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Notch activated protective signaling in damaged mammalian myocardium

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by

Natalie A. Gude

Committee in charge:

University of California, San Diego

Professor James W. Posakony
Professor David Traver

San Diego State University

Professor Mark A. Sussman, Chair
Professor Christopher C. Glembotski, Co-chair
Professor Robert W. Zeller

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Chair

University of California, San Diego

San Diego State University

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DEDICATION

This dissertation is dedicated to my parents, Marion and Fred Lampe, my father Glauco Cambon, my husband Erick Gude and our daughter Audrey, for their encouragement, support and love. Thank you Erick and Audrey for cheering me on throughout the process. Audrey, you are never too old to learn and grow.

This thesis is also in memory of my father, Glauco Cambon, and my brother-in-law, Richard Cruse, whose lives were cut short by heart disease.

EPIGRAPH

Life is what happens to you while you're busy making other plans.

John Lennon

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LIST OF ABBREVIATIONS

αMyHC	alpha myosin heavy chain
AWD	anterior wall dimension
BrdU	bromodeoxyuridine
BZ	border zone
cDNA	complementary DNA
CPC	cardiac progenitor cell
CSC	cardiac stem cell
DLL4	delta-like 4
EF	ejection fraction
EGFP	enhanced green fluorescent protein
FS	fractional shortening
FGF	fibroblast growth factor
Hes	hairy enhancer of split
HGF	hepatocyte Growth Factor
HRT	hairy related transcriptional repressor
IF	immunofluorescence
IGF1	insulin like growth factor 1
IHC-P	immunohistochemistry paraffin
IP	immunoprecipitation
IZ	infarct zone
KNIC	Kopan Notch intracellular domain

LAD	left anterior descending coronary artery
mER	mutated estrogen receptor
MI	myocardial infarction
mNICD	mouse Notch intracellular domain
mTOR	mammalian target of rapamycin
NICD	Notch intracellular domain
NLSGFP	nuclear-targeted GFP
NRCM	neonatal rat cardiomyocyte
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PFA	paraformaldehyde
qPCR	quantitative real time polymerase chain reaction
SMA	smooth muscle actin
T-ALL	T-cell lymphoblastic leukemias and lymphomas
TAC	trans aortic constriction

LIST OF FIGURES

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VITA

Education:
9/78-6/82
Stanford University, Stanford, CA. B.S. and M.S. in Biology.

9/87-1/88:
University of Washington, Seattle, WA. Completed one term of coursework for the Ph.D. Program in Cellular and Molecular Biology.

09/05-06/10:
SDSU/UCSD San Diego, CA. Joint Doctoral Program. Ph.D. in Cellular and Molecular Biology.

Honors and Awards: Rees-Stealy Research Foundation/SDSU Heart Institute Graduate Student Fellow 5/06-07/10. AHA predoctoral fellowship 07/08-07/10. President's Award, SDSU Student Research Symposium 02/08.

Experience:
12/03-present:
Research Scientist I/Lab Manager. Dr. Mark Sussman, PhD., Department of Biology, SDSU Research Foundation, San Diego, CA. Assist in supervising lab personnel. Coordinate inspections, schedule lab meetings and journal club, write protocols, maintain compliance, assure smooth function of the laboratory. Responsible for setting up, maintaining and training others to use histopathology core equipment. Research entails examination of embryonic signaling in pathologically challenged adult mouse heart, as well as localization and characterization of stem cells in normal, transgenic and pathologic cardiac tissue using biochemistry, molecular biology and confocal scanning laser microscopy.

11/98-12/03:
Staff Research Associate II/Lab Manager. Dr. Maurice Montal, Ph.D., Division of Biology, UCSD, La Jolla, CA. Responsible for maintaining laboratory, keeping records, ordering supplies and preparing lab for government inspection. Hired and trained undergraduates to maintain laboratory and to PCR genotype mouse DNA. Conducted research and wrote manuscript on regulated secretion in PC12 cells transfected with SNARE deletion constructs. Involved in cloning, expression and purification of bacterial voltage gated potassium channels. Began development of a high throughput screening FRET assay for channel activity of the botulinum toxin heavy chain serotype A in collaboration with Vertex Corporation.

7/96-11/98:
Research Assistant III. Dr. Mark Sussman, Ph.D., Molecular Cardiovascular Biology, Children's Hospital, Cincinnati, OH. Helped characterize tropomodulin (TOT) and rac (ROT) overexpressing transgenic mouse models for dilated cardiomyopathy created in the Sussman laboratory. Co-developed method for imaging isolated cardiomyocytes using immunofluorescence and confocal laser microscopy. Isolated, cultured and stained neonatal rat cardiomyocytes infected with tropomodulin and rac adenovirus. Examined signal transduction in TOTs by western blot and immunoprecipitation using antibodies to phospho-proteins. Performed morphometric analysis on scanned sections using Image Space software. Adapted Progeny relational database for documentation of transgenic mouse lineages. Assisted in genotyping mouse lines using PCR and chemifluorescent Southern and Northern blotting technology. Experience with whole mount immunostaining of mouse embryos. Trained coworkers to use the Molecular Dynamics confocal laser microscope.

1/95-1/96:
Laboratory Manager/Research Assistant. Dr. Narsing Rao, MD, Ph.D., Ophthalmic Pathology, Doheny Eye Institute, Los Angeles, CA. Responsible for routine processing, sectioning and staining of patient samples. Supervised and trained assistant to process patient samples and prepare laboratory for government inspection. Research entailed localization of cytokine mRNA and protein in rat eye and other tissues during the progression of uveitis. Performed RT-PCR, in situ hybridization and immunostaining.

4/94-12/94:
Research Associate III. Kevin Stark, Ph.D., Amgen, Inc., Developmental Biology, Thousand Oaks, CA. Set up a small core facility for immunohistochemistry and in situ hybridization. Localized protein tyrosine kinase receptor mRNA and protein in developing and adult mouse brain. Some experience with whole mount in situ hybridization of mouse embryos. Participated in the characterization of novel genes identified at Amgen.

5/89-12/93:
Research Assistant. Steven D. Russell, Novo/Nordisk, Department of Islet Molecular Biology, Bagsvaerd, Denmark. Investigated hepatic and pancreatic islet gene expression and regulation in rodent models of type I and II diabetes. Examined the role of glucose and thyroid hormone in type I 5' deiodinase expression in rodent liver and pancreas. Used Northern analysis and immunoblotting, non-radioactive in situ hybridization and immunohistochemistry to detect mRNA and protein in rodent and human pancreatic islet cells. Performed small animal surgery, dissection, and preparation of paraffin and fresh frozen tissue sections. Trained graduate students.

3/85-5/89:
Research Assistant. Robert G. Whalen, Pasteur Institute, Department of Molecular Biology, Paris France. Analyzed expression of myosin heavy chain isoforms in rodent and human cardiac and skeletal muscle. Characterized thyroid hormone regulation of the developmental B-to-a myosin heavy chain transition in Snell dwarf mice. Performed SDS-PAGE and native gel electrophoresis, peptide cleavage mapping, Western blotting, and ATPase and immunofluorescent staining of cryostat sections.

1/83-3/85:
Research Technician. Dr. Helen B. Burch, Ph.D., Washington University School of Medicine, Department of Pharmacology, St. Louis, MO. Measured metabolic enzyme activity and substrate levels in rodent nephron using quantitative histochemistry techniques developed by Dr. Oliver Lowry. Performed macroassays on kidney homogenates and microassays on discreet regions of freeze-dried individual nephrons.

6/82-9/82:
Teaching Assistant. Dr. Donald P. Abbott, Ph.D., Stanford University, Hopkins Marine Station, Pacific Grove, CA. Prepared laboratory materials, maintained seawater lab, assisted students with lab assignments, graded notebooks, and supervised collecting trips for Marine Invertebrates class.

Other:
6/96-7/96:
Temporary Adjunct Biology Faculty. Dr. Sue Rollins, Continuing Education, Drury College, Springfield, MO. Taught an intensive introductory biology course with laboratory. Lectured, supervised laboratory exercises, designed and graded exams.

Publications:

Borillo GA, Mason M, Quijada P, Völkers M, Cottage C, McGregor M, Din S, Fischer K, **Gude N**, Avitabile D, Barlow S, Alvarez R, Truffa S, Whittaker R, Glassy MS, Gustafsson AB, Miyamoto S, Glembotski CC, Gottlieb RA, Brown JH, Sussman MA. Pim-1 kinase protects mitochondrial integrity in cardiomyocytes. Circ Res. 2010 Apr 16;106(7):1265-74.

Voelkers M, Salz M, Herzog N, Frank D, Dolatabadi N, Frey N, **Gude N**, Friedrich O, Koch WJ, Katus HA, Sussman MA, Most P. Orai1 and Stim1 regulate normal and hypertrophic growth in cardiomyocytes. J Mol Cell Cardiol. 2010 Jun;48(6):1329-34.

Huang C, Zhang X, Ramil JM, Rikka S, Kim L, Lee Y, **Gude NA**, Thistlethwaite PA, Sussman MA, Gottlieb RA, Gustafsson AB. Juvenile exposure to anthracyclines impairs cardiac progenitor cell function and vascularization resulting in greater susceptibility to stress-induced myocardial injury in adult mice. Circulation. 2010 Feb 9;121(5):675-83.

Cottage CT, Bailey B, Fischer K, Avitabile D, Collins B, Tuck S, Quijada P, **Gude N**, Alvarez R, Muraski J and Sussman MA. Cardiac progenitor cell cycling stimulated by Pim-1 kinase. Circ Res. 2010 Jan 14.

Belmont PJ, Chen WJ, San Pedro MN, Thuerauf DJ, Gellings Lowe N, **Gude N**, Hilton B, Wolkowicz R, Sussman MA, Glembotski CC. Roles for Endoplasmic Reticulum-Associated Degradation of the Novel Endoplasmic Reticulum Stress Response Gene Derlin-3 in the Ischemic Heart. Circ Res. 2009 Nov. 25.

Fischer KM, Cottage, C.T., Wu, W., Din, S., **Gude, N.A**., Avitabile, D., Quijada, P., Fransioli, J., Sussman, M.A. Enhancement of myocardial regeneration through genetic engineering of cardiac progenitor cells expressing Pim-1 kinase. *Circulation.* 2009, Nov. 24;120(21):2077-87.

Rubio M, Avitabile D, Fischer K, Emmanuel G, **Gude N,** Miyamoto S, Mishra S, Schaefer EM, Brown JH, Sussman MA. Cardioprotective stimuli mediate phosphoinositide 3-kinase and phosphoinositide dependent kinase 1 nuclear accumulation in cardiomyocytes. J Mol Cell Cardiol. 2009 Mar 5.

Whittaker R, Glassy MS, **Gude N**, Sussman MA, Gottlieb RA, Glembotski CC. Kinetics of the Translocation and Phosphorylation of {alpha}B-crystallin in Mouse Heart Mitochondria during Ex Vivo Ischemia. Am J Physiol Heart Circ Physiol. 2009 Feb 27.

Tadimalla A, Belmont PJ, Thuerauf DJ, Glassy MS, Martindale JJ, **Gude N**, Sussman MA, Glembotski CC. Mesencephalic astrocyte-derived neurotrophic factor is an ischemia-inducible secreted endoplasmic reticulum stress response protein in the heart. Circ Res. 2008 Nov 21;103(11):1249-58.

Muraski JA, Fischer KM, Wu W, Cottage CT, Quijada P, Mason M, Din S, **Gude N**, Alvarez R Jr, Rota M, Kajstura J, Wang Z, Schaefer E, Chen X, MacDonnel S, Magnuson N, Houser SR, Anversa P, Sussman MA. Pim-1 kinase antagonizes aspects of myocardial hypertrophy and compensation to pathological pressure overload. Proc Natl Acad Sci U S A. 2008 Sep 16;105(37):13889-94.

Siddiqi S, **Gude N**, Hosoda T, Muraski J, Rubio M, Emmanuel G, Fransioli J,

Vitale S, Parolin C, D'Amario D, Schaefer E, Kajstura J, Leri A, Anversa P, Sussman MA. Myocardial induction of nucleostemin in response to postnatal growth and pathological challenge. Circ Res. 2008 Jul 3;103(1):89-97.

Gude NA, Emmanuel G, Wu W, Cottage CT, Fischer K, Quijada P, Muraski JA, Alvarez R, Rubio M, Schaefer E, Sussman MA. Activation of Notch-mediated protective signaling in the myocardium. Circ Res. 2008 May 9;102(9):1025-35.

Belmont PJ, Tadimalla A, Chen WJ, Martindale JJ, Thuerauf DJ, Marcinko M, **Gude N**, Sussman MA, Glembotski CC. Coordination of growth and endoplasmic reticulum stress signaling by regulator of calcineurin 1 (RCAN1), a novel ATF6-inducible gene. J Biol Chem. 2008 May 16;283(20):14012-21.

Fransioli J, Bailey B, **Gude NA**, Cottage CT, Muraski JA, Emmanuel G, Wu W, Alvarez R, Rubio M, Ottolenghi S, Schaefer E, Sussman MA. Evolution of the c-kit-positive cell response to pathological challenge in the myocardium. Stem Cells. 2008 May;26(5):1315-24.

John A Muraski, Marcello Rota, Yu Misao, Jenna Fransioli, Christopher Cottage, **Natalie Gude**, Grazia Esposito, Francesca Delucchi, Michael Arcarese, Roberto Alvarez, Sailay Siddiqi, Gregory N Emmanuel, Weitao Wu, Kimberlee Fischer, Joshua J Martindale, Christopher C Glembotski, Annarosa Leri, Jan Kajstura, Nancy Magnuson, Anton Berns, Remus M Beretta, Steven R Houser, Erik M Schaefer, Piero Anversa & Mark A Sussman. (2007) Pim-1 regulates cardiomyocyte survival downstream of Akt. Nature Medicine, Vol. 13, pp. 1467-1475.

Natalie Gude, John Muraski, Marta Rubio, Jan Kajstura, Erik Schaefer, Piero Anversa, Mark A. Sussman. (2006) Akt promotes increased cardiomyocyte cycling and expansion of the cardiac progenitor cell population. Circulation Research, Vol. 99, pp. 381-8.

Joshua J. Martindale, Rayne Fernandez, Donna Thuerauf, Ross Whittaker, **Natalie Gude**, Mark A. Sussman, Christopher C. Glembotski. (2006) Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6. Circulation Research, Vol. 98, pp. 1186-93.

Donna J. Thuerauf, Marie Marcinko, **Natalie Gude**, Marta Rubio, Mark A. Sussman, Christopher C. Glembotski. (2006) Activation of the Unfolded Protein Response in Infarcted Mouse Heart and Hypoxic Cultured Cardiac Myocytes. Circulation Research, Vol. 99, pp. 275-82.

Sussman, M.A., Welch, S., **Gude, N**., Khoury, P.R., Daniels, S.R., Kirkpatrick, D., Walsh, R.A., Price, R.L., Lim, H.W., Mollkentin, J.D. (1999) Pathogenesis of dilated cardiomyopathy: molecular, structural, and population analyses in tropomodulin-overexpressing transgenic mice. American Journal of Pathology, Vol. 155, pp. 2101-2113.

Sussman, M.A., Welch, S., **Cambon, N**., Klevitsky, R., Hewett, T.E., Price, R., Witt, S.A. and Kimball, T.R. (1998) Myofibril degeneration caused by tropomodulin overexpression leads to dilated cardiomyopathy in juvenile mice. Journal of Clinical Investigation, Vol. 101, pp. 51-61.

Sussman, M.A., Lim, H.W., **Gude, N**., Taigen, T., Olson, E.N., Robbins, J., Colbert, M.C., Gualberto, A., Wieczorek, D.F., and Molkentin, J.D. (1998) Prevention of cardiac hypertrophy in mice by calcineurin inhibition. Science, Vol. 281, pp. 1690-1693.

Natalie Cambon and Mark Sussman (1997) Isolation and preparation of single mouse cardiomyocytes for confocal microscopy. Methods in Cell Science, Vol. 19, pp. 83-90.

Blume, N., Russell, S.D., **Cambon, N**., Petersen, E.E., Larsson, L.E., Madsen, O.D. (1993) Transplantation of alpha and beta cell tumors selectively suppress corresponding hormone gene expression in rats. Diabetologia, Vol. 36, suppl. 1249.

Russell, S.D., **Cambon, N.A**. and Whalen, R.G. (1993) Two types of neonatal-to-adult fast myosin heavy chain transitions in rat hindlimb muscle fibers. Developmental Biololgy, Vol. 157, pp. 359-370.

Pruliere, G., Butler-Browne, G.S., **Cambon, N**., Toutant, M. and Whalen, R.G. (1989) Induction and stability of an adult myosin phenotype in dwarf mice after repeated injections of thyroid hormone. European Journal of Biochemistry, Vol. 185, pp. 555-561.

Russell, S.D., **Cambon, N**., Nadal-Ginard, B. and Whalen, R.G. (1988) Thyroid hormone induces a nerve-independent precocious expression of fast myosin heavy chain mRNA in rat hindlimb skeletal muscle. Journal of Biological Chemistry, Vol. 263, pp. 6370-6374.

Butler-Browne, G.S., Pruliere, G., **Cambon, N**. and Whalen, R.G. (1987) Influence of the dwarf mouse mutation on skeletal and cardiac myosin isoforms. Journal of Biological Chemistry, Vol. 262, pp. 15188-15193.

Bastin, J., **Cambon, N**., Thompson, M., Lowry, O.H. and Burch, H.B. (1987) Change in the energy reserves in the different segments of the nephron during brief ischemia. Kidney International, Vol. 31, pp. 1239-1247.

Burch, H.B., Brooks, C.A., **Cambon, N**., Bastin, J., and Lowry, O.H. (1985) Effect of ureter obstruction on metabolic enzymes in different parts of the nephron. In “Kidney Function and Metabolism” (Eds R. Dzurik, B. Lichardus, and W. Guder) M. Nijhoff Pub., Dordrecht.

Hintz, C.S., Turk, W.R., **Cambon, N**., Burch, H.B., Nemeth, P.M. and Lowry, O.H. (1985) A method for branched-chain amino acid aminotransferase activity in microgram and nanogram tissue samples. Analytical Biochemistry. Vol. 146, pp. 418-422.

Burch, H.B., **Cambon, N.** and Lowry, O.H. (1985) Branched-chain amino acid aminotransferase along the rabbit and rat nephron. Kidney International, Vol. 28, pp. 114-117.

ABSTRACT OF THE DISSERTATION

Notch activated protective signaling in damaged mammalian myocardium

By

Natalie A. Gude

Doctor of Philosophy in Biology

University of California, San Diego 2010

San Diego State University 2010

Professor Mark A. Sussman, Chair

Professor Christopher C. Glembotski, Co-Chair

Rebuilding the human myocardium following pathological injury remains a significant challenge both at the level of basic research and clinical application. In the last decade, cell based therapy has emerged as a promising option for repairing damaged heart tissue, but a universally available treatment still eludes the medical profession. The work presented here investigates the cardiac response to injury in the context of crosstalk

between stem cell and survival kinase signaling. The hypothesis of this thesis is that activated Notch participates in a HGF/PI3K/Akt mediated survival signaling response to cardiac injury, and that Notch signaling drives cardiac differentiation of cardiac progenitor cells (CPCs).

Notch, a critical cue in the developing heart, is activated in damaged myocardium. Likewise, following myocardial infarction, c-Met, the tyrosine kinase receptor for hepatocyte growth factor (HGF), colocalizes with activated in nuclei of surviving cardiomyocytes. HGF stimulates Notch activity in neonatal rat cardiomyocytes (NRCMs) when applied *in vitro* and when injected into the intact heart. Moreover, exogenous Notch increases levels of cardiac phospho-Akt both *in vitro* and *in vivo*. Genetic tools were developed to manipulate activated Notch signaling and investigate regenerative/survival signaling crosstalk in intact heart as well as cardiac progenitor cells. A regulatable, intracellular Notch (KNIC) fused to the mutated estrogen receptor (mER) was created and tested for induction by tamoxifen. KNICmER was engineered into adenovirus for transient expression and lentivirus for stable integration into cardiac progenitor cells. Likewise, cardiac specific transgenic mice were created expressing KNICmER, and this construct will be subcloned downstream of the c-kit promoter to create a transgenic line with regulatable Notch activity in the progenitor population. These reagents will be applied in future studies aimed at improving the inherent reparative capacity of the

mammalian heart as well as enhancing the regenerative success of adoptive transfer of cardiac progenitor cells into the damaged myocardium.

Overall, this study demonstrates that activated Notch participates in cardiac protection following pathologic injury, in part by activation of the PI3K/Akt survival pathway, and that activated Notch also impacts cardiac progenitors by pushing them toward a cardiogenic lineage.

INTRODUCTION OF THE DISSERTATION

Heart disease statistics are sobering. The American Heart Association estimates that in 2006, 80 million Americans had some form of cardiovascular disease and that cardiovascular disease comprised more than one third of all deaths in the US in 2005. Simply put, one in three Americans have heart disease, and over one in three deaths are a consequence of heart disease. Heart attack, or myocardial infarction, falls into the subset of ailments known as coronary artery disease. Blockage of coronary arteries, the vessels feeding the heart, starves the downstream tissue of oxygen and nutrients, resulting in myocardial death. Eventually, scar tissue replaces what was once contractile myocardium, compromising overall heart function. Treatments currently available for heart disease improve function of the surviving myocardium, but do not repair or replace the heart tissue that is lost following injury. The latest wave of cardiac research focuses on cardiac regeneration and potential cell-based therapies for rebuilding damaged hearts; stem cell therapy for heart disease is at the forefront of cardiovascular medicine. As many laboratories investigate the regenerative potential of what was once thought to be a post-mitotic organ incapable of self repair, understanding the interactions of survival signaling pathways and stem cell regenerative cascades is essential to devising therapies that will exploit the inherent regenerative capacity of the heart.

Notch is a stem cell regulatory protein critical to proper embryonic heart development. 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 HGF/c-Met receptor and PI3K/Akt signaling cascades. The PI3K/Akt survival signaling axis has been well documented in adult myocardium. The work presented in Chapter I of this thesis investigates the role of Notch as a survival and regenerative signal in damaged adult heart with a specific focus on crosstalk between the Notch and PI3K/Akt signaling cascades.

The impact of HGF upon Notch signaling was assessed following myocardial infarction as well as in cultured cardiomyocytes. In the infarcted mouse heart, Notch1 is activated in border zone cardiomyocytes and is 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 that treatment with HGF or insulin increases levels of the Notch effector Hes1, while overexpression of the activated Notch intracellular domain prompts a four-fold increase in phosphorylated Akt. Infarcted hearts injected with recombinant adenovirus encoding Notch intracellular domain (NICD) exhibit improved hemodynamic function compared to control mice four weeks following infarction and treatment with virus, implicating Notch signaling in a cardioprotective role following cardiac injury. These results indicate that Notch

activation in cardiomyocytes is mediated through c-Met and Akt survival signaling pathways, and that Notch1, in turn, enhances Akt activity. This mutually supportive crosstalk suggests a positive survival feedback mechanism between Notch and Akt in the adult myocardium following injury.

In Chapters II and III, a regulatable Notch-mER fusion construct (KNICmER) was developed to provide temporally controlled activation of Notch signaling in cardiac myocytes and progenitor cells *in vitro* and *in vivo.* Activation of KNICmER in NRCMs increased transcripts of known Notch target Hes1 and increased activity of the RBPJ-k luciferase reporter. Interestingly, transcript levels of Notch ligand Jagged1 were also upregulated, implicating Jagged1 as a potential Notch target or as part of a feedback mechanism within the Notch signaling pathway, consistent with the model proposed in Chapter I. The mouse model expressing KNICmER in cardiomyocytes provides an interesting system to explore the role of Notch activation in cardiac regeneration both in myocytes and cardiac progenitors.

In addition to Notch signaling in surviving myocardium, Chapter III addresses the impact of Notch activation in cardiac progenitor cells. Controlling differentiation of CPCs into the desired cardiac lineages to form functional cardiomyocytes and vasculature is an important goal of cardiac stem cell research. As a known effector of stem cell renewal or lineage commitment, Notch serves as an excellent target for manipulation of CPC

differentiation. Extended Notch activity in CPCs pushes them away from the progenitor phenotype, possibly toward a smooth muscle or pre-myocyte lineage. Experiments with the KNICmER construct indicate that transcripts of Notch network members Hes1, Hey1 and Jagged1 all increase in CPCs following induction of Notch activity with tamoxifen. Additionally, smooth muscle actin (SMA), an marker of smooth muscle lineage and recently shown to be a transcriptional target of Notch, increases both at the transcript and protein level in CPCs following KNICmER activation. Conversely, CD31 transcript levels decrease with Notch activation. The KNICmER CPC lines will provide valuable reagents for investigating the role of Notch activity in CPC differentiation and for enhancing reparative capacity of adoptively transferred CPCs used in cardiac cell based therapies. Finally, the transgenic mouse proposed in Chapter III, c-kit-KNICmER, provides an *in vivo* correlate for adoptive transfer experiments. As opposed to limited numbers of KNICmER CPCs applied to a damaged heart, the entire endogenous stem and progenitor population in a c-kitKNICmER system is engineered to respond to Notch activation by tamoxifen. Overall, this dissertation presents Notch as a mediator of cardioprotection and repair both in the myocardium and cardiac progenitor populations.

CHAPTER I:

NOTCH-MEDIATED PROTECTIVE SIGNALING IN THE MYOCARDIUM.

Introduction

Two decades have passed since the Notch gene was originally cloned in Drosophila melanogaster, with pursuant research resulting in a substantial body of literature.[1-7] 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, have recently been touted as a potential approach for enhancing stem cell expansion for regenerative medicine.[8] In the context of the myocardium, studies with embryonic tissues implicate Notch in cell fate determination and suppression of cardiogenic signaling, [9-12] and Notch has a longstanding association with regulation of cardiac development and morphogenesis.[13-16] 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, the relevance of 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 four orthologs in mammals, is a large transmembrane protein important for cell fate decisions, cellular development, differentiation, proliferation, and apoptosis.[17,

[18] Notch signaling plays an important role in embryonic heart development [19-22], while defective Notch1 protein has been linked to aortic valve disease.[23] Notch receptor activation occurs via binding to Notch ligands, the Delta and Serrate families in Drosophila, or Delta-likes and Jagged families in mammals. Target genes of Notch signaling include the basic helix-loop-helix transcription factors Hairy and enhancer of split 1 (Hes1) and the Hairy-related transcription factor family (HRT), which are found in the developing and adult heart.[24] HRT proteins bind GATA transcription factors and inhibit GATA4-mediated transcription of ANP in vitro; Akt1/PKB alleviates this repression through an unknown mechanism.[25]

Notch signaling is triggered through binding to cognate ligands such as Delta. Activation of the c-Met receptor induces expression of Delta in MDCK cells, activating Notch and upregulating expression of Hes1. Hes1, in turn, represses transcription of c-Met, indicating a negative feedback loop for c-Met activity.[26] c-Met is a receptor for hepatocyte growth factor (HGF) that is increased in hypertrophic and infarcted cardiac tissue.[27, 28] Upon 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 [8, 29-32], and the Akt/PKB cascade is a well-known effector of cell survival in the myocardium.[33-35] 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, the presence of c-Met on cardiac stem cells (CSC) may facilitate HGF-dependent migration of these cells to sites of damage, with enhanced survival, thereby directing repair or regeneration of infarcted myocardium.[36] Elucidating interplay between Notch, c-Met and PI3K/Akt signaling in pathologically challenged myocardium constitutes the focus of this study.

Methods

Surgical Procedures

Myocardial infarction (MI). 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, after which 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 a thickness of 4 microns using standard techniques.

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[37]. 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% bromomphenyl 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 4ºC 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 9410 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

NRCMs were isolated and cultured as previously described[38]. 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 MAPK inhibitor (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[39] 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 (Biomeda #V2022), rabbit anti-Hes1 (Santa Cruz # sc-25392).

Results

Notch expression and signaling declines during postnatal development

The relationship between postnatal development and expression levels for cleaved Notch1 or downstream target protein Hes1 protein (indicative of Notch activity) was assessed. Notch1 and Hes1 levels are highest in neonatal heart samples and stabilize by two months postnatal age and thereafter (Figure 1.9A). Quantitation reveals that Hes1 levels decrease 4 to 5-fold from two days to two months (Figure 1.9B), whereas cardiac Notch1 levels decrease approximately 3-fold during postnatal development (Figure 1.9C).

Notch1 is activated in adult injured myocardium

Notch levels decrease in adult myocardium relative to neonatal tissue (Figure 1.1) but increase in response to acute infarction by permanent coronary artery occlusion. Four days after infarction, nuclear Notch accumulates in surviving cardiomyocytes restricted to the border zone of the infarct region (Figure 1.10A, white arrows, Figure 1.1A, 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 1.1B, white arrows, Jagged1, green in overlay), whereas DLL4 is predominantly expressed in interstitial areas (Figure 1.1C, white arrow, DLL4, green in overlay). Interstitial cells labeled for both DLL4 and Notch (Figure 1.1C, arrow) or cardiomyocytes positive for activated Notch (Figure 1.1C, arrowhead) were observed. Corresponding immunoblot analysis

of myocardial lysates taken from the combined infarct and border zone region demonstrates that Notch1, Hes1 and Jagged1 levels are significantly increased four days post infarction (Figure 1.1D, E and F respectively), indicative of Notch activation. Notch signaling is also observed in non-myocyte tissue in infarcted myocardium, as indicated by a c-kit+ cell (Figure 1.10C, green in overlay) expressing activated Notch (NICD, red in overlay) in the tenascin-C rich border zone area or c-kit+ (green) cells lining a vessel wall (Figure 1.10D, 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 signaling prompted examination of infarcted tissue for immunolocalization of HGF and c-Met. HGF levels increase following acute infarction.[40-45] Confocal microscopy reveals strong HGF staining of vessel walls in proximity to the infarct, as well as localization in the infarct region (Figure 1.2A, 1.2B, white arrow, HGF green in overlay, BZ denotes border zone, IZ denotes infarct zone) at four days post injury. In comparison, nuclear staining for c-Met is observed in border zone cardiomyocytes (Figure 1.10B, white arrows, c-Met green in overlay, Figure 1.2B, white arrowheads, c-Met red in overlay, Figure 1.2C, white arrows, c-Met green in overlay). In Figure 1.2B, 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 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, wherein Hes1 levels increased within five minutes and remained elevated for six hours (Figure 1.3A, 1.3B). In comparison, the effect of HGF is similar to insulin-mediated Hes1 induction (Figure 1.3B, Figure S3A). Both HGF and insulin promote Akt activation, so involvement of Akt in induction of Hes1 expression was examined by pretreatment of NRCM with PI3K inhibitor LY294002 prior to 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 1.3C, 1.3D). Inhibition of MAPKK 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 (Figure 1.11B), indicating specific crosstalk between Akt and Notch signaling in NRCMs. These findings demonstrate that the PI3K/Akt pathway mediates activation of Notch1 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 1.4A). 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 two-fold increase in the area of myocardial tissue expressing Notch and a four-fold increase in the average distance extending from the injection lesion to the perimeter of Notch activation (Figure 1.4B). Myocardial samples harvested at multiple time points following HGF injection exhibit a 2.5 fold increase in phospho-Akt^{S473} levels compared to PBS injected controls (Figure 1.4D). Similarly, Notch activation increases two-fold in the same samples (Figure 1.4C). No significant induction of phospho-Akt^{T308} was observed in these samples (data not shown). Previous studies have shown differential phosphorylation of Akt at the T308 and S473 residues.[46] Collectively, these results indicate that HGF activates 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 the human Notch intracellular domain fused to EGFP (adNICDEGFP, gift of K. Tezuka [39]), which accumulates in the nuclei of cardiomyocytes (Figure 1.5A). NRCMs overexpressing adNICDEGFP show induction of Hes1 by immunoblot analysis (Figure 1.5A) relative to cells expressing EGFP (adEGFP), demonstrating that this construct is active in our system. Levels of phospho-Akt^{S473} are increased six-fold in the adNICDEGFP versus noninfected and three-fold over adEGFP infected NRCMs (Figure 1.5A). The ability of adNICDEGFP to activate Akt was confirmed in the adult

myocardium by direct injection of the virus into the apex of hearts. Immunolabeling for EGFP verifies viral expression three days after injection (Figure 1.5B). Immunoblotting reveals elevated phospho-Akt^{S473} levels in adNICDEGFP-injected tissue compared to PBS or adEGFP-injected tissue (Figure 1.5C). In addition to Akt activation, NRCMs overexpressing adNICDEGFP exhibit increased nuclear localization of Pdk1, as well as increased overall levels of Pdk1 compared to noninfected and adEGFP overexpressing controls (Figure 1.12A-C). Interestingly, Pdk1 is required for Notch-mediated trophic and proliferative effects in T-cell development.[47] Additionally, pulldown experiments in lysates of NRCMs overexpressing both adNICDEGFP and nuclear-targeted myc-tagged Akt reveal association between these two exogenously expressed proteins (Figure 1.13D). 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 versus nontransgenic epididymal epithelial cells.[48] PCNA is detected in the nucleus of adNICDEGFP-overexpressing myocytes (Figure 1.6A) but nuclear localization is not seen in adEGFP infected cells (Figure 1.6B), whereas surviving myocytes expressing adenoviral activated Notch

stained positive for Ki67 (Figure 1.6C). 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 (Figs. 1.3 – 1.5) prompted studies to determine if 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 three days post infarction and injection (Figure 1.7A and 1.7B, respectively). Heart function was assessed by *in vivo* hemodynamic analysis four 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 1.7C). Additionally, echocardiographic analysis at 1, 2 and 3 weeks after infarction reveal improved anterior wall thickness in adNICDEGFP versus vehicle treated hearts (Figure 1.13A) and function in adNICDEGFP versus vehicle and adEGFP treated hearts (Figures 1.13B and 1.13C). Infarct area was also decreased in adNICDEGFP versus vehicle or adEGFP-treated hearts four weeks post infarction, as demonstrated in Figure 1.13D. Overall these functional and morphological analyses demonstrate that transient overexpression of exogenous activated Notch in infarcted myocardium attenuates heart failure four weeks post treatment.

Discussion

This study shows that convergence of Notch, c-Met and Akt pathways in border zone cardiomyocytes of infarcted myocardium 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.[49] The Notch pathway crosstalks with other signaling pathways including bidirectional influences upon and from the PI3K/AktB cascade,[29, 30, 47, 50] and HGF/c-Met signaling leads to Notch activity in tumor cell lines.[26] Interplay of these three signaling pathways contributes to cardioprotective effects conferred by activated Notch.

Levels of embryonic and neonatal cardiac proteins such as beta myosin heavy chain, 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 (Figure 1.9). 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.[51-56] Re-expression of fetal genes is an adaptive response following pathological insult in adult myocardium, and increased Notch signaling in the context of acute infarction

(Figure 1.1 and Figure 1.10) is associated with survival of myocardium as well as possibly signaling to regenerate damaged tissue.

c-Met and HGF are expressed during early cardiac development.[57] Multiple studies document elevated HGF levels following myocardial infarction [40-42, 44, 45, 58] as well as protective effects conferred upon myocytes following cardiac ischemic insult.[28, 59-64] Additionally, c-Met nuclear localization occurs in low density cell culture.[65] 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.[26]

HGF/c-Met signaling mediates effects via multiple downstream targets, imparting prosurvival benefits in the heart via PI3K/Akt.[66, 67] Pharmacological inhibition of PI3K and Akt reduces Hes1 levels in neonatal cardiomyocytes stimulated with HGF or insulin (Figure 1.3C and 1.3D, Figure 1.11B), 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^{S473} (Figure 1.4), an outside-

in signal transduction cascade extends from HGF/c-Met to Notch and ultimately PI3K/Akt in the heart (see model, Figure 1.8).

Notch activation supports cell survival via PI3K induction, [8, 30] consistent with overexpression of activated Notch in neonatal rat cardiomyocytes and intact mouse myocardium elevating phospho-Akt^{S473} levels (Figure 1.5). Activated Notch overexpression stimulates proliferative signaling in the mouse male genital tract [48] and in cardiomyocytes infected with adNICDEGP (Figure 1.6). Previous studies have investigated the role of embryonic signaling in myocardial repair.[68] Border zone cardiomyocytes are known to remodel, reenter the cell cycle and re-express genes characteristic of earlier points in development.[69, 70] The relevance of these signals during regeneration is emerging as a focus of regenerative medicine.[68, 71] Relative contributions of anti-apoptotic signaling versus reparative and regenerative activity in the functional improvements resulting from activated Notch1 overexpression in infarcted myocardium (Figure 1.7, 1.8) remain to be determined. Manipulation of Notch expression in the heart will be key to dissecting its potential as a therapeutic agent.

Chapter 1, in full, has been published in Circulation Research 102(9):1025-1035, 2008. Activation of Notch-mediated protective signaling in the myocardium. Gude NA, Emmanuel G, Wu W, Cottage CT, Fischer K, Quijada P, Muraski JA, Alvarez R, Rubio M, Schaefer E, Sussman MA. The dissertation author was the primary investigator and author of this paper.

