Induction of Cardiogenesis in Embryonic Stem Cells via Downregulation of Notch1 Signaling

Mohamed Nemir, Adrien Croquelois, Thierry Pedrazzini, Freddy Radtke

Abstract—Embryonic stem cells represent an attractive source of cardiomyocytes for cell-replacement therapies. However, before embryonic stem cells can be successfully used for the treatment of cardiac diseases, the precise molecular mechanisms that underlie their cardiogenic differentiation must be identified. A network of intrinsic and extrinsic factors regulates embryonic stem cell self-renewal and differentiation into a variety of different cell lineages. Here, we show that Notch signaling takes place in some but not all embryonic stem cells and that the Notch pathway is shut down during the course of differentiation concomitantly with downregulation of Notch receptor and ligand expression. Moreover, gain- and loss-of-function experiments for Notch signaling components show that this pathway is a crucial regulator of cardiomyocyte differentiation within ES cells. Differentiation of ES cells into cardiomyocytes is favored by inactivation of the Notch1 receptor, whereas endogenous Notch signaling promotes differentiation of ES cells into the neuronal lineage. We conclude that Notch signaling influences the cell fate decision between mesodermal and the neuroectodermal cell fates during embryonic stem cell differentiation. These findings should help to optimize the production of specific cell types via modulation of the Notch pathways and, in particular, to improve the production of embryonic stem cell-derived cardiomyocytes. (Circ Res. 2006;98:0-0.)

Key Words: Notch • embryonic stem cells • cardiomyogenesis • differentiation • gene targeting

Heart failure has become the leading cause of death in developed countries. Hundreds of thousands of new cases are diagnosed each year, and despite a large battery of pharmacological agents, heart transplant remains the ultimate therapy for patients with end-stage heart failure. However, the request for organs far exceeds the number of potential donors. As an alternative approach, the regeneration of the myocardium via controlled differentiation of cardiomyocyte progenitors is receiving much attention. Embryonic stem (ES) cells demonstrate several characteristics that suggest that they might serve as a source of cells for the therapeutic regeneration of the heart. First, ES cells can be readily isolated from the inner cell mass of the blastocyst and subsequently maintained indefinitely in vitro. Second, ES cells are totipotent and can be induced to differentiate into a variety of cell types including cardiac myocytes. Third, the recent generation of human ES cell lines has brought further support to the concept of regenerative medicine based on the controlled differentiation of ES cells to replace lost cells in damaged organs. Nevertheless, because of their totipotency, ES cells could paradoxically represent a possible risk of producing teratomas following transfer in vivo. Therefore, knowledge of the precise control of the necessary differentiation processes will be required before ES cells can be used in therapy.

The most commonly used method to induce differentiation of mouse ES cells is the formation of embryoid bodies (EBs), which contain precursors of all 3 embryonic germ layers. Indeed, within EBs, ES cells differentiate into spontaneously beating cardiomyocytes that demonstrate structural and electrophysiological characteristics identical to those observed in cardiomyocytes of the developing heart. Induction of cardiogenesis appears to be, in part, under the control of morphogens of the transforming growth factor (TGF)-β family, such as TGF-β itself, nodal, activin, and bone morphogenetic proteins (BMPs). BMP-dependent pathways, in turn, activate specific transcription factors including Nkx2.5, GATA-4, and mouse embryonic fibroblast (MEF)-2c, which activate cardiac-specific genes such as the α-mysin heavy chain (α-MHC) and the myosin light chain (MLC)-2v. In contrast, totipotency is maintained by culturing ES cells on a feeder layer of MEFs or in the presence of leukemia inhibitory factor (LIF). Two transcription factors, Oct3/4 and Nanog, have been classified as ES cell identity genes, as they are critical for the self-renewal of ES cells. The Notch pathway is involved in many differentiation processes and lineage decisions in fetal and postnatal development, as well as in adult self-renewing organs. Notch receptor genes encode evolutionarily conserved transmembrane proteins, which are activated by 2 types of cell surface...
ligands, named Jagged and Delta. Mouse and humans have 4 Notch receptors (Notch1 to -4) and 5 ligands: Jagged1, Jagged2, Delta-like 1 (Dll-1), Dll-3, and Dll-4.19,20 Signaling is initiated via ligand–receptor interactions on neighboring cells. This interaction leads to the proteolytic cleavage of the receptor, a process that releases the Notch intracellular domain (NIC), which then translocates to the nucleus and heterodimerizes with the transcription factor RBP-Jk, converting it from a transcriptional repressor to an activator.19 Notch signaling plays an important role in the cellularization of the heart during cardiac development, and mice in which the canonical Notch pathway is inhibited die during embryonic life, in part, from heart defects.22 Interestingly, the blockade of the Notch pathways in ES cells was recently shown to favor commitment to a mesodermal cell fate.23 Therefore, in an attempt to define the molecular events controlling the production of ES cell–derived cardiomyocytes, we investigated the ability of the Notch pathway to control ES cell cardiogenic differentiation. We report that Notch signaling mediated by the Notch1 receptor is responsible for suppressing cardiogenesis in ES cells. Notch does not appear to be a general inhibitor of ES cell differentiation but functions as a regulator of cell fate decisions in multipotent ES cells that must choose between mesodermal and neuroectodermal fates.

