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Transcriptional Reversion of Cardiac Myocyte Fate During Mammalian Cardiac Regeneration

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Abstract

Rationale—Neonatal mice have the capacity to regenerate their hearts in response to injury, but this potential is lost after the first week of life. The transcriptional changes that underpin mammalian cardiac regeneration have not been fully characterized at the molecular level.

Objective—The objectives of our study were to determine if myocytes revert the transcriptional phenotype to a less differentiated state during regeneration and to systematically interrogate the transcriptional data to identify and validate potential regulators of this process.

Methods and Results—We derived a core transcriptional signature of injury-induced cardiac myocyte regeneration in mouse by comparing global transcriptional programs in a dynamic model of in vitro and in vivo cardiac myocyte differentiation, in vitro cardiac myocyte explant model, as well as a neonatal heart resection model. The regenerating mouse heart revealed a transcriptional reversion of cardiac myocyte differentiation processes including reactivation of latent developmental programs similar to those observed during de-stabilization of a mature cardiac myocyte phenotype in the explant model. We identified potential upstream regulators of the core network, including interleukin 13 (IL13), which induced cardiac myocyte cell cycle entry and STAT6/STAT3 signaling in vitro. We demonstrate that STAT3/periostin and STAT6 signaling are critical mediators of IL13 signaling in cardiac myocytes. These downstream signaling molecules are also modulated in the regenerating mouse heart.

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COMPETING INTERESTS
The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTION
Conceived and designed the experiments: CCO, JAW, RAG, RTL, and LAB. Performed the experiments: CCO, JAW, RAG, GMF, and JG. Analyzed the data: CCO, JAW, RAG, VLB and AS. Contributed reagents/materials/analysis tools: RTL and LAB. Wrote the paper: CCO, JAW, RTL and LAB.

Conclusions—Our work reveals new insights into the transcriptional regulation of mammalian cardiac regeneration and provides the founding circuitry for identifying potential regulators for stimulating heart regeneration.

Keywords

Cardiac myocyte; gene expression; growth factors/cytokines; myogenesis; regeneration

INTRODUCTION

The adult mammalian heart has a limited capacity for self-renewal following injury.[1–3] Shortly after birth, mammalian cardiac myocytes exit the cell cycle, and subsequent heart growth is achieved primarily by hypertrophy of existing cardiac myocytes.[4] Substantial evidence suggests that even these terminally differentiated adult cardiac myocytes retain some limited ability for cell division.[5, 6] However, the innate ability of the adult mammalian heart to repair itself following injury such as myocardial infarction is inadequate to replace the loss of functional myocardium.[7] In contrast, some vertebrates such as zebrafish and newts can fully regenerate their hearts following amputation throughout their adult lives, primarily by proliferation of mature cardiac myocytes.[8, 9]

Although adult mammalian hearts fail to regenerate after injury, neonatal mice can fully regenerate their heart following resection of the left ventricular apex.[10] Genetic fate mapping demonstrated that new cardiac myocytes in the regenerating apex were derived from preexisting cardiac myocytes as opposed to a resident stem cell or progenitor population. Cardiac myocytes in the regenerating neonatal mouse heart demonstrate loss of distinct sarcomere structures and a significant proportion of these cells enter the cell cycle, as indicated by phosphorylated histone H3 (pH3) expression and up-regulation of aurora B kinase, suggestive of cell fate reversion.[10] Thus, identifying mechanisms by which myocytes naturally undergo cell cycle activity during regeneration is fundamental for elucidating the molecular roadblocks that prevent regeneration in the adult heart. The idea that cardiac myocytes undergo partial reversion of cell fate during mouse heart repair has been based primarily on observations at the ultrastructural level.[10, 11] The transcriptional changes that accompany this phenotypic response to injury remain largely unknown.

Here, we profiled global gene expression patterns over the course of mouse cardiac myocyte differentiation both in vitro (mouse embryonic stem cells differentiated to cardiac myocytes) and in vivo (cardiac myocyte maturation from neonate to adult) and compared this transcriptional signature of differentiation to a cardiac myocyte explant model whereby cardiac myocytes lose the fully differentiated phenotype (mouse adult cardiac myocytes explanted and cultured over 72 hours), to identify genes and gene networks that changed dynamically during these processes. We then examined global expression changes in the neonatal mouse whole heart ventricle as well as in purified cardiac myocytes following apical resection and found that heart regeneration is characterized by a transcriptional reversion of the cardiac myocyte differentiation process, including reactivation of cell cycle genes and developmental programs. We interrogated the RNA sequencing (RNAseq) datasets by using a systematic approach to predict and validate upstream regulators and

associated pathways that can modulate the cell cycle state of cardiac myocytes. We identified interleukin 13 (IL13) as a new regulator of cardiac myocyte cell cycle entry and found that STAT6, STAT3, and periostin are critical mediators of IL13 signaling in cardiac myocytes. As recently described in the zebrafish,[12] STAT3 is predicted to regulate differentially expressed gene networks during heart regeneration in our dataset and STAT3 protein expression is increased in the regenerating neonatal mouse heart. Together, our data suggest that cardiac regeneration involves a transcriptional reversion of the differentiation process, and our systematic approach used to interrogate these RNAseq datasets provides significant insight into signaling pathways of both myocyte cell cycle activity and cardiac regeneration.

METHODS

Animal care and usage

All animal studies were performed in accordance with the local animal care and use committee.

Embryonic stem cell differentiation

RNAseq data for the differentiation of mouse embryonic stem cells into cardiac myocytes through mesodermal and cardiac progenitor intermediates was obtained from Wamstad et. al.[13] Raw data from both biological replicates at each time point (ESC, MES, CP and CM) were reprocessed according to the methodology described below.

Whole heart ventricle isolation

Crl:CD1(CD-1) neonatal mice (Charles Rivers Laboratories, MA) were sacrificed by decapitation at P0, P4, and P7, and by isoflurane overdose at 8–10 weeks of age. Hearts were excised, washed in ice cold PBS, and snap frozen in liquid nitrogen. Heart atria were dissected and discarded, and ventricles were processed for RNAseq. At least two heart ventricles were pooled for each replicate. Two replicates were processed for RNAseq.

Adult cardiac myocyte isolation

Adult mouse cardiac myocytes were isolated from 8–10 week old male CD-1 mice based on a protocol described previously.[14]

Neonatal mouse apical resection

Apical resection surgeries were performed on postnatal day 1 (P1) CD-1 mice as described previously.[10] The chests of sham-operated mice were opened and the hearts were exposed but not resected. Hearts from sham and resected animals were collected at 24 hours and 7 days post surgery, washed in PBS, and snap frozen in liquid nitrogen. The apex of the heart was dissected and processed for RNAseq. Heart apex from 3 animals was pooled for each biological replicate. We consistently observe significantly elevated cardiac myocyte cell cycle entry at 7 days post operation in resected versus sham operated hearts.

Neonatal cardiac myocyte purification

Neonatal cardiac myocytes were dissociated from whole mouse hearts (P0 and P4), and also from sham and resected neonatal mouse hearts at 7 days post surgery using the Neonatal Heart Dissociation Kit (Miltenyi Biotec). Cardiac myocytes were purified from the dissociated cell population using the Neonatal Cardiac myocyte Isolation Kit (Miltenyi Biotec) according to the manufacturers instructions. Three biological replicates were generated per timepoint and experimental treatment for RNAseq. Hearts from five to ten mouse pups were pooled for each biological replicate.

RNA sequencing and analysis

Total RNA was extracted from all samples using Trizol (Invitrogen) according to the manufacturers instructions. Polyadenylated RNA was isolated from 1–10 μg of total RNA using the Dynabeads mRNA purification kit (Invitrogen). Polyadenylated RNA was fragmented and the first strand was synthesized using the Superscript III reverse transcription kit (Invitrogen). Double stranded DNA was then synthesized with DNA polymerase I (Invitrogen). End repair, A-tailing, adapter ligation and size selection were then performed using the SRPI-Works System (Beckman Coulter) followed by minimal amplification and addition of barcodes by PCR. Paired-end 40 base pair read length sequencing was then performed on an Illumina HiSeq 2000. Sequence alignment and assembly was performed as described previously.[15, 16] For the purified neonatal cardiac myocyte samples the TrueSeq (Invitrogen) sample preparation protocol was performed due to the low RNA yield prior to RNAseq.

Isolation, culture and treatment of ventricular neonatal rat cardiac myocytes

Rat ventricular cardiac myocytes were isolated from one-day old Sprague Dawley rats (Charles River) as described previously.[14] Cultured cardiac myocytes were exposed to 100 ng/mL of each growth factor or cytokine followed by quantification of ^{3}H-thymidine incorporation. ^{3}H-thymidine or BrdU incorporation, flow cytometry, expression analysis, siRNA knockdown, qPCR and western blot protocols can be found in the supplementary materials.

