Multicellular Transcriptional Analysis of Mammalian Heart Regeneration

BACKGROUND: The inability of the adult mammalian heart to regenerate following injury represents a major barrier in cardiovascular medicine. In contrast, the neonatal mammalian heart retains a transient capacity for regeneration, which is lost shortly after birth. Defining the molecular mechanisms that govern regenerative capacity in the neonatal period remains a central goal in cardiac biology. Here, we assemble a transcriptomic framework of multiple cardiac cell populations during postnatal development and following injury, which enables comparative analyses of the regenerative (neonatal) versus nonregenerative (adult) state for the first time.

METHODS: Cardiomyocytes, fibroblasts, leukocytes, and endothelial cells from infarcted and noninfarcted neonatal (P1) and adult (P56) mouse hearts were isolated by enzymatic dissociation and fluorescence-activated cell sorting at day 3 following surgery. RNA sequencing was performed on these cell populations to generate the transcriptome of the major cardiac cell populations during cardiac development, repair, and regeneration. To complement our transcriptomic data, we also surveyed the epigenetic landscape of cardiomyocytes during postnatal maturation by performing deep sequencing of accessible chromatin regions by using the Assay for Transposase-Accessible Chromatin from purified mouse cardiomyocyte nuclei (P1, P14, and P56).

RESULTS: Profiling of cardiomyocyte and nonmyocyte transcriptional programs uncovered several injury-responsive genes across regenerative and nonregenerative time points. However, the majority of transcriptional changes in all cardiac cell types resulted from developmental maturation from neonatal stages to adulthood rather than activation of a distinct regeneration-specific gene program. Furthermore, adult leukocytes and fibroblasts were characterized by the expression of a proliferative gene expression network following infarction, which mirrored the neonatal state. In contrast, cardiomyocytes failed to reactivate the neonatal proliferative network following infarction, which was associated with loss of chromatin accessibility around cell cycle genes during postnatal maturation.

CONCLUSIONS: This work provides a comprehensive framework and transcriptional resource of multiple cardiac cell populations during cardiac development, repair, and regeneration. Our findings define a regulatory program underpinning the neonatal regenerative state and identify alterations in the chromatin landscape that could limit reinduction of the regenerative program in adult cardiomyocytes.

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Clinical Perspective

What Is New?

- A transcriptional resource of multiple cardiac cell populations including cardiomyocytes, fibroblasts, endothelial cells, and leukocytes in the neonatal and adult heart with and without myocardial infarction.
- Identification of several developmentally regulated and injury-responsive transcriptional networks associated with neonatal regenerative and adult fibrotic responses to injury.
- Adult cardiomyocytes and endothelial cells do not reactivate a neonatal proliferative program following myocardial infarction.
- Detection of epigenetic modifications associated with loss of regenerative capacity including chromatin compaction around cell cycle genes during postnatal cardiomyocyte maturation.

What Are the Clinical Implications?

- The mechanisms that mediate cardiac regeneration in the neonatal period and that govern loss of regenerative capacity during postnatal development are highly relevant to the clinical management of heart failure and are currently under intensive investigation.
- The present study provides a unique resource for the cardiovascular field by enabling comparative analyses of the regenerative (neonatal) versus nonregenerative (adult) state, thus enabling identification of potential therapeutic avenues for heart regeneration.
- Chromatin modifications in adult myocytes could restrict cardiac regenerative potential after birth and may need to be overcome to facilitate cell cycle reentry in adults.

Ischemic heart disease is the leading cause of death worldwide. Because of the adult heart's extremely limited capacity for regeneration, myocardial infarction (MI) causes irreparable damage to the heart, which often leads to heart failure.[1] Recent evidence suggests that there is a developmental basis for this lack of regenerative capacity in mammals. In contrast with adult mice, neonates can efficiently regenerate the heart following injury, but the capacity to regenerate rapidly diminishes during the first week of life.[2,3] As such, there is substantial interest in defining the hallmarks of the cardiac injury response in neonates and identifying underlying mechanisms that distinguish the neonatal regenerative response from pathological repair processes in adulthood.

A central feature of the cardiac regenerative response in neonatal mice is the activation of cardiomyocyte proliferation following injury.[2–4] Genetic studies in the mouse have identified cardiomyocyte proliferation as the primary source of regenerated cardiomyocytes,[2] which is similar to findings in adult zebrafish.[5,6] The developmental timing of regenerative arrest in rodents coincides with the postnatal window when most cardiomyocytes withdraw from the cell cycle and become terminally differentiated.[7] Accordingly, genetic mutants with defective cardiomyocyte proliferation fail to mount a full regenerative response following injury in the neonatal period.[3,8–10] Reigniting proliferation in the adult heart is being pursued as a promising regenerative strategy,[11] with recent studies augmenting adult cardiomyocyte proliferation rates through manipulation of growth factor receptors,[12] transcription factors,[13] microRNAs,[14] and the cell cycle machinery.[15] However, even with the most potent cardiac mitogens in these studies, cardiomyocyte proliferation rates fail to reach neonatal levels in the adult heart and are generally not sufficient to drive a full regenerative response.[16] As such, there is a fundamental need to define the core biological processes and mechanisms that govern cardiomyocyte proliferative capacity.

It is currently unclear whether these dichotomous responses of neonatal and adult cardiomyocytes to injury are attributable to intrinsic barriers within the myocyte, an absence of extracellular cues for regeneration, or a combination of both. Moreover, it remains unknown whether the neonatal regenerative response is attributable to activation of a specific injury-induced transcriptional program or whether it is a consequence of a developmentally permissive transcriptional state. Although a previous study by Haubner et al[4] identified transcriptional changes in cell cycle genes at day 10 following neonatal MI, their analysis was limited to whole-heart tissue and therefore lacked cellular resolution. The neonatal regenerative response following MI is complex and involves the engagement of multiple cell types following injury.[17] For example, inflammatory cytokines such as interleukin-6 and interleukin-13 signal to drive cardiomyocyte proliferation following neonatal cardiac injury.[18,19] Similarly, macrophages are required for regeneration in the neonate through paracrine effects that drive angiogenesis and cardiomyocyte proliferation following injury.[20,21] More recently, the importance of neural innervation of the heart has also become apparent, with emerging roles for growth factors that signal between parasympathetic nerves and cardiomyocytes during cardiac regeneration in adult zebrafish and neonatal mice.[22] Together, these findings suggest that heart regeneration is regulated by multiple cell types. How these complex relationships between different cellular compartments are established during mammalian development remains an important unanswered question in cardiac biology.

Given the multitude of different regulators of cardiac regeneration, we decided to globally examine the tran-

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scriptional networks driving cardiac injury responses in multiple cell types at different stages of development. Here, we provide a comprehensive transcriptional framework of the major cell populations within the regenerative (P1) and nonregenerative (P56) mouse heart with and without injury. Our analyses identify unique developmentally regulated and injury-induced transcriptional responses in cardiomyocytes, fibroblasts, endothelial cells, and leukocytes that govern distinct cellular behaviors following cardiac injury. Comparative analysis of the cellular transcriptomes showed that adult leukocytes and fibroblasts were characterized by a proliferative neonatal-like transcriptional state following MI, whereas adult myocytes and endothelial cells did not revert to a neonatal-like state. The myocyte and endothelial genes that failed to revert to a neonatal state were chiefly involved in cell cycle regulation. Through extensive epigenetic profiling of cardiomyocytes during postnatal development, we reveal a novel relationship between chromatin accessibility and transcriptional repression of cell cycle genes, which could limit reinduction of the neonatal regenerative program in the adult heart.

METHODS

Please refer to the online-only Data Supplement Methods for a full description of experimental procedures.

Reagents were purchased from Life Technologies unless otherwise indicated. For antibody manufacturer details please see online-only Data Supplement Table I. For polymerase chain reaction primers, please see online-only Data Supplement Table II.

Neonatal and Adult MI Surgeries

Neonatal and adult surgeries were performed as previously described[23] and are detailed in the online-only Data Supplement Methods. Ethical approval for neonatal and adult mouse procedures was obtained from The University of Queensland's Animal Ethics Committee (SBMS/101/13/NHMRC).

Neonatal Heart Cell Isolation

Neonatal CD-1 (ICR) mice were euthanized by decapitation 3 days after surgery. Hearts were then digested at 37°C under constant agitation with a cell stirrer for 30 to 40 minutes with 200 μg/mL Liberase DH (Roche) in neonatal cardiomyocyte digestion buffer (116.3 mmol/L NaCl, 20 mmol/L HEPES, 19 mmol/L NaH2PO4, 5.5 mmol/L glucose, 5.36 mmol/L KCl, 0.83 mmol/L MgSO4). Six ventricles were pooled per sample for cell purification and RNA sequencing (RNA-seq). See online-only Data Supplement Methods for a full description.

Adult Mouse Heart Cell Isolation

Adult CD-1 (ICR) mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazil (12.5 mg/kg) at day 3 postsurgery. Cardiac cells were dissociated by Langendorff reverse coronary perfusion with 200 μg/mL Liberase DH (Roche). See online-only Data Supplement Methods for a full description.

