Activation of Notch-Mediated Protective Signaling in the Myocardium

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Abstract—The Notch network regulates multiple cellular processes, including cell fate determination, development, differentiation, proliferation, apoptosis, and regeneration. These processes are regulated via Notch-mediated activity that involves hepatocyte growth factor (HGF)/c-Met receptor and phosphatidylinositol 3-kinase/Akt signaling cascades. The impact of HGF on Notch signaling was assessed following myocardial infarction as well as in cultured cardiomyocytes. Notch1 is activated in border zone cardiomyocytes coincident with nuclear c-Met following infarction. Intramyocardial injection of HGF enhances Notch1 and Akt activation in adult mouse myocardium. Corroborating evidence in cultured cardiomyocytes shows treatment with HGF or insulin increases levels of Notch effector Hes1 in immunoblots, whereas overexpression of activated Notch intracellular domain prompts a 3-fold increase in phosphorylated Akt. Infarcted hearts injected with adenoviral vector expressing Notch intracellular domain treatment exhibit improved hemodynamic function in comparison with control mice after 4 weeks, implicating Notch signaling in a cardioprotective role following cardiac injury. These results indicate Notch activation in cardiomyocytes is mediated through c-Met and Akt survival signaling pathways, and Notch1 signaling in turn enhances Akt activity. This mutually supportive crosstalk suggests a positive survival feedback mechanism between Notch and Akt signaling in adult myocardium following injury. (Circ Res. 2008;102:1025-1035.)

Key Words: Notch • Akt • cardioprotection • infarction • myocardium

Two decades have passed since cloning of the Notch gene, with pursuant research resulting in a substantial body of literature.1–6 Functional activities of Notch have been linked to diverse cellular processes such as differentiation, proliferation, apoptosis, adhesion, and epithelial-to-mesenchymal transition. Deregulation of the Notch pathway results in a variety of tumor types that can arise from either abundance or lack of appropriate activity. Properties of increased cellular survival and proliferation via manipulating Notch activity has recently been touted as a potential approach for enhancing stem cell expansion for regenerative medicine.7 In the context of the myocardium, studies with embryonic tissues implicate Notch in cell fate determination and suppression of cardiogenic signaling,8–11 and Notch has a longstanding association with regulation of cardiac development and morphogenesis.12–15 However, the function of Notch signaling in the mature myocardium has been largely overlooked. As has been reported in research related to tumorigenesis and stem cell proliferation, relevance for Notch in the heart may lie with survival or proliferative signaling in response to pathological damage.

The Notch receptor, originally identified in Drosophila and having 4 orthologs in mammals, is a large transmembrane protein important for cell fate decisions, cellular development, differentiation, proliferation, and apoptosis.16,17 Notch signaling plays an important role in embryonic heart development,18–21 whereas defective Notch1 protein has been linked to aortic valve disease.22 Notch receptor activation occurs via binding to Notch ligands, the Serrate family in Drosophila or Jagged/Delta family in mammals. Target genes of Notch signaling include the basic helix-loop-helix transcription factors Hes1 (Hairy and enhancer of split 1) and the HRT (Hairy-related transcription) factor family, which are found in the developing and adult heart.23 HRT proteins bind GATA transcription factors and inhibit GATA4-mediated transcription of ANP in vitro; this repression is alleviated by Akt1/protein kinase B (PKB) through an unknown mechanism.24 Notch activity is triggered by cognate ligands such as Delta that bind to Notch receptor. Expression of Notch ligand Delta is induced by activation of c-Met receptor in MDCK cells, leading to activation of Notch and expression of Hes1 that, in turn, represses transcription of c-Met, indicating a negative-feedback loop for c-Met activity.25 c-Met is a receptor for hepatocyte growth factor (HGF) that is increased in hypertrophic and infarcted cardiac tissue.26,27 On activation by...
HGF, c-Met participates in numerous downstream signaling pathways, including activation of ERK and Akt/PKB survival signaling in the heart. Notch and Akt/PKB signaling pathways interact through complex molecular webs in developing, adult, and neoplastic tissues, and the Akt/PKB cascade is a well-known effector of cell survival in the myocardium. These precedents for a network of crosstalk between Notch, HGF, c-Met, and Akt/PKB implicate Notch-driven signaling as a mediator of cell survival. Furthermore, presence of c-Met on cardiac stem cells may facilitate migration of these cells to sites of damage by HGF release with enhanced survival, thereby directing repair or regeneration of infarcted myocardium. Elucidating interplay between Notch, c-Met, and phosphatidylinositol 3-kinase (PI3K)/Akt signaling in pathologically challenged myocardium constitutes the focus of this study.

