A Transcriptomic and Epigenomic Comparison of Fetal and Adult Human Cardiac Fibroblasts Reveals Novel Key Transcription Factors in Adult Cardiac Fibroblasts

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VISUAL ABSTRACT

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SUMMARY

Cardiovascular disease remains the number one global cause of death and presents as multiple phenotypes in which the interplay between cardiomyocytes and cardiac fibroblasts (CFs) has become increasingly highlighted. Fetal and adult CFs influence neighboring cardiomyocytes in different ways. Thus far, a detailed comparison between the two is lacking. Using a genome-wide approach, we identified and validated 2 crucial players for maintaining the adult primary human CF phenotype. Knockdown of these factors induced significant phenotypical changes, including senescence and reduced collagen gene expression. These may now represent novel therapeutic targets against deleterious functions of CFs in adult cardiovascular disease. (J Am Coll Cardiol Basic Trans Science 2016;1:590–602) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Although cardiomyocytes (CMs) occupy most of the tissue volume and provide the mechanical force delivered by the heart, they are largely outnumbered by nonmyocyte cells (30% vs. 70%), part of which are cardiac fibroblasts (CFs) (1,2). Cross-sectional confocal microscopy of ventricular tissue reveals that each CM is in the direct vicinity of at least 1 CF (3), reflecting a significant role for CFs in the heart; that is, to create and hold a supportive environment for CMs, such as by regulation of the extracellular matrix (ECM). More than simply a “scaffold cell,” CFs are understood to communicate with CMs in 3 different ways. The first method is through direct cell-to-cell contact, in which the formation of adherens junctions (cadherins) and gap junctions (connexins) play a crucial role (4). The second method is by paracrine or autocrine secretion of growth factors such as FGF-2/basic FGF and transforming growth factors, such as IL-6 and transforming growth factor-β (9).

Fibroblasts are abundant throughout all tissues in the body, and the population is heterogeneous, with diverse appearances and functions depending on where the cells reside (10). Apart from the expression of common core fibroblast genes, a specific gene expression profile involving the cardiogenic transcriptional network has been described uniquely for CFs (11). Furthermore, regional differences exist in which CFs from the atrium and the ventricle express different cardiogenic transcription factors (TFs) (11,12). Important differences have also been found between rat CFs from the embryonic heart compared with the adult heart, with a differential response in insulin-like growth factor-induced collagen production (13). The influence of CFs on co-cultured CMs also varies depending on age. Embryonic CFs increase proliferation of CMs, whereas adult CFs induce hypertrophy (6). Notably, this finding is consistent with the growth of a fetal or neonatal heart, which depends on the proliferation of CMs, whereas the adult heart responds to stress by hypertrophy. The diversity of the CF population is also underlined by the finding that CF may be derived from at least 3 sources: from the proepicardium, from endocardial- or epicardial-to-mesenchymal transformation (EMT), and from bone marrow (14–16).

Despite increasing attention to the potential role that CF may play in novel disease therapeutics (17), a detailed genome-wide characterization of the genetic and epigenetic profiles of CF has yet to be performed. In the present study, we conducted next-generation sequencing experiments with primary ventricular fetal human cardiac fibroblasts (fHCFs) and adult human cardiac fibroblasts (aHCFs) to carefully map their respective transcriptomic and epigenomic...
profiles, with the aim of unraveling key players responsible for maintaining the CF phenotype.

**METHODS**

Detailed Methods are available in the Supplemental Appendix. aHCFs were purchased from PromoCell (Heidelberg, Germany; catalog no. C-12375, lot no. 3042901.1, lot no. 3042902.1, and lot no. 1051601.5), and fHCFs were purchased from Cell Applications, Inc. (San Diego, California; catalog no. 306-05g, lot no. 1916, and lot no. 2112). The fibroblasts were of ventricular origin, and all donors were free of cardiac disease. Profiling experiments were performed with human cardiac fibroblasts (HCFs) between passages 3 and 4. Cellular, molecular, and next-generation sequencing experiments were performed as described in the Supplemental Appendix. Primer

