RNA-sequencing indicates immune cell signaling and inflammatory gene expression in cardiac fibroblasts increases with developmental age

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Abstract:

Background: Cardiac fibroblasts are responsible for extracellular matrix turnover and repair in the cardiac environment and serve to help facilitate immune responses. However, it is well established that they have significant phenotypic heterogeneity with respect to location, physiological conditions, and developmental age. The goal of this study was to provide an in-depth transcriptomic profile of cardiac fibroblasts derived from rat hearts at fetal, neonatal, and adult developmental ages to ascertain variations in gene expression that may drive functional differences in these cells at these specific stages of development.

Results: We performed RNA-seq of cardiac fibroblasts isolated from fetal, neonatal, and adult rats was performed and compared to the rat genome. Principal component analysis of RNA-seq data suggested data variance was predominantly due to developmental age. Differential expression and Gene set enrichment analysis against Gene Ontology and Kyoto Encyclopedia of Genes and Genomes datasets indicated an array of differences across developmental ages, including significant decreases in cardiac development and cardiac function-associated genes with age, and a significant increase in immune and inflammatory-associated functions - particularly immune cell signaling, and cytokine and chemokine production - with respect to increasing developmental age.

Conclusion: These results reinforce established evidence of diverse phenotypic heterogeneity of fibroblasts with respect to developmental age. Further, based on our analysis of gene expression, age-specific alterations in cardiac fibroblasts may play a crucial role in observed differences in cardiac inflammation and immune response observed across developmental ages.

Keywords: Cardiac fibroblast, RNA-seq, Developmental Age

Introduction:

While cardiomyocytes (CMs) have historically been the therapeutic target for bioengineering approaches to heart repair (1-3), non-cardiomyocyte cell populations in the heart often play a critical role in the heart's response to injury and, as such, have been subject to increased appreciation and study over the past few decades. Non-myocyte cell populations, which include vascular cells, endothelial cells, immune cells, and cardiac fibroblasts (CFs), comprise approximately 70% of the total cell population in the adult mammalian heart (4-6), and CFs are the major fraction of these non-myocyte cells. CFs, in particular, play a major role in mediating cardiac homeostasis, facilitating the development and maturation of cardiac ECM and promoting cardiomyocyte proliferation *in utero* (7), and yet they are also integral in the initiation and progression of various cardiac pathologies (8-11).

CFs are classically defined as mesenchymal-like cells responsible for extracellular matrix (ECM) production and remodeling (11). These cells are largely quiescent in the adult mammalian heart but exhibit extensive phenotypic plasticity, able to react to stimuli following injury and transition to a myofibroblast phenotype. In contrast to CFs in a resting state, myofibroblasts are highly active, migrating into and proliferating rapidly in the wound environment, where they extensively upregulate collagen I production to produce mature fibrotic scar tissue in adult mammals (12, 13). However, the role of CFs in responding to injury extends beyond ECM regulation and fibrosis. CFs are an important immune mediator and contributor to the inflammatory process (14, 15). One study by Kawaguchi *et al* indicated that mouse CFs exposed to lipopolysaccharide (LPS), an inflammatory stimulus, promoted interleukin-1 β (IL-1 β ,

a major inflammatory mediator) expression in those cells, but not in CMs. The CFs were found to act as “sentinel” cells in the heart, deploying a broad range of chemokines including MCP-1, macrophage inflammatory protein-1, and RANTES (15) to aid immune cell influx into a wound environment (16).

Despite their critical roles in the heart, CFs are loosely defined with respect to their phenotype, which varies considerably across developmental ages. The cell type traditionally considered a fibroblast is now understood to be a heterogeneous population of cells arising from different progenitors (17, 18), with a lack of specific markers to establish their identity *in vitro*, although CFs from both fetal and adult sources express periostin, vimentin, and discoidin domain receptor 2 (DDR2) (19). Further, through crosstalk with cardiomyocytes, CFs influence cardiac response to injury differently with respect to developmental age. Fetal CFs are implicated in driving cardiomyocyte growth and proliferation via secretion of paracrine factors, whereas adult CF-secreted factors are implicated in inducing cardiac hypertrophy, findings that reflect the regenerative healing observed in fetal and neonatal hearts, and fibrosis and hypertrophy observed in injured adult cardiac tissue (20, 21). As there is a significant, rapid shift in ECM composition (22, 23) and mechanical function of the heart pre- to post-birth, studying the phenotype of neonatal CFs would also be valuable, although there is scarce literature currently available on CFs of this specific developmental age. A single-cell RNA-seq study has established that transcriptomic changes in mouse CFs from a neonatal to adult state promote concomitant CM maturation, connecting age-specific CF phenotypic changes with cardiac maturation (24).

Studies of CF behavior across various developmental ages are invaluable to further elucidating the identities and functions of this heterogeneous cell population and how it changes with age, particularly with respect to the healing response of cardiac tissue. Many analyses have investigated how CF developmental age impacts cardiac structure and function or induces functional change in CMs (3, 25). By contrast, an understanding of how CF gene expression related to immune infiltration and the inflammatory response change with age is not well-established, despite the critical roles of these cells in cardiac inflammation and immunity and how critical these processes are to the healing response (15, 26). In this study, we employ RNA-seq transcriptomic analysis to evaluate rat cardiac fibroblast gene expression at fetal, neonatal, and adult developmental ages. This transcriptomic profile presents a broad view of CFs across development and aging to facilitate age-specific analyses of these cells. However, we specifically investigate age-dependent changes in immune and inflammatory gene expression, speculating that CF involvement in these processes increases with development. We found that compared to postnatal day 1 neonatal CFs, fetal CFs display significantly downregulated immune- and inflammatory-implicated genes and pathways, whereas adult CFs markedly increased expression of immune and inflammatory proteins. Taken together, this suggests CFs adopt an inflammatory/immune-support role rapidly after birth, that is further enhanced with continued development.

Results:

Fibroblast sequencing, culture, and characterization

Cardiac fibroblasts (CFs) were isolated from fetal (E18), neonatal (P1), and young adult (~7-week-old, at which point the rats are sexually mature (27)) rat hearts. After pre-plating the

isolated mixed cardiac cells (28, 29), isolates were cultured to confluency and used for RNA-sequencing or expanded out to passages 2-4 for additional experiments and culture validation (Fig. 1).

Figure 1: Pipeline for RNA isolation and differential expression analysis. RNA is mapped to Rn6 rat genome via STAR and reads counted, yielding 17337 distinct observations. These are normalized and used in differential expression analysis in DESeq2, and then used directly in GSEA for enrichment analysis, or filtered via LogFC and adjusted p-value to evaluate significantly expressed genes. Insert: Schematic depicting isolation of three distinct ages of rat cardiac fibroblasts, followed by culture and RNA isolation. Schematic creating using art acquired from BioRender.

RNA-seq mapping to the Rn6 rat genome yielded a total of 17337 discrete genes mapped. Differential expression analysis yielded a total of 12786 genes of interested between comparison conditions (adult vs fetal, adult vs neonatal, and fetal vs neonatal) (Fig. 1).

CFs grown in culture were imaged following immunofluorescence labeling and fibroblast lineage was confirmed by demonstration that the cells were negative for markers of endothelial (CD31) (Fig 2D-F) or hematopoietic (CD45) lineages (Fig 2G-I), respectively (30, 31). Cells were positive for vimentin (Fig 2A-C), a broad CF marker (31, 32). Cultured fibroblasts all displayed similar elongated morphology and positive vimentin expression throughout. (Fig 2).

Figure 2: Immunofluorescence microscopy characterization of primary P2-P4 cardiac fibroblast cultures. A-C) Vimentin staining of fetal (A), neonatal (B) and adult (C) cells, in order to identify cardiac fibroblasts. D-F) CD-31 staining of fetal (D), neonatal (E), and adult (F) cells to identify endothelial-lineage cells in culture. G-I) CD-45 staining of fetal (G), neonatal (H), and adult (I) cells to identify hematopoietic-lineage cells in culture. Scale bars = 100um.

Clustering analysis reveals broad variances in gene expression based on CF age

Sequencing data of fetal, neonatal, and adult CFs (groups referred henceforth as FCF, NCF, and ACF, respectively) were analyzed and normalized before differential expression analysis was executed, all in the *DESeq2* package in R. Sample-to-sample Manhattan distance was computed and a heatmap showing sample distance and correlation was produced (Fig 3A).

Figure 3: Normalized RNA-seq sample data from adult cardiac fibroblasts (ACF), neonatal cardiac fibroblasts (NCF) and fetal cardiac fibroblasts (FCF). A) Sample-to-sample distance heatmap utilizing Manhattan-distance calculation, showing close clustering of FCF and NCF samples. Heatmap produced in pcaExplorer in R. B) Heatmap of normalized gene counts from all identified genes, showing clustering of genes the three age groups. Genes are row-normalized, and heat-map color is based on z-score. Heatmap produced with R software.