Figures

Figure 1.1: Notch signaling proteins are expressed in injured myocardium.
A. Paraffin sections from hearts subjected to acute infarction harvested four days post 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 = border zone, IZ = infarcted zone. **(D-F)** Mouse hearts subjected to acute infarction with border zone regions harvested at four days post-infarction. gapdh is used as a loading control and quantitation is in relative fluorescence units. **D.** Activated Notch increases approximately three-fold post infarction. **E.** Hes1 levels increase 1.8-fold at four days post infarction. **F.** Jagged1 increases four-fold at four days post infarction. Significance is represented as $p<.05$ = *, $p<.01$ = **, $p<.001$ = ***.

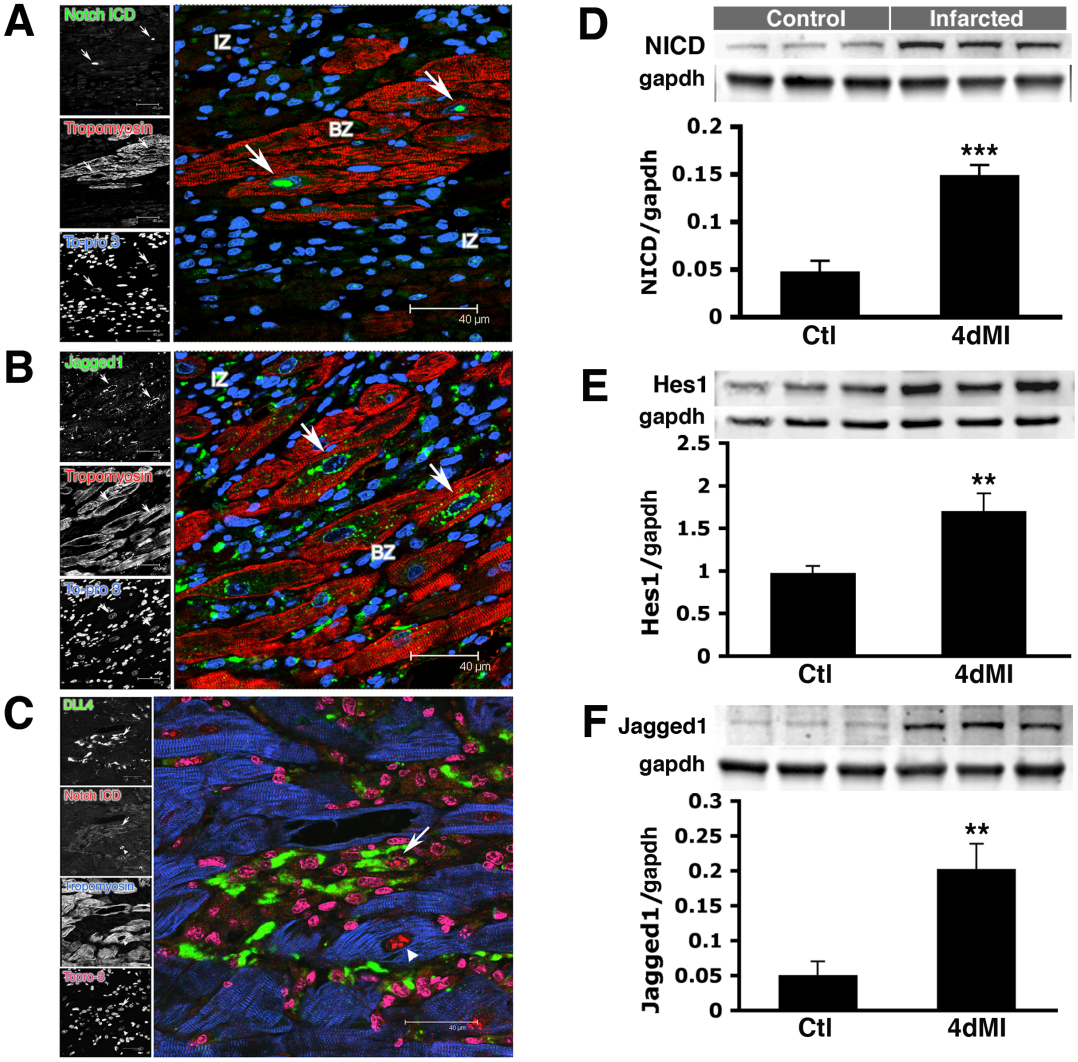
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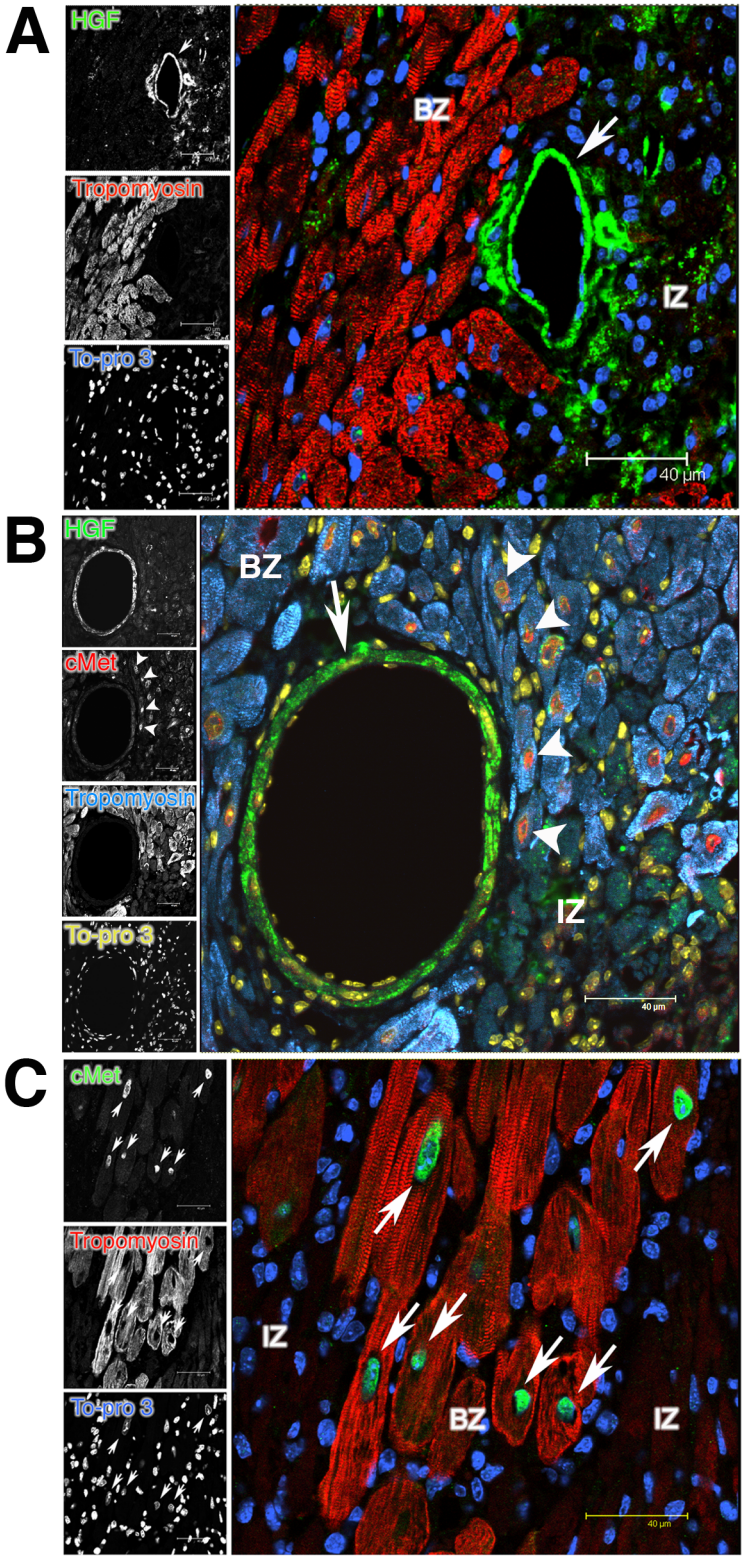
Figure 1.2: Immunolocalization of HGF and c-Met in infarcted myocardium.
Paraffin sections of mouse hearts harvested four days post infarction. BZ = border zone. IZ = infarct zone. **A.** HGF (green) is expressed in vessels (arrow) and other non-myocyte tissues in the infarct zone (IZ). **B.** HGF (green, white arrow) expression in the vessel wall of an infarcted heart adjacent to border zone myocytes with nuclear c-Met (red, white arrowheads), tropomyosin (blue) and To-pro 3 iodide (gold). **C.** c-Met (green) expression in the nuclei of border zone cardiomyocytes (arrows) with tropomyosin (red) and To-pro 3 iodide (blue).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cMet
Tropomyosin
To-pro 3
IZ
BZ
IZ
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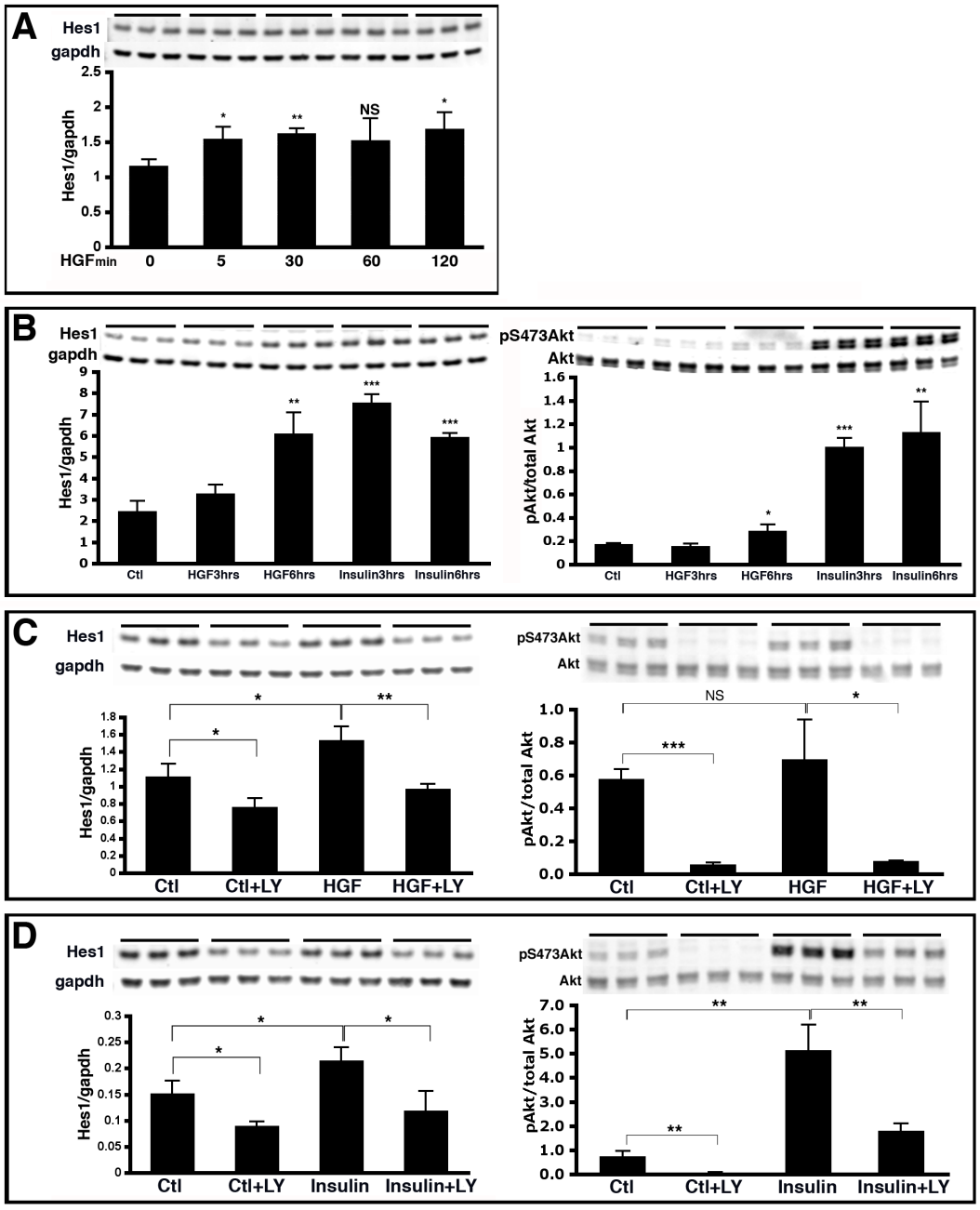
Figure 1.3: HGF and insulin induce Hes1 in cultured neonatal cardiomyocytes.
A. Neonatal rat cardiomyocytes (NRCMs) serum-starved overnight and treated with 50ng/ml recombinant mouse HGF or 100mU/ml insulin as a positive control for the time points indicated. **B.** NRCMs treated with HGF and insulin, harvested after 3 and 6 hours and immunoblotted for Hes1, showing Hes1 levels increase by approximately 2.5 fold following either HGF or insulin treatment by six hours. Total Akt and induction of phospho-Akt^{S473} by HGF and insulin are shown to verify stimulation by exogenous treatments. Hes1 levels are normalized to gapdh and phospho-Akt^{S473} levels normalized to total Akt. **C.** HGF and insulin treatment of NRCMs for six hours in the presence of PI3K inhibitor LY294002 shows reduction of phospho-Akt^{S473} level as well as Hes1 protein levels in untreated and HGF or insulin stimulated NRCMs. Significance is represented as $p<.05$ = *, $p<.01$ = **, $p<.001$ = ***. NS = not significant.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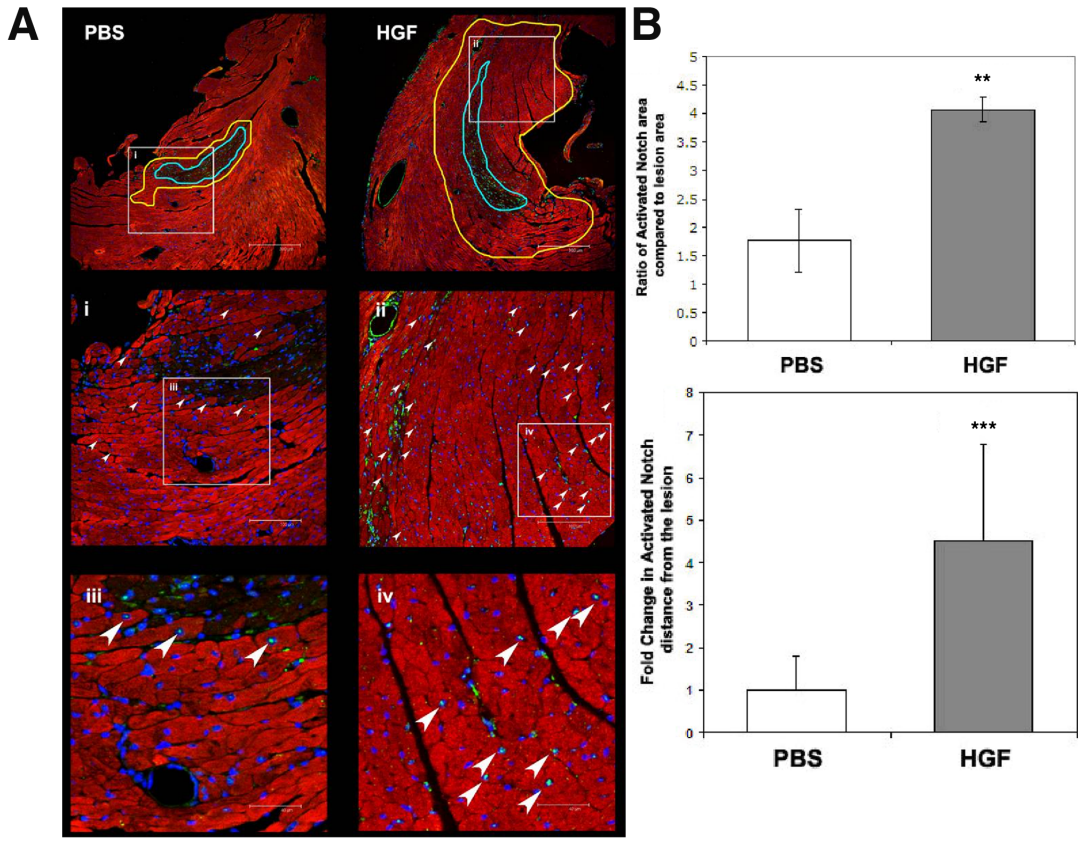
Hes1/gapdh
pAkt/total Akt
Ctl Ctl+LY Insulin Insulin+LY

Figure 1.4: HGF stimulates Notch activation in adult mouse myocardium.

A. Mouse hearts injected intramyocardially with recombinant mouse HGF (rmHGF) or PBS vehicle at several sites in the apex, harvested at three days post injection, and immunostained for activated Notch. The distance and area surrounding the injection sites were measured for Notch activation by HGF (panels labeled HGF, ii, iv) versus PBS (panels labeled PBS, i, iii) treated samples. Cardiomyocytes expressing activated Notch1 (white arrowheads) were used to mark the boundary of activation. Blue lines delineate circumference of injection sites, 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 were injected intramyocardially with rmHGF as described in A, then harvested at the time points as shown. Lysates were immunoblotted for activated Notch1 and normalized to gapdh. Hes1 and Notch levels increase by three days after HGF treatment relative to PBS injected controls. **D.** HGF-injected heart lysates were immunoblotted for total Akt and phospho-Akt^{S473}. Blots show HGF induction of Akt phosphorylation. Phospho-Akt^{S473} levels were normalized to total Akt. Values are average +/- standard deviation, n = 3. Significance is represented as $p<.05$ = *, $p<.01$ = **, $p<.001$ = ***. NS = not significant.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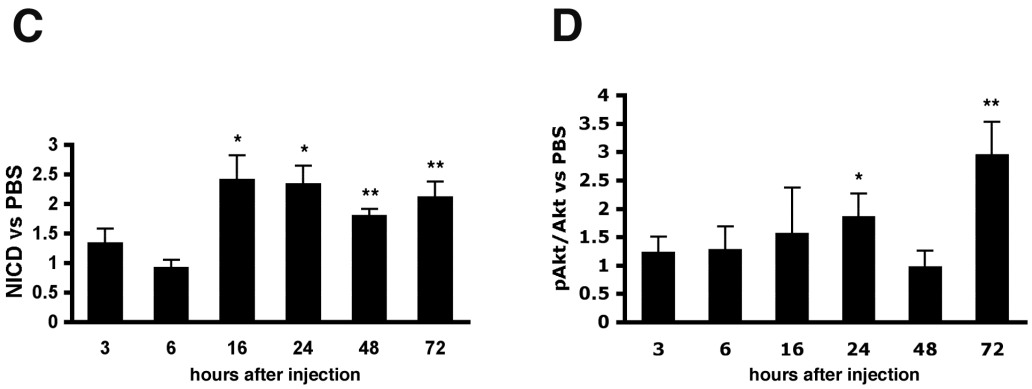
C
NICD vs PBS
hours after injection
3
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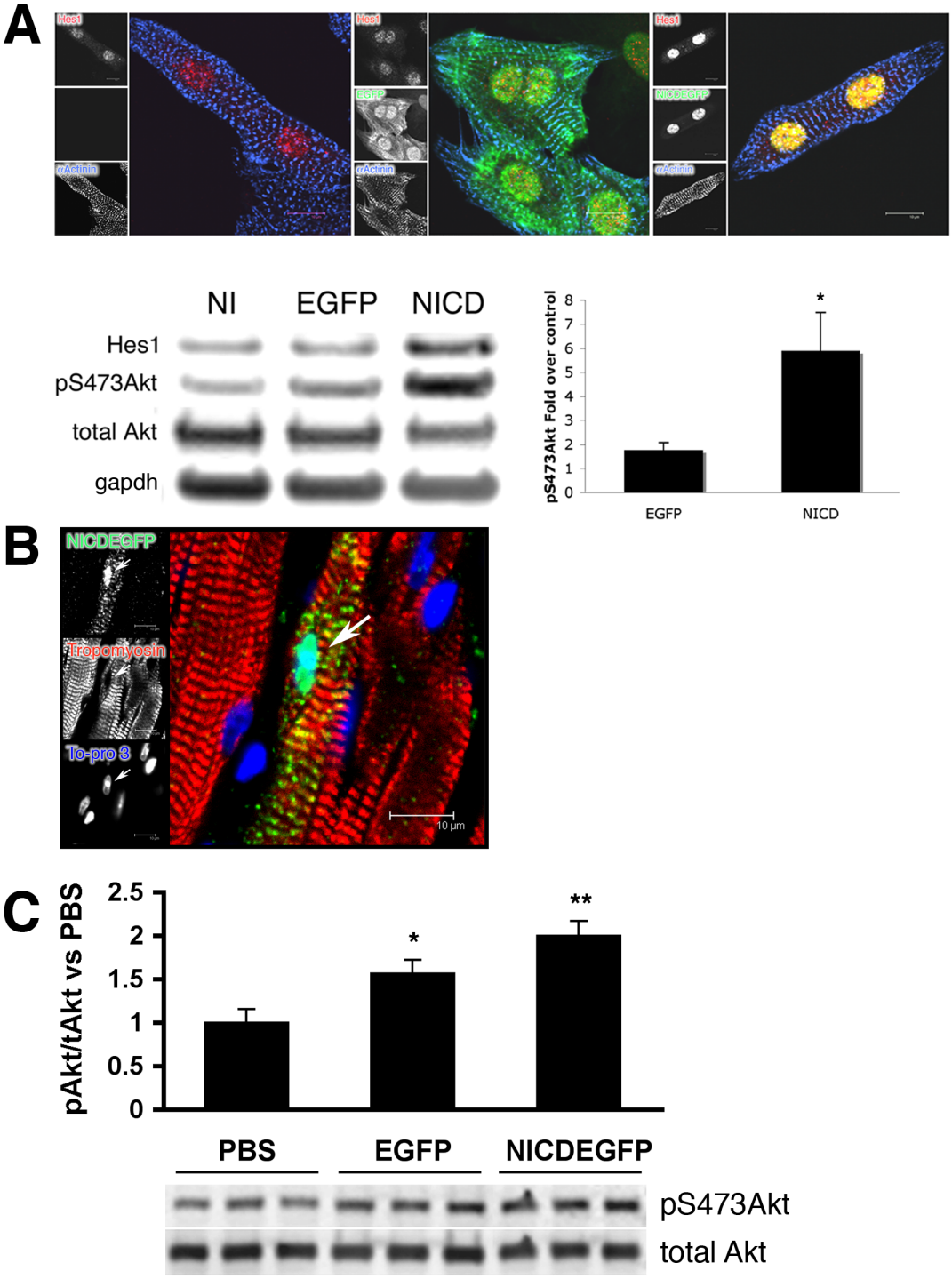
pAkt/Akt vs PBS
hours after injection

Figure 1.5. Notch signaling activates Akt *in vitro* and *in vivo*.
A. NRCMs infected with adenovirus encoding EGFP or NICDEGFP or left untreated and harvested 24 hours later for immunoblot analysis. Quantitation for phospho-Akt^{S473} levels relative to total Akt indicates a three-fold increase over EGFP infected NRCMs. n=4. **B.** Mouse hearts injected intramyocardially with PBS or adenovirus encoding either EGFP or NICDEGFP. Samples collected three days post injection were either processed for immunohistochemistry or as whole cell lysates for immunoblotting. Tissue sections immunolabeled for EGFP (green) verify adenoviral expression with tropomyosin (red) and To-pro 3 iodide (blue). **C.** Immunoblotting for phospho-Akt^{S473}, total Akt and α-sarcomeric actinin shows exogenous activated Notch increases phospho-Akt^{S473} levels two-fold over PBS and EGFP-expressing controls after three days of treatment. Significance is represented as $p<.05$ = *, $p<.01$ = **.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Hes1
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NICDEGFP
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pS473Akt
total Akt
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pS473Akt Fold over control
EGFP
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total Akt

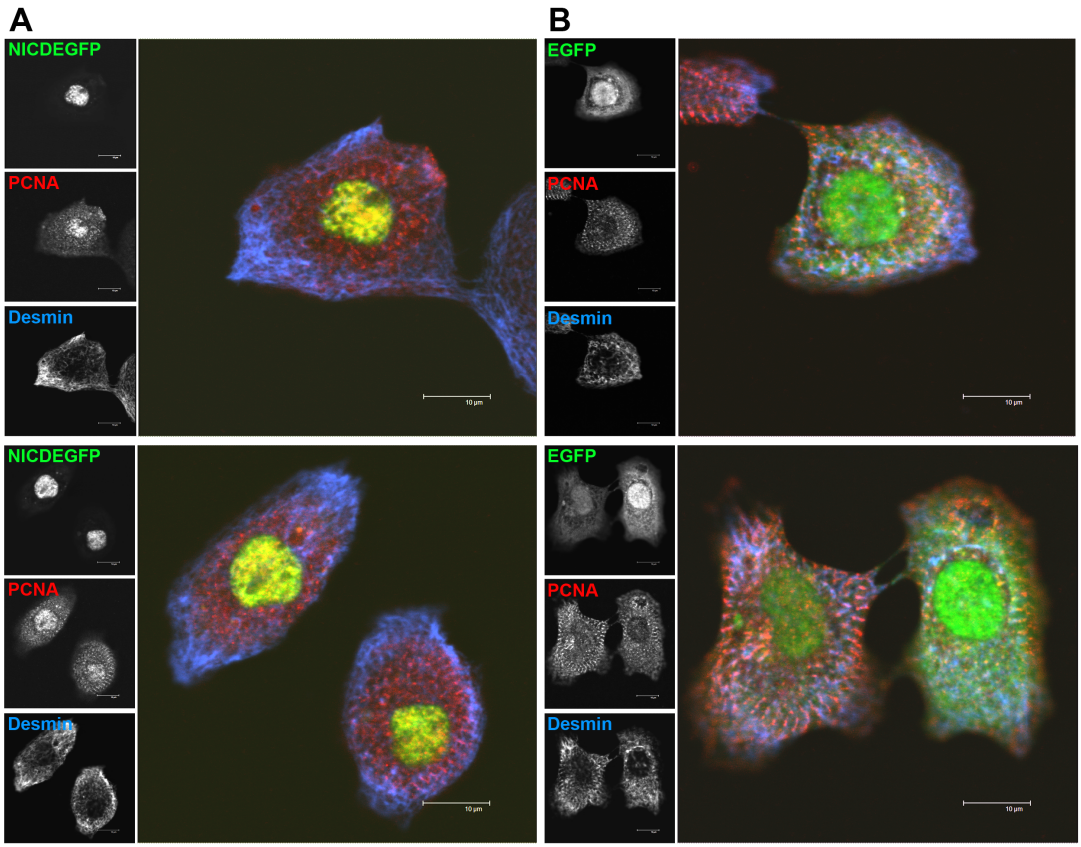


Figure 1.6. Activated Notch stimulates proliferative signaling in cardiomyocytes.
NRCMs infected with adNICDEGFP (**A**, green) or adEGFP (**B**, green) were immunostained for Proliferating Cell Nuclear Antigen (PCNA, red) and desmin (blue). **A.** PCNA localizes to the nucleus in NICDEGFP infected myocytes. **B**. PCNA localization remains cytoplasmic in EGFP infected myocytes. **C**. Mouse hearts subjected to acute infarction and intramyocardial injection of adNICDEGFP were harvested four 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).

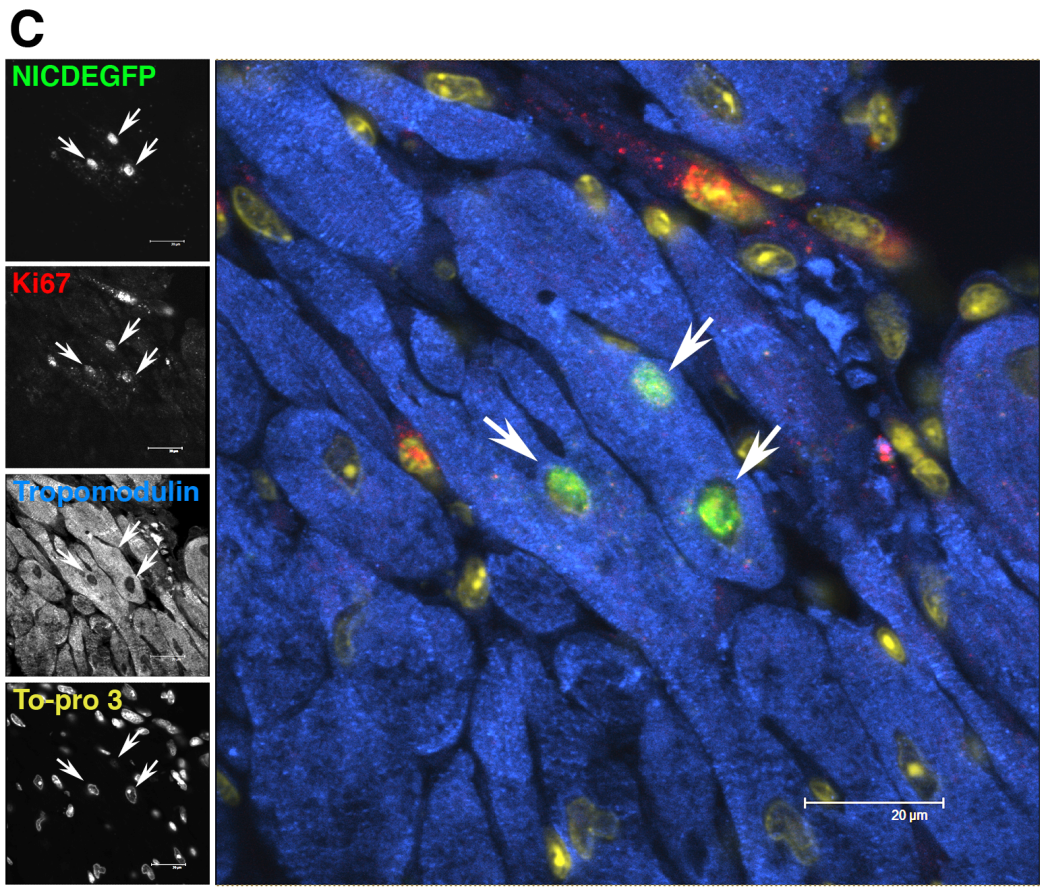


Figure 1.6 continued.

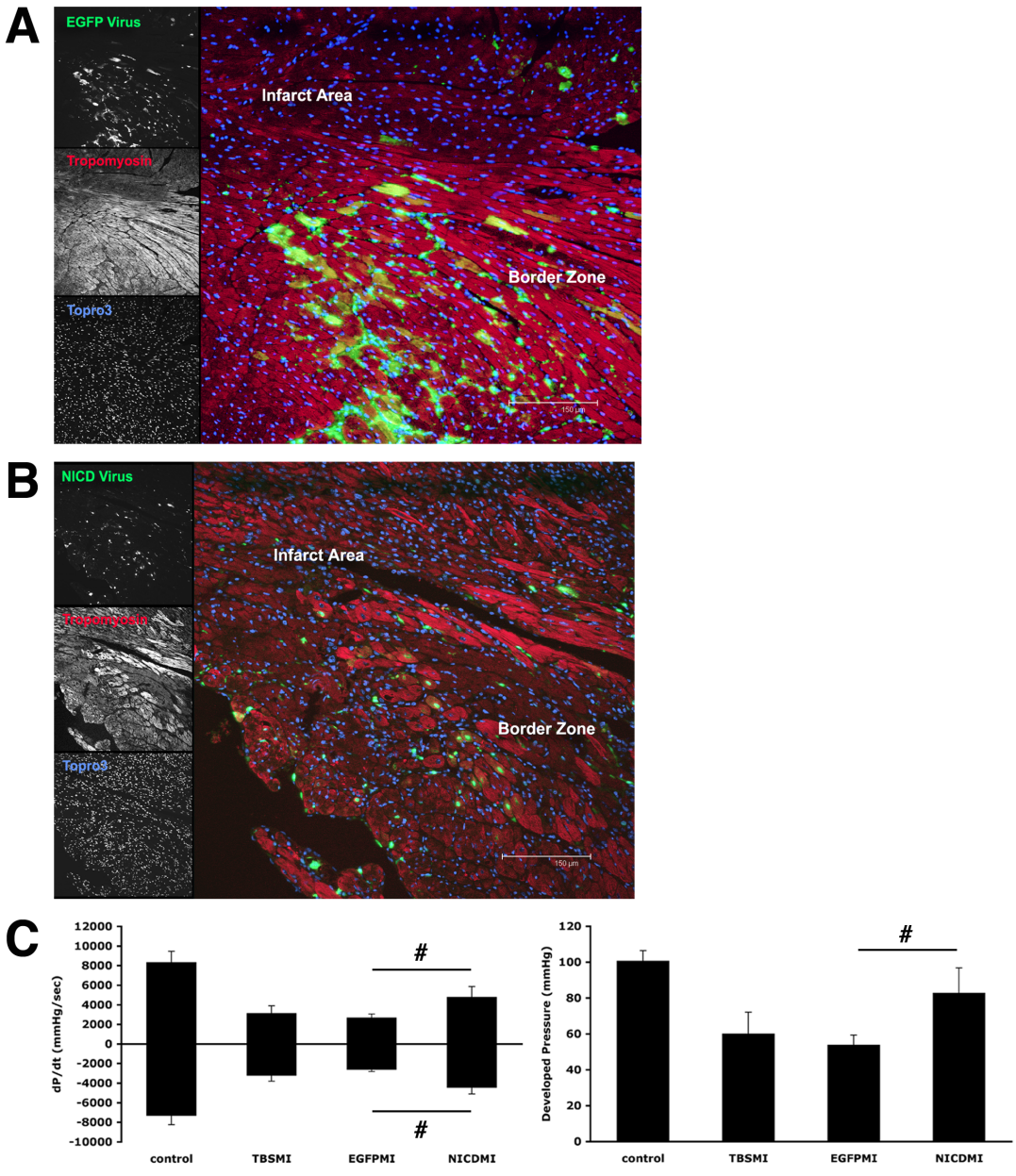


Figure 1.7. Notch signaling is cardioprotective in injured myocardium. Mouse hearts were subjected to acute infarction and injected 10 minutes post infarction with vehicle or adenovirus encoding either EGFP or NICDEGFP. **A** and **B.** Hearts harvested three days post infarction demonstrate expression of EGFP (green, A) and NICDEGFP (green, B) in border zone heart tissue with tropomyosin (red) and nuclei labeled by To-pro 3 iodide (blue). **C.** Hearts injected with virus as described in A,B with cardiac function measured by *in vivo* hemodynamic assessment at 28 days post infarction. Significance measured by one-way Anova with Tukey's post-hoc test. $p<0.05$ = #.

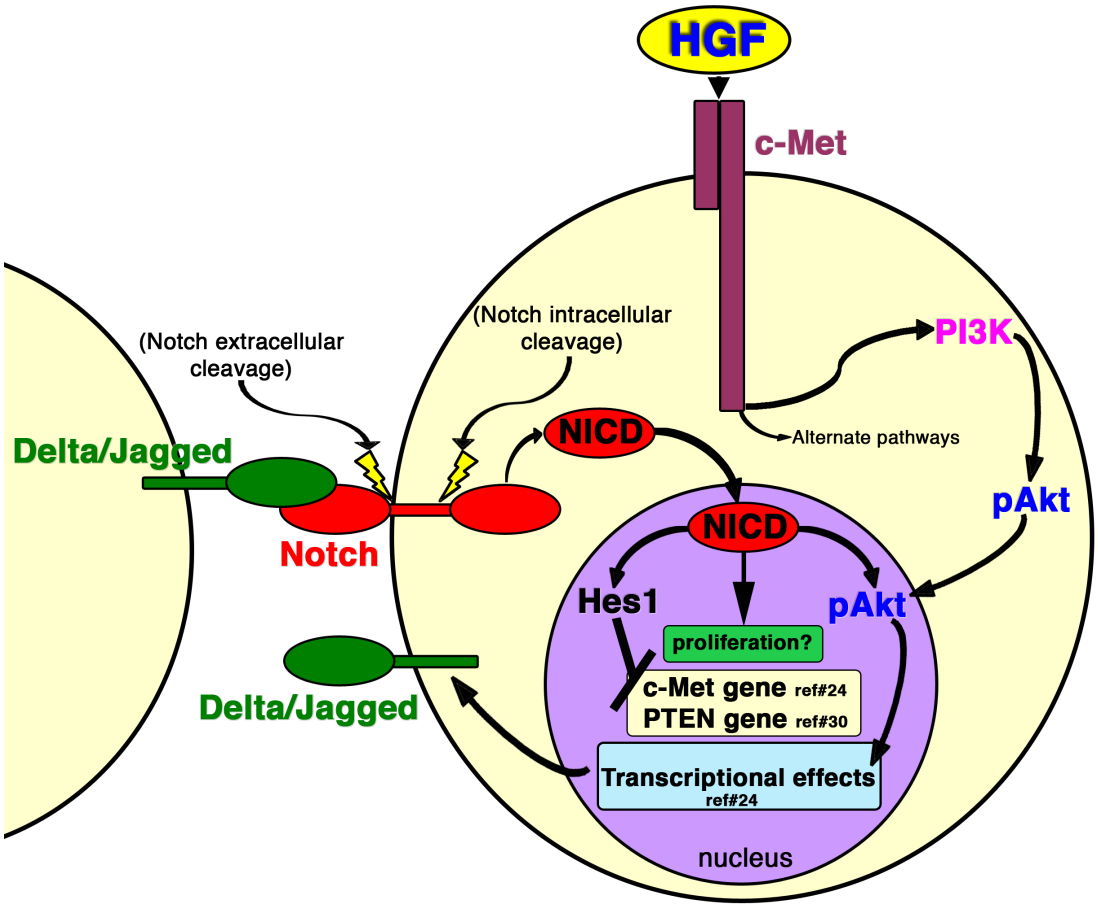


Figure 1.8. Schematic representation of pathway interactions among HGF/c-Met, PI3K/Akt and Notch signaling pathways.
Activation of the Notch receptor (red) by Delta/Jagged ligands (green) results in translocation of NICD (red oval) to the nucleus (purple), where the Hes1 repressor (black) is upregulated, leading to downregulation of PTEN and c-Met transcription [26, 32]. NICD also induces proliferative signaling (green box). Activation of c-Met receptor (brown) by ligand HGF (yellow oval) stimulates PI3K (pink) and pAkt (blue) signaling. Phospho-Akt may interact with NICD in the nucleus, potentially increasing expression of Delta/Jagged ligands [26].