| Company               | Catalog No./Lot No. | Ethnicity | Sex       | Age          | Clinical Profile            | Site of Isolation |
|-----------------------|--------------------|-----------|-----------|--------------|-----------------------------|-------------------|
| fHCF                  |                    |           |           |              |                             |                   |
| Cell Applications, Inc.| 306-05f/1916       | Black     | Female    | 16 weeks gestation | Normal human fetal heart    | Ventricle         |
| Cell Applications, Inc.| 306-05f/2112       | Unknown   | Female    | 21 weeks gestation | Normal human fetal heart    | Ventricle         |
| aHCF                  |                    |           |           |              |                             |                   |
| PromoCell             | C-12375/3042901.1  | White     | Male      | 54 yrs       | Histologically normal tissue| Ventricle         |
| PromoCell             | C-12375/397Z030.3  | White     | Female    | 30 yrs       | Histologically normal tissue| Ventricle         |
| PromoCell             | C-12375/1051601.5  | White     | Male      | 48 yrs       | Histologically normal tissue| Ventricle         |

Isolation methods are proprietary.

aHCFs = adult human cardiac fibroblasts; fHCFs = fetal human cardiac fibroblasts.
sequences for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) are listed in Supplemental Table 1.

**STATISTICAL ANALYSIS.** Ribonucleic acid-seq (RNA-seq) data were aligned to the Hg19 reference genome by using TopHat version 2.0.11 (Center for Computational Biology at Johns Hopkins University, Baltimore, Maryland). The transcriptome was assembled by using Cufflinks version 2.2.1 (Laboratory for Mathematical and Computational Biology at UC Berkeley; Computational Genomics at the Institute of Genetic Medicine at Johns Hopkins University, Baltimore, Maryland; Caltech, Pasadena), and differential expression of genes was analyzed in GENCODE gene annotation version 16 (Wellcome Trust Sanger Institute, Cambridge). Differentially expressed genes were selected on the basis of q value (false discovery rate-corrected p value) and log2fold-change produced by Cuffdiff. Chromatin immunoprecipitation-sequencing (ChIP-seq) and assay for transposase accessible chromatin (ATAC-seq) peaks were called by using DFilter (18). Filter types for H3K4me3, H3K27me3, and ATAC were as follows: 8 to 10 kb, -ks = 100, -bs = 100; 1.5 to 3 kb, -ks = 25, -bs = 100; and 4 to 5 kb, -ks = 50, -bs = 100, respectively. Expected peak cutoff p values for H3K4me3, H3K27me3, and ATAC were set at 1E-6, 1E-3, and 1E-2 according to Dfilter recommendations. Peak annotation was performed by using ChiPpeakAnno (19). Known motifs and de novo motifs from ChIP-seq data were obtained by HOMER Motif Analysis (20).

Two-tailed unpaired Student t tests were used to assess significance in the RT-qPCR and immunostaining analysis. Results are presented as mean ± SD, and statistical significance is denoted in the graphics as p ≤0.05.

**RESULTS**

**CELLULAR CHARACTERIZATION OF CFs.** fHCFs and aHCFs were characterized with the use of immunocytochemistry and flow cytometry. Clinical characteristics of the cell isolates are listed in Table 1. Immunocytochemical labeling was performed for vimentin and Ki67 (proliferation marker). Representative images are shown in Figure 1A and are quantified in Figure 1B. Image analysis of more than 2,000 cells in independent cultures showed that fHCFs were consistently smaller than aHCFs (802 ± 46 μm² vs. 2,127 ± 131 μm²) and proliferated faster. Ki67-positive cells for fHCFs and aHCFs were 63% and 52%, respectively, representing higher proliferation in the former. A higher turnover of fHCFs compared with aHCFs was also substantiated by a shorter doubling time of 34.6 h and 68.9 h, respectively (data provided by the supplier). More than 98% of cells were α-SMA negative (Supplemental Figure 1), ensuring that the majority of the study CFs were not activated to myofibroblasts by stress conditions of cell culture. Flow cytometry results proved that both fHCFs and aHCFs expressed THY-1 and PDGFRA. Moreover, FACS also allowed us to exclude the possibility that the study cells were contaminated with other cells of endothelial (PECAM1 negative) or hematopoetic (CD45 negative) origin (Figure 1C).

