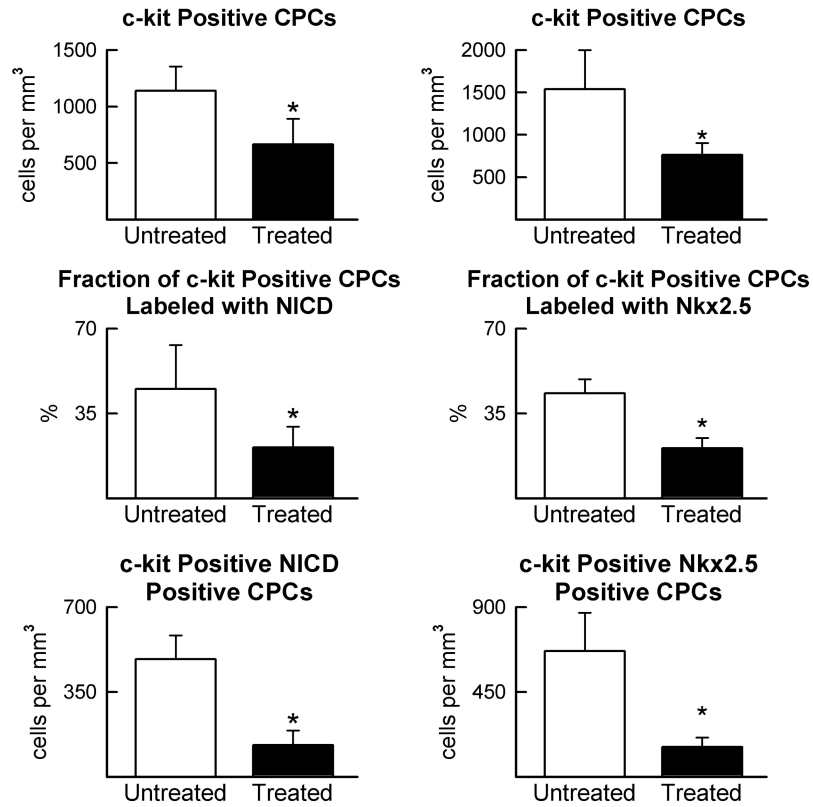


Figure 31. Number of *c-kit*-positive CPCs, fraction and number of CPCs expressing NICD and Nkx2.5 in the border zone of control animals (Untreated) and mice treated with g-secretase inhibitor (Treated). Values are mean \pm SD. *, P < 0.05 vs. Untreated. (G and H) Formation of new myocytes documented by BrdU incorporation.

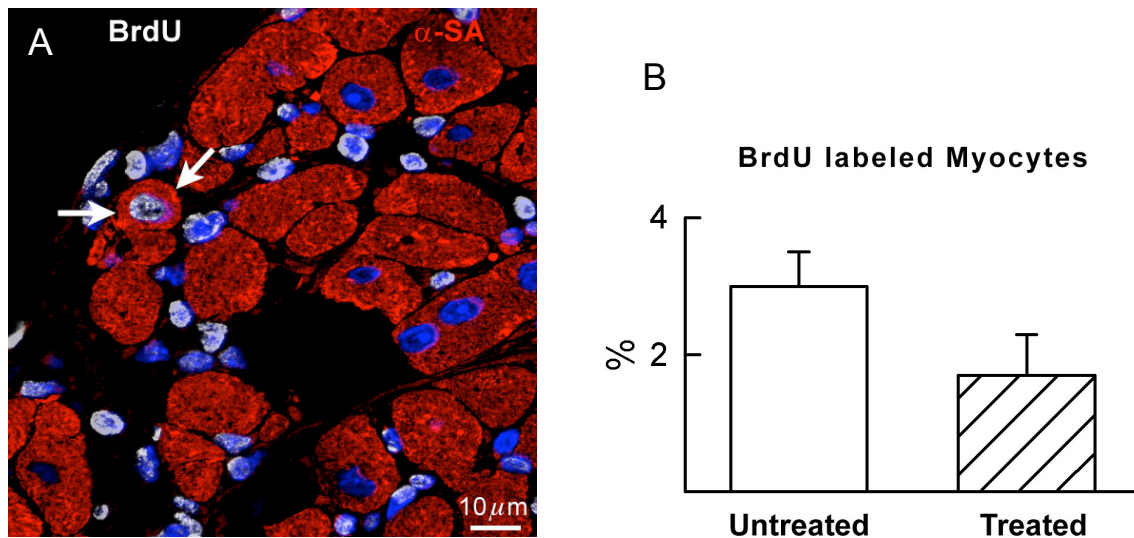


Figure 32. (A) Small myocyte (α -SA, red, arrows) with BrdU labeled nucleus (white) is present. (B) Treatment with γ -secretase inhibitor results in a reduction in BrdU labeling of myocytes. .

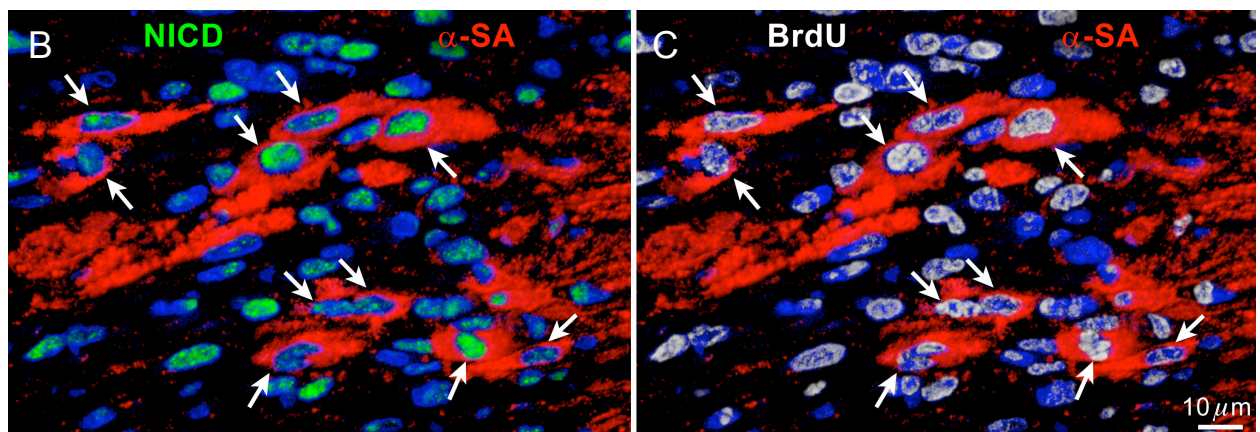
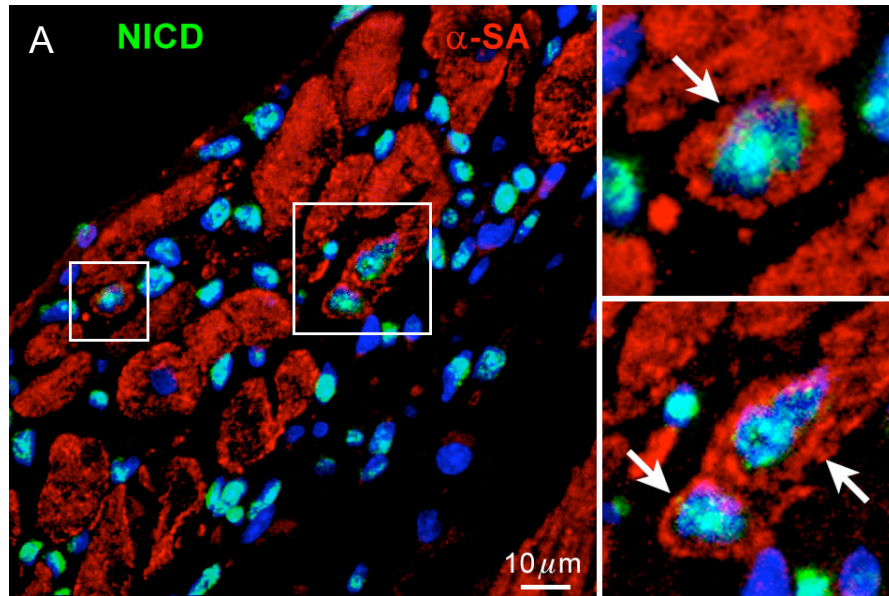


Figure 33. (A-C) Spontaneous myocardial regeneration after infarction in untreated mice. (A) Small myocytes (α -SA, red) included in the squares and shown at higher magnification in the adjacent panels are positive for *NICD* (green, arrows). (B and C) Cluster of small regenerating myocytes (arrows) in the infarcted area that express *NICD* (J, green) and are labeled with BrdU (K, white).

Foci of spontaneous myocardial regeneration have been found within the infarcted heart in humans (Urbanek et al., 2005). In agreement with these observations, these sites of cardiomyogenesis were detected in a few cases of control animals and were absent in animal exposed to γ -secretase inhibition. In the damaged region of non-treated infarcted mice small clusters of newly formed BrdU-positive cardiomyocytes were found to be positive for *NICD* at the level of their nuclei (Fig. 33). Importantly, BrdU positive myocytes showed consistently a nuclear co-localization of *NICD* and *Nkx2.5* (Fig. 34 and 35).