FACS for Purification of Nonmyocyte Fraction

Following cell extraction, the nonmyocyte fraction was resuspended in 100 μL of 5% bovine serum albumin/phosphate-buffered saline solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.8 mmol/L KH_2PO_4, 10 mmol/L Na_2HPO_4, and 75 μmol/L bovine serum albumin). Cell isolates were then incubated at 4°C for 20 minutes with CD90-APC (1:33, A14727, ThermoFisher), CD45-FITC (1:10, 130-102-778, Miltenyi Biotec), CD31-BV421 (1:33, 102423, BioLegend), and Podoplanin-PE/Cy7 (1:66, 127411, BioLegned) in 100 μL of 5% bovine serum albumin/phosphate-buffered saline. The stained cells were washed in 5% bovine serum albumin/phosphate-buffered saline solution and sorted using a BD fluorescence-activated cell sorting (FACS) ARIA cell sorter. Each purified cell population was centrifuged at 1000*g* for 5 minutes, cell media were aspirated, and 1 mL Trizol was added to isolate RNA.

RNA-seq of Enzymatically Isolated Cardiac Cell Populations

For enzymatically isolated cells, ribosomal RNA was depleted with Ribo Zero Gold (Illumina), RNA quality ascertained using a MultiNA bioanalyzer (Shimadzu), and cDNA generated with SuperScript II Reverse Transcriptase (ThermoFisher). Libraries were created with TruSeq Stranded Total RNA kits (Illumina) and read with HiSeq SR Cluster v4 kit (Illumina) on a HiSeq 2500 sequencer. Each sample contained ≈45 million 50-bp single-end reads.

Bioinformatics, Statistics, and Data Availability

See online-only Data Supplement Methods for a full description of bioinformatics and statistical analysis methods. Statistical analyses were performed using GraphPAD Prism 6 (Graphpad Software Inc) using 2-tailed unpaired *t* tests, with a *P* value of <0.05 considered significant. All data are displayed as mean±SEM unless otherwise indicated. For RNA-seq, differential expression analysis was performed with EdgeR, and the false discovery rate was controlled at 5% by using the Benjamini-Hochberg method. All data have been deposited at the Gene Expression Omnibus[24] under the accession numbers GSE95755 and GSE95764.

RESULTS

Isolation of Purified Cardiac Cell Populations From Infarcted and Noninfarcted Neonatal and Adult Mouse Hearts

Recent analyses of the cellular composition of the murine heart have revealed that fibroblasts, leukocytes, and vascular endothelial cells comprise the majority of nonmyocyte cell populations in the heart.[25] Of relevance to this study, each of these cell populations has been implicated in neonatal cardiac proliferative

or regenerative processes.[20,26] To perform transcriptional profiling of the different cardiac cell populations under regenerative versus nonregenerative conditions, we devised a strategy to isolate cardiomyocytes, fibroblasts, leukocytes, and vascular endothelial cells from regenerative neonatal (postnatal day 1; P1, online-only Data Supplement Figure I) or nonregenerative adult (postnatal day 56; P56) mice following MI or sham surgery (Figure 1A). Cardiomyocytes were immediately isolated for RNA extraction following differential density fractionation on a Percoll gradient for neonatal cardiomyocytes or low-speed centrifugation for adult cardiomyocytes (see Figure 1A and Methods). FACS was performed on the nonmyocyte fraction to isolate leukocytes ($CD45^+/CD31^-/CD90^{+/-}$), $CD90^+$ fibroblasts ($CD90^+/CD45^-/CD31^-$), and vascular endothelial cells ($CD31^+/CD45^-/Podo^-$) (Figure 1A). All cell types were viable (>90%) before RNA isolation (online-only Data Supplement Figure II). Consistent with recent findings,[25] the largest population of nonmyocyte cells from noninfarcted adult hearts were endothelial cells (51.8±4.7%) followed by $CD90^+$ fibroblasts (26.5±4.3%) and leukocytes (19.9±0.7%) (Figure 1B). Furthermore, 96.7±0.5% of all $CD31^+/CD45^-$ cells were vascular endothelial cells ($CD31^+/Podo^-$), whereas the remaining 3.3±0.5% were lymphatic endothelial cells ($CD31^+/Podo^+$) (Figure 1B), which is also in accordance with a recent report.[24] Vascular and lymphatic endothelial cells were separated because they exhibit differential physiological and transcriptional behaviors.[27] However, we did not sequence the lymphatic endothelial cells (CD31+/Podo+) or the negative population ($CD45^-/CD90^-/CD31^-$) because they yielded too little RNA for analysis using conventional RNA-seq pipelines. Therefore, we focused our RNA-seq analysis on the major cell types within the neonatal and adult heart (ie, cardiomyocytes, vascular endothelial cells, $CD90^+$ fibroblasts, and leukocytes).

As would be expected, infarcted neonatal and adult hearts had a proportional increase in leukocytes in comparison with noninfarcted controls (Figure 1B). As development progressed from neonatal to adult stages, steady-state (noninfarcted) leukocyte populations within the heart also increased as a proportion of the total number of nonmyocytes in the heart (Figure 1B). Conversely, the percentage of $CD90^+$ fibroblasts relative to other nonmyocyte populations in the heart decreased during development and was markedly reduced after adult infarction (Figure 1B). The percentage of $CD90^+$ fibroblasts in noninfarcted adult mice in this study (26.5±4.3%) is similar to the previously reported percentage of $CD90^+/CD45^-/CD31^-$ fibroblasts (≈30%) published by Ali et al.[28] Therefore, our findings are highly congruent with other recent reports and suggest that endothelial cells are the most numerous nonmyocyte population in the heart and that $CD90^+$ fibroblasts comprise ≈30% of nonmyocytes in the adult mouse heart.

Multicellular RNA-seq Reveals Distinct Transcriptional Programs in Different Cell Types During Cardiac Regeneration or Repair

To determine cell type–specific transcriptional programs deployed during cardiac development, repair, and regeneration, we performed RNA-seq on cardiomyocytes, $CD90^+$ fibroblasts, leukocytes, and vascular endothelial cells isolated from the neonatal and adult heart before or after MI. RNA-seq libraries were prepared from each cell type and provided exceptionally high-quality reads with a mean phred quality score of >35 and an average number of 46 million reads per sample. Analysis of cell type–specific transcripts confirmed that our purified cell populations were highly enriched for cell-specific gene markers (Figure 1C). For example, myosin light chain 7 (*Myl7*) and myosin light chain 2 (*Myl2*) were specifically expressed in the cardiomyocyte fraction and were highly enriched at neonatal and adult stages, respectively (Figure 1C). The nonmyocyte populations were also highly pure. $CD90^+$ fibroblasts, leukocytes, and endothelial cells were enriched for the marker genes Discoidin domain–containing receptor 2 (*Ddr2*), epidermal growth factor–like module-containing mucin-like hormone receptor-like 1 (*Adgre1*) and Von Willebrand factor (*Vwf*), respectively (Figure 1C). Other marker genes also exhibited cell type–specific enrichment (online-only Data Supplement Figure III). In total, >9964 differentially expressed genes were identified in at least 1 comparison within a cell type during development (ShP1.d3 versus ShP56.d3) or following MI (MIP1.d3 versus MIP56.d3; log2(fold-change) ≥1 or ≤ –1, false discovery rate ≤0.05). Hundreds of differentially regulated genes were identified within each cell population in neonatal versus adult hearts, with the clear majority of differences accounted for by the cardiomyocyte compartment (Figure 1D).

To visualize the transcriptional relatedness and reproducibility of each RNA-seq sample, we used principal coordinate analysis (Figure 2A). Each cell type within the RNA-seq data set grouped into a defined cluster within the principal coordinate analysis plot, and RNA-seq samples within each experimental group were also tightly clustered together (Figure 2A). Major transcriptional differences were apparent between neonatal and adult samples for all cell populations (in the absence of injury) (Figure 2A). Pearson correlations and unsupervised hierarchical clustering of RNA-seq samples further confirmed the high reproducibility of transcriptional signatures within groups (Figure 2B and 2C). It is striking that clustering of each sample also revealed that

