Notch1 signaling stimulates proliferation of immature cardiomyocytes

Chiara Collesi,1 Lorena Zentilin,1 Gianfranco Sinagra,2 and Mauro Giacca1

1Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy
2SC Cardiologia, Azienda Ospedaliero-Universitaria “Ospedali Riuniti di Trieste,” Ospedale di Cattinara, Trieste, Italy

The physiological identification of the molecular mechanisms controlling cardiomyocyte proliferation during the embryonic, fetal, and early neonatal life appears of paramount interest in regard to exploiting this information to promote cardiac regeneration. Here, we show that the proliferative potential of neonatal rat cardiomyocytes is powerfully stimulated by the sustained activation of the Notch pathway. We found that Notch1 is expressed in proliferating ventricular immature cardiac myocytes (ICMs) both in vitro and in vivo, and that the number of Notch1-positive cells in the heart declines with age. Notch1 expression in ICMs paralleled the expression of its Jagged1 ligand on non-myocyte supporting cells. The inhibition of Notch signaling in ICMs blocked their proliferation and induced apoptosis; in contrast, its activation by Jagged1 or by the constitutive expression of its activated form using an adeno-associated virus markedly stimulated proliferative signaling and promoted ICM expansion. Maintenance or reactivation of Notch signaling in cardiac myocytes might represent an interesting target for innovative regenerative therapy.

Introduction

The Notch signaling pathway plays a key role at multiple steps of morphogenesis during embryonic development as well as in a wide variety of processes during adult life. In particular, Notch activation appears to finely tune the balance between proliferation and differentiation of stem and progenitor cells in several different settings, including hematopoietic and nervous systems, skin, gut, and heart (for reviews see Bray, 2006; Chiba, 2006; Hurlbut et al., 2007; Nemir and Pedrazzini, 2008; Niessen and Karsan, 2008). Physiological activation of Notch signaling requires cell–cell contact and occurs through binding of the Notch receptor to one of its ligands (Delta and Jagged in vertebrates and Serrate in invertebrates) followed by the proteolytic release of the intracellular domain (ICD) of Notch (Notch-ICD) and its translocation into the nucleus (De Strooper et al., 1999). Once inside the nucleus, Notch-ICD interacts with transcription regulators of the CSL family (CBF1, Su(H), and Lag-1), triggering the activation of genes of the hairy and enhancer of split (HES) family (Jarriault et al., 1995; Iso et al., 2003). Experimental evidence obtained in *Xenopus laevis*, zebrafish, chicken, and mammals has demonstrated that Notch signaling controls mesodermal commitment, including cardiogenic differentiation, during development (Rones et al., 2000; Loomes et al., 2002; Beis et al., 2005; Rutenberg et al., 2006; Del Monte et al., 2007; Grego-Bessa et al., 2007). Of interest, mice lacking the genes encoding either Notch receptors or their ligands show early embryonic lethality, which is mainly the consequence of cardiovascular abnormalities. This observation supports the notion that the Notch pathway essentially participates in the process of cardiac and vascular development (Gridley, 1997; Gridley, 2003; Shawber and Kitajewski, 2004; Del Monte et al., 2007; Grego-Bessa et al., 2007). Several other observations also favor this conclusion. In humans, the Alagille syndrome, a congenital disorder associated with heart abnormalities, has been linked to haploinsufficiency of Jagged1 (Li et al., 1997; Oda et al., 1997; McElhinney et al., 2002). In mice, combined haploinsufficiency of Jagged1 and Notch2 results in cardiac defects reminiscent of this syndrome (McCright et al., 2002). The Notch signaling pathway also appears to be involved in cardiac valve development (Timmerman et al., 2004): in humans, heterozygous mutations disrupt normal development of cardiac valve formation.

Correspondence to Mauro Giacca: giacca@icgeb.org

Abbreviations used in this paper: AAV, adeno-associated virus; CKI, cyclin-dependent kinase inhibitor; DAPT, N-(3,5-difluorophenacetyl)-L-alanyl-S-phenylglycine t-butyl ester; Fc, fragment crystallizable; HES, hairy and enhancer of split (HES) family; ICD, intracellular domain; ICM, immature cardiac myocyte; PI, propidium iodide; rFc-sJ1, recombinant Fc-fused soluble Jagged1; sJ1, soluble Jagged1.

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myocytes rely on Notch1 activity for cell renewal, thus raising the question whether the Notch pathway is also involved in tissue renewal in other organs. Indeed, our results indicate that immature, still proliferating cardiac stromal cells express Notch1. The levels of expression of Notch1 were found to decline together with the loss of the cardiomyocyte progenitor cell phenotype, consistent with this observation, the sustained activation of Notch1 determined a remarkable expansion of cardiac cells in vitro. Similar to the role of the Notch pathway in preserving progenitor cells in other tissues, we propose that the sustained activation of Notch1 might lead to the expansion of the pool of poorly differentiated, committed cardiac cells.

