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RESEARCH LETTER

Defining the Fetal Gene Program at Single-Cell Resolution in Pediatric Dilated Cardiomyopathy

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A central dogma in cardiac biology is that the postnatal heart adopts a fetal-like transcriptional state in response to stress.[1] Consequently, the so-called "fetal gene program" is frequently used as a surrogate marker of adverse cardiac remodeling and heart failure in preclinical models.[2] Fetal gene reactivation in heart failure is traditionally studied in cardiomyocytes; however, the extent to which the fetal gene program is recapitulated in other cardiac cell types is unknown. Here we define the human fetal gene program at single-cell resolution in dilated cardiomyopathy (DCM), a common cause of heart failure in children and adults.

Single-nucleus RNA sequencing profiles of apical left ventricle tissue from fetal (19–20 weeks), nondiseased (ND; 4–14 years), and early-onset DCM samples (5–10 years) were captured (Figure [A]; sample processing and data analysis based on previous work[3]). Methodological details, sample details, source data (GSE185100), code, and quality control data including removal of doublets and ambient RNA are available at https://www.Heart-Explorer.org. Informed consent was obtained from all human subjects with approval from the Royal Children's Hospital Human Research Ethics Committee (HREC 38192 and HREC 36358).

On the basis of known marker genes,[3] single-nucleus RNA sequencing analysis revealed 7 cell clusters across fetal, ND, and DCM samples including cardiomyocytes, fibroblasts, immune cells, smooth muscle cells, endothelial cells, neurons, and pericytes (Figure [B and C]). Postnatal cardiac maturation was associated with a significant increase in the relative proportion of fibroblasts and immune cells, accompanied by a significant decrease in the proportion of cardiomyocytes (Figure [D]; Benjamini-Hochberg false discovery rate <0.05 using propeller function in speckle R package). No statistically significant shifts in cellular composition were observed between DCM and ND hearts (Figure [D]). However, DCM samples were characterized by lower average expression levels of immune markers, suggesting that dysregulation of the immune system may be a feature of pediatric DCM.

To identify transcriptional pathways perturbed in DCM, we used pseudo-bulk profiling to define differentially expressed gene sets across shared cell clusters (Figure [E through H]). DCM-related gene expression alterations were predominantly observed in cardiomyocytes, fibroblasts, and immune cells (Figure [E]). To define the fetal gene program, we identified differentially expressed genes that were developmentally regulated between fetal and ND (fetal versus ND, false discovery rate <0.05, $\log_2$fold change < −1.5 or >1.5). This gene set was intersected with DCM-regulated differentially expressed genes (DCM versus ND, false discovery rate <0.05, $\log_2$fold change < −1.5 or >1.5) across all cell clusters, which showed reactivation of fetal genes predominantly in cardiomyocytes and fibroblasts (Figure [F]). Only a small fraction (~8%) of the fetal gene program was reinstated in cardiomyocytes in DCM (163 of 2079 developmentally regulated genes; Figure [G]). The fetal gene program in DCM cardiomyocytes was involved