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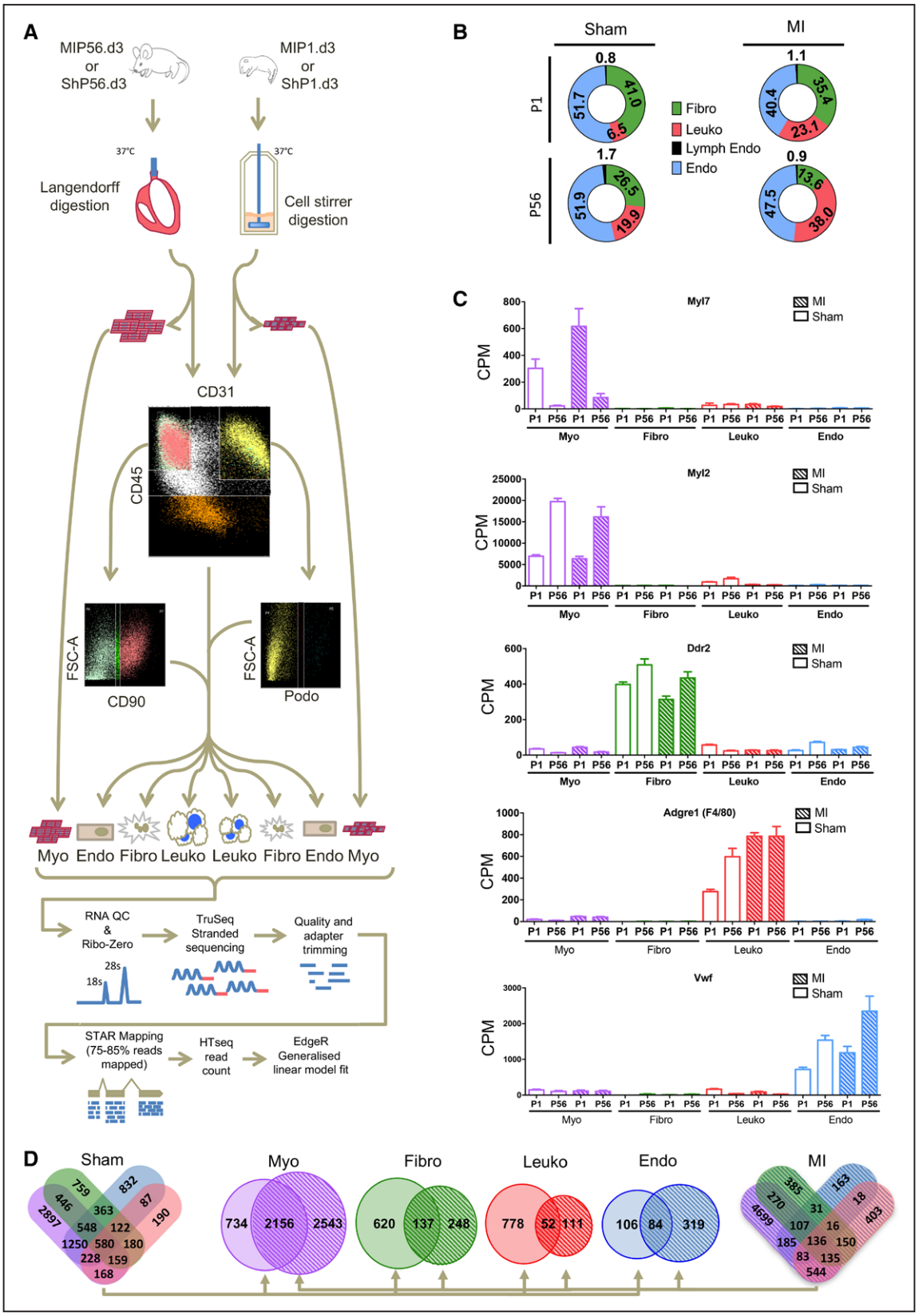

Figure 1. Isolation, sorting, and RNA-sequencing analysis of multiple cardiac cell populations during development and regeneration.

A, Schematic of cardiac cell–sorting strategy and RNA-sequencing pipeline. Myo (purple) indicates cardiomyocytes; Fibro (green), CD90$^+$ fibroblasts; Leuko (red), leukocytes; Endo (blue) , endothelial cells; ShP1.d3, sham surgery at P1 and collected at day 3 postsurgery; MIP1.d3, MI at P1 and collected at day 3 postsurgery; ShP56.d3, sham surgery at P56 and (*Continued*)

neonatal myocytes were more transcriptionally related to endothelial cells and leukocytes than they were to adult myocytes (Figure 2B and 2C). In fact, neonatal and adult myocytes were so transcriptionally distinct (6276 differentially expressed transcripts) that they could be considered as 2 distinct cell types.

As a further validation of our cell-sorting strategy, we performed unsupervised hierarchical clustering of RNA-seq samples followed by gene ontology (GO) analysis for each cluster. As would be expected, upregulated genes within each specific cluster were highly enriched for GO terms associated with that specific cell type (Figure 2C). For example, cluster 11 contained genes that were highly expressed in leukocytes, and this cluster was enriched for GO terms such as inflammatory response and T-cell activation (Figure 2C). Similarly, genes highly expressed in adult cardiomyocytes were contained within cluster 2, which was highly enriched for terms associated with heart development, regulation of heart contraction, and mitochondrial organization (Figure 2C). Clusters 8 and 9 contained genes that were highly enriched in CD90$^+$ fibroblasts and endothelial cells, respectively (Figure 2C).

Defining the Transcriptional Programs Governing Cellular Identity

Cellular identity is governed by a core transcriptional program, which is epigenetically cemented during development. We postulated that core cell identity programs similarly exist at both neonatal and adult stages. To test this, we isolated genes enriched for a particular cell type relative to other cell types in all conditions including infarcted and sham-operated neonates and adults. In these cellular identity data sets many transcription factors, signaling pathways, and gene ontologies classically associated with each cell type were highly enriched (Figure 3). For example, both neonatal and adult myocytes were highly enriched for transcriptional networks controlled by cardiogenic transcription factors, including *Nkx2-5*, *Gata4*, *Mef2c*, and *Mef2a*, and genes associated with cellular metabolic processes, as well (Figure 3A). Similarly, CD90$^+$ fibroblasts were enriched for extracellular matrix proteins (Figure 3A). Our data set revealed 3 overconnected transcription factors within the fibroblast identity network: *Nr2f1*, *Prdm5*, and *Zfp384*, which are all associated with extracellular matrix deposition and tumor suppression.[29–31] However, the relationship between these transcription factors and the cardiogenic fibroblast transcription factor network identified by Furtado et al[32] is currently unclear. The leukocyte-enriched transcription factors all play important roles in immune cells including B cells,[33] T cells,[34] dendritic cells,[35] monocytes,[35] and macrophages[35] (Figure 3A). We also identified immune cell subtypes that were present within the leukocyte fraction. Genes that were specifically enriched within leukocytes from each treatment group (ShP1.d3, ShP56.d3, MIP1.d3, and MIP56.d3) were isolated and compared with the leukocyte cell expression profiles available from the ImmGen compendium[36] (https://www.immgen.org/). This analysis indicated that the leukocyte fraction comprised predominantly dendritic cells, macrophages, monocytes, neutrophils, and eosinophils (online-only Data Supplement Figure IV). Endothelial cells were enriched for Notch and Vascular endothelial growth factors (Vegf), which are important regulators of angiogenesis.[37] Therefore, this data set provides a rich and highly predictive resource for interrogation of transcriptional circuits controlling cardiac cell identity, which could be leveraged for cell-reprogramming studies.

Adult Cardiomyocytes, Endothelial Cells, and Fibroblasts Deploy an Injury-Induced Transcriptional Program That Is Distinct From the Neonatal Injury Response

We next attempted to dissociate developmentally regulated transcripts from injury-induced gene expression changes within each cardiac cell population. Many differentially expressed genes between neonatal and adult MI (MIP1.d3 versus MIP56.d3) were also regulated during development in the absence of injury (ShP1.d3 versus ShP56.d3) (Figure 1D). This finding was also apparent in the GO analysis of these gene sets, with the majority of GO terms shared between both MI (P1 versus P56) and Sham (P1 versus P56) data sets (online-only Data Supplement Figure VA and VB). Strikingly, although all neonatal cell types were highly transcriptionally active (online-only Data Supplement Figure VA), when specifically isolating neonatal and adult cellular responses to MI (MIP1.d3 versus ShP1.d3 and MIP56.d3 versus ShP56.d3), we noted that neonatal cardiomyocytes, CD90$^+$ fibroblasts, and vascular endothelial cells failed to mount a robust transcriptional response to injury (Figure 4A). In contrast, adult cardiomyocytes, CD90$^+$ fibroblasts, and vascular endothelial cells activated a distinct injury-induced transcriptional program in response to MI (Figure 4B), while leukocytes displayed injury-induced transcriptional shifts following MI at both

Figure 1 Continued. collected at day 3 postsurgery; and MIP56.d3, MI at P56 and collected at day 3 postsurgery. **B,** Purity of isolated cardiac cell populations based on expression of known cardiac cell type–specific markers. n=4 per group. CPM indicates counts per million. **C,** Proportion of nonmyocyte populations in each group measured by fluorescence-activated cell sorting. **D,** Differentially expressed genes for each cell type between sham-operated and infarcted mice at neonatal or adult stages (ShP1.d3 versus ShP56.d3 and MIP1.d3 versus MIP56.d3).

