Notch1 regulates the fate of cardiac progenitor cells

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The Notch receptor mediates cell fate decision in multiple organs. In the current work we tested the hypothesis that Nkx2.5 is a target gene of Notch1 and raised the possibility that Notch1 regulates myocyte commitment in the adult heart. Cardiac progenitor cells (CPCs) in the niches express Notch1 receptor, and the supporting cells exhibit the Notch ligand Jagged1. The nuclear translocation of Notch1 intracellular domain (N1ICD) up-regulates Nkx2.5 in CPCs and promotes the formation of cycling myocytes in vitro. N1ICD and RBP-Jk form a protein complex, which in turn binds to the Nkx2.5 promoter initiating transcription and myocyte differentiation. In contrast, transcription factors of vascular cells are down-regulated by Jagged1 activation of the Notch1 pathway. Importantly, inhibition of Notch1 in infarcted mice impairs the commitment of resident CPCs to the myocyte lineage opposing cardiomyogenesis. These observations indicate that Notch1 favors the early specification of CPCs to the myocyte phenotype but maintains the newly formed cells in a highly proliferative state. Dividing Nkx2.5-positive myocytes correspond to transit amplifying cells, which condition the replicative capacity of the heart. In conclusion, Notch1 may have critical implications in the control of heart homeostasis and its adaptation to pathologic states.

Notch1 and CPC Commitment. In freshly isolated CPCs, Notch1 was found to be expressed in nearly 60% of the cells (Fig. 1 G and H). Notch1 was restricted to CPCs negative for the transcription factor GATA4 (Fig. 1 F, which was used as a marker of cell commitment (11). Conversely, GATA4 was detected in 20% Notch1-negative CPCs. The Notch2–4 receptors were present in ~10% of CPCs (Fig. 1 J), pointing to Notch1 as the major Notch isoform in cardiac progenitors.

To characterize the effects of Notch1 on cell differentiation in vitro, CPCs were analyzed at baseline [see supporting information (SI) Fig. S1] and subsequently were exposed to immobilized Jagged1 in the presence or absence of a γ-secretase inhibitor; γ-secretase cleaves Notch1 and releases N1ICD, activating transcription of Notch1-dependent genes (12). Because Hes1 is a target gene of Notch1, its expression was used to document the activation or inhibition of Notch1. Jagged1 resulted in a time-dependent increase in Hes1 transcript whereas γ-secretase inhibition attenuated but did not abrogate Hes1 expression (Fig. 2 A). The short half-life of the γ-secretase inhibitor may have prevented the complete repression of Notch function.

The localization of N1ICD in CPCs was measured at 2, 5, and 8 days with an antibody that recognized only the cleaved form of Notch1. Loss of Notch1 extracellular domain resulted in translo-

The Notch pathway is an evolutionary conserved intercellular and intracellular system that controls stem cell fate (1). The Notch gene family encodes transmembrane receptors that are activated by ligands and proteolytic cleavage with release of the Notch intracytoplasmic domain (NICD). To date, four Notch isoforms have been identified (1). Upon translocation to the nucleus, NICD forms a complex with the DNA recombinant binding protein RBP-Jk, which then loses its repressor function and initiates transcription (2). Targets of NICD/RBP-Jk include the Hes family of proteins, which are transcriptional repressors of Notch-dependent genes (3). Thus, Notch modulates transcription in several cell types, but whether it exerts an inhibitory or inductive role in the growth and differentiation of cardiac progenitor cells and myocardial homeostasis is unknown.

Notch ligands are membrane-bound proteins, and Notch activation links the fate decision of one cell to that of its neighboring cell through lateral inhibition or inductive interaction (1). The effects of Notch on the maintenance of stemness or initiation of differentiation are context- and time-dependent (1, 2). Notch signaling ensures a proper balance between a stem cell and its progeny and 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 (4). Similarly, the effects of Notch on bone marrow cells vary according to the state of maturation of the stimulated cells. Synthesis of the Notch ligand Jagged1 by osteoblasts increases the number of hematopoietic stem cells in bone marrow niches (5). Conversely, Notch activation in hematopoietic progenitors induces T lymphocyte formation (2). In the brain, Notch expands the pool of immature precursors and enhances glial formation (6).