Data analysis and statistics

Data was analyzed by either one-way ANOVA or unpaired t-test unless otherwise stated. All data is presented as mean ± standard error of the mean (SEM). Statistical analysis was carried out with the Prism Graphpad Software.

RESULTS

Determining the transcriptional signature of cardiac myocyte differentiation

To elucidate the transcriptional changes that drive the switch from a proliferative mode of growth to cell cycle exit during cardiac myocyte terminal differentiation and maturation, we first established a reference transcriptional signature of this process by identifying a common set of differentially expressed genes during in vitro differentiation and in vivo cardiac myocyte maturation. We analyzed RNAseq data from cells collected at the

embryonic stem cell (ESC), mesoderm (MES), cardiac progenitor (CP), and cardiac myocyte (CM) stages as described previously[13] (Figure 1A, FPKM values for all RNAseq datasets in Online Table I A). We then analyzed gene expression in an in vivo maturation model and compared these data to the in vitro differentiation model. Within the first week of life, and into adulthood, mammalian cardiac myocytes undergo a maturation process that is characterized by development of a rigid and organized sarcomeric structure, binucleation, increased metabolic demand, and exit from the cell cycle.[17] We collected and analyzed mouse heart ventricles from postnatal day 0, 4, and 7 (P0, P4, and P7) and from 8–10 week old adult mice (Figure 1A). We defined the transcriptional changes that occur during both in vitro and in vivo differentiation as the core gene network and postulated that these genes, at least in part, underpin critical transcriptional determinants of cardiac myocyte differentiation.

As expected, differentially expressed genes during in vitro and in vivo differentiation showed primarily up-regulation of sarcomere and mitochondrial related genes and down-regulation of cell cycle genes as determined by analysis of enriched gene ontology (GO) terms[18, 19] (Online Figure I A and B). Although the ESC differentiation model and in vivo cardiac myocyte maturation have been studied previously, the full set of genes dynamically changing that are common to both datasets has not been described. In total, 927 genes were commonly up-regulated in both datasets (Figure 1B, Online Table I B) which function in mitochondrial, sarcoplasm, and sarcomere-specific processes, among others (Figure 1C,D, Online Table I D). Genes critical for sarcomere assembly, such as Titin Cap and Cardiac troponin I type 3, showed pronounced increases in expression over the course of differentiation reflecting sarcomere assembly and organization during cardiac myocyte differentiation and maturation.

Genes whose expression decreased in both differentiation datasets function primarily in RNA processing and cell cycle (Figure 1C,D, Online Table I C, E). RNA processing encompasses mechanisms such as RNA transport (e.g. Thoc1, 3, 4, and 5, Ncbp1, and Khsrp), RNA splicing (e.g. Xab2, Wbp11, Aqr), and RNA capping (e.g. Rnmt, Rngtt, Ncbp1, and Tgs1), among others. Post-transcriptional modification is a critical mechanism that regulates cell fate commitment and differentiation,[20] and our data support that RNA processing is necessary during the process of cardiac myocyte differentiation both in vitro and in vivo. Cell cycle exit is a hallmark of mature cardiac myocytes and a failure to re-enter the cell cycle is thought to contribute to the lack of cardiac regeneration in adult mammals. We found that cell cycle regulators such as E2F2, Brca2, and a number of cell division genes (e.g. Cdc6, 7, 23, 26, and 27) were consistently down-regulated in both differentiation datasets. Importantly, differentially expressed genes unique to either in vitro or in vivo differentiation did not strongly reflect hallmarks of myocyte differentiation (Online Table I; F, G, H, I).

Explanted cardiac myocytes display a transcriptional reversion of differentiation

Adult mammalian cardiac myocytes are terminally differentiated cells that rarely proliferate in vivo; however, adult rodent cardiac myocytes isolated and cultured ex vivo over several days can revert to a more immature state and re-enter the cell cycle suggesting that adult

cardiac myocytes retain some plasticity.[21] We hypothesized that the transcriptional response of explanted mouse cardiac myocytes would result in a loss of the differentiated phenotype, providing clues to the critical factors required for maintaining the myocyte differentiated state. Thus, we analyzed gene expression patterns by RNAseq from adult mouse cardiac myocytes explanted and cultured for 0, 24, 48, and 72 hours (Figure 1A, Online Figure I; C) to model the early transcriptional changes that occur during destabilization of the cardiac myocyte state.

To compare changes in gene expression programs in ex vivo cultured cardiac myocytes to the differentiation process, we performed hierarchical clustering and identified groups of genes with common functions (Figure 2A, Online Table II; A). Strikingly, we found gene clusters that were repressed during differentiation were re-activated during the cardiac myocyte explant time course, specifically those with functions in RNA processing and chromatin modification (Figure 2A- green, brown and light blue, Online Table II; A). We next used principle component analysis (PCA) to gain further insights into the relationship between the differentiation models and ex vivo time course (Figure 2B). The cardiac myocyte explant samples showed a directional reversion along the PC1 axis, represented by a down-regulation of sarcomere components and activation of the cell cycle. The shift along the PC2 axis in the in vivo samples likely shifts in the opposite direction of the ESC dataset as a result of transcriptional changes involved in blood vessel morphogenesis and extracellular matrix processes or perhaps in response to early developmental genes turned on during in vitro differentiation and turned off during in vivo maturation.

The vast majority (2,253, 93%) of genes differentially expressed during ex vivo culture showed changes in the opposite direction during the differentiation process (Figure 2C, Online Table II; B,C). Genes deactivated during differentiation overlapped those genes that are activated during explant culture, and function in RNA processing, cell cycle, transcription, and DNA repair. Moreover, genes activated during differentiation and down-regulated during the ex vivo time course have roles in cell metabolism and regulation of heart contraction (Figure 2 C,D, Online Table II; D and E). Several sarcomere related genes highly expressed in the adult heart (i.e. Tnnt2, Tcap, Myl3, Tnni3, Tpm1, Myh7, Myh6, Mybpc3, and Tmod1) were downregulated upon explanation (Online Figure II).

Neonatal mouse cardiac regeneration involves a transcriptional reversion of cardiac myocyte differentiation

Neonatal mice maintain the capacity to regenerate their hearts following resection of up to 15% of the apex; however, this ability is lost by 7 days after birth.[10] During this critical developmental window, cardiac myocytes develop rigid and highly organized sarcomere structures, and many of these cells become multi-nucleated.[17] Upon injury, some cardiac myocytes display apparent loss of clear sarcomere structures at 1 and 7 days post resection (dpr),[10] suggesting that cardiac myocyte fate changes during neonatal mouse heart regeneration. However, the process of cardiac myocyte regeneration has not been elucidated at the transcriptional level in mammals. Thus, we used the transcriptional signature derived from the in vitro differentiation, in vivo maturation and ex vivo culture models to characterize the molecular events that occur in the neonatal mouse heart during regeneration.

We analyzed transcripts by RNAseq from the resected hearts 1 and 7 dpr and compared these data to gene expression profiles measured from hearts of sham-operated animals to determine differential gene expression during regeneration. The biological processes modulated during these two time points are strikingly different, with only a small percentage of differentially expressed genes common at both time points (Figure 3A). At 1 dpr, genes with roles in inflammatory response and wound healing processes were primarily up-regulated reflecting an acute infiltration of immune cells. Conversely, at 7 dpr, cell cycle genes were activated and sarcomere and heart contractility-related genes showed lower expression, consistent with a transition of cardiac myocytes to a less mature state (Figure 3A, Online Figure ID).

We next compared the expression of the 2,253 genes inversely expressed during differentiation and ex vivo culture (see Figure 2C) with those genes that showed significant change during regeneration. In total, 22% (14.8% down-regulated and 23.1% up-regulated) of all differentially expressed genes at 7 dpr significantly overlapped with this core gene set ($p=0.002$), whereas differentially expressed genes at 1 dpr did not significantly overlap these genes (Figure 3B), suggesting that a transcriptional reversion occurs by 7 dpr in the regenerating neonatal mouse heart. Genes found only differentially expressed during regeneration included genes related to inflammatory response (e.g. Cxcl15 and Cxcl13) and adhesion molecules (e.g. Fn1 and Cldn18) (Online Table III). These may provide important insight into regenerative specific factors.