The heatmap indicated a stronger correlation between the FCF and NCF samples. FCF samples had the lowest level of similarity to ACF samples, followed by NCFs, suggesting the sample variability was strongly age dependent. This was supported by unsupervised hierarchical clustering of the DESeq2-normalized geneset across all samples, visually presented in the

Figure 4: Principal component analysis with associated loadings with respect to gene expression in fetal, neonatal, and adult rats. A) Table of 50 top contributing genes to the PCA analysis, with associated x-coordinates and y-coordinates on the associated PCA graph in (B). B) PCA of top 50 genes contributing most variance to the first two principal components, showing relationship and location with respect to fetal (FCF), neonatal (NCF), adult (ACF) samples. C) Loadings plot for top/bottom ten highest contributing genes of first and second principal components. D) PCA of samples with respect to first two principal components, showing broad distribution with respect to age on the first PC axis. All graphs were generated in `pcaExplorer` package in R.

Analysis of the 50 genes that most significantly contributed to the PCA variance produced two distinct clusters with respect to the first principal component, which appeared to be associated with CF age. This suggested greater variance between ACFs and the two juvenile CF groups (FCF and NCF), with the juvenile groups associated with genes that clustered closer together. Genes associated with the ACF cluster were broadly immune associated, including chemokines *Ccl22*, *Ccl17*, and *Ccl3*, and cell surface receptor *Cd74*. The NCF/FCF cluster was observed to be predominantly associated with genes related to cardiac and embryonic development, including *Myh6*, *Myh7*, *Nppa*, *Myl2*, *Myl3*, and *Actc1* (Fig 4B-D).

Differential expression analysis further supported the transcriptomic variance between adult CFs and neonatal and fetal CFs: post-hoc filtering ($\log_2FC > 1.5$, $padj > 0.05$) produced 2794 significant differentially expressed genes in ACFs vs FCFs and 2003 significant differentially expressed genes in ACFs vs NCFs, versus 784 genes differentially expressed between FCFs and NCFs (Fig. 1). The top 25 most significantly upregulated and downregulated genes from each comparison are presented in Table 1.

Table 1: List of 25 most significantly upregulated and 25 most significantly downregulated genes from Fetal vs. Neonatal, Adult vs. Neonatal, Adult vs. Fetal comparisons

Upregulated						Downregulated					
Fetal Vs Neo		Adult vs Neo		Adult vs Fetal		Fetal Vs Neo		Adult vs Neo		Adult vs Fetal	
Genes	log2FoldChange	Genes	log2FoldChange	Genes	log2FoldChange	Genes	log2FoldChange	Genes	log2FoldChange	Genes	log2FoldChange
Upk3bl	5.919187	Ccl22	10.28234	Clec10a	11.58615	Akr1c14	-5.566468285	Myh7	-11.30766504	Myh7	-12.48441723
Tbx4	5.711532	Cd74	9.176081	Serpina3n	11.07736	LOC24906	-4.802399558	Actc1	-11.19711678	Actc1	-12.31689974
Hoxb7	5.665582	Ccl17	8.783643	Asgr2	10.40074	Figf	-4.586929021	Myh6	-10.97836111	Myh6	-11.8069547
Igf1bp5	5.59857	Asgr2	8.239064	Ccl22	10.36736	Mrgprf	-4.403228606	Nppa	-10.7449663	Myl2	-11.49213976
Myog	5.505488	Serpina3n	8.080356	Ccl17	9.922081	Clec10a	-4.186947411	Myl2	-9.924278413	Nppa	-11.4027724
Dhrs9	5.362969	Mmp7	7.744266	Pla2g2a	9.609374	Cpxm2	-4.011002786	Myl3	-9.308555082	Tnni1	-10.60890482
Tenn4	5.193547	Pla2g2a	7.512271	Akr1c14	9.354025	Slc47a1	-3.90131184	Sh3bgr	-8.980427683	Sh3bgr	-10.41421189
Gldn	5.130201	RT1-Ba	7.50239	Ccl4	9.265844	Dpysl4	-3.846230821	Tnni1	-8.978986082	Myl3	-10.37610834
Hoxb5	5.100569	Irg1	7.486625	Ccl3	9.027888	Mnda	-3.77006226	Csrp3	-8.766944839	Csrp3	-9.909283343
Mfap2	5.052972	Chi3l1	7.470716	LOC24906	9.026394	Scn7a	-3.679321021	Acan	-8.489888441	H19	-9.635111663
B3gnt5	4.977134	Rgs9	7.464147	Nrros	8.722336	Acot1	-3.536174658	Actn2	-8.410946329	Actn2	-9.256275492
Fendrr	4.923848	Clec10a	7.399204	Cd300lf	8.613316	Mfrp	-3.489356287	Nrap	-8.299109006	Smpx	-9.170751224
Mybpc1	4.773853	Cst7	7.368542	Chi3l1	8.451384	Ceacam1	-3.467994934	Casq2	-8.111326289	Tnni1	-9.157876842
Hoxb8	4.760618	Plet1	7.300047	Serpina1	8.380459	Mageb4	-3.46534003	Smpx	-7.849079831	Unc45b	-9.056882513
Stmn2	4.745606	Ccl4	6.978046	Arhgap9	8.349699	Nrros	-3.444183605	Myl7	-7.810108718	Nrap	-8.993720954
Mstn	4.73928	Irf4	6.931538	Ch25h	8.338975	Upk1a	-3.432813169	Pln	-7.761398734	Casq2	-8.713779547
Wnt16	4.575537	RT1-Da	6.926247	Arhgap25	8.31574	Car1	-3.422033795	Unc45b	-7.643272419	Sympo2l	-8.684113331
Ppp2r2c	4.567238	Milr1	6.76698	Stap1	8.28389	Sepp1	-3.373467452	Tnni1	-7.550449335	Acan	-8.642440402
Cdh8	4.46549	Ccl20	6.640746	Irg1	8.245699	Abca8a	-3.270056117	Nppb	-7.548924581	Ryr2	-8.530608706
Chrna1	4.405294	RT1-Bb	6.541552	Lbp	8.199643	Lbp	-3.26664172	Reg3b	-7.544072558	Nppb	-8.448727672
Tnni2	4.373668	Ccl9	6.532978	Mmp7	8.183537	Krt24	-3.239943831	Xlirp2	-7.541372318	Alpk3	-8.439659492
Fam150a	4.267047	Klkb1	6.498334	Irf4	8.140108	Pmfbp1	-3.198896228	Ryr2	-7.398237956	Dusp27	-8.40961941
Krt15	4.174717	Ccl3	6.464289	Atp6v0d2	8.12108	Tlr7	-3.187350765	Ldb3	-7.351657713	Pln	-8.380649219
Glp1r	4.155335	C3	6.40599	Mcomp1	8.063229	Clec7a	-3.179790031	Sympo2l	-7.268467268	Ldb3	-8.365997859
Hmga2	4.136327	Atp6v0d2	6.403729	Ccl7	8.046836	Akr1c1	-3.122931155	Tnni3	-7.157721088	Reg3b	-8.229578394

Additional descriptions of functions of the top 10 most significantly upregulated and

downregulated genes from each comparison are presented in Additional File 1. A preliminary

investigation of gene distribution within these comparisons was conducted using volcano plots

generated in the *EnhancedVolcano* R package, using a logFC cutoff of 1.5 and p-value cutoff of

0.001 as parameters. The plot of FCFs vs NCFs indicated that immune-associated genes such as

Ifitm1 and *Lbp*, and matrix regulation-associated genes such as *Ctsc*, *Figf*, and *Itgb3* were

downregulated in FCFs compared to NCFs, whereas developmental genes such as *TBX4*, *Mdk*,

Hoxb7, and *Hoxb8* were upregulated (Fig. 5A, Table 1). ACF comparisons to NCFs and FCFs

yielded similar gene distributions in the volcano plots (Fig. 5B-C). In both comparisons, cardiac

development-associated genes such as *Actc1*, *Actg2*, *Tnni1*, and *Myh7* were downregulated in

ACFs versus the younger group, while immune- and inflammatory-associated genes such as

Tgfb1, *Ccl3*, and *Ccl4* were observed in ACFs to be comparatively upregulated (Fig. 5B & 5C,

Table 1).

Figure 5: Volcano plots of significant differentially expressed genes. A) fetal vs neonatal, B) adult vs neonatal, C) adult vs fetal, with boundaries set at logFC of 1.5, and p-value of 0.001. Genes color-coded yellow indicate significant LogFC but not statistically significant. Purple genes indicate statistical significance but genes under absolute value of LogFC threshold. Brown color-coded genes meet LogFC threshold and statistical significance. Volcano plots generated in EnhancedVolcano package in R.

Differential expression indicates age-specific shifts in immune signaling and cardiac development

Differentially expressed gene lists from the three separate comparisons (FCF vs NCF, ACF vs NCF, ACF vs FCF) were ranked based on fold change, and subsequently analyzed using Gene Set Enrichment Analysis (GSEA) methodology (33), within the *clusterProfiler* package in R (34). The full lists of differentially expressed genes identified in these analyses are available in Additional File 3 (Fetal vs Neonatal), Additional File 5 (Adult vs Neonatal), and Additional File 7 (Adult vs Fetal). These datasets were subjected to analysis utilizing Gene Ontology (GO, analyzing for Biological Process, Cellular Component, and Molecular Function terms) (35) and Kyoto Encyclopedia of Genes and Genomes (KEGG) curated datasets (36). Analysis of fetal CFs vs neonatal CFs against GO datasets yielded an array of significantly downregulated ontologies associated with immune response and immune cell signaling (Fig 6A, Table 2), including

“leukocyte mediated immunity,” “positive regulation of immune response,” and “activation of immune response.”