Notch regulates transcription of Mash1, MyoD, and GATA2, which are the master genes of neurogenic, myogenic, and hematopoietic stem cell commitment, respectively (7–9). The equivalent gene in the heart has not been identified, and differentiation of cardiac progenitors into myocytes is mediated by the coordinated function of several transcription factors. However, Nkx2.5 has been viewed as the early determinant of myocyte formation in development (10). Here we tested the hypothesis that Nkx2.5 is a target gene of Notch1 and raised the possibility that Notch1 is the key regulator of myocyte differentiation in the adult heart.

Results

c-kit and Notch1 Receptor. In the mouse heart, c-kit-positive cardiac progenitor cells (CPCs) are organized in niches located preferentially in the atria and apex (11). Niches contain Notch1-positive CPCs, which are nested in the interstitium and are connected to adjacent myocytes by connexin 43 and N-cadherin (Fig. 1 A–D). The Notch ligand Jagged1 is present in proximity of Notch1-positive CPCs, at the interface between CPCs and between CPCs and myocytes, which function as supporting cells (Fig. 1 E). Conversely, Delta-like4 ligand is distributed predominantly in coronary vessels (Fig. 1 F).

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cation of N1ICD to the nucleus. In contrast, N1ICD was absent in cells that displayed Notch1 on the plasma membrane (Fig. 2B). In the presence of Jagged1, CPCs positive for Notch1 extracellular domain decreased dramatically from 2 to 5 days and remained low at 8 days (Fig. S2).

To define the function of Notch1 in the differentiation of CPCs in myocytes, the expression of N1ICD, Nkx2.5, and GATA4 was determined. At baseline, Nkx2.5 coexpressed consistently with N1ICD, and Jagged1 markedly enhanced this colocalization in CPCs at 5 and 8 days (Fig. 2C–G). With Jagged1, the up-regulation of N1ICD at 2 days was not accompanied by a comparable response of Nkx2.5. Because the induction of these two proteins was essentially identical at 5 and 8 days, this observation suggests that the transcriptional regulation of Nkx2.5 in CPCs may depend partly on Notch1 activation. However, we cannot exclude that a transient up-regulation of N1ICD independent of Nkx2.5 occurred at an earlier time point. Importantly, Notch1 inhibition abrogated the effect of Jagged1 on Nkx2.5 at 5 and 8 days.

Notch activation had a negative impact on GATA4; the number of GATA4-positive CPCs increased with γ-secretase inhibition pointing to a repressive role of Notch1 in the transcription of GATA4 (Fig. 2H–J). These results were confirmed by quantitative RT-PCR (Fig. 2K). Although GATA4 transcript increased with time under control conditions, CPCs exposed to Jagged1 continued to show a lower level of GATA4 up to 8 days (Fig. S3). This finding is consistent with the progression of CPC commitment toward a more mature phenotype and with the inhibitory function of Notch1 in late CPC differentiation.

**Cardiac Cell Differentiation.** The recognition that Notch1 is linked to Nkx2.5 and myocyte commitment raised the question of whether Notch1 represses or promotes the differentiation of CPCs in endothelial cells (ECs) and smooth muscle cells (SMCs). The expression of the EC transcription factor Vezf1 and the SMC transcription factor GATA6 was determined in CPCs. In the presence of Jagged1, at 5 days, Vezf1 and GATA6 mRNA decreased significantly in CPCs, and this effect was reversed by γ-secretase inhibition (Fig. 3A). As GATA4, Vezf1, and GATA6 increased markedly in untreated cells at 8 days, Jagged1 attenuated the up-regulation of vascular markers (Fig. S3).