Materials and Methods
Cell Culture
In this study, Notch1−/− ES cell clones and the parental Notch1lox/lox cell line, the E14/T, and the 293T cell lines were used. The culture conditions, transfections, and differentiation protocols are described in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org. Details of cloning and expression of DNA constructs, immunofluorescence, fluorescence-activated cell sorting (FACS), Western blot, and RT-PCR analyses are also described therein.

Results
Notch Signaling Is Downregulated During ES Cell Differentiation
To explore the functions of Notch signaling in ES cells, expression of the different Notch receptors and ligands was determined in undifferentiated ES cells as well as in EBs that exhibited spontaneous contraction, an indication of cardiomyocyte differentiation. Undifferentiated ES cells expressed relatively high levels of Notch1 and Notch4, whereas Notch2 was expressed to a lesser extent (Figure 1). Notch3 did not appear to be expressed (data not shown). Among the Notch ligands, Jagged1 and Dll-1 were expressed at high levels, whereas Jagged2 was expressed at high levels, whereas Jagged1 and Dll-1 were only detected at moderate levels (Figure 1). During ES cell differentiation Notch1, Notch4, and Jagged2 expression appeared to be downregulated similar to that of Oct3/4 and Nanog (Figure 1). In contrast, expression of the other Notch family members such as Notch2, Jagged1, or Dll-1 was not significantly altered. Similarly, no differences in the expression of putative target genes of Notch pathways, such as Hes1, Hey-1, and Hey-2, were observed in undifferentiated or differentiated ES cells, indicating that regulation of these target genes is independent of Notch signaling or mediated by Notch2, the only receptor that is not down regulated during differentiation (Figure 1).

To visualize Notch signaling in ES cells, a reporter gene expressing the red fluorescent protein DsRed under the control of 12 Notch responsive RBP-J DNA binding elements was constructed (pCAGIP-12RBP-DsRed). The specificity of this reporter was first assessed by cotransfection experiments in 293T cells using constructs encoding a constitutively active form of the Notch1 receptor (NIC, pCAGIPNIC) and a dominant negative mutant of the RBP-J protein lacking DNA binding activity (RBP-JDN, pCAGIPRBP-JDN; Figure 2A). Figure 2B shows that 293T cells display low levels of Notch signaling under basal conditions. In contrast, DsRed fluorescence markedly increased with the constitutive activation of the Notch pathway. Finally, reporter expression was totally shut down after cotransfecting the dominant negative RBP-JDN gene. Taken together, these results demonstrate that the DsRed reporter system is Notch responsive and specific.

Next, we transfected this reporter plasmid into ES cells. Representative pictures of undifferentiated ES cell colonies growing in the presence of LIF and of early EBs and a terminally differentiated EBs are shown in Figure 3A. The punctuate expression pattern of DsRed in undifferentiated ES cell colonies and in early EBs indicates that Notch signaling...
occurred only in small, defined areas. Immunostaining for Notch1-IC also demonstrated a dotted staining pattern in wild-type ES cells, consistent with the results obtained with the Notch-DsRed reporter system (Figure 3C). Furthermore, in agreement with the expression data depicted in Figure 1, terminally differentiated EBs showed little or no fluorescence, indicating that Notch signaling is shut down during differentiation. To ascertain that the DsRed cells were indeed receiving a Notch signal, fluorescent (DsRed cells) and nonfluorescent (DsRed cells) were FACS sorted and analyzed for the expression of Notch1, which is itself a direct target gene of Notch signaling. Figure 3B shows that the DsRed cells receiving a Notch signal expressed higher levels of Notch1 mRNA compared with DsRed ES cells, confirming that activation of the reporter is a valid readout of Notch signaling. Interestingly, the expression of Hes1, and Hey-2, but not Hey-1 was moderately increased in DsRed relative to DsRed ES cells (Figure 3B), suggesting that these genes are only partially regulated by Notch in ES cells.