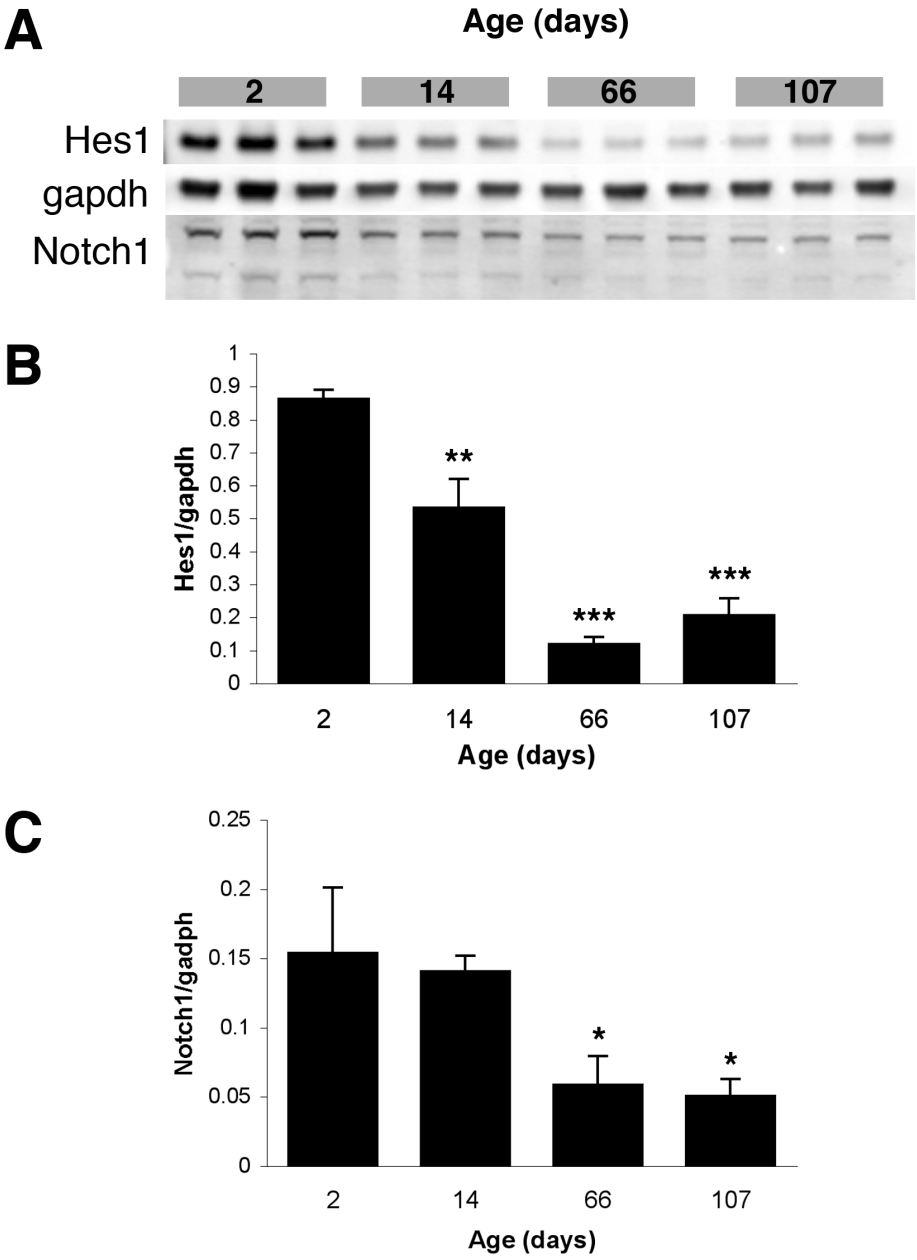
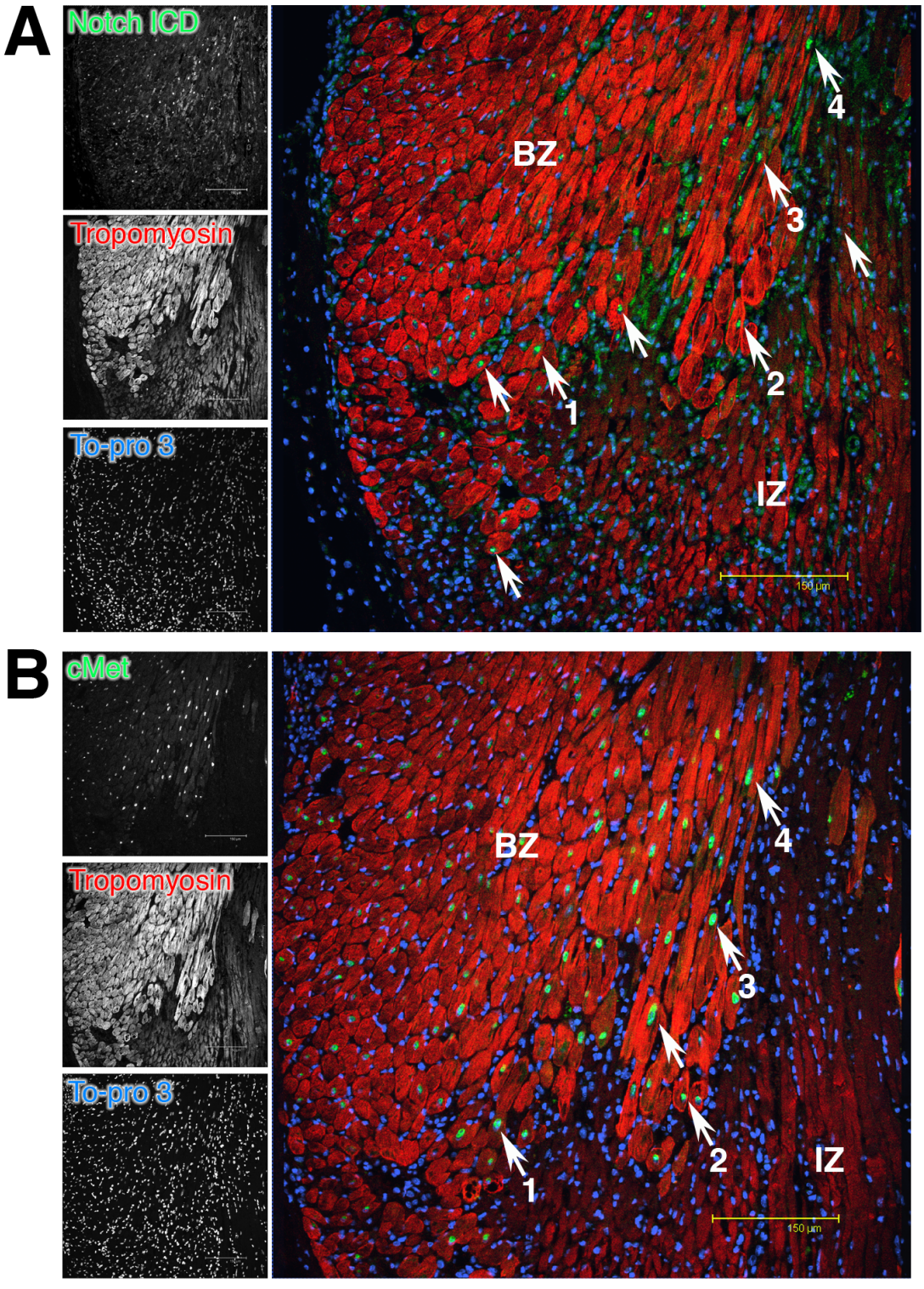


Figure 1.9: Notch1 and Hes1 levels decrease in mouse myocardium during postnatal development.

A. Heart lysates from 2, 14, 66 and 107 day-old mice were immunoblotted 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 $p<.05$ = *, $p<.01$ = **, $p<.001$ = ***.

Figure 1.10: Overview of activated Notch and nuclear c-Met localization in myocardium and non-myocyte tissues 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 c-Met (green), tropomyosin (red) and To-pro 3 (blue) in serial section of A. White arrows point to border zone myocytes containing nuclear c-Met. BZ and IZ denote border zone and infarct zone, respectively. Numbers next to arrows in A and B denote activated Notch and nuclear c-Met 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).


A
Notch ICD
Tropomyosin
To-pro 3
BZ
IZ
1
2
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150 µm
B
cMet
Tropomyosin
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IZ
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C

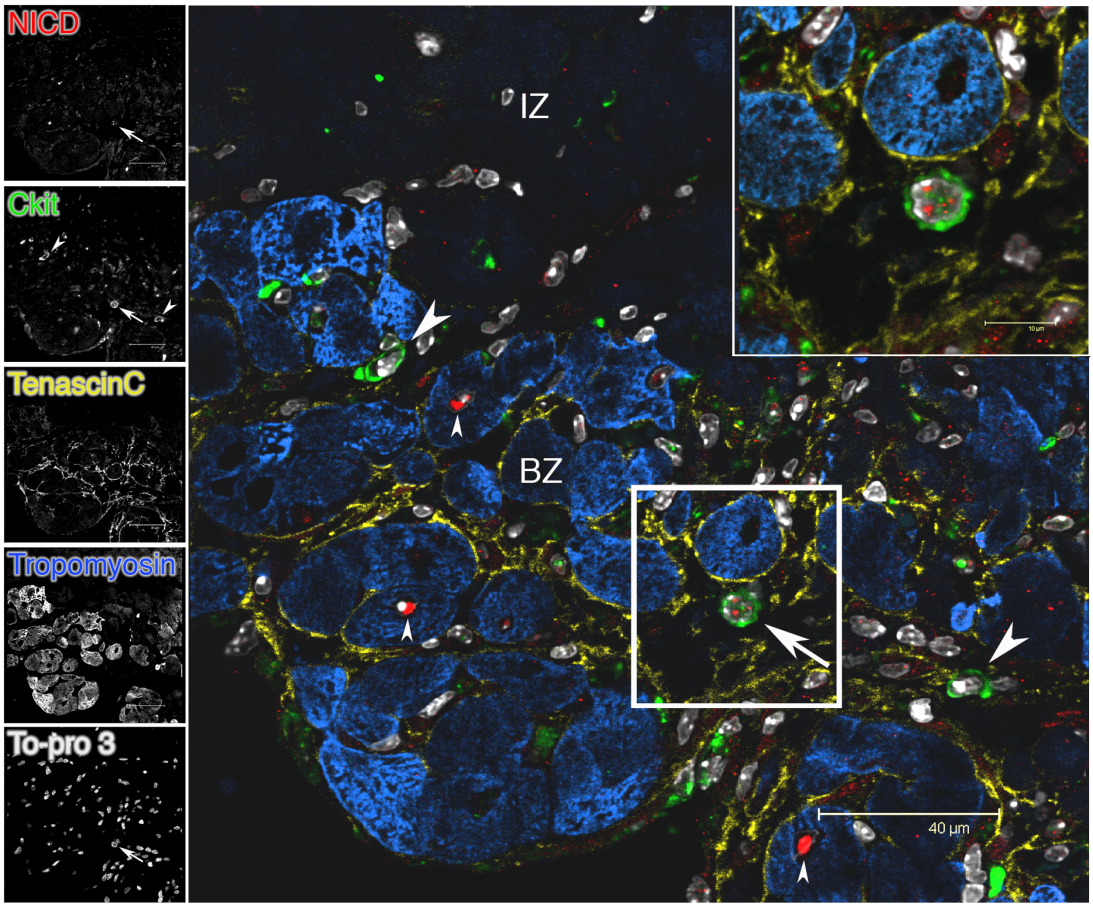
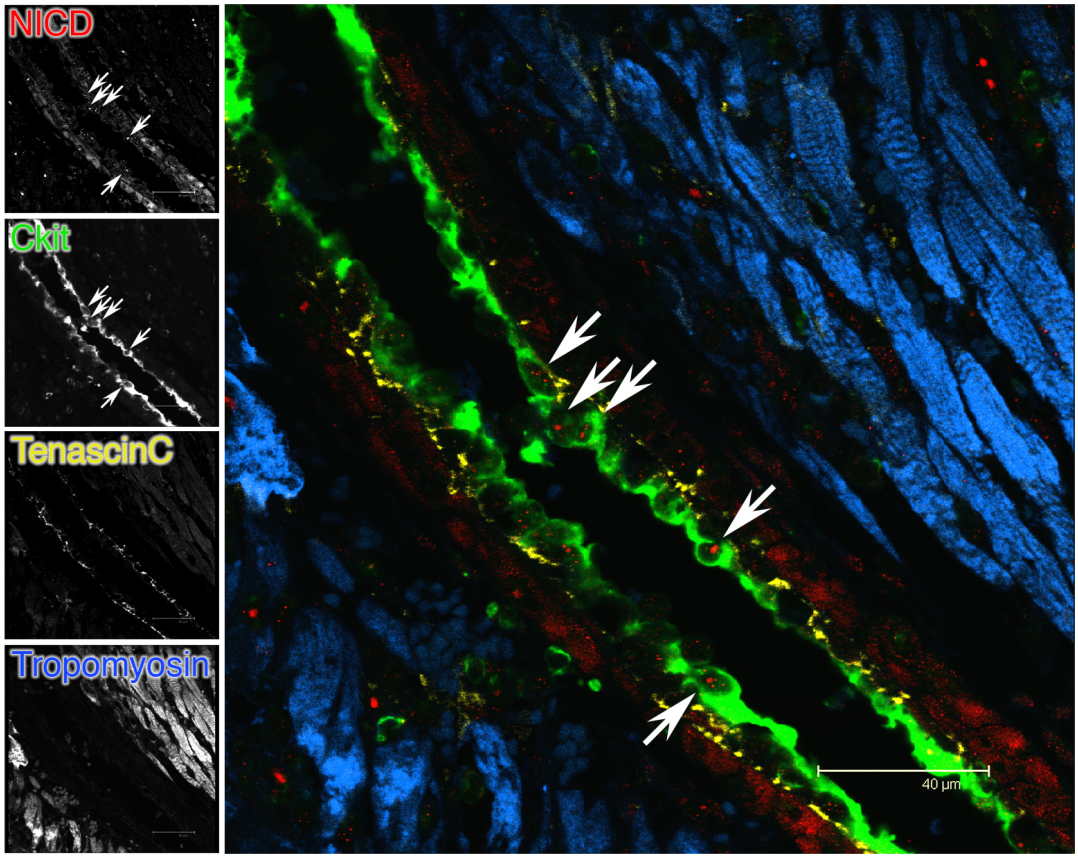


Figure 1.10 continued.

D



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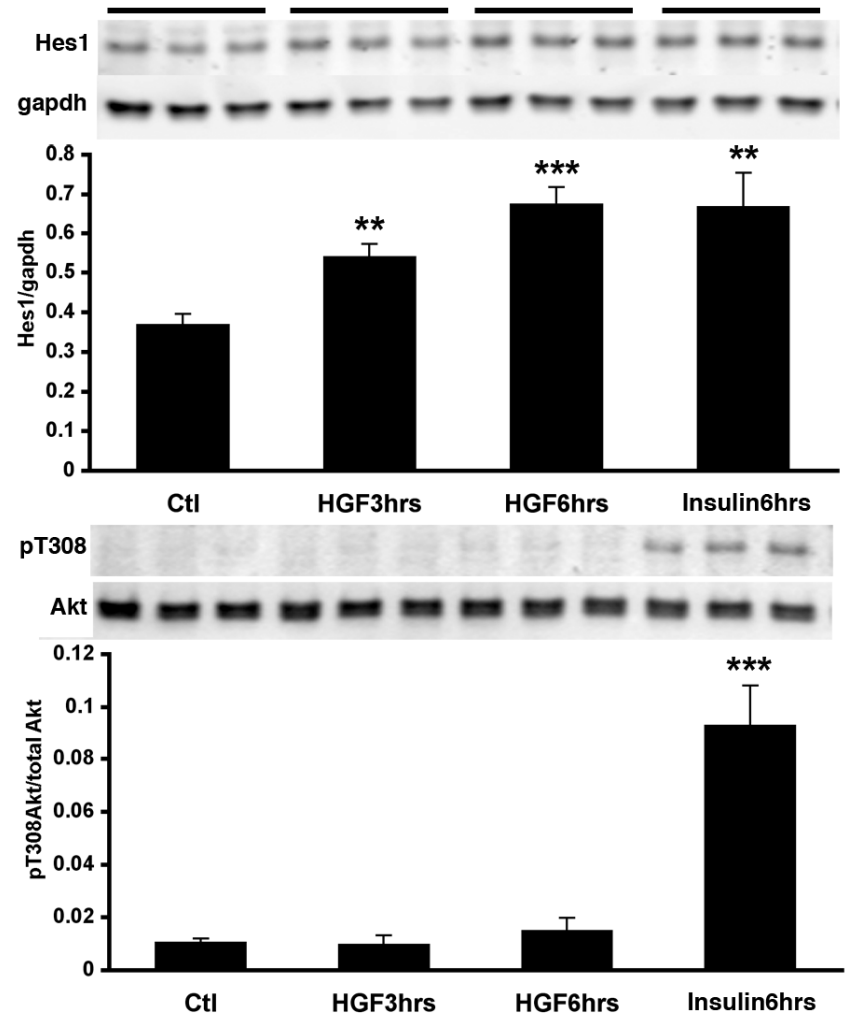


Figure 1.11: Phospho-Akt^{T308} in HGF and insulin treated NRCMS, Hes1 and phospho-Akt^{S473} in NRCMs treated with signaling pathway inhibitors. **A.** Immunoblot detecting Hes1 and phospho-Akt^{T308} in NRCMs treated with HGF (50ng/ml) or insulin (100mU/ml) for the times indicated. Hes1 levels increase two-fold following six hours of treatment with HGF or insulin. Phospho-Akt^{T308} levels are elevated only in insulin treated NRCMS after six hours. **B.** Immunoblot detecting Hes1 and phospho-Akt^{S473} in NRCMs treated for five hours with vehicle (Ctl), 20μM PD98059 (MAPKK inhibitor), 10μM triciribine (Akt inhibitor V) and 10μM rapamycin (mTOR inhibitor). Hes1 levels are decreased 3.5-fold following specific inhibition of Akt by triciribine. Phospho-Akt^{S473} levels decrease almost two-fold following triciribine treatment and increase in both PD98059 and rapamycin treated cells $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

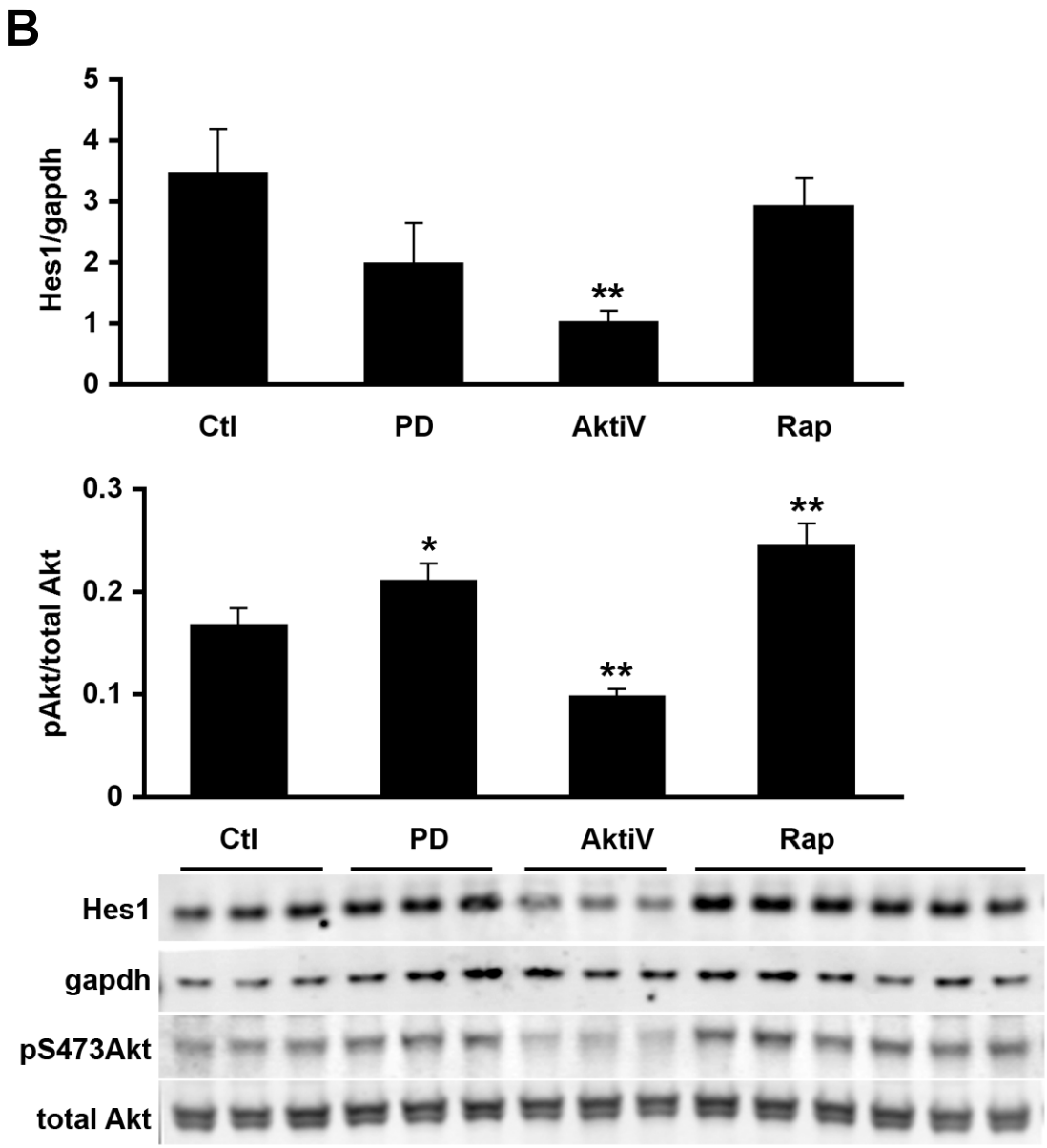
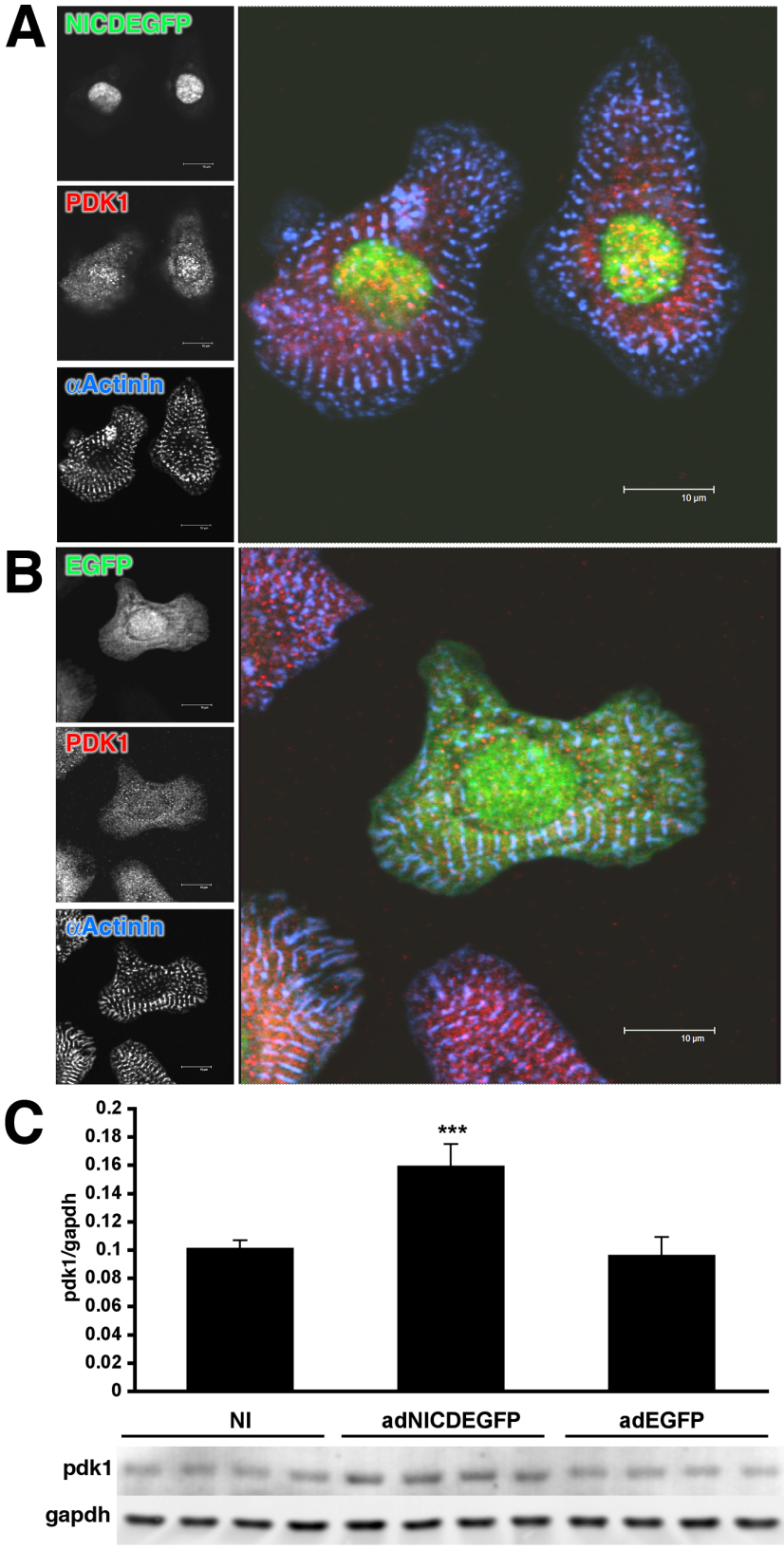


Figure 1.11 continued.

Figure 1.12: 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.


A
NICDEGFP
PDK1
αActinin
10 μm
B
EGFP
PDK1
αActinin
10 μm
C
pdk1/gapdh
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
***
NI
adNICDEGFP
adEGFP
pdk1
gapdh

D

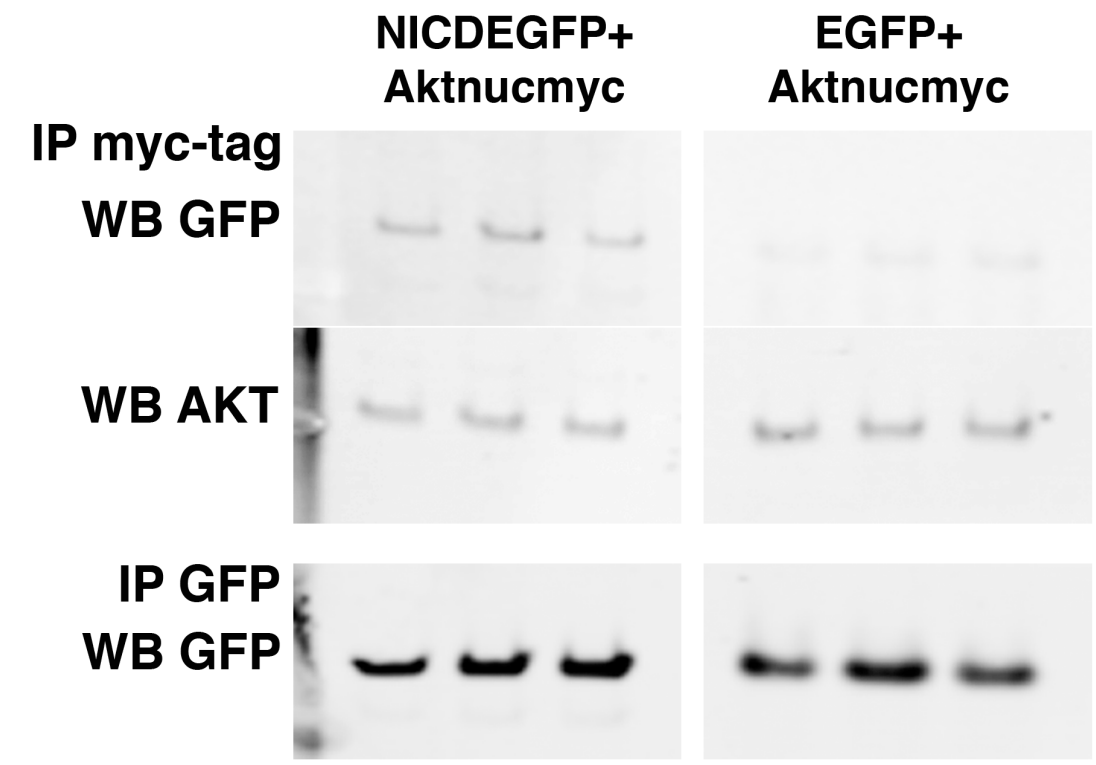
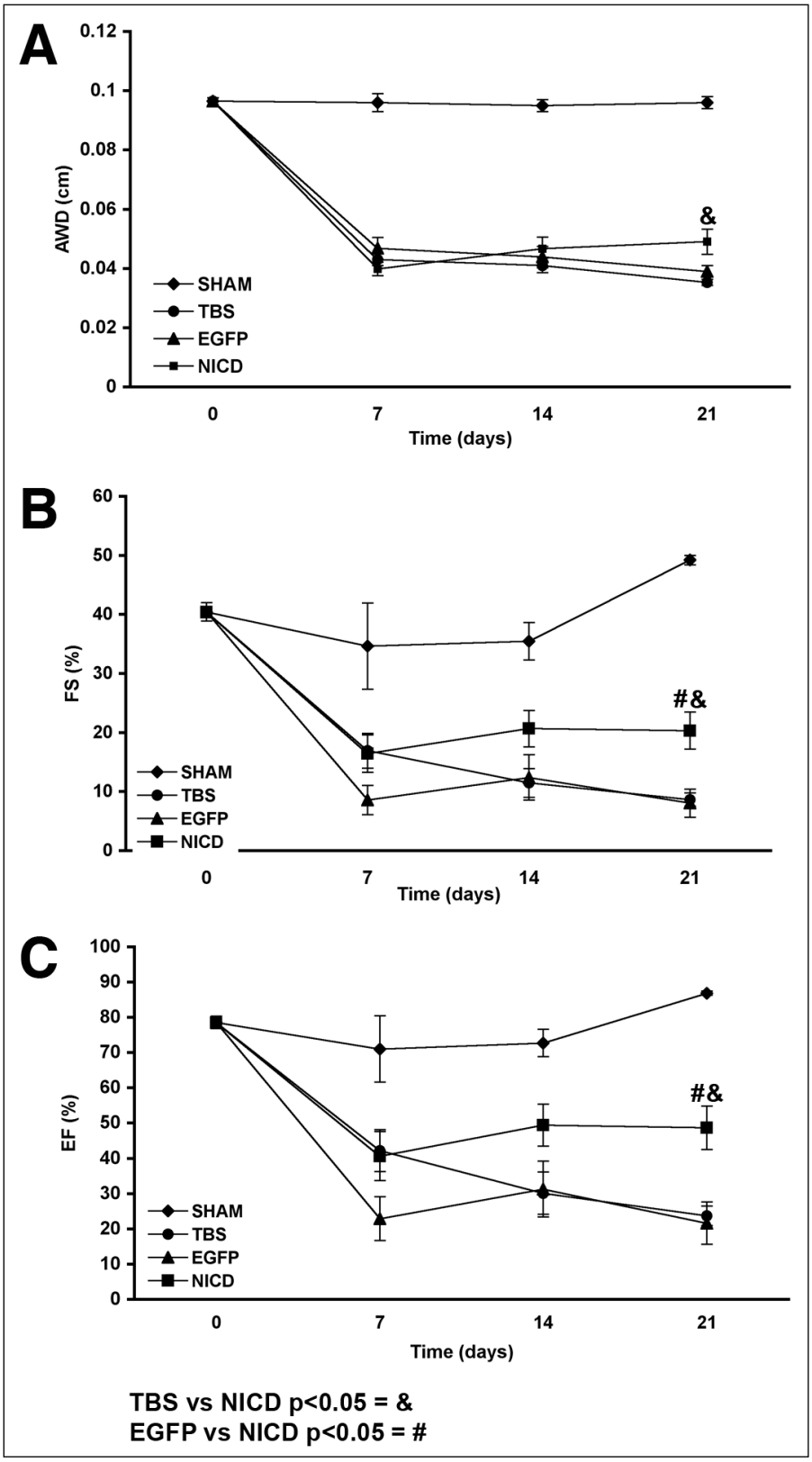


Figure 1.12 continued.

Figure 1.13. 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 immunostained for tropomyosin. Infarct area was divided by total left ventricle area. $p<0.05$=* adNICDEGPF vs. TBS, $p< 0.01$ = ** adEGFP vs. adNICDEGFP.


A
AWD (cm)
0.12
0.1
0.08
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0.04
0.02
0
&
SHAM
TBS
EGFP
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0
7
14
21
Time (days)
B
FS (%)
60
50
40
30
20
10
0
#&
SHAM
TBS
EGFP
NICD
0
7
14
21
Time (days)
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EF (%)
100
90
80
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Time (days)
TBS vs NICD p<0.05 = &
EGFP vs NICD p<0.05 = #

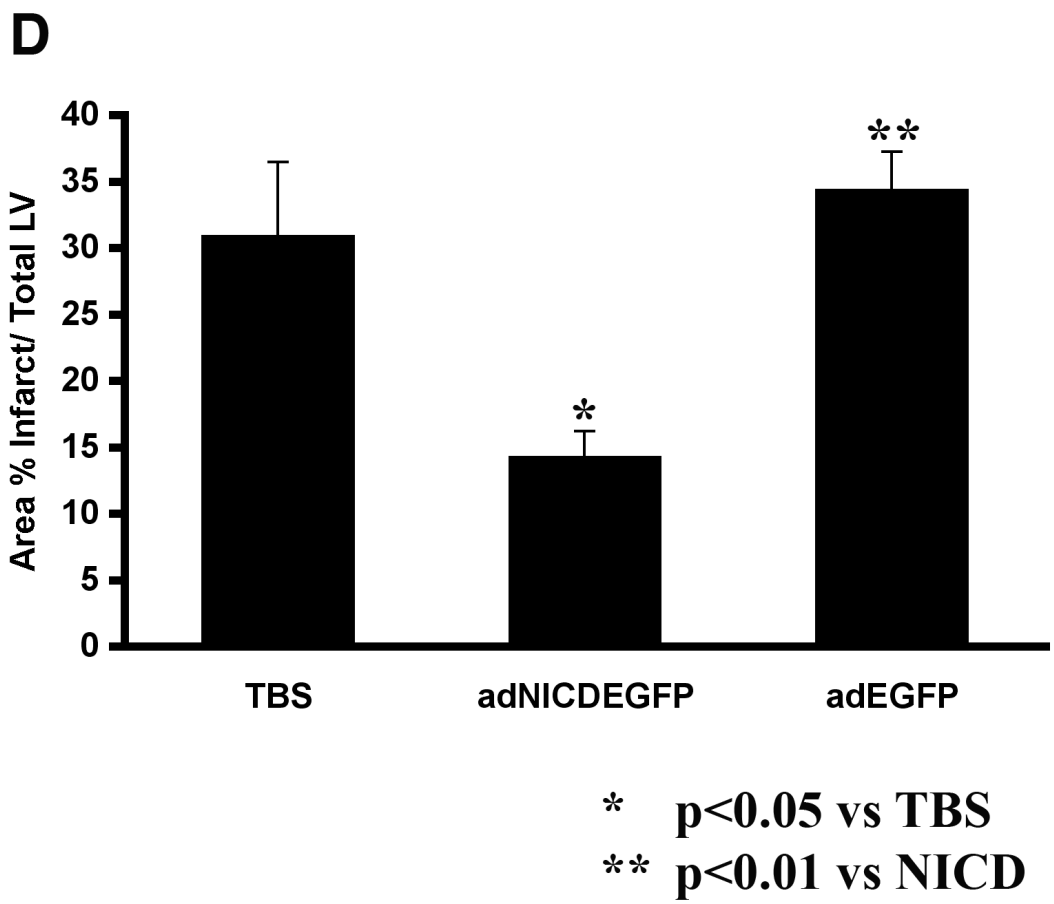


Figure 1.13 continued.