We next developed a metric to assess whether differentially expressed genes at each time point relative to sham controls were associated with genes driving a differentiated or un-differentiated state (Figure 3C). Genes that changed during in vitro differentiation, in vivo maturation and ex vivo explant cardiac myocyte data sets were classified according to how strongly they were expressed at different stages of differentiation (y-axes in Figure 3C, see Experimental Procedures). Genes up-regulated at 7 dpr showed a significant shift toward a less differentiated state, with the strongest shift occurring toward the P0 neonatal state. In contrast, genes down-regulated at 7 dpr displayed the opposite trend, largely shifting towards genes that defined a more differentiated state. Moreover, genes expressed at 7dpr more closely resembled a less differentiated state than at 1dpr.

The majority of genes up-regulated during ex vivo culturing of cardiac myocytes that showed higher expression in the regenerating mouse heart have functions in cell cycle, mitosis, and RNA processing, consistent with the increased cardiac myocyte cell cycle activity in the regenerating mouse heart [10] (Online Figure III A). Genes up-regulated during differentiation and down-regulated in the explant model that were also significantly down-regulated during regeneration included titin-associated genes (e.g. Fhl2, Ank1) (Online Figure III B), calcium regulating genes (e.g. Dmpk, Asph and Hrc) (Online Figure III C) and transcriptional or translational regulators (e.g. Tsc22d3, Zbtb16). Titin and titin-associated genes are critical components of the sarcomere and it is thought that sarcomeres are physically disassembled during regeneration based on ultrastructure analysis; however, this process has not been characterized at the molecular level. Another cardiac myocyte specific gene, atrial natriuretic peptide (Nppa), showed significant increased expression in the regenerating heart. Nppa is a secreted protein involved in heart development and is re-

activated upon heart injury such as myocardial infarction [22] suggesting a functional role for this developmental gene during heart injury. Our data suggests a transcriptional reversion consistent with a change in cardiac myocyte fate to an immature state occurs during mammalian heart regeneration.

Neonatal mouse cardiac regeneration involves a transcriptional reversion in isolated cardiac myocytes

Given the potential for cell heterogeneity, we isolated cardiac myocytes (iCMs) from whole hearts and compared these data to our gene expression analysis in ventricular samples. Myocytes were specifically isolated from neonatal hearts (P0 and P4) and adult cardiac myocytes resulting in ~95% purified cardiac myocytes. We analyzed differentially expressed genes in these samples and used Ingenuity Pathway Analysis to map gene networks that dynamically change over the course of cardiac myocyte maturation from P0 to Adult. Consistent with a more proliferative state, many of the top differentially expressed gene networks in P0 iCMs relative to those isolated from adult hearts involved cell cycle, cellular assembly and organization, DNA replication, and recombination and repair (Online Figure IV). Importantly, differentially expressed genes in whole ventricular P0 tissue relative to adult heart samples overlapped the majority of genes (51 out of 70) present in the network generated from iCMs data sets (Online Figure IV outlined in pink). We observed an up-regulation of representative sarcomere and mitochondrial genes and down-regulation of cell cycle genes in P0 relative to adult iCMs, similar to the patterns observed in whole heart tissue as displayed in figure 1 (Online Figure V A). Principal component analysis of iCMs at P0 and P4 was highly similar to results obtained using ventricular heart tissue. Additionally, hierarchical clustering demonstrated that gene clusters up-regulated during differentiation and maturation (i.e. oxidation reduction) were down-regulated during in vitro explantation and genes down-regulated during differentiation and maturation (i.e. cell cycle) were activated upon explantation (Online Figure V B,C). These analyses demonstrate that the core transcriptional changes are replicated between ventricular samples and purified cardiac myocytes, suggesting that the transcriptional changes are largely specific for cardiac myocytes.

We next isolated cardiac myocytes at 7 days post resection from hearts of mice that had undergone sham or resection at 1 day of age to further test the hypothesis that genes differentially expressed in regenerating myocytes revert to a less differentiated state. Genes up-regulated in regenerating iCMs at 7 dpr showed a shift toward a less differentiated state compared to sham iCMs, with the strongest shift occurring toward the P0 neonatal state. Genes down-regulated at 7 dpr displayed the opposite trend specifically shifting towards genes that defined a more differentiated state in the in vitro differentiation dataset (Online Figure V D). Of interest, atrial natriuretic peptide was also significantly elevated in iCMs during regeneration suggesting a strong candidate factor involved in the myocyte response during regeneration. While some differentially expressed genes (i.e. down-regulation of sarcomere related genes) were not robustly reproduced in the iCMs datasets (likely due to the sensitivity of day 7 mouse hearts to the digestion and purification process), our data support the conclusions derived from the sham and resected ventricle tissue that genes

turned on in regenerating myocytes shift to a less differentiated state at the transcriptional level.

Identification of candidate regulators of cardiac myocyte cell state

We hypothesized that by examining genes differentially expressed in each of our data sets we could predict factors that regulate cardiac myocyte cell cycle state. Differentially expressed genes from in vitro and in vivo differentiation, ex vivo culture, and 7 dpr regeneration datasets were selected as seed genes to predict upstream regulators using Ingenuity Pathway Analysis (www.ingenuity.com). In total, 150 factors were predicted to be upstream regulators of differentially expressed genes in all four of the datasets (Online Table IV). To test whether putative upstream regulators play a role in modulating cell cycle state, we investigated the impact of the exogenous addition of growth factors and cytokines predicted in our analysis on the capacity of cultured neonatal mammalian cardiac myocytes to undergo cell cycle activity. Of the 15 growth factors and cytokines predicted as upstream regulators (Figure 4A), two factors, Neuregulin 1 (Nrg1) and Oncostatin M (OSM), are known to play a significant role in inducing cardiac myocyte cell cycle activity. Nrg1 induces cell division of mature mammalian cardiac myocytes via ErbB4 receptor signaling and protects against myocardial infarction scar formation in vivo.[23] Cardiac myocytes exposed to the cytokine OSM undergo differentiation reversion characterized by loss of sarcomere structure and re-expression of alpha smooth muscle actin.[24] In vivo, OSM signaling results in a dilated cardiomyopathy phenotype. These studies demonstrate that our systematic approach successfully identified factors with critical functions in regulating cardiac myocyte cell cycle activity or differentiated state, leading us to examine other candidates from our analysis.

We screened the effect of several of the candidate cytokines and growth factors on cardiac myocyte DNA synthesis. Exposure of cultured neonatal rat cardiac myocytes to Nrg1, IL3, IL13, CTGF, and Fgf1 significantly stimulated DNA synthesis as detected by ^{3}H-thymidine incorporation (Figure 4B). Among these, Interleukin-13 (IL13) most consistently stimulated DNA synthesis in a dose dependent manner (data not shown). Importantly, IL13 was also predicted to be an upstream regulator of differentially expressed gene networks in the iCMs from resected versus sham mice at 7 days post resection (Online Figure V E). Immunohistochemistry confirmed that cardiac myocytes exposed to IL13 had significantly increased DNA synthesis indicated by BrdU incorporation (Figure 4C). Furthermore, IL13 treated cardiac myocytes showed almost a two-fold increase in Ki67 (Figure 4D) and increased nuclear pH3 expression. The number of neonatal rat cardiac myocytes appearing to undergo cell cycle entry as determined by absence of clear sarcomere structures and strong pH3 staining in the nucleus (Figure 5A right panel) was significantly higher in IL13 treated cells compared to control cells (Figure 5A,B). Furthermore, cardiac myocytes with absence of clear sarcomere structures stained positive for aurora b kinase (Figure 5C), a marker of cell cycle entry. 24 hours post IL13 treatment a significantly higher percentage of total cultured cells stained positive for cardiac troponin T compared to untreated cells (Figure 5D) suggesting that IL13 preferentially induces either proliferation or survival of cardiac myocytes compared to other cell types (such as fibroblasts). Collectively, these data suggests that IL13 stimulates cardiac myocyte cell cycle entry in vitro. IL13 is a cytokine

secreted primarily by activated T_H2 immune cells suggesting that inflammatory factors could play an important role in initiating cardiac myocyte cell cycle activity during heart regeneration via IL13 signaling.

IL13 signals through IL4R/IL13Ra1 in cardiac myocytes

We next sought to elucidate the IL13 signaling mechanism in cardiac myocytes. IL13 can transmit signal to target cells through the IL4Ra/IL13Ra1 receptor heterodimer or through IL13Ra2.[25] We compared expression of these three receptors across whole mouse tissues that are known to respond to IL13, including lung, skeletal muscle, and brain. IL4Ra is expressed at similar levels in all tissues examined but showed highest expression in the lung (Figure 6A) whereas IL13Ra1 demonstrated significantly higher expression in the heart compared to other tissues examined (Figure 6B). We found that both IL4Ra and IL13Ra1 are expressed in cultured neonatal rat cardiac myocytes whereas IL13Ra2 transcripts could not be detected (data not shown), suggesting that IL13 signals on cardiac myocytes via the IL4/IL13Ra1 receptor heterodimer. Knockdown of IL4Ra by siRNA slightly but significantly diminished cardiac myocyte ^{3}H-thymidine incorporation in response to IL13 exposure, whereas knockdown of IL13Ra1, or both IL13Ra1 and IL4Ra, substantially decreased ^{3}H-thymidine incorporation to baseline levels (Figure 6C, Online Figure VI). Thus, IL13Ra1, and to a lesser extent IL4Ra, plays a significant role in mediating the DNA synthesis response to IL13 signaling.