**Figure 2.** Characterization of Notch reporter system. A, Western blot analysis showing NIC-myc and RBP-JDN protein expression in HEK 293T cells transfected with the indicated combinations of CAGIP-NIC-myc and CAGIP-RBP-JDN plasmids, in addition to the Notch reporter p12RBPDsRed and the pEGFPN3 plasmid, used as a control for transfection efficiency. B, Representative micrographs of HEK 293T cells transfected as in A. The bottom panels with red fluorescence show cells with Notch reporter activity, whereas the top panels with green fluorescence show equal transfection efficiency. Scale bar=50 μm.

**Figure 3.** A, The Notch reporter plasmid pCAG-12RBPDsRed was transfected into E14/T ES cells that were selected with puromycin and photographed 48 to 96 hours posttransfection. E14/T cells were grown on gelatin or on MEFs, as indicated. Top row, right, shows transfected cells that were photographed at high magnification to indicate alternating DsRed and DsRed cells representing Notch signaling and responding cells, respectively. B, RT-PCR analysis of Notch target genes in signaling and responding cells. Transfected ES cells were fluorescence-activated cell sorted based on DsRed fluorescence, and RT-PCR analysis was preformed on DsRed and DsRed cells as indicated. Scale bars=100 μm; scale bar in inset in A, 25 μm. C, Immunostaining for activated NIC on wild-type and Notch1 ES cells grown on either gelatin or MEFs.

**Inhibition of Notch Signaling in ES Cells Favors Cardiomyocyte Differentiation**

To investigate whether Notch signaling can control the differentiation of ES cells into particular cell lineages, we modulated the levels of Notch signaling in differentiating ES cells by transfecting either the RBP-JDN inhibitory protein or the NIC constitutively active domain into ES cells. Quantitative analysis showed that 40% of the EBs expressing the RBP-JDN protein showed spontaneous beating activity as early as 24 hours after plating on gelatin (day 7) compared with only 10% in controls (Figure 4 A). After 4 days, the beating activity was observed in nearly 100% of the RBP-JDN expression EBs and only in 50% to 60% of controls (Figure 4A). In additional experiments, we tested whether forced Notch signaling would inhibit cardiogenesis. As shown in Figure 4B, forced expression of the NIC construct significantly inhibits differentiation of ES cells into cardiomyocytes. To determine whether Notch-mediated inhibition preferentially affected the cardiac lineage or blocked commitment toward a mesodermal fate, a time-course experiment was performed to quantify the expression of 2 mesodermal markers, Brachyury and fibroblast growth factor (FGF)-8, in wild-type EBs as well as in NIC-expressing, RBP-JDN-expressing (Figure 4C and 4D), and Notch1-deficient EBs (supplemental Figure I). Inhibition of Notch signaling or loss of Notch1 function resulted in upregulation of Brachyury and FGF-8 expression. In contrast, forced Notch-IC expression produced inhibition of Brachyury and FGF-8 expression (Figure 4C and 4D). Taken together, these data indicated that Notch signaling operated in mesodermal progenitors.
Notch Signaling and Cardiomyocyte Differentiation Are Mutually Exclusive

The experiments described above suggest that the activation of Notch signaling pathways is incompatible with commitment to a cardiac fate. To further address this question, we cotransfected ES cells with the Notch reporter as well as with a cardiac-specific reporter plasmid expressing EGFP gene under the control of the cardiac-specific α-MHC promoter. Figure 4D shows that in early EBs, Notch signaling (red fluorescence) and cardiac differentiation (green fluorescence) occurred in distinct areas and never colocalized, indicating that these 2 events cannot occur in the same cell simultaneously. To exclude the possibility that the ES cells had lost the episomal reporter constructs during differentiation, EGFP+ and DsRed+ cells were sorted from primary EBs and assessed for their ability to express the reporters in secondary EBs. Both EGFP+ and DsRed+ cells were able to form secondary EBs showing green and red fluorescence, respectively, indicating that they had not lost the reporter plasmids (Figure 4E).

Notch1 Mediates Inhibition of Cardiogenic Differentiation in ES Cells

We next investigated which of the Notch receptors might mediate inhibition of cardiomyocyte differentiation. Because Notch1 was downregulated in differentiating EBs, this receptor represented an attractive candidate. Therefore, Notch1-deficient ES cells were generated from blastocysts of floxed Notch1 mice that had been transfected with a Cre-combinase expression plasmid (Figure 5A and 5B). Loss of Notch1 protein and mRNA (Figure 5C and 5F) demonstrated the successful generation of Notch1-deficient ES cell clones. The growth rates (Figure 5D), morphology (Figure 5E), and undifferentiated status of Notch1-deficient ES cells, as indicated by the expression levels of Oct3/4 and Nanog (Figure 5F), were similar to those of parental control ES cells. The
expression of neuronal neurofilament 200 (NF200) and endodermal α-fetoprotein (AFP) markers during ES cell differentiation was indistinguishable between control and Notch1-deficient ES cells (Figure 6E), indicating that Notch1+/− ES cells are still pluripotent.