**TRANSCRIPTOMIC CHARACTERISTICS.** RNA-seq was performed for both fHCFs and aHCFs (n = 3) (Supplemental Figure 2A). We used the stringent cutoff of fragments per kilobase per million fragments mapped >1 to call genes expressed in both aHCFs and fHCFs. A total of 13,400 genes were expressed in both, with 1,639 and 1,354 being exclusively expressed in fHCFs and aHCFs, respectively (Figure 2A). Thus, aHCF and fHCF transcriptomes exhibited marked similarities, raising the important question of whether specific transcriptomic differences might define their unique phenotypic characteristics. Figure 2B shows a heat map of exclusively expressed genes, which expectedly segregate the 2 sets of fHCF and aHCF samples. Supplemental Appendix 2 lists all genes expressed in the 2 sets of HCFs and their corresponding expression values. Correlation comparisons between sample replicates in Figure 2C corroborate the distinction between aHCFs and fHCFs (correlation of fHCF replicates in blue is ~0.98, and the same for aHCF replicates in red, contrasting against lower correlations compared across fHCFs and aHCFs in purple).

We next sought to interpret gene expression signatures that distinguished between fHCFs and aHCFs and started by performing Ingenuity Pathway Analysis (IPA, Qiagen GmbH, Hilden, Germany). Supplemental Table 2 displays the highest significant genes with a log fold-change aHCF > fHCF of >4 or <4. Using the list of differentially expressed genes, a selection was made according to manual curation for relevance on the basis of results of a thorough literature search. Selected genes were validated by using RT-qPCR (Supplemental Figure 2B) and biologically replicated in 3 additional independent HCF cell isolates (2 for adult and 1 for fetal) (Supplemental Figure 2C). Mean gene expression in aHCFs versus fHCFs by using RT-qPCR supported the RNA-seq data for all 24 genes tested.
FIGURE 2  Similarities and Differences Between the Transcriptomes of fHCFs and aHCFs

A

B

C

D

E

Continued on the next page
The top 1,000 differentially expressed genes on the basis of their log-fold difference were fed into IPA to perform a comparison analysis. Canonical pathway analysis of the lists revealed that genes from ephrin receptor signaling, IL-8 signaling, and Notch signaling were statistically enriched in the fetal transcriptome, whereas IL-6 signaling, among others, was enriched in the adult transcriptome. Figure 2D provides the IPA summary for the top canonical pathways and the physiological system development and functions with their respective p values. Both transcriptomes contained genes involved in tissue development and cardiovascular system development, potentially representing a common phenotype for CFs regardless of adult or fetal origins. In contrast, immune cell trafficking was an aHCF hallmark, and embryonic development was an fHCF hallmark. Gene ontology analysis for molecular functions, cellular processes, and biological processes was also consistent with the IPA pathways, and results are listed in Supplemental Figure 3. All together, we curated a list of genes to

**Figure 2 Continued**

**A** A Venn diagram of genes expressed in aHCFs (orange) and fHCFs (blue). **B** Heat map of genes exclusively expressed segregating fHCFs from aHCFs. **C** Correlation between replicates of independent fHCF and aHCF samples (fHCF replicates in blue box, aHCF in red box, and fHCF–aHCF cross-comparison in purple box). **D** Top 5 hits of both canonical pathways analysis and physiological system development and function for transcriptomes of both aHCFs and fHCFs, with p values determined by using Ingenuity Pathway Analysis (Qiagen GmbH, Hilden, Germany). **E** Heat map showing representative genes that are commonly expressed in both fHCFs and aHCFs, as well as genes exclusively expressed in each. fpkm = fragments per kilobase of exon per million fragments mapped; IL = interleukin; other abbreviations as shown in Figure 1.
**Table 2: Summary of 10 Representative Genes, Their Expression Level (RNA-Seq), and Chromatin Profiles (ATAC-Seq, H3K4me3, and H3K27me3)**