Table 2: Ten most significant (based on q-value) GO and KEGG results from GSEA analysis of Fetal vs. Neonatal differential expression.

GO													
ONTOLOGY	ID	Description	setSize	enrichmentScore	NES	pvalue	p.adjust	qvalues	rank	count	GeneRatio	(-)LOG10(qvalues)	
1 BP	GO:0048706	embryonic skeletal system development	98	0.66782739	2.211347	3.09E-10	1.41E-07	1.22E-07	1774	41	0.41836735	6.914235563	
2 BP	GO:0048704	embryonic skeletal system morphogenesis	77	0.673441241	2.163555	1.20E-08	2.97E-06	2.57E-06	1173	27	0.35064935	5.589746169	
3 BP	GO:0030239	myofibril assembly	63	0.685639515	2.132596	1.46E-07	2.24E-05	1.94E-05	1413	31	0.49206349	4.712815643	
4 BP	GO:0045214	sarcomere organization	47	0.711616313	2.115637	1.49E-06	0.00014	0.000121	1413	25	0.53191489	3.915725028	
5 BP	GO:0010226	response to lithium ion	38	0.737469517	2.110208	2.41E-06	0.000205	0.000178	705	11	0.28947368	3.749588093	
6 BP	GO:0002443	leukocyte mediated immunity	269	-0.542219601	-2.25599	1.00E-10	6.20E-08	5.37E-08	1818	105	0.39033457	7.269805204	
7 BP	GO:0050776	regulation of immune response	457	-0.516923697	-2.25641	1.00E-10	6.20E-08	5.37E-08	1430	144	0.31509847	7.269805204	
8 BP	GO:0050778	positive regulation of immune response	334	-0.535040447	-2.28475	1.00E-10	6.20E-08	5.37E-08	1430	107	0.32035928	7.269805204	
9 BP	GO:0045087	innate immune response	372	-0.535070216	-2.2875	1.00E-10	6.20E-08	5.37E-08	2068	144	0.38709677	7.269805204	
10 BP	GO:0002253	activation of immune response	174	-0.5822796	-2.30488	1.00E-10	6.20E-08	5.37E-08	1254	51	0.29310345	7.269805204	
KEGG													
ID	Description	setSize	enrichmentScore	NES	pvalue	p.adjust	qvalues	rank	count	GeneRatio	(-)LOG10(qvalues)		
rno04260	Cardiac muscle contraction	70	0.557570837	1.774523	0.000675	0.008918	0.006495	2372	33	0.47142857	2.187440803		
rno05217	Basal cell carcinoma	44	0.597575616	1.74177	0.001055	0.01126	0.0082	1309	14	0.31818182	2.086175282		
rno04390	Hippo signaling pathway	128	0.488247152	1.68712	0.000218	0.0048	0.003496	1309	28	0.21875	2.456440873		
rno04080	Neuroactive ligand-receptor interaction	192	0.462846994	1.672178	3.42E-05	0.000943	0.000687	1704	78	0.40625	3.163098363		
rno03040	Spliceosome	113	0.486982499	1.659018	0.000701	0.008918	0.006495	4787	79	0.69911504	2.187440803		
rno05152	Tuberculosis	137	-0.530667627	-2.01828	1.02E-07	6.33E-06	4.61E-06	1595	45	0.32846715	5.336554399		
rno04145	Phagosome	131	-0.535473922	-2.03847	4.06E-08	4.04E-06	2.95E-06	1964	48	0.36641221	5.530908671		
rno04621	NOD-like receptor signaling pathway	130	-0.555866396	-2.11026	7.46E-09	2.47E-06	1.80E-06	2242	55	0.42307692	5.745279883		
rno05323	Rheumatoid arthritis	65	-0.641598006	-2.1916	1.15E-07	6.33E-06	4.61E-06	1520	28	0.43076923	5.336554399		
rno04620	Toll-like receptor signaling pathway	77	-0.6261727	-2.21556	4.89E-08	4.04E-06	2.95E-06	1096	24	0.31168831	5.530908671		

Core enriched genes in these datasets included pro-inflammatory chemokines such as *Ccl3*, *Ccl4*, *Ccl11*, and immune-associated receptors such as *Ddx58* and *Lbp*. This downregulation in immune-associated ontologies was supported by KEGG results, which indicated downregulation of Nod-like receptor signaling pathways and Toll-like receptor signaling pathways (37) (Fig. 6D, Table 2, Additional File 2).

Upregulated GO terms in FCFs vs NCFs included developmental terms such as “embryonic skeletal system morphogenesis,” and cardiac tissue-associated ontologies including “myofibril assembly” and “sarcomere organization,” (Fig. 6A) supported by KEGG pathways including “cardiac muscle contraction” and “Hippo signaling pathway.” Identified core enriched genes included homeobox proteins (*Hoxb5/7/8*, *Nkx2.5*), T-box transcription factors (*Tbx1/15*), and several cardiac tissue proteins including *Actc1*, *Actn2*, and *Myh6* (Fig 6D, Table 2, Additional File 2).

Figure 6: Analysis of functionally enriched GO and KEGG gene sets comparing three ages of cardiac fibroblasts, via Gene Set Enrichment Analysis (GSEA). Dot color is indicative of $-\log_{10}(q\text{-value})$, and size is indicative to number of identified core enriched genes. Gene ratio is ratio of identified core enriched genes vs the total number of genes associated with that curated gene set. A, B, C) Dot plots indicating top 5 upregulated and downregulated general GO gene sets for fetal vs neonatal, adult vs neonatal, and adult vs fetal, respectively. GSEA analysis cutoff p-value was 0.05. D, E, F) Dot plots indicating top 5 upregulated and downregulated KEGG gene sets for fetal vs neonatal, adult vs neonatal, and adult vs fetal, respectively. GSEA analysis parameters were set to filter out gene sets with less than 3 genes or greater than 500, or functionally enriched gene sets with p-value > 0.05. Graphs produced in the R programming environment using the clusterProfiler software package.

Similar patterns emerged in analysis of adult CFs compared to both neonatal CFs and fetal CFs. Differential expression in adult vs neonatal and adult vs fetal indicated upregulation of ontology terms associated with adaptive and innate immune response, leukocyte function, and regulation of immune response (Fig 6B & 6C) and in both cases, KEGG pathways including “cytokine-cytokine receptor interaction,” and “viral protein interaction with cytokine and cytokine receptor” were upregulated (Fig 6E & 6F). This was supported by examination of core enriched genes: an array of chemokines including *Ccl3*, *Ccl4*, *Ccl7*, *Ccl11* and *Ccl22* were identified as enriched in both adult vs neonatal (Table 3, Additional File 4) and adult vs fetal (Table 4,

Additional File 6) analyses, suggesting significant variances in chemokine expression with respect to age.

Table 3: Ten most significant (based on q-value) GO and KEGG results from GSEA analysis of Adult vs. Neonatal differential expression.