To determine whether Notch1 activation and myocyte commitment reduced the replicative potential of CPCs, the effect of Jagged1 on cell division was evaluated at 5 days when the peak of Nkx2.5-positive cells was detected. With respect to untreated CPCs, Jagged1 produced a 2.2-fold increase in CPC proliferation from 9% to 48% at 5 days. Additionally, 58% of Nkx2.5-positive cells was cycling (Fig. 3B–D). Collectively, our observations support the notion that Notch1 regulates the transition of CPCs from the immature phenotype to the compartment of amplifying myocytes (Fig. 3 B–F). This cell category has the ability to divide and simultaneously differentiate (13).

**RBP-Jk and the Nkx2.5 Promoter.** The mechanism by which Notch1 regulates CPC differentiation into myocytes is unclear. A binding site for RBP-Jk may be present in the Nkx2.5 promoter, and Notch1 activation may lead to the formation of a protein complex between N1ICD and RBP-Jk. The repressive activity of RBP-Jk would then be inhibited initiating Nkx2.5 transcription. To test this hypothesis, fibronectin (D, yellow) and collagen (D, light blue). (E) Jagged1 (white, arrows) is present between CPCs (c-kit, green) and myocytes. (F) Delta-like4 (yellow) is detected in the wall of a small coronary vessel (α-smooth muscle actin, α-SMA, magenta). (G) Dot plots showing the distribution of c-kit and Notch1 in CPCs. (H) Cytospin of FACS-sorted CPCs illustrating the coexpression of c-kit (green) and Notch1 (red). (I) CPCs (c-kit, green) that are positive for Notch1 (red) do not express GATA4. Two CPCs that are negative for Notch1 express GATA4 (white dots, arrows). (J) Distribution of Notch1–4 isoforms in CPCs.
Western blotting for N1ICD and its target gene Hes1 was performed in untreated and Jagged1-treated CPCs. The level of N1ICD and Hes1 was 1.4-fold and 2.0-fold higher in Jagged1-treated than in untreated CPCs, respectively. Then, cell lysates were immunoprecipitated with an antibody against N1ICD and subjected to Western blotting for RBP-Jk. This pull-down assay documented that N1ICD and RBP-Jk generated a complex in untreated CPCs that was 4-fold greater than in untreated cells (Fig. 3G and Fig. S4).

To determine whether the Nkx2.5 promoter contained a functional binding site for RBP-Jk, studies were performed in CPCs and in the embryonic cell line P19, which has been used for the analysis of gene expression and myocyte differentiation (14). A perfect consensus site for RBP-Jk in the Nkx2.5 promoter was identified at position −2083/−2089. To establish whether RBP-Jk and the promoter of Nkx2.5 form a protein–DNA complex, P19 cells were transfected with a plasmid expressing RBP-Jk in the absence of Jagged1. Nuclear extracts from nontransfected and transfected P19 cells were then used in an electrophoretic mobility shift assay. An oligonucleotide of 17 bp, including the binding site for RBP-Jk, was used as a probe, and a shifted RBP-Jk band was found (Fig. 3H).

We performed a gene reporter assay in P19 cells transfected with a β-Gal reporter construct controlled by the Nkx2.5 promoter. Cells were also cotransfected with an expression plasmid carrying constitutively active N1ICD. Despite the high level of expression of N1ICD, β-Gal activity was not detected, suggesting that the N1ICD/RBP-Jk complex alone did not promote Nkx2.5 transcription (Fig. S5). Because transcription of Nkx2.5 in P19 cells requires the physical interaction of Nkx2.5 promoter with GATA4 and Smad4 (14), these cells were transfected with N1ICD, GATA4, and Smad4 expression plasmids in various combinations. Maximal Nkx2.5 promoter activity was achieved with the three plasmids, which led to a 3-fold increase in β-Gal activity (Fig. S5).

The requirement of GATA4 and Smad4 as cofactors for the transcriptional activation of Nkx2.5 in P19 cells was not consistent with the results obtained in CPCs in which the expression of Nkx2.5 was not coupled with GATA4 up-regulation. CPCs were then transfected with reporter plasmids carrying the wild-type Nkx2.5 promoter or an Nkx2.5 promoter in which the RBP-Jk binding site was mutated in a single nucleotide. Reporter gene activity was ~3-fold higher in CPCs transfected with the wild-type promoter than with its mutated form (Fig. 3K). Importantly, Nkx2.5 transcription in CPCs did not necessitate cotransfection with GATA4 and Smad4. We cannot exclude that noncanonical effects of
Jagged1 on RBP-Jk-independent pathways may contribute to Nkx2.5 transcription in CPCs, although depressed Nkx2.5 activation with a mutated promoter documents that RBP-Jk is crucial for Nkx2.5 expression in these cells.