Materials and Methods
FVB/N male and C57Bl/6 female mice between 8 and 14 weeks of age were used. All animal protocols were approved by the Institutional Animal Care Committee of San Diego State University. Experimental protocols are described in the online data supplement, available at http://circres.ahajournals.org.

Figure 1. Notch signaling proteins are expressed in injured myocardium. A, Paraffin sections from hearts subjected to acute infarction harvested 4 days after infarction and stained for activated Notch1 (Notch ICD) (green), tropomyosin (red), and To-pro 3 iodide nuclear stain (blue). White arrows indicate nuclei in surviving myocytes expressing Notch ICD. B, Sections stained for Jagged1 ligand (green), tropomyosin (red), and To-pro 3 iodide (blue). White arrows point to border zone cardiomyocytes containing perinuclear Jagged1. C, Immunolocalization of a cell (arrow) positive for Delta4 (DLL4) (green), as well as Notch ICD (red) expressed alone in a neighboring myocyte (arrowhead) with tropomyosin (blue) and To-pro 3 (magenta). DLL4 primarily localizes to cells other than myocytes. BZ indicates border zone; IZ, infarcted zone. D through F, Mouse hearts subjected to acute infarction with border zone regions harvested at 4 days after infarction. GAPDH is used as a loading control, and quantitation is in relative fluorescence units. D, Activated Notch increases ~3-fold after infarction. E, Hes1 levels increase 1.8-fold at 4 days after infarction. F, Jagged1 increases 4-fold at 4 days after infarction. Significance is represented as *P<0.05, **P<0.01, ***P<0.001.
Results

Notch Expression and Signaling Declines During Postnatal Development

The relationship between postnatal development and expression levels for Notch1 or downstream target protein Hes1 (indicative of Notch activity) was assessed. Notch1 and Hes1 levels are highest in neonatal heart samples and stabilize by 2 months postnatal age and thereafter (Figure 1A in the online data supplement). Quantitation reveals that Hes1 levels decrease 4- to 5-fold from 2 days to 2 months (supplemental IB), whereas cardiac Notch1 levels decrease approximately 3-fold during postnatal development (supplemental Figure IC).

Notch1 Is Activated in Adult Injured Myocardium

Notch levels decrease in adult myocardium relative to neonatal tissue (supplemental Figure I) but increase in response to acute infarction by permanent coronary occlusion. Four days after infarction, nuclear Notch accumulates in surviving cardiomyocytes restricted to the border zone of the infarct region (supplemental Figure IIA, white arrows, Figure 1A, white arrows, Notch ICD, green in overlay). Similarly, immunoreactivity for Notch ligands Jagged1 or Delta4 is also observed. Jagged1 appears within cardiomyocytes in a perinuclear distribution (Figure 1B, white arrows, Jagged1, green in overlay), whereas Delta4 is predominantly expressed in interstitial areas (Figure 1C, white arrow, DLL4, green in overlay). Interstitial cells labeled for both Delta4 and Notch (Figure 1C, arrow) or cardiomyocytes positive for activated Notch (Figure 1C, arrowhead) were observed. Corresponding immunoblot analysis of myocardial lysates taken from the infarcted region demonstrates that Notch1, Hes1, and Jagged1 levels are significantly increased 4 days after infarction (Figure 1D, 1E, and 1F, respectively), indicative of activated Notch signaling. Notch signaling is also observed in nonmyocyte tissue in infarcted myocardium, as indicated by a c-kit+ cell (supplemental Figure IIC, green in overlay) expressing activated Notch (Notch intracellular domain [NICD], red in overlay) in the tenascin-C–rich border zone area or c-kit+ (green) cells lining a vessel wall (supplemental Figure IID, red in overlay, white arrows).

c-Met and HGF Localization in Infarcted Myocardium

Interplay between HGF receptor c-Met and expression of HGF in relation to Notch-based signaling prompted examination of infarcted tissue for immunolocalization of HGF and c-Met. HGF levels increase following acute infarction. Confocal microscopy reveals strong HGF staining of vessel walls in proximity to the infarct as well as localization within the infarct region (Figure 2A and 2B, white arrow, HGF green in overlay) at 4 days after injury. In comparison, nuclear staining for c-Met is observed in border zone cardiomyocytes (supplemental Figure IIB, white arrows, c-Met green in overlay, Figure 2B, white arrowheads, c-Met red in overlay, Figure 2C, white arrows, c-Met green in overlay). In Figure 2B, border zone myocytes (blue) with nuclear staining pattern for c-Met (red) are localized in proximity to an HGF-positive vessel (green).