Notch1-deficient and control ES cells were then assessed for their differentiation potential into cardiomyocytes (Figure 6). On average, 70% of EBs formed by Notch1-deficient ES cells showed differentiation into cardiomyocytes with beating activity, compared with only 30% of their control counterparts (Figure 6A). In addition, we also noted control EBs showed an average of 2 to 3 beating areas per EB, whereas Notch1-deficient EBs had 6 to 12 beating areas per EB (Figure 6B). Intracellular staining and subsequent FACS analysis of single cell suspensions derived from Notch1-deficient beating EBs showed an average of 66% α-actinin positive cells compared with 38% of control EBs (Figure 6C). Furthermore, to visualize cardiomyocytes within the beating areas, differentiated EBs were fixed and stained for α-actinin. This staining revealed that beating areas of a Notch1-deficient EB was much larger and contained on average 3 times more cardiac myocytes than that of control EBs (Figure 6D, right column). Notch1-deficient early EBs also showed a more mature morphology characterized by elongated and spread-out cardiac myocytes. These also had well-organized myofibrils with aligned sarcomeres (Figure 6D, inset). In contrast, control EBs contained relatively more round cells with weak α-actinin staining, indicative of immature cardiomyocytes (Figure 6D, left column). These results are further supported by gene expression analysis for cardiac-specific markers such as MLC-2v, MLC-2a, and MEF-2c in wild-type and Notch1-deficient ES cells, 6-day EBs, and 12-day EBs. Absence of Notch1 results in increased and/or earlier onset of expression of these cardiac specific markers (Figure 6E). In addition, quantitative RT-PCR analysis of early mesodermal expression markers, such as GATA4, Nkx-2.5, or α- and β-MHC, but not α-skeletal actin (ActaA), shows increased expression during ES cell differentiation in absence of Notch1 (Figure 6F). Moreover, the ratios between α- and β-MHC expression shows increased and earlier onset of α-MHC expression confirming the more mature sarcomeric pattern of cardiomyocytes derived from Notch1+/− ES cells. Taken together, these results clearly show that Notch1 signaling inhibits cardiomyocyte differentiation.

**Notch Signaling in ES Cells Facilitates Differentiation Toward the Neuroectodermal Lineage**

Because Notch signaling has been shown to influence binary cell fate decisions in many developmental systems, it could conceivably play a similar role during ES cell differentiation. To assess whether Notch-mediated prevention of cardiomyocyte differentiation occurs at the expense of another cell lineage, ES cells were transfected with either the Notch signaling or the cardiac differentiation reporter plasmids and differentiated into primary EBs. Cells receiving a Notch signal (red) or cells starting to differentiate toward cardiomyocytes (green) were fluorescence-activated cell sorted and used to form secondary EBs. These secondary EBs were then assessed for their ability to differentiate into either mesodermal (cardiomyocyte) or neuroectodermal (neuronal) precursors. Secondary EBs formed by DsRed+ cells were negative for α-actinin (Figure 7C), indicating that they were no longer able to differentiate into cardiomyocytes. In contrast, a large proportion of these secondary EBs stained positive for nestin (Figure 7E), suggesting that they have adopted a neuronal cell...
understanding of the molecular mechanisms controlling ES cells might represent a valuable source of cardiomyocytes to be used in regenerative medicine. However, a precise understanding of the molecular mechanisms controlling ES cell differentiation is a prerequisite before induction of cardiac repair via transfer of ES-derived cardiomyocytes can be achieved. In this context, the expression pattern of Notch receptors and ligands in heart tissue of Xenopus, zebrafish, and mouse embryos suggests a role for the Notch pathway during cardiac development.25–27 The early embryonic lethal-6 embryonic lethal-10 mutation (embryonic stem cells, ES cells) in ES cells is caused by a critical function of the Notch pathway and its ligands, attributable in part to heart defects, also supports a role for these pathways in cardiac differentiation and morphogenesis.28,29 A key function of Notch signaling is its ability to influence cell fate decisions during development.19 Therefore, to better understand the role of Notch pathways during cardiogenesis, we have studied differentiating ES cells in EBs, a system that has contributed tremendously to our understanding of cardiogenesis.