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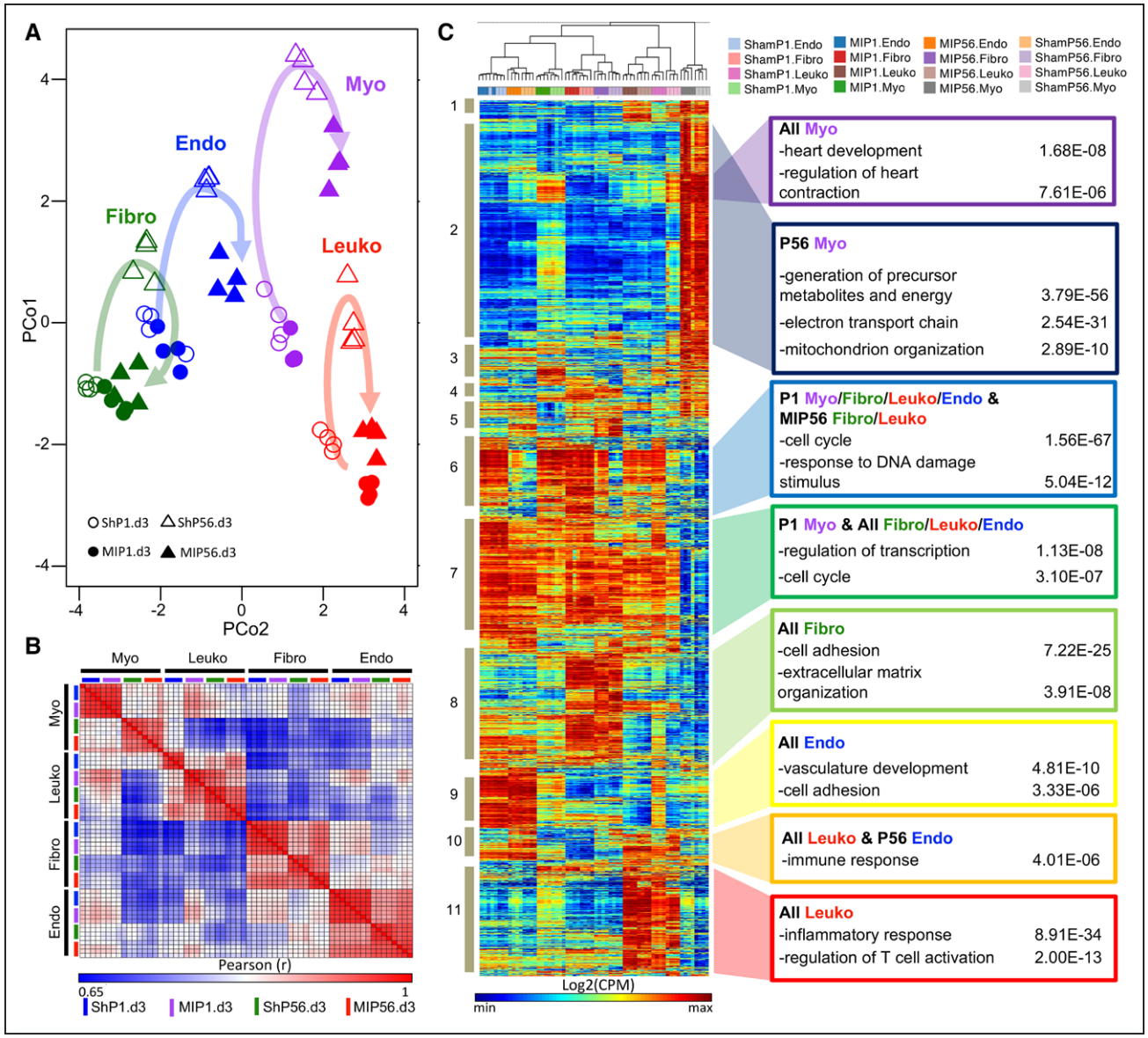

Figure 2. RNA-sequencing analysis reveals that cardiac cell types have distinct transcriptional behaviors during development and myocardial infarction.

A, Principal coordinate analysis for each cell population. The arrows emphasize the developmental transition from neonatal to adult stages in the absence of injury (ie, ShP1.d3 versus ShP56.d3) followed by their transition following myocardial infarction at P56. **B,** Pearson correlations between RNA-sequencing samples for each cell type. Blue indicates ShP1.d3; purple, MIP1.d3; green, ShP56.d3; and red, MIP56.d3. **C,** Hierarchical clustering of significantly regulated genes. Gene ontology analysis of cell-specific clusters reveals enrichment of cell-specific gene ontology terms. Headings in each box denote cell type–specific enrichment of each cluster. Statistical significance is represented by *P* value adjusted for multiple comparisons with a false discovery rate <0.05. Myo indicates cardiomyocytes; Leuko, leukocytes; Fibro, CD90$^+$ fibroblasts; and Endo, endothelial cells.

neonatal and adult stages (Figure 4). An important implication of this result is that neonatal cardiomyocytes, CD90$^+$ fibroblasts, and endothelial cells following MI are almost transcriptionally identical to their sham-operated controls (Figure 4A). In comparison, our analyses reveal enormous transcriptional differences between neonatal and adult cell types, with the vast majority of differences occurring during postnatal maturation (Figure 1, Figure 2B and 2C, online-only Data Supplement Figure V).

Defining Transcriptional Networks Governing Neonatal and Adult Cell States in the Heart

We next aimed to understand the cellular processes, signaling pathways, and transcriptional networks that distinguish adult from neonatal cellular states (ShP56.d3 versus ShP1.d3 or MIP56.d3 versus MIP1.d3). Consistent with enhanced cardiomyocyte proliferation in the

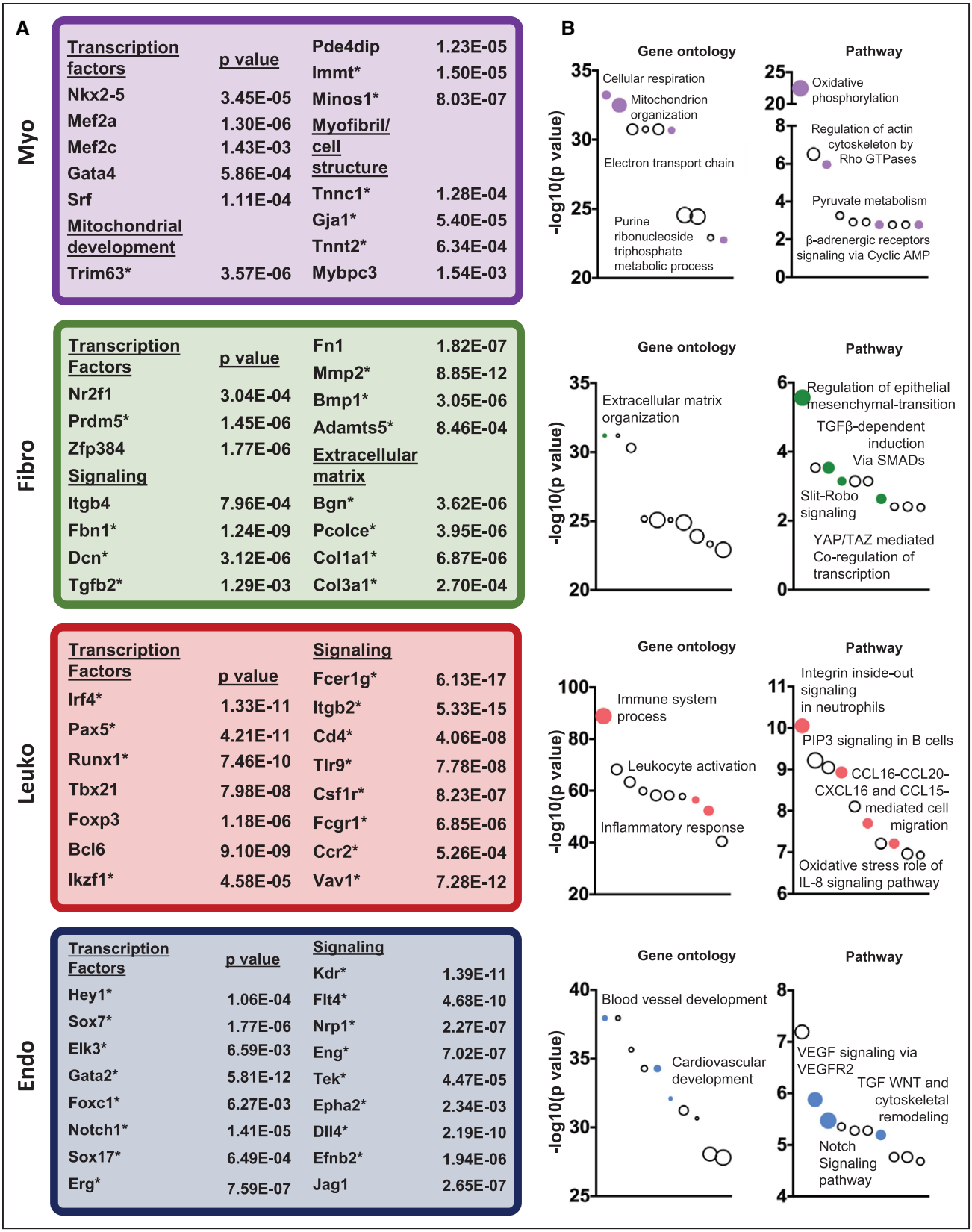

Figure 3. Identification of transcription factors and signaling pathways governing cardiac cell identity.
A, Representative subset of overconnected genes for each cell type. Each data set was collated by identifying genes specifically enriched in each cell type at all time points. MetaCore was used to identify core genes in each cellular data set. * denotes genes that were not only overconnected but also differentially expressed in the data set. **B,** Enriched pathways and (*Continued*)