| Gene   | HCF RNA-Seq | ATAC-Seq Open Chromatin | H3K4me3 Active Promoter | H3K27me3 Repressed Genomic Domain |
|--------|-------------|--------------------------|--------------------------|----------------------------------|
| VIM    | Fetal       | +++                      | +++                      | -                                |
|        | Adult       | +++                      | +++                      | +                                |
| POSTN  | Fetal       | +++                      | +/−                      | +/−                              |
|        | Adult       | +++                      | +/−                      | +/−                              |
| HBEGF  | Fetal       | +/−                      | +/−                      | +/−                              |
|        | Adult       | +/−                      | +/−                      | +/−                              |
| IL6    | Fetal       | +++                      | +++                      | +                                |
|        | Adult       | +++                      | +++                      | +                                |
| FGF7   | Fetal       | −                        | −                        | −                                |
|        | Adult       | +                        | −                        | −                                |
| ITGA8  | Fetal       | −/−                      | +                        | +++                              |
|        | Adult       | +/−                      | ++                      | +++                              |
| ELN    | Fetal       | −/−                      | −/−                      | −/−                              |
|        | Adult       | +/−                      | +/−                      | +/−                              |
| CRYAB  | Fetal       | −/−                      | +/−                      | +/−                              |
|        | Adult       | +/−                      | +/−                      | +/−                              |
| IL1B   | Fetal       | +/−                      | +++                      | +/−                              |
|        | Adult       | +/−                      | +++                      | +/−                              |
| VCAM1  | Fetal       | +/−                      | +/−                      | +/−                              |
|        | Adult       | +/−                      | +/−                      | +/−                              |

The intensity of the signal is ranked from − (no signal) to +++ (strong signal) according to the criteria grouped as noted here. Ribonucleic acid sequencing (RNA-seq): ++++, >800.00; +++, 100.00 to 799.99; ++, 10.00 to 99.99; +, 2.00 to 9.99; and −, <2.00. Assay for transposase accessible chromatin-sequencing (ATAC-seq): histone chromatin immunoprecipitation-sequencing (ChIP-seq): +++, >15; ++, 10 to 15; +, 5 to 10; +/−, 2 to 5; and −, <2.

**Identification of TFs from Motif Analysis.** Having confirmed the consistency and validity of our genome-wide transcriptomic and epigenomic profiles, we investigated whether TF regulators could be identified with an upstream role unique to aHCFs or fHCFs. A de novo motif analysis was performed with Homer on H3K4me3 loci (taken to represent active gene promoters) associated with differentially expressed genes in aHCFs and fHCFs. Two motifs and their corresponding TFs were enriched in differential H3K4me3 loci of each cell type (Figure 4A). Notably, the 2 TFs identified in fHCFs (SOX17 and SOX4) and the 2 for aHCFs (PLAGL1 and STAT1) were also strongly differentially up-regulated in their corresponding cells on the basis of their corresponding RNA-seq data. We accurately replicated these findings further in 3 (2 adult and 1 fetal) additional independent HCF cell isolates (Supplemental Figure 6A). These TFs now represent novel regulators that may play an important role in their respective cells.

**TF Validation by Gapmer Knockdown.** The therapeutic potential of targeting aHCFs in adult cardiac disease led us to choose PLAGL1 and STAT1 for validation by in vitro knockdown using Gapmer oligonucleotides. We performed this procedure in all 3 aHCF lines (Table 1). PLAGL1 and STAT1 knockdown led to >80% reduced expression levels when targeted with Gapmers (Figure 4B, Supplemental Figure 6B). Interestingly, PLAGL1 knockdown also led to reduced
STAT1 levels, suggesting an upstream effect of PLAGL1 on STAT1 that has not been previously reported. Negative A GapmeR construct, vehicle transfection, and nontransfected control specimens were used in all assessments. Cells were stained with antibodies against vimentin and Ki67 and analyzed for cell size, roundness, cell cycle, and cell number (n = 3). Cell size, morphology, or cell number was unchanged in all control specimens (Figures 4C and 4D). Instead, knockdown of STAT1 significantly increased cell roundness, reduced cell number without increasing cell proliferation, and knockdown of either TF changed aHCF cell size (Figure 4D, replicated in independent cell isolates in Supplemental Figure 6C). Changes at the transcriptional level revealed that whereas VIM and SMA were unchanged, both STAT1 and PLAGL1 knockdown caused significant down-regulation of other genes previously noted as being characteristic of the aHCF phenotype (ELN, TNFRSF11B, COL1A1, and COL1A2). Conversely, HBEGF associated with the fetal gene profile was up-regulated (Figure 5A, also replicated in independent cell isolates in Supplemental Figure 6D).