CHAPTER II:

REGULATABLE NOTCH ACTIVITY IN MOUSE MYOCARDIUM

Introduction

Traditional thinking dictates that the heart is a terminally differentiated organ with little to no regenerative capacity. However, in the last decade this perception has been challenged by a growing body of literature investigating myocardial regeneration and cardiac stem cell biology [36, 66, 67, 72-75]. Several studies demonstrate clusters of self-renewing cardiac progenitor cells in discreet locations within the heart comprising cardiac stem cell niches. These cardiac precursor cells respond to cardiac tissue injury by proliferating and migrating to sites of damage, where they replace damaged myocardium by differentiating into new functional cardiac tissue. On the receiving end of this process resides the surviving myocardium adjacent to the site of injury, the border zone. Cells in this region have been known to remodel, re-enter the cell cycle and re-express genes characteristic of earlier points in development [49, 69, 70, 76]. Many of the precise molecular signals received and generated by surviving cardiomyocytes remain uncharacterized. Since this region acts as both recipient and target of stem cell mediated repair following infarction, understanding the signaling behavior of border zone cardiomyocytes is crucial to further understanding and manipulating myocardial regeneration.

The Notch receptor, originally identified in Drosophila and having four orthologs in mammals, is a large cell-surface transmembrane protein important for cell fate decisions, cellular development, differentiation, proliferation, and apoptosis (reviewed in [17, 18]). Notch signaling plays an

important role in heart development [20], while defective Notch1 protein expression has been linked to aortic valve disease [77]. Activation of Notch signaling is achieved through binding to its ligands, the Delta and Serrate families in Drosophila or the Delta-like and Jagged families in mammals, which are also transmembrane proteins. When the extracellular domains of Notch and its ligands interact through adjacent or possibly same-cell contact, an initial cleavage event by the protease Adam/TACE releases the extracellular portion of Notch. A second proteolytic cleavage by gamma secretase/presenilin within the membrane releases the Notch intracellular domain (NICD), which translocates to the nucleus and binds the CSL transcription factors, activating target genes through displacement of corepressors and recruitment of target gene activators. Target genes of Notch include the basic helix-loop-helix transcriptional repressors Hairy and enhancer of split 1 (Hes1) and the Hairy-related transcriptional repressor family (HRT), which are found in the developing and adult heart [24]. HRT proteins have been shown to bind GATA transcription factors and inhibit GATA4-mediated transcription of ANP in vitro; this repression is alleviated by Akt1 through an unknown mechanism [25].

The Notch signaling pathway is important for cell fate determination and differentiation in the developing heart, but the significance of Notch expression in adult cardiomyocytes is less clear. Evidence exists for Notch activity in

regenerating tissue (skeletal muscle, zebrafish heart, vessels, pancreas, liver, bone and tooth) [4, 49, 53, 55, 78-81]. These studies indicate that Notch signaling confers tissue plasticity by contributing to regenerating cell growth and differentiation, adhesion and migration, precursor cell activation, injured cell dedifferentiation, restoration of regenerative potential and overall activation of the regeneration process. Recently, application of the Notch ligand Delta-like 4 (DLL4), combined with fibroblast growth factor (FGF) to injured rat brain has been shown to improve motor function compared to untreated controls, presumably through activation of the Notch signaling pathway and enhanced proliferation of adult neuronal progenitor cells [51]. Notch signaling has been implicated in the pro-survival effects or Akt as well as mTOR [30, 82]; moreover, crosstalk between the Notch and PI3K pathways has recently been demonstrated in T-cell leukemia [32] and receptor tyrosine kinases, integrins and Notch are postulated to interact in lipid rafts in neural stem cells [83]. Although activated Notch has been reported in regenerating zebrafish heart [49], the significance of Notch activity and its effectors in injured and regenerating adult mammalian myocardium remains uncharacterized.

Notch expression during cell fate determination is tightly regulated spatially, temporally and quantitatively [84, 85]. Constitutive overexpression of Notch in mouse cardiomyocytes in vivo does not simulate the endogenous temporal and quantitative regulation of Notch activity needed to examine the

impact of this protein in damaged heart tissue. The mutated estrogen receptor (mER) can block protein activity when fused to transgenes of interest such that activity can be induced at desired times and levels using tamoxifen [86-89]. The mER fusion protein binds to heat shock protein 90 (hsp90), which envelopes the overexpressed protein of interest and blocks activity until application of tamoxifen, resulting in release of hsp90 from mER [89]. The protein of interest is then available to interact with upstream or downstream effectors. This approach has been successfully used by our colleagues in Dr. Glembotski's laboratory to study cardiac ER stress response in transgenic mice overexpressing an ATF6-mER construct under control of the alpha-myosin heavy chain (αMyHC) promoter [86]. Therefore, this strategy was adapted for generating inducible activation of intracellular Notch1 in intact mouse myocardium. A schematic representation of the mER fusion constructs created for this study is illustrated in Figure 2.1.

Methods

Subcloning and creation of adenovirus

To create KNICmER and mNICDmER adenoviruses, FLAGmER was PCR cloned from pCDNA3.1FLAGmER (kindly provided by Dr. Chris Glembotski, SDSU) into pDC315 shuttle vector at the NheI site using SpeI containing primers (see Appendix). Both the full-length intracellular domain of Notch (mNICD, V1744-K2531) and the Kopan Notch Intracellular (KNIC, V1744-S2184) domain were cloned upstream of the FLAGmER fusion construct into the EcoRI site (Figure 1). The mNICD fragment was PCR amplified with gene specific primers using mouse heart cDNA as template, and KNIC was PCR amplified from an established Notch clone generously provided by Dr. Raphael Kopan at Washington University. As a positive control for constitutively active Notch, KNIC was also PCR cloned upstream of EGFP in a pDC315EGFP fusion intermediate at the EcoRI site. Adenovirus was then generated through cotransfection with the pGlox adenovirus genomic plasmid into HEK293 cells. Cells were monitored for lysis for two weeks, indicating recombination of the shuttle and pGlox plasmids to generate virus, then expanded in HEK293 cells and purified by cesium chloride gradient centrifugation followed by dialysis. Titer was measured both by absorbance at OD 260 and 280 nm (pu/ml) or plaque assay (pfu/ml).

Quantitative real time PCR

RNA was extracted from cells using the Zymo micro RNA II kit according to manufacturer instructions. Briefly, for a six well dish, media was removed and cells were lysed in 600 microliters per well of RNA buffer. Extract was purified through a column, washed, eluted and quantitated by OD260 and 280 readings. First strand synthesis of cDNA was performed on total RNA (between 0.1 and 1.0 micrograms) using Applied Biosystems reverse transcriptase and OligodT18 primer. Quantitative real time PCR was performed with Qiagen SybrGreen reaction mix and a Bio-Rad/MJ Opticon thermal cycler using 10 to 50 ng cDNA template. Signals were normalized to GAPDH or B-actin transcript levels and fold induction of transcript level was measured both with respect to untreated NLSGFP as well as vehicle treated sample within each infection group. Primer sequences were designed using Primer Express software to span two exons in order to avoid amplification of genomic DNA. Each primer set was initially tested for efficiency by running standard curves of fourfold serial dilutions ranging from 50 nanograms to 0.8 nanograms of positive control cDNA template per 25 microliter reactions. Product integrity was checked by a melting curve programmed at the end of 40 cycles.

Transgenesis

The KNICmER and KNICGFP constructs were PCR cloned into the HindIII site of C26αMyHC, a backbone designed for cardiac-specific

expression of transgenes in mouse heart under control of the alpha myosin heavy chain promoter (gift of Dr. Jeffrey Robbins, Cincinnati Children's Hospital) [90, 91]. The injection fragments for transgenesis were generated by digestion with BamHI and gel purification of the 5.5 kb αMyHC-KNICmER or αMyHC-KNICGFP fragment. Transgenesis was performed in FVB mice by standard methods practiced at the SDSU Genomics facility. Briefly, the linearized αMyHC-KNICmER or αMyHC-KNICGFP construct was injected into 0.5 dpc embryos, which were transferred into pseudopregnant females. All pups were screened at 2 – 3 weeks of age by PCR using primers specific to the αMyHC promoter and KNIC transgene (see list of mouse primers in Appendix).

Immunoblotting

Whole cell lysates were separated on Bis-Tris gradient gels (Invitrogen) and transferred to Immobilon-P membranes (Millipore). Membranes were briefly stained with PonceauS to confirm uniform transfer, then washed in TBST (150 mM NaCl,50 mM Tris, 0.1% Tween-20, pH 7.5) and blocked for one hour at room temperature in 10% nonfat milk reconstituted in TBST. Blots were rinsed with TBST and primary antibodies applied in blocking buffer overnight at 4C. After washing in TBST, secondary antibodies were applied for 90 minutes at room temperature in blocking buffer, washed in TBST and scanned for fluorescent signal on a Typhoon 9410 (Molecular Dynamics).

Immunostaining

Neonatal rat cardiomyocytes were washed with PBS, fixed in 4% PFA (4% paraformaldehyde in McIlvane's buffer) for at least 30 minutes, washed in PBS, permeabilized in PBS/0.1M Glycine/0.1% TritonX-100 for five to ten minutes, washed twice in PBS and blocked for one hour at room temperature in 10% horse serum in PBS (HS/PBS). Primary antibodies were applied in blocking buffer at 4C overnight, slides were then washed in PBS and secondaries applied in blocking buffer for 90 minutes at room temperature. To label nuclei, To-pro 3 iodide was included in the final wash at a concentration of 1/5000, then slides were coverslipped with Vectashield mounting media and visualized using a Leica SP2 confocal scanning laser microscope.

Results

The Notch Intracellular Domain mER fusion constructs exhibit nuclear expression in vitro

KNICmER, KNICGFP and mNICDmER-expressing adenoviruses infect neonatal rat cardiomyocytes and exhibit nuclear localization as indicated by immunostaining for FLAG (KNICmER, mNICDmER) or GFP fluorescence (KNICGFP) and activated Notch (Figure 2.2A-C). Immunostaining for Hes1, a downstream target of Notch, is elevated in KNICmER cells treated with tamoxifen compared to uninfected cells and KNICmER expressing cells treated with vehicle. Interestingly, cells expressing mNICDmER exhibit strong Hes1 staining in the nucleus, with and without tamoxifen, suggesting that *in vitro*, the mNICDmER fusion construct exhibits less regulation by tamoxifen than KNICmER. (Figure 2.2D-I). Immunoblotting of KNICmER expressing NRCMs reveals only slightly elevated levels of Hes1 in untreated KNICmER cells versus NLSGFP expressing cells, and significantly lower Hes1 levels than cells infected with the constitutively active KNICGFP expressing adenovirus (Figure 2.2J). Collectively, these data indicate that the KNICmER fusion construct is regulated by tamoxifen, as evidenced by tamoxifen-dependent Hes1 expression, whereas mNICDmER may retain elevated Notch activity under basal conditions.

KNICmER but not mNICDmER activity is responsive to tamoxifen treatment in vitro

NRCMs infected with adenovirus encoding Nuclear Localized GFP (NLSGFP), KNICmER, mNICDmER and KNICGFP were treated with vehicle or 1 μM tamoxifen for 24 hours, then harvested for qPCR analysis. As shown in Figure 2.3, tamoxifen treated samples infected with KNICmER, but not mNICDmER, exhibited elevated transcript levels of the Notch target Hes1. Interestingly, the transcript for Jagged1, a Notch ligand expressed in heart, was clearly upregulated in KNICmER expressing myocytes treated with tamoxifen, but less so in tamoxifen treated cells expressing mNICDmER.

Activated Notch does not suppress PTEN transcription in NRCMs

PTEN transcript levels were measured by qPCR in NRCMs expressing NLSGFP, mNICDmER, KNICmER and KNICGFP, with or without tamoxifen treatment. As indicated in Chapter I, Notch activation stimulates the PI3K/Akt pathway in cardiomyocytes *in vitro* and *in vivo*, yet the mechanism of this stimulation remains unclear. In the cancer literature, suppression of PTEN gene transcription by Hes1 serves as a mechanism for enhanced PI3K/Akt activity in T-cell lymphoblastic leukemias and lymphomas (T-ALL)[32]; however PTEN transcript levels do not change in NRCMs expressing constitutively active or mER-regulatable intracellular Notch, as shown in Figure 2.4. No apparent suppression of PTEN mRNA by activated Notch is apparent in these cells, suggesting that this mechanism does not apply in normal cardiac

myocytes which lack gain-of-function Notch mutations such as T-ALL cells. Another potential target to examine is the IGF1 receptor, which is upregulated at the transcriptional level by Notch activity in adenocarcinoma cells[92].

αMyHC-KNICmER is expressed in the mouse myocardium

The αMyHC-KNICGFP and αMyHC-KNICmER fragments were injected into mouse embryos and transferred to pseudopregnant females in order to generate transgenic lines expressing KNICGFP or KNICmER in the mouse heart. After twelve embryo transfers (roughly 350 embryos), no founders were established with the αMyHC-KNICGFP fragment. Furthermore, litter sizes were unusually small, averaging three to four pups per litter as opposed to ten to twelve seen in standard mouse litters. This suggests that the αMyHC-KNICGFP construct is lethal if expressed during development, consistent with a recent publication in which overexpression of activated Notch in the developing heart resulted in congenital heart defects [93]. In contrast, three founder lines were established for the αMyHC-KNICmER transgene. Three founders out of twenty-two pups born following 4 embryo transfers falls well within the normal transgenic founder rate of 5 – 25% of viable pups. These founders are currently being bred back into the FVB/N line purchased from Jackson Labs. Germline transmission was confirmed by western blotting of heart lysates from all three lines; antibodies to FLAG and NICD detect a band of the appropriate size for the KNICmER fusion protein (Figure 2.5). KNICmER transgenic hearts at one month appeared normal at the gross

morphology level. Further characterization of these lines will include immunolocalization of the FLAG and NICD signals in paraffin sections as well as tamoxifen treatment to confirm induction Notch activity *in vivo*. The difference in viability between αMyHC-KNICGFP and αMyHC-KNICmER transgenics suggests that Notch activity is sufficiently suppressed in the KNICmER line to prevent embryonic lethality.

Discussion

Notch is a potent stem cell signal critical for proper cardiac development. Several recent studies have examined the role of endogenous activated Notch following pathological challenge and have shown that Notch can confer protective and proliferative signaling in differentiated cardiomyocytes. Genetic manipulation of activated Notch reveals that, as with many developmental proteins, precise timing and dose of the signal is essential for proper heart formation and perhaps also following cardiac insult (i.e. infarction, pressure overload) in adulthood [21, 93-98]. Thus far, inducible constructs for activated Notch are irreversible Cre-recombinase systems in which, once the signal is turned on, it cannot be reversed. The ligand-regulated mER fusion protein described in this chapter incorporates simplicity and reversibility into its design, as well as the potential for titration of the Notch signal using varying doses or times of tamoxifen treatment. Induction of Notch activity with tamoxifen has been demonstrated in primary cultures of neonatal rat cardiomyocytes using KNICmER encoding adenoviruses, and these reagents will be used in future studies to dissect further the crosstalk between Notch and other signaling pathways, namely PI3K/Akt in cardiomyocytes. For *in vivo* analysis, the αMyHC-KNICmER line has been established and protein expression in the heart confirmed. Pending appropriate induction of the transgene by tamoxifen *in vivo*, this mouse model will serve as a tool to examine the role of Notch signaling in cardiac injury and repair, confirming

how the Notch pathway interacts with survival signals in the heart following pathological challenge. The tools are in place to pursue the biological questions of cardioprotection and signaling crosstalk outlined in the following future directions:

Activation of the KNICmER fusion confers protection against apoptotic stimuli in neonatal rat cardiomyocytes

The hypothesis put forth is that Notch signaling is cardioprotective in neonatal rat cardiomyocytes. Regulation of timing and levels of KNICmER expression by tamoxifen treatment will allow precise control of protective Notch signaling, enabling further dissection of Notch and PI3K/Akt pathway interactions. NRCMs will be infected with the KNICmER or control adenoviruses and treated with vehicle or tamoxifen at varying times and doses, and assayed by immunostaining and immunoblotting for downstream effectors of Notch as well as components of the PI3K/Akt signaling pathway, namely PDK1, phospho-PDK1, Akt and phospho-Akt. To determine whether Notch signaling confers protection from apoptotic stimuli via stimulation of PI3K/Akt, NRCMs infected with AdKNICmER or control adenovirus will be subjected to apoptotic insult and assayed for cell death by TUNEL and AnnexinV FACS assay. Apoptotic stimuli will include treatment with staurosporine, hydrogen peroxide and doxorubicin. Specificity of Akt signaling will be demonstrated through use of Akt inhibitors. Activation of KNICmER in NRCMs is expected to increase pS473Akt and these effects are expected to

be reversible upon withdrawal of tamoxifen. Induction of Notch activity in myocytes should confer protection from apoptosis via PI3K/Akt signaling; thus, inhibitors of PI3K/Akt are expected to blunt these protective effects.

Mice expressing cardiac-specific, conditionally active intracellular Notch exhibit enhanced regenerative capacity in response to pathological challenge

As illustrated in Chapter I, Notch is activated in the damaged mouse myocardium, implicating this pathway in the cardiac response to injury. Additionally, exogenous Notch can ameliorate the decline in cardiac function in mice following infarction. The precise mechanism by which Notch exerts these effects remains undefined, although intramyocardial injection of adNICDEGFP into intact mouse heart increases levels of pS473Akt in whole heart lysates. Synergy between the Notch and PI3K/Akt signaling pathways has been demonstrated in cancer cell lines, providing a template to follow while investigating crosstalk between these pathways in the mammalian myocardium.

Time and dose dependent activation of αMyHC-KNICmER by tamoxifen

Once αMyHC-KNICmER expression is confirmed in cardiac tissue for at least three generations, experiments for activation of NICD with tamoxifen will be performed. As published in previous studies, 20mg/kg tamoxifen or vehicle will be injected intraperitonealy for five days, after which hearts will be harvested for paraffin embedding or immunoblot analysis [86]. Paraffin sections will be stained for NICD, FLAG tag and Hes1. Western blots will be probed for

NICD, FLAG tag, Hes1 as well as Akt and pS473Akt. A time course will be performed between 1 and 10 days to determine maximal activation by tamoxifen, and a dose response curve will be performed to determine optimal tamoxifen dosage to elicit NICD activation.

Infarction and treatment with tamoxifen

Once the αMyHC-KNICmER mouse line is established, acute myocardial infarction will be surgically induced by ligating the left anterior descending coronary artery. Tamoxifen will be injected for at least three days, after which animals will be monitored by echocardiography along with sham operated αMyHC-KNICmER and nontransgenic infarcted counterparts. After a minimum of four weeks, heart function will be assessed by in vivo hemodynamics and hearts harvested for paraffin processing. Paraffin sections will be immunostained for tropomyosin and infarct area measured to determine the protective effects of temporary NICD activation following infarction. The αMyHC-KNICmER construct expresses the fusion protein specifically in mouse cardiomyocytes. Notch activity is expected to increase in cardiomyocytes following treatment with tamoxifen and to subside following cessation of treatment. Mice carrying this transgene are anticipated to withstand cardiac insult better than nontransgenic counterparts; αMyHC-KNICmER hearts will exhibit improved function following infarction and

tamoxifen treatment than vehicle treated, αMyHC-KNICmER infarcted controls.

Potential problems/alternative approaches

The αMyHC-KNICmER construct may not function as anticipated in vivo. The MER fusion may not sufficiently repress Notch activity, or conversely tamoxifen treatment may not sufficiently de-repress the fusion protein. In the former case, it may be necessary to put a MER module upstream and downstream of the KNIC protein, or to remove residues in KNIC module known to interact with the canonical RBPJk direct target, potentially reducing basal activity while preserving sufficient regulation such that tamoxifen will activate the KNICmER fusion. The KNICGFP construct has been cloned into a CMV-Tet-On construct and tested in vitro. Preliminary results in cells cotransfected with tetO-KNICGFP and transactivator plasmid tTA indicate that basal activity is low and that detectable induction of expression occurs following application of doxycycline. In addition to boosting PI3K signaling, activated KNICmER may also stimulate proliferation in neonatal rat myocytes and treated hearts. Immunostaining for Ki67 and PCNA will be performed on neonatal myocytes and KNICmER hearts treated with tamoxifen versus vehicle treated controls to localize proliferating cells. Additionally BrdU incorporation will be used to measure increased DNA synthesis in tamoxifen versus vehicle treated KNICmER hearts.

Figures

Figure 2.1: Schematic representation of recombinant Notch constructs for adenoviral recombination (A,B,C) or cardiac transgenesis (D).
Kopan Intracellular Notch fused to GFP (KNICGFP,A) or mER (KNICmER, B) or full length intracellular Notch fused to mER (mNICDmER, C) were cloned into adenoviral shuttle vector pDC315. KNICmER was cloned downstream of the cardiac specific α-myosin heavy chain promoter (αMyHC-KNICmER) to generate transgenic mice expressing regulatable Notch in cardiomyocytes (D). Protein sequences for full length (mNICD) and truncated intracellular Notch (KNIC) are illustrated in (E). The red "v" residue represents the cleavage site Valine 1744 of Notch by presenilin/gamma secretase.

A

B

C

D

E

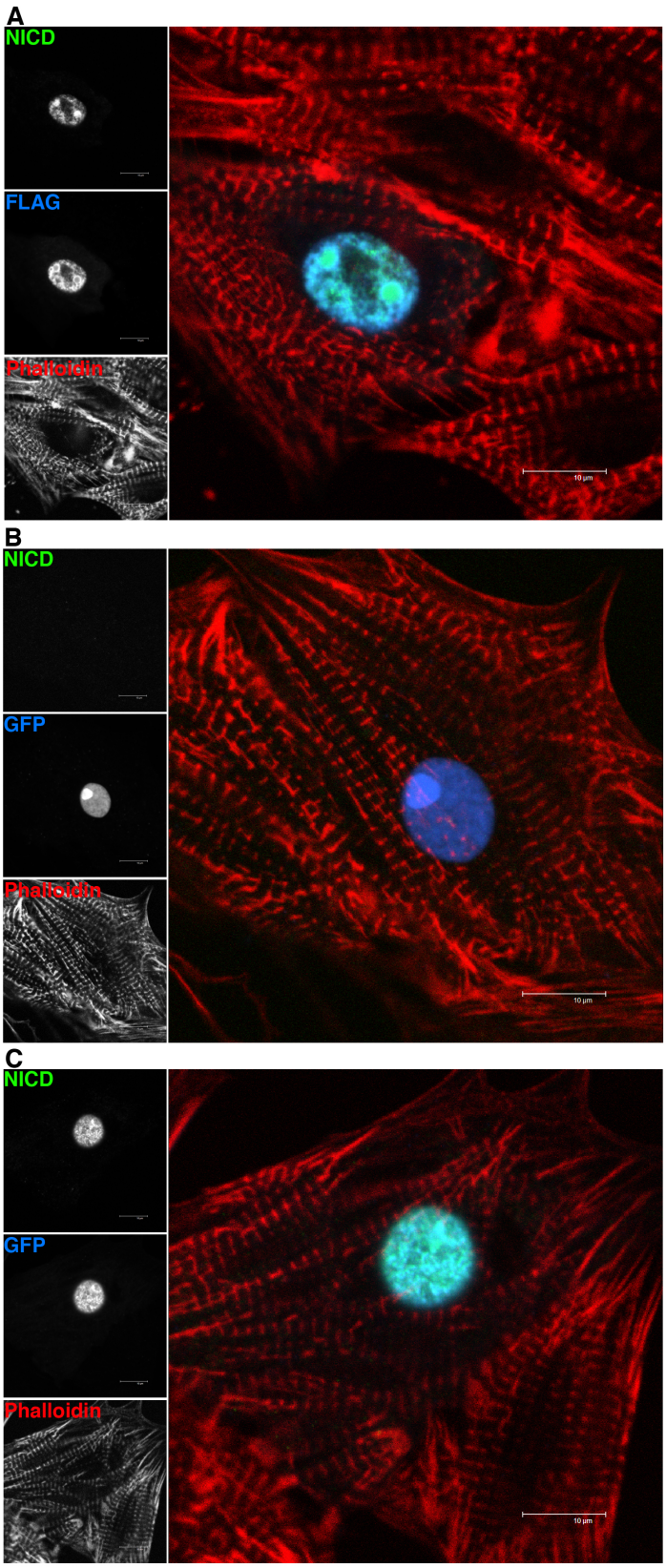
mNICD V**1744**-K**2531**

```
1741 gcgvllsrkr rrqhgqlwfp egfkvseask kkrreplged svglkplkna sdgalmddnq
1801 newgdedlet kkfrfeepvv lpdlsdqtdh rqwtqqhlda adlrmsamap tppqgevdad
1861 cmdvnvrgpd gftplmiasc sgggletgns eeeedapavi sdfiyqgasl hnqtdrtget
1921 alhlaarysr sdaakrllea sadaniqdnm grtplhaavs adaqgvfqil lrnratdlda
1981 rmhdgttpli laarlavegm ledlinshad vnavddlgks alhwaaavnn vdaavvllkn
2041 gankdmqnnk eetplflaar egsyetakvl ldhfanrdit dhmdrlprdi aqermhhdiv
2101 rlldeynlvr spqlhgtalg gtptlsptlc spngylgnlk satqgkkark pstkglacgs
2161 keakdlkarr kksqdgkgcl ldsssmlspv dslesphgyl sdvaspplp spfqqspsmp
2221 lshlpgmpdt hlgishlnva akpemaalag gsrlafeppp prlshlpvas sastvlstng
2281 tgamnftvga paslngqcew lprlqngmvp sqynplrpgv tpgtlstqaa glqhsmmgpl
2341 hsslstntls piiyqglpnt rlatqphlvq tqqvqpqnlq lqpqnlqpps qphlsvssaa
2401 nghlgrsfls gepsqadvqp lgpsslpvht ilpqesqalp tslpssmvpp mtttqfltpp
2461 sqhsyssspv dntpshqlqv pehpfltpsp espdqwssss phsnisdwse gissppttmp
2521 sqithipeaf k
```

KNIC V**1744**-S**2184**

```
1741 gcgvllsrkr rrqhgqlwfp egfkvseask kkrreplged svglkplkna sdgalmddnq
1801 newgdedlet kkfrfeepvv lpdlsdqtdh rqwtqqhlda adlrmsamap tppqgevdad
1861 cmdvnvrgpd gftplmiasc sgggletgns eeeedapavi sdfiyqgasl hnqtdrtget
1921 alhlaarysr sdaakrllea sadaniqdnm grtplhaavs adaqgvfqil lrnratdlda
1981 rmhdgttpli laarlavegm ledlinshad vnavddlgks alhwaaavnn vdaavvllkn
2041 gankdmqnnk eetplflaar egsyetakvl ldhfanrdit dhmdrlprdi aqermhhdiv
2101 rlldeynlvr spqlhgtalg gtptlsptlc spngylgnlk satqgkkark pstkglacgs
2161 keakdlkarr kksqdgkgcl ldss
```

Figure 2.2: KNICmER, KNICGFP and mNICDmER are expressed in the nucleus following adenoviral infection of neonatal rat cardiomyocytes. Myocytes infected with adenovirus expressing KNICmER (A), EGFP (B) or KNICGFP (C) were immunolabeled for NICD (green), FLAG (blue, A) or scanned for GFP fluorescence (blue, B,C). NRCMs infected with KNICmER (E,G,H), mNICDmER (F,I) or no virus (D) were treated with vehicle (E,F), 0.1μM tamoxifen (G) or 1μM tamoxifen (D,H,I,) for 24 hours and immunolabeled for FLAG (blue) Hes1 (green) or Phalloidin (red). Levels of Hes1 in NRCMs infected with NLSGFP, KNICmER and KNICGFP were detected by immunoblot (J). Hes1 signal was normalized to GAPDH as a loading control. T-test was used to measure significance between KNICmER and KNICGFP signals. *** = p<.001.


A
NICD
FLAG
Phalloidin
10 µm
B
NICD
GFP
Phalloidin
10 µm
C
NICD
GFP
Phalloidin
10 µm

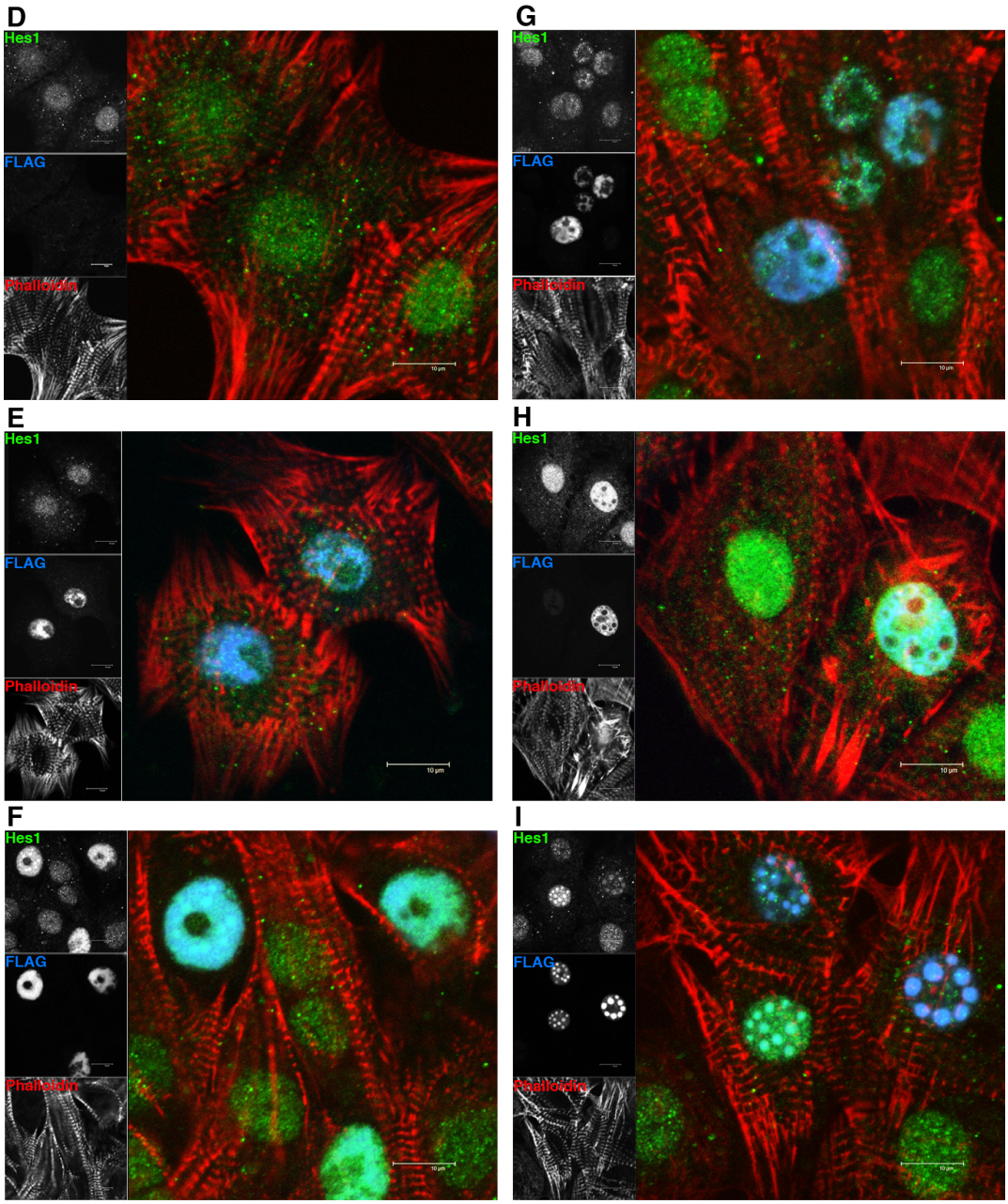


Figure 2.2 continued.

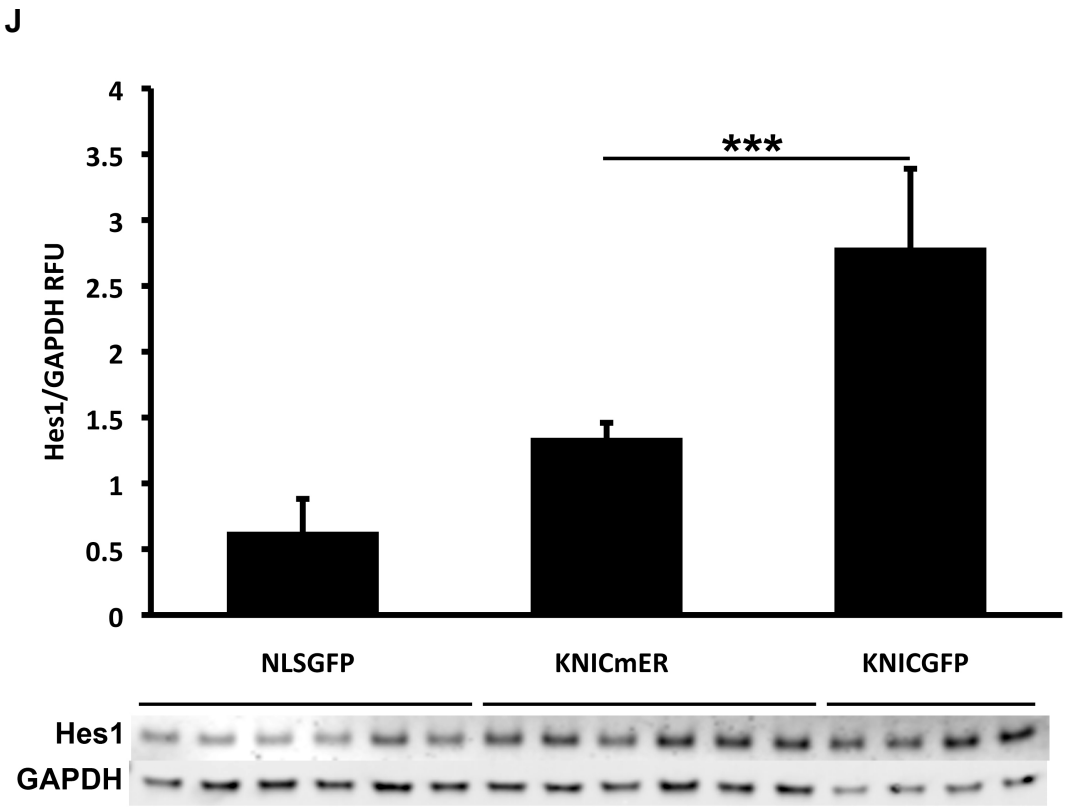
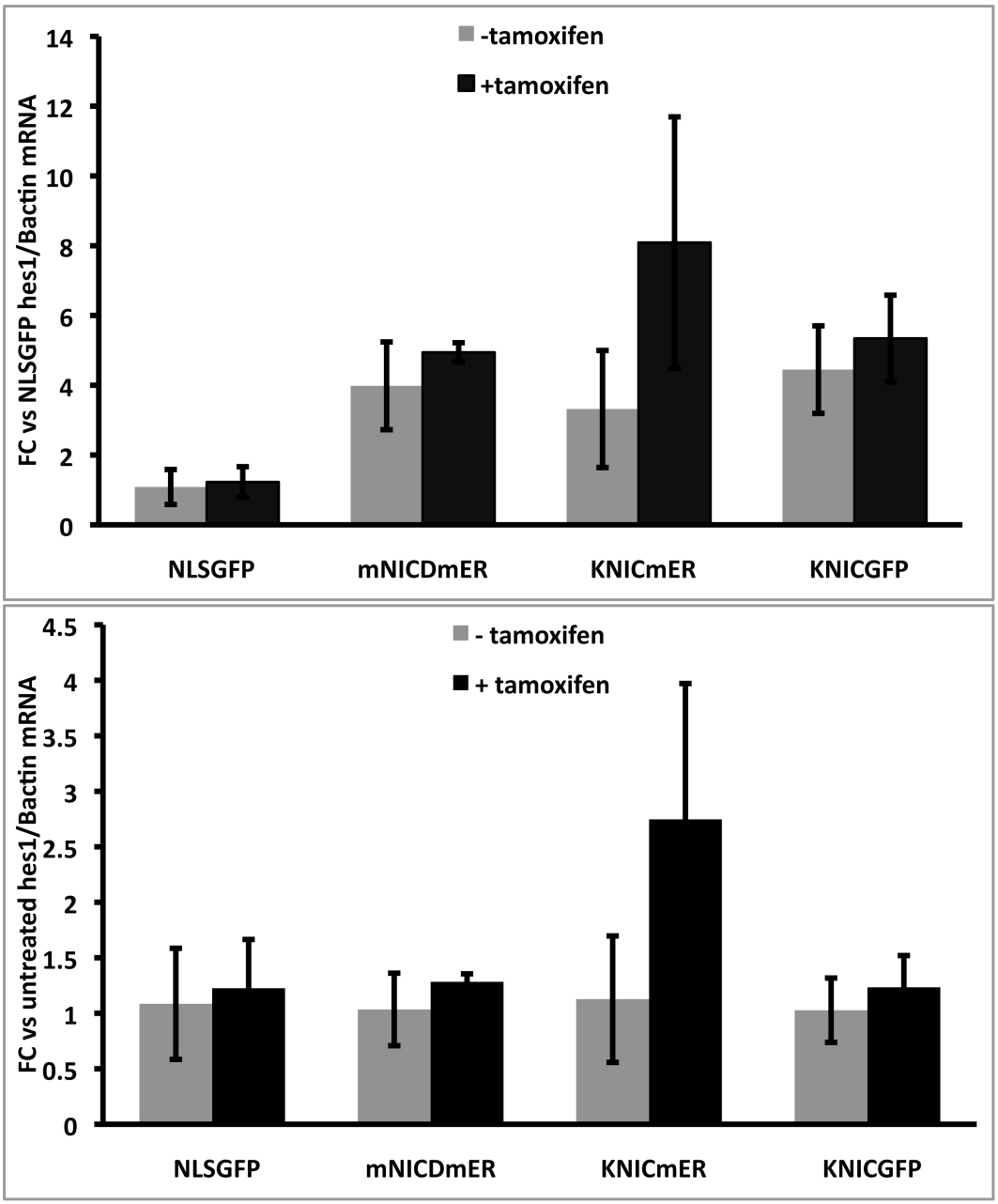


Figure 2.2 continued.