HGF Stimulates Notch Signaling In Vitro and In Vivo

The relationship between HGF and Notch-mediated signaling was assessed using cultured neonatal rat cardiomyocytes (NRCMs) treated with recombinant HGF protein, wherein Hes1 levels increased within five minutes and remained...
Figure 3. HGF and insulin induce Hes1 in cultured neonatal cardiomyocytes. A, NRPMs serum-starved overnight and treated with 50 ng/mL recombinant mouse HGF or 100 mIU/mL insulin as a positive control for the time points indicated. NRPMs treated with HGF or insulin, harvested after 3 and 6 hours, and immunoblotted for Hes1 showed an increase in Hes1 levels of ~2.5-fold following either HGF or insulin treatment by 6 hours. Total Akt and induction of phospho-AktS473 by HGF and insulin are shown to verify stimulation by exogenous treatments. Hes1 levels are normalized to GAPDH and phospho-AktS473 levels normalized to total Akt. C, HGF and insulin treatment of NRPMs for 6 hours in the presence of PI3K inhibitor LY294002 (LY) shows reduction of phospho-AktS473 level as well as Hes1 protein levels in untreated and HGF- or insulin-stimulated NRPMs. Significance is represented as *P<0.05, **P<0.01, ***P<0.001.
Figure 4. HGF stimulates Notch activation in adult mouse myocardium. A, Mouse hearts injected intramyocardially with recombinant mouse HGF or PBS vehicle at several sites in the apex, harvested at 3 days after injection, and immunostained for activated Notch. Distance and area surrounding the injection sites were measured for Notch activation by HGF-treated (images labeled HGF, ii, and iv) vs PBS-treated (images labeled PBS, i, and iii) samples. Cardiomyocytes expressing activated Notch1 (white arrowheads) were used to mark the boundary of activation. Blue lines delineate circumference of injection sites, and area of Notch activation is depicted by a yellow boundary. B, Quantitation shows that HGF injection increased the average area of Notch activation around the lesion. C, Mouse hearts injected intramyocardially with recombinant mouse HGF as described in A and harvested at time points as shown. Lysates were immunoblotted for activated Notch1 and normalized to GAPDH. Hes1 and Notch levels increase by 3 days after HGF treatment relative to PBS-injected controls. D, HGF-injected heart lysates immunoblotted for total Akt and phospho-Akt(S473) show HGF induction of Akt phosphorylation. Phospho-Akt(S473) levels were normalized to total Akt. Values are averages±SD (n=3). Significance is represented as *P<0.05, **P<0.01, ***P<0.001.
elevated for six hours (Figure 3A and 3B). In comparison, the effect of HGF is similar to insulin-mediated Hes1 induction (Figure 3B and supplemental Figure IIIA). Both HGF and insulin promote Akt activation; therefore, involvement of Akt in induction of Hes1 expression was examined by pretreatment of NRCMs with PI3K inhibitor LY294002 before stimulation with HGF or insulin. Inhibition of PI3K by LY294002 significantly suppresses both basal and stimulated expression of Hes1 protein without significantly altering Akt protein expression levels (Figure 3C and 3D). Inhibition of mitogen-activated protein kinase kinase by PD95089 or mTOR by rapamycin did not reduce Hes1 levels significantly, whereas treatment with triciribine (Akt inhibitor V) reduced Hes1 levels by more than half (supplemental Figure IIIB), indicating specific crosstalk between Akt and Notch signaling in NRCMs. These findings demonstrate that the PI3K/Akt pathway mediates activation of Notch1 signaling in NRCMs and that the stimulatory effect of HGF is regulated in part by PI3K.

Induction of Notch activity by HGF in the intact myocardium was confirmed by intramyocardial injection of HGF (Figure 4A). The area of myocardium surrounding the injection site positive for activated Notch (yellow line) relative to the area of the injection site (blue line) was measured, revealing a 2-fold increase in the area of myocardial tissue expressing Notch and a 4-fold increase in the average distance extending from the injection lesion to the perimeter of Notch activation (Figure 4B). Myocardial samples harvested at multiple time points following HGF injection exhibit a 2.5-fold increase in phospho-AktS473 levels compared with PBS injected controls (Figure 4D). Similarly, activated Notch expression increases 2-fold in the same samples (Figure 4C). No significant induction of phospho-AktT308 was observed in these samples (data not shown). Previous studies have shown differential activation of Akt at the T308 and S473 residues. Collectively, these results indicate that HGF activation induces Notch signaling in intact myocardium and that this effect is associated with Akt activation.

