A Dynamic Notch Injury Response Activates Epicardium and Contributes to Fibrosis Repair

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Rationale: **Transgenic Notch reporter mice express enhanced green fluorescent protein in cells with C-promoter binding factor-1 response element transcriptional activity (CBF1-RE$_{x4}$-EGFP), providing a unique and powerful tool for identifying and isolating "Notch-activated" progenitors.**

Objective: **We asked whether, as in other tissues of this mouse, EGFP localized and functionally tagged adult cardiac tissue progenitors, and, if so, whether this cell-based signal could serve as a quantitative and qualitative biosensor of the injury repair response of the heart.**

Methods and Results: **In addition to scattered endothelial and interstitial cells, Notch-activated (EGFP$^+$) cells unexpectedly richly populated the adult epicardium. We used fluorescence-activated cell sorting to isolate EGFP$^+$ cells and excluded hematopoietic (CD45$^+$) and endothelial (CD31$^+$) subsets. We analyzed EGFP$^+$/CD45$^-$/CD31$^-$ cells, a small (<2%) but distinct subpopulation, by gene expression profiling and functional analyses. We called this mixed cell pool, which had dual multipotent stromal cell and epicardial lineage signatures, Notch-activated epicardial-derived cells (NECs). Myocardial infarction and thoracic aortic banding amplified the NEC pool, increasing fibroblast differentiation. Validating the functional vitality of clonal NEC lines, serum growth factors triggered epithelial–mesenchymal transition and the immobilized Notch ligand Delta-like 1–activated downstream target genes. Moreover, cardiomyocyte coculture and engraftment in NOD-SCID (nonobese diabetic–severe combined immunodeficiency) mouse myocardium increased cardiac gene expression in NECs.**

Conclusions: **A dynamic Notch injury response activates adult epicardium, producing a multipotent cell population that contributes to fibrosis repair. (*Circ Res*. 2011;108:51-59.)**

Key Words: Notch ■ epicardium ■ myocardial infarction ■ adult progenitors ■ repair

Cardiovascular disease leading to heart failure is the most common and costly cause of death and disability in the modern world. The adult mammalian heart responds to biomechanical stress and injury with fibrosis. Cardiac fibrosis could have several cellular inputs: (1) preexisting interstitial fibroblasts, (2) circulating fibrocytes, (3) fibroblast progenitors arising by endothelial–mesenchymal transition of endocardial or microvascular coronary endothelial cells, or (4) fibroblast progenitors arising by epithelial–mesenchymal transition (EMT) of epicardial mesothelial cells.[1–3] Recently, there has been keen focus on the epicardium as a candidate source of adult heart repair fibroblasts and other cells.

The origin of the epicardium from the proepicardial organ and its essential role in cardiovascular development have been elegantly elucidated. However, until recently, the biology of the adult epicardium has been largely ignored. Traditionally viewed as a fibrous mesothelial covering, mechanically insulating and lubricating the outer surface of the heart muscle, the adult epicardium is now believed to have a more complex and active role in myocardial homeostasis and repair. The epicardium is a common residence for advanced metastatic cancers and infectious, inflammatory, and rheumatologic diseases; a host for (and possibly source of) unique epicardial adipose tissue; and, most importantly, a potential cardiac stem/progenitor cell niche.[4] Interestingly, recent electron and immunofluorescence microscopy studies identified at least 10 distinct cell types, including putative early cardiomyocyte precursors, in specialized niche-like structures in adult epicardium.[5,6] When "activated" by injury, the epicardium develops organ-wide thickening, with increased cellularity and extracellular matrix, and complex regional topography. New investigative tools and approaches are needed to

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Non-standard Abbreviations and Acronyms	
CBF1	C-promoter binding factor-1
copGFP	copepod green fluorescent protein
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
EGFP	enhanced green fluorescent protein
EMT	epithelial–mesenchymal transition
FACS	fluorescent-activated cell sorting
LAD-MI	left anterior descending myocardial infarction
MI	myocardial infarction
MSC	multipotent stromal cell
NEC	Notch-activated epicardial-derived cell
NICD	Notch intracellular domain
NOD-SCID	nonobese diabetic–severe combined immunodeficiency
NRCM	neonatal rat cardiomyocyte
TAB	thoracic aortic banding
TNR	transgenic Notch reporter

explore the structure and function of this unique and clinically important tissue microenvironment.

One of the key unanswered questions in the field is whether adult epicardium is a birthplace of newly born cardiomyocytes.[5,7–10] Recent fate-mapping studies have provided genetic evidence that new cardiomyocytes are produced in the adult mammalian heart following myocardial injury.[11] The origin of these cells remains unknown. The regenerative capacity of the adult mammalian heart is poor, yet this organ is richly endowed with a variety of molecularly distinct native progenitor cell subtypes.[12] To successfully engineer adult myocardial regeneration, we need to find common threads that link these various progenitor cell subpopulations together and identify mechanisms that control progenitor fate decisions in microenvironments like the epicardium. Signaling pathways like Notch, a key regulator of cardiovascular cell fate decisions, is one of the most important mechanisms.

The Notch signaling pathway plays a crucial role in cardiac development,[13] regulating growth and differentiation in all major cardiovascular cell lineages.[14] After birth, Notch pathway signaling is crucial for postnatal cardiomyogenesis, and pharmacological disruption of Notch signaling leads to dilated cardiomyopathy in newborn mice.[15] In adult animals, the Notch pathway regulates heart regeneration in zebrafish[16] and has been implicated in the injury repair response of the mammalian heart.[17,18]

Here, we report on studies of the transgenic Notch reporter (TNR) mouse heart, a unique model providing a functional signature of Notch pathway activation.[19,20] All previous studies of Notch in the heart have relied on Notch-1 intracellular domain (NICD1) antibody staining, which demonstrates Notch-1 receptor cleavage at the cell surface but not Notch pathway activity at the genome. The CBF1RE$_{x4}$-EGFP transgene, however, reports downstream activity at the level of Notch target genes and is inclusive of other Notch receptor isoforms (2 and 3).[19] We hypothesized that Notch activity in TNR mice might provide a highly stringent experimental tag to identify and localize native progenitor-like cell populations, to isolate them for mechanistic studies in vitro, and, most importantly, to evaluate their function in the adult heart's tissue repair response following myocardial injury.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Adult TNR mouse hearts were perfused retrograde via the aorta for 5 minutes with ADS buffer (116 mmol/L NaCl, 20 mmol/L HEPES, 10 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 5 mmol/L KCl, 0.8 mmol/L $MgSO_4$, pH 7.4) followed by digestion with 20 mL of 0.225 mg/mL Liberase Blenzyme-1 (Roche) in ADS buffer supplemented with 12.5 μmol/L $CaCl_2$ for 20 minutes at 37°C. Outflow tract and atria were removed and tissues gently pulled apart. Following gentle trituration, the Liberase was inactivated with 10% CM media (10% Hyclone FBS, 3:1 DMEM/M-199, 1% 1 mol/L HEPES, 1.2% antibiotic/antimycotic), and the suspension was sequentially filtered through tissue strainers. Cells were collected by centrifugation (400*g* for 5 minutes) and resuspended in 10% CM media. Cells were stained for 20 minutes on ice with phycoerythrin-conjugated anti-CD31 and anti-CD45 antibodies (BD Bioscience Pharmingen). After washing with PBS, the cells were resuspended in 10% CM media, filtered and analyzed and/or sorted with a MoFlo flow cytometer (Cytomation Inc) using Summit software. A fraction of each sample was collected into PBS for postsort assessment of purity. For transcript analysis, EGFP$^+$/CD45$^-$/CD31$^-$ cells were sorted directly into TRIzol for RNA extraction. For cell culture, 5K cells were collected in 10% CM media and plated on gelatin-coated 24-well dishes, and the media were changed every 3 days. After several weeks in culture cells, proliferating cells were passaged and cloned by serial dilution. Cultures were passaged every third day and have been maintained indefinitely.