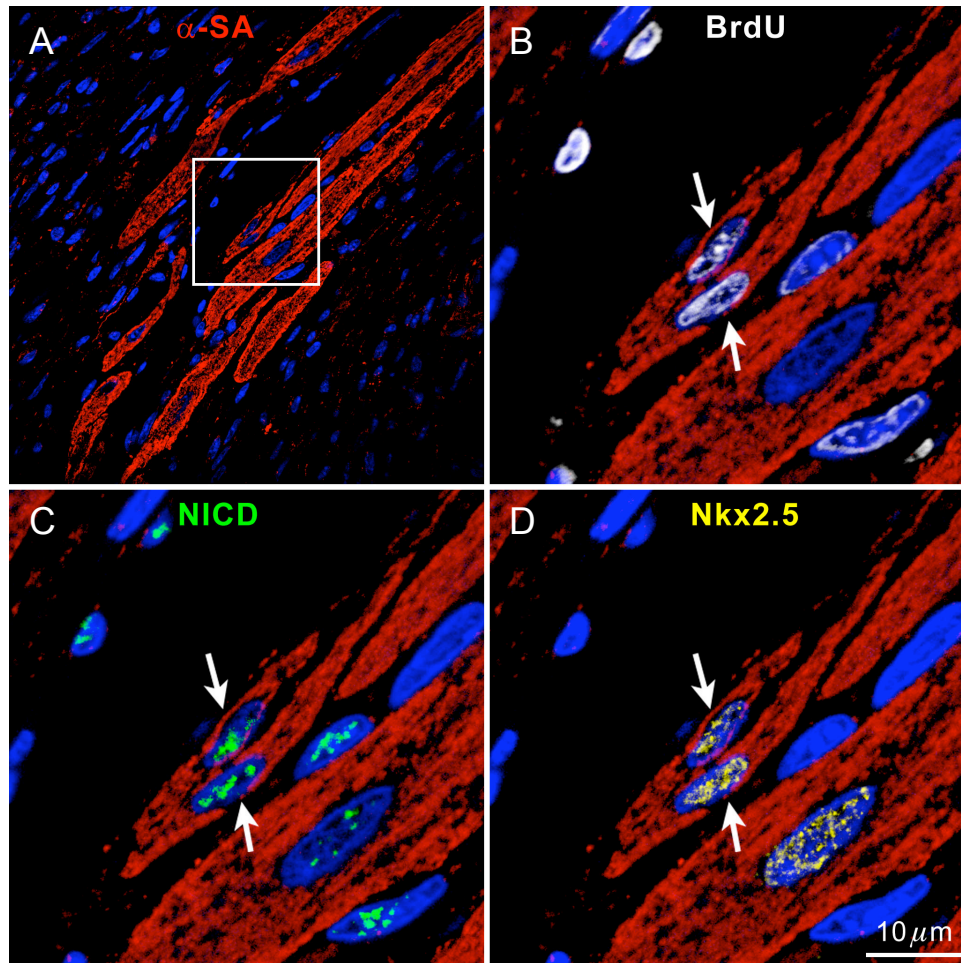


Figure 34. (A-D) Small myocytes (α -SA, red) included in the square (A) are shown at higher magnification in panels B-D (arrows). Newly formed myocytes are labeled with BrdU (B, white) and express *NICD* (C, green) together with *Nkx2.5* (D, yellow).

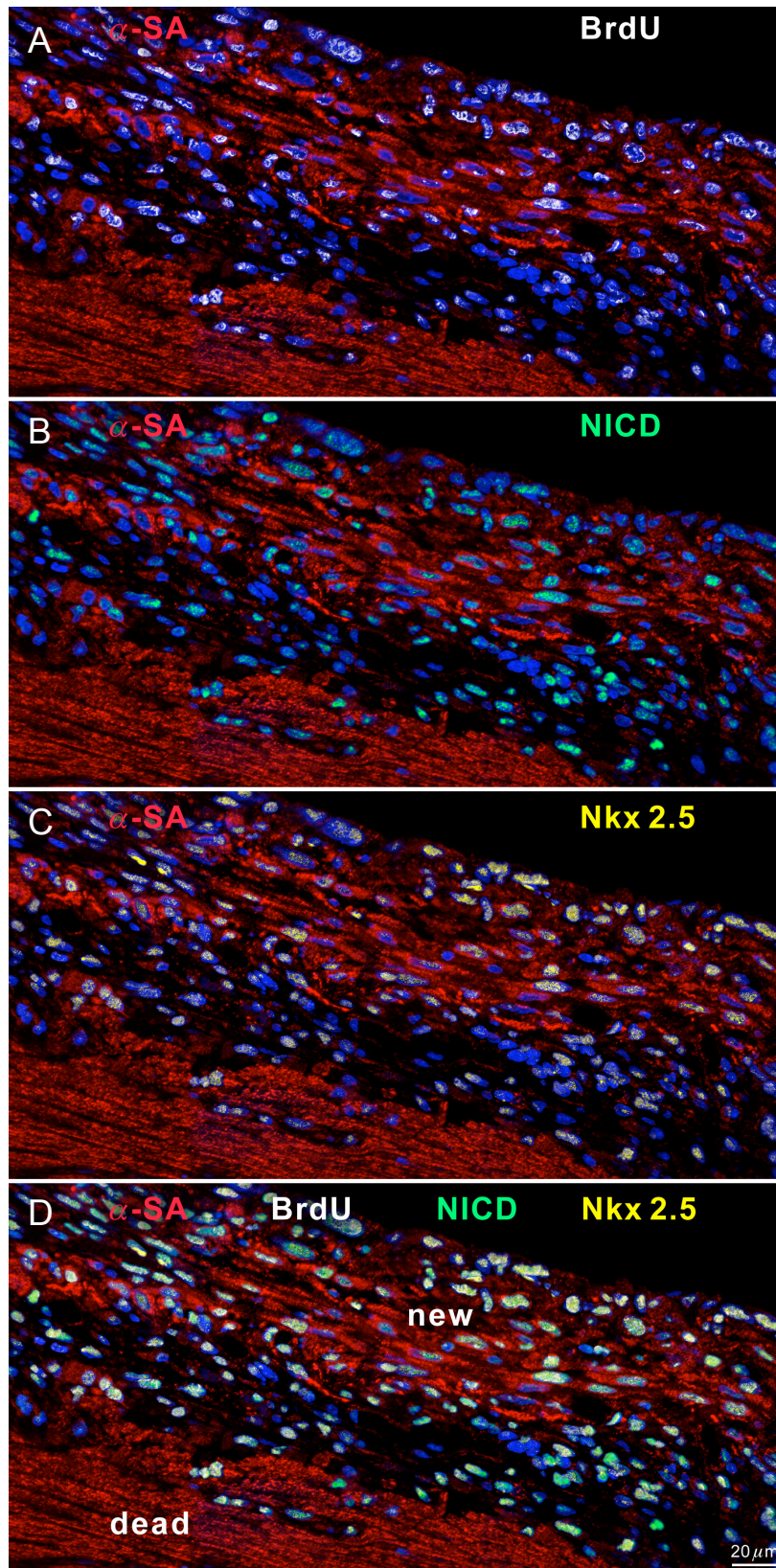


Figure 35. (A-D) Large focus of myocardial regeneration (new) in proximity to dead myocytes (dead). Newly formed myocytes (α-SA, red) are labeled with BrdU (A, white) and express *NICD* (B, green) together with *Nkx2.5* (C, yellow). (D) merge.

CONCLUSIONS.

The results of the present study document that the activation of the Notch1 pathway favors the commitment of CPCs to the myocyte lineage and controls the size of the compartment of transient amplifying myocytes *in vitro* and *in vivo*. This behavior may correspond to a model of differentiation delay in which sustained up-regulation of Notch1 signaling prolongs the amplifying state of CPC-derived myocytes and prevents terminal differentiation and growth arrest. This function of Notch1 involves the expression of the transcription factor Nkx2.5, which represent a novel target gene of Notch1. The induction of Nkx2.5 is an early event in the growth of embryonic myocytes (Schwartz and Olson, 1999), but its function in the postnatal heart remains unclear. Our findings indicate that Nkx2.5 drives the commitment of resident CPCs in the adult heart possibly controlling the activation or repression of the complex transcriptional network that modulates myocyte formation and cardiac homeostasis (Cripps and Olson, 2002; Olson, 2006). Notch function in progenitor cells is generally coupled with the preservation of the primitive phenotype (Androutsellis-Theotokis et al., 2006; Artavanis-Tsakonas et al., 1999; Calvi et al., 2003; Wilson et al., 2001). Conversely, Notch ligands promote the maturation of stem cells in the basal layer of the skin and satellite cells of the skeletal muscle (Blanpain et al., 2006; Kitamura et al., 2007; Lowell et al., 2000). In a similar manner, Jagged1 stimulation coupled with the up-regulation of Nkx2.5 favors the entry of CPCs into a pool of cells characterized by the acquisition of the myocyte phenotype and the ability to divide. The presence of Nkx2.5 does not appear to interfere with the capacity of these cells to proliferate. Nkx2.5 positive cells expressed NICD in their nuclei and are consistently labeled by BrdU and Ki67. They also have a thin layer of cytoplasm containing sarcomeric proteins. Together these cellular properties define the compartment of transient amplifying myocytes (Watt, 1998). In this regard, self-renewing organs contain two populations of replicating cells: stem cells and amplifying cells. The latter corresponds to the progeny of stem cells, which is destined to acquire complete maturation but prior to cell cycle withdrawal experiences a limited number of divisions (Jones and Watt, 1993; Watt, 1998). Amplifying cells are short-lived cells with low self-renewal ability and high differentiation potential. This cell pool increases the number of daughter cells resulting from stem cell division; this growth effect ensures tissue homeostasis, allows stem cells to divide rarely and protect the stem cell compartment from depletion (Booth and Potten, 2000). Our data after myocardial infarction are consistent with this possibility since Notch1 upregulation *in vivo* expands the population of amplifying myocytes. Successive rounds of Notch1 inhibition and activation may results in a controlled exodus of CPCs from the undifferentiated to the committed state in which CPCs assume the myogenic fate. The expression of the Notch1 receptor on the plasma membrane in the absence of the nuclear translocation of its intracellular domain may be linked to a permissive state in which the multipotentiality of CPCs is preserved.