**Notch1 Function in Vivo.** Our *in vitro* results suggested that Notch1 signaling could have significant implications in the myocardial response to infarction. To test this hypothesis, a γ-secretase inhibitor was administered i.p. to mice for 3 days before coronary artery ligation. At surgery, the γ-secretase inhibitor was also injected intramyocardially, and mice were continuously treated for 9 days up to the time they were killed. Control mice were injected with vehicle. Both groups of infarcted mice were exposed to BrdU. Individual and small clusters of CPCs were detected in the viable myocardium of the border zone (Fig. 4A and Fig. S6). Jagged1 was distributed in the same region adjacent to myocytes and CPCs (Fig. 4B). The nuclear localization of N1ICD in CPCs was accompanied by the presence of Nkx2.5 (Fig. 4C and Fig. S7); this coexpression was significantly attenuated by γ-secretase inhibition. Notch blockade reduced the number of CPCs and the fraction of CPCs positive for N1ICD and Nkx2.5 in the border zone (Fig. 4D). Furthermore, to clarify the role of Notch1 in CPC fate, the extent of cardiomyogenesis in the border zone was measured. BrdU-positive myocytes showed invariably a nuclear localization of N1ICD. In control infarcted mice, several regenerated myocytes were identified whereas the generation of new myocytes was 50% lower in animals exposed to γ-secretase inhibition (Fig. 4 E–G and Fig. S8).

Clusters of newly formed myocytes together with small bands of myocardial regeneration were detected in the infarcted region of untreated mice (Fig. 5A–H). These sites of cardiomyogenesis were absent in mice exposed to γ-secretase inhibition. These molecular and cellular modifications were associated with better indices of ventricular function, which, however, did not reach statistical significance (Fig. 5I). This may be because of the short time period after infarction examined here or the modest amount of spontaneous tissue repair present after ischemic injury (15, 16). However, attenuation of myocyte regeneration may have dramatic effects on chronic ventricular remodeling of the infarcted heart (17).

**Discussion**

The results of the present study document that activation of Notch1 favors the commitment of CPCs to myocytes and regulates the compartment of transit amplifying myocytes *in vitro* and *in vivo*. This molecular control may correspond to a model of differentiation delay in which sustained up-regulation of Notch1 prolongs the amplifying state of CPC-derived myocytes and prevents terminal differentiation and growth arrest. Notch1 function involves the expression of Nkx2.5, which represents a novel target gene of Notch1. Nkx2.5 is an early event in the growth of embryonic expression of Nkx2.5, which favors the commitment of CPCs to myocytes and regulates the compartment of transit amplifying myocytes and CPCs (10), but its role in the postnatal heart remained unclear. Our findings indicate that Nkx2.5 drives CPCs in the acquisition of the myocyte lineage, possibly influencing the activation or repres-
sion of the complex transcriptional network that modulates myocyte formation and tissue homeostasis in the adult heart (10).

In progenitor cells Notch is generally linked to the preservation of the primitive phenotype (1, 5, 6), although it promotes the maturation of stem cells in the basal layer of the skin and satellite cells of the skeletal muscle (8, 18). Jagged1 stimulation of Notch1 in CPCs up-regulates Nkx2.5 and favors the acquisition of the myocyte phenotype. Nkx2.5 does not interfere with the capacity of the cells to proliferate. Developing myocytes express N1ICD, have a thin layer of sarcomeric proteins, and are consistently labeled by BrdU or Ki67; these properties define the compartment of transit-amplifying myocytes, which are short-lived cells with low self-renewal capacity and high differentiation potential (13). This cell pool increases the number of daughter cells resulting from stem cell division; this growth effect ensures tissue homeostasis, allows stem cells to replicate rarely, and protects the stem cell compartment from depletion. Our data after myocardial infarction are consistent with this possibility because Notch1 inhibition decreased the population of forming myocytes. Successive rounds of Notch1 inhibition and activation may result in a controlled exodus of CPCs from the undifferentiated to the committed state. The expression of Notch1 on the plasma membrane in the absence of the nuclear translocation of its intracellular domain may reflect a permissive state in which the multipotentiality of CPCs is retained. Additionally, Notch1 enhances myocyte survival (19) and is implicated in the transdifferentiation of endothelial progenitor cells into myocytes via an Nkx2.5-independent mechanism (20).