Results

Localization and Isolation of Adult Notch-Activated Epicardial-Derived Cells

We evaluated CBF1RE$_{x4}$-EGFP transgene activity in histological sections of adult TNR mouse hearts, comparing control and left anterior descending myocardial infarction (LAD-MI) or thoracic aortic banding (TAB) at day 7 after injury. In accordance with previous studies, we detected enhanced green fluorescent protein–positive (EGFP$^+$) endothelial and interstitial cells (Online Figure I), but, quite unexpectedly, we also observed strongly localized epicardial signals (Figure 1A). EGFP marked many epicardial–mesothelial cells in control hearts and many cells in the thickened epicardium of LAD-MI or TAB injured hearts (Figure 1A). LAD-MI and TAB generated divergent patterns of EGFP staining (Figure 1A). EGFP$^+$ cells prominently localized to the LAD-MI border zone (BZ), extending to the left ventricular apex (Figure 1A). In contrast, EGFP$^+$ cells were more diffusely localized in TAB hearts and, surprisingly, even collected on the surface of the right ventricle (Figure 1A). Thus, CBF1RE$_{x4}$-EGFP$^+$ cells distinctly localized to epicardium of control and injury-activated adult TNR mice.

Next, we purified CBF1RE$_{x4}$-EGFP$^+$ cells from adult TNR hearts by flow cytometry. We dissociated TNR ventricles into single cells, mechanically destroyed cardiomyocytes by trituration and straining, and analyzed the remainder by fluorescence-activated cell sorting (FACS) for EGFP. For these experiments, we excluded EGFP$^+$ endothelial and hematopoietic cell subsets by costaining with phycoerythrin-conjugated CD31 and CD45

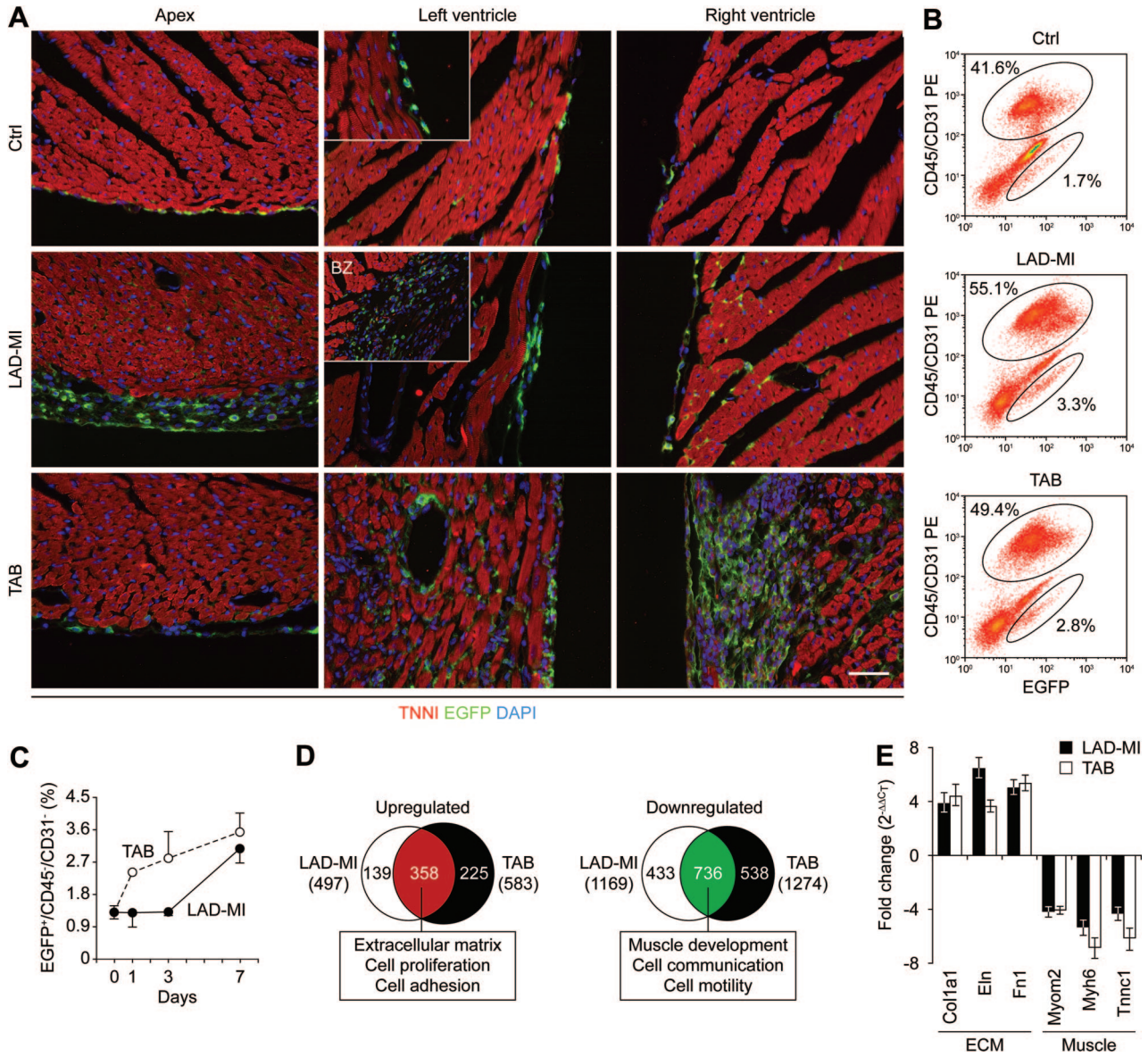

Figure 1. Dynamic and profibrotic epicardial injury response marked by Notch activity. A, EGFP immunohistochemistry **(green)** of TNR hearts (apex, left and right ventricles) of control, LAD-MI, and TAB (day 7), counterstained for troponin I **(red)** and DAPI **(blue)**. **Scale bar,** 40 μm. BZ indicates border zone. **B,** Percentage of EGFP$^+$/CD31$^-$/CD45$^-$ cells by FACS profiling, 7 days after LAD-MI or TAB. Ctrl indicates control. **C,** Time course of increased EGFP$^+$/CD45$^-$/CD31$^-$ cells following LAD-MI and TAB. **D,** Comparison of gene numbers and categories coordinately up- or downregulated in NECs from LAD-MI and TAB hearts (day 7). **E,** Independent quantitative polymerase chain reaction confirming microarray results.

monoclonal antibodies, respectively. Scattergrams routinely displayed a shoulder of EGFP$^+$/CD45$^-$/CD31$^-$ cells, a minor but distinctive subpopulation, comprising ≈1% to 2% of total EGFP$^+$ cells, equivalent to ≈10 to 20 000 cells/control heart (Figure 1B). We called these Notch-activated epicardial-derived cells (NECs).

Corresponding to the increased EGFP staining in injured TNR hearts (Figure 1A), NEC percentage (measured by FACS as [EGFP$^+$/CD45$^-$/CD31$^-$]/[total EGFP$^+$]×100) increased with LAD-MI and TAB (Figure 1B). Indeed, by day 7 after injury, the NEC percentage had nearly doubled in LAD-MI and TAB TNR mouse hearts (Figure 1C). Moreover, the kinetics of NEC accumulation was distinctive in LAD-MI– and TAB-injured mice. NEC percentage rapidly increased following TAB (within 24 hours) but remained steady for 72 hours after acute MI, doubling at some point between days 3 and 7 (Figure 1C). We concluded that counting NEC percentage by flow cytometry was a reliable, valid, and sensitive biosensor, directly correlating with the increased number of EGFP$^+$ cells observed by immunohistochemistry.

