Comparative transcriptomic analysis identifies genes differentially expressed in human epicardial progenitors and hiPSC-derived cardiac progenitors

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The adult mammalian heart has limited ability to regenerate, and after myocardial infarction (MI) the lost myocardium is typically replaced with fibrotic scar tissue. Although the scar can withstand elevated filling pressure, it does not conduct electricity or actively contract, leading to adverse cardiac remodeling and eventually to heart failure. Once heart failure develops, the prognosis for the patient is poor. Despite therapeutic advances in recent decades, the five-year mortality rate of heart failure is close to 50% (2). Therapies that facilitate survival or regeneration of damaged myocardium after ischemic injury in the human heart are urgently needed and would have enormous social and economic impact.

The adult mammalian heart has been traditionally regarded as a postmitotic organ with no regenerative capacity. However, this dogma has been refuted by some recent landmark studies. For example, compelling data demonstrated that new cardiomyocyte formation takes place at an annual rate of 0.45–1.0% in the adult human heart (4, 5). Moreover, a number of putative cardiac progenitor cell (CPC) populations have been described in the adult mammalian heart by many groups (12, 23, 28, 30). Some of the CPCs are shown to be capable of differentiating into cardiomyocytes, endothelial cells, and smooth muscle cells and thus may represent promising cell populations for cardiac regeneration. However, CPCs are rare in the adult mammalian heart, and they are difficult to isolate and expand in vitro, making it challenging to use these cells routinely in drug discovery. Recent advances in induced pluripotent stem cell (iPSC) technologies have now made it possible to produce human CPCs on a large scale (29). These cells can be used for interrogation of relevant human biology and in screening campaigns for drug discovery as well as for cell therapy (26, 27, 31). Different markers have been used to identify CPCs, and it is currently unclear which set of markers is the most appropriate to use to identify the most potent CPCs. When a comparison of many of the various populations of CPCs that have been described, the cardiac transcription factor NKX2.5 emerges as a common key marker (7, 12, 35). Based on these data, human iPSC-derived NKX2.5-positive CPCs have been used in a robust phenotypic screening platform to identify molecules capable of inducing CPC proliferation and differentiation to the cardiac lineage (9). Moreover, recent studies have shown that a few selected key cardiac transcription factors can directly reprogram mouse fibroblasts into induced CPCs. These multipotent induced progenitors have the ability to differentiate to cardiomycocytes, smooth muscle cells, and endothelial cells in vitro as well as in vivo in post-MI mouse hearts (20).

Another cell type relevant to cardiac regeneration is epicardium-derived cells (EPDCs), which have been shown to be an important cell and signaling source for heart development and post-MI heart repair (25). During heart development in mice, EPDCs contribute to formation of different cardiac cell types and secrete trophic factors for myocardial maturation (25). In the adult heart, EPDCs are quiescent under normal conditions and become activated after myocardial injury. In zebrafish, organ-wide activation of epicardium in response to injury has
been shown to be critical for heart regeneration, through facilitation of new vasculature and stimulation of cardiomyocyte proliferation via release of paracrine factors (15–17, 21). Following MI in mice, EPDCs undergo an epithelial-to-mesenchymal transition, characterized by expression of Wilms tumor protein 1 (WT-1), and they have been shown to have the capacity to differentiate into several lineages (44), although the default lineage is smooth muscle/fibroblast (45). In addition to contributing to the formation of new cells, EPDCs have been demonstrated to secrete various paracrine factors, such as vascular endothelial growth factor-A (VEGF-A), FGF2, and PDGF-CC, which promote the growth of blood vessels, protect the myocardium, and improve heart function in a mouse model of acute MI (45).

Both CPCs and EPDCs are activated after MI, and they may influence each other through paracrine mechanisms or direct interactions. Winter et al. (43) reported that cotransplantation of CPCs and EPDCs in mice resulted in better improvement of cardiac function than either cell type alone, suggesting a synergistic effect. Currently, efforts are being made to discover and develop therapeutic molecules to increase the number of CPCs and EPDCs (9, 25). To better understand the characteristics and therapeutic potential of CPCs and EPDCs, as well as the regulatory mechanisms, we performed transcriptomic analysis of human iPSC-derived CPCs and human primary EPDCs and discovered unique gene expression profiles for each cell type.