Figure 2.3: Induction of hes1 and jagged1 mRNA in NRCMs expressing regulatable activated Notch.
NRCMs were infected with adenovirus encoding NLSGFP, mNICDmER, KNICmER and KNICGFP, treated with vehicle or 1 μM tamoxifen for 24 hours and harvested for RNA. Transcript levels for hes1 (A) or jagged1 (B) were measured by qPCR using the ddCT method with B-actin as in internal control. Fold change was calculated relative to vehicle treated NLSGFP (top panel) or to vehicle treated for each construct. Jagged1 transcripts were compared between NRCMs expressing KNICmER and mNICDmER treated with 0, 0.1 and 1.0 μM tamoxifen (C). Levels were calculated using the ddCT method with gapdh as an internal control. Fold change (FC) was relative to the vehicle treated samples. T-test was used to measure significance between vehicle and treated samples. * = $p<.05$, ** = $p<.01$, *** = $p<.001$.

A


-tamoxifen
+tamoxifen
FC vs NLSGFP hes1/Bactin mRNA
14
12
10
8
6
4
2
0
NLSGFP
mNICDmER
KNICmER
KNICGFP
- tamoxifen
+ tamoxifen
FC vs untreated hes1/Bactin mRNA
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
NLSGFP
mNICDmER
KNICmER
KNICGFP

B

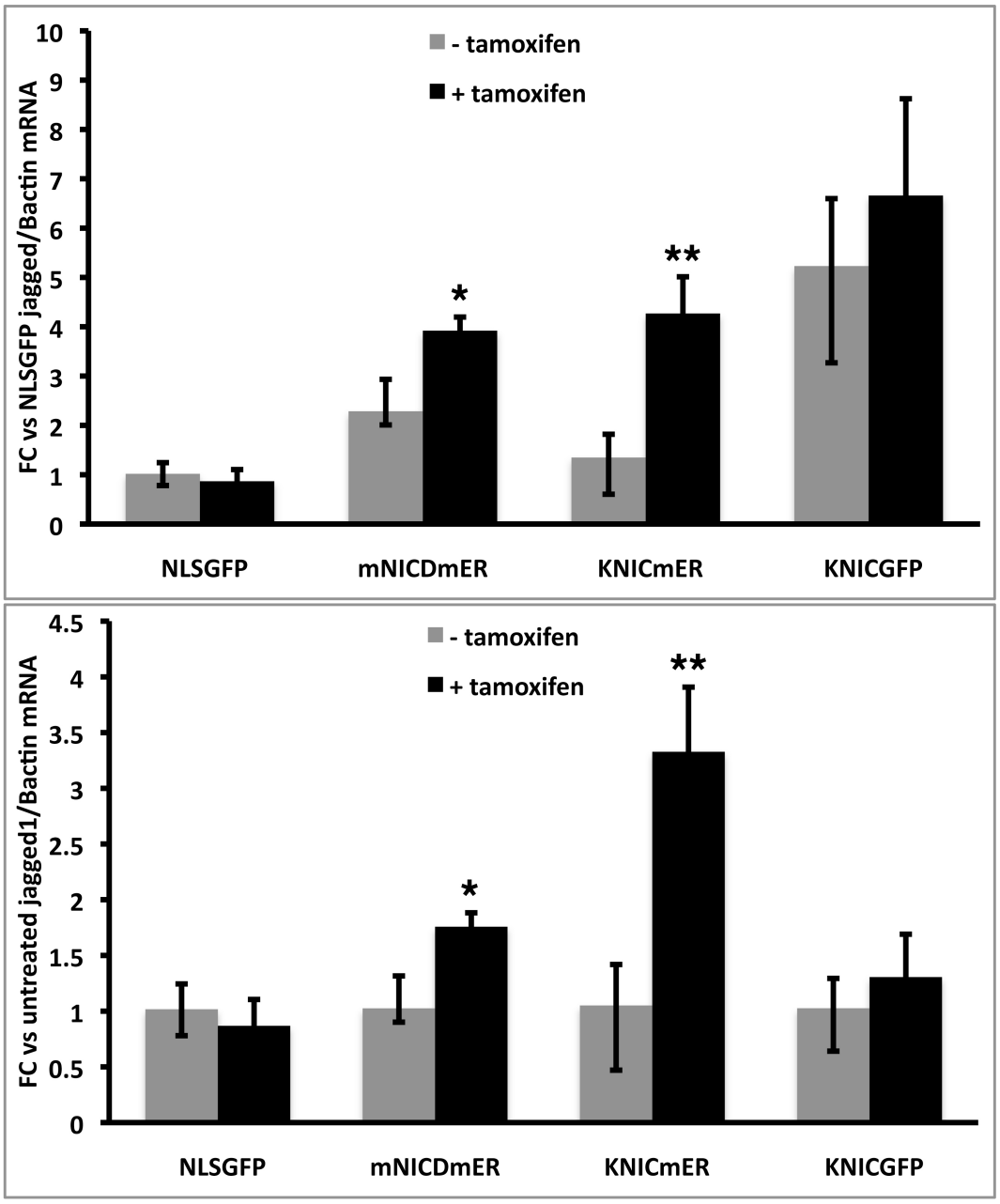


Figure 2.3 continued.

C

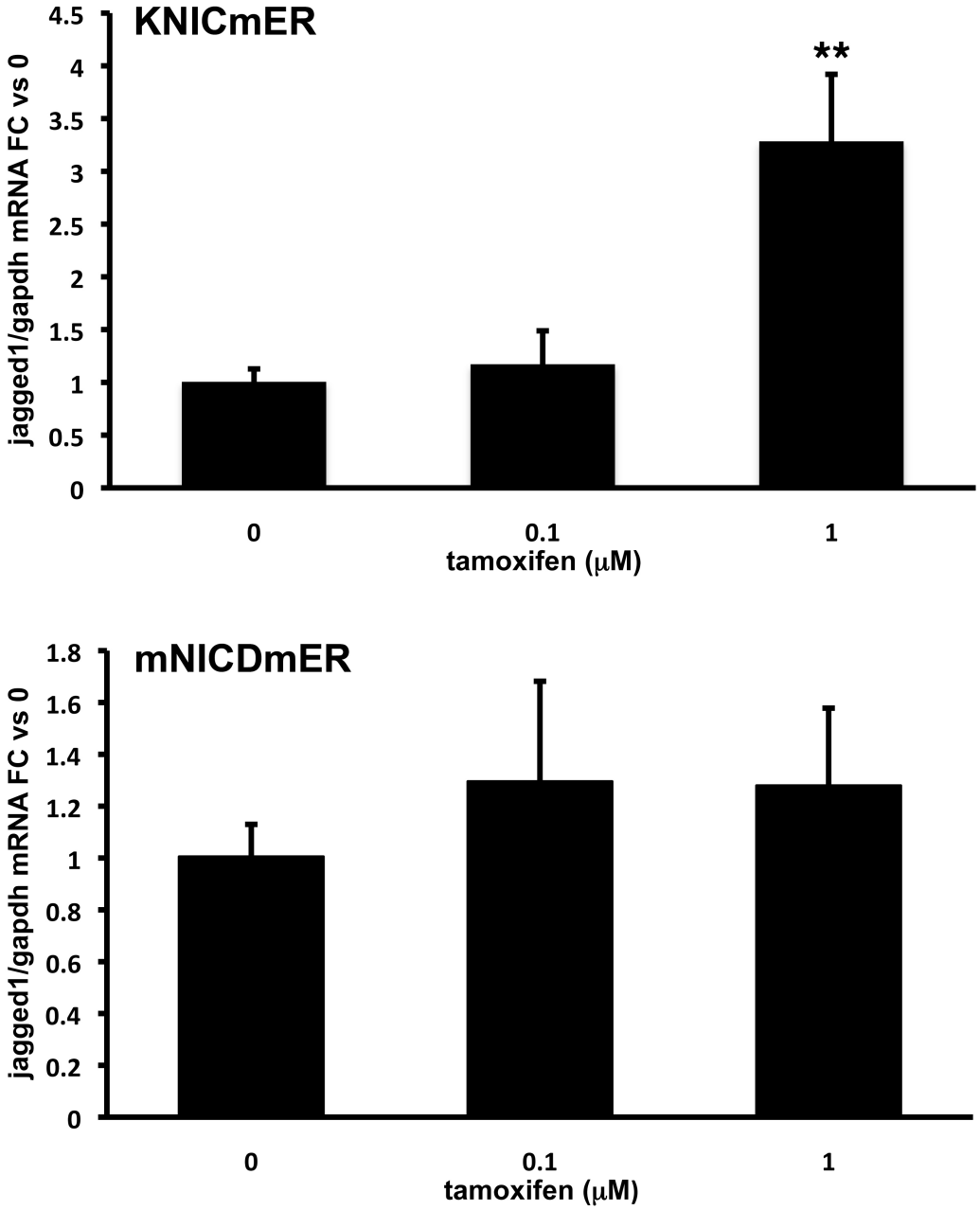


Figure 2.3 continued.

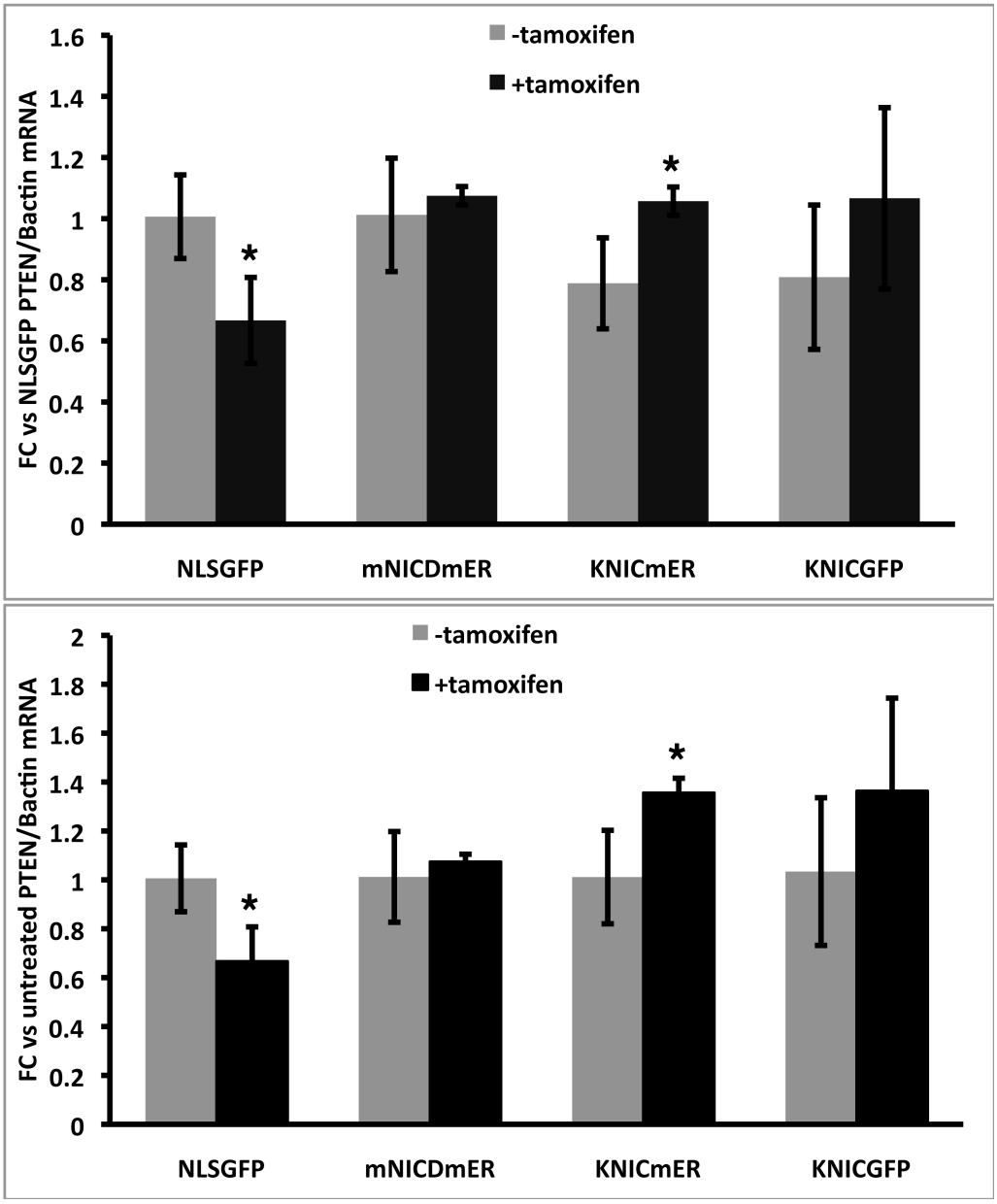


Figure 2.4: PTEN transcript is not suppressed by Notch in NRCMs. NRCMs infected with NLSGFP, mNICDmER, KNICmER and KNICGFP were treated with 1 μM tamoxifen for 24 hours. Transcript levels of PTEN were measured by qPCR using the ddCT method with β-actin as an internal control. Fold change was calculated relative to untreated NLSGFP sample (top panel) or untreated sample for each construct (bottom panel). Significance between vehicle and tamoxifen treatment for each sample was measured by T-test, n=3. * = p<.05.

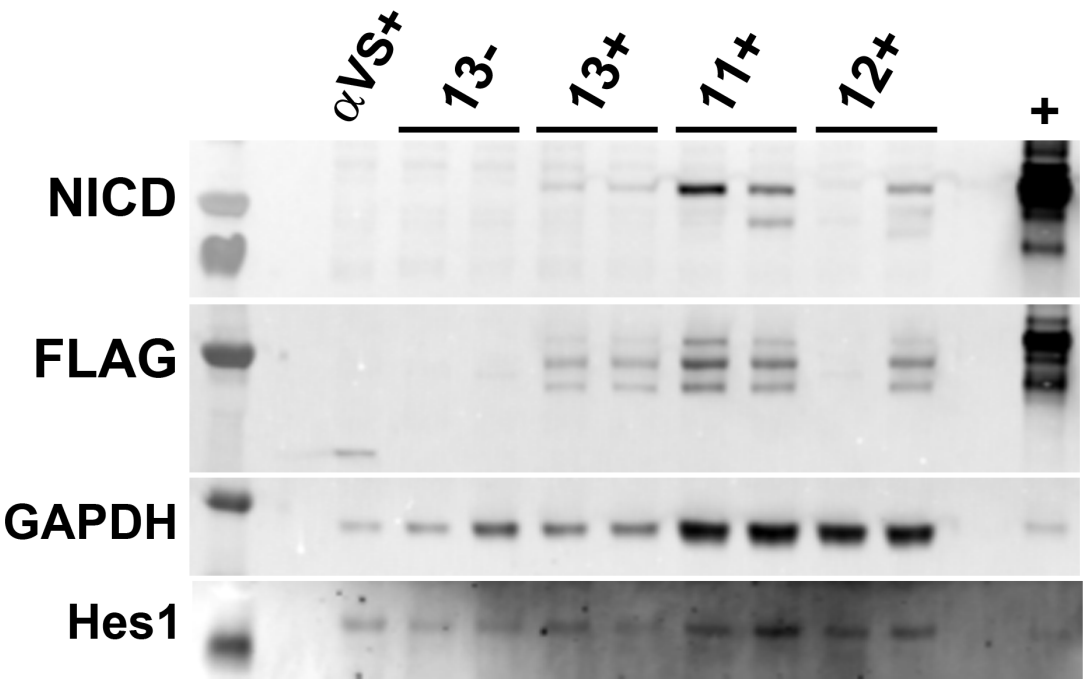


Figure 2.5: FLAG and NICD are expressed in cardiac tissue of αMyHC-KNICmER transgenic mice.
Hearts from F1 descendants of three αMyHC-KNICmER founder lines were prepared for immunoblotting. Samples from PCR negative (13-) and positive (13+, 11+, 12+) offspring and an unrelated FLAG expressing transgenic line (aVS+) were probed for NICD, FLAG and Hes1 expression. NRCMs infected with KNICmER encoding adenovirus served as a positive control (+). GAPDH was used as a loading control.

Appendix

Antibodies used for immunohistochemistry (IHC-P or IF), immunoblotting (Western) and immunoprecipitation (IP):

Antibody	Application	dilution	Catalog number
Goat anti-mouse c-kit	IHC-P, Western	5 μg /ml, 0.5 μg/ml	RNDSystems AF1356
Rat anti-mouse Ki67	IHC-P	1/100	DAKO #M7249
Rabbit anti-NICD1	IHC-P, Western	1/100, 1/1000	Rockland #100-401-407
Goat anti-Jagged1	IHC-P, Western	5 μg /ml, 0.5 μg/ml	RNDSystems #AF599
Goat anti-DLL4	IHC-P	5 μg /ml	RNDSystems #AF1389
Mouse anti-Tropomyosin	IHC-P	1/100	Sigma #T9283
Rabbit anti-Desmin	IHC-P	1/200	Biomeda #V2022
Goat anti-HGF	IHC-P	5 μg /ml	RNDSystems #AF2207
Rabbit anti-c-Met	IHC-P	1/50	Santa Cruz #sc-161
Mouse anti-GAPDH	Western	1/3000	Millipore/Chemicon #MAB374
Goat anti-Akt1/2	Western	1/400	Santa Cruz #sc-1619
Rabbit anti-p(S473)Akt	Western	1/1000	CST #9271
Rabbit anti-Hes1	Western, IF	1/400, 1/40	Santa Cruz #sc-25392
Goat anti-Notch1	Western	1/400	Santa Cruz #sc-6015
Mouse anti-Flag M2	Western, IF	1/1000, 1/100	Stratagene #200472

Antibody	Application	dilution	Catalog number
Goat anti-Nucleostemin	IF	1/100	RNDSystems AF1638
Rabbit anti-NICD	IF, Western	1/100, 1/1000	CST #2421
Mouse anti-sarcomeric actinin	IF	1/100	Sigma # A7811
Rat anti-Tenascin C	IHC-P	4 μg /ml	RNDSystems #MAB2138
Rabbit anti-PDK1	IF, Western	1/50, 1/500	Biosource #AHZ0512
Goat anti-GFP	IHC-P, Western	1/200, 1/1000	Rockland #600-101-215
Rabbit anti-p(T308)Akt	Western	1/1000	CST# 9275
Rabbit anti-c-myc	IP	1 μg /ml	Sigma #C3956
Rabbit anti-GFP	IP	1 μg /ml	Molecular Probes #A11122
Rabbit anti-tropomodulin	IF, IHC-P	1/50	Sussman lab
Mouse anti-PCNA	IF	1/40	Santa Cruz #sc-56
Rat anti-Tubulin	IF	1/50	Chemicon #CBL270
Rabbit anti-GATA4	IF, Western	1/50, 1/500	Santa Cruz #sc-9053
Rabbit anti-Smooth Muscle Actin (SMA)	IF	1/100	Anaspec #29553

Primers for cloning and genotyping:

To clone into pDC315 shuttle vector for adenovirus:

3xFlag SpeI Forward
5'-GG **ACTAGT** CGA CTA CAA AGA CCA CGA CGG TG-3'

mER Spe1 Stop Reverse
5'-GG **ACTAGT** TCA GAT CGT GTT GGG GAA G-3'

mNICD EcoRI Kozak Forward
5'-GCC **GAATTC** ACC ATG GTG CTG CTG TCC CGC AAG CGC-3'

mNICD EcoRI Fusion Reverse
5'-GCC **GAATTC** TTT AAA TGC CTC TGG AAT GTG G-3'

Kopan NIC (KNIC) EcoRI Fusion Reverse
5'-GCC **GAATTC** CGA GCT GTC CAA CAG GCA GC-3'

To clone into C26αMyHC for transgenic mice:

KNIC HindIII Kozak Forward:
5'-GCC **AAGCTT** ACC ATG GTG CTG CTG TCC CGC A-3'

EGFP HindIII Stop Reverse:
5'-GATC **AAGCTT** TTA CTT GTA CAG CTC GTC CAT GCC GA-3'

mER HindIII Reverse Stop
5'-GG **AAGCTT** TCA GAT CGT GTT GGG GAA G-3'

To genotype KNICmER mice:

Genotyping primer for αMyHC Forward:
5'- GGG TGC TCT CTT ACC TTC CTC-3'

Genotyping primer for KNIC Reverse
5'-GAG GGG CTC TCT CCG CTT C-3'

Primers for rat qPCR:

Rat hes1, Accession # NM_024360.3
For: 5'-GAAATTACGAATGGCTGTTCGGT-3'
Rev: 5'- CGCGGTACTTCCCCAACAC-3'

Rat hey1, Accession # XM_342216.3
For: 5'- GAAGCGCCGACGAGACCGAATCAA-3'
Rev: 5'- CAGGGCGTGCGCGTCAAAATAACC-3'

Rat jagged1, Accession # NM_019147.1
For: 5'- TTGCAGCGAATTGAGGAATCT-3'
Rev: 5'- GGATGTCTTCGGCAGAAATGG-3'

Rat pten, Accession # NM_031606.1
For: 5'- CAGTGGCGGAACTTGCAATC-3'
Rev: 5'- CGCGTGGGTCCTGAGTTG-3'

Rat gapdh, Accession # (several)
For: 5'- GACATGCCGCCTGGAGAAAC-3'
Rev: 5'- AGCCCAGGATGCCCTTTAGT-3'

CHAPTER III

REGULATABLE NOTCH ACTIVITY IN MOUSE CARDIAC PROGENITOR CELLS

Introduction

Cell-based therapy for treatment of heart disease continues to gain momentum as the future for myocardial regenerative medicine. Multiple studies over the last decade have established the existence of cardiac progenitors (CPCs) and demonstrated functional improvement in damaged hearts following adoptive transfer of a wide range of cell types in experimental models of heart failure and human subjects[99]. Despite the optimism and promise associated with these findings, improvements are often modest and transient depending upon the cell type or disease model under investigation. To overcome this limitation, genetic modification of progenitor cells to improve survival, engraftment and persistence into mature heart tissue shows remarkable results for enhancing cell-based myocardial therapeutics [100-105]. However, directing appropriate lineage commitment of adoptively transferred cells while maintaining survival and proliferative capacity remains elusive. The Notch pathway is a well-documented stem cell signaling network known to be critical in heart development, as well as stem cell self-renewal and specification. Recent studies of Notch signaling in mouse hearts and progenitor cells suggest that prudent manipulation of this critical signaling trigger will boost myocardial regeneration.

Notch participates in multiple cellular processes in various tissues throughout the cellular life cycle and as such is subject to several layers of regulation. Notch expression during cell fate determination is tightly regulated

spatially, temporally and quantitatively [84, 85]. Recent publications delve into the regulatory complexity of this pathway by analyzing posttranslational regulation or by creating computational models of context-dependent Notch signaling [106, 107]. A recurring theme that emerges from these publications is that levels and timing of Notch expression are subject to sophisticated controls in order to achieve appropriate cellular specification and tissue formation. As such, it is increasingly apparent that constitutive overexpression of activated Notch does not adequately emulate *in vivo* dynamics for this potent arbiter of intercellular communication. Optimal utilization of Notch for therapeutic molecular intervention rests upon a new generation of constructs capable of reversible and titratable expression, in conjunction with tissue-targeted regulatory elements incorporated into vector design. This dissertation delineates a strategy to create a system facilitating reversible regulation of Notch activity in CPCs with two parallel approaches: 1) a transgenic mouse possessing regulatable Notch activation under control of the mouse c-kit promoter, and 2) genetically engineered CPC lines with regulatable Notch activity for adoptive transfer.

To incorporate regulation of Notch activity, an inducible and reversible expression system is essential to achieve a physiologically relevant outcome. As a fusion protein, the mutated estrogen receptor (mER) confers control of protein activity via application of tamoxifen [86-89]. The mER fusion protein binds to heat shock protein 90 (hsp90), thereby enveloping the overexpressed

protein and blocking activity until displaced by treatment with tamoxifen [89]. The protein of interest is then available to interact with upstream or downstream effectors. This approach has been successfully used by the Glembotski laboratory here at San Diego State [86]. In this study, Kopan Notch Intracellular domain (KNIC) fused to mER will be targeted to CPCs *in vivo* using the c-kit promoter as previously described [108, 109]. This KNICmER fusion construct has been cloned into a lentiviral expression plasmid and transduced into CPCs to create stable cell lines. These cells will be used in adoptive transfer experiments to boost cardiac repair following myocardial infarction in mice with or without tamoxifen treatment. A schematic representation of the proposed constructs is illustrated in Figure 3.1.

Methods

Subcloning and creation of lentivirus

KNICmER was cloned by PCR amplification from pDC315KNICFLAGmER (Chapter II) into the CGW and PGK lentiviral vector backbones at the PacI and BamHI sites, respectively. See Primers for cloning, Chapter III, Appendix for primer sequences. CGWKNICmER, PGKGFP and PGKKNICmER constructs shown in Figure 3.1C-E were each co-transfected with three packaging plasmids pMDLg/pRRE, pRSV-rev, and vesicular stomatitis virus-G (VSVG) into 293T cells. Viral supernatant was harvested and either concentrated using ultracentrifugation as previously described (PGKGFP) [110] or retained as unconcentrated virus (PGKKNICmER and CGWKNICmER) and then applied directly to CPC cultures. Cells expressing PGKGFP and CGWKNICmER are detectable by EGFP expression, the latter being driven off the lentiviral IRES. After four passages cells were tested for protein expression of intracellular Notch and FLAG in order to verify integration of the transgene and establishment of the cell line (Fig. 3.8).

Luciferase assay

HEK293 or CPCs were seeded in six well dishes at approximately 50 percent confluency. The following day cells were transfected with mammalian expression plasmids encoding NLSGFP, KNICGFP, KNICmER or mNICDmER together with either control or RBPJk-luciferase encoding plasmid (Superarray). Cells were maintained in growth medium for 24 - 48 hours in

order to allow for plasmid protein expression, treated with either vehicle or 1 μM tamoxifen for 24 hours and then harvested in extraction buffer for measurement of luciferase activity according to manufacturer's instructions (Promega Dual Luciferase Kit) as follows: Luciferase activity was measured on a Tecan plate reader using the _ settings. Signals were normalized to luciferase transfection controls.

Immunoblotting

Whole cell lysates were separated on Bis-Tris gradient gels (Invitrogen) and transferred to Immobilon-P membranes (Millipore). Membranes were briefly stained with PonceauS to confirm uniform transfer, then washed in TBST (150 mM NaCl,50 mM Tris, 0.1% Tween-20, pH 7.5) and blocked for one hour at room temperature in 10% nonfat milk reconstituted in TBST. Blots were rinsed with TBST and primary antibodies applied in blocking buffer overnight at 4C. After washing in TBST, secondary antibodies were applied for 90 minutes at room temperature in blocking buffer, washed in TBST and scanned for fluorescent signal on a Typhoon 9410 (Molecular Dynamics). Refer to list of Antibodies in Appendix, Chapter II for antibodies used in immunoblotting.

Quantitative real time PCR

RNA was extracted from cells using the Zymo micro RNA II kit according to manufacturer instructions. Briefly, for a six well dish, media was removed and cells were lysed in 600 microliters per well of RNA buffer.

Extract was purified through a column, washed, eluted and quantitated by OD260 and 280 readings. First strand synthesis of cDNA was performed on total RNA (between 0.1 and 1.0 micrograms) using Applied Biosystems reverse transcriptase and OligodT18 primer. Quantitative real time PCR was performed with Qiagen SybrGreen reaction mix and a Bio-Rad/MJ Opticon thermal cycler. Primer sequences were designed using Primer Express software to span two exons in order to avoid amplification of genomic DNA. Some primer sequences were obtained from publications and verified by Primer Express[111]. Each primer set was initially tested for efficiency by running standard curves of fourfold serial dilutions ranging from 50 nanograms to 0.8 nanograms of positive control cDNA template per 25 microliter reaction. Product integrity was checked by a melting curve programmed at the end of 40 cycles. See primers for mouse qPCR, Chapter III, for primer sequences used in real time PCR.

Immunostaining

Cardiac progenitors were washed with PBS, fixed in 4% PFA (4% paraformaldehyde in McIlvane's buffer) for at least 30 minutes, washed in PBS and blocked for one hour at room temperature in 10% horse serum in PBS (HS/PBS). Primary antibodies were applied in blocking buffer at 4C overnight, slides were then washed in PBS and secondaries applied in blocking buffer for 90 minutes at room temperature. To label nuclei, To-pro 3 iodide was included in the final wash at a concentration of 1/5000, then slides were

coverslipped with Vectashield mounting media and visualized using a Leica SP2 confocal scanning laser microscope. Refer to List of Antibodies in Appendix, Chapter II for antibodies used for immunostaining.

Results

Constructs for expression in adenovirus, lentivirus and in c-kit progenitors in vivo

Murine NIC was obtained from Dr. Raphael Kopan (Washington University) as a cDNA (KNIC) and spans V1744-S2184, omitting the PEST domains that target intracellular Notch for degradation (Chapter II, Figure 2.1). KNIC was cloned by PCR amplification into the pDC315 shuttle vector for adenoviral expression to create both a constitutively active green fluorescent fusion protein (KNICGFP, Figure 3.1A) and a ligand regulated Notch mER fusion protein (KNICmER, Figure 3.1B). KNICmER was subcloned by PCR amplification into lentiviral constructs under control of a viral promoter (Figure 3.1C) or the mammalian PGK promoter (Figure 3.1E). As a control for creating stable cell lines, the PGK-EGFP lentivirus was used (Figure 3.1D). Figure 3.1F illustrates the construct that will be used to generate transgenic mice expressing regulatable intracellular Notch under control of the c-kit promoter in order to manipulate Notch signaling in c-kit expressing stem and progenitor cells.

Protein expression of KNICGFP and KNICmER in CPCs and induction of RBPJ-k luciferase reporter

CPCs were transfected with pDC315 KNICGFP and KNICmER constructs. Fusion protein expression was confirmed by immunoblot analysis for FLAG (KNICmER), GFP (KNICGFP) and Notch Intracellular Domain (NIC,

Figure 3.2A). Regulation of KNICmER by tamoxifen was demonstrated using a RBPJk-luciferase reporter system (SuperArray) combined with the Dual Luciferase assay (Promega). CPCs were cotransfected with the RBPJk-luciferase construct and either KNICmER or a negative control EGFP-encoding vector. As an additional negative control, cells were cotransfected with KNICmER and a control vector provided by the manufacturer. After 24 hours, cells were treated with vehicle or 1 μM tamoxifen, harvested and assayed for luciferase activity. The top panel represents activity as absolute values of luminescence units, while the bottom panel represents tamoxifen induction of luciferase activity in each sample set as normalized to vehicle treated control (Figure 3.2B). The KNICmER construct is regulation by tamoxifen, with tamoxifen treated luciferase activity two and a half fold higher than vehicle treated control (bottom panel). Basal levels of RBPJk-luciferase in vehicle treated KNICmER samples were elevated above the KNICmER and GFP negative controls.

Tamoxifen induces Notch activity in KNICmER expressing CPCs

CPCs were transfected with pDC315 plasmids encoding nuclear-targeted GFP (GFPNLS), KNICGFP and KNICmER (Figure 3.1A, 3.1B), and after 24 – 48 hours were treated with vehicle or 1μM tamoxifen for 24 hours. Samples extracted for RNA and qPCR was performed on cDNA, as described in Materials and Methods. Expression of both jagged1 and hey1 mRNA was

increased in the KNICmER-expressing cells following tamoxifen treatment, as measured by qPCR. In this assay, β-actin served as the internal control and signal strength was based on a standard curve using mouse embryo cDNA template for each primer set (Figure 3.3A). KNICmER was subcloned into two constructs for creating lentivirus to generate CGWKNICmER and PGKKNICmER (Figure 3.1C and 3.1E, respectively). CGWKNICmER contains a viral MND promoter, whereas PGKKNICmER utilizes the mammalian PGK promoter. Cell lysates of transient transfections for both constructs were tested by immunblot for FLAG and NICD to confirm appropriate expression of the KNICmER fusion (data not shown). The PGKKNICmER construct was transfected into CPCs and tested for tamoxifen regulation via qPCR analysis of hey1, jagged1 and smooth muscle actin (SMA) transcripts (Figure 3.3B, 3.3C, 3.3D, respectively). GAPDH served as the internal control for each sample, and fold change was calculated relative to either the vehicle treated PGKGFP control sample (PKGFP, top panel) or to the vehicle treated sample for each construct (bottom panel). Fold change was calculated using the delta CT method. Jagged1, hey1 and SMA transcripts were all upregulated following tamoxifen treatment of the PGKKNICmER-expressing cells versus cells expressing PGKGFP or control cells (PKntg). While jagged1 and hey1 represent Notch signaling family members, smooth muscle actin is a differentiation marker for either vascular

smooth muscle cells or a differentiation intermediate in the transition from cardiac progenitor to cardiac myocyte.

Sustained Notch activation pushes CPCs away from progenitor phenotype

CPCs were infected with adenovirus expressing either constitutively active or regulatable NIC for at least five days. Cells were monitored for gross morphological changes and analyzed by qPCR for changes in Notch target transcripts and cardiac differentiation markers. Initially cells were infected with adenovirus expressing either EGFP or NICDEGFP (human NICD, gift of Dr. Ken Tezuka, Japan). After six days, cells were fixed and stained for nucleostemin (white), a nucleolar protein present in stem cells and lost following differentiation, GATA4 (red), a transcription factor associated with cardiac lineage commitment, and tubulin (blue) to mark the cell body. EGFP fluorescence (green) was scanned directly. After six days of NICDEGFP expression, CPCs were larger and flatter than EGFP expressing counterparts which retained the rounder, more spindle shaped morphology typical of progenitor cells. Cells expressing high NICDEGFP had less intense nucleostemin and GATA4 staining (Figure 3.4B, arrowheads) than cells with lower levels of NICDEGFP (arrows). Immunoblots for c-kit and hes1 illustrate biochemical changes in CPCs overexpressing activated Notch for one or six days versus EGFP controls (Figure 3.4C). Hes1 levels were elevated in NICD-expressing cells after one and six days, whereas c-kit and GATA4 levels were unchanged compared to EGFP controls at one day but decreased

noticeably after six days of NICD expression. CPCs were then infected with adenovirus encoding EGFP, KNICGFP or KNICmER and treated with either PBS vehicle or 1 μM tamoxifen for five days. Cells were monitored for morphological changes and harvested for analysis by qPCR. KNICmER-expressing cells treated with vehicle resembled EGFP-expressing cells treated with tamoxifen after five days. By contrast, KNICmER cells treated with tamoxifen for five days were similar on a macroscopic level to cells expressing KNICGFP for five days (Figure 3.5). A parallel set of cells were plated on chamber slides and received the same treatment, vehicle or tamoxifen for five days in growth medium. Immunostaining for smooth muscle actin (SMA, red) and Notch (green) shows that vehicle treated KNICmER cells remain smaller and have nominal staining for SMA similar to EGFP cells under the same conditions. However KNICmER cells receiving tamoxifen have many large, SMA positive cells consistent with stainings of KNICGFP expressing cells (Figure 3.6). At the molecular level, real time qPCR performed on cDNA extracts revealed similar results: transcripts for hey1, jagged1 and SMA were increased in KNICmER cells treated with tamoxifen while vehicle treated KNICmER cells retained transcript levels near those of the EGFP controls. Interestingly, mRNA levels of CD31, which is a differentiation marker for endothelial lineage, were decreased in response to elevated Notch activity. Collectively, these results indicate that elevated Notch activity is impacting

CPC morphology and lineage commitment. In this set of experiments the cells were maintained in complete growth medium, which may influence the direction in which Notch "pushes" the progenitor cells. Future experiments will combine induction of Notch activity with media conditions favoring progenitor differentiation, such as adding dexamethasone and removing serum, coculture with NRCMs, or manipulating other developmental cues such as Wnt or noncanonical Wnt signaling known to be important in cardiac development and lineage commitment.