The current work has led to the identification of a functional RBP-jk consensus site in the promoter region of Nkx2.5 pointing to the critical role that Notch1 signaling has in the regulation of myocyte turnover in the adult mouse heart. This possibility is consistent with observations in Nkx2.5 null mouse embryos in which the expression of multiple cardiac transcription factors, contractile proteins and myocyte membrane proteins is markedly reduced (Lyons et al., 1995; Tanaka et al., 1999). Collectively, these findings indicate that Nkx2.5 plays an essential role in the transcriptional regulation of the cardiac gene program during development and in the adult heart, as suggested by our results. The upregulation of Nkx2.5 with pressure-overload has been interpreted as part of the induction of the fetal cardiac phenotype in the stressed myocardium (Heineke and Molkentin, 2006). However, Nkx2.5 expression may reflect the activation of resident CPCs and the formation of a myocyte progeny rather than being restricted to the synthesis of fetal proteins in terminally differentiated post-mitotic cardiomyocytes. Myocyte regeneration occurs in animals and human following aortic stenosis, systemic hypertension and pulmonary artery banding (Anversa et al., 1990; Olivetti et al., 1988; Urbanek et al., 2003). This notion is supported by the function of Nkx2.5 that contributes to the activation of the hypertrophic program in the overloaded heart, although this homeobox gene does not participate (is not sufficient) in the increase of myocyte size with cardiac hypertrophy (Akazawa and Komuro, 2003). Understanding the cellular and molecular events involved in the Nkx2.5-dependent specification of cell fate may provide important insights on the mechanisms of myocardial regeneration in the pathologic heart (Schwartz and Olson, 1999). Modulation of the Notch1 pathway may restore the balance between uncommitted and committed cells and maintain physiologic cardiac homeostasis in chronic cardiac diseases delaying the onset of heart failure.

The recognition that Jagged1 stimulation and Notch1 signaling in CPCs promote myocyte formation and concurrently interfere with the acquisition of the EC and SMC lineages and vasculogenesis, emphasizes the importance of this effector pathway in the myocardial response to growth stimuli. This model of selective specification of cell fate mimics the function of Notch in other stem cell-regulated organs (Artavanis-Tsakonas et al., 1999; Blanpain et al., 2006; Tanigaki and Honjo, 2007). Notch may act as a lineage switch between myocyte and vascular fates by positively regulating Nkx2.5 and negatively influences the genes required for vascular differentiation. The down regulation of the SMC transcription factor GATA6 is consistent with the activity of the Notch-dependent repressors of transcription Hey1 and Hey2 on members of the GATA family of proteins in the heart and hematopoietic system (Fischer et al., 2005). A similar mechanism may be operative for the EC transcription factor Vezf1. The observation that transient amplifying myocytes express Nkx2.5 but lack GATA4 raises the possibility that Notch1

upregulation is necessary for early myocyte commitment but opposes the acquisition of the adult phenotype.

Several fundamental biologic issues have been addressed by the present investigation. Cell fate decision in Stem Cells is crucial for the structural and functional integrity of organs and systems. Thus, unrevealing the mechanisms regulating cardiac homeostasis may result in new therapeutic approaches to prevent or delay the onset and development of heart failure.

METHODS.

Cell isolation and CPCs enrichment.

Cardiac small cells were isolated by enzymatic digestion of the mouse heart and plated o/n in F12K plus 20% FBS (high serum). On the following day, cells were labelled with a monoclonal antibody anti c-kit conjugated with magnetic beads (Miltenyi Biotech Inc, Auburn, CA) and c-kit positive CPCs were selected using MACS Separation Columns (Miltenyi Biotech Inc, Auburn, CA) according to the manufacturer instructions. After selection c-kit positive CPCs were expanded and starting from three days before the experiment CPCs were gradually starved to 5% of FBS (low serum). On the day of the experiment the cells were reselected for c-kit as above and plated in dishes (40×10^3 cells for 2 days time point and 20×10^3 cells for 5 and 8 days time point) and in chamber slides (4×10^3 cells/chamber for 2 days time point and 2×10^3 cells/chamber for 5 and 8 days time point).

Stimulation with ligand and g-secretase inhibition.

Petri dishes and chamber slides were pre-coated with an antibody anti-human IgG1 [25mg/ml] (Sigma, St. Louis, MO) and, after washing (PBS + BSA 0.1%), treated with blocking buffer (PBS + BSA 1%) for 1h RT. After the removal of the excess of blocking buffer, dishes and chamber slides were coated with the human Fc-Jagged1 chimera [6mg/ml] (R&D Systems, Minneapolis, MN) for 2h RT.

g-secretase inhibitor (Calbiochem, San Diego, CA) was dissolved in DMSO according to the manufacturer instructions [2mM]. For the in vitro experiment the treated samples were cultured in the presence of g-secretase inhibitor [final: 10 nM] and the control samples with 0.0005% DMSO only. Medium was replaced after 2 and 5 days.

Animal treatments.

Female C57BL/6 mice of two months of age (n=10) were injected intraperitoneally with g-secretase inhibitor for 3 days before myocardial infarction (MI). After left coronary occlusion the animals were treated for additional 9 days and then sacrificed. Treated animals received 80 nmols of g-secretase inhibitor per day and, on the day of surgery, 0.25 nmols of g-secretase inhibitor by two intra myocardial injections in the border zone of the infarct. Controls animals were infarcted and treated with the vehicle only. In both groups of animals BrdU was delivered by IP injections (50mg/Kg) twice a day for 9 days after MI and in the drinking water (1g/L).

With mice under tribromoethanol anesthesia, the abdominal aorta was cannulated with a polyethylene catheter, PE-50, filled with a phosphate buffer, 0.2 M, pH 7.4, and heparin, 100 units/ml. In rapid succession, the heart was arrested in diastole by the injection of 0.15 ml of CdCl_2 , 100 mM, through the aortic catheter; the thorax was opened; perfusion with phosphate buffer was started; and the vena cava was cut to allow the drainage of blood and perfusate.

The aortic catheter was connected to a pressure reservoir to adjust perfusion pressure to mean arterial blood pressure measured *in vivo*. Simultaneously, the left ventricular chamber (LV) was filled with fixative, 4% formaldehyde in phosphate buffer from a pressure reservoir set at a height equivalent to end-diastolic pressure determined *in vivo*.

This was accomplished by inserting a 25G3/4 needle into the LV through the apex. The needle was connected to a pressure reservoir. After perfusion with buffer for 2 min, the coronary vasculature was perfused for 15 min with fixative. Subsequently, the heart was excised, and weights were recorded. The volume of the myocardium in each anatomical region of the heart was determined by dividing the weight by the specific gravity of muscle tissue, 1.06 g/ml.

ICC and IHC.

Cells were fixed with 4% PFA and treated with Triton X100 0.01% for the detection of intracellular antigens. Unspecific binding was reduced incubating the cell in 10% BSA for 30' RT.

The following antibodies were employed for the detection of the antigens of interest: a rabbit polyclonal antibody able to recognize NICD, the active form of the Notch1 receptor (Cell Signalling Technology, Beverly, MA), a goat polyclonal anti-GATA4 (Santa Cruz Biotechnology, Santa Cruz, CA), a goat polyclonal anti-Nkx2.5 (Santa Cruz Biotechnology, Santa Cruz, CA), a pre-diluted mouse monoclonal antibody able to recognize the extracellular domain of Notch1 (Abcam, Cambridge, MA), a goat polyclonal antibody anti-c-kit (R&D Systems, Minneapolis, MN), a rabbit polyclonal antibody anti-Ki67 (Vector laboratories Inc., CA) and a mouse monoclonal antibody anti- α -sarcomeric actin (Sigma, St. Louis, MO).

The cell body was revealed by incubation with phalloidin directly conjugated with a fluorochrome (Invitrogen Corporation, Carlsbad, CA).

Paraffin-embedded tissue sections were stained with the following antibodies: a rabbit polyclonal antibody anti-c-kit (R&D Systems, Minneapolis, MN), rabbit polyclonal antibody anti-Nkx2.5 (Santa Cruz Biotechnology, Santa Cruz, CA), a pre-diluted mouse monoclonal antibody able to recognize the extracellular domain of Notch1 (Abcam, Cambridge, MA) were utilized.

For the detection of the active form of Notch1 a rabbit polyclonal antibody anti-NICD (Cell Signaling) and the Tyramide Signal Amplification Kit (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA) were employed according to the manufacturer instructions.

BrdU incorporation was detected with a mouse monoclonal antibody (Roche Applied Bioscience, Indianapolis, IN).

Secondary antibodies, conjugated with the appropriate fluorochrome, (Jackson ImmunoResearch, West Grove, PA) were employed. Primary and secondary antibodies were utilized following the manufacturer instructions.

Real time RT-PCR.

Total RNA was extracted using the RNeasy plus mini kit (Qiagen Inc., Valencia, CA) and RNA concentration was measured with the NanoDrop system (NanoDrop Technologies, Wilmington, DE). The reactions were performed using the ABI Prism 7300 Real Time PCR Systems (Applied Biosystems, Valencia, CA). After DnaseI treatment, an amount of 25 ng of total RNA/well was amplified in the presence of the One Step RT-PCR Master Mix Reagent (Applied Biosystems, Valencia, CA). 40 amplification cycles were performed. Differently, cDNA to identify Nkx2.5 transcript level was generated from 1ug total RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Valencia, CA) following manufacturer's protocol. Real-time PCR for Nkx2.5 was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Valencia, CA) according to manufacturer's instructions. Primers and probes were designed using the Integrated DNA Technologies software (IDT, Inc. Coralville, IA).