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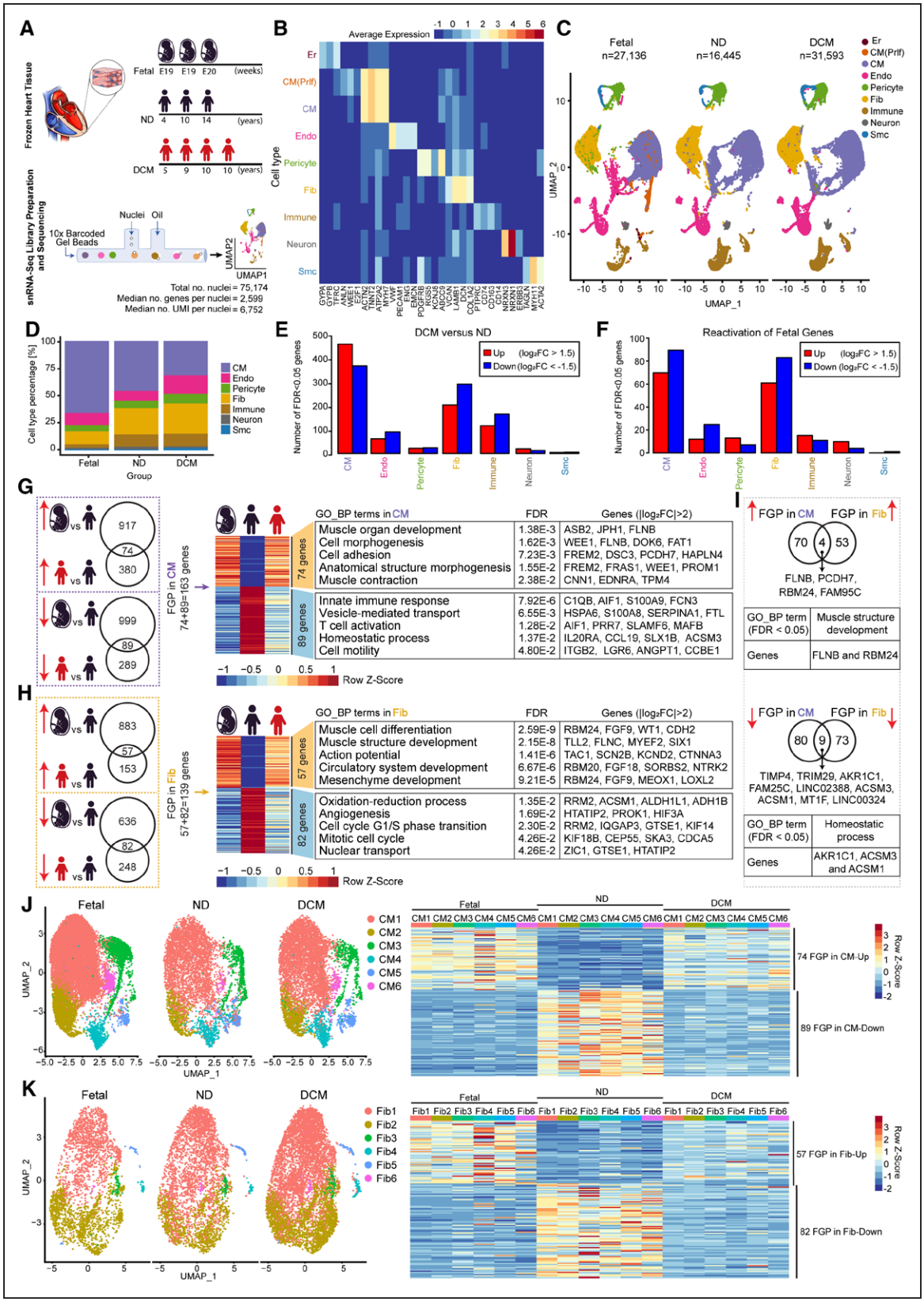

Figure. snRNA-seq analysis of fetal, pediatric nondiseased (ND) and pediatric dilated cardiomyopathy (DCM) hearts.
A, Summary of experimental design showing total number of biological samples and nuclei sequenced, as well as the median number of genes and unique molecular identifiers (UMI) detected per nucleus. **B**, Heatmap showing expression of cell type–specific marker genes across broad cell types. **C**, Uniform Manifold Approximation and Projection (UMAP) plot of nuclei showing distinct clusters of cardiac cell types across fetal, nondiseased (ND), and dilated cardiomyopathy (DCM) samples. **D**, Bar plot of cell type proportions for each group. (*Continued*)