Cell lines of cardiac progenitors transduced with lentivirus encoding KNICmER express FLAG and NICD

CPCs were transduced with crude stock of CGWKNICmER (Figure 3.1C) or PGKKNICmER (Figure 3.1E). After one day, cells were washed in PBS, re-fed with cardiac stem cell growth media, and monitored for expression of GFP (CGWKNICmER contains an IRES driving GFP expression) and density. Cells were passaged at least four times before freezing and storage as established cell lines. Immunostaining and immunoblotting indicate that these lines retain the transgene after four passages as shown in Figure 3.8. Cells were fixed in paraformaldehyde and immunostained for FLAG tag (green), intracellular Notch (NICD, red) or tubulin (blue). Additionally, cell lysates were immunoblotted for FLAG tag and NICD to confirm expression of the transgene (Figure 3.8G). Nontransgenic cells clearly lack the FLAG and NICD bands, while both CGWKNICmER cells at passage 3 and

PGKKNICmER cells at passage 4 retain expression of the transgene. Future experiments will focus on induction of Notch targets in these cells following tamoxifen treatment, as well as changes in morphology and cardiac lineage markers.

KNICGFPTetOn constructs are inducible with doxycycline

A common concern with inducible systems is "leakiness" or high basal levels of transgene expression prior to the inductive stimulus. As an alternative method for expressing inducible Notch activity, KNICGFP was subcloned by PCR amplification into the pTight Tet-On plasmid (Clontech) at the HindIII site and tested for inducible NICD activity in parallel with the PGKKNICmER lentiviral construct (Figure 3.9). For the KNICTetOn construct, cells were cotransfected with the transactivator plasmid. Whereas the KNICmER construct is expressed prior to activation by tamoxifen, the KNICTetOn protein is not expressed until treatment with doxycycline. Cells expressing KNICmER, KNICGFP and PGKGFP were treated with vehicle or tamoxifen, and KNICTetOn cells received 1μM doxycycline for 24 hours. Within a few hours of doxycycline treatment, KNICTetOn transfected cells began to exhibit green fluorescence, indicative of KNICGFP expression. Cells were harvested for qPCR analysis, and as shown in Figure 3.9, transcripts of jagged1 were elevated in KNICTetOn induced cells as well as PGKKNICmER tamoxifen treated cells, although not to the same absolute extent. Nonetheless, KNICTetOn cells exhibited lower basal activity compared to

PGKKNICmER samples, which could be an important feature if slightly elevated basal levels of Notch activity in CPCs over time would be sufficient to push them away from self renewal and toward lineage commitment.

Discussion

This chapter investigates novel approaches for enhancing the effectiveness of cardiac stem cell therapy through development of *in vitro* and *in vivo* models of genetically engineered cardiac progenitor cells (CPCs) expressing regulatable activated Notch, a potent regenerative stem cell signal. The overall hypothesis is that temporal and reversible control of Notch signaling in CPCs will improve their reparative function in damaged myocardium. The overall goal is to manipulate key cellular signals driving survival and differentiation to strengthen the regenerative capacity of CPCs as reagents for cell-based therapeutics in the treatment of heart disease.

Carefully timed Notch signaling is expected to improve cardiogenic potential in CPCs. Regulation of timing and levels of inducible Notch activation by tamoxifen treatment will allow more precise control of regenerative Notch signaling as well as enable further dissection of Notch and survival signaling pathway interactions. Using this regulatable system we will optimize differentiation conditions under which Notch activity pushes CPCs into the mature cardiac lineages: endothelial, vascular smooth muscle and cardiomyocyte. The purpose of harnessing inducible Notch activity is to improve CPC survival, engraftment and commitment to cardiac lineages following adoptive transfer into injured myocardium. Therefore it will be important to assess the impact of pulsatile Notch activation on KNICmER CPC proliferation and differentiation *in vitro* and *in vivo*. CPCs are cultured in

growth medium to promote proliferation and pluripotency. Alternatively, by removing cytokines, serum and factors which promote “stemness” and adding reagents such as dexamethasone or coculturing with NRCMs in low serum myocyte medium, CPCs can be induced to stop dividing and assume a differentiated phenotype. In future studies, control and KNICmER CPCs will be treated with tamoxifen in either growth or differentiation medium in order to activate KNICmER in both conditions and determine the impact on CPC differentiation.

In addition to characterization of KNICmER CPCs *in vitro*, it will be necessary to test their efficacy in myocardial repair *in vivo*. To this end, mouse KNICmER CPCs will be delivered via intramyocardial injection into FVB mouse models of acute myocardial infarction. Groups will include sham operated mice and mice with infarcted hearts receiving intramyocardial injection of buffer, control CPCs or KNICmER CPCs. All animal groups will receive intraperitoneal injection of either vehicle or tamoxifen at varying times and dosages as estimated by the induction parameters determined in the KNICmER CPC cultures to promote cardiogenic differentiation of the transgenic cells *in vivo*. Collectively, these studies will determine if activated KNICmER CPCs regenerate damaged myocardium more effectively than control CPCs. Additionally, these experiments will define conditions under which the potent regenerative Notch signal can be manipulated to promote cardiac progenitor proliferation, differentiation and cardiac lineage commitment

in a cell based therapy application. This will provide an important advancement to optimizing cardiac progenitor efficiency for treatment of damaged myocardium.

Recent work from our group reveals elevation of endogenous NIC in damaged mouse myocardium and demonstrates that exogenous NIC overexpression ameliorates the decline in cardiac function following infarction [95]. A transgenic mouse model in which cardiomyocyte-specific KNICmER is expressed under control of the α-MyHC promoter [112] has recently been created in our laboratory to extend understanding of myocyte-specific effects (Chapter II). In contrast, the mouse model described here expresses conditionally activated Notch under control of the c-kit promoter, which has previously been used by our group to track c-kit+ cells via GFP expression[109] [108]. KNICmER fusion protein will be expressed in c-kit+ cells throughout the body and will be activated following administration of tamoxifen either by injection or in the food supply. This transgenic line will provide a tool to determine the effect of temporally controlled activated Notch on c-kit+ stem cell activation, proliferation, engraftment and differentiation *in vivo*. Allowing for KNICmER expression in all c-kit+ cells provides the advantage that stem cells outside the heart are included in the regulatable system, allowing for a more “whole body” approach to stem cell repair of the heart. Instead of constitutive Notch activation by an irreversible genetic recombination event, the mER fusion system has the built in flexibility of timing Notch activation with

application of tamoxifen, as well as diminishing activity by withdrawal of the tamoxifen stimulus. Additionally, it may be possible to titrate the activation response by varying the dose as well as duration of tamoxifen.

The overall purpose of creating this mouse model is to examine the benefit of increased Notch activity in CPCs *in situ* following myocardial injury. To assess how enhanced activated Notch in the c-kit+ progenitor population impacts cardiac repair at the functional and immunohistochemical level, acute MI will be surgically induced by ligating the left anterior descending coronary artery (LAD). Tamoxifen (20mg/kg) will be injected intraperitoneally for up to 14 days, after which heart function and morphology will be monitored by echocardiography along with sham operated ckit-KNICmER and nontransgenic infarcted counterparts treated with vehicle or tamoxifen. After a minimum of four weeks, heart function will be assessed by *in vivo* hemodynamics and hearts harvested for paraffin processing.

In the c-kitKNICmER mouse KNIC will be expressed in c-kit positive cells throughout the body and subject to mER regulation. However, we posit this transgene will demonstrate the impact of controlled pulses of Notch activity in stem cells following cardiac injury. Since the activation of KNICmER will be temporary, it is not predicted that other tissues will be severely affected and that the injured myocardium will be the primary site of stem cell activity. Indeed, other tissues such as intestine, testes or ovaries, bone marrow and other sites of active c-kit expression will serve as “in house” positive controls

for construct expression and induction. The ideal cardiac progenitor specific expression construct would target KNICmER only to stem cells residing in the heart, however to date there is no universally approved cardiac progenitor marker available to drive expression in these cells in the adult heart. The Tet inducible system in which a cardiac specific transactivator line is crossed with a tet-inducible c-kit line (or vice versa) provides an alternate inducible approach to the mER fusion system proposed here. Currently c-kit–rtTA, αMyHCmin^{TetO}, αMyHC-tTA and αMyHC-rTA lines exist [113, 114], however only cardiac progenitors with αMyHC activity would express the transgene in the last three. Although expression of αMyHC driven transgenes has been detected in CPCs[115] all potential CPCs including circulating c-kit+ cells will be transgenic in the proposed model.

Figures

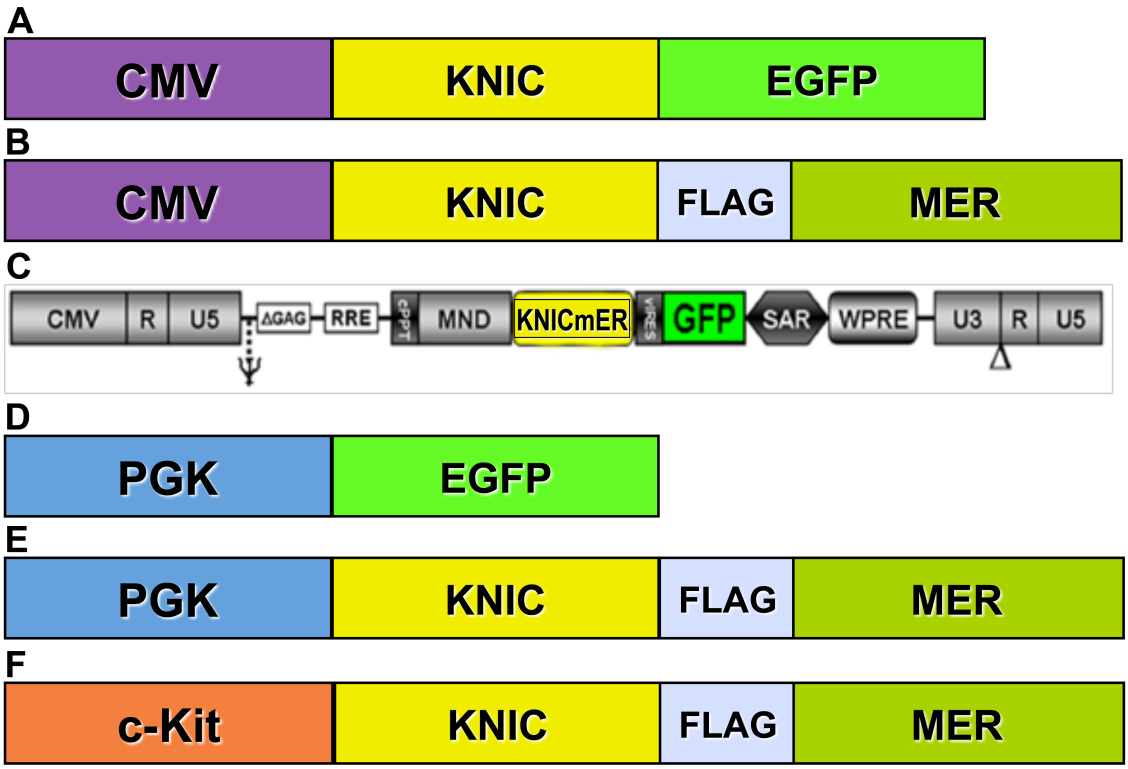


Figure 3.1: Schematic representation of recombinant DNA constructs
Vectors to be used for adenoviral recombination (A,B) lentiviral transduction (C,D,E) and progenitor transgenesis (F)

Figure 3.2: Regulatable Notch clones express NICD and increase luciferase reporter activity following tamoxifen treatment.
CPCs transfected with control FLAGmER, KNICGFP and KNICmER plasmids were immunoblotted and probed for activated Notch (NICD), GFP or FLAG fusion tags (A). Lysates from HEK293 cells transfected with control pDC315 or mNICDmER plasmid were immunoblotted and probed for NICD and FLAG fusion tag. GAPDH was used as a loading control (B). Dual luciferase reporter assay of CPCs cotransfected with negative control or RBPJk-luciferase reporter and plasmids encoding KNICmER or GFP treated with PBS or 1 μM tamoxifen for 24 hours (C).

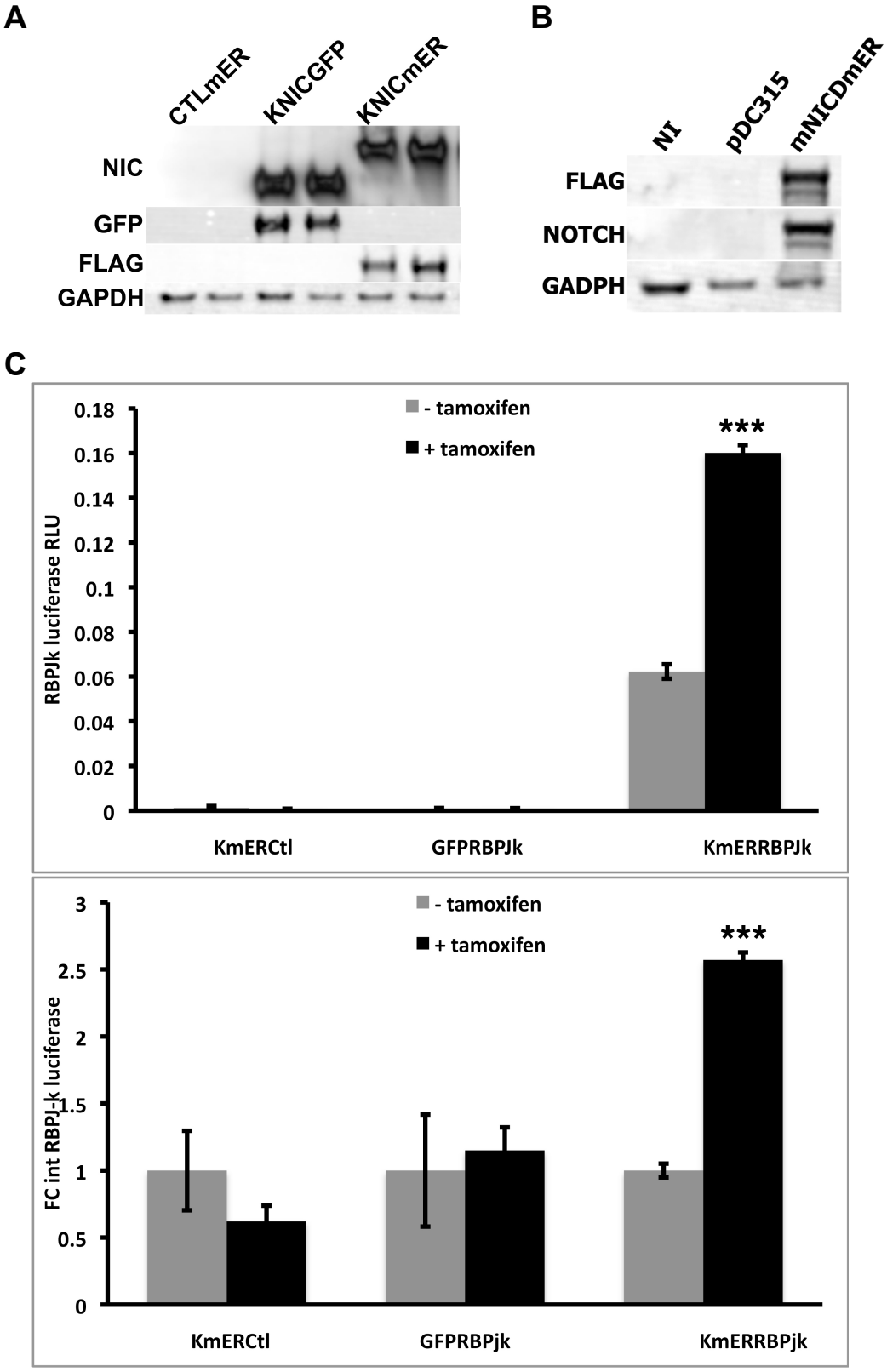
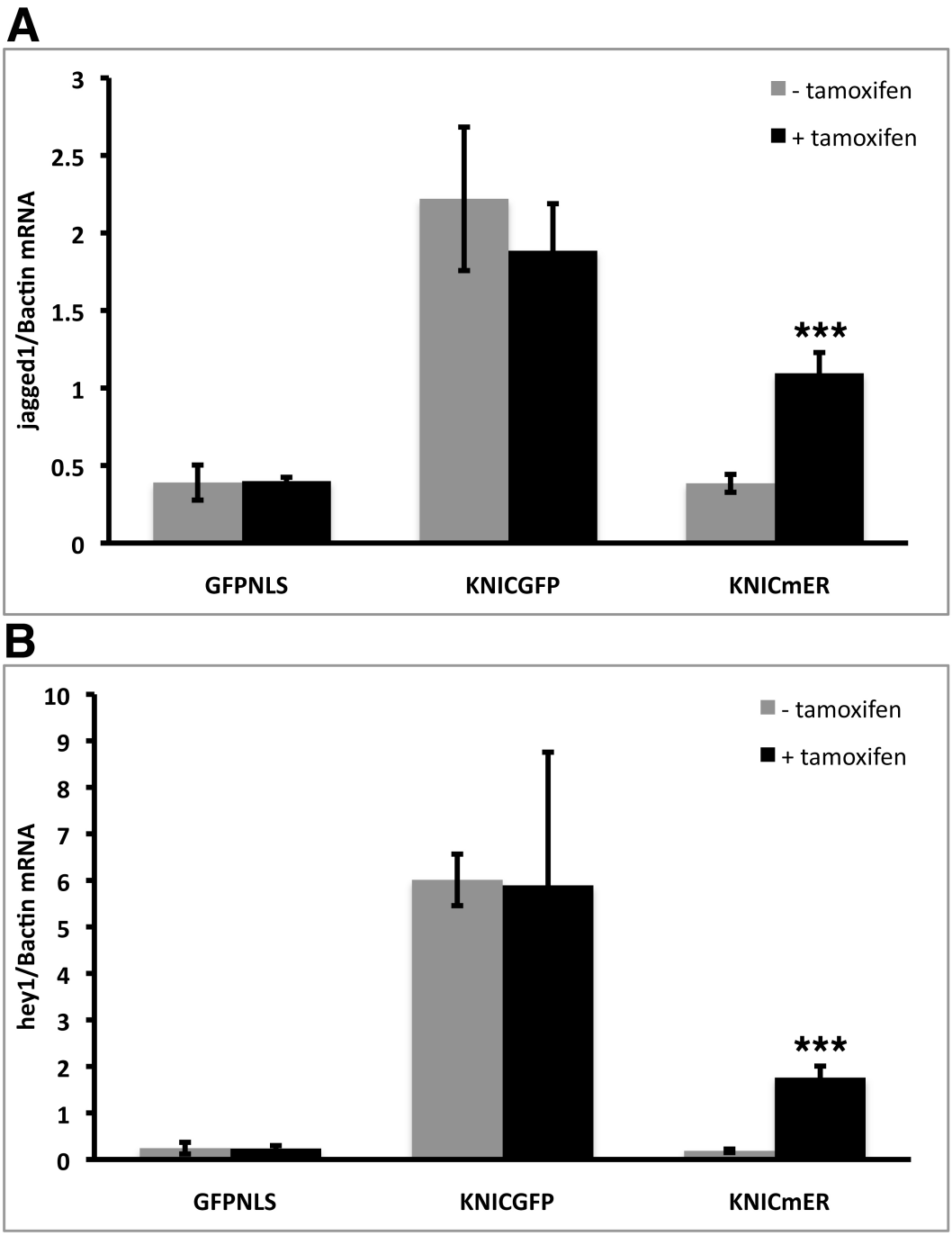

A
CTLmER
KNICGFP
KNICmER
NIC
GFP
FLAG
GAPDH
B
NI
pDC315
mNICDmER
FLAG
NOTCH
GADPH
C
- tamoxifen
+ tamoxifen
***
RBPJk luciferase RLU
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
KmERCtl
GFPRBPJk
KmERRBPJk
- tamoxifen
+ tamoxifen
***
FC int RBPJ-k luciferase
3
2.5
2
1.5
1
0.5
0
KmERCtl
GFPRBPjk
KmERRBPjk

Figure 3.3: Induction of jagged1, hey1 and smooth muscle actin (SMA) mRNA in tamoxifen treated CPCs expressing inducible activated Notch. Quantitative PCR of jagged1 (A) and hey1 (B) using cDNA derived from nontransgenic CPCs transfected with adenoviral plasmids encoding nuclear targeted GFP (GFPNLS), intracellular Notch GFP fusion (KNICGFP) and intracellular Notch mER fusion (KNICmER) treated with PBS or 1 μM tamoxifen for 24 hours standardized to B-actin control. Transcript levels for A and B are based on a standard curve derived from embryonic cDNA control. Transcript levels of jagged1 (C), hey1 (D) and SMA (E) were measured by qPCR of cDNA derived from CPCs transfected with lentiviral constructs encoding GFP (PKGFP), regulatable Notch (PKKNICmER) or buffer (PKntg) treated with vehicle (- tamoxifen) or 1 μM tamoxifen (+ tamoxifen) for 24 hours. GAPDH was used as an internal control. Signals were expressed as fold change (FC) relative to the vehicle treated PKGFP (top panel) or to the vehicle treated sample for each vector (bottom panel) using the ddCT method. T-test was used to measure significance. * = $p<.05$, ** = $p<.01$, *** = $p<.001$. n per sample = 3.


A
jagged1/Bactin mRNA
3
2.5
2
1.5
1
0.5
0
- tamoxifen
+ tamoxifen
***
GFPNLS
KNICGFP
KNICmER
B
hey1/Bactin mRNA
10
9
8
7
6
5
4
3
2
1
0
- tamoxifen
+ tamoxifen
***
GFPNLS
KNICGFP
KNICmER

C

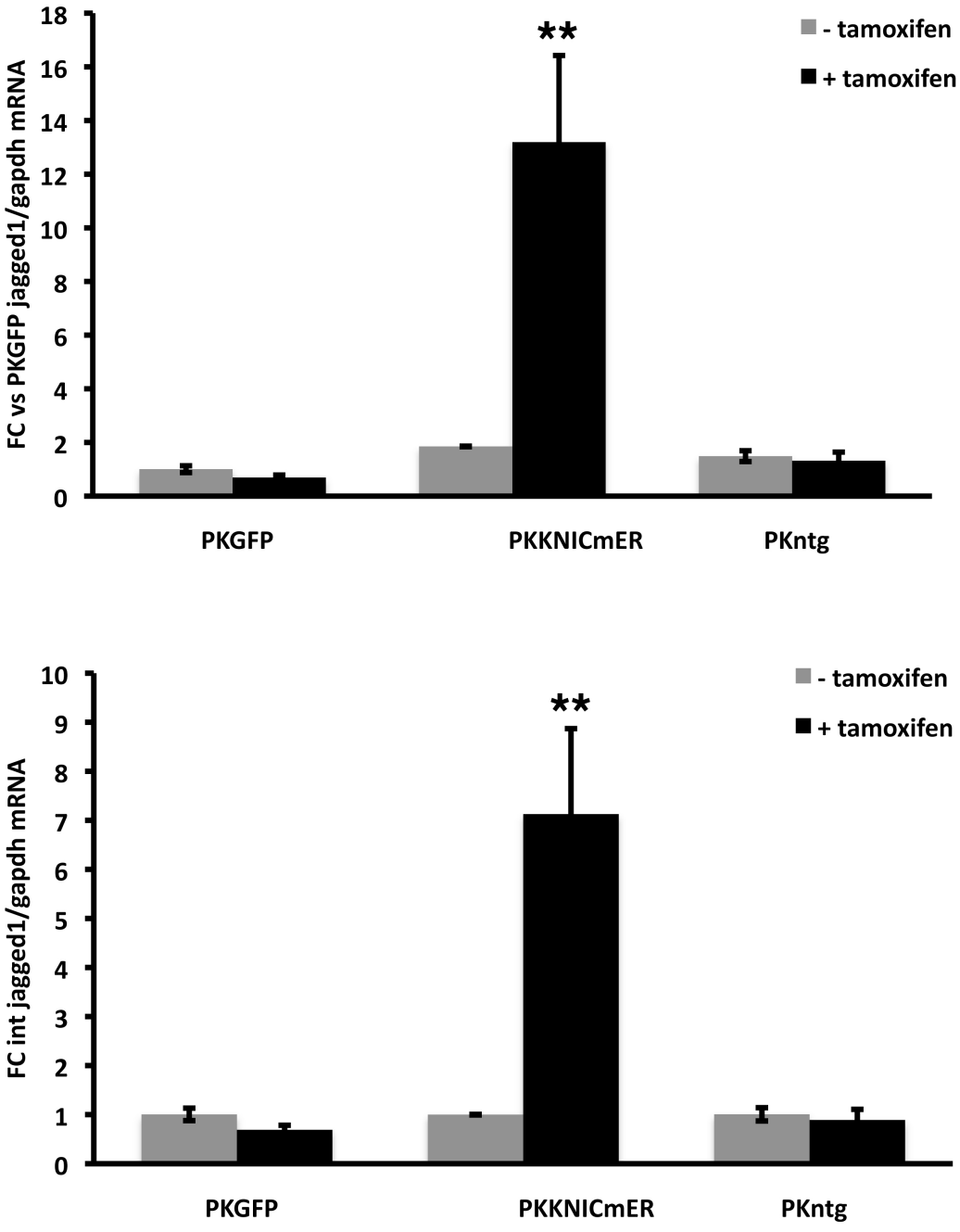


Figure 3.3 continued.

D

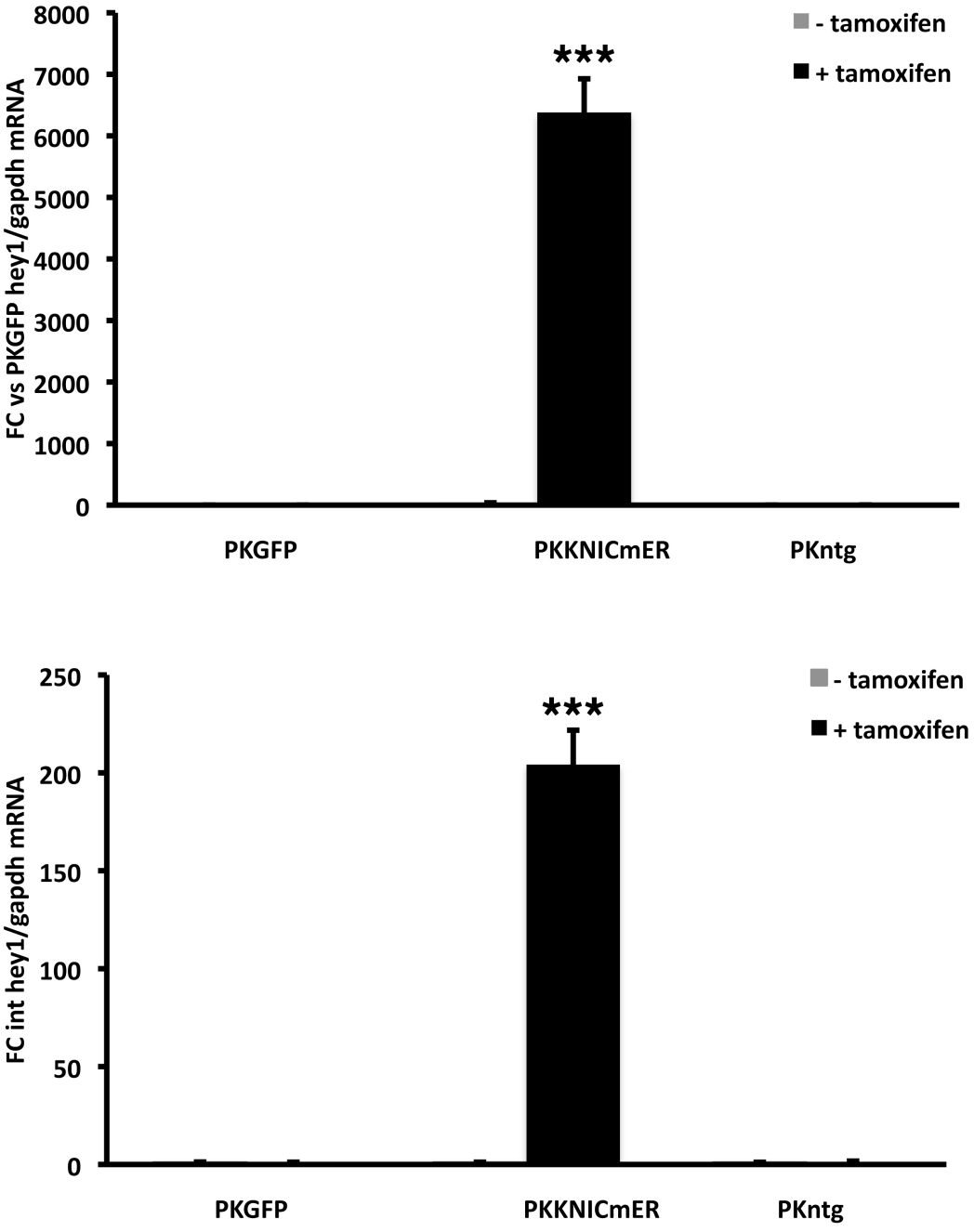


Figure 3.3 continued.

E

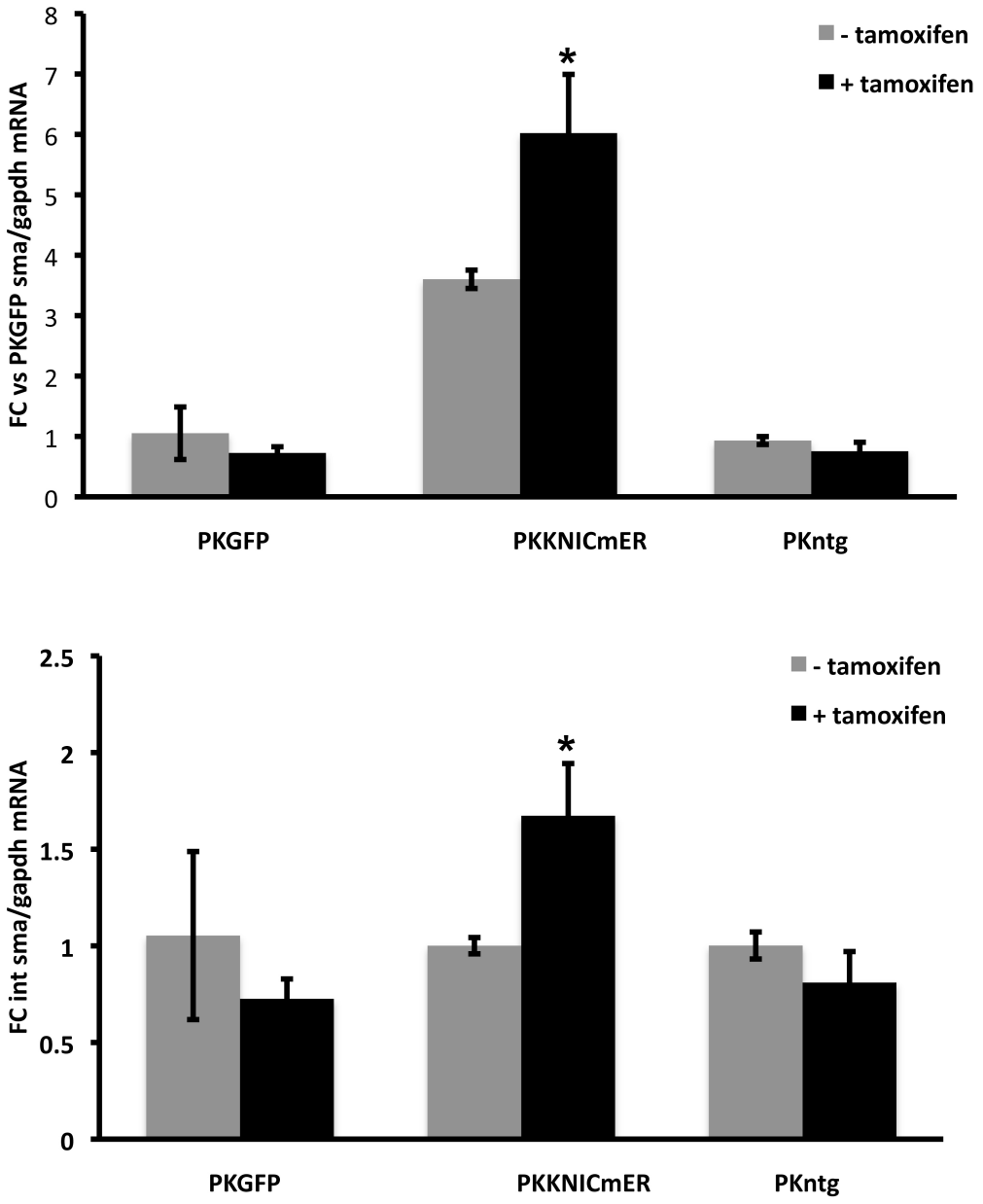
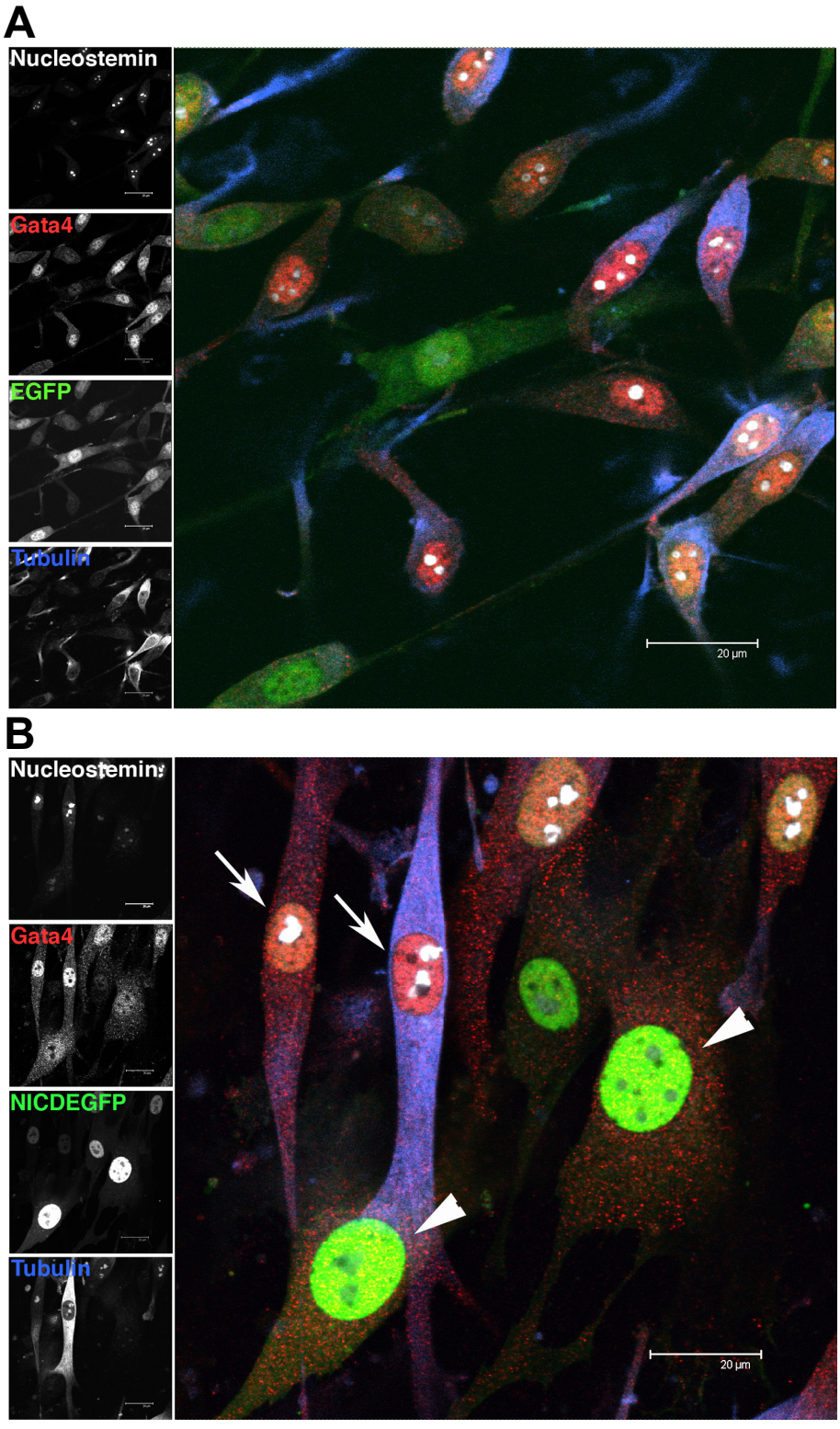


Figure 3.3 continued.