Transcriptome Analysis of Adult Notch-Activated Epicardial-Derived Cells From Injured Hearts

We asked whether NECs are involved in injury repair by evaluating freshly isolated cells from control and LAD-MI or TAB hearts by gene expression microarray. Myocardial injury markedly changed the in vivo gene expression profiles of NECs. Compared to control, NECs from LAD-MI and

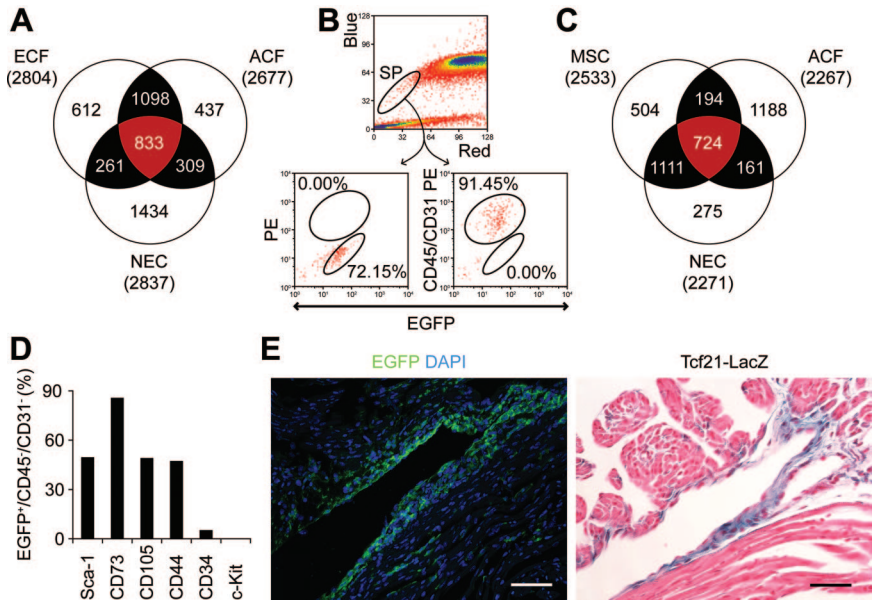

Figure 2. Adult Notch-activated epicardial-derived cells are multipotent stromal cells. A, Comparison of genes commonly expressed in NECs and adult (ACF) and embryonic cardiac (ECF) fibroblasts. **B,** FACS profile of Hoechst-stained TNR heart cells to distinguish side population cells and NECs. **C,** Three-way comparison of genes commonly expressed in NECs, bone marrow–derived multipotent stromal cells (MSCs) and adult cardiac fibroblasts (ACF). **D,** Expression of cell surface markers by FACS profiling in NECs (percentage of cell population positive for marker). **E,** Colocalization of EGFP and Tcf21-LacZ immunohistochemistry to annulus fibrosis of adult double transgenic mouse heart. **Scale bar,** 40 μm.

TAB hearts (at day 7 after injury) both activated a fibrosis repair gene program (gene ontology groups: extracellular matrix, cell proliferation, and cell adhesion) (Figure 1D; Online Tables I and II). We validated this array data by quantitative polymerase chain reaction, confirming upregulation of candidate extracellular matrix genes, collagen-I, elastin, and fibronectin (Figure 1E; Online Table II). Although most of the expression was overlapping, LAD-MI and TAB also generated unique gene expression signatures in NECs (Figure 1D). We concluded that LAD-MI and TAB strongly promoted fibroblast differentiation in NECs in vivo.

We also observed downregulation of muscle genes (gene ontology groups: muscle development, cell communication, and cell motility) in NECs from LAD-MI and TAB hearts (at day 7 after injury) (Figure 1D; Online Tables I and II). Reciprocal regulation of fibrosis and muscle genes in injury-activated NECs is consistent with the propensity of the mammalian heart to repair by fibrosis and not regenerate muscle, suggesting that NECs have an injury-regulated gene/fate switch.

Phenotypic Characterization of Adult Notch-Activated Epicardial-Derived Primary Cells

We set out to characterize NECs in greater detail by examining gene expression and cell surface marker profiles. We compared NEC gene expression profiles with published microarray results from other mouse heart cell subpopulations.[21,22] First of all, NECs were not cardiac fibroblasts (Figure 2A). Whereas embryonic and adult cardiac fibroblasts shared a majority of transcripts with each other (≈70%), ≈50% of NEC transcripts were unique and not detected in cardiac fibroblasts (Figure 2A).[22] We also evaluated NECs by Hoechst dye exclusion FACS assay. Although >72% of adult TNR heart side population cells were EGFP$^+$, the reciprocal was not true: NECs did not efflux Hoechst dye (Figure 2B). Finally, we compared the NEC gene expression profile with published arrays from multipotent stromal cells (MSCs) (previously known as mesenchymal stem cells), and, indeed, NECs shared ≈80% transcript identity with MSCs (Figure 2C).[21]

NECs were a mixed cell population by cell surface marker analysis (Figure 2D). Confirming our bioinformatics categorization, ≈50% of NECs expressed typical MSCs markers: Sca-1, CD105 (endoglin, a cell surface glycoprotein associated with the TGFβ receptor complex) and CD44 (a cell surface glycoprotein involved in cell-cell interactions), and ≈80% expressed CD73 (an ectoplasmic 5′-nucleotidase enzyme) (Figure 2D). Very few NECs expressed CD34 (a cell surface glycoprotein that functions in cell–cell adhesion); moreover, c-Kit was undetectable in NECs (Figure 2D). We present additional characterization of this unique and complex native cell population in Online Table III.

Multiple lines of molecular and genetic evidence established that NECs were derived from the epicardium. First, we confirmed that NECs had an epicardial gene expression signature by transcriptome analysis, detecting Wt1, Tbx18, Mesothelin, and Capsulin (Epicardin/Tcf21) mRNAs in NECs by microarray (Online Table III). The Capsulin-LacZ knock-in mouse is an established tool for epicardial lineage tagging.[23] We crossbred TNR and Capsulin-LacZ mice and confirmed by immunochemistry and LacZ histochemistry that CBF1RE$_{x4}$-EGFP$^+$ and Capsulin-LacZ signals colocalized to the same regions of the heart (Figure 2E). In particular, the annulus fibrosis, a naturally thickened area of epicardium, was strongly positive for both markers (Figure 2E). Thus, we concluded that NECs comprised a small but distinct population of epicardium-derived MSC-like cells, their immediate predecessors (epicardial mesothelial cells), and differentiated progeny, all regulated by Notch activity.

Characterization of Adult Notch-Activated Epicardial-Derived Clones

We developed a stepwise cloning/expansion strategy to obtain NEC lines originating from individual cells (Online Figure II, A and B); the efficiency of cloning NEC lines directly from the heart (without preexpanding) was very low