To test whether knockdown fibroblasts underwent mesenchymal epithelial transition (21), key markers for epithelial cells (CDH1, KRT9, and ATP2C2) were quantified, but these remained poorly expressed or undetected, rejecting the possibility that this transition had taken place (Supplemental Figure 6D). We next assessed for apoptosis by using the ApopTag-Red assay. Baseline apoptosis of aHCFs in culture was 3% (untreated cells, n = 5,237), 5% (cells treated with vehicle or negative A GapmeR, n = 5,217), and 7% (PLAGL1 or STAT1 knockdown, n = 5,217).

(A) Transcription factor (TF) motifs enriched at active promoters (marked by H3K4me3) of differentially expressed genes for each aHCF and fHCF. Expression of each corresponding TF gene taken from RNA-seq data is also shown. (B) Effective >90% GapmeR-mediated knockdown (k.d.) of PLAGL1 and STAT1 in aHCFs shown by reverse transcription quantitative-polymerase chain reaction, normalized to GAPDH and PPIA housekeeping gene control and subsequently to negative A (NegA) control. Values are mean ± SD. (C) Knockdown of either PLAGL1 or STAT1 leads to changes in the aHCF phenotype compared with control specimens. (D) Quantification of cellular parameters after knockdown of either TF shows that aHCFs become smaller and rounder after STAT1 knockdown. PLAGL1 knockdown also results in increased proliferation. All experiments were conducted as n = 3. Biological repeats in independent human cardiac fibroblast cell isolates are shown in Supplemental Figure 6. Values are mean ± SD. *p < 0.05 compared with NegA control. Abbreviations as shown in Figures 1 and 3.

**FIGURE 4** TF Identification and Validation

| Motif       | P-value | Probable TF | FPKM aHCF | FPKM fHCF |
|-------------|---------|-------------|-----------|-----------|
| Fetal       |         |             |           |           |
| 1 E-16 GAGATGAAATGCC | 1.0   | SOX17      | 16.23     | 0.74      |
| 1 E-12 CCGAAACGC   | 1.0   | SOX4       | 21.17     | 3.66      |
| Adult       |         |             |           |           |
| 1 E-18 TCCGCTAGCC | 1.0   | PLAGL1     | 4.79      | 14.23     |
| 1 E-23 AGTCCATGC   | 1.0   | STAT1      | 65.25     | 343.70    |

**TABLE 1**

**A** Transcription factors predicted for fetal and adult HCF

**B** STAT1 expression after k.d. in aHCF(2)

**C** PLAGL1 expression after k.d. in aHCF(2)

**D** Cell size and Roundness

**E** Quantitation of cellular parameters after knockdown of either TF shows that aHCFs become smaller and rounder after STAT1 knockdown. PLAGL1 knockdown also results in increased proliferation. All experiments were conducted as n = 3. Biological repeats in independent human cardiac fibroblast cell isolates are shown in Supplemental Figure 6. Values are mean ± SD. *p < 0.05 compared with NegA control. Abbreviations as shown in Figures 1 and 3.
FIGURE 5  STAT1 Knockdown Induces Fibroblast Senescence and Reduced Collagen Gene Expression

A

- **general markers**
  - VIM
  - SMA

- **fibrosis markers**
  - COL1A1
  - COL1A2

- **adult markers**
  - ELN
  - TNFRSF11B

- **fetal marker**
  - HBEGF

- **senescence markers**
  - MMP3
  - RB1
  - TP53

- **apoptosis markers**
  - BAX
  - CASP3
  - PCNA
  - MCM3

B

- **DAPI**
- **ApopTag-Red**
- **Overlay**

C

- **DAPI**
- **ApopTag-Red**

D

- **DAPI**
- **Ki67**

Continued on the next page.
with control vehicle lipofectamine alone, n = 4,992),
and 6% (cells treated with control negative A
GapmeR, n = 3,710). In contrast, knockdown of
PLAGL1 or STAT1 led to significant up-regulation
of apoptosis to 29% in PLAGL1 knockdown cells
(n = 2,500) and 156% in STAT1 knockdown cells
(n = 2,739); >100% apparent apoptosis is due to
automated detection of multiple ApopTag-Red-
stained granules in many cells) (Figure 5B).

Interestingly, apart from the classical ApopTag-
Red-positive staining of nuclei to mark apoptotic
cells, an overwhelming number of STAT1 knockdown
cells were also noted that showed cytoplasmic frag-
mentation labeling. This finding was replicated more
than 3 times and was absent both in PLAGL1 knock-
down cells and in DNase1-treated positive control
cells. At this time, we considered the possibility that
cytoplasmic labeling represents advanced apoptosis
in STAT1 knockdown cells or evidence of widespread
mitochondrial damage.