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neonatal heart, neonatal cardiomyocytes were highly enriched for transcription factors and GO terms associated with cell cycle in both the sham-operated and infarcted states (online-only Data Supplement Figure V, Figure 5A). In addition, we constructed matrices of genes for well-known signaling pathways implicated in cardiac regeneration and development (online-only Data Supplement Figure VI). It is interesting to note that neonatal cardiomyocytes, endothelial cells, and CD90$^+$ fibroblasts were all highly enriched for a gene network associated with Wnt signaling following MI at P1 versus P56 (Figure 5B). The highest ranked transcriptional networks in neonatal cardiomyocytes were related to core cell cycle transcription factors (E2f1 and Foxm1), and the Wnt signaling pathway, as well (Figure 5B). Many pathways and transcription factors related to metabolism and mitochondrial organization were highly enriched in adult myocytes in the infarcted and noninfarcted state, with autophagic (*Tfeb*, *Amfr*, *Gabarap*, *Gabarapl1*) and oxidative stress response (*Sod1*) genes specifically enriched in the adult infarcted state (Figure 5A). Similar to neonatal myocytes, neonatal vascular endothelial cells were also enriched for GO terms and transcription factors associated with cell cycle (online-only Data Supplement Figure VA, Figure 5A). In contrast, adult endothelial cells were highly enriched for transcriptional networks induced by the C3 complement signaling pathway (Figure 5A and 5B). Adult leukocytes and CD90$^+$ fibroblasts from the infarcted heart were also enriched for genes related to the immune response and inflammatory signaling pathways (Figure 5A and 5B), with infarcted adult leukocytes specifically characterized by expression of H2-Aa and H2-Ab1 (Figure 5A). It is interesting to note that H2-Aa and H2-Ab1 are both highly expressed in B cells, dendritic cells, and monocytes,[36] which display unique cell recruitment profiles following neonatal MI.[20] Thus, neonatal and adult cardiomyocytes and nonmyocytes are characterized by unique transcriptional networks and signaling pathways, most of which arise during the normal course of development and some of which are deployed specifically in response to MI.

Adult Cardiomyocytes Fail to Reactivate Neonatal Transcriptional Networks Following MI

Adult cardiac pathologies are commonly associated with reversion to a fetal/neonatal gene program.[38] However, few studies have directly assessed this phenomenon using genome-wide methods with multicellular resolution. Hence, in the majority of whole-heart transcriptomic studies, reversion to a neonatal-like gene program under pathological conditions could be attributable to contaminating nonmyocyte populations. We sought to understand global transcriptional responses to MI at neonatal and adult stages. Following MI, adult CD90$^+$ fibroblasts and leukocytes underwent a transcriptional reversion to a "neonatal-like" state, with their transcriptomes clustering more closely with infarcted and noninfarcted neonatal cells than noninfarcted adult cells (Figure 2A). A similar reversion phenomenon did not occur in adult cardiomyocytes and vascular endothelial cells following MI. The postinfarction reversion of adult leukocytes and adult CD90$^+$ fibroblasts to a neonatal-like state was largely associated with the reinduction of cell cycle genes (Figure 6B and 6C). In contrast, cardiomyocytes (and to a lesser extent endothelial cells) failed to reactivate the same set of cell cycle genes following MI (Figure 6A). Further analysis of this cell cycle network revealed that all cardiac cell populations downregulated a common set of 644 genes during development from neonatal to adult stages but only CD90$^+$ fibroblasts and leukocytes could reactivate this gene set in adulthood following MI (Figure 6A). Conversely, we identified a common set of 217 genes involved in cellular metabolic processes that were upregulated in all cardiac cell populations during development from neonatal to adult stages and specifically repressed in adult fibroblasts and leukocytes following MI.

Developmental Remodeling of the Cardiomyocyte Epigenetic Landscape Is Correlated With Postnatal Silencing of the Neonatal Proliferative Network

To identify whether chromatin remodeling is associated with the inability of adult cardiomyocytes to reengage the neonatal proliferative program, we analyzed the genome-wide chromatin accessibility landscape of murine cardiomyocytes. For these studies, cardiomyocyte nuclei were isolated from heart tissue specimens and FACS sorted using the cardiomyocyte nuclear antigen, Pcm1, according to recently published methods (Figure 7A).[39] We then subjected purified cardiomyocyte nuclei from P1, P14, and P56 mouse hearts to next-generation sequencing to assess the transcriptome (RNA-seq) and chromatin accessibility landscape (Assay for Trans-

Figure 3 Continued. gene ontologies in each cell type. Pathways and ontologies are ranked based on –log(*P* value) from left to right. Representative gene ontologies and pathways are highlighted with colored circles. Endo indicates endothelial cells; Fibro, CD90$^+$ fibroblasts; IL-8, interleukin 8; Leuko, leukocytes; Myo, cardiomyocytes; PIP3, phosphatidylinositol 3,4,5-trisphosphate; TGFβ, transforming growth factor β; and VEGF, vascular endothelial growth factor.

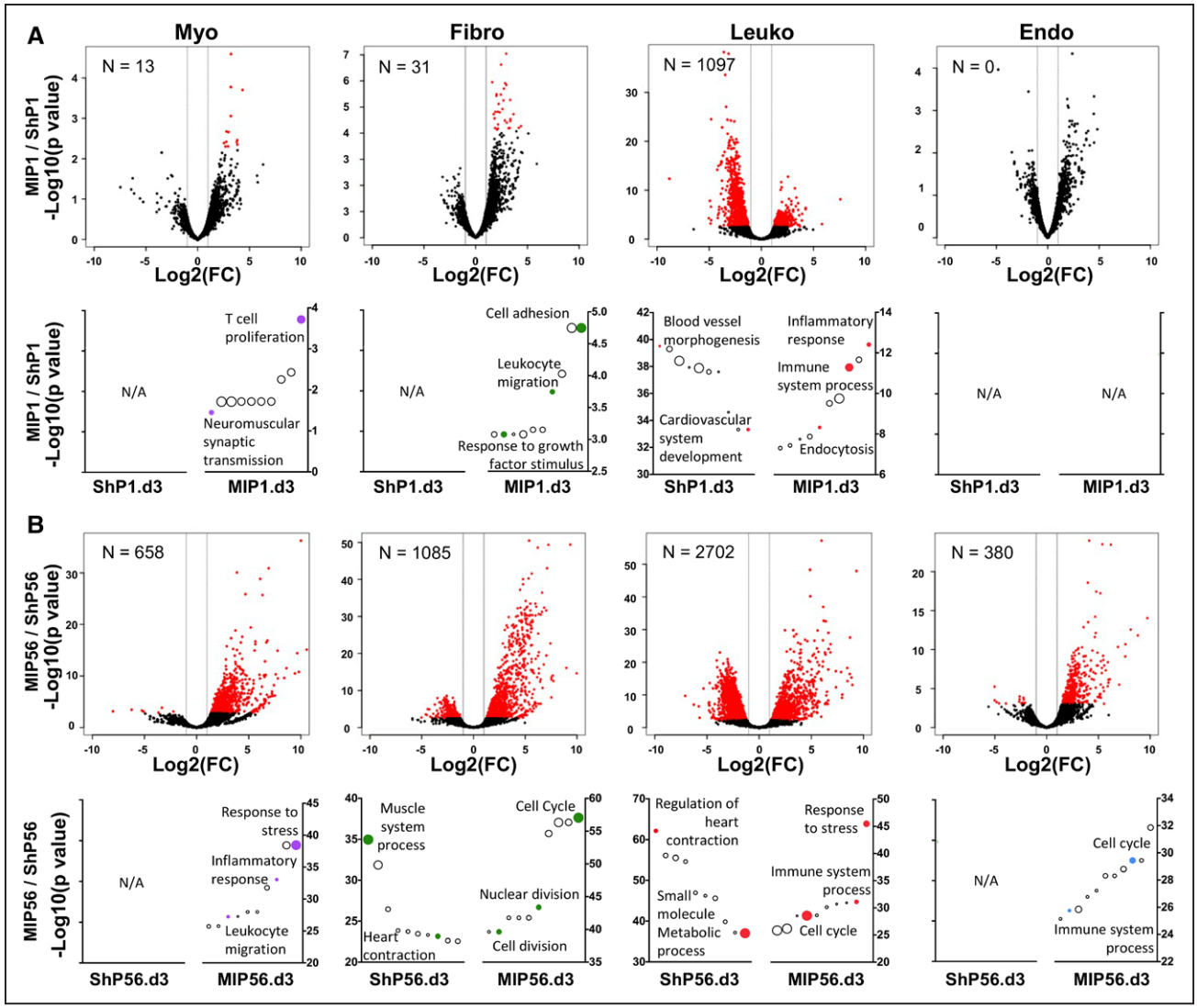

Figure 4. Injury-induced transcriptional responses in multiple cardiac cell populations following neonatal or adult myocardial infarction.