**Notch1 Signaling Supports Akt Activation In Vitro and In Vivo**

The impact of Notch signaling on Akt activity was assessed using an adenoviral vector that expresses human NICD fused to enhanced green fluorescent protein (EGFP) (adNICDEGFP; gift of K. Tezuka, PhD, Gifu University...
Graduate School of Medicine, Gifu, Japan\textsuperscript{43}), which accumulates in the nuclei of cardiomyocytes (Figure 5A). NRCMs overexpressing adNICDEGFP show induction of Hes1 by immunoblot analysis (Figure 5A) relative to cells expressing EGFP (adEGFP), demonstrating that this construct is active in our system. Levels of phospho-Akt\textsuperscript{S473} are increased 6-fold in the adNICDEGFP versus noninfected and 3-fold over adEGFP-infected NRCMs (Figure 5A). The ability of adNICDEGFP to induce Akt activation was confirmed within adult myocardium by direct injection of the virus into the apex of hearts. Immunolabeling for EGFP verifies viral expression 3 days after injection (Figure 5B). Immunoblotting reveals elevated phospho-Akt\textsuperscript{S473} levels in adNICDEGFP-injected tissue compared with PBS or adEGFP-injected samples (Figure 5C). In addition to Akt activation, NRCMs overexpressing adNICDEGFP exhibit increased nuclear localization of Pdk1, as well as increased overall levels of Pdk1, compared with noninfected and adEGFP-overexpressing controls (supplemental Figure IVA through IVC). Interestingly, Pdk1 is required for Notch-mediated trophic and proliferative effects in T-cell development.\textsuperscript{44} Additionally, pull-down experiments in lysates of NRCMs overexpressing both adNICDEGFP and nuclear-targeted myc-tagged Akt reveal an association be-

Figure 6. Activated Notch stimulates proliferative signaling in cardiomyocytes. NRCMs infected with adNICDEGFP (green) (A) or adEGFP (green) (B) were immunostained for PCNA (red) and desmin (blue). A, PCNA localizes to the nucleus in adNICDEGFP-infected myocytes. B, PCNA localization remains cytoplasmic in EGFP-infected myocytes. C, Mouse hearts subjected to acute infarction and intramyocardial injection of adNICDEGFP were harvested 4 days postoperatively for paraffin embedding. Immunolocalization of adNICDEGFP to visualize viral expression (green), Ki67 to indicate proliferation (red), tropomodulin as a sarcomeric marker (blue), and To-pro 3 iodide to stain nuclei (gold) reveals colocalization of exogenous activated Notch and Ki67 (white arrows).
tween these 2 exogenously expressed proteins (supplemental Figure VD). Taken together, these results provide further evidence for crosstalk between the Notch and PI3K/Akt signaling pathways in cardiac myocytes.

**Activated Notch Stimulates Proliferative Signaling In Vitro and In Vivo**

Transgenic mice overexpressing activated Notch in the male reproductive tract exhibit higher numbers of proliferating cell nuclear antigen (PCNA)-positive cells in transgenic versus nontransgenic epididymal epithelial cells. PCNA is clearly detected in the nucleus of adNICDEGFP-overexpressing myocytes (Figure 6A), but nuclear localization is not seen in adEGFP-infected cells (Figure 6B), whereas surviving myocytes expressing adenoviral activated Notch stained positive for Ki67 (Figure 6C). Collectively, these results suggest activated Notch stimulates proliferative signaling in cardiomyocytes.