Results

The number of Notch1-positive, BrdU-positive cardiomyocytes in vivo declines with age

We started by analyzing the number of cycling cells in neonatal and adult rat hearts. For this purpose, animals at birth and at 9 mo of age (adults) were treated with BrdU, and after 1 wk, the presence of cells that had incorporated the nucleotide analogue was assessed by immunofluorescence. In agreement with published observations (Chen et al., 2007; Kang and Koh, 1997), >30% of nuclei of the α-actinin–positive neonatal cardiomyocytes scored positive for BrdU incorporation, whereas <3% of cells had incorporated BrdU in the adult hearts (Fig. 1, A and B, for representative images at different magnifications; and Fig. 1 C for quantification). To confirm the cardiac lineage specificity of the proliferating cells, double labeling with the cardiomyocyte-specific nuclear marker GATA-4 and BrdU was performed on sections from heart sections from newborn rats showing strong immunoreactivity for Notch1 (red) of α-actinin (green)-positive cardiomyocytes. Arrows indicate Notch1-positive myocardial cells. (H and I) High-magnification images of representative cells from a newborn heart section showing positivity of BrdU incorporation (green) and expression of the Notch1 cell membrane receptor (red). Nuclei were stained with DAPI. (J) Representative images of both newborn (top) and adult (bottom) rat heart sections showing a patchy staining for Jagged1 (red) in stromal areas of myocardial tissue, the latter positive for α-actinin staining (green). Nuclei were stained with DAPI. In A, E, and G–L, the largest figure in each panel shows fluorescence in all three channels; the two smaller panels show the red/green/channels. Bars: (A, top) 200 μm; (A, bottom) 10 μm; (B and D) 10 μm; (E) 100 μm; (F, top) 100 μm; (E, bottom) 10 μm; (G, top) 200 μm; (G, bottom) μm; (H and I) 10 μm. (J) 100 μm.
These observations raised the intriguing possibility that the Notch1-positive, BrdU-positive cells might represent immature cardiomyocytes that were still in proliferation in the neonatal hearts.

**Notch-1 expression is down-regulated during cardiomyocytes differentiation**

To further investigate the role of Notch in the regulation of cardiomyocytes proliferation, the expression of the different Notch receptors and ligands was analyzed in primary cultures of cells extracted from neonatal rat myocardium. To specifically distinguish between cardiac myocytes and cardiac stromal cells, we obtained cultures highly enriched in α-actinin-positive cardiomyocytes by three subsequent sub-plating steps (>90% purity; see Materials and methods) along with cultures of α-actinin-negative cardiac muscle fibers (Fig. 1 L and not depicted).
time points, these cells expressed high levels of Jagged1, Notch2, and its specific downstream effector Hey1 (Rutenberg et al., 2006). No significant differences in the expression of the putative target genes of the Notch pathway, such as Hes1-5, Hey1, and Hey2, were observed in immature and fully differentiated cardiomyocytes, as well as in stromal cells, which indicates that regulation of these target genes is also dependent on pathways different from Notch (also see the following paragraph).

The expression profiles of the Notch1 and Jagged1 proteins in differentiating cardiomyocytes and in the stromal cells were further analyzed by Western blotting. A polyclonal antiserum directed against the C terminus of Notch1 detected both the 300-kD, full-length protein and the p120 cleaved transmembrane Notch1 subunit in ICMs at day 1 of culture, whereas positivity progressively decreased at days 3 and 7 (Fig. 2F). No specific signals were detected in stromal cells at days 1 and 3, although these became apparent at day 7. Consistent with the RT-PCR findings, the Jagged1 protein was not expressed in ICMs (day 1), though it progressively increased at days 3 and 7. In stromal cells, the levels of the protein remained relatively high at all time points (Fig. 2F).

Finally, the levels of activated Notch1 (ICD) paralleled the expression pattern of the full-length protein (Figs. 2G and Fig. S1A, available at http://www.jcb.org/cgi/content/full/jcb.200806091/DC1).

The cellular localization of Notch1 and Jagged1 proteins during cardiomyocytes differentiation was further studied by immunofluorescence (Fig. 2H). Staining with an antibody that recognizes the C terminus of Notch1 detected both the 300-kD, full-length protein and the p120 cleaved transmembrane Notch1 subunit in ICMs at day 1 of culture, whereas positivity progressively decreased at days 3 and 7 (Fig. 2F). No specific signals were detected in stromal cells at days 1 and 3, although these became apparent at day 7. Consistent with the RT-PCR findings, the Jagged1 protein was not expressed in ICMs (day 1), though it progressively increased at days 3 and 7.

In stromal cells, the levels of the protein remained relatively high at all time points (Fig. 2F). Finally, the levels of activated Notch1 (ICD) paralleled the expression pattern of the full-length protein (Figs. 2G and Fig. S1A, available at http://www.jcb.org/cgi/content/full/jcb.200806091/DC1).

The cellular localization of Notch1 and Jagged1 proteins during cardiomyocytes differentiation was further studied by immunofluorescence (Fig. 2H). Staining with an antibody that recognizes the C terminus of Notch1 confirmed the expression of the protein in ICMs (day 1) and its progressive disappearance afterward. Consistent with these findings, very strong immunoreactivity for activated Notch1-ICD (an antibody against Val1744) was found in small, round-shaped ICMs at day 1 (Figs. 2H and Fig. S1B), whereas differentiated cardiomyocytes at day 7 were almost completely negative. In agreement with the RT-PCR and
Western blot analyses, and in contrast to cardiomyocytes, stromal cells were positive for both anti-Notch1 antibodies at days 3 and 7. The Notch ligand Jagged1 was not detectable in ICMs at day 1, whereas an increasingly stronger signal was detected at later times, as the cardiomyocyte acquired a fully differentiated and beating phenotype. Stromal cells showed a strong immunoreactivity for Jagged1 at all time points.