A, Volcano plots portray differentially expressed genes in different cell types in the neonate (P1) following myocardial infarction or sham surgery. Red dots denote significantly regulated genes (false discovery rate ≤ 0.05, log2(fold change) ≥ 1 or ≤ –1). N signifies the number of differentially expressed genes. Gene ontology analysis was performed for each comparison. **B,** Volcano plots portray differentially expressed genes in different cell types in the adult (P56) following myocardial infarction or sham surgery. Gene ontology analysis was performed for each comparison. Endo indicates endothelial cells; Fibro, CD90$^+$ fibroblasts; Leuko, leukocytes; Myo, cardiomyocytes; and N/A, not available.

posase-Accessible Chromatin [ATAC-seq])[40] (Figure 7, online-only Data Supplement Figures VII through XIII).

Consistent with our transcriptional profiling of enzymatically isolated cardiomyocytes, RNA-seq of cardiomyocyte nuclei revealed a repression of cell cycle–associated genes and activation of metabolic and sarcomeric genes during postnatal development (Figure 7B, online-only Data Supplement Figures IX through XI). In addition, ATAC-seq analysis of cardiomyocyte nuclei uncovered successive waves of chromatin remodeling during postnatal development (Figure 7C, online-only Data Supplement Figures VIII, XII, and XIII). At P1, genomic regions associated with genes involved in cell cycle, inflammation, and extracellular matrix deposition were in a euchromatic state, but these regions became progressively condensed by P14 and P56 (Figure 7C; cluster 1). Conversely, gene promoters associated with metabolism, muscle development, and contraction transitioned from a closed to an open state between P1 and P56 (Figure 7C; cluster 2). These alterations in chromatin accessibility appear to largely mirror developmental changes in the transcriptional landscape of cardiomyocytes, which is evident by the strong positive correlation between open chromatin regions at promoters (+/–1 kb transcription start site) and transcriptionally active genes (Figure 7D).

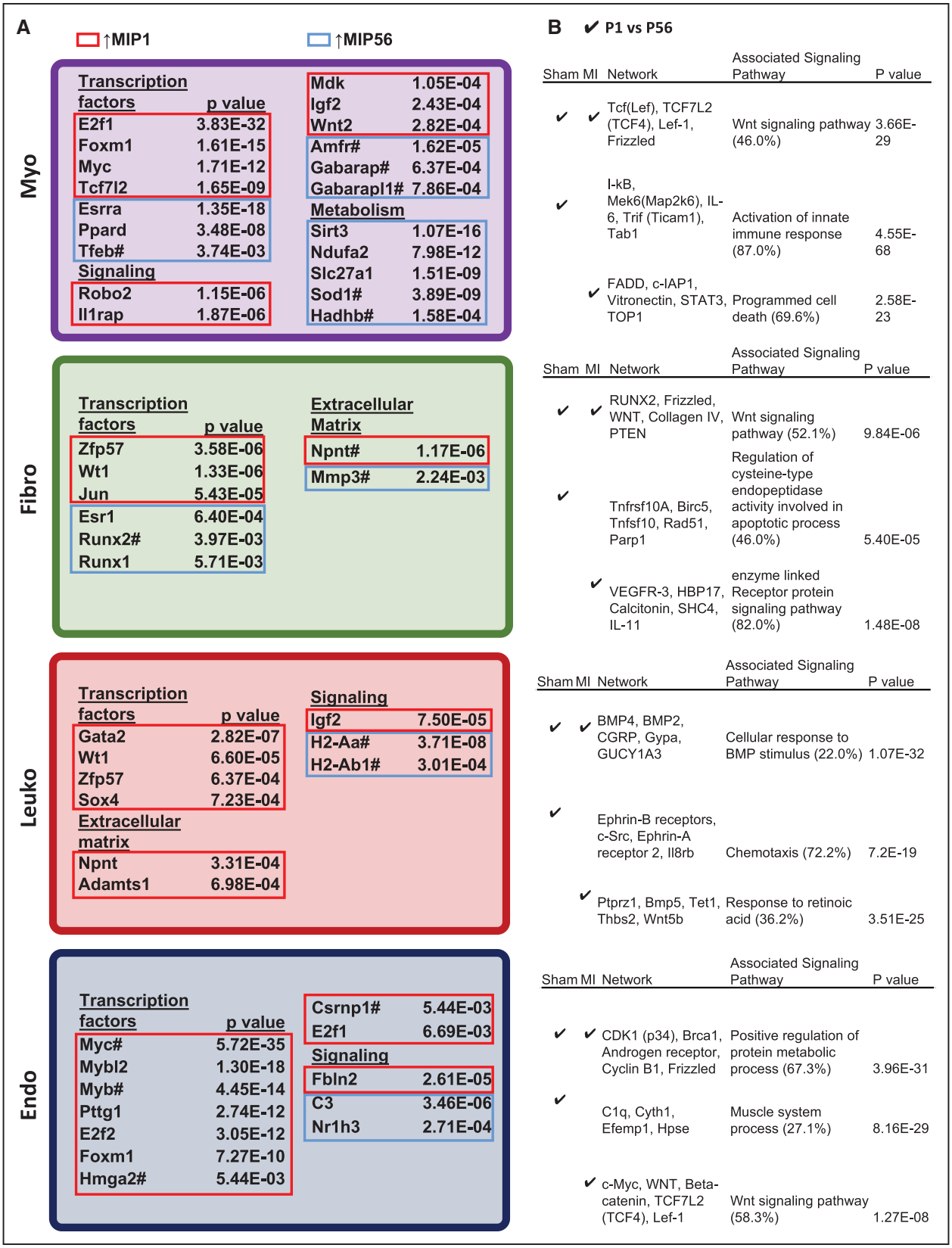

Figure 5. Different cardiac cell types exhibit unique transcription factor and gene network usage during cardiac development and following MI.

A, Overconnected transcription factors and signaling molecules are shown for each cellular data set. Overconnected genes following MI in neonatal cell types are shown in red boxes, whereas overconnected adult genes are shown in blue boxes. # denotes genes that are specifically regulated following MI (MIP1.d3 versus MIP56.d3), whereas genes without # are (*Continued*)

We next intersected our RNA-seq and ATAC-seq data sets from purified Pcm1+ nuclei to identify transcriptional networks that were epigenetically silenced in cardiomyocytes during postnatal development. We first isolated genes whose expression levels were positively correlated with an open chromatin state (+/–1 kb transcription start site) in either P1 or P56 myocytes and then determined which transcription factor targets were enriched at these promoters by using Gene Set Enrichment Analysis. It is striking that the open chromatin state for actively transcribed genes in P1 cardiomyocytes displayed strong signatures for the core cell cycle–related transcription factors, including E2f4 and Foxm1 (Figure 7E). Moreover, these were the same transcription factors underlying the set of cell cycle genes in cardiomyocytes that failed to revert to a neonatal state following MI (Figure 6B and Figure 7G). These results suggest that chromatin compaction occurs around cell cycle genes during cardiomyocyte maturation, indicative of a possible epigenetic mechanism for silencing of the neonatal cardiomyocyte proliferative gene network.

DISCUSSION

Despite decades of concerted effort, complete regeneration of the adult heart following injury remains an elusive goal of cardiovascular medicine. Recent studies have suggested that dedifferentiation and proliferation of resident cardiomyocytes is the primary mechanism contributing to cardiac regeneration in multiple vertebrate species.[41] Consequently, there has been intensive interest in uncovering the molecular programs that control myocyte proliferative capacity, with the ultimate goal of augmenting adult cardiomyocyte proliferation rates to facilitate endogenous cardiac regeneration. However, the heart is not solely composed of cardiomyocytes but also consists of numerous nonmyocyte populations, which have been poorly studied and often overlooked with respect to their role in cardiac regeneration. In this study, we addressed this issue by seeking to understand the transcriptional changes and contributions of the major cell types present in the heart including cardiomyocytes, CD90+ fibroblasts, leukocytes, and endothelial cells during cardiac maturation, regeneration, and repair. Our comprehensive transcriptional framework of multiple cardiac cell populations in the neonate and adult with and without myocardial infarction provides a unique resource for interrogation of transcriptional networks controlling cellular identity, maturation, and the response to ischemic injury. We used this rich resource to unveil previously unappreciated cellular behaviors, to define a transcriptional signature for neonatal heart regeneration, and to identify key drivers and barriers to mammalian heart regeneration.