We first show that inhibition of Notch signaling by means of expressing a RBP-JDN protein enhances cardiac differentiation and that constitutive activation of the Notch pathway significantly reduces cardiac differentiation. This is in agreement with a previous report showing that inactivation of RBP-J in ES cells results in increased cardiomyocyte differentiation.23 However, our data also demonstrate that blockade of cardiogenesis is mediated by the Notch1 receptor. Interestingly, the expression of this particular receptor is markedly downregulated during differentiation. Other Notch receptors do not compensate for the lack of Notch1, suggesting that suppression of cardiomyocyte differentiation of ES cells is specifically mediated by Notch1 in an RBP-J–dependent manner.

Figure 7. Differentiating ES cells having received a Notch signal are predisposed toward the neuronal lineage and can no longer differentiate into cardiomyocytes. E14/T cells were transfected with the Notch and the cardiac reporter plasmids. Cells were differentiated into EBs for 6 days before they were dissociated into single cells and sorted for DsRed– (A, C, and E) and EGFP– (B, D, and F) cells to generate secondary EBs. Forty-eight hours postplating on gelatin-coated plates, secondary EBs were stained with antibodies against sarcomeric α-actinin (C and D) or against Nestin (E and F) or with secondary antibodies (2nd Ab) alone (A and B). At left panel (A, C, and E), red fluorescence (DsRed) indicates Notch signaling, and green fluorescence shows Nestin-expressing cells. At right (B, D, and F), green fluorescence indicates α-MHC–driven EGFP expression, whereas the red fluorescence shows α-actinin–expressing cells. Scale bar=500 μm.

Figure 8. Notch signaling enhances neural progenitor formation and neuronal differentiation. A, Parental E14/T cells and E14/T cells stably expressing NIC and dominant negative RBP-J (RBP-JDN) were grown in N2B27 differentiation medium in presence of FGF4 and stained for Nestin (red; Nestin) at day 9 and for β-III-tubulin (red; β-III-tubulin) and for neurofilament 160 (red; NF-160) at day 12 and counterstained with DAPI (blue). Two different clones were used with identical results. Representative micrographs are shown. Scale bar in A, 100 μm. B, Quantitation of neuronal differentiation. β-III-tubulin+ cells were counted on 4 representative micrographs for each clone and are expressed as a percentage of total cells counted on DAPI. At least 1000 DAPI-stained nuclei were counted per micrograph. Error bars indicate SD. P < 0.005 for wild-type (WT) vs NIC and wild-type vs RBP-JDN.
manner. Transfection of a Notch reporter construct into ES cells also shows that Notch signaling is reduced during differentiation, concomitantly with the reduction of the expression of Notch1 itself. In addition, cotransfection of Notch and cardiac differentiation reporter constructs shows that cells that have received a Notch signal are prevented from committing to a cardiac fate. These data indicate that Notch signaling inhibits cardiac differentiation of ES cells through the action of Notch1. However, we also show that the expression of Brachyury and FGF-8, 2 markers for mesodermal cell lineage, are also repressed in Notch-responsive cells and, together with other mesodermal markers such as GATA4, Nkx-2.5, or α- and β-MHC, markedly increased in cells lacking Notch signaling, suggesting that Notch influences commitment of mesodermal progenitors.

The expression of Notch1, Notch4, and Jagged2 is down-regulated during ES cell differentiation, similar to that of the 2 ES cell identity genes Oct3/416 and Nanog17 (Figure 1). However, it is unlikely that Notch pathways play a role in ES cell maintenance or self-renewal. This is supported by the punctuate pattern of Notch signaling that is restricted to some areas of ES cell colonies, making it unlikely that an entire colony could be maintained in an undifferentiated state through only a few Notch signal–receiving cells (Figure 3). Second, expression of Oct3/4 and Nanog are not affected by the presence or absence of Notch1. Third, the morphology and growth rate of Notch1-deficient ES cells, as well as their pluripotency, is identical to wild-type cells (Figures 5 and 6). Similarly, RBP-J–deficient or RBP-J+ES cell lines have characteristics identical to wild-type cells. Finally, embryos lacking components of the Notch cascade develop beyond gastrulation to a late stage (embryonic day 9.5) (reviewed previously28,29).

Notch signaling could inhibit cardiogenesis either by blocking mesodermal differentiation per se or by inhibiting a mesodermal progenitor from adopting its final cardiomyocyte fate. Analysis of Brachyury and FGF-8 expression, 2 markers of mesodermal differentiation, suggests that Notch pathways act rather at an early step in the differentiation process to inhibit commitment toward the mesodermal lineage. The increase in MLC-2a, as well as in α- and β-MHC, expression in Notch1-deficient ES cells, together with upregulation of early cardiac markers such as GATA4 and Nkx2.5, likely reflects a general relief of a block in mesodermal and then cardiac differentiation, generating in turn cardiac cells not yet specified in ventricular or atrial myocytes.