To further characterize the in vitro differentiation of neonatal ICMs, we analyzed the pattern of expression of Numb, an inhibitor of Notch signaling that interacts with the intracellular portion of Notch and prevents its nuclear translocation (Guo et al., 1996; Wakamatsu et al., 1999). At day 1, Numb expression was almost undetectable in ICMs, although it gradually increased at day 3 and 7, as detected by both transcript-specific PCR and Western blotting (Fig. 3, A and B, respectively).

The levels of Numb along cardiomyocyte differentiation were further analyzed by immunofluorescence staining using specific antibodies. The protein was expressed at very low levels at day 1, though its levels progressively increased at days 3 and 7 (Fig. 3 C). Interestingly, asymmetrical intracellular localization of Numb was detected in dividing cardiomyocytes at day 1 (Fig. 3 D), which suggests that Numb-mediated Notch down-regulation might participate in the loss of replicative potential and differentiation of ICMs.

Together, these results show that the loss of proliferative potential of isolated cardiac myocytes parallels the down-regulation of expression of the Notch1 receptor and the progressive expression of Jagged1. In contrast, Jagged1 is constantly expressed in supporting stromal cells, whereas the levels of Notch1 increase over time and parallel their increased proliferation rate.

Inhibition of Notch signaling blocks cardiomyocyte proliferation and induces apoptosis

To further investigate the role of Notch1 activation in primary cardiomyocytes, cells at different days of culture were transfected with a luciferase construct under the control of two different Notch1-responsive promoters: the natural HES1 promoter (Jarriault et al., 1995) and an artificial promoter containing four repeats of the CBF1 binding sites (HES1 and CBF1 reporters, respectively; Hsieh et al., 1996). Parallel to the Notch1 transcript and protein levels, activation of the natural HES1 reporter was high at day 1, although it progressively decreased at later times (an approximately fivefold decrease in promoter activation between day 1 and 7; Fig. 4 A). Interestingly, this promoter turned out to be inhibited by the γ-secretase inhibitor only in differentiated cardiomyocytes at days 3 and 7, whereas it was totally unaffected by N-[3,5-difluorophenacetyl]-L-alanyl-S-phenylglycine t-butyl ester (DAPT) treatment in ICMs (Fig. 4 A). This observation prompts us to speculate that, in immature cardiomyocytes, the HES1 promoter might be a direct target of CREB activation, as demonstrated in other cell types (not further investigated here; Herzig et al., 2003). The CBF1 reporter showed an identical response at the various time points (>40 decrease in promoter activation between day 1 and day 7; Fig. 4 B); cell treatment with DAPT markedly suppressed promoter activation (a more than fivefold decrease at day 1), which is consistent with the conclusion that CBF1 reporter activation in cultured cardiomyocytes was strictly dependent on Notch1 activity.

To assess whether the block of Notch activation by γ-secretase inhibition might impair cardiomyocyte proliferation, the levels of BrdU incorporation at days 1, 3, and 7 of culture were analyzed. Treatment with DAPT determined a marked decrease in the number of BrdU-positive cardiomyocytes at days 1 and 3, respectively (Fig. 4 C).

When the same analysis was performed in cultures of stromal cells, DAPT was also found to significantly decrease the proliferative potential of these cells at day 7 only (Fig. 4 D), which is consistent with a role of Notch1 activation in sustaining also the proliferation of these cells at later time points.
Gene transfer in primary cardiac myocytes can be efficiently achieved using viral vectors based on the adeno-associated virus serotype 8 (AAV8), as shown in Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200806091/DC1). The constitutive expression of the activated Notch1 ICD (N1-ICD), driven by AAV8, and immobilized IgG. The transactivation effect was evaluated at the indicated time points after reporter transfection. The histogram represents mean values ± SEM, n = 6. **, P < 0.001. [B] Treatment with sJ1 markedly enhances expression of the CBF1-luciferase reporter. Control or sJ1-conditioned medium (sJ1) was added to cardiomyocytes for 24 h after reporter transfection; the transactivation effect was evaluated at the indicated time points. The histogram represents mean values ± SEM, n = 6; ***, P < 0.001. [C] sJ1 stimulation increases BrdU incorporation in cardiac myocytes. Neonatal cardiomyocytes were cultured in the presence of either sJ1- or control-conditioned medium, or either hIgG or rFc-sJ1 ligand preclustered on Sepharose beads, and pulsed for BrdU incorporation. In a mirror experiment, neonatal cardiomyocytes were seeded onto rFc-sJ1 (or normal h-IgG as a control) precoated slides. After BrdU immunostaining, the total number of BrdU+ cells per well was scored. The histograms show mean ± SEM of the percentage of BrdU+ nuclei from at least three fields of ~200 cells per field. Results are representative of at least three independent experiments. ***, P < 0.001. [D] Low (panels a, b, g, h, o, and p)- and high (panels c–f and i–n)-magnification representative images of BrdU+ cardiomyocytes at different time points, after treatment with control- or sJ1-conditioned medium. Green, α-actinin; red, BrdU; blue, DNA (DAPI). Bars: (low magnification) 50 μm; (high magnification) 10 μm. The examples shown are representative of more than five experiments with similar outcomes. (E) sJ1 remarkably stimulates cardiomyocyte proliferation. Cardiomyocytes were cultured in the presence of sJ1 (sJ1)- or control-conditioned media. After specific immunostaining, the total number of α-actinin–positive cells per well was scored. The histogram shows mean ± SEM of at least 8 wells per time point from three independent experiments. ***, P < 0.001. [F] AAV8-N1ICD strongly stimulates BrdU incorporation in cardiomyocytes. Neonatal cardiomyocytes were infected either with AAV8-LacZ or AAV8-N1ICD and pulsed for BrdU incorporation at different time points. After BrdU immunostaining, the total number of BrdU+ cells per well was scored. The histograms show mean ± SEM of the percentage of BrdU+ nuclei from at least three fields of ~200 cells per field. Results are representative of at least three independent experiments; ***, P < 0.001. (G) SJ1 stimulates BrdU incorporation in stromal cells. Stromal cells were cultured in the presence of sJ1 (sJ1)- or control-conditioned medium and pulsed for BrdU incorporation. After BrdU immunostaining, the total number of BrdU+ cells per well was scored. The histograms show mean ± SEM of the percentage of BrdU+ nuclei from at least three fields of ~200 cells per field. ***, P < 0.001.