**Notch Signaling Is Cardioprotective in Injured Myocardium**

Relationships established between Notch activity and Akt (Figures 3 through 5) prompted studies to determine whether Notch activity confers cardioprotection in the wake of infarction challenge. AdNICDEGFP was injected into the ischemic border region of mouse heart tissue at the time of infarction. Both the EGFP and NICDEGFP viruses express protein in myocytes of the border zone region 3 days after infarction and injection (Figure 7A and 7B, respectively). Heart function was assessed by in vivo hemodynamic analysis 4 weeks after infarction. Hearts treated with adNICDEGFP exhibit improved function, with maximum and minimum rates of change in pressure over time (dP/dt) and developed pressure of adNICDEGFP-treated hearts significantly improved over adEGFP-injected hearts (Figure 7C). Additionally, echocardiographic analysis at 1, 2, and 3 weeks after infarction reveal improved anterior wall thickness in adNICDEGFP versus vehicle-treated hearts (supplemental Figure VA) and function in adNICDEGFP versus vehicle and adEGFP-treated hearts (supplemental Figure VB and VC). Infarct area was also decreased in adNICDEGFP versus vehicle or adEGFP-treated hearts 4 weeks after infarction, as demonstrated in supplemental Figure VD. Overall, these functional and morphological analyses demonstrate that transient overexpression of exogenous activated Notch in infarcted myocardium attenuates heart failure 4 weeks after treatment.

**Discussion**

Convergence of Notch, c-Met, and Akt pathways in cardiomyocytes of infarcted myocardium at the border zone of...
damage protects from acute pathological injury. Integrated and coordinated activity of the HGF/Notch/c-Met/Akt signaling axis is a survival response of border zone cardiomyocytes. Our findings point to a protective role for Notch signaling in damaged myocardium, reminiscent of lower vertebrates.46 The Notch pathway crosstalks with other signaling pathways, including bidirectional influences on and from the PI3K/AktB cascade,28,29,44,47 and HGF/c-Met signaling leads to Notch activity in tumor cell lines.25 Interplay of these 3 signaling pathways contributes to cardioprotective effects conferred by activated Notch.

Embryonic protein levels decrease in postnatal heart as the myocardium hypertrophies and matures to the adult hormonal and contractile state. Similarly, Notch1 and Hes1 levels decline steadily after birth (supplemental Figure I). Enhanced Notch signaling following tissue injury has been documented in various tissues. Elevated levels of Notch ligands and receptors have been reported in damaged and regenerating teeth, liver, pancreas, brain, and vessels.48–53 Reexpression of fetal genes is an adaptive response following pathological insult in adult myocardium, and increased Notch signaling in the context of acute infarction (Figure 1 and supplemental Figure II) is associated with survival of myocardium as well as possible signaling to regenerate damaged tissue.

c-Met and HGF are expressed during early cardiac development.54 Multiple studies document elevated HGF levels following myocardial infarction,36–38,40,41,55 as well as protective effects conferred on myocytes following cardiac ischemic insult.27,56–61 Additionally, c-Met nuclear localization occurs in low-density cell culture.62 Border zone cardiomyocytes experience disruption of cell–cell contacts and may sense this as a “low-density” environment that, combined with elevated HGF stimulation, results in nuclear c-Met localization. Given the molecular signaling between HGF/c-
Met and Notch that leads to negative feedback of c-Met expression by Notch activation, it is tempting to speculate that a similar cascade occurs in border zone cardiomyocytes.65

HGF/c-Met signaling mediates effects via multiple downstream targets, imparting prosurvival benefits in the heart via PI3K/Akt.63,64 Pharmacological inhibition of PI3K and Akt reduces Hes1 levels in neonatal cardiomyocytes stimulated with HGF or insulin (Figure 3C and 3D and supplemental Figure IIIB), indicating that HGF-induced increases in Hes1 are mediated through PI3K/Akt signaling, reinforcing the connection of Notch activity to known survival signaling networks in the heart. Together with HGF-mediated increases in Notch activation leading to higher levels of phospho-Akt(473) (Figure 4), an outside-in signal transduction cascade extends from HGF/c-Met to Notch and ultimately PI3K/Akt in the heart (see model, Figure 8).

Notch activation supports cell survival via PI3K induction,7,29 consistent with overexpression of activated Notch in neonatal rat cardiomyocytes and intact mouse myocardium elevating phospho-Akt(473) levels (Figure 5). Activated Notch overexpression stimulates proliferative signaling in the mouse male genital tract65 and in cardiomyocytes infected with adNICDEGP (Figure 6). Previous studies have investigated the role of embryonic signaling in myocardial repair.66 Border zone cardiomyocytes are known to remodel, reenter the cell cycle, and reexpress genes characteristic of earlier points in development.66,67 The relevance of these signals during regeneration is emerging as a focus of regenerative medicine.65,68 Relative contributions of antiapoptotic signaling versus reparative and regenerative activity in the functional improvements resulting from activated Notch1 overexpression in infarcted myocardium (Figures 7 and 8) remain to be determined. Manipulation of Notch expression in the heart will be key to dissecting its potential as a therapeutic agent.