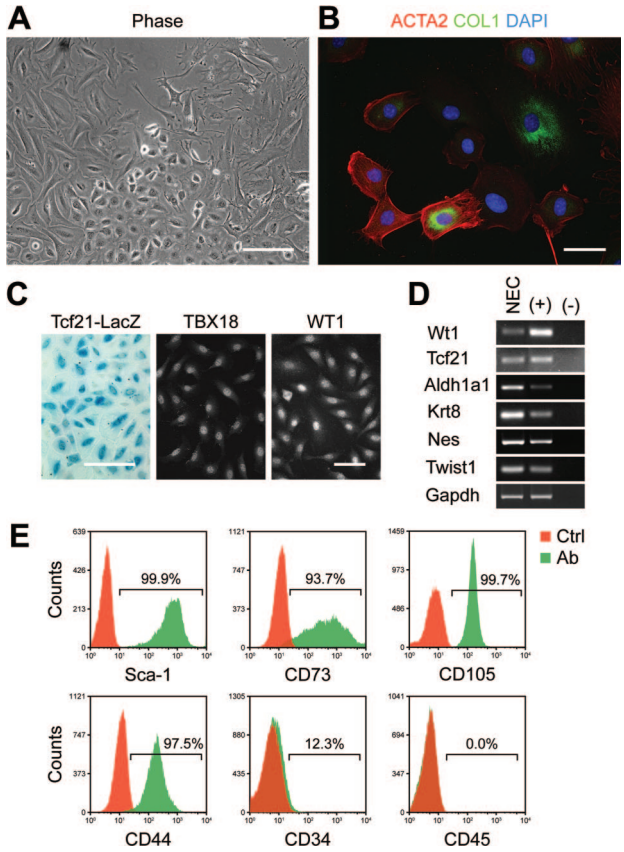

Figure 3. Confirmation of epicardial and multipotent phenotype in adult Notch-activated epicardial-derived cell clones. A, Heterogeneity of cell types in NEC clones demonstrated by phase-contrast microscopy. **Scale bar,** 200 μm. **B,** Cell type heterogeneity in NEC clones demonstrated by immunocytochemistry for smooth muscle actin **(red)** and collagen I **(green)** with DAPI. ACTA2, smooth muscle actin; COL1, collagen I. **Scale bar,** 40 μm. **C,** Expression of epicardial markers in NEC clone from TNR/Capsulin-LacZ double reporter mouse by LacZ histochemistry (**scale bar,** 200 μm) and TBX18 and WT1 immunostaining (**scale bar,** 40 μm). **D,** Expression of epicardial mRNAs in a NEC clone by RT-PCR. **E,** Expression of cell surface markers by FACS profiling in NEC clone.

(Online Figure II, C). A constant feature of NEC lines, despite clonality, was morphological heterogeneity, which is a hallmark of multipotent stromal cells maintained in vitro. With routine passage, NECs spontaneously formed neighboring colonies with divergent cell phenotypes, seen by phase-contrast microscopy (Figure 3A). We also observed the intrinsic heterogeneity of these cultures by immunocytochemistry for smooth muscle actin and collagen I (Figure 3B). In addition to the stepwise cloning strategy (Online Figure I, A and B), we have cloned lines directly from freshly isolated EGFP$^+$/CD45$^-$/CD31$^-$ cells sorted directly into 96-well plates (Online Figure II, C). The majority of these cells did not grow under our experimental conditions: using the most stringent criterion of >20 cells to qualify as a single cell-derived colony at 1 month, we calculated a colony-forming efficiency for freshly isolated NECs of <5%.

Like primary NECs, clonal lines also had the signature of epicardial lineage. We cloned NEC lines from double reporter TNR/Capsulin-LacZ mice and confirmed they were Capsulin-LacZ$^+$ and expressed nuclear WT1 and TBX18 protein by immunocytochemistry (Figure 3C). Even further, NEC clones expressed multiple epicardial transcripts (Wt1, Tcf21, Aldh1a1, Krt8, Nestin, and Twist1) by RT-PCR (Figure 3D).

We also reevaluated clonal NEC lines by flow cytometry using Sca-1, CD73, CD105, CD44, CD34, and CD45 antibodies (Figure 2E). As expected, clonally derived NEC populations were now homogeneous, ≈100% Sca1-, CD73-, CD105-, and CD44-positive (Figure 3E). Clonally derived NEC populations were CD34$^+$ (low) and remained CD45$^-$ (Figure 3E). With this cell surface marker signature (Sca-1$^+$, CD73$^+$, CD105$^+$, CD44$^+$, CD34$^-$, and CD45$^-$), we can identify NEC-like cells from any mouse heart.

Biology of Adult Notch-Activated Epicardial-Derived Cells In Vitro

EMT is a central feature of epicardium-derived cell biology.[24,25] As noted above, NEC cultures were dynamic and heterogeneous, often demonstrating adjacent epithelial- and mesenchymal-like cell clusters, suggesting local spontaneous EMT (Figure 3A) and perhaps even the reverse, mesenchymal–epithelial transition. Experimentally, we efficiently drove EMT in NECs by exposing cultures to epidermal growth factor (EGF), which redistributed β-catenin, a functional differentiation hallmark of EMT (Figure 4A). Although already expressed at baseline in these cultures, epidermal growth factor increased 2 mesenchymal markers, Twist1 and

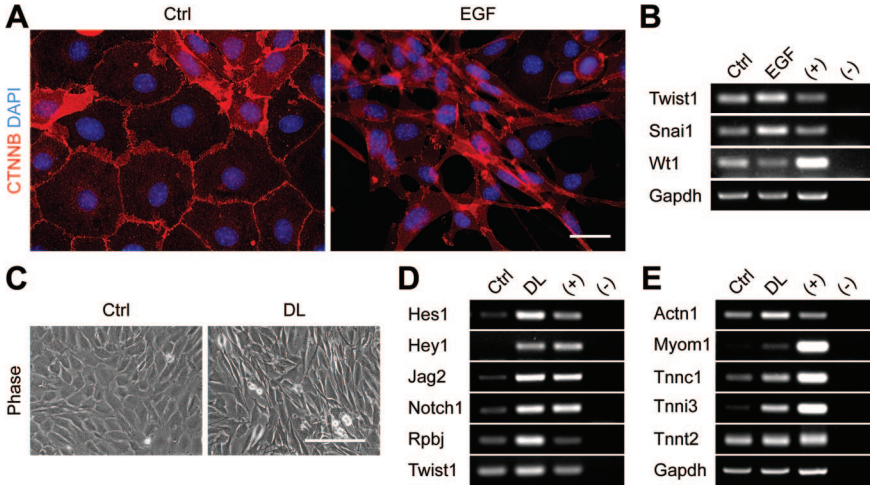

Figure 4. Adult Notch-activated epicardial-derived cell clones undergo EMT and display intact Notch signaling cascade. A, EMT morphological change and β-catenin **(red)** relocalization induced by epidermal growth factor (EGF) in NEC clone, with nuclei stained by DAPI. **Scale bar,** 40 μm. **B,** Reciprocal gene regulation of Twist1 and Snai1 (mesenchymal markers) vs Wt1 (epithelial marker) in NECs treated with EGF. **C,** Morphological changes induced by immobilized Delta-like 1 (DL) in NEC clones demonstrated by phase-contrast microscopy. **Scale bar,** 200 μm. **D and E,** Activation of Notch target genes (Hes1, Hey1, Jag2, Notch1, Rbpj) and EMT marker (Twist1) **(D)** and cardiac genes (Actn1, Myom1, and troponins) **(E)** by immobilized Delta-like ligand in an NEC clone by RT-PCR. Ctrl indicates control.

Snai1, and reciprocally decreased Wt1, an epithelial marker, in NECs (Figure 4B). We also studied Notch pathway signaling in NECs using the immobilized ligand Delta-like 1. Delta-like 1 triggered epithelial-to-mesenchymal-like morphological changes in NECs shown by phase-contrast microscopy (Figure 4C) and also activated downstream Notch target genes (Hes1, Hey1, Jag2, Notch1, and Rpbj/CBF1), as well as Twist1, a marker of EMT (Figure 4D). Notably, immobilized Delta-like 1 treatment of NECs induced cardiac gene expression (Actn1, Myom1, and cardiac troponins) (Figure 4E). Taken together, these results demonstrated growth factor-induced EMT and confirmed Notch pathway function in NECs, leading to expression of cardiac genes in vitro.