Moreover, the observation that cardiac differentiation and Notch signaling are mutually exclusive suggests that Notch-expressing cells are not direct precursors of cardiomyocytes. In contrast, the presence of cells expressing nestin but not α-actinin in secondary EBs formed by Notch responsive cells (DsRed+) indicates that cells receiving Notch signals have adopted a neuronal fate and are no longer able to differentiate into cardiomyocytes. This interpretation is further supported by the ability of a dominant active form of Notch to induce and enhance neuronal differentiation, whereas inhibition of Notch signaling results in suppression of neurogenesis. These observations are compatible with the ability of Notch to influence binary cell fate decisions, among the best-documented roles of Notch signaling in many developmental systems (reviewed previously30). Although our data clearly demonstrate that Notch signaling influences neuronal versus cardiac differentiation, it remains to be shown whether this control is exerted on 1 common or 2 distinct neural/mesodermal progenitors. Although such a common neural/cardiac progenitor has not yet been described in ES cells, bipotent progenitors have been shown to exit in the neural crest–derived cardiac side population.31

Several endoderm-derived factors, such as TGF-β32 BMPs,33 and FGFs,34 that regulate cardiogenesis in the adjacent mesoderm have been used to enhance cardiac differentiation of ES cells in vitro.35 Indeed, a recent report shows that timely inhibition of BMP signaling in ES cells by Noggin greatly enhances cardiogenesis via induction of Brachyury and Nkx2.5.33 Our study demonstrates that inhibition of Notch signaling promotes cardiogenesis and simultaneously enhances Brachyury expression. Interestingly, in several model systems, such as in differentiating myoblasts, Notch and BMP pathways cooperate to block downstream differentiation events.36 Whether or not Notch signaling can directly modulate Noggin expression and/or release to further enhance cardiogenesis in ES cells requires further investigation. Nevertheless, the present data support the hypothesis that inhibition of Notch1-mediated signaling is essential for mesodermal induction of ES cells and is, therefore, a mandatory step for their cardiogenic differentiation.

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Disclosures

None.

References

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Expanded Materials and Methods

Floxed Notch1 (N1^{lox/lox}) ES cells were derived from blastocysts carrying floxed Notch1 gene and Notch1^{−/−} (N1^{−/−}) ES cells were obtained by transfecting N1^{lox/lox} cells with pPGK-Cre plasmid. Notch1 deletion was detected by PCR analysis as described. E14/T ES cell line stably expressing the polyoma large T antigen allows maintenance of episomal vectors carrying a polyoma origin of replication was a generous gift from Dr. Austin Smith (Stem cell Laboratory, Edinburgh, Scotland). Cells were cultured on mouse embryonic fibroblast feeders (N1^{lox/lox} and N1^{−/−} cells) or on gelatinized plates (E14/T cells) in standard ES cell medium supplemented with 1000U/ml of LIF. Cardiac differentiation of ES cells was induced by aggregating aliquots containing ~500 cells in hanging drops to form embryoid bodies. Quantitation of cardiac differentiation was done by counting the number of beating EBs and counting the number of beating areas per EB. Directed differentiation of ES cells into neurones was carried out in monolayer cultures as described with the exception of the addition of 1 ng/ml of FGF4 (Sigma). After 3, 6, 9 or 12 days of growth in differentiation medium, cells were processed for immunofluorescence analysis.

Plasmids and transfections

Notch and cardiac reporter plasmids: To avoid silencing observed with stably transfected DNA in ES cells, we used episomal DNA constructs in E147T ES cells described above. For Notch reporter plasmids, DsRed cDNA was isolated from pDsRedN1 (Clontech) using XhoI and NotI and cloned downstream of a DNA
fragment containing 12 RBP DNA-binding sites. The fragment containing the Notch responsive promoter plus DsRed was cut with PvuI (blunted) and SfiI and cloned into pPyCAGIP (a kind gift from Dr. Austin Smith) via SfiI and PstI (blunted) resulting in pCAGIP-12RBPDsRed. For the Cardiac reporter plasmid, Alpha myosin heavy chain (α−MHC) promoter was isolated from the Cl22-MHC plasmid (a kind gift from Dr. Jeffery Robbins; University of Cincinnati, College of Medicine, Cincinnati, Ohio, USA) using BamHI and XhoI and the 2XHS4 insulator fragment of chicken β-globin gene was cut from pJC13-1 plasmid (a kind gift from Dr. Adam West; NIDDK, NIH, Bethesda, Maryland, USA) using NdeI and BamHI. These two fragments have been subsequently cloned into pEGFPN1 digested with AseI- SalI using a 2-insert ligation. The fragment containing the HS4 insulator, the α-MHC promoter and EGFPN1 cDNA were digested with SfiI and PvuI (blunted) and cloned into pPyCAGIP vector digested with PstI (blunted) and SfiI to yield pCAGIP-Tri.