Figure 5. sJ1 and Notch-ICD transduction enhance cardiomyocyte proliferation. [A] Treatment with sJ1 activates HES1 promoter transcription. Neonatal cardiomyocytes were assayed for HES1 reporter transactivation upon stimulation with conditioned medium from NIH-3T3 cells secreting sJ1 or rFc-sJ1 either preclustered on Sepharose beads or immobilized on plates. The respective controls were the supernatant from mock-transfected NIH-3T3 cells (Control), Sepharose-IgG, and immobilized IgG. The transactivation effect was evaluated at the indicated time points after reporter transfection. The histogram represents mean values ± SEM, n = 6. **, P < 0.001. (B) Treatment with sJ1 markedly enhances expression of the CBF1-luciferase reporter. Control or sJ1-conditioned medium (sJ1) was added to cardiomyocytes for 24 h after reporter transfection; the transactivation effect was evaluated at the indicated time points. The histogram represents mean values ± SEM, n = 6; ***, P < 0.001. (C) sJ1 stimulation increases BrdU incorporation in cardiac myocytes. Neonatal cardiomyocytes were cultured in the presence of either sJ1- or control-conditioned medium, or either hIgG or rFc-sJ1 ligand preclustered on Sepharose beads, and pulsed for BrdU incorporation. In a mirror experiment, neonatal cardiomyocytes were seeded onto rFc-sJ1 (or normal h-IgG as a control) precoated slides. After BrdU immunostaining, the total number of BrdU+ cells per well was scored. The histograms show mean ± SEM of the percentage of BrdU+ nuclei from at least three fields of ~200 cells per field. Results are representat...
apoptotic cells detected at the same time points in the presence of DAPT; Fig. 4 E). Of notice, the proapoptotic effect of DAPT was not rescued by AAV8-N1ICD transduction at day 1, a result that is consistent with the kinetics of transgene expression after AAV8 transduction, which reaches its maximum at 72 h after infection (Fig. S2). This finding is consistent with the biology of AAV transduction in a variety of other cell types in vitro and in vivo (Palomeque et al., 2007; Zincarelli et al., 2008).

Together, these results strongly indicate that Notch1 activation at the early time points not only triggers cell proliferation but is also required to maintain cell viability and prevent apoptosis.

Soluble Jagged1 enhances cardiomyocyte proliferation

The observation that the inhibition of the Notch pathway impairs cardiomyocyte proliferation suggested that, on the contrary, its stimulation might lead to their increased proliferation. To directly assess this possibility, we cultured freshly isolated neonatal cardiomyocytes in the presence of different sources of its Jagged1 ligand, including conditioned medium from NIH-3T3 cells secreting a soluble form of Jagged1 (sJ1; Wong et al., 2000) and recombinant fragment crystallizable (Fc)-fused soluble Jagged1 (rFc-sJ1) either preclustered on Sepharose beads (Vas et al., 2004) or immobilized on plates (Koyanagi et al., 2007).