Modest Cardiogenic Potential of NECs

Even if fibrosis repair is the default program, a crucial question remains whether NECs have any potential, if appropriately instructed, to differentiate into cardiomyocytes; if so, future therapeutic strategies might strive to enhance this potential. We have begun to explore this possibility in 2 models: (1) coculture of NECs with primary cells (including cardiomyocytes) from newborn rat hearts (neonatal rat cardiomyocytes [NRCMs]); and (2) direct injection of NECs into immunocompromised mouse ventricular myocardium. To provide a stable immunofluorescence tracer for these experiments, we tagged NECs with a constitutively expressed copepod GFP (copGFP). We cocultured copGFP-NECs with NRCMs for 2 months and then evaluated these mixed cell cultures by immunocytochemistry. The overall morphology of copGFP-NECs/NRCM cocultures was clearly distinct from control NRCM cultures. Most importantly, only copGFP-NECs/NRCM cocultures maintained spontaneous beating and high-level sarcomeric organization of cardiac α-actinin; as expected, these features were lost over time in routine NRCM cultures (Figure 5A). We concluded that NECs provided essential cues to maintain long-term survival and functional differentiation of NRCMs in coculture. Consistent with this observation, we detected numerous secreted cardiotropic growth factor mRNAs in NECs by microarray (data not shown).

In the reciprocal experiment, cocultures induced cardiac differentiation in copGFP-NECs. We dissociated 2-month-old cocultures and used FACS to reisolate adult mouse copGFP-NECs and verified the postsort purity of this population (>98%) (Figure 5B). We used mouse-specific primers to evaluate NECs for muscle genes. Indeed, cocultured copGFP-NECs increased expression of a number of mouse muscle genes, up to 6-fold, including cardiac actin, α-actinin, myomesin-1, and troponin-C (Figure 5C). These gene expression results confirmed that NECs had modest cardiogenic potential when cocultured with NRCMs.

Finally, we evaluated the cardiac differentiation potential of copGFP-NECs in vivo by returning them to their native microenvironment. We transplanted copGFP-NECs (100K cells/heart) into the (uninjured) myocardium of NOD-SCID (nonobese diabetic severe combined immunodeficiency) mice, and, after 1 week, harvested recipient hearts and obtained histological sections. We observed numerous colonies of surviving copGFP-NECs (Figure 5D). Most remarkably, by double immunohistochemistry, almost all surviving copGFP-NECs expressed α-actinin (Figure 5D), corroborating the induction of mouse α-actinin (Actn2) mRNA observed in copGFP-NEC/NRCM cocultures (Figure 5C). We

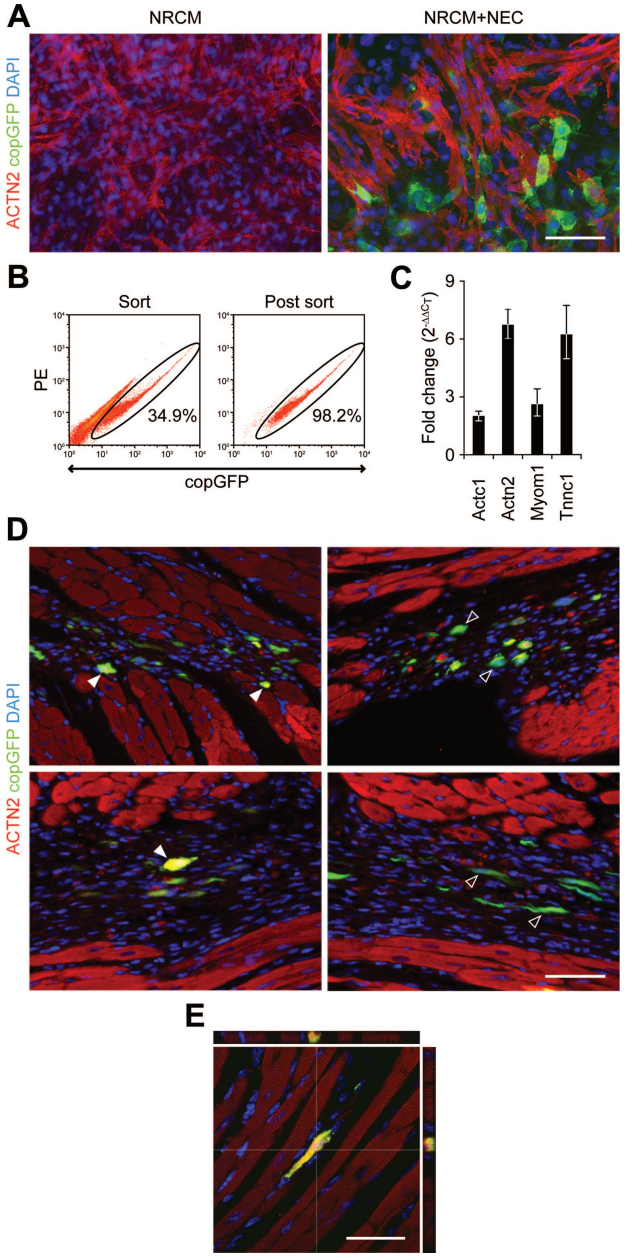

Figure 5. Adult Notch-activated epicardial-derived cells express cardiac genes after NRCM coculture or engraftment in ventricular myocardium. A, Double immunostaining for copGFP **(green)** and α-actinin **(red)**, with DAPI staining of nuclei **(blue)** of 2-month-old primary newborn rat heart/NRCM cultures, alone or cocultured with copGFP-NECs. **Scale bar,** 40 μm. **B,** Scattergram of copGFP-NECs retrieved from long-term cocultures by FACS and the postsort confirming purity. **C,** Quantitative PCR using mouse-specific primers demonstrating increased expression of cardiac genes in copGFP-NECs retrieved from NRCM cocultures compare to control cells. **D,** Identification of copGFP-NECs colonies engrafted in NOD-SCID ventricular myocardium (day 7 after injection) by double staining for α-actinin (ACTN2) **(red)** and copGFP **(green)**. **Scale bar,** 40 μm. **E,** Expression of α-actinin by engrafted copGFP-NEC as in **D** by confocal microscopy. **Scale bar,** 40 μm.

confirmed the specific expression of α-actinin in the copGFP cells by confocal microscopy (Figure 5E). Together, the NEC coculture and engraftment data provided compelling evidence that these cells have modest but potentially significant cardiac differentiation potential.

Discussion

We have taken a novel approach to exploring the adult mouse heart for resident progenitor-like cells. We set out to define a fluorescent protein-tagged cellular "module" for monitoring the response of the adult mouse heart to pathophysiological stimuli and direct mechanical or ischemic injury. Ideally, this module would allow us to quantitatively measure and qualitatively evaluate the injury repair response of the adult mouse heart at cellular and molecular levels. In future studies, this system will provide an in vivo pharmacological target for evaluating the efficacy of cardiogenic gene-activating, small-molecule drugs. Importantly, we sought a new approach that would be unbiased with the regard to previously characterized endogenous cardiac stem cells; that being said, any overlap with c-Kit$^+$, Sca-1$^+$, side population, or other putative adult cardiac stem cells would be welcomed and would validate our new and as yet untested model. We based our strategy on Notch, a signaling pathway fundamentally important for many cardiovascular developmental processes and already shown to play a role in the myocardial injury repair response.

We used transgenic reporter mice (TNR; CBF1RE$_{x4}$-EGFP), an indicator strain that monitors downstream transcriptional activity of the Notch pathway, a complex inter- and intracellular signaling cascade. The TNR model has already been validated in brain, bone marrow, and other tissues.[19,20] In adult TNR heart, the EGFP signal allowed us to identify, localize, isolate, and characterize a specific subpopulation of resident cells that we further subfractionated by depleting hematopoietic (CD45) and endothelial (CD31) subsets. We called these EGFP$^+$/CD45$^-$/CD31$^-$ cells, Notch-activated epicardial-derived cells (NECs). Cell surface marker analysis revealed that the NEC population was heterogeneous (eg, ≈50% Sca1$^+$; Figure 2D). However, transcriptome analysis exposed a dominant profile: matching the NEC gene expression signature to multipotent stromal cells.