Acknowledgments

We thank members of the laboratory of M.A.S. for helpful discussions and comments.

Sources of Funding

M.A.S. is supported by NIH grants RO1HL067245, 1RO1HL091102, and 1P01HL085577. Piero Anversa is the Principal Investigator of program project grant 1P01AG023071. N.A.G. is a Fellow of the Rees-Steady Research Foundation and the San Diego State University Heart Institute. G.E. and W.W. were supported by a summer stipend funded by AHA Scientific Counsels Student Scholarship in Cardiovascular Disease and Stroke.

Disclosures

None.

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Circ Res. 2008;102:1025-1035; originally published online March 27, 2008;
doi: 10.1161/CIRCRESAHA.107.164749
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/102/9/1025

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Materials and Methods

Surgical Procedures. 1) Myocardial infarction. Adult male FVB mice (2 – 3 Months Old) under isoflurane anesthesia were intubated and ventilated. A lateral thoracotomy was performed, exposing the anterior surface of the heart. The anterior descending branch of the left coronary artery was ligated using 8-0 suture (Ethicon). Sham operations were conducted by passing the suture under the coronary artery at the position used for ligation. Cyanosis and akinesia of the affected area were observed to confirm complete ligation of the coronary vessel, then the chest was closed. Hearts were harvested following cervical dislocation and the margin of ischemic and non-ischemic area was carefully dissected and snap frozen for western blot analysis. For immunohistochemistry analysis, hearts were arrested in diastole with relaxation buffer (333 mM KCl, 67 mM CdCl₂, 33 mM Tris pH 7.0) and were fixed in 4% Neutral Buffered Formalin for 24 hours. Tissues were embedded in paraffin and sectioned at 4 micron thickness using standard techniques. 2) Intramyocardial injection. Adult male mice were anesthetized using 2% Isoflurane, intubated, and an incision made between the 4th and 5th left ribs to expose the heart. Adenovirus encoding activated Notch-EGFP (NICDEGFP) or EGFP purified by FPLC to a titer of 1.0 x 10⁹ in PBS, HGF diluted in PBS (200 ng/mL) or PBS alone were delivered directly to the mouse myocardium. The pericardium was carefully removed and a 32 gauge blunt needle attached to a 10 microliter glass syringe (Hamilton Co., Reno) was advanced into the myocardium at a shallow angle to the surface of the heart. Five microliters of solution per injection were administered with six injections per
animal. Upon sacrifice, hearts were collected and the apical region was isolated and either snap frozen for western blot analysis or embedded in paraffin as described above. For intramyocardial injections of adenovirus following myocardial infarction, female C57Bl/6 mice were used. Injection of virus or vehicle occurred immediately after ligation, with five injections of five microliters placed at the margin of ischemic and non-ischemic tissue. Echocardiography was performed weekly to assess gross cardiac structure and function. After four weeks, in vivo hemodynamics was performed and hearts were harvested for paraffin embedding. Signals were quantitated using EMKA, Microsoft Excel and Prizm software. Values are expressed as average +/- standard deviation using one-way Anova with Tukey’s post hoc test to determine significance.

