Single-Cell Transcriptional Profiling Reveals Cellular Diversity and Intercommunication in the Mouse Heart

Graphical Abstract

Highlights
- Comprehensive transcriptional profiling of non-myocyte cells in the adult mouse heart
- Detection of major cell types and characterization of transcriptional heterogeneity
- Development of tools for isolation of understudied cells, including mural cells and glia
- Characterization of intercellular communication networks

Authors
Daniel A. Skelly, Galen T. Squiers, Micheal A. McLellan, Mohan T. Bolisetty, Paul Robson, Nadia A. Rosenthal, Alexander R. Pinto

Correspondence
nadia.rosenthal@jax.org (N.A.R.), alex.pinto@jax.org (A.R.P.)

In Brief
Skelly et al. carry out comprehensive gene expression profiling of non-myocyte cells of the mouse heart. These data allow detection of a diverse and complex ensemble of cells characterized by specific gene expression signatures. They present an analysis of cell type markers, heterogeneity within cell types, and communication between cell types.

Data and Software Availability
E-MTAB-6173
Single-Cell Transcriptional Profiling Reveals Cellular Diversity and Intercommunication in the Mouse Heart

Daniel A. Skelly,1 Galen T. Squiers,1,7 Micheal A. McLellan,1,2,7 Mohan T. Bolisetty,3 Paul Robson,1,3,4 Nadia A. Rosenthal,1,2,3,5,6,8,* and Alexander R. Pinto1,5,8,9,*

1The Jackson Laboratory, Bar Harbor, ME, USA
2Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, MA, USA
3The Jackson Laboratory for Genomic Medicine, Farmington, CT, USA
4Department of Genetics and Genome Sciences, University of Connecticut, Farmington, CT, USA
5Australian Regenerative Medicine Institute, Monash University, Melbourne, VIC, Australia
6National Heart and Lung Institute, Imperial College London, London, United Kingdom
7These authors contributed equally
8Senior author
9Lead Contact
*Correspondence: nadia.rosenthal@jax.org (N.A.R.), alex.pinto@jax.org (A.R.P.)

https://doi.org/10.1016/j.celrep.2017.12.072

SUMMARY

Characterization of the cardiac cellulome, the network of cells that form the heart, is essential for understanding cardiac development and normal organ function and for formulating precise therapeutic strategies to combat heart disease. Recent studies have reshaped our understanding of cellular cardiac composition and highlighted important functional roles for non-myocyte cell types. In this study, we characterized single-cell transcriptional profiles of the murine non-myocyte cardiac cellular landscape using single-cell RNA sequencing (scRNA-seq). Detailed molecular analyses revealed the diversity of the cardiac cellulome and facilitated the development of techniques to isolate understudied cardiac cell populations, such as mural cells and glia. Our analyses also revealed extensive networks of intercellular communication and suggested prevalent sexual dimorphism in gene expression in the heart. This study offers insights into the structure and function of the mammalian cardiac cellulome and provides an important resource that will stimulate studies in cardiac cell biology.

INTRODUCTION

Recent studies have challenged assumptions about both the cellular composition (Pinto et al., 2016) and functional significance of the cardiac non-myocyte cell pool, with unexpected roles identified for resident fibroblasts (Furtado et al., 2014) and immune cell populations (Hulsmans et al., 2017; Monnerat et al., 2016). To acquire a high-resolution map of the non-myocyte cardiac cellulome, we prepared a single-cell suspension of viable, metabolically active, nucleated non-myocyte cells from heart ventricles of female and male mice for single-cell RNA sequencing (scRNA-seq) analysis. To minimize any potential alterations in transcriptional profiles due to enzymatic digestion and cell sorting (van den Brink et al., 2017), we chose a protocol with a relatively short 45-min dissociation incubation and endeavored to maintain cell suspensions on ice for all subsequent steps (Experimental Procedures). To avoid oversampling of endothelial cells, which form the largest cardiac cell population and principally comprise microvascular endothelial cells (Pinto et al., 2016), we reduced the proportion of endothelial cells to ~10% of total non-myocytes (Figure 1A). We obtained transcriptional profiles of single cells using the 10x Chromium platform and analyzed 10,519 cells that passed quality control and filtering, for which an average of 1,897 genes per cell were measured (Figure S1A).