STAT6 and STAT3/periostin mediate the IL13 response in cardiac myocytes

A recent study demonstrated Jak1/STAT3 signaling is critical for myocyte proliferation during zebrafish heart regeneration.[12] In response to IL13 stimulation and binding IL4Ra/IL13Ra1, TYK2 activates STAT1 and STAT6, while JAK2 signaling is upstream of STAT3 activation.[26] While STAT6 expression remained constant, IL13 treated cardiac myocytes demonstrated visibly pronounced STAT6 phosphorylation as early as 15 minutes after treatment (Figure 7A). STAT6 phosphorylation decreased from 30 minutes to 24 hours after IL13 exposure. On the other hand, STAT3 phosphorylation was delayed, appearing by 3 hours and increasing at 24 hours post IL13 treatment. We investigated the upstream regulators of differentially expressed gene networks to examine the possibility that STAT6 or STAT3 signaling regulates gene expression in the regenerating mouse heart. STAT3 (Activation score 2.621, $p=7.67x10^{-7}$) was among the 22 transcription factors predicted to significantly activate differentially expressed gene networks during heart regeneration at 7 dpr (Online Table V). Although STAT3 RNA expression did not change during regeneration, STAT3 protein levels were significantly increased in resected hearts compared to sham operated hearts at 1 dpr (Figure 7B).

Delivery of recombinant periostin has been shown to induce cell cycle re-entry in differentiated, mononucleotide mammalian cardiac myocytes in vivo.[27] Interestingly, periostin expression has been used as a marker of IL13 activity in vivo[28] and its expression is regulated by STAT3 activation.[29] Given its role in heart regeneration, we hypothesized that periostin mediates the cardiac myocyte response downstream of the IL13/STAT3 pathway. IL13 exposure stimulated expression of periostin by 24h post treatment in cultured cardiac myocytes (Figure 7C). In vivo, periostin expression shows a trend towards up-

regulation during neonatal mouse heart regeneration 7 dpr, however this difference is not statistically significant (data not shown). Notably, siRNA knockdown of STAT3 in neonatal rat cardiac myocytes decreased periostin expression (Figure 7C), demonstrating that STAT3 mediates expression of periostin in cultured cardiac myocytes. Moreover, both periostin and STAT6 knockdown diminished the DNA synthesis phenotype induced by IL13 (Figure 7D). Our data demonstrate that IL13 induces cardiac myocyte specific expression of several factors involved in the regeneration response including STAT3 and periostin.

DISCUSSION

Heart regeneration in lower organisms as well as in the neonatal mouse has been proposed to occur by proliferation of pre-existing cardiac myocytes.[10, 30] This assumption has been based primarily on observations of loss of clear sarcomeres at the ultrastructural level and expression of cell cycle entry markers such as phosphorylated histone H3; however, the molecular changes that precisely characterize this process have not been studied in detail. We identified a core transcriptional signature of cardiac myocyte differentiation, and found that explanted adult cardiac myocytes cultured over several days demonstrate a transcriptional reversion of the differentiation process. Similarly, our data show that regenerating neonatal mouse heart 7 dpr appear to undergo a transcriptional reversion following apical resection.

Although cardiac myocytes account for the majority of total myocardial mass (between 60–80% depending on the developmental stage[31]), numerous additional cell types may also contribute to the transcriptional profiles obtained via RNA profiling in ventricular tissue and some transcriptional changes may be due to changes in cell type composition. To address this confounding factor, we profiled in vitro systems that specifically model a transient cardiac myocyte state (in vitro differentiation and adult cardiac myocyte explants) to identify expression changes specific to cardiac myocytes during the differentiation and loss of differentiation processes. This method of profiling multiple model systems increases the likelihood that transcriptional changes observed across all four models represent a change in myocyte cell state. To further verify these findings, we profiled purified cardiac myocytes over the same time course during maturation (P0 to adult) and in the regenerating neonatal mouse heart 7 dpr. These datasets support our conclusions that cardiac myocyte regeneration involves a transcriptional reversion of maturation.

Our work suggests that transcriptional alteration of a specific subset of genes that regulate sarcomere organization, RNA processing, and cell cycle progression is likely critical for remodeling the terminally differentiated program of cardiac myocytes. Several genes involved in sarcomere organization and function (e.g. Smyd1, Fhl2, Ankrd1, Tcap, and Ttn) showed increased and sustained expression during differentiation and were suppressed during myocyte explantation and at 7 dpr during heart regeneration. Consistent with our findings, experimental evidence demonstrates that diminished expression of Smyd1b, a methyltransferase involved in sarcomere organization that we found down-regulated during regeneration,[32] causes significant disruption of myofibril organization in zebrafish cardiac muscles.[33] Additional studies will be required to determine if modulating the expression of these genes influences the proliferative state of cardiac myocytes.

Accumulating evidence suggests that cytokines and growth factors trigger regeneration in other cell types such as skeletal muscle,[34] optic nerve,[35] and hepatocytes[36, 37]. Thus, we were particularly interested in identifying novel cytokines and growth factors that could initiate cell cycle entry of cardiac myocytes. Our systematic approach identified Nrg1 and OSM, two factors demonstrated to induce cardiac myocyte proliferation and de-differentiation both in vitro and in vivo.[23, 24] The identification of Nrg1 and OSM establishes the validity of our model and suggests that other identified factors may also be physiologically relevant for inducing cardiac myocyte cell cycle activity. We found that IL13, a cytokine produced primarily by activated T_H2 cells, can activate cardiac myocyte cell cycle activity in vitro. IL13 acts on non-hematopoietic cell types such as smooth muscle cells, endothelial cells, and fibroblasts and can induce proliferation of cultured vascular smooth muscle cells.[39] Thus, we reasoned that IL13 could play a role in mediating cardiac myocyte cell cycle activity following an inflammatory response. We demonstrate that the IL4Ra/IL13Ra1 heterodimer mediates cardiac myocyte response to IL13. The reduction in DNA synthesis was most pronounced following IL13Ra1 knockdown, which approached the level of untreated cells. These data suggest that IL13Ra1 may play a key regulatory role in transducing IL13 signal compared to IL4Ra.

We demonstrate that IL13 induces myocyte cell cycle entry and the response to IL13 is mediated in part by the STAT3/periostin pathway. STAT3 activation is required for cardiac myocyte proliferation in response to injury in zebrafish[12] and has also been implicated in mammalian muscle regeneration as a critical mediator of myoblast proliferation.[40] Our analysis identified IL13 and STAT3 as regulators of differentially expressed gene networks during mouse heart regeneration and STAT3 protein expression was increased in the regenerating mouse heart, thus, IL13/STAT3 could be an initiating factor in mouse cardiac regeneration. Periostin delivery has been shown to stimulate myocyte proliferation and reduced injury size and fibrosis following experimental myocardial infarction.[27] We found periostin expression to be mediated by STAT3 expression in cultured cardiac myocytes, thus, it is plausible that IL13 initiates STAT3 and periostin induction to facilitate mouse heart regeneration.

To date, STAT6 signaling has not been described in the context of heart regeneration. Our data demonstrate that STAT6 mediates IL13 signaling in cardiac myocytes in vitro, however, we did not find compelling evidence that STAT6 plays a role in heart regeneration in vivo. In isolated cardiac myocytes IL13 induced rapid STAT6 phosphorylation that quickly diminished, whereas STAT3 phosphorylation was strongly detected 24 hours after IL13 exposure. We postulate that STAT3 activity persists several days after the injury event that initiates regeneration, whereas STAT6 signaling is transient and therefore undetectable in the days following resection. Further experiments will be required to determine if STAT6 signaling mediates heart regeneration processes. Together, this work demonstrates that our approach to identify the core transcriptional changes that occur during cardiac myocyte maturation and regeneration can be applied to gain a systems level understanding of the molecular factors that coordinate these critical processes.