Immature cardiomyocytes at day 1, cultured in the presence of any of the three sources of sJ1, showed a marked up-regulation of the HES1 promoter (Fig. 5 A). This transactivation effect was still evident at day 3, although it became almost undetectable in differentiated cardiomyocytes at day 7. A conditioned medium containing the Delta1 ligand had no apparent effect when used under the same conditions (unpublished data). Of interest, the conditioned medium containing sJ1 was even more potent than the two forms of immobilized rFc-sJ1, which is consistent with the possibility that sJ1 might spontaneously cluster on the surface of the target cells by virtue of its interactions with various proteins of the extracellular matrix and cell surface, as already described for other soluble growth factors (for reviews see Taipale and Keski-Oja, 1997; Cool and Nurcombe, 2006; Guerrini et al., 2007). In a consistent manner, sJ1 was also able to activate transcription of the CBF1 promoter in cardiomyocytes at days 1 and 3 (but not at day 7) of culture (Fig. 5 B). The sensitivity to sJ1 stimulation correlated with the presence of the active Notch1 receptor in cardiac myocytes. Upon sJ1 stimulation, day-1 and, to a lesser extent, day-3 cardiomyocyte extracts showed markedly increased levels of N1-ICD, whereas DAPT treatment completely abolished the signal in sJ1-stimulated cardiac myocytes at any time point; no signal was detected using the Val 1744 antiserum in day-7 cardiac myocytes under any condition (Fig. S1 A).

Consistent with the transactivation results, the treatment of neonatal cardiomyocytes with either sJ1 or the two different types of immobilized rFc-sJ1 proteins determined a marked increase in the extent of BrdU incorporation. The number of BrdU-positive cells was ~34% in the sJ1-treated sample versus 7% in the absence of treatment at day 1, and 11% versus 4.4% at day 3; BrdU incorporation was consistently increased upon treatment with immobilized rFc-sJ1 (quantification and repre-

sentative images are shown in Fig. 5 (C and D, respectively). At day 7 of culture, when Notch1 was no longer expressed in cardiomyocytes, sJ1 addition had no apparent effect on BrdU incorporation. Remarkably, the activation of the Notch1 pathway by sJ1 lead to a significant increase in the total number of cardiomyocytes in culture, which, at day 7 of stimulation, was almost fivefold compared to the control (Fig. 5 E). The effect of Notch1 stimulation on cardiomyocyte proliferation was further strengthened by the observation that the forced expression of N1-ICD in cardiomyocytes by AAV8-N1ICD transduction stimulated BrdU incorporation in day-3 and, remarkably, also in day-7 cardiomyocytes (21.1% vs. 2.8% in AAV8-N1ICD vs. AAV-LacZ transduced cardiomyocytes; Fig. 5 F).

Finally, in contrast to cardiomyocytes, sJ1 did not affect BrdU incorporation in stromal cells at day 1, in keeping with the absence of the Notch1 receptor in these cells at this time point (Fig. 5 G). At days 3 and 7, when Notch1 became expressed, the number of cells incorporating BrdU started to increase, although at a level remarkably lower than in early cardiomyocytes (17.2% vs. 11% BrdU-positive stromal cells at day 7 in the presence or absence of sJ1, respectively).
Notch1 activation parallels cell cycle protein expression in immature cardiomyocytes

The levels of several proteins involved in cell cycle regulation were analyzed by immunoblotting in cardiomyocytes at different time points in culture and upon Sj1 stimulation. The arrest in proliferation and decrease in BrdU incorporation over time were found to correlate with the progressive increase of the cyclin-dependent kinase inhibitors (CKIs) p21Cip1 and p27Kip1, and of the Cdt1 inhibitor geminin (Fig. 6 A; Wohlschlegel et al., 2000). Consistent with the increase of these factors, the levels of cyclin D1, cyclin A, and cyclin E were significantly lower in cardiomyocytes at days 7 and 14 of culture. These observations reinforce similar findings of Campa et al. (2008) on the specific role of cyclin D1 regulation in the cardiomyocyte cell cycle. Remarkably, Sj1 stimulation reversed cell cycle exit at days 1 and 3, whereas it had no effect at later stages. Cardiomyocyte proliferation correlated with the presence of high levels of phosphorylated p38 MAPK; conversely, differentiated cells expressed Y705-phosphorylated p-STAT3, a known marker of terminal differentiation in other tissues (Androutsellis-Theoktis et al., 2006). The sensitivity to Sj1 stimulation was strictly dependent on the presence of the Notch1 receptor on cardiomyocytes. At days 1 and 3, both the Notch1 precursor p300 and the cleaved p120 and p110 forms were present, and Sj1 stimulation further increased their levels while inhibiting its own expression (evident at day 3 in Fig. 6 A). At days 7 and 14, p300 was undetectable, and its cleaved forms progressively disappeared. In contrast, expression of Jagged1 became detectable and was negatively regulated by Sj1 stimulation in a negative feedback loop. Collectively, these results support the conclusion that Notch activation powerfully stimulates immature cardiac myocyte proliferation and expansion during the early times of culture, whereas it progressively becomes ineffective along the differentiation process and is incapable on its own to promote reentry of differentiated cardiomyocytes into the cell cycle.

Discussion

In this study, we provide evidence that activation of the Notch1 pathway maintains proliferation and permits expansion of ex vivo cultured ICMs. We found that Notch1 is highly expressed by immature, still dividing, cardiac myocytes, whereas Jagged1 is mainly expressed on supporting stromal cells. The loss of proliferative potential by isolated myocytes parallels the down-regulation of the Notch1 receptor and the progressive expression of Jagged1. On the contrary, we show that the sustained, constitutive activation of Notch1 signaling directly correlates with an increase in ICM proliferation.