To identify distinct cell populations based on shared and unique patterns of gene expression, we performed dimensionality reduction and unsupervised cell clustering using methods implemented in the Seurat software suite (Experimental Procedures; Butler and Satija, 2017; Macosko et al., 2015). This clustering approach requires some user specification of parameters, but is agnostic to known or predicted cell type markers. We identified 12 distinct cell clusters expressing known markers of major cell types (Figures 1B and 1C). The clusters comprised endothelial cells (Cd5h, Pecam1; Breviario et al., 1995; Newman et al., 1990), fibroblasts (2 clusters; Col1a1, Pdgfra, Tcf21; Acharya et al., 2012; Ivey and Tallquist, 2016), granulocytes (Ccr1, Csf3r, S100a9; Bonecchi et al., 1999; Panopoulos and Wato-wich, 2008; Ryckman et al., 2003), lymphocytes (3 clusters; Ms4a1, Cd3e, Kirb1c, Ncr1; Biassoni et al., 2003; Kuijpers et al., 2010; Ryan et al., 1992; Williams et al., 1987), pericytes (P2ry14, Pdgfrb; He et al., 2016), macrophages (Adgre1, Fcgfr1; Gautier et al., 2012), dendritic cell (DC)-like cells (Cd209a; Geijtenbeek et al., 2000), Schwann cells (Pp1, Cnp; Deng et al., 2014; Doerflinger et al., 2003), and smooth muscle cells (SMCs; Acta2, Tagln; Rensen et al., 2007). Prototypical markers that define cell populations (Figure 1C) included Col1a1 (fibroblasts), Ms4a1 (B lymphocytes), Pp1 (Schwann cells), and Acta2 (SMCs). We also identified additional genes that strongly
and specifically marked each major cell population (Figure 1D). Gene Ontology (GO) enrichment analysis of genes with restricted expression in each major cluster supported predicted identities of cell populations (Table S1). For example, fibroblast distinctness is driven by terms associated with extracellular matrix; leukocytes are associated with immune-regulatory terms; endothelial cells and SMCs are associated with vascular development and homeostasis terms; and Schwann cells are associated with neuro-regulatory terms.

To reveal heterogeneity within major cell types, we carried out subclustering of these populations (Figure 2A; Figures S1B and S1C). We used a similar unsupervised approach as described in the figure.
Figure 2. Major Cardiac Cell Types Harbor Subpopulations that Reflect a Hierarchy of Transcriptional Diversity

(A) Subclustering of lymphoid (top) and myeloid (bottom) cell populations reveals structure that is not apparent when clustering at a global level (Figure 1). Left panels depict cells positioned in t-SNE space with the populations of interest in color and all other cells in gray. Right panels are colored to reflect subcluster designations and show cells positioned in subcluster t-SNE space. The inset shows tertiary clustering of T cells to identify CD4+ and CD8+ T cell populations.

(B) Detailed map of cardiac cellular diversity after subclustering. Cells are colored according to subcluster designation.

(C) Gene expression gradients identified within macrophage/DC-like populations. The points show myeloid lineage cells positioned in t-SNE space identically to (A), lower right, with the more intense red hue indicating higher relative expression and gray signifying no expression.

(D) Identification of putative fibrocytes that exhibit gene expression signatures of both fibroblasts and macrophages. Violin plots display gene expression of canonical fibroblast (Col1a1, Pdgfra, and Tcf21) and macrophage/leukocyte (Fcgf1, Cd14, and Ptprc) genes.