Heterogeneity is a hallmark of MSCs, which have a large capacity and aggressive tendency to differentiate. Most importantly, MSCs are tissue repair cells, exactly what we were seeking to find in TNR hearts: a native heart tissue repair module. MSCs already have an established role in heart repair.[26]

NECs had 2 unique phenotypic identifiers that distinguished them from generic (typically bone marrow–derived) MSCs: (1) they expressed all known markers of epicardial lineage and localized to epicardium; and (2) they expressed and regulated muscle transcript levels in several contexts. On the one hand, after LAD-MI or TAB injury, muscle transcript levels were downregulated in NECs in vivo (Online Table II). On the other hand, muscle transcript or protein levels were upregulated in NECs following coculture with NRCMs (Figure 5A through 5C) or engraftment in NOD-SCID ventricular myocardium (Figure 5D and E). Furthermore, in studies to be presented in detail elsewhere, we demonstrated that cardiogenic small-molecules upregulated cardiac muscle genes in NECs, in vitro and in vivo.

The central function of a tissue repair module is a dynamic response to injury, and we confirmed this for NECs in 2 ways. First, LAD-MI and TAB expanded this native cell pool with kinetics appropriate for the type of injury (TAB is an instantaneous mechanical stressor, whereas LAD-MI is a wound-healing process involving an early inflammatory phase) (Figure 1C). Ischemia/reperfusion injury and doxorubicin-mediated chemical injury also expanded NECs, whereas, conversely, differentiation-inducing small molecules contracted this cell pool in vivo (data not shown). Secondly, we demonstrated that NECs, which were undifferentiated MSCs in the uninjured heart, were recruited by LAD-MI and TAB into fibrosis repair pathways, demonstrated by coordinated upregulation of a battery of fibrosis genes (Figure 1D and 1E; Online Tables I and II). Thus, NECs responded to injury with fibrosis, the default repair response of the adult mammalian heart. Our results also show, for the first time, that LAD-MI and TAB activated adult mouse epicardial Notch signaling, organ-wide.

Residing in the connective tissue/mesenchyme of most organs, MSCs repair tissues by secreting trophic growth factors and differentiating into specific lineages, and we confirmed both of these activities for NECs.[27] Paracrine secretion of mitogenic and cardiotropic growth factors is a hallmark of epicardium-derived cells.[28] Indeed, NECs expressed multiple cardiotropic growth factor mRNAs, many at very high levels, including thymosin-β4, PDGF-α, TGF-β3 and -2, BMP-1 and -4, hepatoma-derived growth factor, interleukin-18 and -25, CSF–macrophage, and FGF-7 (data not shown). We confirmed this functionally by showing that NRCMs maintained vigorous spontaneous beating (for several months) only when cocultured with NECs (Figure 5A and data not shown).

Our results are distinctive and provide additional knowledge to the field. Although our NEC studies partially overlapped with previous work on adult mouse heart Sca1$^+$/CD31$^-$ cells, ≈50% of our primary cell population was Sca1$^-$.[29] Similarly, although a subset of cKit$^+$ cells from the newborn heart contained NICD1,[15] we never observed cKit$^+$ cells (by flow cytometry) in NEC populations (Figure 2D). Our Notch-activity localization data are also very different from previous reports.[17,18] We are the first to report that Notch-activated cells are focally enriched in the epicardial zone, especially after injury (Figure 1). This discrepancy can be explained by differences in experimental technique. All earlier studies localized Notch activity in adult heart using an NICD1 (Notch-1) antibody.[17,18] However, the CBF1RE$_{x4}$-EGFP TNR transgene can be activated by multiple receptor isoforms, and microarray analysis confirmed that Notch-2, not Notch-1, was the predominant receptor isoform transcript in NECs. Thus, invisible in previous NICD1 antibody studies for Notch-1, Notch-2 drives unambiguous epicardial CBF1RE$_{x4}$-EGFP activity in TNR mice (Figure 1).

Our studies also had several unique technical considerations. First, the EGFP signal in NECs reflects the Notch-activated state, which is complex and dynamic, determined by

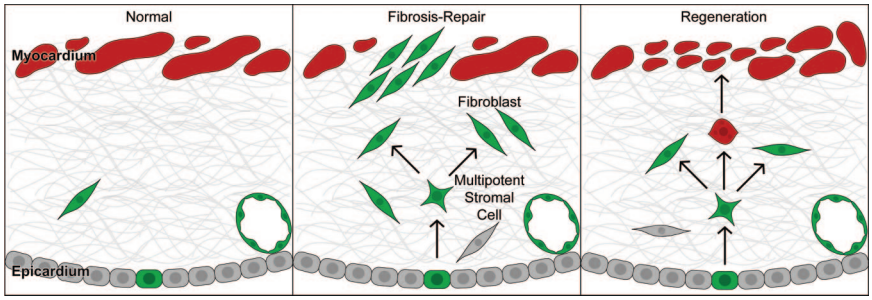

Figure 6. A dynamic adult epicardial injury response marked by Notch activity contributes to fibrosis repair. Model of Notch-activated ($CBF1RE_{x4}$-$EGFP^{+}$) epicardial–mesothelial cell that delaminates into the subepicardial space and undergoes EMT to produce a multipotent stromal cell (MSC) capable of differentiating into fibroblasts (FB), smooth muscle cells (SMC), and other cells, perhaps even cardiomyocytes.

a balance of positive and negative regulatory factors, and by the in vivo half-life of the EGFP polypeptide. In theory, EGFP can mark the full continuum of cells arising in the epicardial microenvironment: starting from mesothelial cells that delaminate and undergo EMT, giving rise to MSCs capable of differentiating into multiple, possibly all, cardiovascular lineages (Figure 6). Thus, Notch-driven EGFP provides a panoramic view of this tissue repair-module. This model is ideal for using TNR mice to screen for pharmacological agents that redirect this wound-healing process from fibrosis toward muscle-rebuilding regeneration. One additional caveat of our studies is that we have intentionally excluded Notch-activated $CD31^{+}$ cells. Undoubtedly, these Notch-activated $CD31^{+}$ cells, which use Notch activity to stabilize arterial endothelial fate and control angiogenesis, will be play a key role in the potential success of regenerative therapies.[30]

In summary, we conclude that Notch activity in TNR mice provides a unique, reliable, and versatile cell-based biosensor. Notch-activated EGFP can be used to identify and purify the continuum of mesothelial cells, MSCs, and their immediate progeny, arising in the epicardial microenvironment of the adult mouse heart. This Notch-activated repair module provides a novel experimental system for studying native multipotent stromal cell function and may provide a practical system for evaluating candidate cardio-regenerative drugs.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Notch pathway signaling is a gatekeeper of cell fate decisions in many cell types, including cardiovascular progenitors, and is a critical regulator of cardiac development and adult heart homeostasis and repair.
- Wound healing after adult heart injury involves dramatic morphological and physiological changes that lead to the formation of a fibrotic scar to maintain homeostasis.

What New Information Does This Article Contribute?

- Notch pathway activation is involved in epicardial hypercellular expansion that accompanies myocardial infarction and pressure overload injury.
- Notch-activated epicardial-derived cells are multipotent stromal cells that transdifferentiate into fibroblast lineages after injury.
- Notch-activated epicardial-derived cells are multipotent and can be cultured indefinitely and experimentally manipulated.

Heart injury is responsible for a quarter of all deaths in the United States. Native progenitor-based repair of heart injury is one of the most promising but challenging areas of regenerative medicine. The adult heart has multiple types of progenitor cells, yet when called to repair, these multipotent cells all produce scar rather than muscle. A greater mechanistic understanding of the barriers that prevent progenitors from regenerating muscle is critically needed. Many cell types involved in heart repair use Notch, an inter- and intracellular signaling pathway critical for sensing and responding to environmental cues like injury. We show that Notch activity identifies and localizes progenitors serving as biosensors of cardiac injury and mediators of repair. Notch-activated progenitors are multipotent stromal cells, derived from epicardium by epithelial–mesenchymal transition and destined to fibroblast differentiation after injury but capable of expressing muscle genes if appropriately instructed. These findings provide new mechanistic insights into native epicardial repair processes and provide a platform for developing new cardioregenerative strategies and drugs.