Figure 3.4: Overexpression of activated Notch1 alters cardiac progenitor cell morphology and c-kit levels.
CPCs infected with adEGFP A. or adNICDEGFP B. for six days stained for GATA4 (red), nucleostemin (white) and tubulin (blue). C. Western blot of uninfected CPCs (NI), CPCs infected with adEGFP (EGFP) or adNICDEGFP (NICD) for six days immunoblotted for GATA-4, c-Kit and Hes1, and GAPDH as a loading control.


A
Nucleostemin
Gata4
EGFP
Tubulin
20 µm
B
Nucleostemin
Gata4
NICDEGFP
Tubulin
20 µm

C

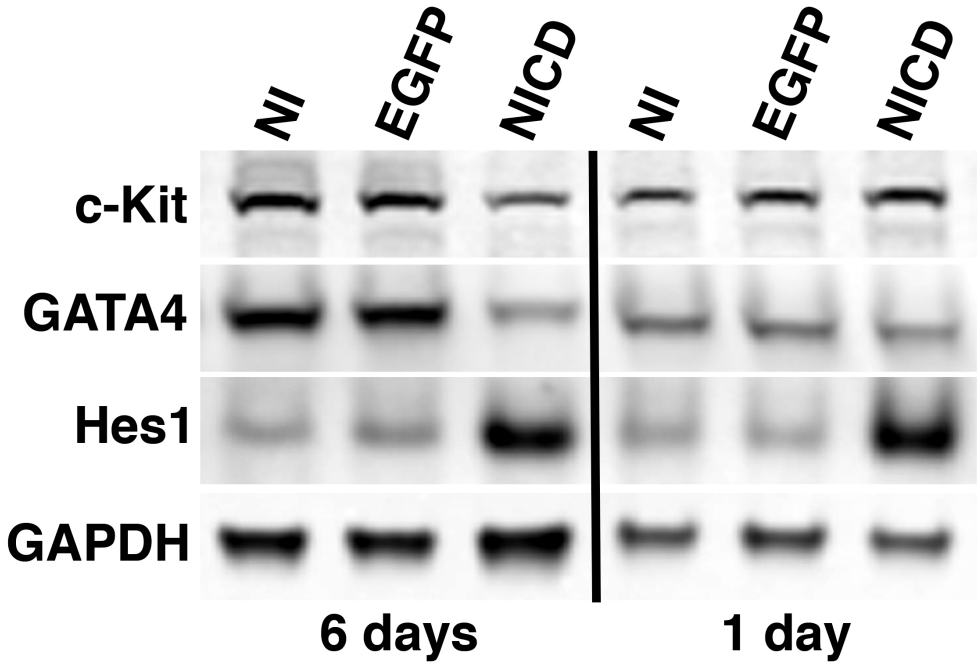


Figure 3.4 continued.

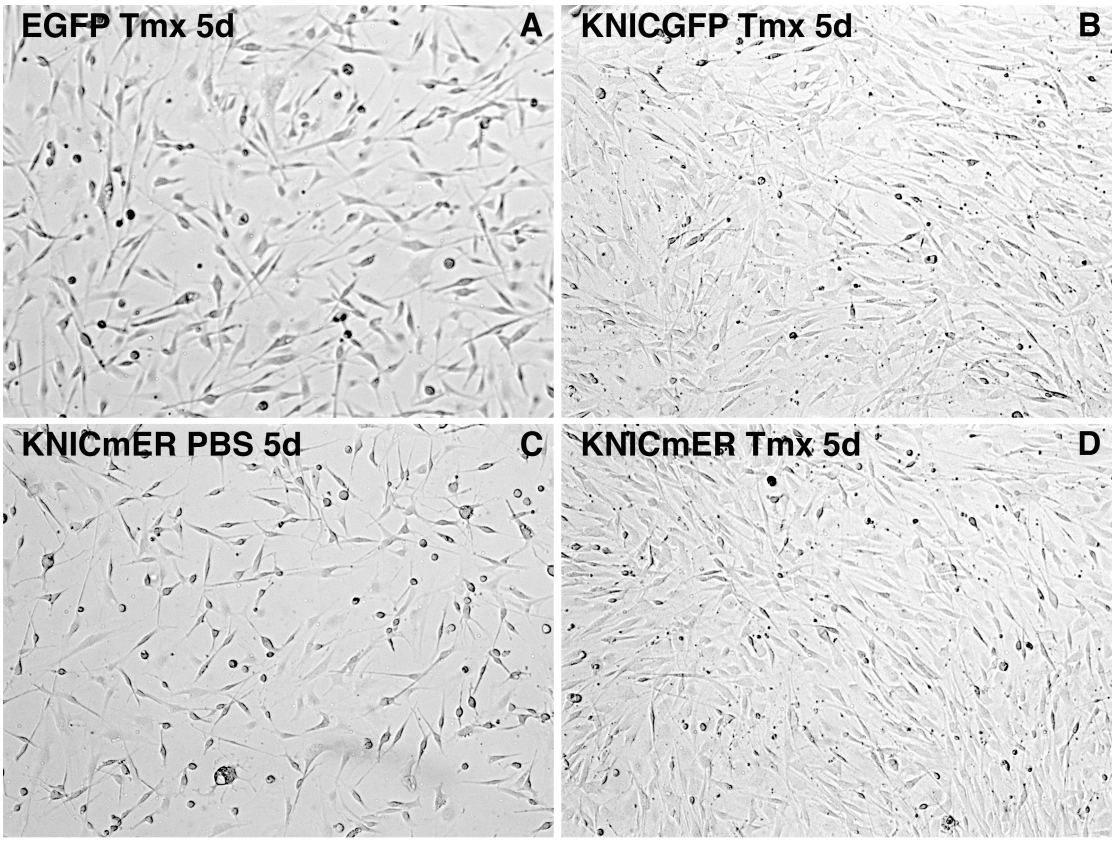


Figure 3.5: CPCs respond to induction of Notch activity.
CPCs were infected with adenoviruses encoding GFP, KNICmER or KNICGFP and treated with either vehicle or 1 μM tamoxifen in growth medium for five days. Mouse CPCs infected with adenovirus encoding A) EGFP B) KNICGFP or C,D) KNICmER.

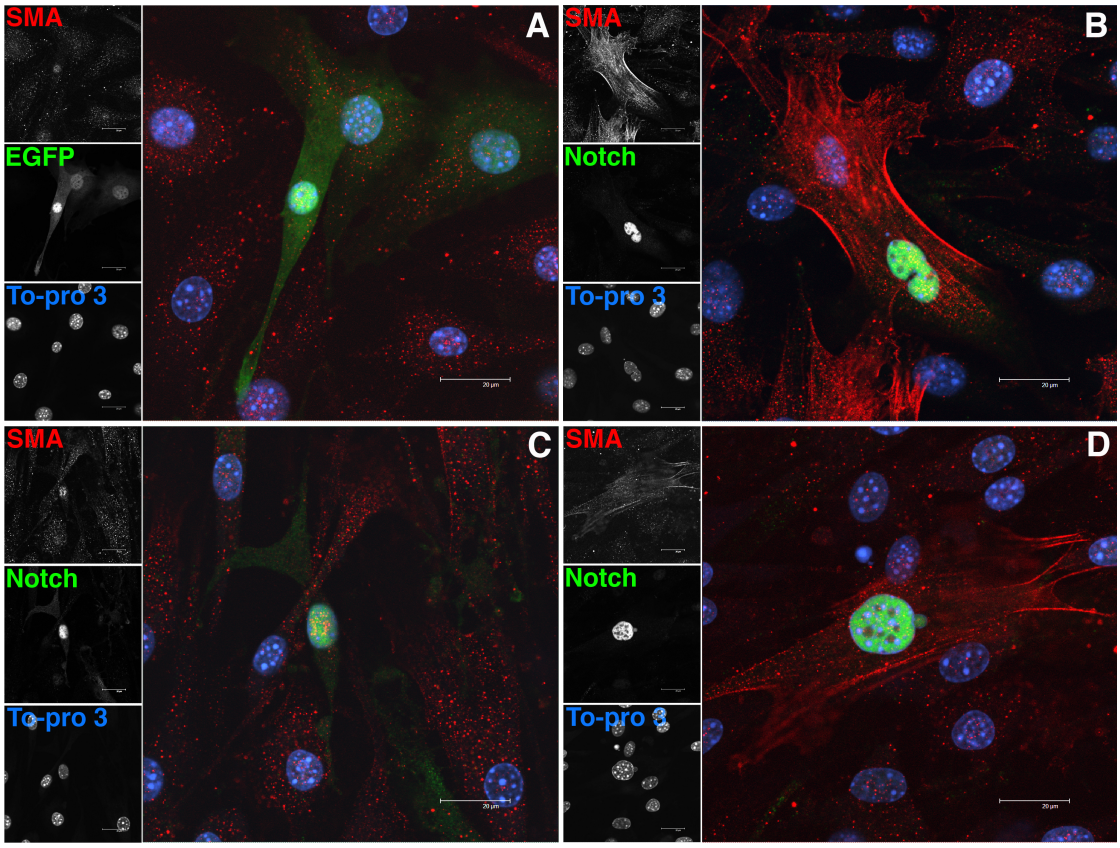
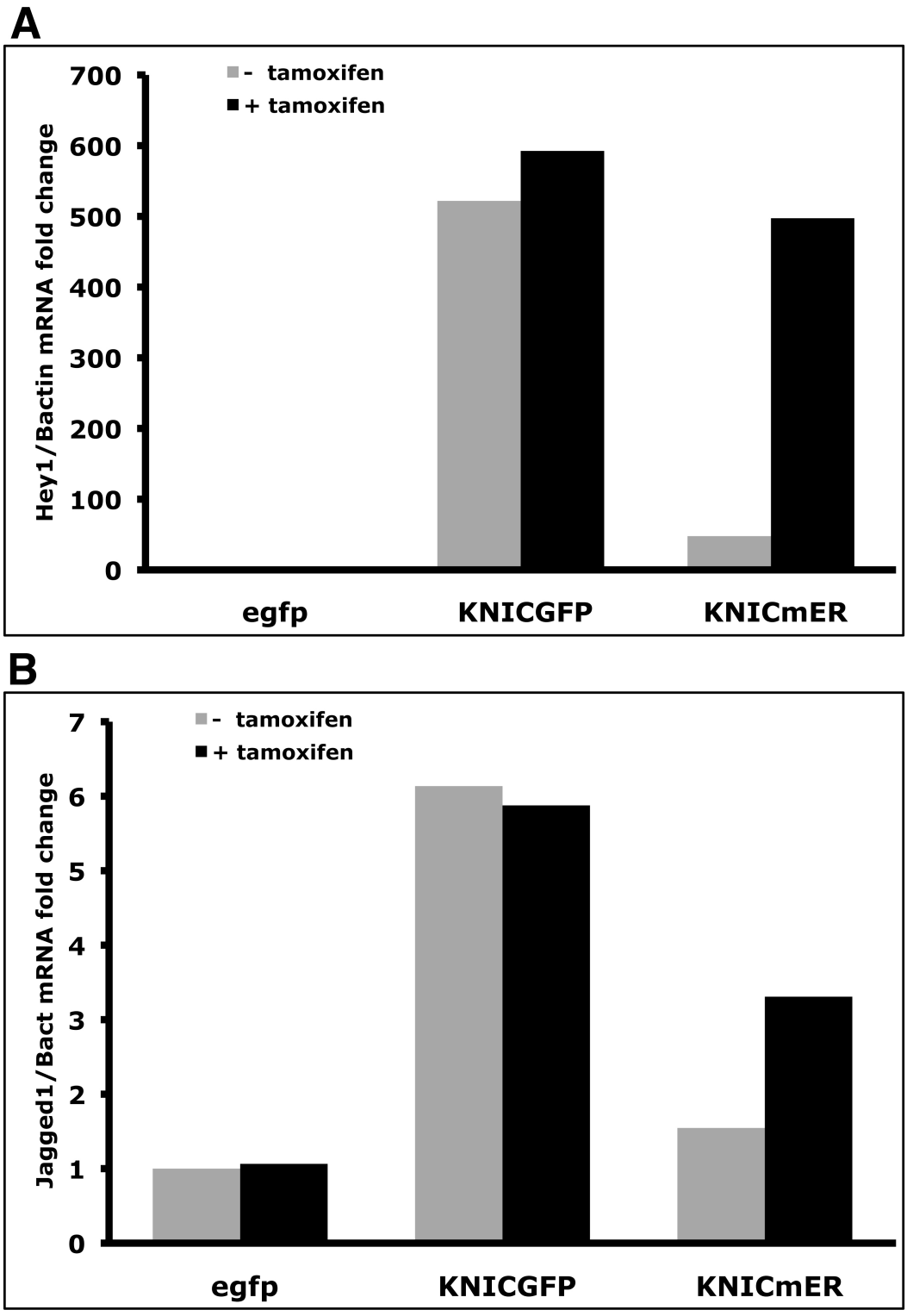


Figure 3.6: CPCs express smooth muscle actin (SMA) following induction of Notch activity.
Mouse CPCs infected with adenovirus encoding A) EGFP B) KNICGFP and C,D) KNICmER were treated with PBS or tamoxifen for five days, fixed and stained for smooth muscle actin (SMA, red), Notch (green) or DNA (To-pro 3). EGFP (A, green) was detected directly. KNICmER cells treated with PBS (C) retained morphology similar to EGFP cells (A) and weak staining for SMA. KNICmER cells treated with tamoxifen (D) became large and flattened and stained strongly for SMA, similar to KNICGFP cells with (B) or without tamoxifen.

Figure 3.7: Induction of jagged1, hey1 and smooth muscle actin (SMA) mRNA in CPCs expressing inducible activated Notch treated with tamoxifen for six days.
Cardiac progenitors were infected with adenovirus encoding GFP (egfp), constitutively active intracellular Notch fused to GFP (KNICGFP), or regulatable intracellular Notch fused to mER (KNICmER). Cells were treated in growth medium with vehicle or 1μM tamoxifen for six days and harvested for RNA. Transcript levels for hey1 (A), jagged1 (B), SMA (C) and CD31 (D) were measured by qPCR using the ddCT method with B-actin as an internal control. Signals were normalized to vehicle treated EGFP expressing samples.


A
- tamoxifen
+ tamoxifen
Hey1/Bactin mRNA fold change
700
600
500
400
300
200
100
0
egfp
KNICGFP
KNICmER
B
- tamoxifen
+ tamoxifen
Jagged1/Bact mRNA fold change
7
6
5
4
3
2
1
0
egfp
KNICGFP
KNICmER

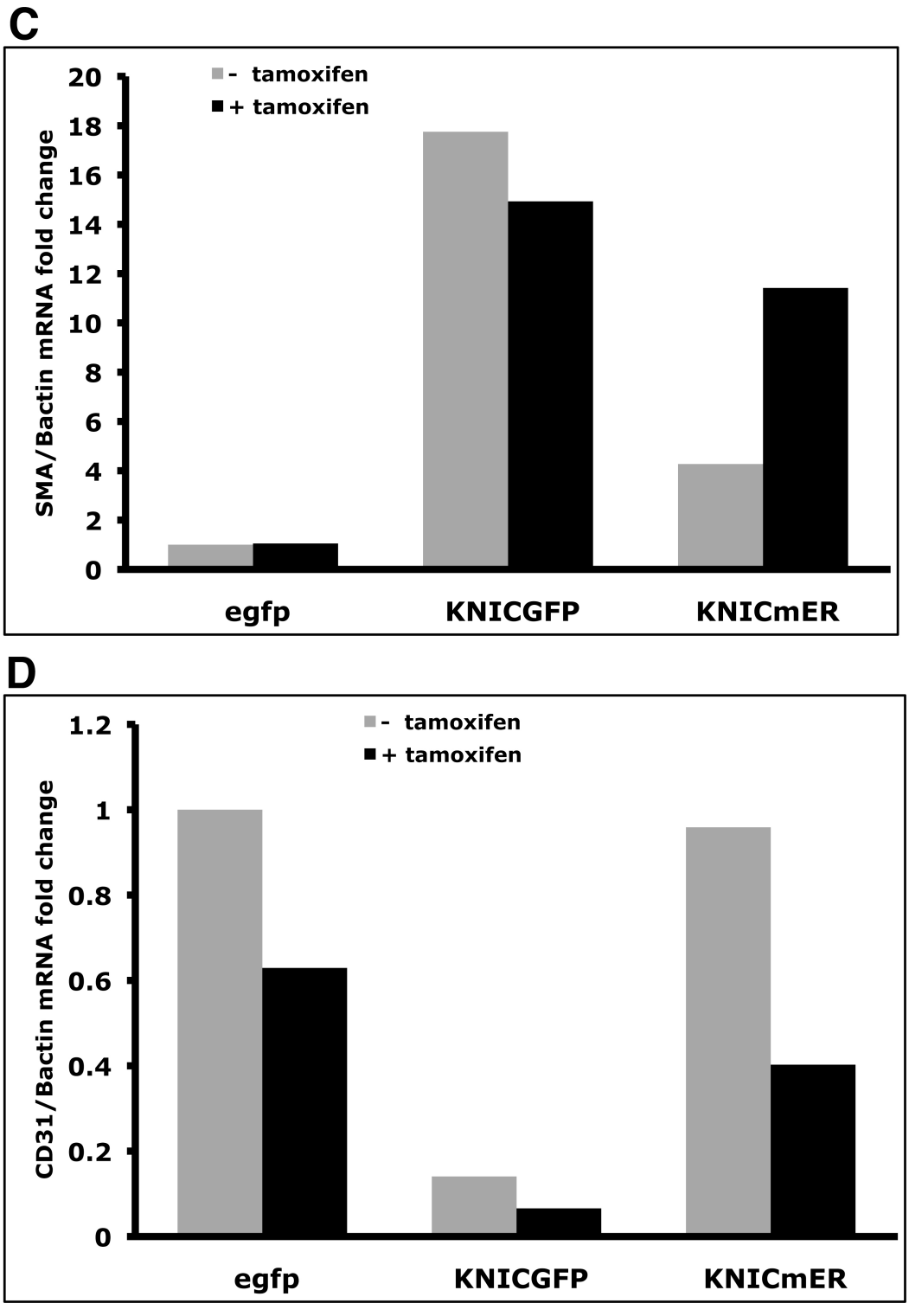
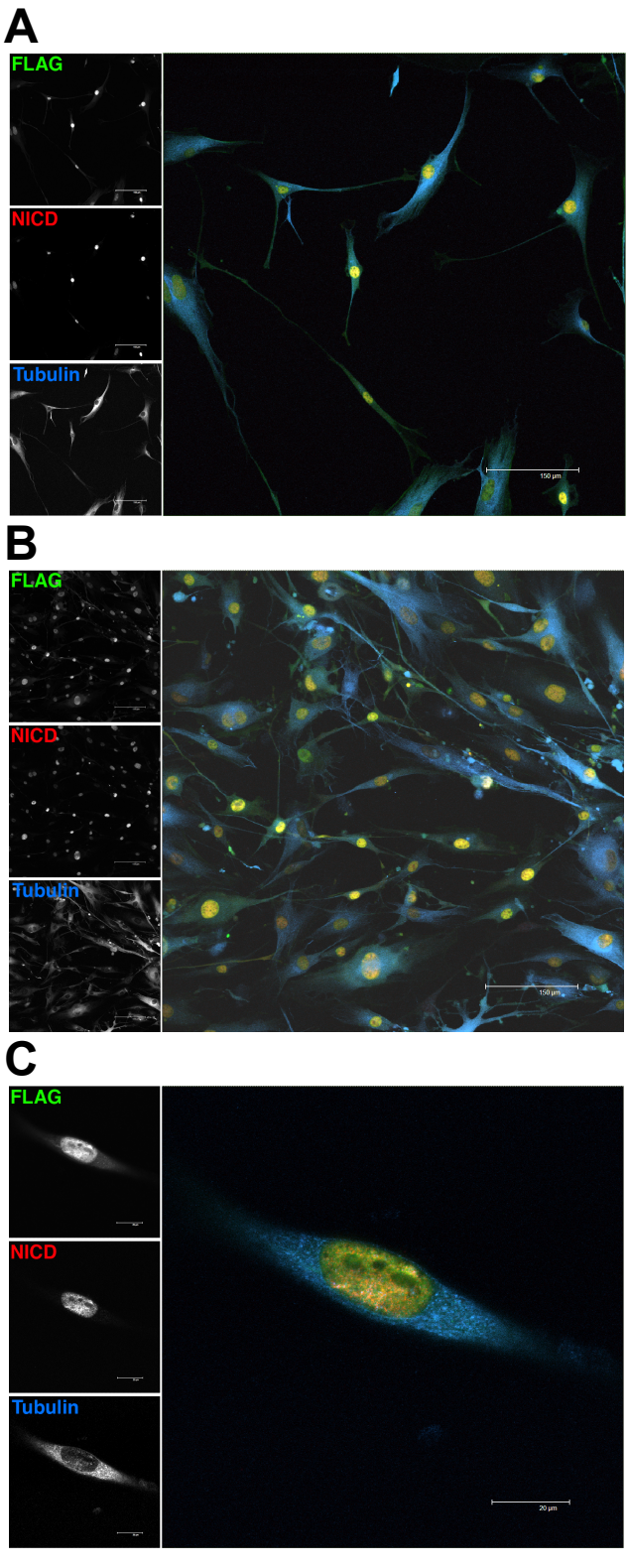
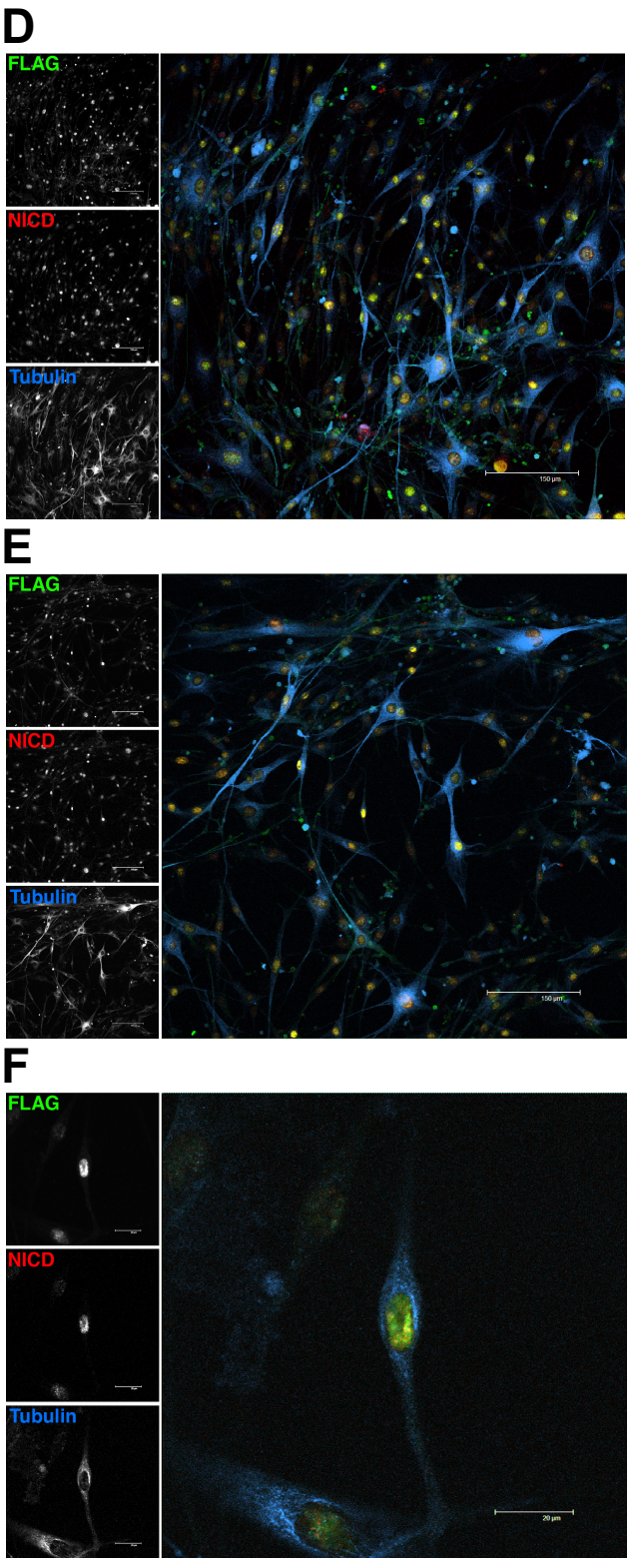


Figure 3.7 continued.

Figure 3.8: Cell lines established from cardiac progenitors transduced with lentivirus encoding regulatable activated Notch:
CPCs transduced with CGWKNICmER (A-C) or PGKNICmER (D-F) express the FLAG tag (green) and intracellular Notch (NICD, red) after at least four passages. Tubulin staining is in blue. Western blotting (G) verifies expression of FLAG and NICD in these cell lines with and without tamoxifen treatment compared to untransduced nontransgenic CPCs (NTG).


A
FLAG
NICD
Tubulin
B
FLAG
NICD
Tubulin
C
FLAG
NICD
Tubulin



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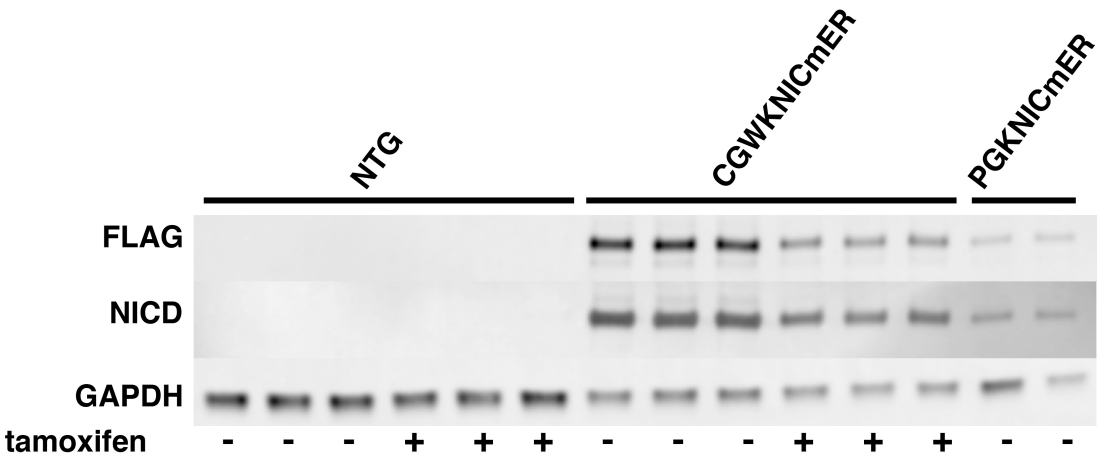

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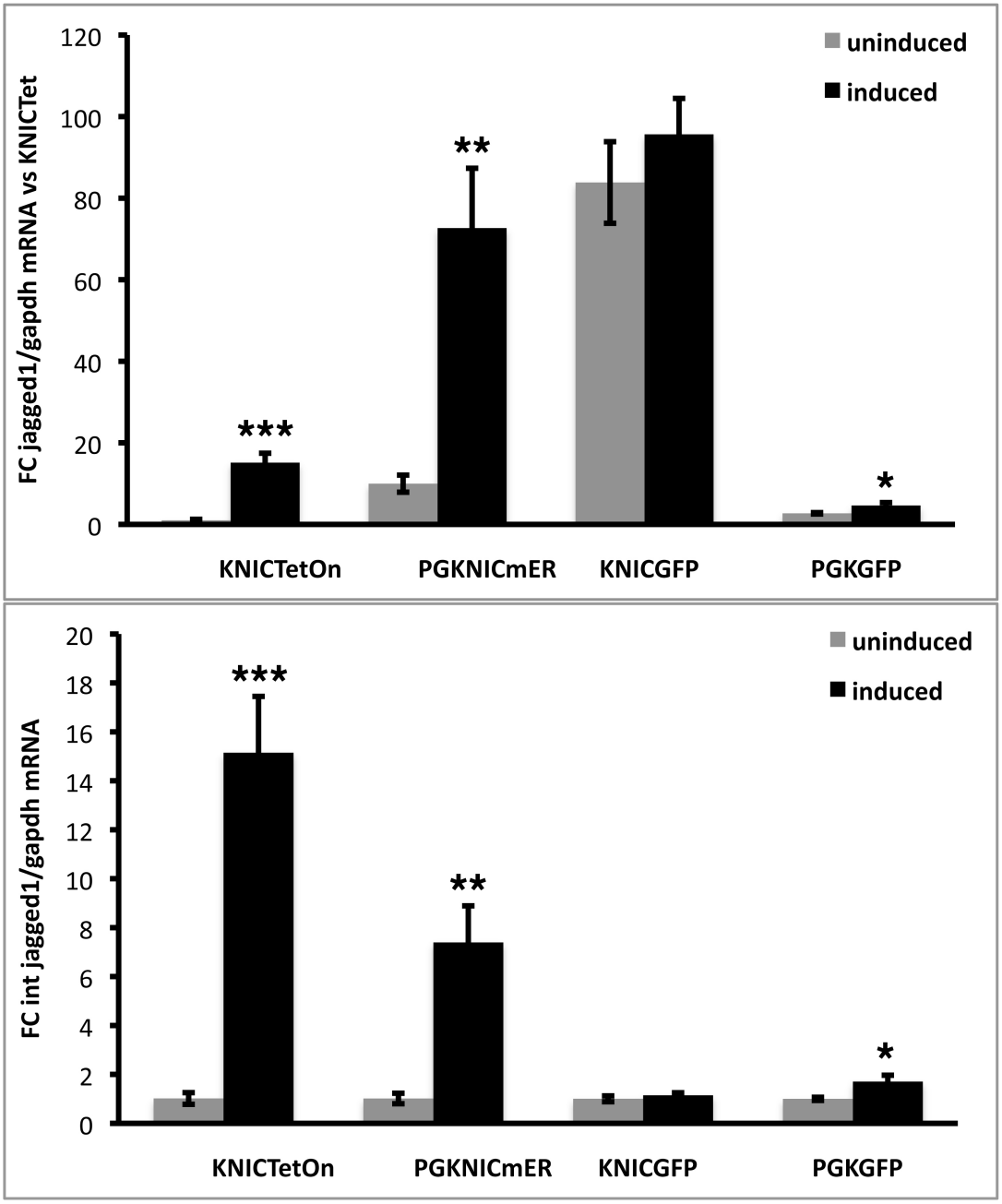

Figure 3.9: Upregulation of jagged1 mRNA expression in CPCs using TetOn inducible or mER fusion regulatable construct.
Mouse CPCs were transfected with KNICTetOn+tTA, PGKNICmER, KNICGFP or PGKGFP plasmids and treated with vehicle or inducing agent (doxycycline for TetOn, tamoxifen for others). Top: fold change relative to uninduced KNICTetOn. Bottom: fold change versus untreated for each construct.

Appendix

Primers for mouse qPCR

Mouse hes1, Accession # NM_008235.2
For: 5'-GAGGCTGCCAAGGTTTTTGG-3'
Rev: 5'-GCTGGTGTAGACCGGGATGA-3'

Mouse hey1, Accession # NM_010423.2
For: 5'-TGAATCCAGATGACCAGCTACTGT-3'
Rev: 5'- TACTTTCAGACTCCGATCGCTTAC-3'

Mouse notch1, Accession # NM_008714
For: 5'- TCAATGCCGTGGATGACCTA-3'
Rev: 5'- CCTTGTTGGCTCCGTTCTTC-3'

Mouse jagged1, Accession # NM_013822
For: 5'- CGGGATGGAAACAGCTCAC-3'
Rev: 5'- CACCAAGCAACAGACCCAAG-3'

Mouse pten, Accession # NM_008960
For: 5'- GACAAGGCCAACCGATACTTC-3'
Rev: 5'- TGCTAGCCTCTGGATTTGATG-3'

Mouse β-actin, Accession # NM_007393
For: 5'- CCGTGAAAAGATGACCCAGA-3'
Rev: 5'- AGGCATACAGGGACAGCACA-3'

Mouse αSMA, Accession # NM_007392.2
For: 5'- GTTCAGTGGTGCCTCTGTCA-3'
Rev: 5'- ACTGGGACGACATGGAAAAG-3'

Mouse CD31, Accession # NM_008816.2
For: 5'- AGTTGCTGCCCATTCATCAC-3'
Rev: 5'- CTGGTGCTCTATGCAAGCCT-3'

Mouse gapdh Accession # XM_001473623.1
For: 5'- CATGGCCTTCCGTGTTCCTA-3'
Rev: 5'- CCTGCTTCACCACCTTCTTGAT-3'

Primers for cloning:

To clone into CGW lentiviral backbone:

KNIC PacI Kozak Forward:
5'-GCC **TTAATTAA** ACC ATG GTG CTG CTG TCC CGC A-3'

mER PacI Stop Reverse:
5'-GG **TTAATTAA** TCA GAT CGT GTT GGG GAA G-3'

To clone into PGK lentiviral backbone:

KNIC BamHI Kozak Forward:
5'-GCC **GGATCC** ACC ATG GTG CTG CTG TCC CGC A-3'

mER BamHI Stop Reverse:
5'-GG **GGATCC** TCA GAT CGT GTT GGG GAA G-3'

DISCUSSION OF THE DISSERTATION

The role of Notch signaling in damaged myocardium and cardiac repair is the focus of increasing investigation. Reactivation of Notch in postmitotic cardiomyocytes has been reported to promote proliferative signaling, blunt hypertrophy and fibrosis, mitigate damage from infarction and stimulate protective signaling cascades[93, 95-98]. Interestingly, constitutive cardiac overexpression of activated Notch during development leads to cardiac defects, arrested cardiomyocyte proliferation and differentiation, and perinatal lethality[93, 94, 116]. Clearly the timing of Notch signaling is critical for proper heart field specification and development, suggesting that candidate progenitor cell populations for cell based therapy will also be sensitive to timing and dose of Notch activity. Several studies have investigated the impact of Notch signaling on cardiac differentiation in various progenitor populations, including circulating endothelial progenitors, CPCs and mouse embryonic stem cells[117-120]. The consensus is that Notch activation pushes these cell types toward a cardiac fate, facilitating their regenerative potential following transplantation into damaged myocardium. The work presented here reports reactivation of the endogenous Notch pathway in adult myocardium following infarction, documenting elevated protein levels of Jagged1 ligand, NICD and downstream target Hes1 in infarcted heart lysates. Furthermore, NICD is localized to border zone cardiomyocytes, a novel finding that was replicated in subsequent studies[93]. Colocalization of c-Met with NICD in nuclei of border zone cardiomyocytes, prompted the investigation of potential crosstalk

between the Notch and HGF signaling cascades. HGF, also upregulated following myocardial infarction, acts as a cardioprotective cytokine that reduces apoptosis, infarct area and fibrosis, and increases vascularization and proliferative signaling in surviving myocytes; this protective effect is mediated via the PI3K/Akt pathway[28, 43, 58-63, 67, 121-125]. The current study demonstrates activation of Notch by HGF in cardiomyocytes *in vitro* and *in vivo,* and reveals that this effect is mediated through the PI3K/Akt signaling axis; inhibitors of PI3K and Akt blunt the elevation of Hes1 in HGF treated NRCMs. Furthermore, transient overexpression of exogenous NICD in NRCMs and intact myocardium increases phospho-Akt levels in these tissues, pointing to supportive signaling between Notch and PI3K/Akt pathways in heart.

Crosstalk between the Notch and PI3K/Akt pathways has been documented in the cancer literature[30-32, 92, 126-129] as well as in noncancerous cell types[29, 130]. Mechanisms to explain stimulation of PI3K signaling include: transcriptional repression of PI3K antagonist PTEN by Hes1, upregulation of IGF-R expression, formation of a complex between Notch1, p56(lck) and PI3K to stimulate Akt, and upregulation of Deltex1, which can stimulate PI3K signaling through recruitment of signaling kinases. The first mechanism addresses canonical Notch signaling whereby the intracellular domain translocates to the nucleus, binds to RBPJ-k and stimulates expression of downstream targets such as Hes1. To test this mechanism in our system, PTEN transcript levels were measured in NRCMs expressing activated Notch

(Figure 4, Chapter II). Contrary to reports in leukemia and adenocarcinoma cells, PTEN transcripts are not decreased by Notch activation in NRCMs. It is interesting to note that Jagged1 transcripts are elevated in KNICGFP and tamoxifen treated KNICmER expressing myocytes and CPCs. Jagged1 has been implicated in the noncanonical activation of PI3K by Deltex1[128]. Based on these cumulative results, future experiments will measure IGF-R transcript and protein levels as well as Deltex1 transcripts in KNICGFP and KNICmER expressing myocytes to uncover the mechanism underlying activation of PI3K/Akt by Notch.