(legend continued on next page)
above and analyzed cells of a single type or multiple related types (e.g., myeloid or lymphoid leukocytes) to discover cell subtypes (Experimental Procedures). For some cell populations, such as endothelial cells, Schwann cells, and SMCs, subclustering did not identify transcriptionally distinct subpopulations (Figure 2B), suggesting that these populations are relatively homogeneous in the mature heart. In contrast, multiple levels of subpopulation heterogeneity emerged within other cell populations, where subclustering clearly and distinctly separated identifiable populations. For example, subclustering of lymphoid cell populations revealed a population of group 2 innate lymphoid cells (ILC2s; Artis and Spits, 2015) expressing genes such as Gata3, Areg, and Rora, which previously clustered with T lymphocytes (Figures 1B, 2A, and 2B). Moreover, further subclustering of T lymphocytes alone produced 2 clusters corresponding to CD4+ and CD8+ T cells (Figure 2A, inset).

In some major clusters, transcriptional heterogeneity that differentiated subpopulations reflected continuous gradients rather than discrete groups of homogeneous cells with clear gene expression boundaries. For example, subclustering all myeloid populations together identified the granulocyte population detected at the global level (Figures 1B and 1C), but macrophage and DC-like subtypes were less clearly separable and were connected by transcriptional gradients (Figures 2A–2C; Figure S2). This presents a more nuanced picture of the macrophage transcriptional landscape than offered by the canonical subtyping of cardiac tissue macrophages based on markers, such as Cx3cr1, H2-AsAb (major histocompatibility complex [MHC] class II), Ccr2, Mrc1, and Adgre1 (F4/80; Epelman et al., 2014; Ilinykh and Pinto, 2017; Pinto et al., 2012). At least five subtypes of macrophages can be classified in our data on the basis of subtle differences in the expression of combinations of several genes (Figures 2A–2C), and in some cases, additional myeloid subpopulations can be discovered after further subclustering (e.g., DC–cell-like subtypes; Figure S2). Similar transcriptional continuities have also been identified in other tissues using scRNA-seq (Gokce et al., 2016). This blending of profiles to form intermediate populations could represent transcriptional heterogeneity that is buffered at the protein level. Alternatively, these hybrid expression signatures could reflect the multifaceted nature of macrophages, with cells poised to respond to a diverse range of stimuli.

Among macrophages, we discovered a subpopulation of cells that exhibit a hybrid molecular signature of both macrophages and fibroblasts, resembling a putative fibrocyte population with cells poised to respond to a diverse range of stimuli. Signatures could reflect the multifaceted nature of macrophages, buffered at the protein level. Alternatively, these hybrid expression continuities included epicardial cells marked by Tbx18, the expression of which was dispersed among cells falling within fibroblast and mural cell clusters (data not shown). Isl1 transcripts marking putative cardiac progenitors were present in 4 cells, but these were dispersed across various cardiac cell populations and did not form a distinct cluster (data not shown). Lymphatic endothelial cells expressing Prox1 and Podpl were also absent from the artificially reduced endothelial population, but these cells normally comprise only ~3% of total non-myocytes (Pinto et al., 2016). We also did not detect a discrete population of monocytes, which are present at low frequency and are likely dispersed among macrophage clusters. Overall, the absence of these cell populations is likely due to their relatively low abundance and lack of highly distinctive transcriptional signatures.

Mural cells (pericytes and SMCs) are essential components of vascular tissue (Armulik et al., 2011; Gaengel et al., 2009), but tools have been lacking for precise isolation of these cells by flow cytometry. Similarly, although Schwann cells are known to be present in the heart (Fregoso and Hoover, 2012), flow cytometric approaches to isolate these cells are limited. Commonly used markers, including Cspg4 (also called NG2), Pdgfrb, and Mcam, are relatively non-specific (Figure 3A); Cspg4 and Mcam are expressed in mural cells and Schwann cells, and Pdgfrb expression is detected in mural cells and fibroblasts (Figure 3A). To develop strategies to discriminate pericytes, SMCs, Schwann cells, and fibroblasts, we identified genes that showed higher expression in one of these cell types relative to the others.