CONCLUSIONS

Our study suggests that cardiac regeneration is not a stochastic loss of the mature cell state, but rather a direct transcriptional reversion of the differentiation process. We observed this trend in the in vitro cardiac myocyte explant model as well as during heart regeneration at 7 dpr. We demonstrate a proof of principal concept by which interrogation of high throughput RNA expression datasets elucidated novel pathways that stimulate in myocyte cell cycle activity, and importantly this signaling occurs during mammalian heart regeneration. Although post-transcriptional events such as transcription factor translocation to the nucleus are also critical mediators the regenerative response[41] our study provides a critical framework for understanding the transcriptional expression changes required for cardiac myocyte repair in response to injury that will be invaluable for ultimately guiding efforts to promote adult mammalian cardiac regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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Nonstandard Abbreviations and Acronyms

Dpr	Days post resection
ESC	embryonic stem cell
MES	mesoderm
CP	cardiac progenitor
CM	cardiac myocyte
pH3	phosphorylated histone H3
BrdU	Bromodeoxyuridine
P1	postnatal day 1
P4	postnatal day 4
P7	postnatal day 7
iCMs	isolated cardiac myocytes

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Novelty and Significance

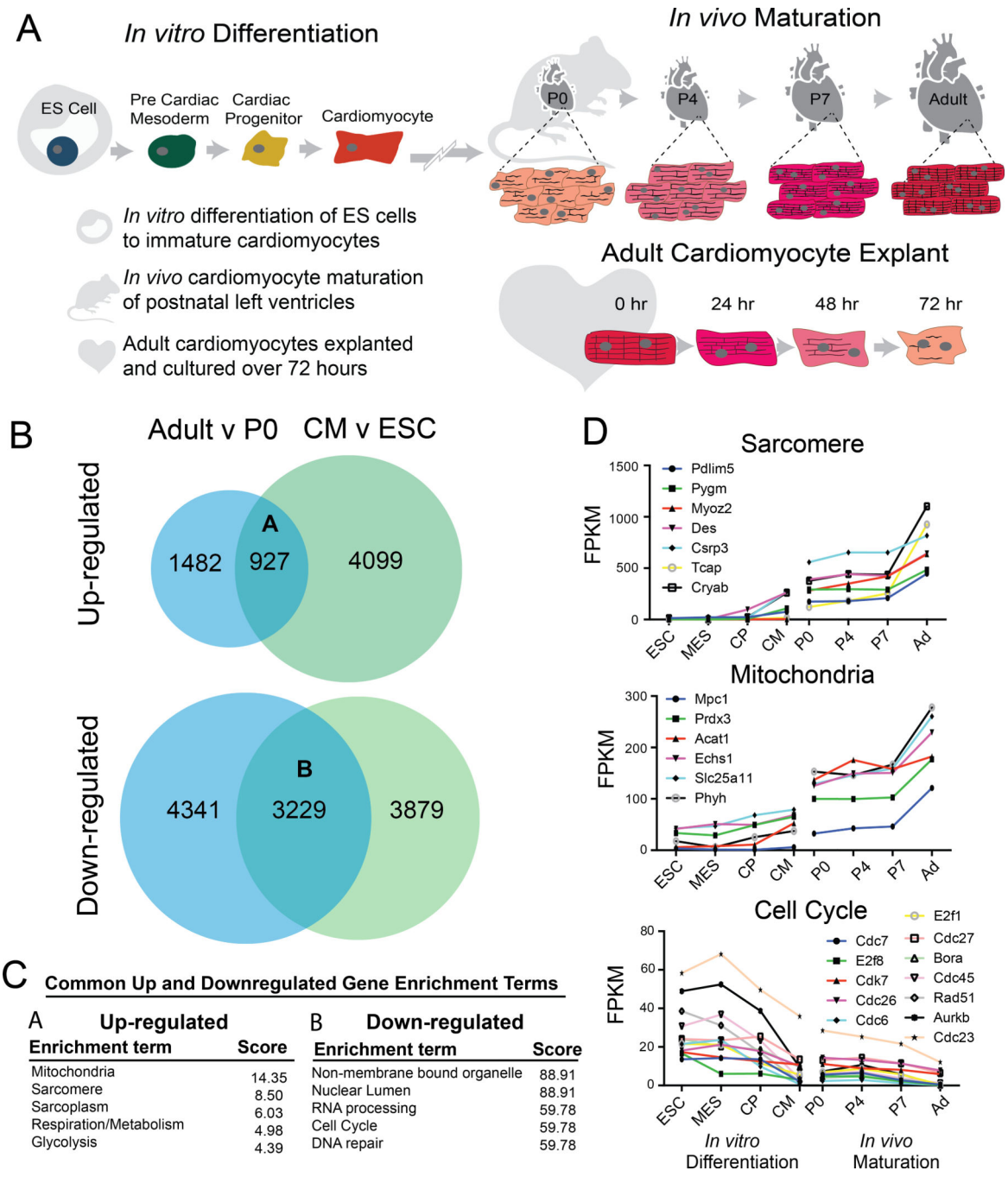
What Is Known?

- Neonatal mice have the capacity to regenerate their hearts within the first week of life.
- Mammalian heart regeneration is thought to occur by proliferation of existing cardiac myocytes yet the transcriptional changes that underpin this process have not been fully characterized at the molecular level.

What New Information Does This Article Contribute?

- The regenerating mouse heart reveals a transcriptional reversion of the cardiac myocyte differentiation processes.
- Systematic analysis of several models of cardiac myocyte differentiation and ex vivo culture identifies potential regulators of cardiac myocyte cell cycle activity and heart regeneration.
- Interleukin-13 is a novel upstream regulator of differentially expressed gene networks during regeneration and induces cardiac myocyte cell cycle entry.

Adult mammalian hearts have limited regenerative capacity following injury such as myocardial infarction, which is likely due to the inability of cardiac myocytes to undergo robust cell cycle activity. However, recent studies demonstrate that neonatal mice regenerate their hearts shortly after birth primarily by proliferation of existing cardiac myocytes. The goal of our study was to determine if regenerating mouse cardiac myocytes revert the transcriptional phenotype to a less differentiated and proliferative state during regeneration and subsequently identify regulators of this process. We identified differentially expressed genes upon injury-induced cardiac myocyte regeneration in mice and compared these to a core network of genes that change during models of in vitro and in vivo cardiac myocyte differentiation, and in vitro adult cardiac myocyte explantation. Regenerating mouse hearts display global expression patterns that begin to resemble immature neonatal cardiac myocytes at the transcriptional level, suggesting a transcriptional reversion of gene expression profiles during regeneration. We interrogated differentially expressed gene networks from all datasets to predict factors that might regulate cardiac myocyte cell cycle activity. Among others, Interleukin 13 induced cell cycle activity of cultured cardiac myocytes. Our work reveals new insights into the transcriptional regulation of mammalian cardiac regeneration and provides the founding circuitry for identifying potential regulators for stimulating heart regeneration.



C **Common Up and Downregulated Gene Enrichment Terms**

A **Up-regulated**

Enrichment term	Score
Mitochondria	14.35
Sarcomere	8.50
Sarcoplasm	6.03
Respiration/Metabolism	4.98
Glycolysis	4.39

B **Down-regulated**

Enrichment term	Score
Non-membrane bound organelle	88.91
Nuclear Lumen	88.91
RNA processing	59.78
Cell Cycle	59.78
DNA repair	59.78

Figure 1. In vitro differentiation and in vivo maturation models share a core transcriptional signature

A) The cardiac myocyte differentiation and explant models selected for RNA sequencing include the four stages of directed differentiation from embryonic stem cell (ESC) to mesoderm (MES), cardiac progenitor (CP), and cardiac myocyte (CM); whole heart ventricle maturation from P0, P4, P7, and adult mice; and explanted adult mouse cardiac myocytes collected after 0, 24, 48, and 72 hours in culture. .B) Venn diagram of common or unique differentially expressed genes during in vivo and in vitro cardiac myocyte differentiation. C) Table of top gene enrichment terms for common up- or down-regulated

genes during differentiation. Letters A and B correspond to the region of the Venn diagram in Figure 1B. D) FPKM values of representative sarcomere, mitochondrial, and cell cycle genes significantly differentially expressed during in vitro differentiation and in vivo maturation.