NotchIC and dominant negative RBPJ expression plasmids: NotchIC-myc Tag was cut from pMI-Notch IC (a kind gift from Professor Mike Bevan; University of Washington, Seattle, USA) using SmaI and a partial digest with ApaI and subsequently cloned into pBSII (Stratagene). pBSII-NIC was then cut with SmaI and KpnI in order to clone NIC into pPGKEGFP. NIC was isolated via SmaI and HpaI and ligated into pPyCAGIP via blunted XhoI, giving rise to the plasmid pCAG-NIC. The cDNA encoding a dominant negative RBP-J protein (RBPJDN) was isolated from pCMX-RBPJ/R218H (a kind gift from Professor Tasuko Honjo, University of Kyoto, Japan) using EcoRI and BamHI, blunted with Klenow DNA polymerase and ligated into the blunted XhoI site of the pPyCAGIP giving rise to pCAG-RBPJDN.

E14/T cells were transfected with plasmid DNA using Lipofectamine2000 reagent (Invitrogen) and were selected for 2 days using 2 µg/ml of puromycin.
(Sigma). Cells were continuously maintained in medium containing 0.5 µg/ml of puromycin. For stable expression of NotchIC and RBPjDN proteins, plasmids were linearized with PvuI and transfected into ES cells by electroporation using BioRad Gene Pulser at 960 µF and 250mV settings. Transfected cells were selected using 2 µg/ml of puromycin and resistant clones were picked and screened for protein expression. Stable transfectants were also maintained under 0.5 µg/ml puromycin selection throughout the experiments.

Immunofluorescence analysis

Differentiated EBs and monolayer cultures were fixed for 10 minutes in 4% paraformaldehyde in PBS and permeabilised with 0.2% Triton X100 in PBS. After blocking in blocking buffer (PBS containing 0.001% Triton X100, 1% BSA and 1% FCS) they were incubated overnight at 4 °C with the following antibodies: anti α-actinin (1:500, Sigma A7811), anti β-III-tubulin (1:200, Sigma, T8660); anti Neurofilament 160 (NF160; 1:100, Sigma N5264) anti-nestin (1:200, Chemicon MAB 363) and anti-activated Notch1 IC (Abcam ab8925). For quantification of neuronal differentiation, β-III-tubulin-positive cells were counted on 4 representative micrographs containing each 1000-3000 cells using the NIH Image software.

FACS analysis

Differentiated EBs were trypsinized into single cells, fixed in 2% PFA, permabilized with 0.5% Saponin and intracellularly stained with Alexa488-conjugated anti sarcomeric α-actinin antibody (made in house and diluted 1:30 in PBS/0.5% Saponin/3%FCS) for 45 min. Cells were analyzed using a BD FACS Calibur. Analysis was carried out on an FSC/SSC gate to select for cells resembling newborn cardiomyocytes.
Western blot analysis

Total cellular proteins (80 µg) were detected by Western blotting using following antibodies: anti-Notch1, 1:200 (C20; Santa Cruz), anti-myc Tag 1:200 (9E10; Santa Cruz) and anti-RBPJ-k protein 1:250 (T6709; Institute of Immunology Co. Tokyo, Japan). Antibody binding was detected using HRP-conjugated antibody and the ECL reagent (Amersham).

RT-PCR and quantitative RT-PCR analysis

RNA was isolated using Trizol reagent (Invitrogen). Aliquots containing 250-500 ng of total cellular RNA were analyzed using the GeneAmp kit (Applied Biosystems). The sequences of specific primers used for Notch1, Notch2, Notch3, Notch4, Jagged1, Jagged2, Dll4, Nanog, Oct3/4, Brachyury, MLC2v, MLC2a, MEF2c, NF200, AFP, and tubulin are listed in the supplementary Table 1 below. Each RT-PCR was performed with optimized conditions allowing amplification within the exponential phase of the PCR reaction. For quantitative Real-Time RT-PCR, total cellular RNA was reverse transcribed using Omniscript RT Kit (Qiagen) and oligo dT primers (Promega). Quantitative PCR amplification of the resulting cDNAs was performed using primers, probes and the Universal Master Mix of TaqMan Gene Expression Assays on an ABI PRISM 7700 Sequence Detection System following the manufacturer’s instructions (PE, Applied Biosystems). The data represent an average of at least two independent experiments done in duplicates. The values of the relative expression levels of individual genes are normalized to those of GAPDH.