Immunohistochemistry and immunoblot analyses. Paraffin sections were treated for immunohistochemistry as previously described. For immunoblotting, neonatal rat cardiomyocytes (NRCMs) or mouse heart samples were lysed in sample buffer (150mM Tris pH6.8, 8M Urea, 50 mM DTT, 2% SDS, 15% sucrose, 2 mM EDTA, 0.01% bromophenyl blue, Sigma protease and phosphatase inhibitor cocktails), sonicated, boiled and run through a 4-12% Bis/Tris gel using 1X MES buffer (Invitrogen), transferred to Immobilon PVDF membrane (Millipore) and blocked in 10% nonfat milk in TBST (150 mM NaCl, 50 mM Tris pH 7.5, 0.1% Tween-20). Membranes were probed with primary antibodies overnight at 4°C in blocking buffer, washed in TBST, probed with secondary antibodies in blocking buffer at room temperature for 90 minutes, washed, and fluorescent signal detected using a Typhoon 9400 imaging system.
(Molecular Dynamics). Signals were quantitated using ImageQuant (Molecular Dynamics) and Microsoft Excel software. Values are expressed as average +/- standard deviation using Student’s T-test to determine significance. Antibodies used for immunoblot analyses include: goat anti-Notch1 (Santa Cruz #sc-6015), rabbit anti-Hes1 (Santa Cruz #sc-25392), goat anti-Jagged1 (RNDSystems #AF599), goat anti-Akt1/2 (Santa Cruz #sc-1619), rabbit anti pS473Akt (Biosource #44-621G or CST #9271), rabbit anti-pT308Akt (CST# 9275), rabbit anti-PDK1 (Biosource #AHZ0512), mouse anti-GAPDH (Chemicon #MAB374). Immunoprecipitation was performed using rabbit anti-c-myc (Sigma #C3956) or rabbit anti-GFP (Molecular Probes #A11122). Antibodies used for immunohistochemistry include: rabbit anti-cleaved Notch1 (Rockland #100-401-407), mouse anti-tropomyosin (Sigma #T9283), goat anti-DLL4 (RNDSystems #AF1389), goat anti-GFP (Rockland #600-101-215), goat anti-HGF (RNDSystems #AF2207), goat anti-Jagged1 (RNDSystems #AF599), rabbit anti-c-Met (Santa Cruz #sc-161), rat anti-Ki67 (Dako #M7249), rabbit anti-tropomodulin (Sussman), rat anti-Tenascin-C (RNDSystems #MAB2138), goat anti-c-kit (RNDSystems #AF1356),

Cell culture and treatments. NRCM were isolated and cultured as previously described. For activation of Notch, cells were incubated overnight in serum free medium containing 0.1% BSA and treated with recombinant mouse HGF (RNDSystems #1389-D4) or insulin (Sigma I5500) at the concentrations and times described. For inhibition of PI3K in insulin or HGF stimulated NRCMs, 30 µM LY294002 (Sigma L9908) was applied one hour prior to stimulation and
included in serum free/0.1% BSA media with HGF or insulin. For baseline inhibition, NRCMs were incubated in 20 µM PD98059 (Sigma P215), 10 µM Tricirbine (Akt inhibitor V, Calbiochem 124012) or 10 µM rapamycin (Alexis ALX-380-004) in media containing 2% FBS for three hours prior to harvest of whole cell lysates for immunoblot analysis. Adenoviruses used include adNICDEGFP courtesy of Dr. Ken Tezuka 3 and adEGFP from the University of Pittsburgh Cancer Institute Vector Core Facility. Antibodies used for immunocytochemistry include: mouse anti-α-actinin (Sigma # A7811), rabbit anti-PDK1 (Biosource #AHZ0512), mouse anti-PCNA (Santa Cruz # sc-56), rabbit anti-desmin (Biomed #V2022), rabbit anti-Hes1 (Santa Cruz # sc-25392).

Supplemental Figure Legends

Supplemental Figure 1: Notch1 and Hes1 levels decrease in mouse myocardium during postnatal development. A. Heart lysates from mice at 2, 14, 66 and 107 days postnatal age probed for cleaved Notch1 and Hes1. Gadph was used as a loading control. B. Quantitation of Hes1 normalized to gapdh expressed as relative fluorescence units. C. Quantitation of Notch1 normalized to gapdh expressed as relative fluorescence units. Values are shown as average +/- standard deviation, n=3, Significance is represented as p<.05 = *, p<.01 = **, p<.001 = ***.

Supplemental Figure 2: Overview of activated Notch and nuclear cMet localization in myocardium. (A,B) and non-myocyte tissues (C,D) four days post acute infarction. A. Immunostaining for endogenous activated Notch (Notch ICD, green), tropomyosin (red) and To-pro 3 (blue) in mouse myocardium four days post infarction. White arrows indicate border zone myocytes expressing activated Notch. B. Immunolocalization of nuclear cMet (green), tropomyosin (red) and To-pro 3 (blue) in serial section of A. White arrows point to border zone myocytes containing nuclear cMet. BZ and IZ denote border zone and infarct zone, respectively. Numbers next to arrows in A and B denote activated Notch and nuclear cMet in corresponding cells. C. Immunolocalization of activated Notch (NICD, red), c-kit (green), tenascin-C (yellow), tropomyosin (blue) and To-pro 3 (white) infarcted mouse myocardium four days post infarction. White arrow points to c-kit+ cell expressing activated Notch, shown enlarged in upper right corner. Larger white arrowheads point to c-kit+ cells.
lacking NICD, and small white arrowheads point to border zone myocytes expressing NICD. BZ and IZ denote border zone and infarct zone respectively. **D.** Immunolocalization of activated Notch (NICD, red), c-kit (green), tenascin-C (yellow) and tropomyosin (blue) in cardiac vasculature endothelium four days post infarction. White arrows point to c-kit+ endothelial cells expressing activated Notch (NICD, red).