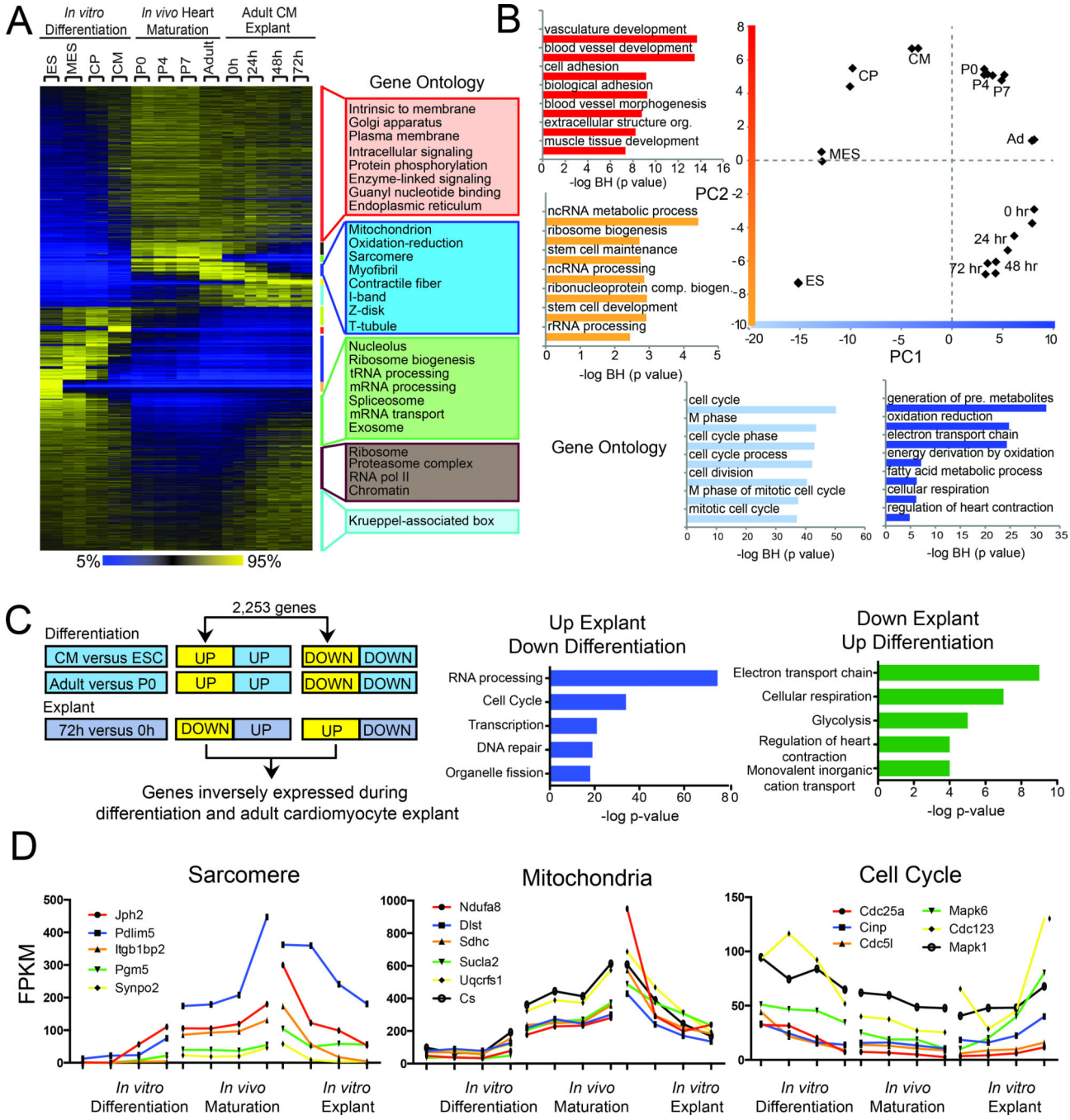


Figure 2. Explanted cardiac myocytes display a transcriptional reversion of differentiation

A) Hierarchical clustering of all expressed genes over the course of in vitro differentiation, in vivo maturation, adult cardiac myocyte (CM) explants. Biological process annotated by DAVID for selected gene clusters are provided. B) Principle component analysis of all genes differentially expressed during in vitro differentiation, in vivo differentiation, and adult cardiac myocyte explantation. ES= Embryonic stem cell, MES=Mesoderm, CP= Cardiac progenitor, CM= cardiac myocyte, P0, P4, P7 = postnatal day 0, 4, and 7 respectively, Ad=adult. BH= Benjamini Hochberg. Two replicates are presented for each

timepoint; the ES cell datapoints overlap. C) Prioritization scheme used to determine genes that may be critical to maintaining the stable state of mature cardiac myocytes. Yellow highlighting indicates genes whose expression is inverted during differentiation and explantation. Representative Gene Ontology (GO) terms of up- and down-regulated cardiac myocyte differentiation genes, which are defined as genes differentially expressed in the opposite direction during differentiation (in vitro and in vivo) and adult cardiac myocyte explantation. D) FPKM values of representative mitochondrial, sarcomere, and cell cycle genes significantly differentially expressed during in vitro differentiation, in vivo maturation, and adult cardiac myocyte explantation.

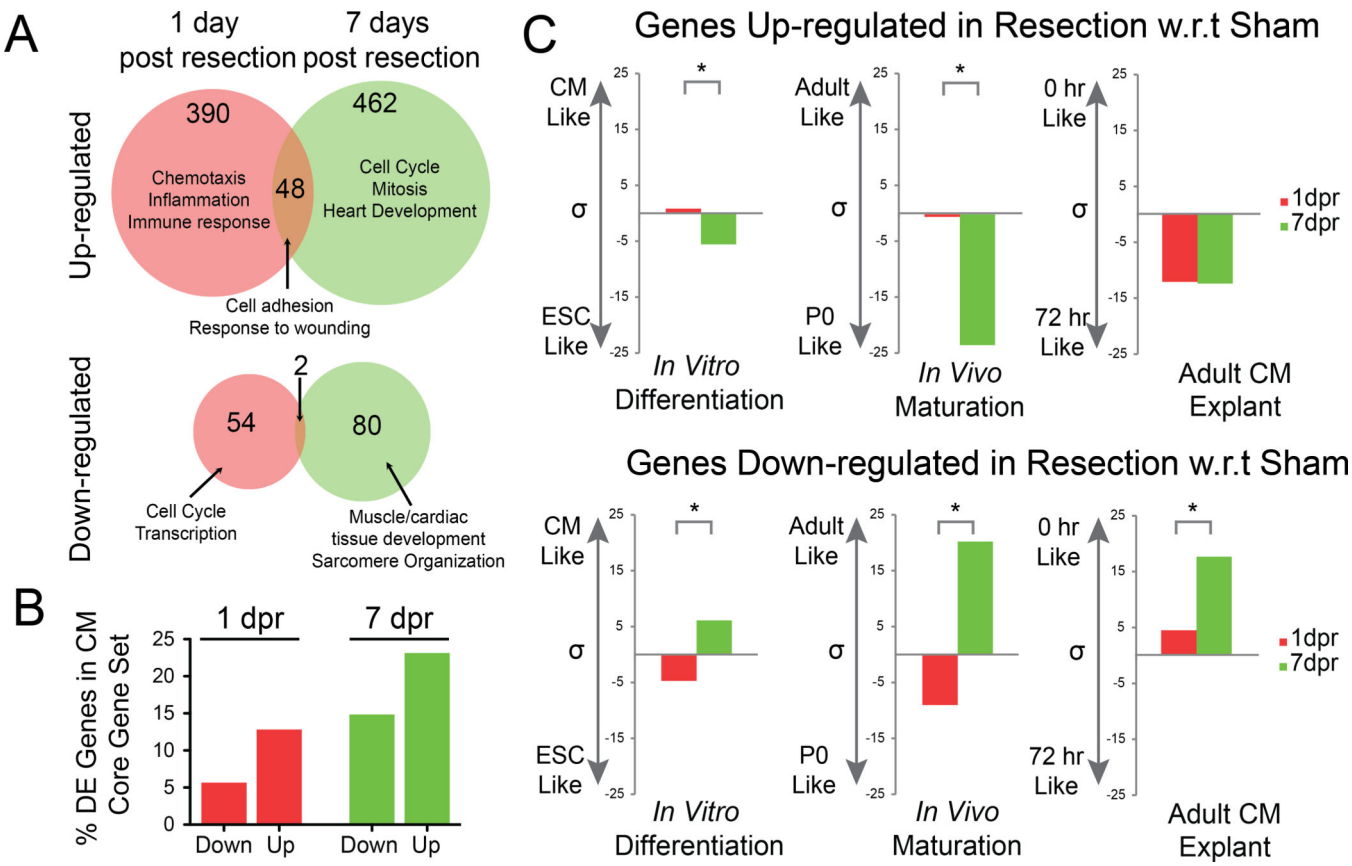


Figure 3. Neonatal mouse heart regeneration involves a transcriptional reversion of cardiac myocyte differentiation

A) Venn diagram of the genes differentially expressed in resected hearts versus sham at 1 and 7 days post resection (dpr). The number of genes differentially expressed are presented for each group along with representative biological processes related to each set of genes. B) Genes differentially expressed (DE) (up or down) during regeneration at 1 and 7 dpr were intersected with the set of genes inversely expressed during cardiac myocyte differentiation and adult cardiac myocyte explantation. In total 12% of differentially expressed genes at 1 dpr overlapped with our core set of cardiac myocyte stability genes established in Figure 2C, whereas 22% overlapped this gene set at 7 dpr. C) Z-transformed scores for the top 500 genes up-regulated/down-regulated in 1dpr and 7dpr samples as compared to a random background. Positive scores indicate the genes tend to be expressed in cells with a differentiated phenotype and negative scores indicate they are expressed in cells with an undifferentiated phenotype. Scores were computed separately for the in vitro differentiation, in vivo maturation and adult cardiac myocyte explant samples. w.r.t =with respect to.