Hes1

Fw: 5'-CAC GAC ACC GGA CAA ACC A-3'

Rev: 5'-GCC GGG AGC TAT CTT TCT TAA GTG-3'

Probe: 5'-/56-FAM/AGC CTA TCA TGG AGA AGA GGC GAA GGG CAA/3BHQ_1/-3'

Notch1

Fw: 5'-GTG GAT GAC CTA GGC AAG TCG-3'

Rev: 5'-GTC TCC TCC TTG TTG TTC TGC AT-3'

Probe: 5'-/56-FAM/TGC TGT TGT GCT CCT GAA GAA CGG AGC CAA/3BHQ_1/-3'

GATA4

Fw: 5'-GAA TAA ATC TAA GAC GCC AGC AGG TCC-3'

Rev: 5'-GAC AGC TTC AGA GCA GAC AGC A-3'

Probe: 5'-/56-FAM/ATG TCC CAG ACA TTC AGT ACT GTG TCC GGC CA/3BHQ_1/-3'

GATA6

Fw: 5'-AAG CGC GTG CCT TCA TCA C-3'

Rev: 5'-GAG CCA CTG CTG TTA CCG GA-3'

Probe: 5'-/56-FAM/ATG CTG AGG GTG AGC CTG TGT GCA ATG CTT/3BHQ_1/-3'

Vezf1

Fw: 5'-CCA GGG AAG CAG GTA GAG ACA C-3'

Rev: 5'-TTT GAC ATA GTC CCA GAC GAC ACA G-3'

Probe: 5'-/56-FAM/AGA AAG AAA GAA GCT GCC AAC CTG TGC CAA/3BHQ_1/-3'

Nkx2.5

Fw: 5'-CCC AAG TGC TCT CCT GCT TTC C-3'

Rev: 5'-ACA GCT CTT TCT TAT CCG CCC GA-3'

β-Actin

Fw: 5'-CTG AAC CCT AAG GCC AAC CG-3'

Rev: 5'-CAG CCT GGA TGG CTA CGT AC-3'

Probe: 5'-/56-FAM/TGA CCC AGA TCA TGT TTG AGA CCT TCA ACA CCC/3BHQ_1/-3'

Immunoprecipitation-Western blotting.

c-kit positive CPCs whole cell extracts were obtained using the M-PER Mammalian Protein Extraction Reagent with the addition of the Halt Proteases Inhibitors Cocktail Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer instructions.

Equivalent of 500 mg of protein extracts were incubated with a rabbit polyclonal anti-RBPjk antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C on a rotor followed with an incubation with G protein-agarose (Invitrogen Corporation, Carlsbad, CA) for 1h at 4°C on a rotor. The immunoprecipitates (IPs) were separated by 8% SDS-PAGE and transferred onto a nitrocellulose membrane.

Blots were incubated overnight at 4°C with rabbit polyclonal anti-cleaved Notch1 (Val 1744) antibody (Cell Signaling Technology, Beverly, MA).

Antibodies were used at a concentration suggested by the manufacturer instructions in TBS containing 0.1% Tween 20 and 5% non-fat milk. HRP-conjugated IgG were used as secondary antibodies. Proteins were detected with ECL (Pierce Biotechnology, Rockford, IL).

Electrophoretic mobility shift assay.

P19CL6 cells were transfected with an expression vector for RBP-jk and nuclear extracts from non-transfected and transfected cells were used in this assay. Single-stranded oligonucleotides containing the conserved RBP-jk consensus site (GTGGGAA) of the mouse wild type *Nkx2.5* promoter (wild sense: 5'-TAGGGGTGGGAAAGTCA-3' and wild antisense: 5'-TGACTTCCCACCCCTA-3') were end-labeled with T4 polynucleotide kinase (Promega, Madison, WI) and [$\gamma^{32}\text{P}$]-ATP (PerkinElmer Life and Analytical Sciences, Shelton, CT). After precipitation, the wild labelled oligonucleotides and the complementary oligonucleotides containing a mutated RBP-jk consensus site of the mouse *Nkx2.5* promoter (mutated sense: 5'-TAGGGAGGTAGAGTCA-3' and mutated antisense 5'-GACTCTACCTCCCCTA-3') were annealed and purified by precipitation. Binding reactions (20 μl) were prepared in binding buffer (Promega, Madison, WI) with 30 μg of nuclear extract, 4 mCi of labelled oligonucleotide probe and incubated 20 min at room temperature. Where indicated, 4 mg of rabbit polyclonal anti-RBPjk antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding reactions.

For competition assays (specific and unspecific), unlabelled wild type and mutant probes were added at the same concentration of the labelled probe. The complexes were resolved by electrophoresis on 4% polyacrylamide gels (acrylamide / bisacrylamide at 29:1) in 0.5x Tris Buffered EDTA (TBE) buffer containing 2.5% glycerol at 350 V for 40' RT. The gel was dried and exposed to a film overnight at – 80 °C with intensifying screens.

Chromatin immunoprecipitation.

CPCs and P19CL6 cells were expanded in F12K plus 5% FBS and a-MEM plus 10% FBS, respectively to reach ~80-90% confluency at the time of the experiment. On the day of the experiment the cells were crosslinked with 1% formaldehyde for 10 min room temperature; cells were collected and cell extracts were sonicated for DNA shearing (Artek Sonic Dismembrator 150). Chromatin was immunoprecipitated with a rabbit polyclonal anti-RBPjk antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After decrosslinking, immunoprecipitated DNA fragments were treated with RNase and Proteinase K and they were purified with spin columns (QIAGEN Inc., Valencia, CA). For PCR analysis three different sets of primers were utilized: Fw: 5'-GAGGTCACCCCAATTCTGTT-3' and Rev: 5'-GGGCTTCCA GAGTTGACAAA-3' for *Nkx2.5*; Fw: 5'-GCCTCTGAGCACAGAAAGGT-3' and Rev: 5'-CGCCTCTTCTCCCTG ATAGG-3' for *HES1* (positive ctrl); Fw: 5'-GACCGACCTGCTTTACTTGA-3' and Rev: 5'-TGACACACCAGGCTG TTCAAC-3' for *Mef2c* (negative ctrl).

35 cycles of PCR was performed using 20ng of template.

The DNA amplicons corresponding to each specific target were eluted from the gel and sequenced.

Gene reporter activity assays.

P19CL6 cells were grown in a-MEM plus 10% FBS and antibiotics. 24h before transfection the medium was replaced with antibiotic-free medium. On the day of the experiment (~80% confluency), the cells were transfected using Lipofectamine transfection reagent (Invitrogen Corporation, Carlsbad, CA); a plasmid carrying the mouse *Nkx2.5* upstream of the *b-gal* reporter gene (1mg) was used in three different set of co-transfections along with expression vectors carrying the mouse *NICD*, *GATA4* and *Smad4* coding sequences (1mg each). The Promega pGL3-*luc* control vector (1mg) was used to define the efficiency of transfection and to normalize the data. After transfection the cell extracts were obtained and b-galactosidase and luciferase enzyme activity assays were performed (Promega, Madison, WI) according to the manufacturer instructions.

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Curriculum vitae:



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I was born in Cremona (Italy) on the 5th of January 1973. After the achievement of the High School degree at “Liceo Scientifico G. Aselli” in 1992, I started studying Ecological and Environmental Sciences at the University of Padova (Italy).

Although my innate passion for natural sciences and botany, during the first years of study I developed a strong interest in cell biology and molecular biology. As a result, I started studying Biological Sciences at the University of Parma (Italy) in 1996 and I obtained my degree (103/110) in Biochemistry and Molecular Biology in March 2001. Dissertation title: “Recombinant expression and functional characterization of proteins belonging to the isoflavone-reductase family” (Advisors: Prof. Ottonello S, Dr. Petrucco S and Prof. Percudani R).

In September 2001 I obtained a 2 year-fellowship provided by the “Associazione Italiana contro le Leucemie (A.I.L.) at the Dept. of Immuno-hematology and Transplant Medicine, Azienda Ospedaliera, Cremona (Italy) for the molecular and cytofluorimetric studies of the minimal residual disease in patients following bone marrow transplantation. During this period I developed my skills in flow cytometry attending an advanced training at the Italian Society of Flow Cytometry, Ospedale Policlinico, Milano (Italy).

In January 2003 I started a 4 years PhD program in the laboratory run by Prof. Federico Quaini at the Dept. of Internal Medicine and Biomedical Sciences, University of Parma (Italy) studying cardiac stem cells and heart regeneration. According to my PhD program, after one year of training I moved to the Cardiovascular Research Institute of the New York Medical College, New York (USA) to join the laboratory run by Prof. Piero Anversa to continue my research on cardiac muscle regeneration. During the last three years I developed my research on three main topics: cardiomyocyte contractility, cardiac stem cell niches and molecular pathways involved in the differentiation of cardiac progenitor cells. In particular I have been focused on the demonstration of a possible role of the Notch pathway on the early commitment of cardiac progenitor cells to the cardiomyocyte lineage.

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APPENDIX.

Stem cell niches in the adult mouse heart

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Cardiac stem cells (CSCs) have been identified in the adult heart, but the microenvironment that protects the slow-cycling, undifferentiated, and self-renewing CSCs remains to be determined. We report that the myocardium possesses interstitial structures with the architectural organization of stem cell niches that harbor long-term BrdU-retaining cells. The recognition of long-term label-retaining cells provides functional evidence of resident CSCs in the myocardium, indicating that the heart is an organ regulated by a stem cell compartment. Cardiac niches contain CSCs and lineage-committed cells, which are connected to supporting cells represented by myocytes and fibroblasts. Connexins and cadherins form gap and adherens junctions at the interface of CSCs–lineage-committed cells and supporting cells. The undifferentiated state of CSCs is coupled with the expression of α_4 -integrin, which colocalizes with the α_2 -chain of laminin and fibronectin. CSCs divide symmetrically and asymmetrically, but asymmetric division predominates, and the replicating CSC gives rise to one daughter CSC and one daughter committed cell. By this mechanism of growth kinetics, the pool of primitive CSCs is preserved, and a myocyte progeny is generated together with endothelial and smooth muscle cells. Thus, CSCs regulate myocyte turnover that is heterogeneous across the heart, faster at the apex and atria, and slower at the base–midregion of the ventricle.