Genetic tools for manipulating Notch signaling in intact heart and cell culture systems were designed to probe these molecular interactions further. The mutated estrogen receptor fusion system was chosen for its simplicity and flexibility, allowing direct activation by tamoxifen. Previously an intracellular Notch estrogen receptor fusion construct was utilized in studies of Notch activation on B cell gene expression[131]. The mutated estrogen receptor, mER, is unresponsive to estrogen but is activated by tamoxifen, a distinct advantage for *in vivo* models susceptible to hormonal fluctuations. Additionally, our collaborators in the laboratory of Professor Christopher Glembotski have used the mER fusion system successfully to discover targets of ATF6-mediated endoplasmic reticulum stress in myocardium[86, 132-135]. Of the intracellular Notch mER fusion constructs tested, KNICmER exhibits the best induction of Notch activity following tamoxifen treatment as evidenced by RBPJ-k

luciferase reporter activity, quantitative real time PCR measurements of Notch target transcripts, and immunostaining and blotting for Hes1 protein. Therefore, this construct was chosen to confer regulatable Notch activity in adenovirus, lentivirus and the cardiac specific transgenic systems.

Constitutive Notch activation in developing heart results in cardiac malformation and lethality. The transgenic mouse line expressing KNICmER under control of the alpha myosin heavy chain promoter has been verified for NICD protein expression in conjunction with the FLAG tag. Given that the corresponding positive control construct, αMyHC-KNICGFP, which expresses constitutively active Notch in cardiomyocytes, produced no founders, it would appear that the mER fusion is suppressing Notch activity of KNICmER sufficiently to allow viable offspring. Preliminary analysis of hearts from KNICmER F1 offspring reveals no gross defects in cardiac morphology; heart and body weights are comparable to nontransgenic littermates and KNICmER transgenic mice seem healthy. The finding that αMyHC-KNICGFP produced no viable founders is consistent with previous reports of constitutive Notch activity during development leading to cardiac defects and lethality[93]. Once tamoxifen regulation is established in this line, pathological challenge will be surgically induced (infarction via LAD ligature, pressure overload via TAC banding) and mice treated with vehicle or tamoxifen. Since Notch activation in cardiomyocytes is thought to play a key role in zebrafish heart regeneration[49],

it is anticipated that a well timed burst of Notch signaling in KNICmER mouse myocardium following injury will result enhanced cardiac repair.

The final chapter of this thesis investigates the role of Notch signaling in cardiac progenitor cell differentiation and cardiogenesis. The regulatable KNICmER construct was introduced into CPCs and induction of Notch target genes confirmed by qPCR. Notch is a known regulator of cardiac specification, so not surprisingly, CPCs expressing activated Notch for five days changed shape and gene expression profiles, losing progenitor markers c-kit and nucleostemin, and upregulating SMA. Two KNICmER expressing lines were created using lentiviral transduction, the first of which utilizes the viral MND promoter and has been successfully in this laboratory to establish Pim-1 expressing CPCs used in adoptive transfer experiments. The second line, PGKKNICmER, utilizes the ubiquitous mammalian PGK promoter in order to avoid potential transgene silencing that can occur with viral promoters introduced into mammalian systems[136]. The KNICmER CPC lines will be used to manipulate Notch signaling as it pertains to CPC differentiation into cardiac lineages both *in vitro* and *in vivo*. The companion *in vivo* model, a transgenic mouse expressing KNICmER under control of the c-Kit promoter, will serve as a whole body system for elucidating the mechanisms through which Notch impacts CPC differentiation and repair in the heart.

In conclusion, the ultimate goal of this research is to define conditions, which enhance cardiogenesis and cardiac repair following pathologic challenge. The reagents described here have all been designed with that endpoint in mind and will be employed to dissect molecular mechanisms of Notch and survival signaling crosstalk, and to enhance myocyte and progenitor based cardiac regeneration *in vivo*.

REFERENCES

1. Artavanis-Tsakonas S, Muskavitch MA, Yedvobnick B. Molecular cloning of Notch, a locus affecting neurogenesis in Drosophila melanogaster. *Proc Natl Acad Sci U S A.* 1983;80(7):1977-1981.

2. Iso T, Hamamori Y, Kedes L. Notch signaling in vascular development. *Arterioscler Thromb Vasc Biol.* 2003;23(4):543-553.

3. Kanwar R, Fortini ME. Notch signaling: a different sort makes the cut. *Curr Biol.* 2004;14(24):R1043-1045.

4. Luo D, Renault VM, Rando TA. The regulation of Notch signaling in muscle stem cell activation and postnatal myogenesis. *Semin Cell Dev Biol.* 2005;16(4-5):612-622.

5. Sjolund J, Manetopoulos C, Stockhausen MT, Axelson H. The Notch pathway in cancer: differentiation gone awry. *Eur J Cancer.* 2005;41(17):2620-2629.

6. von Boehmer H. Coming to grips with Notch. *J Exp Med.* 2001;194(7):F43-46.

7. Yoon K, Gaiano N. Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat Neurosci.* 2005;8(6):709-715.

8. Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD. Notch signalling regulates stem cell numbers in vitro and in vivo. *Nature.* 2006.

9. Kwon C, Han Z, Olson EN, Srivastava D. MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. *Proc Natl Acad Sci U S A.* 2005;102(52):18986-18991.

10. Nemir M, Croquelois A, Pedrazzini T, Radtke F. Induction of cardiogenesis in embryonic stem cells via downregulation of Notch1 signaling. *Circ Res.* 2006;98(12):1471-1478.

11. Watanabe Y, Kokubo H, Miyagawa-Tomita S, Endo M, Igarashi K, Aisaki K, Kanno J, Saga Y. Activation of Notch1 signaling in cardiogenic mesoderm induces abnormal heart morphogenesis in mouse. *Development.* 2006;133(9):1625-1634.

12. Han Z, Bodmer R. Myogenic cells fates are antagonized by Notch only in asymmetric lineages of the Drosophila heart, with or without cell division. *Development.* 2003;130(13):3039-3051.

13. Kokubo H, Miyagawa-Tomita S, Johnson RL. Hesr, a mediator of the Notch signaling, functions in heart and vessel development. *Trends Cardiovasc Med.* 2005;15(5):190-194.

14. Kokubo H, Miyagawa-Tomita S, Nakazawa M, Saga Y, Johnson RL. Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. *Dev Biol.* 2005;278(2):301-309.

15. Kokubo H, Miyagawa-Tomita S, Tomimatsu H, Nakashima Y, Nakazawa M, Saga Y, Johnson RL. Targeted disruption of hesr2 results in atrioventricular valve anomalies that lead to heart dysfunction. *Circ Res.* 2004;95(5):540-547.

16. Park M, Yaich LE, Bodmer R. Mesodermal cell fate decisions in Drosophila are under the control of the lineage genes numb, Notch, and sanpodo. *Mech Dev.* 1998;75(1-2):117-126.

17. Greenwald I. LIN-12/Notch signaling: lessons from worms and flies. *Genes Dev.* 1998;12(12):1751-1762.

18. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science.* 1999;284(5415):770-776.

19. Chau MD, Tuft R, Fogarty K, Bao ZZ. Notch signaling plays a key role in cardiac cell differentiation. *Mech Dev.* 2006.

20. Rones MS, McLaughlin KA, Raffin M, Mercola M. Serrate and Notch specify cell fates in the heart field by suppressing cardiomyogenesis. *Development.* 2000;127(17):3865-3876.

21. Pedrazzini T. Control of cardiogenesis by the notch pathway. *Trends Cardiovasc Med.* 2007;17(3):83-90.

22. High FA, Epstein JA. The multifaceted role of Notch in cardiac development and disease. *Nat Rev Genet.* 2008;9(1):49-61.

23. Garg V, Muth AN, Ransom JF, Schluterman MK, Barnes R, King IN, Grossfeld PD, Srivastava D. Mutations in NOTCH1 cause aortic valve disease. *Nature.* 2005;437(7056):270-274.

24. Nakagawa O, McFadden DG, Nakagawa M, Yanagisawa H, Hu T, Srivastava D, Olson EN. Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of Notch signaling. *Proc Natl Acad Sci U S A.* 2000;97(25):13655-13660.

25. Kathiriya IS, King IN, Murakami M, Nakagawa M, Astle JM, Gardner KA, Gerard RD, Olson EN, Srivastava D, Nakagawa O. Hairy-related transcription factors inhibit GATA-dependent cardiac gene expression through a signal-responsive mechanism. *J Biol Chem.* 2004;279(52):54937-54943.

26. Stella MC, Trusolino L, Pennacchietti S, Comoglio PM. Negative feedback regulation of Met-dependent invasive growth by Notch. *Mol Cell Biol.* 2005;25(10):3982-3996.

27. Fiaccavento R, Carotenuto F, Minieri M, Fantini C, Forte G, Carbone A, Carosella L, Bei R, Masuelli L, Palumbo C, Modesti A, Prat M, Di Nardo P. Stem cell activation sustains hereditary hypertrophy in hamster cardiomyopathy. *J Pathol.* 2005;205(3):397-407.

28. Ueda H, Nakamura T, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. A potential cardioprotective role of hepatocyte growth factor in myocardial infarction in rats. *Cardiovasc Res.* 2001;51(1):41-50.

29. McKenzie G, Ward G, Stallwood Y, Briend E, Papadia S, Lennard A, Turner M, Champion B, Hardingham GE. Cellular Notch responsiveness is defined by phosphoinositide 3-kinase-dependent signals. *BMC Cell Biol.* 2006;7:10.

30. Mungamuri SK, Yang X, Thor AD, Somasundaram K. Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. *Cancer Res.* 2006;66(9):4715-4724.

31. Calzavara E, Chiaramonte R, Cesana D, Basile A, Sherbet GV, Comi P. Reciprocal regulation of Notch and PI3K/Akt signalling in T-ALL cells In Vitro. *J Cell Biochem.* 2007.

32. Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, Caparros E, Buteau J, Brown K, Perkins SL, Bhagat G, Agarwal AM, Basso G, Castillo M, Nagase S, Cordon-Cardo C, Parsons R, Zuniga-Pflucker JC, Dominguez M, Ferrando AA. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med.* 2007;13(10):1203-1210.

33. Welch S, Plank D, Witt S, Glascock B, Schaefer E, Chimenti S, Andreoli AM, Limana F, Leri A, Kajstura J, Anversa P, Sussman MA. Cardiac-

specific IGF-1 expression attenuates dilated cardiomyopathy in tropomodulin-overexpressing transgenic mice. *Circ Res.* 2002;90(6):641-648.

34. Rota M, Boni A, Urbanek K, Padin-Iruegas ME, Kajstura TJ, Fiore G, Kubo H, Sonnenblick EH, Musso E, Houser SR, Leri A, Sussman MA, Anversa P. Nuclear targeting of Akt enhances ventricular function and myocyte contractility. *Circ Res.* 2005;97(12):1332-1341.

35. Shiraishi I, Melendez J, Ahn Y, Skavdahl M, Murphy E, Welch S, Schaefer E, Walsh K, Rosenzweig A, Torella D, Nurzynska D, Kajstura J, Leri A, Anversa P, Sussman MA. Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. *Circ Res.* 2004;94(7):884-891.

36. Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A, Hosoda T, Chimenti S, Baker M, Limana F, Nurzynska D, Torella D, Rotatori F, Rastaldo R, Musso E, Quaini F, Leri A, Kajstura J, Anversa P. Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. *Circ Res.* 2005;97(7):663-673.

37. Gude N, Muraski J, Rubio M, Kajstura J, Schaefer E, Anversa P, Sussman MA. Akt promotes increased cardiomyocyte cycling and expansion of the cardiac progenitor cell population. *Circ Res.* 2006;99(4):381-388.

38. Kato T, Muraski J, Chen Y, Tsujita Y, Wall J, Glembotski CC, Schaefer E, Beckerle M, Sussman MA. Atrial natriuretic peptide promotes cardiomyocyte survival by cGMP-dependent nuclear accumulation of zyxin and Akt. *J Clin Invest.* 2005;115(10):2716-2730.

39. Tezuka K, Yasuda M, Watanabe N, Morimura N, Kuroda K, Miyatani S, Hozumi N. Stimulation of osteoblastic cell differentiation by Notch. *J Bone Miner Res.* 2002;17(2):231-239.

40. Ono K, Matsumori A, Shioi T, Furukawa Y, Sasayama S. Enhanced expression of hepatocyte growth factor/c-Met by myocardial ischemia and reperfusion in a rat model. *Circulation.* 1997;95(11):2552-2558.

41. Sato T, Tani Y, Murao S, Fujieda H, Sato H, Matsumoto M, Takeuchi T, Ohtsuki Y. Focal enhancement of expression of c-Met/hepatocyte growth factor receptor in the myocardium in human myocardial infarction. *Cardiovasc Pathol.* 2001;10(5):235-240.

42. Sato T, Fujieda H, Murao S, Sato H, Takeuchi T, Ohtsuki Y. Sequential changes of hepatocyte growth factor in the serum and enhanced c-Met expression in the myocardium in acute myocardial infarction. *Jpn Circ J.* 1999;63(11):906-908.

43. Matsumori A, Ono K, Okada M, Miyamoto T, Sato Y, Sasayama S. Immediate increase in circulating hepatocyte growth factor/scatter factor by heparin. *J Mol Cell Cardiol.* 1998;30(10):2145-2149.

44. Sato T, Yoshinouchi T, Sakamoto T, Fujieda H, Murao S, Sato H, Kobayashi H, Ohe T. Hepatocyte growth factor(HGF): a new biochemical marker for acute myocardial infarction. *Heart Vessels.* 1997;12(5):241-246.

45. Matsumori A, Furukawa Y, Hashimoto T, Ono K, Shioi T, Okada M, Iwasaki A, Nishio R, Sasayama S. Increased circulating hepatocyte growth factor in the early stage of acute myocardial infarction. *Biochem Biophys Res Commun.* 1996;221(2):391-395.

46. Yamada T, Katagiri H, Asano T, Inukai K, Tsuru M, Kodama T, Kikuchi M, Oka Y. 3-phosphoinositide-dependent protein kinase 1, an Akt1 kinase, is involved in dephosphorylation of Thr-308 of Akt1 in Chinese hamster ovary cells. *J Biol Chem.* 2001;276(7):5339-5345.

47. Kelly AP, Finlay DK, Hinton HJ, Clarke RG, Fiorini E, Radtke F, Cantrell DA. Notch-induced T cell development requires phosphoinositide-dependent kinase 1. *Embo J.* 2007;26(14):3441-3450.

48. Lupien M, Dievart A, Morales CR, Hermo L, Calvo E, Kay DG, Hu C, Jolicoeur P. Expression of constitutively active Notch1 in male genital tracts results in ectopic growth and blockage of efferent ducts, epididymal hyperplasia and sterility. *Dev Biol.* 2006;300(2):497-511.

49. Raya A, Koth CM, Buscher D, Kawakami Y, Itoh T, Raya RM, Sternik G, Tsai HJ, Rodriguez-Esteban C, Izpisua-Belmonte JC. Activation of Notch signaling pathway precedes heart regeneration in zebrafish. *Proc Natl Acad Sci U S A.* 2003;100 Suppl 1:11889-11895.

50. Sade H, Krishna S, Sarin A. The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. *J Biol Chem.* 2004;279(4):2937-2944.

51. Givogri MI, de Planell M, Galbiati F, Superchi D, Gritti A, Vescovi A, de Vellis J, Bongarzone ER. Notch signaling in astrocytes and neuroblasts of the adult subventricular zone in health and after cortical injury. *Dev Neurosci.* 2006;28(1-2):81-91.

52. Jensen CH, Jauho EI, Santoni-Rugiu E, Holmskov U, Teisner B, Tygstrup N, Bisgaard HC. Transit-amplifying ductular (oval) cells and their hepatocytic progeny are characterized by a novel and distinctive expression of delta-like protein/preadipocyte factor 1/fetal antigen 1. *Am J Pathol.* 2004;164(4):1347-1359.

53. Jensen JN, Cameron E, Garay MV, Starkey TW, Gianani R, Jensen J. Recapitulation of elements of embryonic development in adult mouse pancreatic regeneration. *Gastroenterology.* 2005;128(3):728-741.

54. Lovschall H, Tummers M, Thesleff I, Fuchtbauer EM, Poulsen K. Activation of the Notch signaling pathway in response to pulp capping of rat molars. *Eur J Oral Sci.* 2005;113(4):312-317.

55. Mitsiadis TA, Fried K, Goridis C. Reactivation of Delta-Notch signaling after injury: complementary expression patterns of ligand and receptor in dental pulp. *Exp Cell Res.* 1999;246(2):312-318.

56. Tsukumo S, Hirose K, Maekawa Y, Kishihara K, Yasutomo K. Lunatic Fringe Controls T Cell Differentiation through Modulating Notch Signaling. *J Immunol.* 2006;177(12):8365-8371.

57. Andermarcher E, Surani MA, Gherardi E. Co-expression of the HGF/SF and c-met genes during early mouse embryogenesis precedes reciprocal expression in adjacent tissues during organogenesis. *Dev Genet.* 1996;18(3):254-266.

58. Yasuda S, Goto Y, Baba T, Satoh T, Sumida H, Miyazaki S, Nonogi H. Enhanced secretion of cardiac hepatocyte growth factor from an infarct region is associated with less severe ventricular enlargement and improved cardiac function. *J Am Coll Cardiol.* 2000;36(1):115-121.

59. Jin H, Yang R, Li W, Ogasawara AK, Schwall R, Eberhard DA, Zheng Z, Kahn D, Paoni NF. Early treatment with hepatocyte growth factor improves cardiac function in experimental heart failure induced by myocardial infarction. *J Pharmacol Exp Ther.* 2003;304(2):654-660.

60. Kitta K, Day RM, Ikeda T, Suzuki YJ. Hepatocyte growth factor protects cardiac myocytes against oxidative stress-induced apoptosis. *Free Radic Biol Med.* 2001;31(7):902-910.

61. Tomita N, Morishita R, Higaki J, Ogihara T. Novel molecular therapeutic approach to cardiovascular disease based on hepatocyte growth factor. *J Atheroscler Thromb.* 2000;7(1):1-7.

62. Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. *J Clin Invest.* 2000;106(12):1511-1519.

63. Ueda H, Sawa Y, Matsumoto K, Kitagawa-Sakakida S, Kawahira Y, Nakamura T, Kaneda Y, Matsuda H. Gene transfection of hepatocyte growth factor attenuates reperfusion injury in the heart. *Ann Thorac Surg.* 1999;67(6):1726-1731.

64. Kondoh H, Sawa Y, Fukushima N, Matsumiya G, Miyagawa S, Kitagawa-Sakakida S, Imanishi Y, Kawaguchi N, Matsuura N, Matsuda H. Combined strategy using myoblasts and hepatocyte growth factor in dilated cardiomyopathic hamsters. *Ann Thorac Surg.* 2007;84(1):134-141.

65. Pozner-Moulis S, Pappas DJ, Rimm DL. Met, the hepatocyte growth factor receptor, localizes to the nucleus in cells at low density. *Cancer Res.* 2006;66(16):7976-7982.

66. Forte G, Minieri M, Cossa P, Antenucci D, Sala M, Gnocchi V, Fiaccavento R, Carotenuto F, De Vito P, Baldini PM, Prat M, Di Nardo P. Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. *Stem Cells.* 2006;24(1):23-33.

67. Wang Y, Ahmad N, Wani MA, Ashraf M. Hepatocyte growth factor prevents ventricular remodeling and dysfunction in mice via Akt pathway and angiogenesis. *J Mol Cell Cardiol.* 2004;37(5):1041-1052.

68. Kusano KF, Pola R, Murayama T, Curry C, Kawamoto A, Iwakura A, Shintani S, Ii M, Asai J, Tkebuchava T, Thorne T, Takenaka H, Aikawa R, Goukassian D, von Samson P, Hamada H, Yoon YS, Silver M, Eaton E, Ma H, Heyd L, Kearney M, Munger W, Porter JA, Kishore R, Losordo DW. Sonic hedgehog myocardial gene therapy: tissue repair through transient reconstitution of embryonic signaling. *Nat Med.* 2005;11(11):1197-1204.

69. Kajstura J, Leri A, Castaldo C, Nadal-Ginard B, Anversa P. Myocyte growth in the failing heart. *Surg Clin North Am.* 2004;84(1):161-177.

70. Kajstura J, Leri A, Finato N, Di Loreto C, Beltrami CA, Anversa P. Myocyte proliferation in end-stage cardiac failure in humans. *Proc Natl Acad Sci U S A.* 1998;95(15):8801-8805.

71. Stoick-Cooper CL, Moon RT, Weidinger G. Advances in signaling in vertebrate regeneration as a prelude to regenerative medicine. *Genes Dev.* 2007;21(11):1292-1315.

72. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell.* 2003;114(6):763-776.

73. Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. *Physiol Rev.* 2005;85(4):1373-1416.

74. Urbanek K, Cesselli D, Rota M, Nascimbene A, De Angelis A, Hosoda T, Bearzi C, Boni A, Bolli R, Kajstura J, Anversa P, Leri A. Stem cell niches in the adult mouse heart. *Proc Natl Acad Sci U S A.* 2006;103(24):9226-9231.

75. Vandervelde S, van Luyn MJ, Tio RA, Harmsen MC. Signaling factors in stem cell-mediated repair of infarcted myocardium. *J Mol Cell Cardiol.* 2005;39(2):363-376.

76. Flink IL. Cell cycle reentry of ventricular and atrial cardiomyocytes and cells within the epicardium following amputation of the ventricular apex in the axolotl, Amblystoma mexicanum: confocal microscopic immunofluorescent image analysis of bromodeoxyuridine-labeled nuclei. *Anat Embryol (Berl).* 2002;205(3):235-244.

77. Garg V. Molecular genetics of aortic valve disease. *Curr Opin Cardiol.* 2006;21(3):180-184.

78. Conboy IM, Rando TA. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev Cell.* 2002;3(3):397-409.

79. Kohler C, Bell AW, Bowen WC, Monga SP, Fleig W, Michalopoulos GK. Expression of Notch-1 and its ligand Jagged-1 in rat liver during liver regeneration. *Hepatology.* 2004;39(4):1056-1065.

80. Lindner V, Booth C, Prudovsky I, Small D, Maciag T, Liaw L. Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell-cell interaction. *Am J Pathol.* 2001;159(3):875-883.

81. Nobta M, Tsukazaki T, Shibata Y, Xin C, Moriishi T, Sakano S, Shindo H, Yamaguchi A. Critical regulation of bone morphogenetic protein-

induced osteoblastic differentiation by Delta1/Jagged1-activated Notch1 signaling. *J Biol Chem.* 2005;280(16):15842-15848.

82. Nair P, Somasundaram K, Krishna S. Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway. *J Virol.* 2003;77(12):7106-7112.

83. Campos LS, Decker L, Taylor V, Skarnes W. Notch, epidermal growth factor receptor, and beta1-integrin pathways are coordinated in neural stem cells. *J Biol Chem.* 2006;281(8):5300-5309.

84. Guentchev M, McKay RD. Notch controls proliferation and differentiation of stem cells in a dose-dependent manner. *Eur J Neurosci.* 2006;23(9):2289-2296.

85. Armstrong EJ, Bischoff J. Heart valve development: endothelial cell signaling and differentiation. *Circ Res.* 2004;95(5):459-470.

86. Martindale JJ, Fernandez R, Thuerauf D, Whittaker R, Gude N, Sussman MA, Glembotski CC. Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6. *Circ Res.* 2006;98(9):1186-1193.

87. Kohn AD, Barthel A, Kovacina KS, Boge A, Wallach B, Summers SA, Birnbaum MJ, Scott PH, Lawrence JC, Jr., Roth RA. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J Biol Chem.* 1998;273(19):11937-11943.

88. Scott PH, Brunn GJ, Kohn AD, Roth RA, Lawrence JC, Jr. Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *Proc Natl Acad Sci U S A.* 1998;95(13):7772-7777.

89. Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res.* 1995;23(10):1686-1690.

90. Palermo J, Gulick J, Ng W, Grupp IL, Grupp G, Robbins J. Remodeling the mammalian heart using transgenesis. *Cell Mol Biol Res.* 1995;41(6):501-509.

91. Robbins J, Palermo J, Rindt H. In vivo definition of a cardiac specific promoter and its potential utility in remodeling the heart. *Ann N Y Acad Sci.* 1995;752:492-505.

92. Eliasz S, Liang S, Chen Y, De Marco MA, Machek O, Skucha S, Miele L, Bocchetta M. Notch-1 stimulates survival of lung adenocarcinoma cells during hypoxia by activating the IGF-1R pathway. *Oncogene.*

93. Kratsios P, Catela C, Salimova E, Huth M, Berno V, Rosenthal N, Mourkioti F. Distinct Roles for Cell-Autonomous Notch Signaling in Cardiomyocytes of the Embryonic and Adult Heart. *Circ Res.* 2009.

94. Varadkar PA, Kraman M, McCright B. Generation of mice that conditionally express the activation domain of Notch2. *Genesis.* 2009.

95. Gude NA, Emmanuel G, Wu W, Cottage CT, Fischer K, Quijada P, Muraski JA, Alvarez R, Rubio M, Schaefer E, Sussman MA. Activation of Notch-mediated protective signaling in the myocardium. *Circ Res.* 2008;102(9):1025-1035.

96. Croquelois A, Domenighetti AA, Nemir M, Lepore M, Rosenblatt-Velin N, Radtke F, Pedrazzini T. Control of the adaptive response of the heart to stress via the Notch1 receptor pathway. *J Exp Med.* 2008;205(13):3173-3185.

97. Collesi C, Zentilin L, Sinagra G, Giacca M. Notch1 signaling stimulates proliferation of immature cardiomyocytes. *J Cell Biol.* 2008;183(1):117-128.

98. Campa VM, Gutierrez-Lanza R, Cerignoli F, Diaz-Trelles R, Nelson B, Tsuji T, Barcova M, Jiang W, Mercola M. Notch activates cell cycle reentry and progression in quiescent cardiomyocytes. *J Cell Biol.* 2008;183(1):129-141.

99. Chamuleau SA, Vrijsen KR, Rokosh DG, Tang XL, Piek JJ, Bolli R. Cell therapy for ischaemic heart disease: focus on the role of resident cardiac stem cells. *Neth Heart J.* 2009;17(5):199-207.

100. Haider H, Jiang S, Idris NM, Ashraf M. IGF-1-overexpressing mesenchymal stem cells accelerate bone marrow stem cell mobilization via paracrine activation of SDF-1alpha/CXCR4 signaling to promote myocardial repair. *Circ Res.* 2008;103(11):1300-1308.

101. Zhang D, Fan GC, Zhou X, Zhao T, Pasha Z, Xu M, Zhu Y, Ashraf M, Wang Y. Over-expression of CXCR4 on mesenchymal stem cells

augments myoangiogenesis in the infarcted myocardium. *J Mol Cell Cardiol.* 2008;44(2):281-292.

102. Shujia J, Haider HK, Idris NM, Lu G, Ashraf M. Stable therapeutic effects of mesenchymal stem cell-based multiple gene delivery for cardiac repair. *Cardiovasc Res.* 2008;77(3):525-533.

103. Gnecchi M, He H, Melo LG, Noiseaux N, Morello F, de Boer RA, Zhang L, Pratt RE, Dzau VJ, Ingwall JS. Early beneficial effects of bone marrow-derived mesenchymal stem cells overexpressing Akt on cardiac metabolism after myocardial infarction. *Stem Cells.* 2009;27(4):971-979.

104. Noiseux N, Gnecchi M, Lopez-Ilasaca M, Zhang L, Solomon SD, Deb A, Dzau VJ, Pratt RE. Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. *Mol Ther.* 2006;14(6):840-850.

105. Fischer KM, Cottage CT, Wu W, Din S, Gude NA, Avitabile D, Quijada P, Collins BL, Fransioli J, Sussman MA. Enhancement of myocardial regeneration through genetic engineering of cardiac progenitor cells expressing Pim-1 kinase. *Circulation.* 2009;120(21):2077-2087.

106. Agrawal S, Archer C, Schaffer DV. Computational models of the Notch network elucidate mechanisms of context-dependent signaling. *PLoS Comput Biol.* 2009;5(5):e1000390.

107. Fortini ME. Notch signaling: the core pathway and its posttranslational regulation. *Dev Cell.* 2009;16(5):633-647.

108. Fransioli J, Bailey B, Gude NA, Cottage CT, Muraski JA, Emmanuel G, Wu W, Alvarez R, Rubio M, Ottolenghi S, Schaefer E, Sussman MA. Evolution of the c-kit-positive cell response to pathological challenge in the myocardium. *Stem Cells.* 2008;26(5):1315-1324.

109. Cairns LA, Moroni E, Levantini E, Giorgetti A, Klinger FG, Ronzoni S, Tatangelo L, Tiveron C, De Felici M, Dolci S, Magli MC, Giglioni B, Ottolenghi S. Kit regulatory elements required for expression in developing hematopoietic and germ cell lineages. *Blood.* 2003;102(12):3954-3962.

110. Swan CH, Buhler B, Steinberger P, Tschan MP, Barbas CF, 3rd, Torbett BE. T-cell protection and enrichment through lentiviral CCR5 intrabody gene delivery. *Gene Ther.* 2006;13(20):1480-1492.

111. Crews L, Mizuno H, Desplats P, Rockenstein E, Adame A, Patrick C, Winner B, Winkler J, Masliah E. Alpha-synuclein alters Notch-1 expression and neurogenesis in mouse embryonic stem cells and in the hippocampus of transgenic mice. *J Neurosci.* 2008;28(16):4250-4260.

112. Subramaniam A, Jones WK, Gulick J, Wert S, Neumann J, Robbins J. Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. *J Biol Chem.* 1991;266(36):24613-24620.

113. Sanbe A, Gulick J, Hanks MC, Liang Q, Osinska H, Robbins J. Reengineering inducible cardiac-specific transgenesis with an attenuated myosin heavy chain promoter. *Circ Res.* 2003;92(6):609-616.

114. Kerrison JB, Duh EJ, Yu Y, Otteson DC, Zack DJ. A system for inducible gene expression in retinal ganglion cells. *Invest Ophthalmol Vis Sci.* 2005;46(8):2932-2939.

115. Bailey B, Izarra A, Alvarez R, Fischer KM, Cottage CT, Quijada P, Diez-Juan A, Sussman MA. Cardiac stem cell genetic engineering using the alphaMHC promoter. *Regen Med.* 2009;4(6):823-833.

116. Grego-Bessa J, Luna-Zurita L, Del Monte G, Bolos V, Melgar P, Arandilla A, Garratt AN, Zang H, Mukouyama YS, Chen H, Shou W, Ballestar E, Esteller M, Rojas A, Perez-Pomares JM, de la Pompa JL. Notch signaling is essential for ventricular chamber development. *Dev Cell.* 2007;12(3):415-429.

117. Adler ED, Chen VC, Bystrup A, Kaplan AD, Giovannone S, Briley-Saebo K, Young W, Kattman S, Mani V, Laflamme M, Zhu WZ, Fayad Z, Keller G. The cardiomyocyte lineage is critical for optimization of stem cell therapy in a mouse model of myocardial infarction. *Faseb J.* 2009.

118. Boni A, Urbanek K, Nascimbene A, Hosoda T, Zheng H, Delucchi F, Amano K, Gonzalez A, Vitale S, Ojaimi C, Rizzi R, Bolli R, Yutzey KE, Rota M, Kajstura J, Anversa P, Leri A. Notch1 regulates the fate of cardiac progenitor cells. *Proc Natl Acad Sci U S A.* 2008;105(40):15529-15534.

119. Chen VC, Stull R, Joo D, Cheng X, Keller G. Notch signaling respecifies the hemangioblast to a cardiac fate. *Nat Biotechnol.* 2008;26(10):1169-1178.

120. Koyanagi M, Bushoven P, Iwasaki M, Urbich C, Zeiher AM, Dimmeler S. Notch signaling contributes to the expression of cardiac markers in human circulating progenitor cells. *Circ Res.* 2007;101(11):1139-1145.

121. Kondo I, Ohmori K, Oshita A, Takeuchi H, Fuke S, Shinomiya K, Noma T, Namba T, Kohno M. Treatment of acute myocardial infarction by hepatocyte growth factor gene transfer: the first demonstration of myocardial transfer of a "functional" gene using ultrasonic microbubble destruction. *J Am Coll Cardiol.* 2004;44(3):644-653.

122. Jin H, Wyss JM, Yang R, Schwall R. The therapeutic potential of hepatocyte growth factor for myocardial infarction and heart failure. *Curr Pharm Des.* 2004;10(20):2525-2533.

123. Li Y, Takemura G, Kosai K, Yuge K, Nagano S, Esaki M, Goto K, Takahashi T, Hayakawa K, Koda M, Kawase Y, Maruyama R, Okada H, Minatoguchi S, Mizuguchi H, Fujiwara T, Fujiwara H. Postinfarction treatment with an adenoviral vector expressing hepatocyte growth factor relieves chronic left ventricular remodeling and dysfunction in mice. *Circulation.* 2003;107(19):2499-2506.

124. Jayasankar V, Woo YJ, Bish LT, Pirolli TJ, Chatterjee S, Berry MF, Burdick J, Gardner TJ, Sweeney HL. Gene transfer of hepatocyte growth factor attenuates postinfarction heart failure. *Circulation.* 2003;108 Suppl 1:II230-236.

125. Ahmet I, Sawa Y, Yamaguchi T, Matsuda H. Gene transfer of hepatocyte growth factor improves angiogenesis and function of chronic ischemic myocardium in canine heart. *Ann Thorac Surg.* 2003;75(4):1283-1287.

126. Graziani I, Eliasz S, De Marco MA, Chen Y, Pass HI, De May RM, Strack PR, Miele L, Bocchetta M. Opposite effects of Notch-1 and Notch-2 on mesothelioma cell survival under hypoxia are exerted through the Akt pathway. *Cancer Res.* 2008;68(23):9678-9685.

127. Liu ZJ, Xiao M, Balint K, Smalley KS, Brafford P, Qiu R, Pinnix CC, Li X, Herlyn M. Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. *Cancer Res.* 2006;66(8):4182-4190.

128. Veeraraghavalu K, Subbaiah VK, Srivastava S, Chakrabarti O, Syal R, Krishna S. Complementation of human papillomavirus type 16 E6 and E7 by Jagged1-specific Notch1-phosphatidylinositol 3-kinase signaling

involves pleiotropic oncogenic functions independent of CBF1;Su(H);Lag-1 activation. *J Virol.* 2005;79(12):7889-7898.

129. Ma J, Meng Y, Kwiatkowski DJ, Chen X, Peng H, Sun Q, Zha X, Wang F, Wang Y, Jing Y, Zhang S, Chen R, Wang L, Wu E, Cai G, Malinowska-Kolodziej I, Liao Q, Liu Y, Zhao Y, Xu K, Dai J, Han J, Wu L, Zhao RC, Shen H, Zhang H. Mammalian target of rapamycin regulates murine and human cell differentiation through STAT3/p63/Jagged/Notch cascade. *J Clin Invest.*120(1):103-114.

130. Liu ZJ, Xiao M, Balint K, Soma A, Pinnix CC, Capobianco AJ, Velazquez OC, Herlyn M. Inhibition of endothelial cell proliferation by Notch1 signaling is mediated by repressing MAPK and PI3K/Akt pathways and requires MAML1. *Faseb J.* 2006;20(7):1009-1011.

131. Strobl LJ, Hofelmayr H, Marschall G, Brielmeier M, Bornkamm GW, Zimber-Strobl U. Activated Notch1 modulates gene expression in B cells similarly to Epstein-Barr viral nuclear antigen 2. *J Virol.* 2000;74(4):1727-1735.

132. Thuerauf DJ, Marcinko M, Belmont PJ, Glembotski CC. Effects of the isoform-specific characteristics of ATF6 alpha and ATF6 beta on endoplasmic reticulum stress response gene expression and cell viability. *J Biol Chem.* 2007;282(31):22865-22878.

133. Belmont PJ, Chen WJ, San Pedro MN, Thuerauf DJ, Gellings Lowe N, Gude N, Hilton B, Wolkowicz R, Sussman MA, Glembotski CC. Roles for endoplasmic reticulum-associated degradation and the novel endoplasmic reticulum stress response gene Derlin-3 in the ischemic heart. *Circ Res.*106(2):307-316.

134. Tadimalla A, Belmont PJ, Thuerauf DJ, Glassy MS, Martindale JJ, Gude N, Sussman MA, Glembotski CC. Mesencephalic astrocyte-derived neurotrophic factor is an ischemia-inducible secreted endoplasmic reticulum stress response protein in the heart. *Circ Res.* 2008;103(11):1249-1258.

135. Belmont PJ, Tadimalla A, Chen WJ, Martindale JJ, Thuerauf DJ, Marcinko M, Gude N, Sussman MA, Glembotski CC. Coordination of growth and endoplasmic reticulum stress signaling by regulator of calcineurin 1 (RCAN1), a novel ATF6-inducible gene. *J Biol Chem.* 2008;283(20):14012-14021.

136. Loser P, Jennings GS, Strauss M, Sandig V. Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: involvement of NFkappaB. *J Virol.* 1998;72(1):180-190.