A major finding of the present study is that all cell types within the heart undergo drastic transcriptional alterations during postnatal development. These results highlight the potential relevance of all cell types in the heart, not only myocytes, in postnatal maturation and loss of regenerative capacity. Our analysis of the leukocyte RNA-seq data sets suggests the involvement of dendritic cells, macrophages, monocytes, neutrophils, T cells, B cells, and eosinophils in the cardiac response to MI at neonatal and adult stages. Moreover, transcriptional analysis of the CD45+ leukocyte population revealed key differences in the inflammatory response of the infarcted neonatal and adult heart, which potentially trigger regenerative or fibrotic repair processes in the heart. Although it is possible that differences in leukocyte kinetics following injury in the adult versus neonatal heart may have contributed to these transcriptional differences, a previous study showed no differences in the proportions of monocytes, macrophages, dendritic cells, or neutrophils at day 4 post-MI in regenerative versus nonregenerative hearts.[20] In addition, our own FACS analysis (Figure 1B) shows that the proportional increase in CD45+ cells at day 3 post-MI is similar between P1 and P56 hearts (16.6% increase in CD45+ cells following MI at P1 versus 18.1% increase in CD45+ cells following MI at P56). Moreover, analysis of the CD45+ RNA-seq data sets using ImmGen demonstrated that many of the same leukocyte populations were present in the neonatal and adult heart at day 3 post-MI (online-only Data Supplement Figure IV). It is therefore unlikely that differences in the kinetics of leukocyte recruitment alone account for the observed differences between leukocyte transcriptomes. However, the precise contributions of nonmyocyte populations and the interplay between different cell types in the heart during regeneration and postnatal heart maturation require further investigation. Our vast transcriptional data set is an unparalleled resource that could be harnessed to better understand these complex interactions.

A key finding of the present study is that some adult cardiac cell types retain greater transcriptional plasticity than others. Of note, we found that adult CD90+ fibroblasts and leukocytes underwent a large-scale transcriptional reversion to a neonatal-like state following MI. Transcriptional reversion of adult CD90+ fibroblasts

Figure 5 Continued. also regulated during development (ShP1.d3 versus ShP56.d3). **B,** Gene networks and associated signaling pathways were assembled and ranked for each cell type after sham (ShP1.d3 versus ShP56.d3) or MI (MIP1.d3 versus MIP56.d3). Endo indicates endothelial cells; Fibro, CD90+ fibroblasts; Leuko, leukocytes; MI, myocardial infarction; and Myo, cardiomyocytes.

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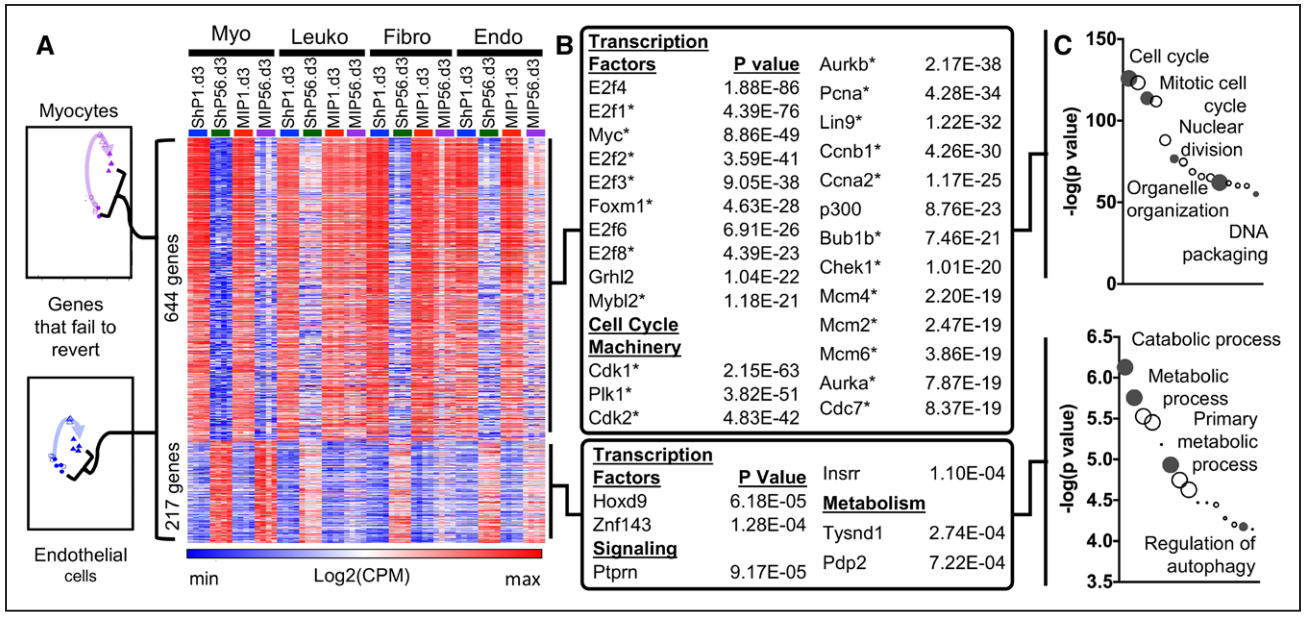

Figure 6. Adult cardiomyocytes and endothelial cells fail to reactivate the neonatal proliferative network following myocardial infarction.

A, Heat map showing a subset of transcripts that are commonly downregulated (n=644) or upregulated (n=217) during postnatal development (ShP1 versus ShP56) in all cardiac cell types. This cell cycle network includes genes that reverted to a neonatal-like signature following myocardial infarction (MIP56 versus MIP1) in adult leukocytes and CD90⁺ fibroblasts, but not myocytes and endothelial cells. Genes that exhibited the inverse expression profile are also shown. **B,** Overconnected transcription factors and key signaling molecules within the network were predominantly associated with cell cycle. The inverse expression profile was predominantly associated with metabolism. * indicates genes that were both overconnected and differentially expressed within the cell cycle network. **C,** Gene ontology analysis indicated that the genes that reverted to a neonatal signature in adult leukocytes and CD90⁺ fibroblasts were associated with cell cycle processes, whereas the inversely regulated genes were associated with autophagy and metabolism. Endo indicates endothelial cells; Fibro, CD90⁺ fibroblasts; Leuko, leukocytes; and Myo, cardiomyocytes.

and leukocytes was accompanied by induction of a large network of cell cycle genes, which failed to revert to a neonatal-like state in adult cardiomyocytes and endothelial cells following MI. This finding is consistent with the proliferative state of adult CD90⁺ fibroblasts and leukocytes during the early inflammatory phase post-MI and contrasts with the nonproliferative state of endothelial cells and cardiomyocytes in the adult heart. Whether this transcriptional reversion was attributable to the influx of cell cycle–competent leukocytes or CD90⁺ fibroblasts into the heart or the expression of cell cycle genes by resident CD90⁺ fibroblasts and leukocytes, or a combination of both, is unclear. Nevertheless, the failure of adult cardiomyocytes to reactivate neonatal transcriptional networks, in particular, those embedded within the cell cycle regulatory network, contrasts with established dogma regarding the transcriptional reversion to a fetal/neonatal gene program during cardiac hypertrophy and heart failure in adult mammals.[38] Numerous studies have established that a subset of genes, such as the classically studied sarcomeric isoforms and some metabolic genes, are clearly capable of being reinduced in adult cardiomyocytes following injury or disease.[38] However, the vast majority of neonatal transcripts, including those governing the cell cycle, appear to be largely refractory to transcriptional reinduction in adulthood. On the basis of our data at 3 days postinjury, we would argue that reversion to a fetal/neonatal gene program in adult myocytes is an exception rather than a rule. This point is further reinforced by the fact that cardiomyocytes undergo, by far, the most extensive transcriptional alterations of any cardiac cell type during postnatal maturation.

Although it is difficult to place the loss of regenerative capacity on a single factor or pathway, gene network analysis revealed a significant enrichment for components of the Wnt signaling pathway in neonatal cardiomyocytes, endothelial cells, and CD90⁺ fibroblasts following MI at P1 versus P56. It is interesting to note that we recently reported that components of the Wnt/β-catenin signaling pathway are methylated and transcriptionally silenced during postnatal heart maturation,[43] which could contribute to the developmental loss of neonatal regenerative capacity. Although the mechanisms mediating epigenetic shutdown of Wnt signaling during postnatal cardiomyocyte maturation are currently unclear, it is noteworthy that the majority of differentially expressed genes during cardiomyocyte