Furthermore, a significant up-regulation of the
classical marker for fibroblast senescence MMP3
(1,400-fold compared with control), as well as a subtle
up-regulation of RBl and TP53 (also markers of cell
senescence), were noted in STAT1 knockdown aHCFs
(Figure 5A). Significant up-regulation of MMP3 was
replicated in 1 of 2 of the additional aHCF cell isolates.
The third cell line instead showed a signifi-
cant up-regulation of RB1 (Supplemental Figure 6D).
High-power magnification of 4',6-diamidino-2-
phenylindole-stained nuclei showed formation of
heterochromatin foci in 9% of STAT1 knockdown
aHCFs (n = 5,595), whereas this outcome was not seen
in untreated or positive control cells and in <1%
after PLAGL1 knockdown (n = 4,899). Cells with
heterochromatin foci were frequently positive for
ApopTag-Red (Figure 5C), although not all ApopTag-
Red-positive cells showed heterochromatin foci.
Instead, we observed a striking exclusivity in which
nuclei displaying the heterochromatin foci were
never positive for Ki67 (Figure 5D). Together, these
findings suggest that whereas 1 subset of knockdown
aHCFs undergoes apoptosis, another appears to
become senescent. Furthermore, STAT1 and PLAGL1
knockdown led to a significant reduction in collagen
COL1A1 and COL1A2 gene expression (Figure 5A).
These results were again supported by RT-qPCR in all
additional sets of aHCF cells.

**DISCUSSION**

Results of previous studies indicated that fHCFs and
aHCFs are different in that they influence neighboring
CMs in distinct ways (6,13). In the present study, we
have further defined their distinct differences by
performing a thorough characterization of primary
fetal and adult human ventricular CFs by using
cellular, molecular, and genome-wide sequencing
approaches. Our results show that fHCFs are smaller
and proliferate more quickly than their adult coun-
terpart. We hypothesized that these characteristics
are explained by differences in their transcriptomes
and epigenomes. Indeed, genome-wide analyses of
RNA-seq, ATAC-seq, and histone ChiP-seq led us to 2
TFs, highly expressed in aHCFs but not in fHCFs. We
performed functional validation to conclude that they
regulate aHCF cell size, cell cycle re-entry, and cell
survival. Knockdown of either of the 2 TFs led to a
destabilization of the aHCF phenotype and reduced
gene expression of collagen genes, a major constituent
of pathological fibrosis in the adult heart.

RNA-seq CONFIRMS FIBROBLAST IDENTITY AND
IDENTIFIES DIFFERENTIAL SIGNALING PATHWAYS.
We first confirmed that “core” genes that define a
fibroblast and that are typically used as fibroblast
markers, such as VIM, THY1, DDR2, and COL1A2, do
not differ in expression between fHCFs and aHCFs.
According to IPA analysis, 3 top functions found in
the overlapping transcriptomes of the fHCFs and
aHCFs are tissue development, cardiovascular
system development and function, and organismal
development, validating our cardiac cell source and