SUPPLEMENTAL MATERIAL

Detailed Methods

RNA extraction/RT-PCR

Total RNA was isolated with Trizol (Invitrogen) and reverse transcription performed using the Superscript First-Stand Synthesis System (Invitrogen). QPCR was performed using SYBR Green QPCR or Taqman Mastermix (ABI) and relative quantification of gene expression was calculated using the comparative method (ΔΔCT) with Gapdh as the endogenous control and specific control calibrator as described [1]. Neonatal heart was used as positive control. Primer sequences are available upon request.

Immunocyto/histochemistry

Cells were fixed for 10 minutes with 4% PFA in PBS, washed and stained with the following antibodies: anti-α smooth muscle actin/ACTA2 (1A4) (Sigma), anti-Type I Collagen (COL1) (SouthernBiotech), anti-TBX18 (C-20) (SCBT), anti-WT1 (C-19) (SCBT), anti-β-Catenin (BD), and anti-copGFP (Evrogen). Paraffin-embedded sections from PFA fixed adult mouse hearts were deparaffinized and treated with Pronase for antigen retrieval. Slides were stained with anit-GFP (3E6) (Invitrogen) using the Tyramide Signal Amplification Kits (Molecular Probes) and co-stained with Anit-Troponin I (H-170) (SCBT) or stained with anti-copGFP and co-stained with anti-sarcomeric α-actinin (EA-53) (Sigma).

Microarray/gene-enrichment analysis

All samples were prepared as previously described [2]. Briefly, RNA was extracted with Trizol (Invitrogen) and labeled with MessageAMP II-Biotin kit and submitted to UT Southwestern Microarray Core for processing, hybridization and scanning. Sorted samples were subjected to an additional round of amplification. Samples were normalized and compared with dChip software 2010 as described [2]. For comparison to previously published data, all samples were standardized and normalized to the baseline sample with SNOMAD (Standardization and Normalization of MicroArray Data) web implementation [3]. A gene list of Probeset identifiers of differentially expressed genes was analyzed with Expression Analysis Systematic Explorer version 2.0 (EASE), which determines enrichment of Gene Ontology terms using an EASE score (a modified Fisher Exact P-Value) [4].

β-galactosidase staining

Animals were perfused with 15 ml PBS followed by 15 ml fixative (1% PFA, 0.2% Glutaraldehyde, 5 mM EGTA, 2 mM MgCl2, and 0.01% NP-40 in PBS), hearts were removed and placed in prewarmed (37°C) staining buffer (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 0.01% NP-40 and 0.1% X-gal) and stained overnight at 37°C in the dark. Samples were then washed and post fixed with 4% PFA in PBS overnight. Hearts were processed, sectioned and counterstained with nuclear fast red. Cells were fixed for 10 minutes and stained as above.

Flow cytometry

Antibodies: BD PE anti-CD45 (30-F11), BD PE anti-CD31 (MEC 13.3), BD PE anti-Ly-6A/E/Sca-1 (E13-16.7), Biolegend Alexa Fluor 647 anti-CD73 (TY/11.8), Biolegend PE/Cy7

anti-CD105 (MJ7/18), BD APC anti-CD44 (IM7), Biolegend Alexa Fluor 647 anti-CD34 (MEC14.7), BD APC anti-CD117/c-Kit (2B8). All samples were stained as recommended by the manufacturer. For Side population discrimination, samples were stained with Hoechst 33342 prior to antibody staining as previously described [5].

Surgical procedures

The UTSW IACUC approved all animal protocols. Adult ICR TNR transgenic male mice were subjected to LAD-MI, TAB or sham surgery. Mice were anesthetized with 2.4% isoflurane and intubated. For MI the LAD was visualized and ligated with a 6-0 prolene suture and for TAB the aorta was constricted to a 27G stenosis. Animals were observed daily and sacrificed at 0, 3 and 7 days post-injury.

Induction of EMT and activation of Notch pathway

For induction of EMT, cells were treated with 50ng/ml EGF (Invitrogen) for four days. For activation with Delta-like ligand, culture dishes were coated with 5 μg/ml RetroNectin (Takara Bio, Inc.) with and without 5 μg/ml Delta-ext-IgG (gift from Irwin Bernstein) overnight at 4°C. The following day plates were washed five times with PBS (without Ca^{2+} and Mg^{2+}) and blocked with serum containing media for 1 hour at 37°C. Plates were washed five more times with PBS and then cells were plated on the coated dishes. Samples were harvested 48 hours after exposure.

Cloning efficiency assay

Adult TNR hearts were dissociated and stained as described. EGF+/CD31-/CD45- cells were sorted one cell per well on 96-well gelatin coated plates. One month after plating plates were washed with PBS+ and fixed for five minutes with 4% PFA. After several PBS washes, the cells were stained with 0.05% Crystal Violet in water for 30 minutes. Cells were then washed two times with water and then allowed to air dry. Number of cells per well was determined.

Supplemental References

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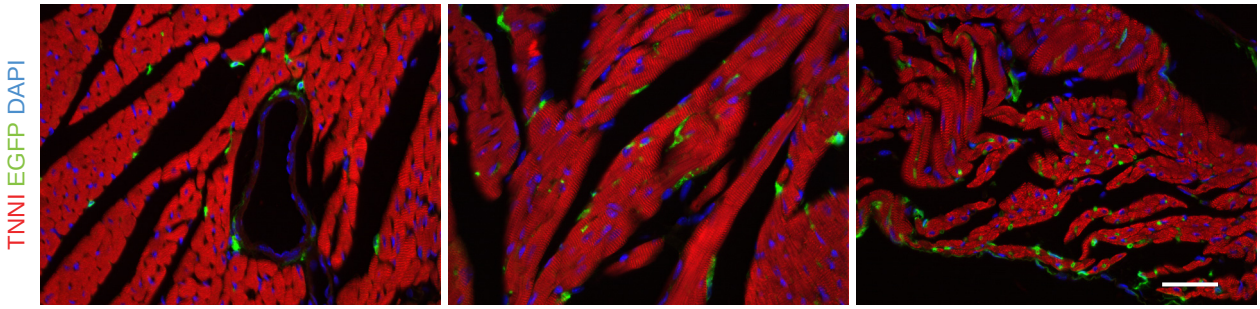
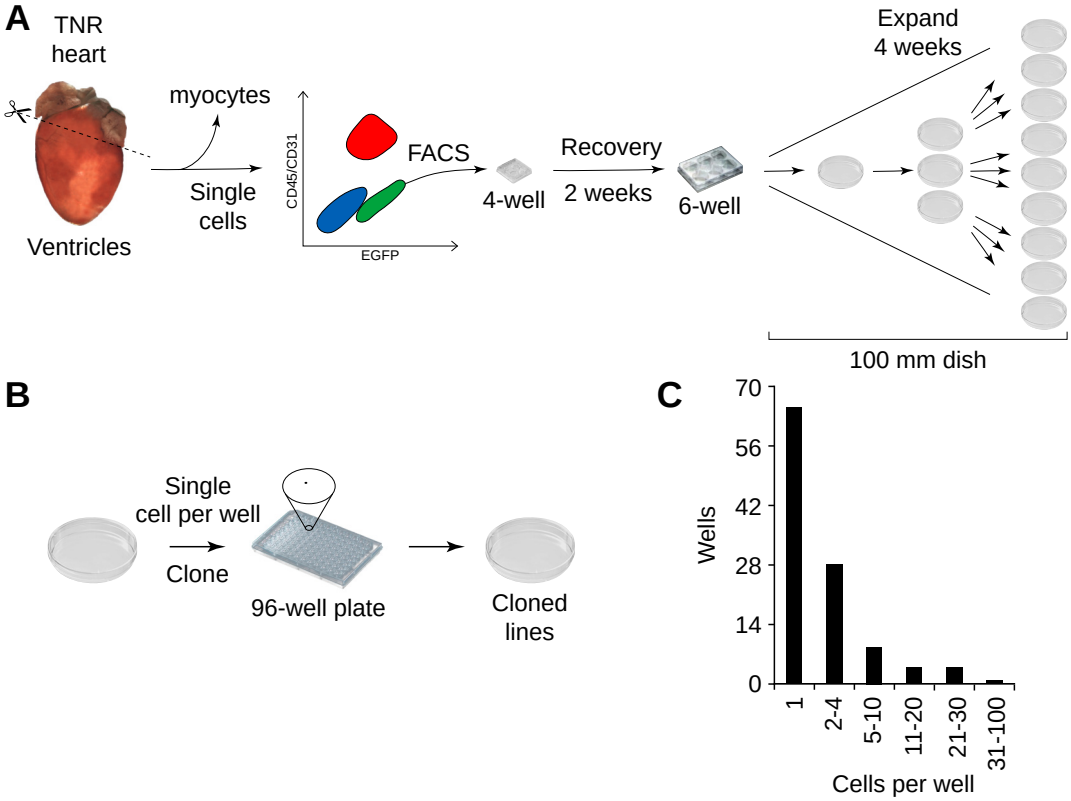
Supplemental Figures and Tables