(E) Identification of fibrocytes within the uninjured hearts of PDGFRαGFP+/+ mice, where GFP expression principally identifies fibroblasts. (F) ImageStream cytometry analysis of GFP+ cells from PDGFRαGFP+/+ mouse hearts labeling for MHC class II (H-2-AsAb), CD64 (FcgR1), and CD45 (Ptprc).

(G) Major cell populations contain minor subsets of cells expressing fibroblast-like gene signatures. Left: expression of Col1a1 (gray = low, red = high) in cells plotted in t-SNE space identical to (B), except the diameter of each point is also proportional to the expression level of Col1a1. Minor subsets of B cells, endothelial cells, macrophages, pericytes, Schwann cells, and SMCs expressing Col1a1 are circled. Right: dot plot shows the expression of a selection of cell-type-specific genes in subsets of cardiac cells. Data outlined in green highlight fibroblast-like cells in this groups by gating in silico on Col1a1 expression. Each row is based on a random sample of 35 cells from the cell type shown. Individual dot size and color reflects the proportion of each cell type expressing marker genes and the mean expression of the gene across all cells (indicated by the key).
using a likelihood-ratio-based test (McDavid et al., 2013) implemented in Seurat (Macosko et al., 2015). Genes identified using this approach with commercially available antibody reagents included *Itga7*, *Entpd1*, and *Cd59a* (Figure 3A, right; Table S2). Examination of resident mesenchymal cells (RMCs; Figure S3A) after ITGA7 staining enabled mural cells to be distinguished from fibroblasts using mEF-SK4 (Pinto et al., 2016) as a secondary marker. This approach identified 3 populations: R1 (mEF-SK4/hiITGA7/C0), R2 (mEF-SK4/intITGA7+), and R3 (mEF-SK4/loITGA7/C0) (Figure 3C). Analysis of PDGFRαGFP and CSPG4-GFP mice that mark expression in fibroblasts (Hamilton et al., 2003) and mural cells (Hughes et al., 2013), respectively (Figures 3B–D), revealed that almost all GFP+ RMCs in the PDGFRαGFP/+ mouse hearts are within R1, whereas almost all GFP+ RMCs in CSPG4-GFP mouse hearts are within R2 (Figure 3D). Conversely, analysis of GFP+ cells in R1–3 reveals that almost all R1 cells are GFP+ in PDGFRαGFP mouse hearts, whereas the majority of R2 cells are GFP+ in CSPG4-GFP mice (Figure 3C). Thus, cardiac fibroblasts (defined here as PDGFRα+ cells) can be identified and isolated with high precision using the mEF-SK4 and ITGA7 markers, where ITGA7 distinguishes mural cells from mEF-SK4low/int fibroblasts. Similar to ITGA7, we found that MCAM separates mural cells from fibroblasts, but also marks Schwann cells. In addition, we validated that ENTPD1...
Figure 4. A Dense Network of Autocrine and Paracrine Signaling Underpins Cardiac Homeostasis

Two fibroblast populations, as well as DC-like cells and macrophages, have ligand and receptor profiles that are substantially similar and are merged for clarity. (A) Capacity for intercellular communication between cardiac cell types. The line color indicates ligands broadcast by the cell population of the same color (labeled). Lines connect to cell populations where cognate receptors are expressed. The line thickness is proportional to the number of ligands where cognate receptors are present in the recipient cell population. Loops indicate autocrine circuits. Map quantifies potential communication, but does not account for anatomic position or boundaries of cell populations.

(B) Detailed view of ligands broadcast by each major cell population and those populations expressing cognate receptors primed to receive a signal. Numbers indicate the quantity of ligand-receptor pairs for each inter-population link.