Our results indicate that the multidirectional Notch receptor/ligand signaling paradigm also extends to cardiac cells. According to this paradigm, it can be envisaged that the stromal microenvironment, expressing Jagged1, triggers an inductive signal to ICMs to expand their number, keeping them in a highly proliferative state. This is supported by our observation that activation of Notch signaling by both exogenous soluble ligand stimulation and intracellular AAV-mediated constitutive expression of N1-ICD enhance ICM proliferation, delay newly formed cardiomyocytes from rapid differentiation into mature beating cells (normally within 24 h from plating), and extend their lifespan in culture. However, Notch receptor activation does not revert newly formed cardiomyocytes back into precursor cells but keeps the ICM proliferating window open for a longer time, stimulating their increase in number.

The early embryonic lethality caused by cardiovascular defects of mice lacking genes of the Notch signaling cascade strongly supports a key function for Notch in the process of cardiac and vascular development (Loomes et al., 1999; Rones et al., 2000; Loomes et al., 2002; for reviews see High and Epstein, 2008; Niessen and Karsan, 2008). Indeed, Notch signaling has been found to exert a suppressive role during myocardial specification in several different species (Park et al., 1998; Rones et al., 2000; Han and Bodmer, 2003; Chau et al., 2006). Consistent with these findings, several in vitro studies have also implied a suppressive role of Notch on mesodermal commitment and cardiac differentiation. RBP-J–deficient embryonic stem cells show enhanced mesodermal and cardiogenic differentiation, which is likely attributable to the lack of Notch signaling (Schroeder et al., 2003; Nemir et al., 2006; Schroeder et al., 2006). In addition, constitutive activation of Notch in mesodermal progenitors blocks cardiomyocyte production in vitro (Schroeder et al., 2006), and forced Notch expression in mesodermal cardiac progenitor cells expressing Mesp1 during development prevents cardiogenesis and results in cardiac abnormalities in transgenic mice (Watanabe et al., 2006).

The contradiction between the findings postulating a suppressive role of Notch during early cardiac development and our results, which clearly show that Notch activation is essential to promote ICM proliferation, is only apparent. In fact, Notch signaling is known to be exquisitely sensitive to dosage, developmental timing, and cellular context (Artavanis-Tsakonas et al., 1999). It is therefore not surprising that, after mesodermal specification and cardiac commitment, the reactivation of Notch signaling is required to sustain the proliferation of the pool of ICMs still able to undergo mitosis.

Our results demonstrate the existence, both in vitro and in vivo in neonatal animals, of a pool of N1-ICD–positive cells that express early cardiac markers (GATA4 and Nkx2.5) and incorporate BrdU. Loss of the proliferative potential of these cells parallels the progressive disappearance of Notch1, which is consistent with the conclusion that Notch1 might primarily act by preventing terminal differentiation in this expanding proliferating compartment. On the basis of the knowledge currently available on Notch function and our results, we would like to propose the following model (Fig. 6 B). Although Notch1 is essential to sustain ICM proliferation, its levels progressively decrease in vitro parallel to cardiomyocyte differentiation. This determines a progressive withdrawal of differentiated cardiomyocytes from the cell cycle. By a positive feedback regulation, the Jagged1 ligand starts to be expressed on the surface of these cells. At this point, by a lateral induction mechanism, cardiac myocytes themselves behave as signal-sending cells to their stromal counterparts, the proliferation of which is also sustained at later stages of culture by activation of de novo expressed Notch1 itself. This model is also consistent with the observation that the levels of expression of the Notch antagonist Numb increase as long as the ICM proliferation diminishes, thus inversely correlating with the levels of Notch1. An asymmetrical localization of Numb has been described during embryogenesis, where it is
involved in cell fate determination in different tissues (Rhyu et al., 1994; Carmena et al., 1998; Park et al., 1998; Artavanis-Tsakonas et al., 1999; Wakamatsu et al., 1999) and in satellite cell activation during skeletal muscle regeneration (Conboy and Rando, 2002). Moreover, high levels of Notch signaling lead to a reduction of Numb protein, revealing a reciprocal negative regulation between Notch and Numb (Chapman et al., 2006). Our results indicate that this regulation also occurs in mammalian cardiomyogenesis. Whether Numb expression might also play a role in vivo still remains to be determined. In this respect, however, it is intriguing to notice that Numb-positive progenitor cells have been found in the adult mouse heart, lying in defined interstitial structures reminiscent of stem cell niches (Urbanek et al., 2006).

ICM cells showed shortening of the G1 phase, showed low or undetectable levels of the p27Kip1 and p21Cip1 CKIs and of the Cdt1 inhibitor geminin, and expressed cyclins D1, A, and E, an overall profile that is characteristic of cells with a high replicative turnover. In addition, cell treatment with sJ1 further enhanced these features. In contrast, fully differentiated cardiomyocytes expressed abundant levels of CKIs, whereas the G1/S cyclins became almost undetectable. These features are perfectly in agreement with the observations presented in the accompanying paper by Campa et al. (2008), which shows that the forced activation of Notch2 by ICD gene transfer promotes re-entry of cardiomyocytes into the cell cycle but does not bypass a checkpoint that blocks these cells in the G2/M phase.