Statistical analysis
For statistical significance of beating EB counts, comparison between different experimental groups was performed by Student’s T-test using two-tailed distribution and unequal variances. For Real time PCR data, Statistical comparisons among values of all groups was performed using Two-way ANOVA with \( p < 0.05 \).

Supplementary Table S1

List of RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (LP) primer sequence</th>
<th>Reverse (RP) primer sequence</th>
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<tr>
<td>Notch1</td>
<td>5’-TGT GAC AGC CAG TGC AAC TC-3’</td>
<td>5’-GCA GTG CTT CCA GAG TGC CA-3’</td>
</tr>
<tr>
<td>Notch2</td>
<td>5’-ACA TCA TCA CAG ACT TGG TC-3’</td>
<td>5’-GGC AGC TGC TGT CAA TAA TG-3’</td>
</tr>
<tr>
<td>Notch3</td>
<td>5’-GAT GTC AAT GCA GTG GAT GAG-3’</td>
<td>5’-CTT TGA GGC CAG GAA GGA AG-3’</td>
</tr>
<tr>
<td>Notch4</td>
<td>5’-CAC CTA GCT GCC AGA TTC TCT C-3’</td>
<td>5’-ACT GTC CTG GGC ATC TTT ATC C-3’</td>
</tr>
<tr>
<td>Jagged1</td>
<td>5’-ATT CGA TCT ACA TAG CCT GTG AG-3’</td>
<td>5’-ACC GGA TGG AAT ACA TCG TAT A-3’</td>
</tr>
<tr>
<td>Jagged2</td>
<td>5’-TGT CAG CCA CGG AGC AGT CAT T-3’</td>
<td>5’-AAG CGC AGG AAA GAA CGT GAG A-3’</td>
</tr>
<tr>
<td>Dll4</td>
<td>5’-GGG TTA ATA CCT GCA CCT GTC TC-3’</td>
<td>5’-CTG ATG TGC AGT TCA CAG TGG-3’</td>
</tr>
<tr>
<td>Nanog</td>
<td>5’-AGG GTC TGC TAC TGA GAT GCT CTG-3’</td>
<td>5’-CAA CCA CTG GTT TTT CTG CCA CCG-3’</td>
</tr>
<tr>
<td>Oct3/4</td>
<td>5’-CTG AGG GCC AGG CAG GAG CAC GAG-3’</td>
<td>5’-CTG TAG GGA GGG CTT CGG GCA CTT-3’</td>
</tr>
</tbody>
</table>
Brachyury LP: 5’-AAG GAA CAA CCG GTC ATC-3’
   RP: 5’-GTG TGC GTC AGT GGT GTG TAA TG-3’
MLC2v LP: 5’-GCC AAG AAG CGG ATA GAA GG-3’
   RP: 5’-CTT GTG GAA ACG TTT CTC TTG CGG-3’
MLC2a LP: 5’-CGT GGC TCT TCT AAT GTC TTC TC-3’
   RP: 5’-AAA CAG TTG CTC TAC CTC AGC AG-3’
MEF2c LP: 5’-GGT TTC CGT AGC AAC TCC TAC TT-3’
   RP: 5’-CTT GAC TGA AGG ACT TTC CCT TT-3’
NF-200 LP: 5’-GAGTGGTTCCGAGTGAGGTTGGAC-3’
   RP: 5’-GAAGCTCCATATCCCTGGGTGGAAAG-3’
AFP LP: 5’-CCTTGGCTGCTCAGTACGACAAGG-3’
   RP: 5’-CCTGCAGACACTCCAGCGAGTTTC-3’
Tubulin LP: 5’-TCA CTG TGC CTG AAC TTA CC-3’
   RP: 5’-GGA ACA TAG CCG TAA ACT GC-3’

Supplementary legend to Figure S1

The expression of the mesodermal marker genes Brachyury and FGF8 is elevated in Notch1−/− ES cells compared to the parental Notch1lox/lox ES cells during differentiation. Time course analysis of Brachyury (A) and FGF8 (B) gene expression was performed using real-time quantitative PCR at the indicated time points. The data represents expression values in Notch1−/− relative to Notch1lox/lox ES cells (set at 1), after normalizing to GAPDH.
Supplementary References


Figure S1

A

Brachyury

Relative expression level

N1lox/lox  N1/-/

ES EBs

24h 48h 72h 96h

B

FGF8

Relative expression level

N1lox/lox  N1/-/

ES EBs

24h 48h 72h 96h