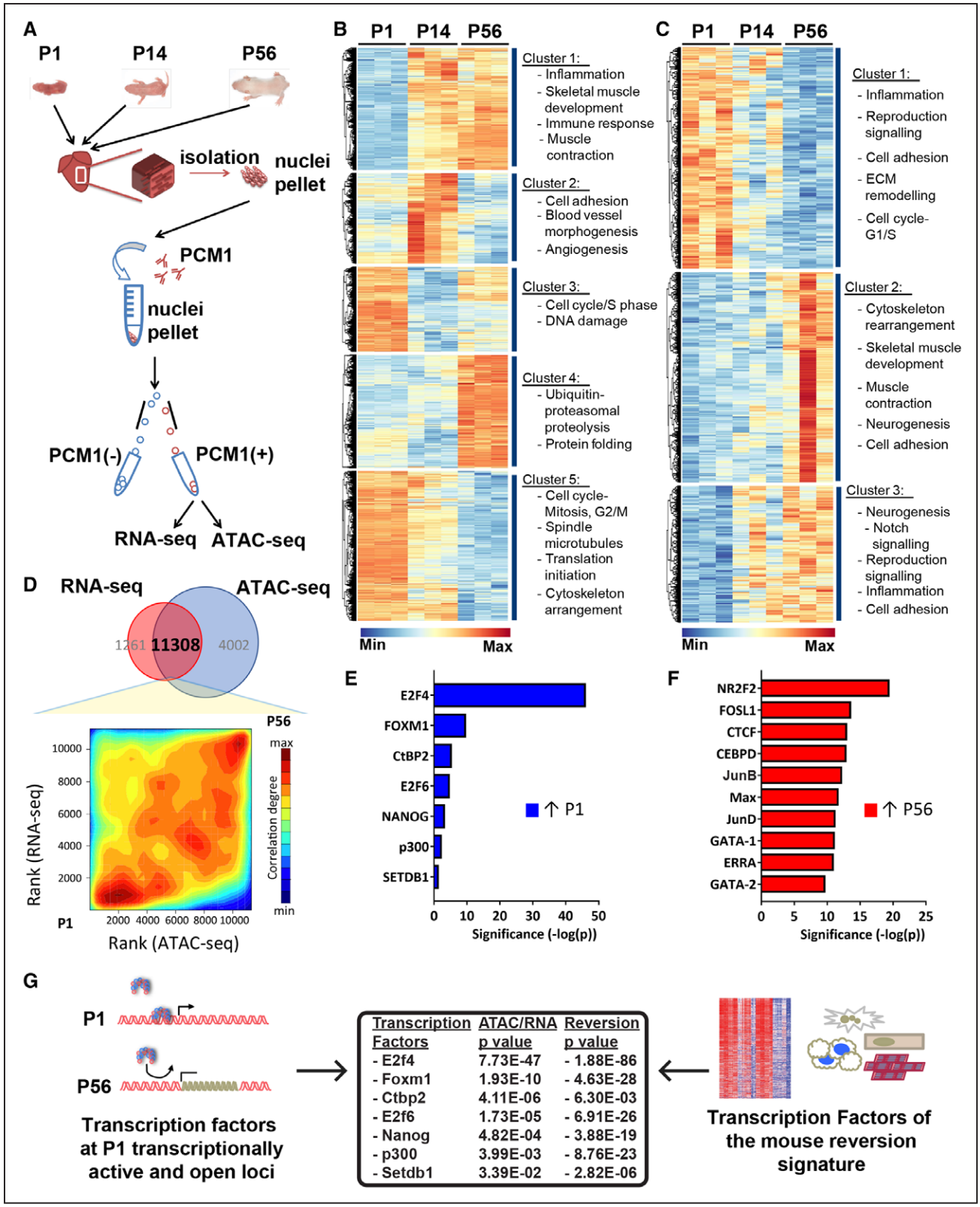

Figure 7. Epigenetic and transcriptional profiling of cardiomyocytes during postnatal development reveals a loss of chromatin accessibility at cell cycle genes after P1.

A, Schematic of Pcm1+ nuclei sorting for RNA-seq and ATAC-seq experiments. **B**, Heatmap of differentially expressed genes (RNA-seq) from P1 to P14 and P56. **C**, Heatmap of differentially accessible chromatin regions (ATAC-seq) from P1 to P14 and P56. **D**, **Top**, Venn diagram of all differentially regulated genes (no *P* value threshold) from RNA-seq and ATAC-seq data sets (1 kb ± transcription start site). A total of 11 308 genes were detected in both data sets. **Bottom**, Heatmap contour (*Continued*)

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development are associated with cell cycle shutdown and induction of metabolic pathways related to oxidative phosphorylation and mitochondrial organization. The inverse relationship between cell cycle and metabolism during cardiomyocyte maturation has been previously observed[42] and a direct role for cardiomyocyte oxidative metabolism in repression of the cardiac cell cycle has been recently proposed.[43] The inability of adult cardiomyocytes to reengage neonatal transcriptional networks and revert to a regenerative state may be, in part, mediated by metabolic processes that repress developmental signaling pathways during postnatal development.

There are many parallels between the cardiac regenerative response of neonatal mice and adult zebrafish.[41] However, our study suggests that there may be a key difference in the regenerative response to injury. Although zebrafish cardiomyocytes reengage developmental programs in adulthood following injury to facilitate regeneration, our data indicate that neonatal cardiomyocytes are already in a permissive regenerative state. Moreover, neonatal cardiomyocytes, endothelial cells, and CD90$^+$ fibroblasts do not deploy a major transcriptional response to MI. The lack of robust transcriptional induction of neonatal myocytes, CD90$^+$ fibroblasts, and endothelial cells is in stark contrast with adult cell types, which engage cell type–specific transcriptional responses following MI. This surprising finding suggests that neonatal heart regeneration is associated with a developmentally permissive transcriptional state in cardiomyocytes, CD90$^+$ fibroblasts, and endothelial cells rather than activation of a regenerative transcriptional program following injury. However, we detected major transcriptional shifts in the leukocyte population following neonatal MI, suggesting that local inflammatory responses also play a key role in driving the regenerative process. Given the distinct inflammatory responses and lack of transcriptional reversion of adult cardiomyocytes and endothelial cells following MI, it seems likely that efficient endogenous regeneration of the adult mammalian heart will require forced transcriptional reversion of multiple cell types into a neonatal-like state.

To uncover the molecular drivers of the regenerative network, we focused on identifying key regulators of a subset of genes that failed to revert to a neonatal-like state in adult cardiomyocytes and endothelial cells, but reverted in both CD90$^+$ fibroblasts and leukocytes following adult MI. In myocytes, predicted drivers of the cell cycle network showed strong overlap with genes that were epigenetically repressed during cardiomyocyte maturation. The chronology of myocyte cell cycle shutdown, the concomitant loss of chromatin accessibility around cell cycle genes, and the inability to reinduce the neonatal proliferative network following MI in adulthood are consistent with an epigenetic mechanism for silencing of the neonatal regenerative program during postnatal life.[16,44]

To date, even the most potent mitogens have stimulated significant but only modest reinduction of adult cardiomyocyte proliferation.[45] Our findings suggest that the heterochromatic state around cell cycle gene loci, which is acquired during preadolescence, could constitute an epigenetic roadblock that prohibits adult myocyte cell cycle reentry and adult heart regeneration. Although a very small subset of cardiomyocytes may evade this developmentally regulated checkpoint and retain the ability to proliferate throughout life,[46] further analysis of cardiomyocyte heterogeneity in the adult heart would require transcriptional and epigenomic analyses at single-cell resolution. A recent single-cell sequencing study identified 2 subpopulations of cardiomyocytes during heart development,[47] highlighting the potential use of this new technology for assessing heterogeneous cellular responses during regeneration. However, there are limitations to single-cell sequencing, such as the inability to interrogate lowly expressed genes. Also, the Fluidigm single-cell RNA-seq methodologies used by the Seidman and Wu groups were designed to only sequence mRNAs.[47,48] Therefore, unlike the data sets presented here, single-cell analysis does not capture any noncoding RNA information. In the future, spatial transcriptomic approaches (such as Tomo-seq[49]) could be combined with single cell sequencing technologies to further resolve transcriptional changes in the infarct and border zone following neonatal or adult MI. Nevertheless, even though transcriptomic heterogeneity exists within the cardiomyocyte population, the developmental shift in gene transcription from neonatal to adult stages was so dramatic that neonatal and adult cardiomyocytes could (and perhaps should) be viewed as 2 transcriptionally distinct cell types. Hence, efforts to

Figure 7 Continued. of integration of RNA-seq and ATAC-seq data sets showing a positive correlation between active transcription and open chromatin structure. **E**, Gene Set Enrichment Analysis for transcription factor targets in open chromatin regions associated with transcriptional activation at P1. Significance is represented as –log10 of *P* value. **F**, Gene Set Enrichment Analysis for transcription factor targets in open chromatin regions associated with transcriptional activation at P56. Significance is represented as –log10 of *P* value. **G**, Overlay of transcription factors that control the cell cycle network and transcription factors associated with genes highly expressed at P1 from euchromatic promoters. The *P* values of the transcription factors in the inset table were calculated from either the cell cycle network (cellular RNA-seq reversion signature) or from highly expressed genes at P1 euchromatic promoters (nuclear intersection of RNA-seq and ATAC-seq). ATAC-seq indicates Assay for Transposase-Accessible Chromatin; and RNA-seq, RNA sequencing.

stimulate adult cardiomyocyte proliferation may require resetting or reprograming of the adult epigenetic landscape into a neonatal-like regenerative state.

The present study provides a comprehensive transcriptional resource of multiple cardiac cell populations during cardiac development, repair, and regeneration. Our findings define a transcriptional program underpinning the neonatal regenerative state and shed light on a potential epigenetic barrier to adult heart regeneration.

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DISCLOSURES

None.

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FOOTNOTES

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