Hypoxia has been reported to affect the survival, proliferation, and differentiation potential of progenitor cells (18, 19). In most in vitro assays, the cells are cultured and maintained in 20% oxygen, whereas in situ the CPCs and EPDCs exist in an environment of much lower oxygen, particularly during myocardial ischemia. To make sure that the biological and pharmacological findings in cell assays under ambient oxygen conditions are relevant to the in vivo situation, it is important to understand the effect of hypoxia on the behavior, gene expression, and paracrine profiles of the cells. In the present study, we also studied the effect of hypoxia on the transcriptome of human iPSC-derived CPCs and human primary EPDCs. Our data demonstrate that the effect of hypoxia was modest and only affected the expression of a limited number of genes related to glycolysis and cell survival.

MATERIALS AND METHODS

Cell Isolation and Culturing

Human iPSC-derived CPCs (hereafter only referred to as CPCs) were obtained from Cellular Dynamics International (iCell Cardiac Progenitor Cells). Briefly, the differentiation process was initiated by formation of aggregates of human iPSCs in Essential 8 medium (Life Progenitor Cells). Briefly, the differentiation process was initiated by expression of Wilms tumor protein 1 (WT-1), and they have been shown to have the capacity to differentiate into several lineages (44), although the default lineage is smooth muscle/fibroblast (45). In addition to contributing to the formation of new cells, EPDCs have been demonstrated to secrete various paracrine factors, such as vascular endothelial growth factor-A (VEGF-A), FGF2, and PDGF-CC, which promote the growth of blood vessels, protect the myocardium, and improve heart function in a mouse model of acute MI (45).

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EPDCs were isolated and cultured from the right atrial appendages excised during cardiac surgery from four adult patients. All experiments with human tissue specimens were carried out according to the official guidelines of the Leiden University Medical Center and with the approval of the institutional ethical committee.

The layer of epicardium was stripped and minced into small pieces. The tissue pieces were cultured in a 1:1 mixture of low-glucose DMEM and medium 199 (M199) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum (FCS, Invitrogen), and 2 ng/ml bFGF (Sigma-Aldrich, St. Louis, MO). The addition of bFGF stimulated outgrowth of cells from the tissue pieces and proliferation of the cells (3). When the outgrowth of the epicardial cells was confluent, the cells were detached from the surface of the culture dish with trypsin/EDTA (Invitrogen) solution. The cells were seeded at high density and cultured in a 1:1 mixture of DMEM and M199 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS and frozen at passage 3. For experiments, cells from each of the four donors were expanded to passage 6–9 and then plated at 40,000 cells/cm². After 24 h, the medium was changed on both the CPCs and the EPDCs, and the cells were placed into hypoxic (3% O₂) or normoxic (20% O₂) incubators for 24 h prior to RNA extraction. Thus, three biological replicates of CPCs in normoxic condition and three corresponding replicates of these samples under hypoxic condition, and four biological replicates of EPDCs under normoxic condition and four corresponding replicates of these samples under hypoxic conditions are included in this study design. In total, the study consists of 14 samples/arrays.

Immunocytochemistry

Cells were fixed for 20 min in 4% formaldehyde and then stained with rabbit anti-NKX2.5 (Abcam, Cambridge, UK), rabbit WT-1 (Abcam), mouse anti-CD73 (Abcam) or Islet-1 (rb, Abcam) overnight before being counterstained with either Alexa Fluor-594 donkey anti-rabbit or Alexa Fluor-488 donkey anti-mouse (Life Technologies) for 1 h and Hoechst 33342 for 10 min. Plates were imaged on an ImageXpress Micro system (Molecular Devices) at ×20.

RNA Extraction and Microarray Experiments

The cell samples were collected using lysis solution (RNeasy Mini prep kit, Qiagen). Total RNA was extracted using semi-automatic preparation (Qiucube, Qiagen) with a DNase digest. Quantification of nucleic acids was performed on NanoDrop ND-1000 (NanoDrop, http://www.nanodrop.com). The quality of the RNA and cDNA, labeled by in vitro transcription, was verified using a 2100 Agilent Bioanalyzer. To measure the mRNA levels, fragmented cDNA was hybridized at 45°C for 16 h to whole transcript HuGene ST 1.0 arrays (Affymetrix, http://www.affymetrix.com) at SweGene Center for Integrative Biology at Lund University (SCIBLU) Genomics (Lund, Sweden). The microarrays were scanned on a GeneChip Scanner 3000 7G (Affymetrix), and expression signals were extracted and normalized by means of the Expression Console (Affymetrix) v.1.1.2 applying the robust multi-chip average (RMA) normalization method. The microarray data follow the MIAME standard. The raw expression dataset is available at ArrayExpress (http://www.ebi.ac.uk/microarray-as/a/e/), accession number E-MTAB-4609.