(C) Production of essential growth factors by cardiac cell populations.

(D) Non-patrolling cardiac cell populations supporting macrophage growth by CSF1 or interleukin-34 (IL-34) production.

(legend continued on next page)
and CD59a identify SMCs and Schwann cells, respectively (Figures S3C and S3D). By combining these markers, we were able to identify and isolate SMCs, pericytes, and Schwann cells (Figures 3E and 3F).

To define intercellular communication networks within the cardiac celluome, we used a dataset of human ligand-receptor pairs (Ramilowski et al., 2015) to develop a list of mouse orthologs comprising 2,009 ligand-receptor pairs (Table S3; Experimental Procedures). Although anatomical barriers between cell types are not modeled in this analysis, expression patterns of ligand-receptor pairs in each cell type revealed a dense intercellular communication network (Figure 4A). GO enrichment analysis of ligands for which cognate receptors are present in cardiac non-myocytes revealed genes involved in cell positioning (locomotion, migration, etc.), expressed by non-patrolling cardiac cell populations (non-B and -T cells or granulocytes), and analysis of receptors showed enrichment for processes such as cell communication and signal transduction (Table S4). Broadcast ligands for which cognate receptors are detected within cardiac non-myocytes (Figure 4A) identified fibroblasts as the most trophic cell population with dense connections to multiple cell types (Figure 4B). These include signaling circuits that support survival of specific cardiac cell populations (Figure 4C). For example, fibroblasts and pericytes express Csf1 and Il34, respectively (Figure 4D), which signal through CSF1R and are essential factors for macrophage growth and survival. Fibroblasts also express growth factors Ngf, Vegfa, Igf1, and Fgf2 (Figure 4C), which support neurons of the autonomous nervous system, endothelial cells, and mural cells (Bach, 2015; Bikfalvi et al., 1997; Glebova and Ginty, 2004; Lohela et al., 2009).

To examine the capacity of cardiac cells to support cell types within the cardiac celluome, we cultured mixed cardiac cells and observed growth of disparate cell populations in vitro. Non-myocyte cultures from LysM-Cre:RosaZsGreen/+ mice (Clarke, 1993; Clausen et al., 1999) generated abundant macrophages labeled by ZsGreen fluorescent protein (Figures 4E and 4F) as well as endothelial cells (Figure 4G). However, macrophages failed to grow when cultures were treated with a CSF1R-blocking antibody (Śudo et al., 1995), synthetic CSF1R inhibitor GW2580 (Conway et al., 2005) (Figure 4H), or when cultured in normal growth media without non-macrophage cells (data not shown). Although these results indicate that fibroblasts support both cardiac macrophage and endothelial cell growth, non-fibroblast sources of essential growth factors point to the complexity of the network of cells that establish the cardiac niche and support resident cell populations.

Our analyses also show that diverse cell populations support nervous innervation of the heart. Consistent with recent findings that cardiac nerves develop along vasculature (Nam et al., 2013), we found that mural cells express Ngf and Ntf3, both important factors for axonal development (Figure 4C). Expression of Ngf and Ntf3 by cardiac pericytes highlights their potentially important role in the development of the autonomic nervous system in the heart. Moreover, cardiac Schwann cells, the myelin-producing cells of the peripheral nervous system, are a discrete cardiac cell population (Fregoso and Hoover, 2012). Thus, multiple cardiac cell types contribute to supporting the development, maintenance, and signal transduction of autonomic nerves.