The notion that the heart exerts its function until the death of the organism with the same myocytes that are present at birth (1) has been challenged. Activation of cyclins and cyclin-dependent kinases, BrdU incorporation, and expression of Ki67, MCM5, cdc6, and phosphohistone-H3 have been shown in myocytes, together with karyokinesis and cytokinesis (2). The search for the origin of dividing myocytes led to the identification of a population of primitive cells with the characteristics of stem cells (SCs) in humans (3) and animals (4–8). In self-renewing organs, SCs are stored in niches that constitute the microenvironment in which SCs are maintained in a quiescent state (9). After activation, SCs replicate and migrate out of the niches to sites of cell replacement where they differentiate and acquire the adult phenotype. Niche homeostasis is regulated by division of SCs, which preserves the ideal proportion of primitive and committed cells within the organ (9, 10).

Although cardiac SCs (CSCs) have been found, the myocardial structure in which CSCs and early lineage-committed cells (LCCs) are nested together remains to be identified. Similarly, the supporting cells that are intimately connected with the CSCs–LCCs within the niches have not been recognized. This identification is of crucial importance, because CSCs cannot exist in the absence of supporting cells, which anchor SCs to the niche and modulate growth signals, resulting in the formation of a cardiac progeny. These biological variables define whether the heart is capable of significant myocyte regeneration. Asymmetric division of CSCs generates CSCs and LCCs that move out of the niches and replace old, poorly contracting myocytes. Conversely, symmetric division of a CSC gives rise to two CSCs or two LCCs. Although asymmetric division may have a prominent impact on niche homeostasis and myocyte turnover, symmetric division could be involved in emergency situations demanding a rapid formation of CSCs or cardiomyocytes. SC destiny depends on cell fate determinants, which include Numb and α -adaplin (9, 10), whose role in CSCs is unknown.

We have identified the components of cardiac niches and the contribution of CSC symmetric and asymmetric division to heart homeostasis. The functional properties and growth kinetics of CSCs have been defined by BrdU-retaining assays, because the long-term label-retaining property of a cell documents its stemness, while the progressive dilution of the label identifies the formed progeny (11).

Results

CSC Clusters. Although a niche can include a single SC, we have directed our search to clusters of CSCs and early LCCs. These nests were present in the atria, base–midregion, and apex, and consisted of lineage-negative (Lin^-) c-kit⁺, MDR1⁺, or Sca-1⁺ cells assembled with LCCs. By definition, CSCs exhibit SC antigens (c-kit, MDR1, Sca-1) but do not express transcription factors or membrane and cytoplasmic proteins of cardiac cells (3, 5). Progenitors correspond to cells in which the epitope of stemness coexists with transcription factors indicative of myocytes, endothelial cells (ECs), or smooth muscle cells. Precursors are a step farther in the differentiation process: The cytoplasm contains specific myocyte, EC, or smooth muscle cell proteins. More differentiated cells possess lineage-related nuclear and cytoplasmic proteins in the absence of SC antigens (Fig. 1 A–C). Capillaries were occasionally seen at the periphery of pockets of CSCs–LCCs, which were all CD45-negative (Fig. 6 and Table 1, which are published as supporting information on the PNAS web site). The sporadic and scattered location of capillaries within the niches, together with the absence of hematopoietic cell markers on CSCs, pointed to the non-bone marrow origin of cardiac primitive cells.

Supporting Cells. Connexin 43 and 45 and N- and E-cadherin were found between two Lin^- CSCs, one Lin^- CSC and one LCC, or two LCCs. These proteins were also detected between CSCs–LCCs and myocytes and fibroblasts (Fig. 1 D–J). However, they were not observed between CSCs–LCCs and ECs or smooth muscle cells. The expression of connexins and cadherins in isolated c-kit⁺ CSCs–LCCs was confirmed by Western blotting. The specificity of this protocol was strengthened by the fact that these cell preparations were negative for markers of myocyte differentiation, such as myosin light chain 2v (Fig. 7, which is published as supporting information on the PNAS web site).

A functional assay was performed to document the role of connexins in the formation of gap junctions between c-kit⁺ CSCs–LCCs and myocytes, fibroblasts, or ECs. Cell coupling was analyzed *in vitro* by two-photon microscopy after preincubation and loading of c-kit⁺ CSCs–LCCs with the green fluorescent dye calcein. The cells were also labeled with the red fluorescent dye 1,1'-dioctadecyl-

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Abbreviations: CSC, cardiac SC; EC, endothelial cell; LCC, lineage-committed cell; Lin^- , lineage-negative; SC, stem cell.

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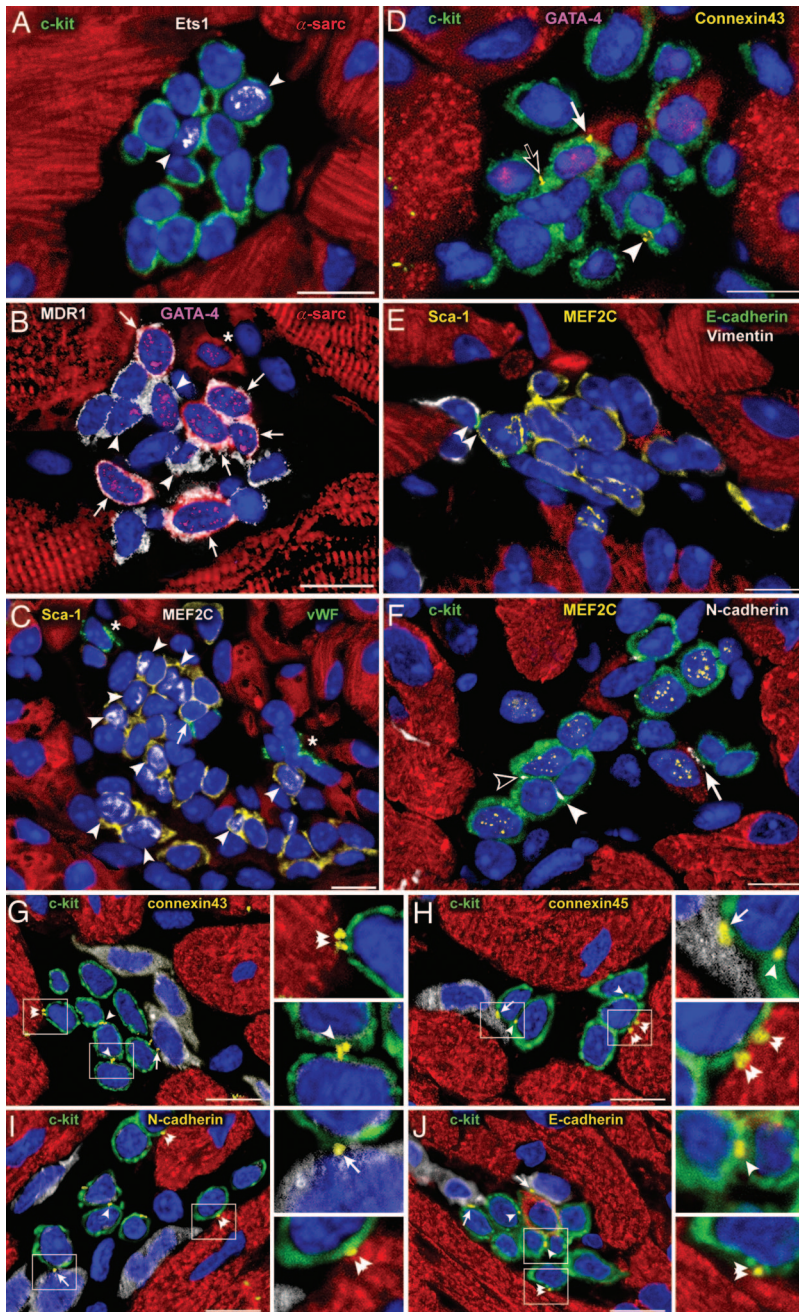


Fig. 1. Cardiac niches and putative supporting cells. Apical (A, B, and F) and atrial (C–E) niches contain CSCs and LCCs. (A) Fourteen c-kit⁺ cells (green) express Ets1 (white dots) in two nuclei (EC progenitors, arrowheads). (B) Fourteen MDR1⁺ cells (white) express GATA-4 (magenta dots) in nine nuclei; six of the nine GATA-4⁺ cells express α -sarcomeric-actin in the cytoplasm (red; myocyte precursors, arrows). Three cells positive for GATA-4 only correspond to cardiac progenitors (arrowheads). One small, developing myocyte is visible (asterisk). Five cells express only MDR1 and are lineage-negative (Lin^-) (CSCs). (C) Twenty-three Sca-1⁺ cells (yellow) express MEF2C (white dots) in 9 nuclei (myocyte progenitors, arrowheads); one Sca-1⁺ cell is labeled by von Willebrand factor (green; EC precursor, arrowheads). ECs are also present (asterisks). (D–F) Localization of connexin 43 and E- and N-cadherin in niches containing c-kit⁺ (D and F, green) and Sca-1⁺ (E, yellow) CSCs and LCCs. CSCs are Lin^- ; LCCs express GATA-4 (D, magenta dots) and MEF2C (E and F, yellow dots). Connexin 43 (yellow dots), E-cadherin (green dots), and N-cadherin (white dots) are located between two CSCs (D and F, arrowheads), a CSC and an LCC (F, open arrowhead), an LCC and a fibroblast (E, vimentin, white, double arrowhead), a CSC or an LCC and a myocyte (D and F, arrows), and two LCCs (D, open arrow). (G–J) Connexin 43 and 45 and N- and E-cadherin in atrial niches containing c-kit⁺ CSCs–LCCs (green) are represented by yellow dots located between two CSCs–LCCs (arrowheads), a CSC–LCC and a fibroblast (arrows), and a CSC–LCC and a myocyte (double arrowheads); see *Insets*. Nuclei are stained by propidium iodide (blue). (Scale bars, 10 μm .)