![FIGURE 5](image-url) Continued

(A) Reverse transcription quantitative-polymerase chain reaction of representative genes after PLAGL1 or STAT1 knockdown in aHCFs. Whereas common human cardiac fibroblast genes VIM and SMA are unchanged, genes associated with the adult phenotype are down-regulated (ELN and TNFRSF11B), and HBEFG (associated with fHCFs) is up-regulated. Apoptosis-related genes such as BAX, CASP3, PCNA, and MCM3 are in particular up-regulated after STAT1 knockdown. Similarly, cellular senescence marker MMP3, as well as RB1 and TP53 showed strong upregulation following STAT1 knockdown. Collagen genes (COL1A1 and COL1A2) are down-regulated upon knockdown of both TFs. Values are mean ± SD and taken from at least n = 3. All p < 0.05, Student t test. Biological repeats from independent aHCF cell isolates are shown in Supplemental Figure 6. (B) Apoptosis was assessed by using the ApopTag-Red assay that detects DNA fragmentation. A low number of cells (6%) exhibited apoptosis under control conditions (control Neg. A GapmeR transfection), and this was significantly up-regulated in the positive control (DNase I-treated cells) as well as after PLAGL1 and STAT1 knockdown. A strikingly high level of cytoplasmic ApopTag-labeling was repeatedly evident in STAT1 knockdown cells, which was not seen in others. (C) Heterochromatin foci were detected in 9% (450 of 5,595 nuclei analyzed) of STAT1 knockdown cells. These foci frequently, but not exclusively, overlapped with the ApopTag-Red stain. (D) Nuclei illustrating that heterochromatin foci were never found in Ki67-positive cells. Abbreviations as in Figures 1, 3, and 4.
supporting the conclusion that fHCF and aHCF transcriptomes do not differ substantially. Conversely, this finding implied that important differences would be decidedly more subtle. Nonoverlapping gene functions are embryonic development and organismal survival for fHCFs, reflecting their fetal origin (22,23), and immune cell trafficking and skeletal and muscular system development and function for aHCFs, suggesting how aHCFs may have a different role in the adult human heart compared with their fetal counterpart. We used 2 additional HCF lines to validate the RNA-seq to show that differential gene expression was confirmed across independent biological replicates. Among these are likely to be bona fide markers that can be used to distinguish between aHCFs and fHCFs.

**Novel and Interesting Genes Identified.** Differences in transcriptomes between cell types may help us to understand and explain their differential functions. For example, we expected that the fHCF transcriptome would contain genes that reveal their role for the developing heart and for inducing appropriate heart growth, whereas the aHCF transcriptome should comprise genes involved in the maintenance of a heart that is already matured. We therefore dissected the gene pathways and functions specific to each differentially expressed transcriptome for this analysis. Genes of the ephrin-receptor and ephrin b-signaling pathways were enriched in fHCFs. Indeed, ephrin B2 is important in the developing heart for ventricular chamber morphogenesis (24). Coherent to this process is the importance of Notch signaling; we also found that the Notch pathway is present in fHCFs but not aHCFs. Ephrin signaling has further been implicated for the development of vasculature (25). The finding of IL-8 signaling and ephrin signaling in fHCFs therefore suggests a role for fHCFs in vascular development as well. VCAM-1 is among the highest differentially expressed genes in fHCFs, and VCAM-1 has known importance for cardiac development because Vcamt null mice embryos do not survive past E13.5 due to retarded heart growth (23). Interestingly, ITGA4 is also among the highest differentially expressed genes in fHCFs, and ITGA4 is an endogenous ligand for VCAM-1, creating the opportunity for inter-fHCF communication that might guide cardiac development. We also noted that analysis of gene ontology cellular processes revealed more profound plasma membrane components in fHCFs, such as integrins and other receptors, whereas aHCF-specific genes seem to focus on extracellular components. HBEGF is yet another gene that is highly expressed in fHCFs and lacking in aHCFs. HBEGF has been reported to promote cardiomyocyte proliferation in a paracrine fashion in a mouse model (6), and our data support its importance exclusively in fHCFs.

Among the top differentially expressed genes in aHCFs is FGF7. A gene network of FGF signaling in IPA also showed that 10 of the 15 genes involved in FGF signaling were highly differentially expressed in aHCFs, with other members of the network, including FGFR2 and FGFR4. Although it is well established that the FGF superfamily of growth factors is important in pathophysiological processes within the cardiac environment (26), a specific role for FGF7 has yet to be defined. Another gene more highly expressed in aHCFs was TNFRSF11B encoding for osteoprotegerin, a mesenchymal marker for EMT, from which a large population of adult CFs is proposed to derive (27). Overexpression of TNFRSF11B in rat CFs increased the expression of fibrosis-related proteins after treatment with angiotensin II, whereas its knockdown produced the reverse effect (28). ELN (elastin) is a third gene highly expressed in aHCFs. Elastin is a major contributor to the ECM, and it functions together with fibrillin to provide elasticity to a range of tissues (29). Interestingly, overexpression of ELN in cells that were transplanted to infarcted cardiac tissue in rats ameliorated the formation of scar tissue, compared with infarcted cardiac tissue that received vector-transduced cells (30). A necessity for crosslinking of elastin fibers is the presence of lysyl oxidases (29), and LOXL4 (lysyl oxidase-like 4) is also one of the highest differentially expressed genes in aHCFs.