GO	ONTOLOGY	ID	Description	setSize	enrichmentScore	NES	pvalue	p.adjust	qvalues	rank	count	GeneRatio	(-)LOG10(qvalues)
	1 BP	GO:0045087	innate immune response	372	0.722149595	2.720167	1.00E-10	6.41E-09	5.36E-09	1464	165	0.44354839	8.270753532
	2 BP	GO:0030595	leukocyte chemotaxis	164	0.763164784	2.653903	1.00E-10	6.41E-09	5.36E-09	1457	92	0.56097561	8.270753532
	3 BP	GO:0097529	myeloid leukocyte migration	157	0.764705641	2.645979	1.00E-10	6.41E-09	5.36E-09	1457	86	0.54777707	8.270753532
	4 BP	GO:0050776	regulation of immune response	457	0.689612673	2.639878	1.00E-10	6.41E-09	5.36E-09	1402	179	0.3916849	8.270753532
	5 BP	GO:0002250	adaptive immune response	256	0.719591097	2.633516	1.00E-10	6.41E-09	5.36E-09	1402	119	0.46484375	8.270753532
	6 BP	GO:0006941	striated muscle contraction	150	-0.714875271	-2.74878	1.00E-10	6.41E-09	5.36E-09	1155	61	0.40666667	8.270753532
	7 CC	GO:0030018	Z disc	108	-0.763253614	-2.78295	1.00E-10	6.41E-09	5.36E-09	1692	57	0.52777778	8.270753532
	8 CC	GO:0031674	I band	118	-0.772573628	-2.85689	1.00E-10	6.41E-09	5.36E-09	1020	50	0.42372881	8.270753532
	9 CC	GO:0043292	contractile fiber	200	-0.739998739	-2.95676	1.00E-10	6.41E-09	5.36E-09	1155	81	0.405	8.270753532
	10 CC	GO:0030017	sarcomere	168	-0.768016426	-2.97554	1.00E-10	6.41E-09	5.36E-09	1155	72	0.42857143	8.270753532
KEGG		ID	Description	setSize	enrichmentScore	NES	pvalue	p.adjust	qvalues	rank	count	GeneRatio	(-)LOG10(qvalues)
	1	mo04061	Viral protein interaction with cytokine and cytokine receptor	54	0.857751975	2.580386	1.00E-10	1.84E-09	1.29E-09	1274	42	0.77777778	8.89057343
	2	mo04610	Complement and coagulation cascades	54	0.819248085	2.464554	1.00E-10	1.84E-09	1.29E-09	902	32	0.59259259	8.89057343
	3	mo05152	Tuberculosis	137	0.720182397	2.443564	1.00E-10	1.84E-09	1.29E-09	1476	57	0.41605839	8.89057343
	4	mo04612	Antigen processing and presentation	60	0.796210676	2.415343	1.00E-10	1.84E-09	1.29E-09	1745	40	0.66666667	8.89057343
	5	mo04060	Cytokine-cytokine receptor interaction	168	0.696654888	2.412105	1.00E-10	1.84E-09	1.29E-09	1363	79	0.4702381	8.89057343
	6	mo04022	cGMP-PKG signaling pathway	137	-0.504111198	-1.91715	9.09E-07	7.16E-06	5.01E-06	1166	42	0.30656934	5.300051701
	7	mo05412	Arrhythmogenic right ventricular cardiomyopathy	54	-0.634212078	-2.08246	1.03E-05	6.99E-05	4.89E-05	1468	25	0.46296296	4.31058563
	8	mo04261	Adrenergic signaling in cardiomyocytes	130	-0.586202053	-2.20062	8.13E-10	1.17E-08	8.18E-09	1242	44	0.33846154	8.087188493
	9	mo05410	Hypertrophic cardiomyopathy	71	-0.686444768	-2.34037	2.76E-09	3.38E-08	2.37E-08	1069	31	0.43661972	7.625837896
	10	mo04260	Cardiac muscle contraction	70	-0.68777467	-2.34201	1.33E-09	1.83E-08	1.28E-08	1069	26	0.37142857	7.891880561

Downregulation of cardiac function and cardiac development terms were also evident in both comparisons. GO terms indicated as functionally downregulated by the analyses included “contractile fiber,” “striated muscle contraction,” and “sarcomere” in the adult vs neonatal comparison, and similarly “muscle cell development,” and “striated muscle development” were downregulated in the adult vs fetal analysis (Fig 6B & 6C). KEGG terms associated cardiac function including “cardiac muscle contraction” and “adrenergic signaling in cardiomyocytes” were similarly downregulated in both adult vs neonatal and adult vs fetal comparisons. This was supported by examination of core enriched genes for these ontologies, which recapitulated this downregulation of cardiac functional and developmental genes: in both cases genes such as *Myh6/7*, *Actn1*, *Actn2*, and *Actc1*, among several others, were identified (Table 3, Table 4, Additional File 4, Additional File 6).

Table 4: Ten most significant (based on q-value) GO and KEGG results from GSEA analysis of Adult vs. Fetal differential expression.

GO	ONTOLOGY	ID	Description	setSize	enrichmentScore	NES	pvalue	p.adjust	qvalues	rank	count	GeneRatio	(-)LOG10(qvalues)
	1 BP	GO:0045087	innate immune response	372	0.730172944	2.85648	1.00E-10	6.53E-09	5.38E-09	1383	171	0.45967742	8.268851562
	2 BP	GO:0002250	adaptive immune response	256	0.713069792	2.700958	1.00E-10	6.53E-09	5.38E-09	1713	127	0.49609375	8.268851562
	3 BP	GO:0050776	regulation of immune response	457	0.679065009	2.700829	1.00E-10	6.53E-09	5.38E-09	1369	180	0.39387309	8.268851562
	4 BP	GO:0097529	myeloid leukocyte migration	157	0.741205865	2.668663	1.00E-10	6.53E-09	5.38E-09	1405	83	0.52866242	8.268851562
	5 BP	GO:0002443	leukocyte mediated immunity	269	0.70320752	2.66696	1.00E-10	6.53E-09	5.38E-09	1363	123	0.45724907	8.268851562
	6 BP	GO:0055001	muscle cell development	181	-0.717141094	-2.72958	1.00E-10	6.53E-09	5.38E-09	1157	69	0.38121547	8.268851562
	7 BP	GO:0055002	striated muscle cell development	169	-0.729352454	-2.75649	1.00E-10	6.53E-09	5.38E-09	1157	66	0.39053254	8.268851562
	8 CC	GO:0031674	I band	118	-0.767269305	-2.76631	1.00E-10	6.53E-09	5.38E-09	1482	59	0.5	8.268851562
	9 CC	GO:0043292	contractile fiber	200	-0.734161239	-2.81494	1.00E-10	6.53E-09	5.38E-09	1190	78	0.39	8.268851562
	10 CC	GO:0030017	sarcomere	168	-0.761170516	-2.8709	1.00E-10	6.53E-09	5.38E-09	1190	70	0.41666667	8.268851562
KEGG		ID	Description	setSize	enrichmentScore	NES	pvalue	p.adjust	qvalues	rank	count	GeneRatio	(-)LOG10(qvalues)
	1	mo04061	Viral protein interaction with cytokine and cyt	54	0.837282252	2.570787	1.00E-10	1.74E-09	1.18E-09	904	37	0.68518519	8.928097603
	2	mo05133	Pertussis	63	0.803432005	2.521358	1.00E-10	1.74E-09	1.18E-09	1024	33	0.52380952	8.928097603
	3	mo05152	Tube rculosis	137	0.708294169	2.507483	1.00E-10	1.74E-09	1.18E-09	1272	54	0.39416058	8.928097603
	4	mo04060	Cytokine-cytokine receptor interaction	168	0.68397845	2.477031	1.00E-10	1.74E-09	1.18E-09	1578	90	0.53571429	8.928097603
	5	mo04145	Phagosome	131	0.703042923	2.468219	1.00E-10	1.74E-09	1.18E-09	1042	53	0.40458015	8.928097603
	6	mo04360	Axon guidance	150	-0.473361751	-1.75765	1.68E-05	0.000101	6.84E-05	2207	61	0.40666667	4.164645951
	7	mo05412	Arrhythmogenic right ventricular cardiomyopa	54	-0.678056354	-2.16521	4.68E-07	3.87E-06	2.62E-06	1289	22	0.40740741	5.581140681
	8	mo04261	Adrenergic signaling in cardiomyocytes	130	-0.600734177	-2.18366	3.93E-10	6.19E-09	4.19E-09	1261	46	0.35384615	8.377526534
	9	mo05410	Hypertrophic cardiomyopathy	71	-0.693004055	-2.31184	7.91E-10	1.09E-08	7.39E-09	1340	30	0.42253521	8.131305082
	10	mo04260	Cardiac muscle contraction	70	-0.724982175	-2.40669	1.00E-10	1.74E-09	1.18E-09	1336	31	0.44285714	8.928097603

Taken together, these data underscore a transcriptomic shift from more development-supporting

genes to predominantly immune- and inflammatory-specific genes. This shift is evident in comparison of young animal-sourced cardiac fibroblasts (NCF and FCF) to ACFs, but transcriptomic differences also exist between FCFs and NCFs as well.

RT-qPCR validates differential expression differences in CFs

Reverse transcriptase quantitative PCR (RT-qPCR) was used to analyze FCFs, NCFs, and ACFs in order to validate the RNA-seq results for selected genes. Results show relative expression normalized to GAPDH for ACFs and FCFs, normalized against results from NCFs. The genes evaluated were *Il6*, *Tbx5*, *Myh7*, *Mmp9*, *Mmp2*, and *Colla1*. *Il6* was selected as Interleukin-6 was shown to be comparatively upregulated in ACF vs NCF (Additional File 5) and is an important inflammatory cytokine. *Tbx5* (T-box transcription factor 5) is a critical cardiac development protein (31) and was observed to be upregulated in FCFs vs NCFs (Additional File 3). *Myh7* (encoding a myosin heavy chain β isoform) was observed to be markedly downregulated in ACFs vs NCFs (Additional File 5). *Mmp2*, *Mmp9*, and *Colla1*, encoding matrix metalloproteinase-2 and -9, and collagen type I, alpha-1, are all critical in cardiac matrix

regulation and crucial proteins expressed by CFs. Of these, *Mmp9* was shown to be upregulated in ACFs vs. NCFs, while *Mmp2* and *Col1a1* were observed to be marginally downregulated in both ACFs and FCFs compared to NCFs (Fig 7A).