Data Analysis

After the RMA normalization, the data were filtered from transcripts that are expressed at close to background level (log2 < 5) in all samples analyzed. The filtering threshold was chosen based on our previous studies including both undifferentiated hPSCs and their differentiated progenies (34, 37, 38). The threshold value is ~5 on the log2 scale and represents the level for several pluripotency markers, which are not expected to be expressed in the differentiated cells. In total 27,679 probes passed this filtering criteria and these were considered for subsequent analysis.
Identification of Differentially Expressed Genes between CPCs and EPDCs

To identify differentially expressed genes between the CPCs and the EPDCs under normal oxygen conditions, the significance analysis of microarrays (SAM) algorithm (40) was applied using the R package siggenes. A false discovery rate (FDR) of ≤ 0.05 was considered significant. The list of significant genes was further filtered on fold change (FC) > 2. Only normoxia samples were included in this analysis.

Identification of Genes Affected by Hypoxic Culture Conditions in CPCs and EPDCs

To investigate the effect of culturing the CPCs and EPDCs under hypoxia vs. normoxia, each cell preparation was maintained under hypoxia for 24 h or under normoxia before harvest. In general, the effects on gene expression from the hypoxic culture conditions varied in magnitude, causing difficulties in using appropriate statistical algorithms for assessment because of high standard deviation. Therefore, instead we used the FC values for each transcript, which were calculated between paired samples of hypoxia and normoxia for both the CPCs and the EPDCs. The transcripts that show >50% induction or repression due to hypoxic stimulation were considered differentially expressed.

Gene Ontology Annotation and Pathway Enrichment Analyses

To further investigate the functional properties among the genes (Suppl. File S1) identified from comparisons of the different groups described above, the Cytoscape ver. 3.2.1 (33) together with the ClueGO plug-in (6) was applied.1 For the Gene Ontology (GO) annotation enrichment analysis, “Grouping” was used as “View style setting” and for the Gene Ontology enrichment analysis the Biological Process annotations (updated per 17th of November 2015) was used with evidence code “All experimental.” Annotations from GO levels 5–8 that were annotated to a minimum of five genes in the input list were considered for further analysis. Only annotations with FDR <0.001 are reported, and “Selected ontologies” was used as the reference set and the “GO term grouping” was applied. GO-term weighting algorithms were also evaluated (1) and showed overlapping results with the ClueGO.

To identify pathways that were significantly overrepresented among the lists of differentially expressed genes, we analyzed pathways represented in the KEGG database (14). The default “kappa score” (0.4) was used to indicate groups of pathways with shared genes and only pathways with FDR < 0.05 and a minimum number of five genes among the input genes were considered. “Significance”

1 The online version of this article contains supplemental material.

Fig. 1. A: hierarchical clustering at the global transcriptional level including all samples in the experiment. As shown in the dendrogram, the most pronounced separation is between cardiac progenitor cells (CPCs) and epicardium-derived cells (EPDCs). Moreover, at the global scale, the variation between individuals is more pronounced than the variation introduced by hypoxia stimulation. B: the expression levels of 10 marker genes of different stages during cardiac differentiation. POUSF1 and NANOG are markers of undifferentiated cells, NXX2.5 and ISL1 and WT1 are markers of mesoderm lineage, ENG, THY1, and ALDH1A2 are markers of cardiac progenitors, and MYH6 and TNNT2 are markers of mature cardiomyocytes. Blue bars represent CPCs, and green bars represent EPDCs. The error bars show SD across replicate samples (n = 3 for CPCs and n = 4 for EPDCs). C: immunocytochemistry confirms the phenotype of EPDCs and CPCs. a. EPDCs: Wilms tumor protein 1 (WT-1, red), alpha smooth muscle actin (α-SMA, green), Hoechst (blue). b. EPDCs: CD73 (red), Hoechst (blue). c. CPCs: Islet-1 (ISL1, red), Hoechst (blue). d. CPCs: NXX2.5 (red), Hoechst (blue).
was used as “View style setting,” and “Selected ontologies” was used as the reference set.