To survey sexual dimorphism in cardiac non-myocyte gene expression, we segregated female and male cells based on expression of female- (Xist) and male-specific genes (6 Y chromosome genes: Ddx3y, Eif2s3y, Erdr1, Gm29650, Kdm5d, and Utv; Figure 5A). Highly similar clustering patterns for female and male cells were observed at the global level, but within individual cell types, we identified many genes that appeared to show sexual dimorphism in their expression (Figure 5A). Of the 396 genes upregulated in female cells and the 430 genes upregulated in males (Experimental Procedures), the vast majority (~97%) were differentially expressed below 2-fold (Figure 5B), with a median fold difference of 1.17 for both females and males. Our analysis is underpowered due to comparisons based on 2 biological replicates of each sex, but these findings present an opportunity to characterize broad sex-dependent shifts in transcriptional networks and to identify candidate sexually dimorphic genes. Specific mechanisms underlying sexual dimorphism in gene expression are likely to be diverse and encompass both subtle shifts in the composition of cell subtypes as well as regulatory responses to hormonal cues or trans-acting sex chromosome factors.

Analysis of the most highly sexually dimorphic genes for each cell population provided evidence that the extent and direction of sexual dimorphism in gene expression is cell-type dependent (Figure 5C; Table S5). Furthermore, we identified 27 genes that exhibited discordant patterns of sexually dimorphic expression between cell types (Figure 5D), revealing the dichotomous effect of biological sex on gene expression in the heart. Among male macrophages, we found evidence that male-upregulated genes tend to play a role in responding to foreign antigens, with significant GO enrichment for terms including antigen processing and presentation via MHC class II molecules as well as the broader immune response (adjusted p < 0.001; Table S6). In contrast, female-upregulated genes in macrophages were enriched for processes involving response to stress and the electron transport chain (Table S6).

These results support epidemiological and experimental data documenting sexually dimorphic responses to cardiac insults (Blenck et al., 2016). Females exhibit cardioprotection with decreased neutrophil infiltration and reduced cardiomyocyte death (Cavasin et al., 2006; Fang et al., 2007), whereas the present analysis reveals that the male cardiac celluome is particularly geared toward sensing inflammatory cues. We observe
sexual dimorphism in cardiac tissue macrophage transcriptional profiles, such as male upregulation of *Irf8*, which is linked to chronic inflammation (Langlais et al., 2016). Conversely, the most upregulated gene in female macrophages and the most sexually dimorphic macrophage gene in both sexes (Figure 5C) is *Tsc22d3* (also known as Gilz), a transcription factor implicated in anti-inflammatory functions and a downstream driver of the potent anti-inflammatory effects of glucocorticoids (Eddleston et al., 2007; Ronchetti et al., 2015; Sevilla et al., 2015). The functional significance of this gene has been demonstrated by the transplantation of cells overexpressing *Tsc22d3* to infarcted murine hearts, which results in dampened inflammation and reduced cardiomyocyte cell death (Baban et al., 2017). However, it is unlikely that macrophage *Tsc22d3* expression alone confers the anti-inflammatory phenotype in female hearts, which also have elevated expression of other genes implicated in anti-inflammatory mechanisms, such as *Cebpb* (Ruffell et al., 2009) (Figure 5C).

This study builds on previous explorations of cardiac cellular diversity (DeLaughter et al., 2016; Pinto et al., 2016) and provides unique insights into the structure and function of the cardiac cellulome. We have characterized single-cell transcriptional profiles of major non-myocyte cell populations within the mouse heart. We observed a dense ligand-receptor network facilitating extensive communication between diverse cell types and documented a contribution of biological sex to the transcriptional programs that govern organ function. An important aspect of the dataset presented here is its utility for identifying and studying cardiac cells. Here, we used our scRNA-seq dataset to develop strategies to more precisely identify cardiac mural cells, fibroblasts, and Schwann cells using flow cytometry. Our results will stimulate research into new avenues of inquiry in cardiac cell biology and provide a resource for in-depth interrogation of the cell-type-specific transcriptional networks that underpin heart development, homeostasis, and disease.

**EXPERIMENTAL PROCEDURES**

Further details and an outline of resources used in this work can be found in Supplemental Experimental Procedures.