Figure Continued. **E**, Bar plot showing the number of DE genes in each cell type that are upregulated (FDR <0.05, log_2FC >1.5, red) or downregulated (FDR <0.05, log_2FC < −1.5, blue) for DCM versus ND. FDR <0.05 with the correction procedure of Benjamini-Hochberg was used. **F**, Bar plot showing the number of reactivated fetal genes in each cell type that are upregulated (FDR <0.05, log_2FC >1.5, red) or downregulated (FDR < 0.05, log_2FC < −1.5, blue). FDR <0.05 with the correction procedure of Benjamini-Hochberg was used. **G**, Venn diagram showing the fetal gene program (FGP) in cardiomyocytes (CM; **left**) and heatmap illustrating the expression of the FGP in CM for each group (fetal, ND, DCM) including gene ontology terms for significantly regulated biological processes and associated genes with |log_2FC| >2 (**right**). **H**, Venn diagram showing the FGP in fibroblasts (Fib; **left**) and heatmap illustrating the expression of the FGP in Fib for each group (fetal, ND, DCM) including gene ontology terms for significantly regulated biological processes and associated genes with |log_2FC| >2 (**right**). **I**, **Top**, Venn diagram showing upregulated fetal genes that are shared between CM and cardiac Fib in DCM including gene ontology terms and associated genes for a significantly regulated biological process. **Bottom**, Venn diagram showing downregulated fetal genes that are shared between CM and cardiac Fib in DCM including gene ontology terms and associated genes for a significantly regulated biological process. **J**, UMAP plot showing 6 distinct subclusters of CM (CM1–CM6) from 3 groups (fetal, ND, DCM; **left**) and heatmap showing expression of the FGP across CM subclusters (**right**). **K**, UMAP plot showing 6 distinct subclusters of cardiac Fib (Fib1–Fib6) from 3 groups (fetal, ND, DCM; **left**) and heatmap showing expression of the FGP across Fib subclusters (**right**). CM (Prlf) indicates cardiomyocyte (proliferative); Endo, endothelial; Er, erythroid; FC, fold change; FDR, false discovery rate; pericyte; and Smc, smooth muscle cell.

in upregulation of genes involved in muscle development and downregulation of genes implicated in the innate immune response (Figure [G]). Similarly, in fibroblasts, ~8% of the fetal transcriptome was re-engaged in DCM (139 of 1658 developmentally regulated genes (Figure [H]). The transcriptional response of DCM fibroblasts was associated with upregulation of a muscle differentiation program and downregulation of oxidative metabolic processes and the cell cycle (Figure [H]). It is interesting that among the most highly upregulated (log_2fold change >2) fetal genes in DCM fibroblasts was *MEOX1* (Figure [H]), a transcription factor recently identified as a central regulator of myofibroblast activation in the heart.[4] In addition, a small subset of fetal genes (13 in total) overlapped between cardiomyocytes and fibroblasts in DCM (Figure [I]), suggesting this developmental transcriptional network is governed by shared regulatory mechanisms in both cell types.

Single-cell transcriptomics uniquely permits analysis of transcriptional heterogeneity across cell populations. We thus investigated whether the fetal gene program is broadly re-engaged in cardiomyocytes and fibroblasts or restricted to specific cell subpopulations in DCM. Subclusters of cardiomyocytes (Figure [J]) and fibroblasts (Figure [K]) were identified as previously described.[3] The fetal gene program was ubiquitously engaged across all subclusters of cardiomyocytes (Figure [J]) or fibroblasts (Figure [K]) in DCM, suggesting that this transcriptional program is not restricted to a specialized subpopulation of cells in the disease state.

The single-cell transcriptomic data sets provided here permit the identification of cell type–specific gene dysregulation in DCM. In contrast with a previous single-cell transcriptional analysis of pediatric DCM,[5] the current study included fetal and nondiseased control tissues, thus enabling precise cellular delineation of the fetal gene programs reactivated in DCM. Surprisingly, the phenomenon of fetal gene reactivation in DCM does not appear to be restricted to cardiomyocytes because a similar proportion of genes (<10%) adopts a fetal-like expression pattern in both cardiomyocytes and fibroblasts. This work provides a multicellular framework to identify the critical gene expression networks that underpin DCM disease pathogenesis in children. It will be important to determine whether these transcriptional mechanisms are also operative in other forms of heart failure including DCM in adults.

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Disclosures

Drs Porrello and Hudson are cofounders, are scientific advisors, and hold equity in Dynomics, a biotechnology company focused on the development of heart failure therapeutics. The other authors report no conflicts.

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