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 crucial role that Notch1 has in the regulation of myocyte turnover in the adult heart. In this regard, the expression of multiple cardiac transcription factors and contractile and myocyte membrane proteins is reduced in Nks2.5-null mouse embryos (21). These data indicate that Nks2.5 is critical for the transcriptional regulation of the cardiac gene network during development (10) and, as shown here, in the adult heart. Up-regulation of Nks2.5 has been interpreted as part of the induction of the fetal gene program in the overloaded heart (22). However, Nks2.5 expression may reflect the activation of CPCs and myocyte formation rather than the synthesis of fetal proteins in postmitotic cardiomyocytes. This homeobox gene is not sufficient to promote an increase of myocyte size with cardiac hypertrophy (23).

The recognition that Jagged1-mediated Notch1 signaling in CPCs induces myocyte commitment but interferes with the acquisition of the EC and SMC lineage is a model of selective specification of cell fate that mimics the function of Notch in other stem-cell-regulated organs (1, 2). Notch may act as a lineage switch between myocytes and vascular cells by positively regulating Nkx2.5 and negatively influencing the genes required for vasculogenesis. Down-regulation of GATA6 in differentiating CPCs is consistent with repression of transcription induced by the Notch-dependent genes Hey1 and Hey2 on the GATA family of proteins (24). A similar mechanism may be operative for the EC transcription factor Vezf1 and GATA4.

The reciprocal relationship between Nks2.5 and GATA4 is not defined. The Nks2.5 promoter/enhancer region is complex and contains binding sites for multiple transcription factors including GATA4 (10). In the embryonic heart, the expression of Nks2.5 and GATA4/5/6 largely overlaps, making it difficult to establish whether GATA4/5/6 lie upstream of Nks2.5 (25). The positive

**Fig. 4.** Notch1 function in vivo. Border zone of untreated (A–C and E) and γ-secretase inhibitor-treated (F) infarcts in which the areas included in rectangles are shown at higher magnification in the Insets. (A) CPCs (c-kit, green, arrows) express Notch1 (yellow). Red, tropomyosin. (B) Jagged1 (white, arrows) is expressed in CPCs and between CPCs and myocytes (γ-H9251-βA, red). (C) CPCs express N1ICD (magenta, arrow) and Nkx2.5 (yellow). (D) Number of CPCs and percentage of CPCs positive for N1ICD and Nkx2.5 in the border zone of untreated and treated infaracts. *P < 0.05 vs. untreated. (E and F) Newly formed BrdU-positive myocytes (white, arrows) in the border zone of untreated (E) and treated (F) infarcts. (G) γ-Secretase inhibition decreases the fraction of BrdU-labeled myocytes. *P < 0.05 vs. untreated.
effect of GATA4 on Nkx2.5 has been documented in vitro in cell systems that may have limited relevance to cardiomyogenesis (14, 26). Nkx2.5 transactivation in CPCs is independent of GATA4, systems that may have limited relevance to cardiomyogenesis (14, 15).

Materials and Methods

CPCs were isolated from the mouse heart by enzymatic digestion and sorted for c-kit. CPCs were expanded and exposed to Jagged1 and Nkx2.5. CPCs were expanded and exposed to Jagged1 and γ-secretase inhibitor was administered in vivo to infarcted mice, and the extent of myocardial regeneration was evaluated 9 days later. Protocols are described in SI Materials and Methods.

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