**Putative Regulatory TFs for Fetal HCFs Determined by Using Motif Analysis.** TFs determined by using de novo motif analysis add another layer of information to the regulation of the differential transcriptomes between aHCFs and fHCFs. We performed motif analysis by using active promoters, demarcated according to H3K4me3, of differentially expressed genes for either aHCFs or fHCFs, thus specifying TFs for the respective transcriptomes. Top motifs were SOX17 and SOX4 in fHCFs. Importantly, these TFs were also all highly expressed genes in fHCFs compared with aHCFs. SOX17 is also expressed in endothelial cells, where it regulates angiogenesis under the influence of Notch signaling (31). Our results show that SOX17 is expressed in fHCFs as well. SOX4 is a master regulator of EMT and is needed for proper formation of the cardiac outflow tract (32,33). Together, the abundant expression of these 2 TFs in fHCFs suggests that they may maintain prefate determination functions, and/or that fHCFs have a role in the development of...
the vasculature of the heart, similar to the ephrin and IL-8 signaling discussed earlier.

**REGULATORY TFs in aHCFs FOUND BY MOTIF ANALYSIS AND VALIDATED IN VITRO.** The top 2 TFs identified for aHCFs were *PLAGL1* and *STAT1*, and their corresponding messenger ribonucleic acid was indeed significantly highly expressed in aHCFs compared with fHCFs. *PLAGL1* is an important TF in the developing heart, but its role has only been linked to CMs rather than CFs (34). In our experiments, knockdown of *PLAGL1* in aHCFs led to reduced cell size and increased apoptosis. *STAT1* activation reportedly increases fibroblast activation and collagen synthesis induced by high glucose levels (35). In our experiments, *STAT1* knockdown in aHCFs also led to reduced cell size and apoptosis. Gene expression analysis after knockdown showed that *VIM* and *SMA* levels remained unchanged, indicating that *STAT1* knockdown cells were not transforming to myofibroblasts. Instead, markers associated with the adult phenotype were down-regulated, and fetal markers were up-regulated. Together with their smaller size and increased apoptosis, we interpret these changes to represent a de-stabilization of the aHCF phenotype and viability.

We also found evidence for cell senescence based on the very high up-regulation of the classical fibroblast senescence marker *MMP3* and concomitant up-regulation of other senescence markers (*RB1* and *TP53*) (36,37). Consistent with this finding was the formation of heterochromatin foci in knockdown cells. Cells with heterochromatin foci were mutually exclusive from Ki67-positive cells, consistent with the description of senescence-associated heterochromatin foci that have already been extensively described (38). Concurrently, the expression of collagen genes *COL1A1* and *COL1A2* as well as *TNFRSF11B* were strongly down-regulated, suggesting that *STAT1* knockdown cells have a diminished ability to stimulate profibrotic processes. Importantly, these findings were confirmed in independent sets of aHCF cells from different donors.

**CONCLUSIONS**

Overall, this report describes for the first time important genome-wide differences between fHCFs and aHCFs. Signals from loci of active promoters led to the identification of important TFs responsible for the differential roles of HCFs. Although the exclusive use of human material limited this research to in vitro studies, these findings nevertheless reveal interesting new explanations for the different ways in which fibroblasts act in the human adult and fetal heart. Our data further suggest that this source of HCFs may be useful for translational research, such as for disease modeling of cardiac fibrosis, or for other uses, such as improving CM maturation in differentiation protocols from human embryonic stem cells. Our knockdown studies for *PLAGL1* and *STAT1* suggest their importance for aHCF viability and phenotype maintenance. Further in vivo studies will be needed to determine whether they do indeed represent potential drug targets for steering away the malevolent behavior of CFs during adult heart disease progression.

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**COMPETENCY IN MEDICAL KNOWLEDGE:** We report important genome-wide differences between HCFs and aHCFs and identified key players responsible for maintaining the adult HCF phenotype. Our data suggest that this source of HCFs is useful in translational research, such as for disease modeling of cardiac fibrosis.

**TRANSLATIONAL OUTLOOK:** The transcription factors identified (*PLAGL1* and *STAT1*) may be potential drug targets for steering away the malevolent behavior of HCFs during progression of adult heart disease.

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**KEY WORDS** cardiac fibroblasts, collagen, histone methylation, transcriptome

**APPENDIX** For supplemental material including tables and figures, please see the online version of this article.