Figure 7: RT-qPCR validation of differentially expressed genes in adult and fetal CFs, with respect to neonatal CFs. A) Log₂FC differential gene expression from RNA-seq data for genes selected for validation via qPCR. B) IL6 gene expression in adult CFs vs. fetal CFs, normalized to neonatal CF controls and relative to GAPDH expression. C-G) TBX5, MYH7, MMP9, MMP2, COL1A1 expression levels, respectively, in adult CFs vs. fetal CFs. Statistical analysis: one-tail t-test, with cutoff p-value threshold of 0.05. *=p<0.05, *=p<0.001, ns = no significance. Error bars indicate standard deviation.**

RT-qPCR results recapitulated these observed expressed differences in part: *Il6* had a relative (to NCF, normalized to GAPDH) expression value of 2.72 ± 0.54 (std. dev) in ACFs, vs expression in FCFs of 1.64 ± 1.67 ($p=0.1320$) (Fig. 5B). *Tbx5* was significantly less expressed (5.33 ± 1.65) in ACFs vs in FCFs (36.38 ± 28.04 , $p=0.0345$) (Fig. 7C) consistent with trends observed in the RNA-seq results, while *Myh7* showed variable, low expression in both cases (ACF: 0.96 ± 0.52 , FCF: 1.34 ± 0.58 , $p=0.1821$) (Fig. 5D). *Mmp9* (ACF: 29.06 ± 17.67 , FCF: 9.74 ± 3.33 , $p=0.0376$), *Mmp2* (ACF: 4.33 ± 0.58 , FCF: 1.76 ± 0.38 , $p=0.0002$), and *Col1a1* (ACF: $1.73 \pm$

0.10, FCF: 1.39 ± 0.30 , $p=0.0387$) all had significantly higher expression in ACFs vs FCFs (Fig. 7E, 7F, 7G).

Discussion:

Analysis of the cardiac fibroblast transcriptome in fibroblasts isolated from fetal, neonatal, and adult developmental ages indicates broad differences in gene expression with respect to age. This data offers new insights into the potential functions of CFs at these developmental ages while reinforcing age-specific gene expression differences in CFs and cardiac tissue that have been previously uncovered in the literature. More specifically, this data supports previous conclusions that indicate that fetal CFs present a gene expression profile reflecting functions in support of embryonic growth and cardiac development, whereas their adult CF counterparts upregulate genes more reflective of immune cell communication and trafficking, and cardiac homeostatic maintenance (19, 21, 31, 38, 39). Our data both supports and expands on these studies by incorporating neonatal CFs into the analysis to evaluate fetal-to-neonatal differences. This establishes a clearer timeline of the phenotypic shifts in CFs, from the primarily developmental role of fetal CFs (21, 38), transitioning to a phenotype associated with rapid expansion and maturation in the neonatal heart (40) to an adult phenotype characterized by immune signaling and tissue maintenance (24, 25, 40, 41).

RNA-seq confirms cardiac fibroblast immune function is age-dependent

Although historically the function of CFs was considered to exclusively ECM maintenance, it is now known these cells possess varied functions fundamental to cardiac function (24, 31, 38), and in particular are vital to cardiac immune and inflammatory processes. In this respect, CFs secrete

an array of growth factors, chemokines, and cytokines that facilitate invasion of immune cells such as macrophages and granulocytes (4, 15, 25, 41, 42), including MCP-1, MIP-1 α , MIP-1 β , IP-10, and RANTES (42, 43).

RNA-seq results in this study reinforces the hypothesis of an immune-regulatory role of cardiac fibroblasts, focusing on how this role varies across organismal development. The lack of a strong immune system in the fetal and neonatal mammalian heart is well-established and hypothesized to contribute to the regenerative wound healing observed in fetal and neonatal mammals (44, 45). It is similarly understood that maturation of the immune system and expression of inflammatory cytokines increases rapidly during post-natal development (46), but an in-depth examination of how the cardiac fibroblast transcriptome changes in an immune context, and how these changes may contribute to immune maturation in the heart has, to our knowledge, not been thoroughly explored. The transcriptomic data presented herein point toward more immune crosstalk and inflammatory signaling in CFs with respect to increasing developmental age, and is worth further investigation and validation. Understanding how CF impact inflammation and immunity at different developmental ages may support the development of regenerative therapies and engineered cardiac tissues by uncovering ways to minimize the tissue inflammatory response and aggressive immune response typical of fibrotic wound repair following injury in adults (11, 47, 48).

We observed an upregulation of genes responsible for macrophage invasion (*Ccl3/Ccl4*) (49) (Table 3, Table 4, Additional File 5, Additional File 7) with respect to developmental age, in addition to increases in the expression of inflammatory mediators (*Il-1 β* , *Il-6*) in adult CFs versus

neonatal and fetal CFs (Additional File 5, Additional File 7). Further, gene ontology analysis results support the hypothesis that immune and inflammatory gene expression differences exist between fetal and neonatal CFs, suggesting cardiac immune maturation progresses rapidly post-birth. These included fetal downregulation (compared to neonatal and adult CFs) of immune response-associated gene ontology terms (Fig 6A), downregulation of Nod-like and Toll-like receptor signaling pathways (Fig 6D), which have both been established as expressed in cardiac fibroblasts and are implicated in triggering an immune response to pathogen-associated molecular patterns (15, 42). We also observed a comparative upregulation of chemokines in adult CFs versus fetal CFs, including *Ccl3/4* immune-associated genes like *Ddx58* (an innate immune response receptor essential in identifying viral-infected cells (50) and *Lbp*, which binds to bacterial lipopolysaccharides and initiates an immune response (51) (Table 1).

Cardiogenic developmental/regenerative potential of cardiac fibroblasts is rapidly lost post-birth

Cardiac fibroblasts, like their counterparts in other bodily tissues, play a vital role in tissue morphology, extracellular matrix regulation and upkeep, mechanical support, and inflammatory response *in vivo* (15, 19, 26, 41, 52, 53). Beyond this, CFs are also implicated in the development and maturation of the heart *in utero* and assist in driving cardiomyocyte proliferation in that regard. This was well-established in work by Ieda *et al.* in 2008 (25), in studies that determined that fibroblast-derived proteins including fibronectin and periostin stimulated cardiomyocyte proliferation via β 1-integrin signaling.

It is well-understood that cardiomyocyte proliferation and cardiac regenerative wound healing diminishes rapidly post-birth (22, 24, 54), and is reflected in a reduced regenerative potential in cardiac fibroblasts, which begin to express a more pro-fibrotic phenotype (3, 55, 56). Our study reinforces the age-dependent transition to a fibrotic phenotype in CFs, and suggests that it begins rapidly after birth, as we observed significant downregulation of several immune-associated GO and KEGG pathways in fetal CFs versus neonatal CFs (Fig 6A & 6D, Additional File 2), implying neonatal CFs begin to upregulate genes necessary to mount significant immune recruitment to initiate an inflammatory response immediately after birth. Our data also indicated functional upregulation of genes associated with muscle cell development in fetal and neonatal CFs versus adult CFs (Fig 6B & 6C, Additional File 4, Additional File 6), which may support the conclusion that early-development CFs play a direct role in the functional development of cardiac tissue. This was particularly evident in FCFs, within which development-associated transcription factors including *Tbx4*, *Hoxb5*, and *Hoxb7* were upregulated, and ontological terms associated with morphogenesis and development were more functionally upregulated than their neonatal counterparts (Fig 6A, 6D, Table 1, Additional File 1). This builds upon previous work investigating transcription factors in cardiac fibroblasts, which concluded that cardiogenic genes including *Tbx20* and *Gata4* expressed by adult fibroblasts contribute to cardiac repair (31, 57). The presence of cardiomyocyte structural genes including *Actc1*, *Actn2*, *Myh6*, and *Myh7* was also observed to be upregulated in FCFs and NCFs versus ACFs, which was surprising as these proteins are conventionally considered cardiomyocyte-specific (57, 58). There is evidence CFs, particularly fetal-derived, express these genes at low levels: a previous microarray assay of fetal versus adult mouse cardiac fibroblast gene expression indicated similar differential expression of

these genes with expression levels consistent with what was observed in this study, specifically upregulation of *Actc1*, *Actn2*, *Myh6*, and *Myh7* in fetal CFs versus adult CFs (25).

Differences in CF expression of ECM and ECM-associated proteins is also strongly linked to the transition to pro-fibrotic wound healing in the heart. In previous studies of fetal versus adult cardiac fibroblasts, gene expression analysis indicated enrichment of fibronectin (*Fn*), collagen (*Col*) genes, heparin-binding EGF-like growth factor (HBEGF), periostin (*Postn*), and tenascin C (*Tnc*) in fetal cardiac fibroblasts but not adult cardiac fibroblasts, an expression profile associated with increased cardiomyocyte proliferation and regenerative healing (21, 25, 59). Within our differential expression analysis, we observed an upregulation of *Col9a1*, *Col26a1*, and *Col4a4*, and *Tnc* in fetal CFs vs neonatal CFs (Additional File 3). Of the collagens, both Col9 (60) and Col4 (61) have potential links to cardiac development. *Tnc*, the gene for protein tenascin-C, is predominantly expressed in embryonic tissue and implicated in tissue morphogenesis and remodeling via modulation of cell adhesion to ECM substrates (62). Taken together, this data provides more evidence that the loss a regenerative phenotype in CFs occurs rapidly after birth.