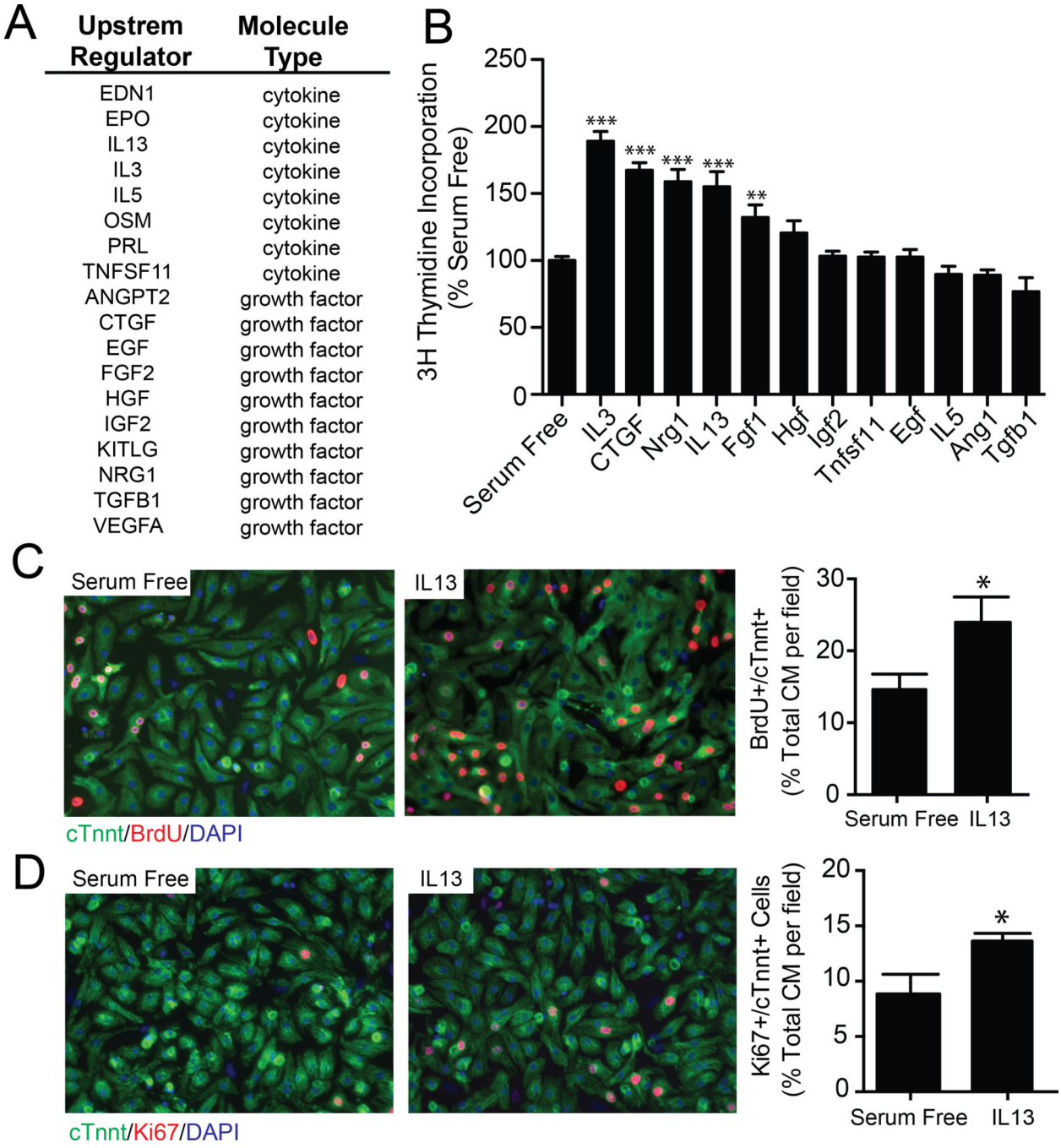


Figure 4. Systematic identification of factors that stimulate in vitro cardiac myocyte cell cycle activity

A) 18 growth factors and cytokines were predicted to be upstream regulators of the differentiated state of cardiac myocytes by Ingenuity network analysis. B) Cultured neonatal rat cardiac myocytes were exposed to twelve of the candidate growth factors and cytokines and cardiac myocytes were assessed for levels of DNA synthesis, as indicated by ^{3}H-thymidine incorporation. ^{3}H-thymidine incorporation is presented as percent change compared to serum free. N for each group is as follows; Serum Free=51, IL3=12 CTGF=12, Nrg1=12, IL13=12, Fgf1=12, Hgf=12, Igf2=13, Tnfsf11=13, Egf=12, IL5=18, Ang1=17 and

Tgfb1=12. Data were pooled from at least two replicated experiments for each factor. C) IL13 treated cardiac myocytes demonstrate significantly increased BrdU incorporation (n= 4 for each group). D) Neonatal rat cardiac myocytes exposed to IL13 demonstrate increased Ki67 expression. (N=4 for each group). All data is presented as mean ± SEM. *$p<0.05$ ** $p<0.01$, ***$p<0.001$ versus serum free.