Our experiments raise the possibility that the Notch pathway might also mediate the expansion of the pool of ICMs through protection from apoptotic cell death. Indeed, we observed that Notch signaling in the regulation of apoptosis in other mammalian systems is not univocal (Mason et al., 2000; Lutolf et al., 2002; Noseda et al., 2004) and might thus be dependent on the differentiation and proliferation state of the cells.

Collectively, in this work, we provide the first evidence that activation of the Notch pathway mediates the expansion of a pool of immature still-dividing cardiac myocytes by promoting their proliferation and protecting them from apoptosis. Our results also show that the in vitro treatment of neonatal cardiomyocyte cultures with an sJ1 agonist, although highly successful in expanding ICM proliferation at earlier stages, becomes totally ineffectual when Notch1 is no longer expressed. These observations are contrary to the possibility that treatment with sJ1 might reverse differentiation but prompt its use for the in vitro expansion of the pool of immature, still-proliferating cardiomyocytes. Thus, sJ1 might turn out to be a powerful growth factor able to expand the dividing cardiac precursor cell population when starting with various sources of cardiac stem cells.

Materials and methods

Constructs, cell lines and antibodies
The insertless pMEXneo and sJ1 constructs, together with stable NIH-3T3 transfecants, were provided by I. Prudovsky (Center for Molecular Medicine, Mainz Medical Center Research Institute, Scarborough, ME) and cultured as described previously (Wong et al., 2000); the pcDNA3-6MyrPlasmid was provided by R. Kapon (Molecular Biology and Pharmacology, Washington University, St. Louis, MO); the Delta1c construct (Wong et al., 1998) was provided by G. Weinmaster (David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA); wild-type (4xIRESdelDelta1-Luc) and mutant (4xIRESdelDelta1-Luc) reporters were provided by S.D. Hayward (John Hopkins University School of Medicine, Baltimore, MD); and the phle1-Luc reporter construct was provided by R. Kageyama (Institute for Virus Research, Kyoto University, Kyoto, Japan).

Anti-Notch-1 (C-20), anti-Jagged1 (H-114), anti-Δ-Alt (F15), anti-cyclin-A (C19), anti-cyclin-D1 (C20), anti-cyclin-E (C19), anti-p27Kip1 (C-19), and anti-GATA-4 antibodies were obtained from Santa Cruz Biotechnology, Inc.; anti-cleaved Notch-1 (Val1744), anti-phospho-p38 MAPK, and anti-phospho-STAT3 (tyr705) were obtained from Cell Signaling Technology; antisera to protein kinase C-α (EA-53) and anti-p21Cip1 antibodies were obtained from Abcam; anti-Numb antibodies were obtained from Millipore; and FITC-coupled anti-βd3 antibodies were obtained from BD Biosciences. Anti-geminin antibodies were a gift from R. Laskey (Hutchinson/Medical Research Council Research Center, Cambridge, UK). BrdU was obtained from Boehringer-Manheim/Roche. Hoechst 33258, PI, RNase A, and all other chemicals were purchased from Sigma-Aldrich.

Production, purification, and characterization of rAAV vectors
rAAV vectors were prepared by the AAV Vector Unit at the International Centre for Genetic Engineering and Biotechnology Trieste (http://www.icgeb.org/RESEARCH/TS/COREFACILITIES/AVU.htm), as described previously (Arsic et al., 2004). In brief, infectious AAV8 vector particles were generated in HEK293 cells by cotransfecting each vector plasmid (pAAV-N1ICD and pAAVLuc2) together with the packaging plasmid (pAAV8-2; Gao et al., 2002) and helper plasmid (pHEPER; Stratagene), expressing AAV and adenovirus helper functions, respectively. Viral stocks were obtained by CsCl gradient centrifugation; rAAV titers, determined by measuring the copy number of viral genomes in pooled, dialyzed gradient fractions, as described previously (Zentilin et al., 2001), were in the range of 1012 to 1013 genome copies per milliliter.

Animals
Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 12, 1987). Wistar rats were purchased from Charles River Laboratories Italia Srl and maintained under controlled environmental conditions. When indicated, animals were treated for 2 d with 20 mg/kg of BrdU before killing.

Culture of neonatal rat cardiomyocytes
Neonatal rat ventricular myocytes were plated at low density in DMEM/4.5 g glucose containing 5% bovine calf serum and 2 mg/ml vitamin B12 and cultured as described previously (Deng et al., 2000). In brief, ventricles from 2–3 litter of neonatal Wistar rats were separated from the atria using scissors and then dissociated in C8I1H (calcium and bicarbonate-free Hanks with Hepes) buffer containing 2 mg/ml of trypsin and 20 μg/ml of DNase II. Cells were preplated for 2 h into 100-mm dishes in DMEM containing 5% defined bovine calf serum (HyClone); Stromal cells were then separated from unattached myocytes that were plated at a density of 500 cells/mm2 into 60-mm dishes (for protein signaling, cytchemistry, RNA, and protein analysis; Falcon), multi-well plates (for biochemical assays; Costar) or multiwell slides coated with 0.2% gelatin (for immunofluorescence staining). For RT PCR and Western blot analyses, three subsequent 1-h-long preplating steps were performed in order to strongly enrich the cell preparations in cardiomyocytes (~90% purity). For the experiments involving cardiomyocyte transduction using AAV vectors, neonatal rat cardiomyocytes, immediately after plating onto 4-well slides coated with 0.2% gelatin at a density of 5x104 cells per well, were infected with 2x105 genome copies per well. After 12 h, the culture medium was changed and cells were subjected to the different treatments and subsequent analyses.