3,3,3',3'-tetramethylindocarbocyanine, which integrates stably within the cell membrane (12). Labeled c-kit⁺ CSCs–LCCs (green and red) were plated with unlabeled myocytes, fibroblasts, or ECs. The appearance of green fluorescence in unlabeled cells, in the absence of red fluorescence, indicated the transfer of calcein through the formation of gap junctions. Calcein translocated from c-kit⁺ CSCs–LCCs to myocytes and fibroblasts but not to ECs, suggesting that myocytes and fibroblasts function as supporting cells. This finding was confirmed by the identification of connexin 43 and 45 between c-kit⁺ CSCs–LCCs and myocytes and fibroblasts by confocal microscopy (Fig. 2). When the gap junction-blocker heptanol was added to the coculture of c-kit⁺ CSCs–LCCs and myocytes, calcein transfer did not occur, despite the expression of connexin at the interface of the two cell types (see Figs. 8 and 9, which are published as supporting information on the PNAS web site).

Integrin Receptors. In the niches, α_4 -integrin defined the plasma membrane of Lin^- CSCs; it was not expressed in LCCs (Fig. 3A and B). β_1 -integrin was detected in both CSCs and LCCs (Fig. 3C and D). The α_2 -chain of laminin paralleled the localization of α_4 -integrin (Fig. 3E–H). Laminin-8/9 and -10/11 were present in the niches and myocardium (data not shown). Niches were surrounded by myocytes, but a basal lamina was not observed. Fibronectin accumulated on the surface of Lin^- CSCs together with α_4 -integrin (see Figs. 10 and 11, which are published as supporting information on the PNAS web site). The colocalization of the α_2 -chain of laminin and fibronectin with α_4 -integrin was restricted to Lin^- CSCs, suggesting that this discrete ligand–receptor interaction was implicated in the preservation of the undifferentiated state of CSCs. In this regard, inhibition of α_4 -integrin promotes mobilization of bone marrow progenitor cells (13), providing evidence for the critical role of α_4 -integrin in CSC homeostasis. Also, α_4 -integrin induces the renewal of hematopoietic SCs (14).

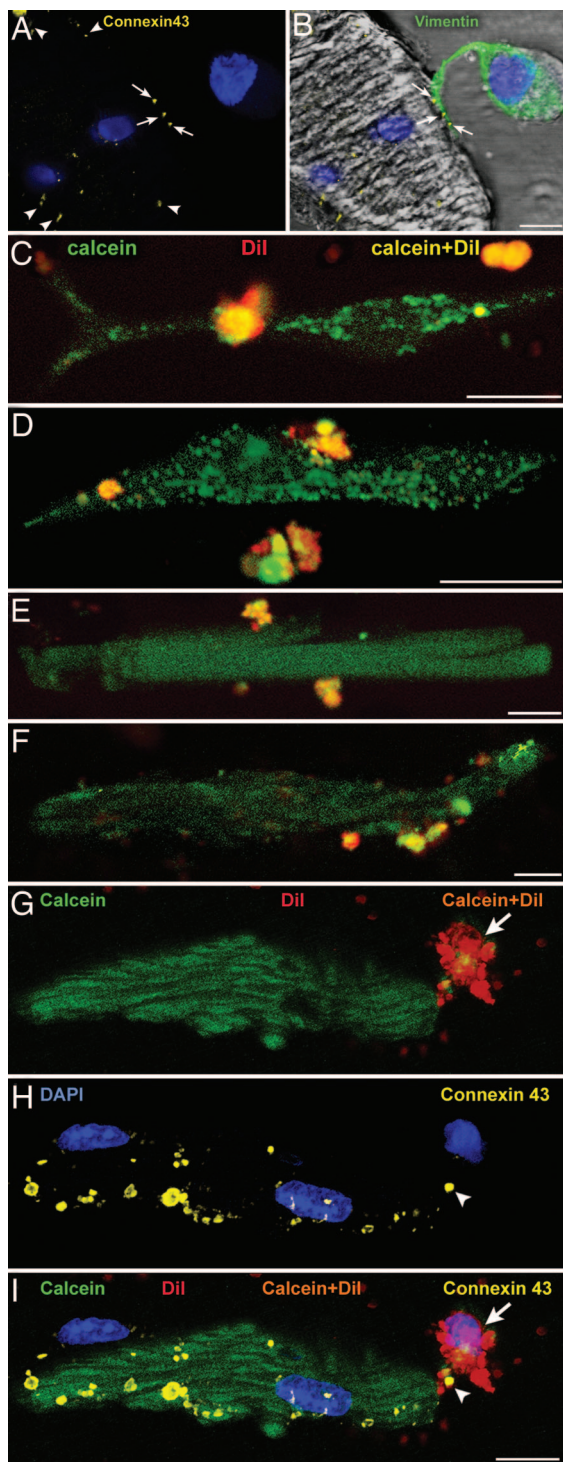


Fig. 2. Formation of gap junctions. (A and B) Localization of connexin 43 (A, yellow dots, arrows) between the projection of a c-kit⁺ CSC-LCC (B, vimentin, green) and an unlabeled myocyte (B, vimentin-negative) in culture. Connexin 43 is present in other areas of the myocyte surface (A, arrowheads). (C-F) Two-photon microscopy of c-kit⁺ CSCs-LCCs loaded with calcein and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (yellow fluorescence) and cocultured with unlabeled fibroblasts or unlabeled myocytes. Green fluorescence in fibroblasts (C and D) and myocytes (E and F) demonstrates the translocation of calcein (green) from CSCs-LCCs to fibroblasts and myocytes. (G-I) Two-photon microscopy of a myocyte and adjacent CSC-LCC (G). Calcein-1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (orange) is apparent in the CSC-LCC (arrows) but not in the myocyte, which is calcein⁺ only (green). The same cells are shown after

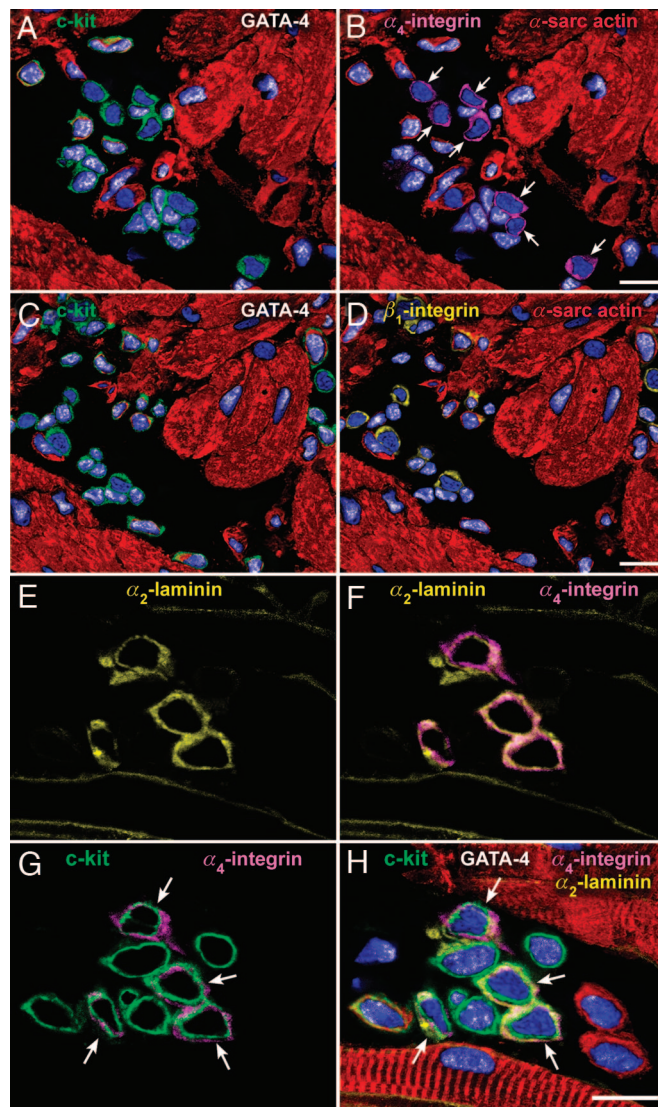


Fig. 3. Cardiac niches, integrin receptors, and extracellular ligands. Apical (A–D) and atrial (E–H) niches contain integrin subunits and laminin. (A–D) The same niche is illustrated at two distinct levels: One (A and B) and two (C and D). c-kit⁺ cells (A, green), which do not express GATA-4 in their nuclei (A and B, white dots), possess α_4 -integrin on the cell surface (B, magenta, arrows). However, c-kit⁺ cells (C, green), which express or do not express GATA-4 (C and D, white dots) possess β_1 -integrin (D, yellow). (E–H) The α_2 -chain of laminin (E, F, and H, yellow) is associated with α_4 -integrin (F–H, magenta) in four of the nine c-kit⁺ cells (G and H, green, arrows). The four α_2 -laminin α_4 -integrin c-kit⁺ cells are negative for GATA-4; GATA-4 is present in the other five c-kit⁺ cells (H, white dots). (Scale bars, 10 μ m.)

Cardiac Niches. Niches were defined as randomly oriented ellipsoid structures constituted by cellular and extracellular components. Fifty niches each were measured in the atria, base–midregion, and apex. The average volume of niches was $\approx 11,000 \mu\text{m}^3$. However, atrial niches were ≈ 2.0 -fold larger than ventricular niches. The number of niches per mm^3 of atrial and apical myocardium was ≈ 8 -fold higher than at the base–midregion (Fig. 12, which is published as supporting information on the PNAS web site). The number of CSCs-LCCs within atrial niches was higher than in other

fixation and staining by confocal microscopy (H). Connexin 43 between the cells is depicted by yellow dots (H and I, arrowheads). (I) Merge of G and H. (Scale bars, 10 μ m.)