**Study Animals and Cardiac Single-Cell Preparation**

All experiments were conducted using C57BL/6J mice (*Mus musculus*; with the exception of NGS2-GFP mice, which were on an FVB background) from...
The Jackson Laboratory that were fed a standard diet ad libitum prior to tissue isolation. All procedures conducted on mice were approved by The Jackson Laboratory Institutional Animal Care and Use Committee (IACUC). Four mice (2 female and 2 male) at 10 weeks of age were used to prepare cardiac single-cell preparations for RNA sequencing. All other mice were between 10 and 15 weeks of age and of varied sex. For lineage reporting experiments PDGFRα-GFP (Hamilton et al., 2003), CSPG4-GFP (Hughes et al., 2013), CNP-mEGFP (Deng et al., 2014) or LysM-Cre/Rosa26Raih1tm mice (Clarke, 1993; Clausen et al., 1999) were used.

Single-cell suspensions from isolated mouse hearts were prepared by tissue mincing and enzyme digestion as previously described (Pinto et al., 2013, 2016). Briefly, mice were euthanized and subsequently perfused, hearts were isolated, and ventricles were minced. Minced tissue was digested with Collagenase IV and Dispase II, filtered, and resuspended as described in Supplemental Experimental Procedures. Vitality dye/antibody staining was carried out using standard procedures (Supplemental Experimental Procedures).

Sequencing and Statistical Methods for Analysis of scRNA-Seq Data
We prepared cells from 2 female and 2 male mouse hearts and then performed flow cytometry to sort metabolically active and nucleated cells and to artificially deplete endothelial cells. We pooled cells from the first female and male and from the second female and male. We loaded ~12,000 cells into 1 channel of the Chromium system for each of these 2 pooled samples and prepared libraries according to the manufacturer’s protocol (10x Genomics) and then performed Illumina sequencing, as detailed in Supplemental Experimental Procedures.

We used Cell Ranger version 1.3 (10x Genomics) to process raw sequencing data and the Seurat suite version 2.0.0 (Butler and Satija, 2017; Macosko et al., 2015) for downstream analysis. For clustering, we first reduced dimensionality by principal-component analysis (PCA). We selected variable numbers of principal components (PCa) using either a permutation-based test or heuristic methods implemented in Seurat and performed clustering using methods implemented in Seurat. We used a likelihood ratio-based test (McDavid et al., 2013) or an area-under-the-curve-based scoring classifier, as implemented in Seurat (Macosko et al., 2015), to identify genes whose expression was enriched in specific clusters. To identify genes showing evidence for sexual dimorphism in gene expression, we used a hybrid 3-test approach in an attempt to overcome weaknesses of individual tests. Detailed descriptions of all statistical procedures are provided in Supplemental Experimental Procedures.

DATA AND SOFTWARE AVAILABILITY
The accession number for the data reported in this study is ArrayExpress: E-MTAB-6173.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, three figures, and six tables.

ACKNOWLEDGMENTS
The authors are grateful for assistance with library preparation and sequencing from The Jackson Laboratory Genomics Technologies facility. A.R.P. was supported by American Heart Foundation grant 17IR03320004. The Australian Regenerative Medicine Institute was supported by grants from the State Government of Victoria and the Australian Government.

AUTHOR CONTRIBUTIONS
D.A.S. and A.R.P. wrote the manuscript with input from all co-authors. A.R.P. designed the experiments with technical assistance from P.R. and D.A.S. M.T.B. analyzed the raw data with supervision from P.R. G.T.S., M.A.M., and A.R.P. carried out cell preparation for RNA-seq and conducted the flow cytometry experiments. M.A.M. and A.R.P. carried out the cell culture experiments. A.R.P., G.T.S., and M.A.M. analyzed the flow cytometry data. D.A.S. analyzed all scRNA-seq data. N.A.R. and A.R.P. provided supervision and guided presentation of the data. A.R.P. conceived the project.

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: October 12, 2017
Revised: November 22, 2017
Accepted: December 20, 2017
Published: January 16, 2018

REFERENCES