**Supplemental Figure 3:** Phospho-Akt<sup>T308</sup> in HGF and insulin treated NRCMS (A) and Hes1 and phospho-Akt<sup>S473</sup> in NRCMs treated with signaling pathway inhibitors (B). **A.** Immunoblot detecting Hes1 and phospho-Akt<sup>T308</sup> in NRCMs treated with HGF (50ng/ml) or insulin (100mU/ml) for the times indicated. Hes1 levels increase 2-fold following six hours of treatment with HGF or insulin. Phospho-Akt<sup>T308</sup> levels are elevated only in insulin treated NRCMS after six hours. **B.** Immunoblot detecting Hes1 and phospho-Akt<sup>S473</sup> in NRCMs treated for five hours with vehicle (Ctl), 20µM PD98059 (PD, MAPKK inhibitor), 10µM triciribine (AktiV, Akt inhibitor V) and 10µM rapamycin (Rap, mTOR inhibitio). Hes1 levels are decreased 3.5-fold following specific inhibition of Akt by triciribine. Phospho-Akt<sup>S473</sup> levels decrease almost two-fold following triciribine treatment and increase in both PD98059 and rapamycin treated cells  p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***.

**Supplemental Figure 4:** Pdk1 accumulates in the nucleus of NICDEGFP infected NRCMs (A-C) and activated Notch and nuclear-targeted Akt associate when overexpressed in NRCMs (D). **A.** Immunostaining for Pdk1 (red) and sarcomeric αactinin (blue) in NICDEGFP infected myocytes (green).
Pdk1 accumulates in the nucleus. **B.** Immunostaining for Pdk1 (red) and sarcomeric actinin (blue) in EGFP infected myocytes (green). Pdk1 localization remains primarily cytoplasmic. **C.** Immunoblot detecting levels of Pdk1 in uninfected, adEGFP or adNICDEGFP NRCMs. Pdk1 levels increase by approximately 60% in whole cell lysates of NICDEGPF infected myocytes versus uninfected and adEGFP expressing controls. **D.** Immunoprecipitation of whole cell lysates coinfected with nuclear-targeted myc-tagged Akt and adNICDEGFP. Pulldown with anti-myc tag and immunoblot for gfp reveals association of nuclear-targeted Akt and NICDEGFP in NRCMs. Loading controls include myc-tag IP blotted for Akt as well NICDEGFP or EGFP infected lysates pulled down with gfp antibody and immunoblotted for gfp.

**Supplemental Figure 5.** Echocardiographic measurements of infarcted hearts receiving intramyocardial injection of TBS vehicle, adEGFP or adNICDEGFP at one, two and three weeks following surgery (A-C). Measurement of infarct size in hearts treated as in A-C harvested for paraffin embedding at four weeks postoperatively (D). **A.** Anterior wall dimension (AWD) is significantly greater in NICDEGFP treated versus vehicle treated hearts four weeks following treatment. **B.** Fractional Shortening (FS) and **C.** Ejection Fraction (EF) are significantly greater in NICDEGFP treated hearts four weeks following treatment compared to vehicle and EGFP treated controls. Significance determined by one-way Anova with Tukey’s post hoc test. TBS vs. NICD \( p < 0.05 = \& \). EGFP vs. NICD \( p < 0.05 = # \). **D.** Area of infarct was measured in paraffin cross sections of midwall left ventricle of hearts treated as
described above and immunostained for tropomyosin. Infarct area was divided by total left ventricle area.  

\[ p < 0.05 = ^* \text{adNICDEGFP vs. TBS, } p < 0.01 = ^{**} \text{adEGFP vs. adNICDEGFP.} \]
Supplemental Figure 1
Supplemental Figure 2A, 2B
Supplemental Figure 2D
Supplemental Figure 3
Supplemental Figure 4A-C
Supplemental Figure 4D
Supplemental Figure 5A-C
Supplemental Figure 5D

- * p<0.05 vs TBS
- ** p<0.01 vs NICD
A

B

C

TBS vs NICD p<0.05 = &
EGFP vs NICD p<0.05 = #
* p<0.05 vs TBS
** p<0.01 vs NICD