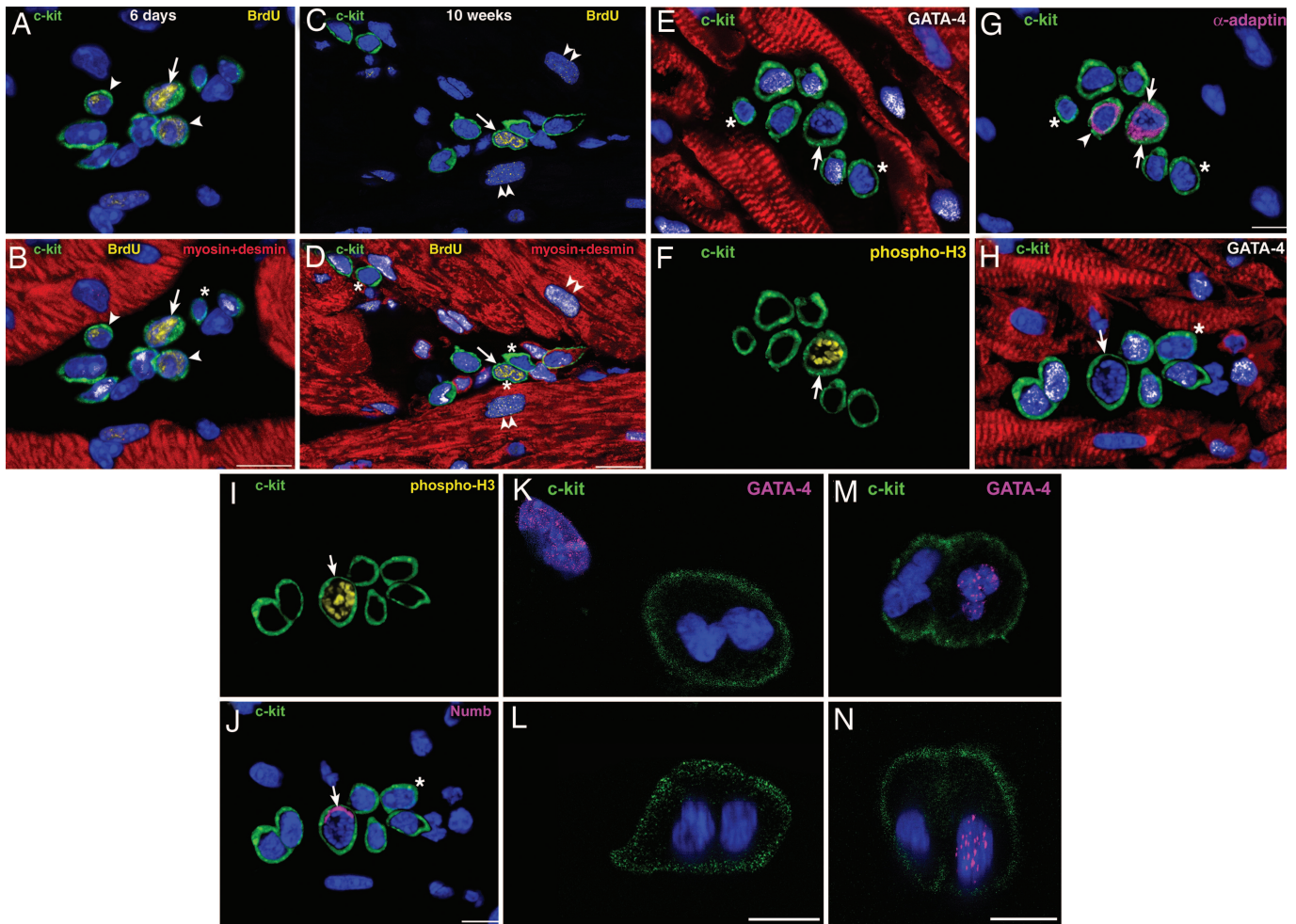


Fig. 4. CSC growth in cardiac niches. (A–D) Atrial niches with bright (arrows) and dim (arrowheads) BrdU⁺ (yellow) c-kit⁺ cells (green) after 6 days of labeling (A and B illustrate the same field) and 10 weeks of chasing period (C and D illustrate the same field). Niches contain c-kit⁺ LCCs (transcription factors, white dots; cardiac myosin and desmin, red) and c-kit⁺Lin[−] CSCs (B and D, asterisks). (D) Three Lin[−] CSCs, one of which is BrdU-bright (arrow). Two dim BrdU-labeled myocytes are present (C and D, double arrowheads). (E–G) Atrial niche with seven c-kit⁺ cells (green), three of which express GATA-4 (E, white dots). One GATA-4[−] c-kit⁺ cell (E, arrow) expresses phospho-H3 (F, yellow, arrow) and shows α -adaptin at both poles of the cell (G, magenta, arrows). The nonmitotic α -adaptin⁺GATA-4[−] cell may represent a recently formed daughter CSC (G, arrowhead). The two remaining c-kit⁺GATA-4[−] cells may correspond to quiescent CSCs (E and G, asterisks). (H–J) Atrial niche with seven c-kit⁺ cells (green), five of which express GATA-4 (H, white dots). One GATA-4[−] c-kit⁺ cell (H, arrow) expresses phospho-H3 (I, yellow) and shows Numb at one pole of the cell (J, magenta, arrow). The remaining c-kit⁺GATA-4[−] cell may correspond to a quiescent CSC (H and J, asterisks). (K–M) Two c-kit⁺ cells (green) in culture at the end of mitosis are illustrated in the xy (K and M) and the xz (L and N) plane. The absence (K and L) and presence (M and N) of GATA-4 (magenta dots) suggest symmetric and asymmetric division, respectively. (Scale bars, 10 μ m.)

areas and depended on the size of the niches. Atria and apex were the preferential storage sites of Lin[−] CSCs (Fig. 12).

Long-Term Label-Retaining Assay. Four and 12 injections of BrdU were performed at 12-hour intervals in two groups of mice over a period of 2 and 6 days, respectively. A third group was treated in an identical manner for 6 days, and animals were killed after a chasing period of 10 weeks. Bright and dim BrdU-labeled Lin[−] CSCs were distinguished on the basis of fluorescence intensity (Fig. 4A–D, and see *Materials and Methods*). The number of bright BrdU-Lin[−] CSCs in the atrial niches increased 140% from 2 to 6 days but decreased 91% after 10 weeks of chasing. Of 257 bright BrdU-Lin[−] CSCs at 6 days, only 23 cells retained this characteristic at 10 weeks, constituting the slow-cycling SC pool in this portion of the heart (Fig. 13, which is published as supporting information on the PNAS web site). At the base–midregion and apex, bright BrdU-Lin[−] CSCs increased 80% and 130% from 2 to 6 days and decreased 88% and 91% after chasing, respectively. Dim BrdU-Lin[−] CSCs increased from 2 to 6 days, but, in contrast to bright BrdU-Lin[−] CSCs, dim

BrdU-Lin[−] CSCs increased dramatically after chasing: 16-fold in the atria, 13-fold at the base–midregion, and 16-fold at the apex (Fig. 13). The aggregate number of Lin[−] CSCs within the niches did not change over a period of 10 weeks in young mice. Thus, the growth kinetics of Lin[−] CSCs tends to preserve the pool of primitive cells within the niches. The recognition of long-term label-retaining cells provides functional evidence of resident CSCs in the adult heart.

Division of CSCs. The long-term label-retaining assay indicated that \approx 10% of Lin[−] CSCs are slow-cycling and conform to the paradigm of SCs. The BrdU pulse/chase protocol, however, did not provide information regarding the pattern of growth at the single SC level. For this purpose, numerous niche profiles were viewed (Table 2, which is published as supporting information on the PNAS web site), and 18, 17, and 20 Lin[−]c-kit⁺ CSCs in mitosis were detected in the atria, base–midregion, and apex, respectively. The presence of Numb and α -adaptin at the two poles of cycling CSCs (for symmetric division, see Fig. 4E–G) and the restricted localization of