RT-PCR analysis
Total mRNA was purified from cultured cardiomyocytes at 1, 3, or 7 d after plating. mRNA was reverse-transcribed using SuperScript II (Invitrogen) with random hexamers (10 μM) as a primer in a 35-μl reaction containing 1x Superscript II RT buffer (Invitrogen), 10 μM dATP, dGTP, dCTP, and dTTP, and 20 U RNasin (Invitrogen). After 2 h at 42°C, the reaction was terminated by adding 365 μl of H2O and boiling for 2 min. For PCR amplification, specific primer pairs (0.5 μM each) were incubated with 50 ng cDNA and 1 U Herculase enhanced DNA polymerase (Stratagene) in a 20-μl reaction mixture that included 1x buffer and 100 μM dATP, dTTP, dCTP, and dGTP. Typical cycle parameters were 1 min at 92°C, 1 min at 55°C, and 3 min
at 72°C for 20 cycles, followed by a cycle at 72°C for 7 min. The whole reaction was then fractionated on 1.2% agarose gel and the PCR product was visualized by ethidium bromide staining.

The primers for RT-PCR analysis were as follows: Notch1 forward primer, 5'-GCCGcccACAGAACTcAACCAcTtg-3', and reverse primer, 5'-TAAgCTGTTACgGATGtgGATGAt-3' (445-bp amplified fragment); Notch2 forward primer, 5'-cGTTcGcGCTAGcGcTGGcGcA-3', and reverse primer, 5'-gAgGGAAGTGGGAgGAAcGAAcGAtGctGcGcATG-3' (505 bp); Notch3 forward primer, 5'-TTCGACgGCTTACcAGGGcAA-3', and reverse primer, 5'-TcGtGcGCTAGcGcTGGcGcA-3' (480 bp); Notch4 forward primer, 5'-cGCcACGAgGcTtgGttGACGAtGCTG-3', and reverse primer, 5'-gAgGGAAGTGGGAgGAAcGAAcGAtGctGcGcATG-3' (397 bp); Hes1 forward primer, 5'-gGgGcGcGgGcCAGAGCAGC-3', and reverse primer, 5'-gAgGGAAGTGGGAgGAAcGAAcGAtGctGcGcATG-3' (302 bp); Hes5 forward primer, 5'-AAGGcCTcAGCAGcCAGCAG-3', and reverse primer, 5'-cGTTcGcGCTAGcGcTGGcGcA-3' (352 bp); Hey1 forward primer, 5'-AggGcCTcAGCAGcCAGCAG-3', and reverse primer, 5'-gAgGGAAGTGGGAgGAAcGAAcGAtGctGcGcATG-3' (352 bp); Hey2 forward primer, 5'-cGCcACGAgGcTtgGttGACGAtGCTG-3', and reverse primer, 5'-tGTCgGcGCTAGcGcTGGcGcA-3' (178 bp); Numb forward primer, 5'-AACAcCTCAGCAGCAGCAG-3', and reverse primer, 5'-TgAgGCCTGgGgGACCCGACcC-3' (266 bp); and GapDH forward primer, 5'-ATGTCGACAGACTGACTTGTCCG-3', and reverse primer, 5'-AgGgCCTGGGTtGCCTGCCT-3' (220 bp). In all experiments, RT-PCR was performed on total embryo RNA as a positive control [+].

Western blotting

For protein analysis, samples were lysed in RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NaDoc, 1% Triton X-100, 0.1% SDS) containing 0.2% SDS and 0.5% PFA and 2% sucrose for 10 min; aldehydes were quenched with 0.1 M glycine in PBS for 15 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 5 min, blocked in 2% BSA and 0.05% sodium azide in PBS (blocking buffer) for 1 h, and incubated with primary antibodies from 2 h to overnight. Alexa Fluor 488– or Alexa Fluor 594– conjugated secondary antibodies (Invitrogen) were incubated for 30 min to 1 h at room temperature. All washes were in PBS 0.2% Tween 20. Cell nuclei were stained with DAPI, and samples were mounted in Vectashield (Vector Laboratories) with ProLong Gold (Invitrogen). For histological analysis, frozen sections (5-μm thick) were fixed in cold acetone (~20°C) and blocked for 30 min with 5% goat or horse serum in PBS, depending on the secondary antibody. Primary antibodies were used at the following dilutions: biotinylated secondary antibodies were incubated for 30 min to 1 h at room temperature. Western blotting

Luciferase assays

To detect Notch1 activity under the different experimental conditions, neona-
tal cardiomyocytes were seeded onto 0.2% gelatin-coated 24-well culture plates [5 × 10^4 cells per well] and cotransfected at the stated time points with 0.2 μg of reporter plasmid and 0.01 μg pRL-Renilla (which was used as a positive control). 1 ml of cold 70% ethanol, and centrifuged (1,500 × g, 10 min). Cell pellets were resuspended in 0.5 ml of 1X PBS containing 0.2 mg/ml RNase-A.
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