Study Limitations

This study contains several potential experimental limitations that should be addressed by future investigations. This study exclusively explores the transcriptomic profile of cardiac fibroblasts, and direct evaluations of genes identified must be performed to identify specific factors responsible for the phenotypic differences in CFs hypothesized. The protocols used to isolate cardiac fibroblasts from its resident tissue differed slightly across developmental ages: specifically, while neonatal and adult CFs were from ventricular tissue, fetal CFs were isolated

from the whole fetal heart, which was necessary to produce a sufficient cell yield. However, cardiac fibroblast phenotype can vary with respect to location in the heart (63), and incorporation of location-dependent CF RNA-seq analyses would likely be of value. Further, additional single-cell RNA-seq associated studies may be valuable to identify crucial cell-cell heterogeneity associated with CFs, and how this likely differs across developmental age and may be partly responsible for observations presented in this study (24, 32, 64). We also note that the qPCR work was completed with fibroblasts cultured *in vitro* at low passage numbers, versus the RNA-seq analysis which was done with confluent P0 CFs. This could explain why the gene expression in the qPCR study is not entirely consistent with the RNA-seq.

Conclusion:

This transcriptomic analysis of the rat cardiac fibroblast at three discrete developmental ages (fetal, neonatal, and adult) using RNA-seq provides significant insights into CF transcriptional heterogeneity. Immune function-related transcriptomic evaluations presented herein suggest age-specific CF heterogeneity may be partly responsible for differences in cardiac inflammation and immune response observed across developmental ages *in vivo*. The results reinforce established knowledge of fibroblast phenotypic heterogeneity across developmental ages, including marked upregulation of cytokines and chemokines in adult CFs vs fetal and neonatal CFs, and an age-dependent transition to a more pro-fibrotic, pro-inflammatory gene expression profile, versus the pro-regenerative gene expression observed in fetal and early-neonatal CFs. The results further suggest that this transition away from a developmental, pro-regenerative phenotype becomes acute quickly after birth.

Methods:

Heart harvest from fetal, neonatal, and adult rats

All animal procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Tufts University. Rat hearts harvested for cell isolation were from fetal, neonatal, and adult Sprague-Dawley rats. Pregnant dams (~3 months old) and adult rats (~7 weeks old) were deeply anesthetized with 3-5% isoflurane and euthanized via heart removal. Fetal pups (embryonic day 18, E18) were isolated from the uterus, euthanized by conscious decapitation and hearts were isolated. Neonatal pups (postnatal day 1, P1) were euthanized by conscious decapitation prior to heart harvest. Hearts were stored in an ice-cold solution of sterile phosphate-buffered saline (PBS) with 20 mM glucose and immediately used for cell isolation, as described below.

Primary cardiac fibroblast isolation and culture

Cardiac fibroblasts were isolated from fetal, neonatal, and adult rat hearts following previously described methods (65). After euthanasia and heart harvest, adult heart left ventricles were separated from the heart and minced to pieces less than 1 mm³ in size. Fetal and neonatal hearts were minced whole. Minced tissue underwent 3-5 serial digestions in collagenase type 2 (Worthington Biochemical Corp, Lakewood, NJ) in sterile PBS with 20 mM glucose, and passed through a 40 µm cell strainer and centrifuged. CFs were separated from other cardiac cells via collection as fast-adhering cells in a 1-hour pre-plating in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Invitrogen), and 0.1 mM ascorbic acid, followed by removal of nonadherent cells and reapplication of media (3, 25).

Fibroblasts for RNA-seq were expanded to confluency in culture and used immediately for RNA isolation. CFs for all other experiments were used at passage 2-4.

Immunofluorescence

Immunofluorescent imaging was used to validate that the cells used in culture were predominantly cardiac fibroblasts, and not from another nonmyocyte cell lineage. To confirm this, cells were stained for vimentin (a broad marker for fibroblasts but also expressed in endothelial cells (31, 32)), and either CD31 or CD45 to identify endothelial or hematopoietic cells, respectively (30, 31). CFs at passage 2-4 were seeded at a density of 50,000 cells/cm² for 24 hours, and then fixed for 10 minutes with ice-cold 4% paraformaldehyde. Cells were permeabilized with 0.05% Triton X-100 in PBS for 10 minutes at room temperature, rinsed in PBS, and blocked with 5% normal donkey serum and 1% bovine serum albumin (BSA) in PBS overnight at 4 °C. After blocking, cells were incubated with primary antibodies, against vimentin (Cell Signaling Technology, #5741, dilution 1:200), CD31 (Abcam, ab119339, dilution 1:500), and CD45 (Abcam, ab10558, dilution 1:250) diluted in 1% BSA in PBS overnight, again at 4 °C. Cells were rinsed in PBS with 0.1% Tween-20 (PBST) before incubation with fluorescently labeled secondary antibodies (ThermoFisher Donkey Anti-Rabbit 488-A21206, Donkey Anti-Mouse 488-A10037) for two hours, followed by Hoechst (10 µg/mL) and phalloidin (Invitrogen 588-A12380, diluted according to manufacturer's instructions, ~125ug/mL) for 30 minutes at room temperature. Cells were rinsed in PBST again and immediately imaged on a Keyence BZ-X710 fluorescent microscope.

RNA Preparation for Sequencing

P0 neonatal, fetal, and adult Sprague Dawley rat cardiac fibroblasts were cultured to confluency in T75 flasks, in serum-containing media with 0.1 mM ascorbic acid. Upon reaching confluent density, the cells were lysed in 1mL of ice-cold TRIzol reagent (Invitrogen) and RNA was isolated via manufacturer's instructions: briefly, 200uL of chloroform was added to each mL of TRIzol, mixed vigorously, incubated on ice for 2-5 minutes, and centrifuged at 4C at 12,000G for 15 minutes. The aqueous upper layer containing RNA was isolated and purified of chemical contaminants and DNA via processing in a SurePrep RNA Cleanup and Concentration Kit, and treatment with ThermoScientific RapidOut DNA Removal Kit, both following manufacturer's protocols. RNA was then quantified on a Nanodrop 2000 UV-vis spectrophotometer (ThermoFisher Scientific) and stored at -80C until further use.

RNA Sequencing

Purified RNA samples from fetal, neonatal, and adult rat hearts (n = 3 for each age group) were delivered to the Tufts University Genomics Core Facility for quality certification, cDNA library construction (Illumina TruSeq Stranded mRNA), and sequencing. Sequencing was performed on the Illumina HiSeq 2500 platform (50-bp single-end lane). Sequencing data in fastq format can be accessed in the Gene Expression Omnibus database (GEO) with the accession number GSE162277. The alignment of sequenced reads to the rat reference genome (Rn6 rat genome from the UCSC Genome Browser) was performed in several steps. First, sequenced data was adapter-trimmed using Trimmomatic 0.39 (66). Pre- and post-trimming, raw sequence data was quality validated using FASTQC (67). Trimmed and quality-assessed data was loaded into STAR (v. 2.5.2b) (68) for read alignment to the Rn6 rat genome. STAR-aligned reads stored as

BAM files loaded into featureCounts (69) for read counting. Raw counts were imported into R for differential gene expression analysis.

Differential Gene Expression Analysis

Raw counts were imported into R for gene expression analysis, using the *DESeq2* package, using the workflow described in the DESeq2 manual (70). Briefly, DESeq2 normalizes counts via a median of ratios method, in which counts are divided by sample-specific size factors determined by the median ratio of gene counts, relative to the geometric mean per gene (71). Principal component analysis (PCA) was performed with the plotPCA function in R, using natural log-transformed expression data from DESeq2. Normalized gene expression data from the three groups was output into a hierarchical clustering heatmap with the *pheatmap* package in R. These analyses were done to check for batch effects, and to broadly evaluate the gene expression data across the three age groups. Calculation of number of reads per sample (in millions of reads), correlation between samples, analysis of loadings of individual genes in the principal components, and clustering of individual genes with respect to principal components was performed in pcaExplorer (72), which utilizes the DESeq2-transformed data in its gene set analyses.

The neonatal cardiac fibroblast group (NCF) was assigned as the control condition for analysis, and differential expression comparisons to adult cardiac fibroblasts (ACF) and fetal cardiac fibroblasts (FCF) were calculated, although the comparison case ACF vs FCF was also analyzed and included in results. The full datasets pre-ranked by log₂FC were imported into the *clusterProfiler* package in R (34), which includes methods incorporating Gene Set Enrichment Analysis (GSEA) (33) and analyzed against Gene Ontology (GO) (35) and Kyoto Encyclopedia

of Genes and Genomes (KEGG) (36) databases to identify enriched gene sets and biological pathways. GSEA was run with gene set size thresholds of a minimum of 3 and maximum of 500, p-value cutoff of 0.05, and p-value adjustment using the Benjamini-Hochberg method (73). Separately, expression results were filtered against a cutoff $\log_2FC$ and adjusted p-value ($abs(\log_2FC) \geq 1.5$, $padj < 0.05$) to create datasets of significantly differentially expressed genes for downstream evaluation.