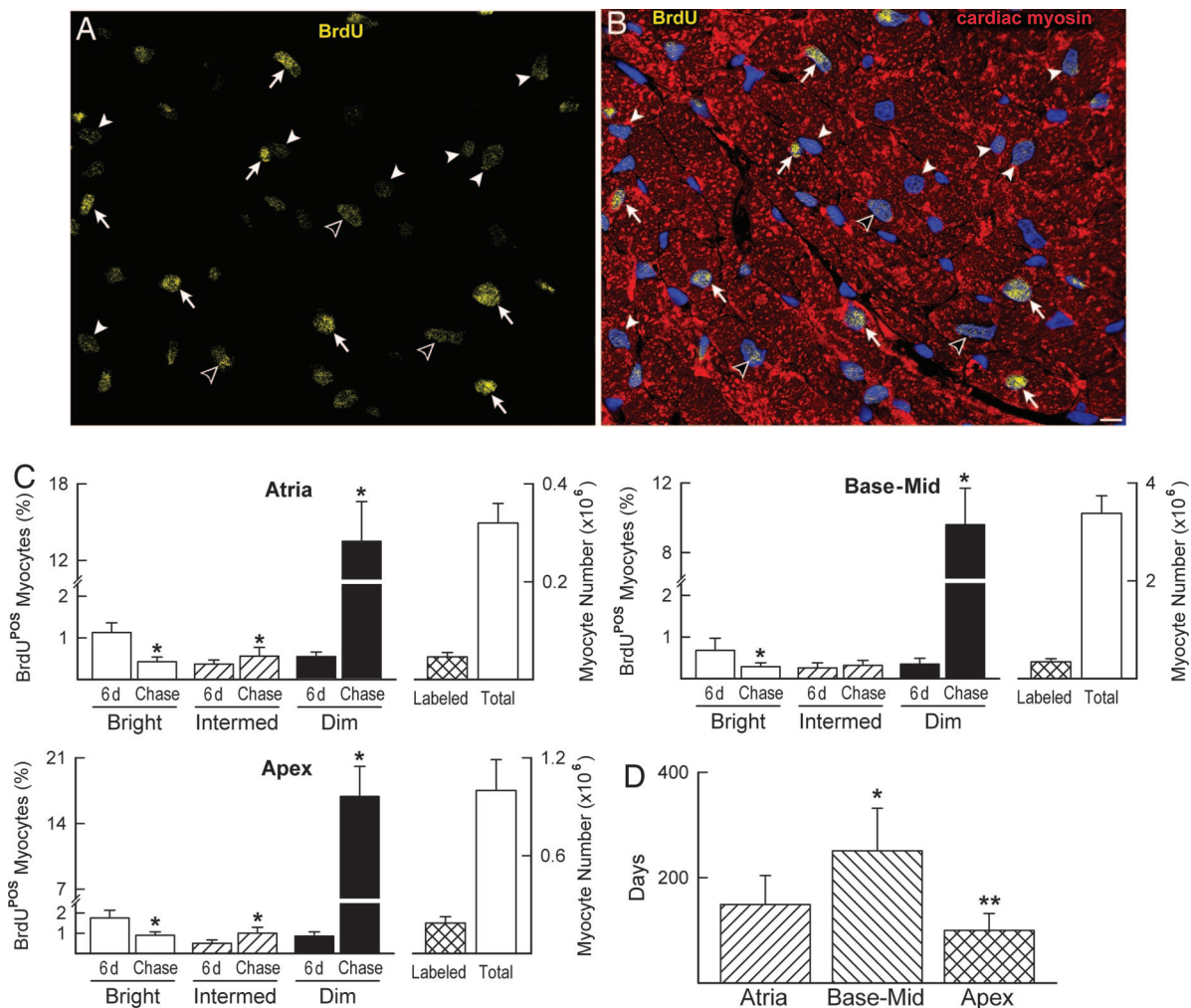


Fig. 5. Myocyte turnover and life span. (A and B) Apical section illustrating bright (arrows), intermediate (open arrowheads), and dim (arrowheads) BrdU⁺ myocyte nuclei (yellow) after 10 weeks of chasing. Myocytes are stained by cardiac myosin (B, red). (Scale bar, 10 μ m.) (C) Bars show the percentage of BrdU-bright, -intermediate, and -dim myocyte nuclei after 6-day labeling and after 10-week chasing. (Right) Bars document the total number of BrdU⁺ myocytes (labeled) and the total number of myocytes in the atria, base-midregion, and apex. *, $P < 0.05$ vs. 6 days. (D) Myocyte half-life. * and **, $P < 0.05$ vs. atria and base-midregion, respectively.

these proteins at one pole (for asymmetric division, see Fig. 4 H–J) were identified; of the 18 dividing atrial CSCs, 14 were undergoing asymmetric division, and 4 were undergoing symmetric division. Corresponding values at the base-midregion were 10 asymmetric and 7 symmetric and, at the apex, 14 asymmetric and 6 symmetric. Thus, asymmetric division was more prevalent than symmetric division ($P < 0.02$).

The CSC mitotic index did not vary in the three portions of the heart, and, because of the low frequency of CSC mitosis, it was difficult to establish whether groups of niches were preferentially activated. Similarly, the limited sampling of mitotic CSCs in each cardiac region did not allow us to establish whether this process had a privileged anatomical localization. To obtain additional information about CSC proliferation, c-kit⁺ cells were cultured in growth medium to promote cell division. On the basis of the compartmentalization of α -adaptin and Numb, symmetric division accounted for $38 \pm 7\%$ and asymmetric division for $62 \pm 7\%$. These patterns of cell growth were confirmed by the detection of GATA-4 as a marker of cell commitment (Fig. 4 K–N).

Myocyte Turnover. Myocyte formation was assessed by measuring the fraction of BrdU⁺ cells 6 days after BrdU administration and after 10 weeks of chasing. BrdU-bright myocytes at 10 weeks were

cells that experienced a limited number of divisions, whereas more rounds of doublings had to occur in myocytes with intermediate levels of labeling. These myocyte classes were assumed to correspond to amplifying myocytes, which incorporated BrdU at the time of injection and continued to divide and differentiate. Conversely, BrdU-dim myocytes were considered the progeny of cycling CSCs, which became BrdU⁺ at the time of injection and gave rise to a large number of committed cells. Clusters of BrdU-dim myocytes, together with BrdU-bright myocytes, were observed in the ventricle, whereas a more dispersed pattern of labeling was seen in the atria and apex (Fig. 5 A and B). The percentage of BrdU-bright myocytes detected at 6 days decreased markedly after 10 weeks, whereas BrdU-dim myocytes increased (Fig. 5 C).

The apex had 19% BrdU-labeled myocytes, the atria 15%, and the base-midregion 10%. The BrdU data at 6 days and at 10 weeks provided us with the magnitude of cell turnover in the myocardium (see *Materials and Methods*). These results were complemented with the measurements of myocyte progenitors-precursors (LCCs) (Table 3, which is published as supporting information on the PNAS web site), which, together with the CSC number, were used to evaluate the half-life of myocytes. Myocytes located at the base-midregion have a half-life 2-fold longer than atrial and apical myocytes, suggesting that myocyte turnover is high (Fig. 5 D).

Discussion

The heart typically shows discrete structures in the interstitium where CSCs and LCCs are clustered together, forming cardiac niches. The documentation that SC niches are present in the adult myocardium has been based on the identification of these pockets of CSCs–LCCs and the detection of cell-to-cell contact between CSCs–LCCs and myocytes and fibroblasts. In the heart and other self-renewing organs, SCs are connected structurally and functionally to the supporting cells by junctional and adhesion proteins represented by connexins and cadherins (15). Connexins are gap junction channel proteins that mediate passage of small molecules involved in cell-to-cell communication (16). Cadherins are calcium-dependent transmembrane adhesion molecules, which have a dual function: They anchor SCs to their microenvironment and promote a cross-talk between SCs and between SCs and nurse cells (15). Cardiac niches create the necessary, permissive milieu for the long-term residence, survival, and growth of CSCs.

The arrangement of CSCs–LCCs and supporting cells in the cardiac niches is similar to that found in the bone marrow (17) and brain (18), providing elements of analogy for these organs. In the bone marrow, osteoblasts and stromal cells function as supporting cells (18, 19). They can be considered the equivalent of myocytes and fibroblasts found here in the heart. ECs do not operate as nurse cells in the heart, but ECs are the candidate supporting cells in the brain (20) and may have a comparable role in the bone marrow (21). An uninterrupted basal lamina delimits neural and epithelial niches, but it is not present in the bone marrow (9) or, as shown here, in the heart.

Integrins are adhesion receptors for the attachment of cells to the extracellular proteins fibronectin and laminin (22). β_1 -Integrin is commonly found on the surface membrane of primitive and committed cells within the niches (23). Fibronectin and the α_2 -chain of laminin are ligands for $\alpha_4\beta_1$ -integrin (23, 24). The colocalization of the α_2 -chain of laminin and fibronectin with α_4 -integrin was restricted to Lin⁻ CSCs, suggesting that α_4 -integrin binding may be typically involved in the preservation of stemness of CSCs. β_1 -Integrin is highly expressed on the interfollicular SCs of the epidermis (11) and in neural SCs (23), whereas α_4 -integrin has been linked to the renewal of hematopoietic SCs (14). The α_2 -chain of laminin is detected in SCs of the skin and intestinal crypts (25) but is not seen in bone marrow SCs (26). Laminin-8/9 and -10/11 are typical components of bone marrow niches (26) but lack this preferential localization in the heart. By inference, in cardiac niches, the α_2 -chain of laminin and fibronectin transduce mechanical signals from the extracellular compartment to the $\alpha_4\beta_1$ -integrin receptor, which activates effector pathways opposing CSC commitment and differentiation.

Homeostasis of cardiac niches is mediated by asymmetric and symmetric division of CSCs. Asymmetric division predominates, and, by this mechanism, CSCs protect their pool and generate a committed progeny. The uniform and nonuniform localization of Numb and α -adaplin, together with the identification of phosphohistone-H3 expression, (3) was determined to define symmetric and asymmetric division, respectively. The intracellular segregation of these proteins at the time of mitosis constitutes the intrinsic determinant of SC fate. Numb and α -adaplin interact to enable primitive cells to produce differently destined sibling cells (27). Numb is expressed from late prophase to telophase and in the early stages of life of the daughter cell (28). Numb localizes to endocytic vesicles, where it binds to the endocytic protein α -adaplin, promoting the internalization and inactivation of the Notch receptor (29). Numb-negative cells retain their responsiveness to Notch and adopt the fate associated with Notch activation (30).

The prevailing inhomogeneous localization of Numb and α -adaplin in cycling CSCs controls the production of daughter cells, which follow divergent paths. The BrdU pulse/chase assay conformed to this pattern of asymmetric CSC division. Over time, the number of CSCs–LCCs confined to the niches remains constant, suggesting that asymmetric kinetics characterizes most of the growth of CSCs in the myocardium. This mechanism of cell renewal has been described as “invariant” (11), and it typically occurs in an organ in a steady state. Pathological conditions, including ischemia-reperfusion injury lead to the formation of reactive oxygen species. Oxidative stress triggers distinct cellular responses; low levels initiate growth, intermediate degrees promote apoptosis, and high magnitudes mediate cell necrosis (31). Whether CSCs–LCCs within the niches react to the oxidative challenge in a similar manner is a critical, unanswered question.

Materials and Methods

Hearts of FVB/N mice at 3 months of age were studied. The volume, composition, and number of cardiac niches were measured. A subgroup of animals was injected with BrdU for 2 and 6 days and killed at 2 days, 6 days, and after 10 weeks of chasing. By this approach, bright and dim BrdU⁺ CSCs and myocytes were counted (see *Supporting Materials and Methods* and Table 4, which are published as supporting information on the PNAS web site).

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