Online Figure I. EGFP localization in the uninjured adult myocardium. EGFP IHC (green) of ventricular myocardium (left two panels) and atrium (right panel) counterstained for Troponin-I (red) and DAPI (blue) showing positive endothelial and interstitial cells (bar=40 µm).



Online Figure II. Cell Isolation Strategies. **A**, Strategy used to isolate and pre-expand NECs before single cell cloning. **B**, Strategy to generate single cell clones from pre-expanded NECs. **C**, Frequency of wells with cell number as listed after sorting single NECs directly into individual wells of a 96-well plate.

Online Table I. Gene categories upregulated in NECs 7 days post LAD-MI and TAB.

Gene Ontology term	EASE score*
Cellular Component	
extracellular matrix	1.55E-10
extracellular	1.10E-08
extracellular space	8.95E-07
collagen	4.82E-05
microfibril	1.35E-04
Molecular Function	
glycosaminoglycan binding	1.18E-06
extracellular matrix structural constituent	5.27E-06
growth factor activity	1.35E-05
extracellular matrix structural constituent conferring tensile strength	2.04E-05
heparin binding	2.16E-05
cell adhesion molecule activity	2.52E-05
Biological Process	
cell proliferation	1.43E-08
mitosis	2.12E-08
M phase of mitotic cell cycle	2.42E-08
cell cycle	6.65E-08
nuclear division	1.49E-07
mitotic cell cycle	1.87E-07
M phase	2.42E-07
skeletal development	7.27E-06
regulation of cell cycle	1.41E-05
cytokinesis	4.44E-05
cell adhesion	4.50E-05
obsolete biological process	7.11E-05
cellular process	1.58E-04
cell communication	4.09E-04
development	4.31E-04
DNA replication and chromosome cycle	7.45E-04
mitotic prophase	7.76E-04
mitotic chromosome condensation	7.76E-04

*modified Fisher exact probablility

Online Table I. Categories of genes upregulated in NECs 7 days post LAD-MI and TAB. Gene enrichment analysis showed ECM and cell proliferation categories as significant after injury.

Online Table II. Fold change of ECM and muscle transcripts following injury.

Probe set	Gene Title	Gene Symbol	LAD-MI	TAB
Upregulated				
1449827_at	aggrecan	Acan	2.79	2.62
1455494_at	collagen, type I, alpha 1	Col1a1	3.59	3.31
1416741_at	collagen, type V, alpha 1	Col5a1	5.56	6.65
1418441_at	collagen, type VIII, alpha 1	Col8a1	2.56	3.15
1434667_at	collagen, type VIII, alpha 2	Col8a2	7.24	8.54
1418599_at	collagen, type XI, alpha 1	Col11a1	34.47	44.13
1434411_at	collagen, type XII, alpha 1	Col12a1	7.63	11.41
1427168_a_at	collagen, type XIV, alpha 1	Col14a1	2.36	2.57
1420855_at	elastin	Eln	4.34	2.77
1425896_a_at	fibrillin 1	Fbn1	2.57	3.02
1456084_x_at	fibromodulin	Fmod	2.84	3.56
1426642_at	fibronectin 1	Fn1	3.72	3.94
1416121_at	lysyl oxidase	Lox	10.01	11.15
1449254_at	secreted phosphoprotein 1	Spp1	20.13	17.11
1460302_at	thrombospondin 1	Thbs1	2.35	3.05
1460227_at	tissue inhibitor of metalloproteinase 1	Timp1	3.01	2.87
Downregulated				
1444152_at	CUG triplet repeat, RNA binding protein 2	Cugbp2	-2.76	-5.17
1460318_at	cysteine and glycine-rich protein 3	Csrp3	-3.14	-4.95
1449283_a_at	mitogen-activated protein kinase 12	Mapk12	-2.72	-2.85
1457435_x_at	myomesin 2	Myom2	-4.15	-4.18
1448826_at	myosin, heavy polypeptide 6, cardiac muscle, alpha	Myh6	-3.83	-4.8
1448554_s_at	myosin, heavy polypeptide 7, cardiac muscle, beta	Myh7	-4.53	-5.5
1448394_at	myosin, light polypeptide 2, regulatory, cardiac, slow	Myl2	-3.38	-3.93
1427768_s_at	myosin, light polypeptide 3	Myl3	-4.57	-6.21
1449465_at	reelin	Reln	-2.77	-3.04
1437523_s_at	sarcoglycan, gamma (dystrophin-associated glycoprotein)	Sgcg	-5.98	-4.03
1447655_x_at	SRY-box containing gene 6	Sox6	-4.06	-3.13
1418370_at	troponin C, cardiac/slow skeletal	Tnnc1	-4.02	-5.82
1422536_at	troponin I, cardiac	Tnni3	-6.62	-8.34
1418726_a_at	troponin T2, cardiac	Tnnt2	-3.27	-3.44

Online Table II. Fold change of ECM and muscle transcripts following injury. Reciprocal regulation of ECM-fibrosis and muscle genes in NECs post LAD-MI and TAB (day 7).

Online Table III. NEC phenotypic characterization

	Marker		Method
Stem/progenitor	CBF1REx4-EGFP	+	FACS
	Sca-1	+	FACS/Array
	c-Kit	-	FACS/Array
	Isl1	-	Array
	Oct3/4	-	Array
	Nanog	-	Array
	Abcg2	+	Array
Epicardial	Wilms tumor 1	+	RTPCR/ICC/Array
	T-box18	+	RTPCR/ICC/Array
	RALDH2	+	RTPCR/Array
	PDGF-Rα	+	FACS/Array
	PDGF-Rβ	+	FACS/Array
	Podoplanin	+	FACS/Array
	Integrin alpha 4	+	RTPCR/Array
	Mesothelin	+	RTPCR/Array
	Twist 1	+	RTPCR/Array
	Snai1	+	RTPCR/Array
	Capsulin	+	RTPCR/Array
	Keratin 8	-	RTPCR/Array

Online Table III. Phenotypic characterization of NECs. Summary of data, emphasizing stem/progenitor- and epicardium-related markers.

A Dynamic Notch Injury Response Activates Epicardium and Contributes to Fibrosis Repair

Jamie L. Russell, Sean C. Goetsch, Nicholas R. Gaiano, Joseph A. Hill, Eric N. Olson and Jay W. Schneider

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