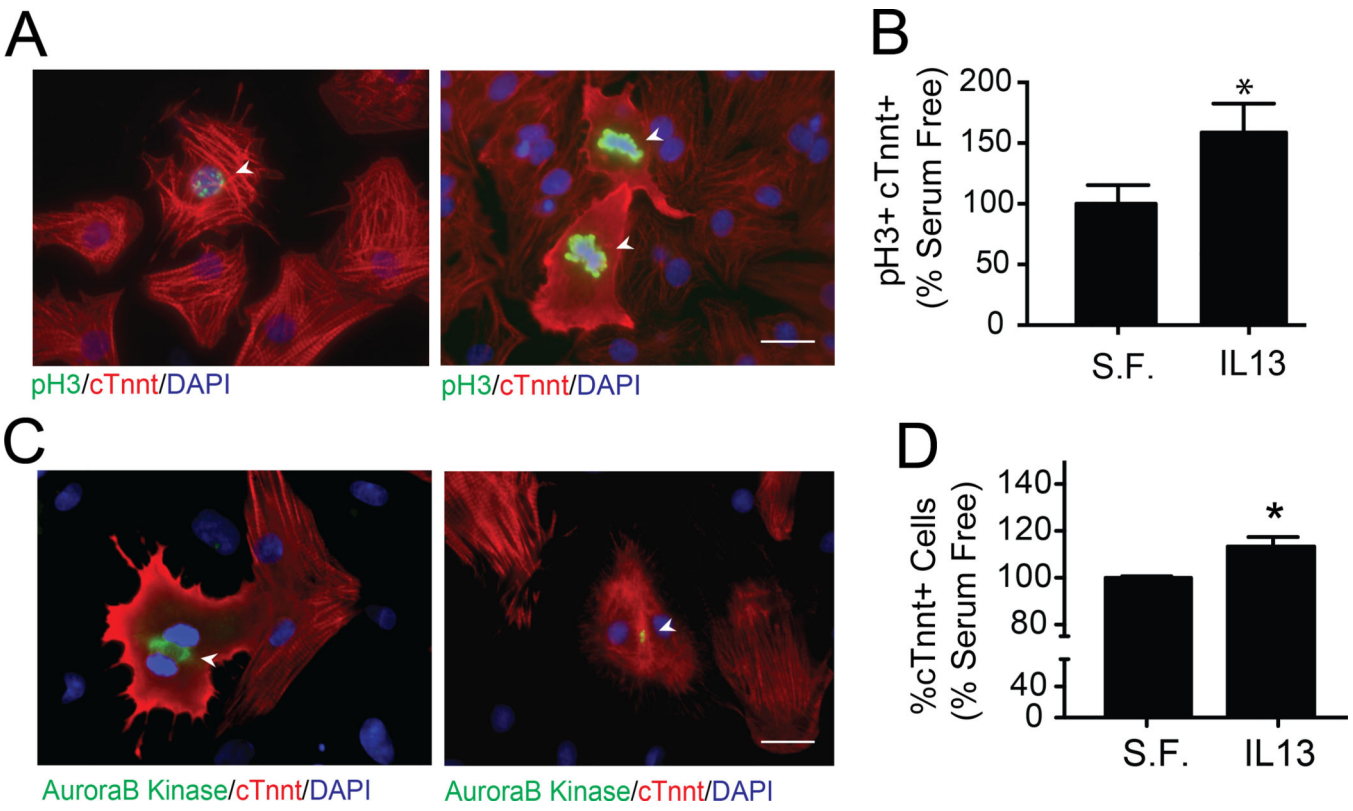


Figure 5. Interleukin-13 stimulates cardiac myocyte cell cycle entry

A) 60X representative images of pH3+ cardiac myocytes (left panel) and pH3+ cardiac myocytes undergoing apparent sarcomere disassembly (right panel). Scale Bar= 20 μM B) IL13 treatment significantly increased the number of pH3+ cardiac myocytes with absence of clear sarcomere structure at 48 hours post treatment relative to serum free treatment (n=8 per group). C) Cardiac myocytes with absence of clear sarcomere structures stained positive for aurora B kinase. Scale bar= 20 μM. D) Neonatal rat cardiac myocytes stained for cTNT were quantified by flow cytometry (n=3 per group). All data is presented as mean ± SEM. *p<0.05 ** p<0.01, ***p<0.001 versus serum free.

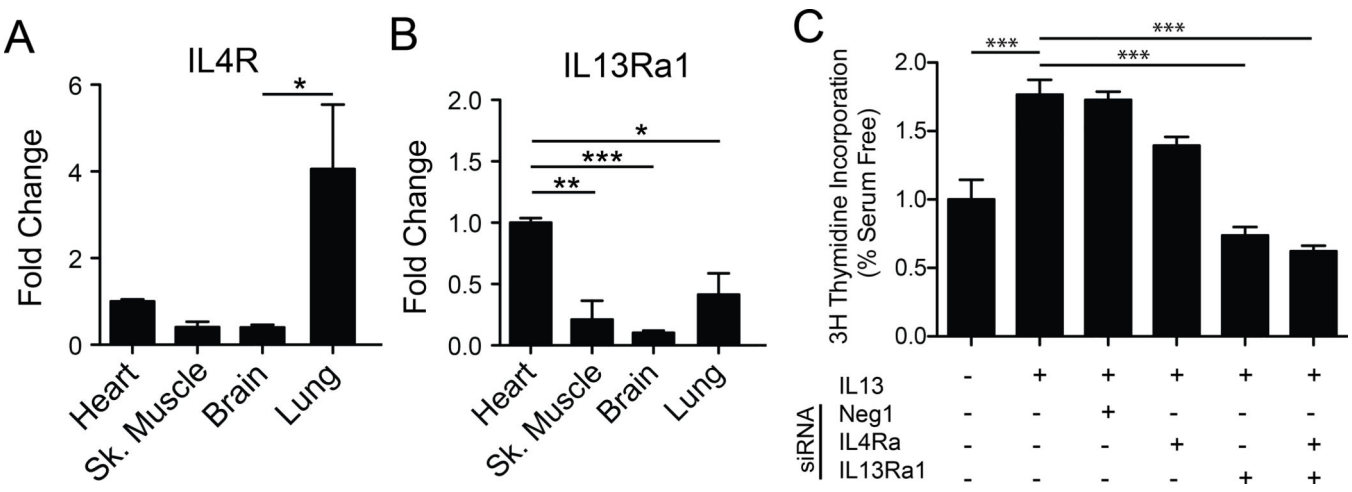


Figure 6. IL13Ra1/IL4Ra receptor heterodimer mediates IL13 proliferative phenotype in cardiac myocytes

RNA expression of A) IL4Ra and B) IL13Ra1 across adult mouse tissues that can to respond to IL13 signaling (n=4 per group). C) siRNA knockdown of IL4Ra and IL13Ra1 demonstrates DNA synthesis in response to IL13 is mediated through this receptor heterodimer (n=6–8 per group). All data is presented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus serum free.

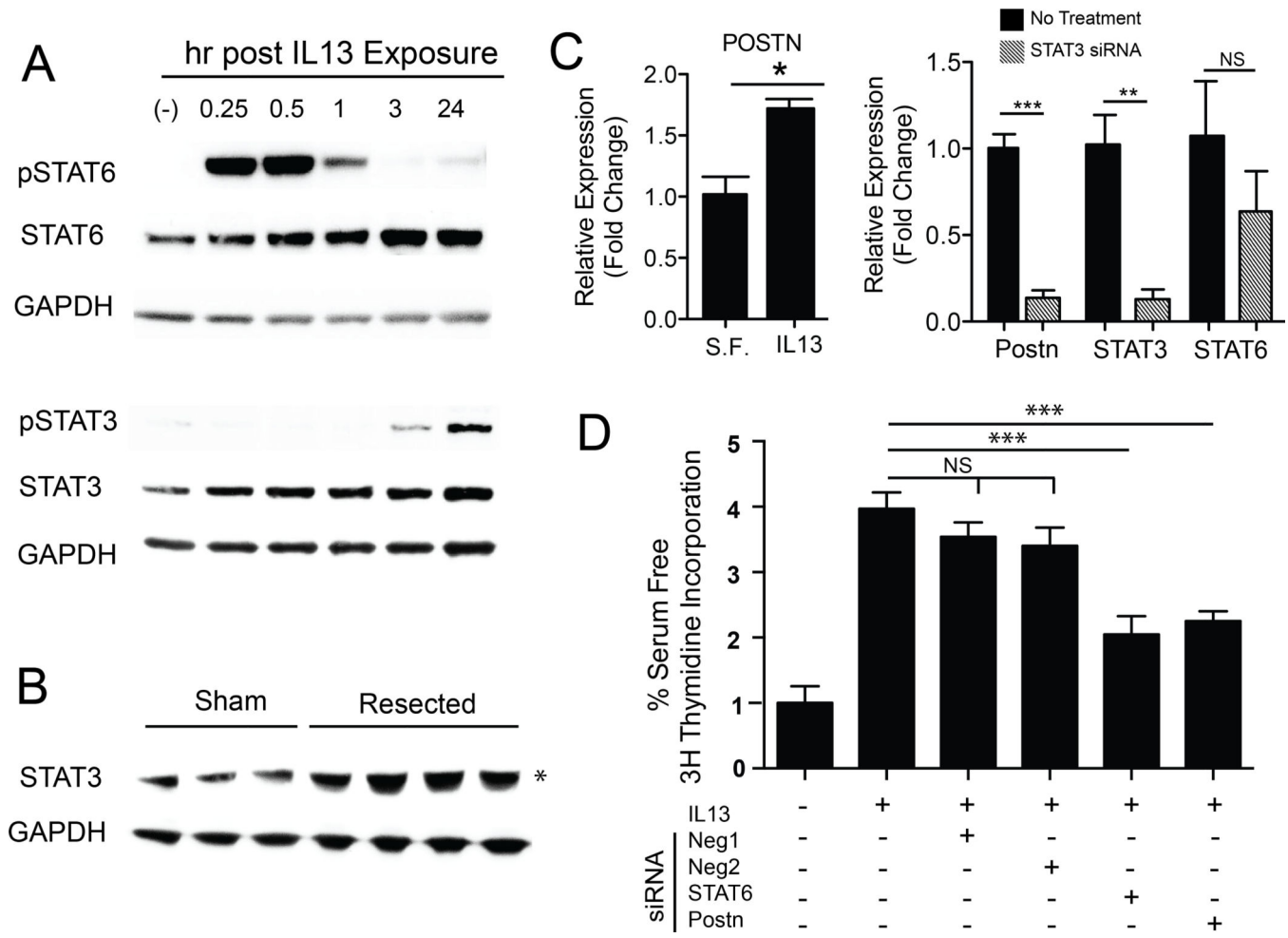


Figure 7. STAT phosphorylation and periostin mediate IL13 signaling in cardiac myocytes

A) STAT6 and STAT3 are phosphorylated in a biphasic manner in response to IL13 treatment B) STAT3 protein expression is significantly increased in regenerating mouse hearts compared to sham. C) Periostin RNA expression is induced by IL13 in isolated cardiac myocytes. (n=3 per group) and STAT3 knockdown by siRNA diminishes periostin RNA expression (n=3 per group) G) Periostin and STAT6 knockdown by siRNA diminishes DNA synthesis in isolated cardiac myocytes. (n=6–8 per group). All data is presented as mean ± SEM. *p<0.05, ** p<0.01, ***p<0.001 versus serum free.