RT-qPCR Gene Expression Validation

CFs at passage 2-4 were lysed and RNA isolated and purified using a SurePrep DNA/RNA/Protein Concentration Kit, following manufacturer's protocols. RNA was quantified on a Nanodrop and stored at -80C until use. Following RNA purification, RNA was converted to cDNA using a ThermoFisher Scientific High Capacity cDNA Reverse Transcriptase Kit and cDNA was stored at -80C until further analysis. Reverse transcriptase quantitative PCR (RT-qPCR) was performed using an Applied Biosystems TaqMan Gene Expression Master Mix, carried out on a BioRad CFX96 Real-Time System using TaqMan PCR primers (ThermoFisher Scientific) according to manufacturer instructions using 50ug of cDNA per reaction. The genes assayed were COL1A1 (Collagen 1 $\alpha 1$ chain, Rn01463848_m1), MMP2 (matrix metalloproteinase 2, Rn01538170_m1), MMP9 (Rn00579162_m1), IL6 (interleukin 6, Rn01410330_m1), myosin heavy chain beta (MYC7, Rn01488777_g1), and GAPDH (Rn01775763_g1). Genes of interest were normalized to GAPDH expression, with comparative C_T used for analysis using neonatal cardiac fibroblasts as a control group.

Statistical Analysis

Statistical analysis of differential expression data was performed entirely in the R programming environment. As stated above, when identifying significantly expressed genes, a cutoff adjusted p-value of 0.05 was applied. For qPCR evaluation, statistical significance was determined via a one-tailed t-test, with significance set at $p < 0.05$. Statistical analysis and data visualization of qPCR data was done in the GraphPad Prism environment.

Additional Files:

Additional File 1: Top 10 most upregulated and most downregulated ($\log_2FC$) genes in each differential expression comparison, with associated functional descriptions.

Additional File 2: Full Lists of GSEA-identified GO and KEGG functionally enriched terms for Fetal vs. Neonatal comparison

Additional File 3: Full List of DESeq2-calculated differentially expressed genes in Fetal vs. Neonatal comparison.

Additional File 4: Full Lists of GSEA-identified GO and KEGG functionally enriched terms for Adult vs. Neonatal comparison

Additional File 5: Full List of DESeq2-calculated differentially expressed genes in Adult vs. Neonatal comparison.

Additional File 6: Full Lists of GSEA-identified GO and KEGG functionally enriched terms for Adult vs. Fetal comparison

Additional File 7: Full List of DESeq2-calculated differentially expressed genes in Adult vs. Fetal comparison.

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Abbreviations:

cDNA – complementary deoxyribonucleic acid
 CF – cardiac fibroblast
 CM – cardiomyocyte
 DAPI – 4',6-diamidino-2-phenylindole
 DDR2 – discoidin domain receptor 2
 DNA – deoxyribonucleic acid
 ECM – extracellular matrix
 FBS – fetal bovine serum
 GAPDH – glyceraldehyde 3-phosphate dehydrogenase
 GO – gene ontology
 IL – interleukin
 KEGG – Kyoto Encyclopedia of Genes and Genomes
 logFC – log fold change
 LPS – lipopolysaccharide
 MMP – matrix metalloproteinase
 MYH – myosin heavy chain

593 mRNA – messenger ribonucleic acid
 594 PBS – phosphate buffered saline
 595 PCA – principal component analysis
 596 RNA – ribonucleic acid
 597 RNA-seq – RNA sequencing
 598 RT-qPCR – reverse transcriptase quantitative polymerase chain reaction

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600 **References:**

- 601 1. Gwak SJ, Bhang SH, Kim IK, Kim SS, Cho SW, Jeon O, et al. The effect of cyclic strain on
 602 embryonic stem cell-derived cardiomyocytes. *Biomaterials*. 2008;29(7):844-56.
- 603 2. Parikh AS. Engineering cardiomyocytes from diverse human pluripotent stem cell lines for
 604 cardiac cell therapy with optimized cell yield and efficiency. 2014.
- 605 3. Li YA, Huda; Bursac, Nenad. Age-dependent functional crosstalk between cardiac fibroblasts and
 606 cardiomyocytes in a 3D engineered tissue. *Acta Biomaterialia*. 2017;55:120-30.
- 607 4. Fujii K, Nagai R. Contributions of cardiomyocyte–cardiac fibroblast–immune cell interactions in
 608 heart failure development. *Basic Research in Cardiology*. 2013;108(4):357.
- 609 5. Camelliti P, Borg TK, Kohl P. Structural and functional characterisation of cardiac fibroblasts.
 610 *Cardiovascular Research*. 2005;65(1):40-51.
- 611 6. Zhou P, Pu WT. Recounting Cardiac Cellular Composition. *Circulation research*. 2016;118(3):368-
 612 70.
- 613 7. Lajiness JD, Conway SJ. The Dynamic Role of Cardiac Fibroblasts in Development and Disease.
 614 *Journal of Cardiovascular Translational Research*. 2012;5(6):739-48.
- 615 8. Masur SK, Dewal HS, Dinh TT, Erenburg I, Petridou S. Myofibroblasts differentiate from
 616 fibroblasts when plated at low density. *Proceedings of the National Academy of Sciences of the United*
 617 *States of America*. 1996;93(9):4219-23.
- 618 9. Diaz-Araya G, Borg TK, Lavandero S, Loftis MJ, Carver W. IGF-1 Modulation of Rat Cardiac
 619 Fibroblast Behavior and Gene Expression is Age-Dependent. *Cell Communication & Adhesion*.
 620 2003;10(3):155-65.
- 621 10. Porter KE, Turner NA. Cardiac fibroblasts: At the heart of myocardial remodeling. *Pharmacology*
 622 *& Therapeutics*. 2009;123(2):255-78.
- 623 11. Shinde AV, Frangogiannis NG. Fibroblasts in myocardial infarction: a role in inflammation and
 624 repair. *J Mol Cell Cardiol*. 2014;70:74-82.
- 625 12. Pakshir P, Hinz B. The big five in fibrosis: Macrophages, myofibroblasts, matrix, mechanics, and
 626 miscommunication. *Matrix Biology*. 2018.
- 627 13. Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. *Pharmacol*
 628 *Ther*. 2009;123(2):255-78.
- 629 14. Turner NA. Inflammatory and fibrotic responses of cardiac fibroblasts to myocardial damage
 630 associated molecular patterns (DAMPs). *Journal of Molecular and Cellular Cardiology*. 2016;94:189-200.

15. Díaz-Araya G, Vivar R, Humeres C, Boza P, Bolivar S, Muñoz C. Cardiac fibroblasts as sentinel cells in cardiac tissue: Receptors, signaling pathways and cellular functions. *Pharmacological Research*. 2015;101:30-40.
16. Kawaguchi M, Takahashi M, Hata T, Kashima Y, Usui F, Morimoto H, et al. Inflammasome Activation of Cardiac Fibroblasts Is Essential for Myocardial Ischemia/Reperfusion Injury. 2011.
17. Crawford JR, Haudek SB, Cieslik KA, Trial J, Entman ML. Origin of Developmental Precursors Dictates the Pathophysiologic Role of Cardiac Fibroblasts. *Journal of Cardiovascular Translational Research*. 2012;5(6):749-59.
18. Liao B, Jackman CP, Li Y, Bursac N. Developmental stage-dependent effects of cardiac fibroblasts on function of stem cell-derived engineered cardiac tissues. *Scientific Reports*. 2017;7:42290.
19. Moore-Morris T, Cattaneo P, Puceat M, Evans SM. Origins of cardiac fibroblasts. *Journal of Molecular and Cellular Cardiology*. 2016;91:1-5.
20. Liao B, Jackman CP, Li Y, Bursac N. Developmental stage-dependent effects of cardiac fibroblasts on function of stem cell-derived engineered cardiac tissues. *Scientific Reports*. 2017;7(1):42290.
21. Jonsson MKB, Hartman RJG, Ackers-Johnson M, Tan WLW, Lim B, van Veen TAB, et al. A Transcriptomic and Epigenomic Comparison of Fetal and Adult Human Cardiac Fibroblasts Reveals Novel Key Transcription Factors in Adult Cardiac Fibroblasts. *JACC: Basic to Translational Science*. 2016;1(7):590-602.
22. Williams C, Quinn KP, Georgakoudi I, Black LD, 3rd. Young developmental age cardiac extracellular matrix promotes the expansion of neonatal cardiomyocytes in vitro. *Acta Biomater*. 2014;10(1):194-204.
23. Williams C, Black L, III. The Role of Extracellular Matrix in Cardiac Development. In: Suuronen EJ, Ruel M, editors. *Biomaterials for Cardiac Regeneration*: Springer International Publishing; 2015. p. 1-35.
24. Wang Y, Yao F, Wang L, Li Z, Ren Z, Li D, et al. Single-cell analysis of murine fibroblasts identifies neonatal to adult switching that regulates cardiomyocyte maturation. *Nature communications*. 2020;11(1):2585-.
25. Ieda M, Tsuchihashi T, Ivey KN, Ross RS, Hong T-T, Shaw RM, et al. Cardiac Fibroblasts Regulate Myocardial Proliferation through β 1 Integrin Signaling. *Developmental Cell*. 2009;16(2):233-44.
26. Frangogiannis NG. Cardiac fibrosis: Cell biological mechanisms, molecular pathways and therapeutic opportunities. *Molecular Aspects of Medicine*. 2019;65:70-99.
27. Sengupta P. The Laboratory Rat: Relating Its Age With Human's. *International journal of preventive medicine*. 2013;4(6):624-30.
28. Naito H, Melnychenko I, Didié M, Schneiderbanger K, Schubert P, Rosenkranz S, et al. Optimizing Engineered Heart Tissue for Therapeutic Applications as Surrogate Heart Muscle. 2006;114(1_supplement):I-72-I-8.
29. Zimmermann WH, Melnychenko I, Wasmeier G, Didié M, Naito H, Nixdorff U, et al. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nat Med*. 2006;12(4):452-8.
30. Moore-Morris T, Guimarães-Camboa N, Yutzey KE, Pucéat M, Evans SM. Cardiac fibroblasts: from development to heart failure. *Journal of Molecular Medicine*. 2015;93(8):823-30.
31. Furtado MB, Costa MW, Rosenthal NA. The cardiac fibroblast: Origin, identity and role in homeostasis and disease. *Differentiation*. 2016;92(3):93-101.
32. Tarbit E, Singh I, Peart JN, Rose'Meyer RB. Biomarkers for the identification of cardiac fibroblast and myofibroblast cells. *Heart Failure Reviews*. 2019;24(1):1-15.
33. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. 2005;102(43):15545-50.

34. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. 2012;16(5):284-7.
35. Consortium GO. The Gene Ontology (GO) database and informatics resource. Nucleic Acids Research. 2004;32(suppl_1):D258-D61.
36. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. 2000;28(1):27-30.
37. Fukata M, Vamadevan AS, Abreu MT. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) in inflammatory disorders. Seminars in Immunology. 2009;21(4):242-53.
38. Reiss K, Cheng W, Kajstura J, Sonnenblick EH, Meggs LG, Anversa P. Fibroblast proliferation during myocardial development in rats is regulated by IGF-1 receptors. American Journal of Physiology-Heart and Circulatory Physiology. 1995;269(3):H943-H51.
39. Lajiness JD, Conway SJ. Origin, development, and differentiation of cardiac fibroblasts. Journal of Molecular and Cellular Cardiology. 2014;70:2-8.
40. Souders CA, Bowers SLK, Baudino TA. Cardiac Fibroblast. The Renaissance Cell. 2009;105(12):1164-76.
41. Shinde AV, Frangogiannis NG. Fibroblasts in myocardial infarction: A role in inflammation and repair. Journal of Molecular and Cellular Cardiology. 2014;70:74-82.
42. Van Linthout S, Miteva K, Tschöpe C. Crosstalk between fibroblasts and inflammatory cells. Cardiovascular Research. 2014;102(2):258-69.
43. Xia Y, Pauza ME, Feng L, Lo DJ. RelB regulation of chemokine expression modulates local inflammation. 1997;151(2):375.
44. Haubner BJA-BMK, Sanjay; Tiefenthaler, Viktoria; Metzler, Bernhard; Aitman, Tim; Penninger, JM. Complete cardiac regeneration in a mouse model of myocardial infarction. Aging. 2012;4(12):966-77.
45. Haubner BJ, Schneider J, Schweigmann U, Schuetz T, Dichtl W, Velik-Salchner C, et al. Functional Recovery of a Human Neonatal Heart After Severe Myocardial Infarction. Circulation Research. 2016;118(2):216-21.
46. Sattler S, Rosenthal N. The neonate versus adult mammalian immune system in cardiac repair and regeneration. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2016;1863(7, Part B):1813-21.
47. Fivenson DP, Faria DT, Nickoloff BJ, Poverini PJ, Kunkel S, Burdick M, et al. Chemokine and inflammatory cytokine changes during chronic wound healing. Wound Repair and Regeneration. 1997;5(4):310-22.
48. Voss S, Krüger S, Scherschel K, Warnke S, Schwarzl M, Schrage B, et al. Macrophage Migration Inhibitory Factor (MIF) Expression Increases during Myocardial Infarction and Supports Pro-Inflammatory Signaling in Cardiac Fibroblasts. Biomolecules. 2019;9(2):38.
49. Maurer M, von Stebut E. Macrophage inflammatory protein-1. The International Journal of Biochemistry & Cell Biology. 2004;36(10):1882-6.
50. Identification of DDX58 and CXCL10 as Potential Biomarkers in Acute Respiratory Distress Syndrome. 2019;38(12):1444-51.
51. Jack RS, Fan X, Bernheiden M, Rune G, Ehlers M, Weber A, et al. Lipopolysaccharide-binding protein is required to combat a murine Gram-negative bacterial infection. Nature. 1997;389(6652):742-5.
52. Ravanti L, Toriseva M, Penttinen R, Crombleholme T, Foschi M, Han J, et al. Expression of human collagenase-3 (MMP-13) by fetal skin fibroblasts is induced by transforming growth factor β via p38 mitogen-activated protein kinase. The FASEB Journal. 2001;15(6):1098-100.
53. Sampson N, Koziel R, Zenzmaier C, Bubendorf L, Plas E, Jansen-Dürr P, et al. ROS Signaling by NOX4 Drives Fibroblast-to-Myofibroblast Differentiation in the Diseased Prostatic Stroma. Molecular Endocrinology. 2011;25(3):503-15.

54. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, et al. Transient Regenerative Potential of the Neonatal Mouse Heart. *Science*. 2011;331(6020):1078.
55. Lindsey ML, Goshorn DK, Squires CE, Escobar GP, Hendrick JW, Mingoia JT, et al. Age-dependent changes in myocardial matrix metalloproteinase/tissue inhibitor of metalloproteinase profiles and fibroblast function. *Cardiovascular Research*. 2005;66(2):410-9.
56. Brown RD, Ambler SK, Mitchell MD, Long CS. THE CARDIAC FIBROBLAST: Therapeutic Target in Myocardial Remodeling and Failure. *Annual Review of Pharmacology and Toxicology*. 2005;45(1):657-87.
57. Furtado MB, Costa MW, Pranoto EA, Salimova E, Pinto AR, Lam NT, et al. Cardiogenic Genes Expressed in Cardiac Fibroblasts Contribute to Heart Development and Repair. 2014;114(9):1422-34.
58. Quaife-Ryan GA, Sim CB, Ziemann M, Kaspi A, Rafehi H, Ramialison M, et al. Multicellular Transcriptional Analysis of Mammalian Heart Regeneration. *Circulation*. 2017;136(12):1123-39.
59. Hortells L, Johansen AKZ, Yutzey KEJ, disease. Cardiac Fibroblasts and the Extracellular Matrix in Regenerative and Nonregenerative Hearts. 2019;6(3):29.
60. Gil-Cayuela C, Rivera M, Ortega A, Tarazón E, Triviño JC, Lago F, et al. RNA sequencing analysis identifies new human collagen genes involved in cardiac remodeling. 2015;65(12):1265-7.
61. Pöschl E, Schlötzer-Schrehardt U, Brachvogel B, Saito K, Ninomiya Y, Mayer UJD. Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. 2004;131(7):1619-28.
62. Imanaka-Yoshida K, Hiroe M, Nishikawa T, Ishiyama S, Shimojo T, Ohta Y, et al. Tenascin-C Modulates Adhesion of Cardiomyocytes to Extracellular Matrix during Tissue Remodeling after Myocardial Infarction. *Laboratory Investigation*. 2001;81(7):1015-24.
63. Burstein B, Libby E, Calderone A, Nattel S. Differential behaviors of atrial versus ventricular fibroblasts: a potential role for platelet-derived growth factor in atrial-ventricular remodeling differences. *Circulation*. 2008;117.
64. Avery D, Govindaraju P, Jacob M, Todd L, Monslow J, Puré E. Extracellular matrix directs phenotypic heterogeneity of activated fibroblasts. *Matrix Biology*. 2018;67:90-106.
65. Au - Ye KY, Au - Sullivan KE, Au - Black LD. Encapsulation of Cardiomyocytes in a Fibrin Hydrogel for Cardiac Tissue Engineering. *JoVE*. 2011(55):e3251.
66. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014;30(15):2114-20.
67. Andrews S. FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom; 2010.
68. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)*. 2013;29(1):15-21.
69. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-30.
70. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*. 2014;15(12):550.
71. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biology*. 2010;11(10):R106.
72. Marini F, Binder H. pcaExplorer: an R/Bioconductor package for interacting with RNA-seq principal components. *BMC Bioinformatics*. 2019;20(1):331.
73. Benjamini Y, Hochberg YJ, JotRssB. Controlling the false discovery rate: a practical and powerful approach to multiple testing. 1995;57(1):289-300.