**Protein-protein Interaction Analysis**

To further investigate the functional properties of the differentially expressed genes in each cell type we performed a protein-protein interaction analysis to identify putative functional modules and hub proteins among the most highly upregulated genes (FC > 7). STRING software (http://string-db.org) (39) version 10 was used for this analysis with medium confidence score (0.400) and excluding interactions based on text mining.

**Analysis of Gene Expression of Paracrine Factors in CPCs and EPDCs**

A detailed investigation of the mRNA expression of genes that code for some key paracrine factors was performed in CPCs and EPDCs. In total 92 predefined biomarkers for cardiovascular diseases (http://www.olink.se, Proseek Multiplex CVD I) were selected and analyzed. Only the data from the normoxia culturing conditions were used in this analysis, and the genes that significantly differ (FDR < 0.05) in expression between the two cell populations are reported.

**RESULTS**

**Hierarchical Clustering of Global Transcriptomics Data**

To investigate the variability in the data, we performed hierarchical clustering, which showed a clear separation between the CPC and the EPDC samples. Moreover, paired samples of normoxia and hypoxia for each of these cell types were analyzed. Paired samples of normoxia and hypoxia for each of these cell types cluster closely together with only one exception (Fig. 1A). This result indicates rather small transcriptional effects of the cells after the 24 h hypoxia stimulation compared with the more distinct differences between the CPCs and the EPDCs.

**Expression of Key Markers in CPCs and EPDCs**

We selected 10 marker genes representing different stages of cardiac differentiation, and their mRNA expression was measured using microarrays in the CPCs and EPDCs obtained from normoxic culture conditions. POUSF1 and NANOG are markers of undifferentiated pluripotent cells; NKX2.5 and ISL1 represent cardiogenesis-related markers; WT1, ENG, THY1, and ALDH1A2 are markers of EPDCs; and MYH6 and TNNT2 are cardiomyocyte markers. As shown in Fig. 1B, some of these genes showed similar expression levels in both cell types (NKX2.5 and MYH6). However, ENG and THY1 were substantially more highly expressed in EPDCs than in CPCs. WT1 and ALDH1A2 were also more highly expressed in EPDCs than in CPCs, but the differences were less dramatic. On the other hand, ISL-1 and TNNT2 showed higher expression in the CPCs. The mRNA expression of both POUSF1 and NANOG was detected in CPCs and EPDCs, although at very low levels compared with what is typically observed in undifferentiated human pluripotent stem cells. Moreover, expression of MYH6 was also detected at similarly low levels, suggesting that these cells are neither pluripotent nor differentiated cardiomyocytes, typical characteristics of progenitor cells. Overall, the patterns of expression for the 10 marker genes align well to what previously has been reported for CPCs and EPDCs (8, 12, 27).

**Table 1. The top 30 differentially expressed genes from Suppl. Table S1 displaying significantly higher expression in the CPCs than in the EPDCs**

<table>
<thead>
<tr>
<th>UniGene ID</th>
<th>Symbol</th>
<th>Gene Description</th>
<th>Mean CPC</th>
<th>Mean EPDC</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs.542050</td>
<td>EPCAM</td>
<td>epithelial cell adhesion molecule</td>
<td>2,776</td>
<td>17</td>
<td>168</td>
</tr>
<tr>
<td>Hs.128453</td>
<td>FRZB</td>
<td>frizzled-related protein</td>
<td>3,805</td>
<td>25</td>
<td>150</td>
</tr>
<tr>
<td>Hs.227752</td>
<td>TECRL</td>
<td>trans-2,3-enoyl-CoA reductase-like</td>
<td>1,565</td>
<td>11</td>
<td>142</td>
</tr>
<tr>
<td>Hs.130957</td>
<td>PRTG</td>
<td>protogenin</td>
<td>7,350</td>
<td>71</td>
<td>104</td>
</tr>
<tr>
<td>Hs.23616</td>
<td>LIN28B</td>
<td>lin-28 homolog B (C. elegans)</td>
<td>749</td>
<td>8</td>
<td>89</td>
</tr>
<tr>
<td>Hs.118127</td>
<td>ACTC1</td>
<td>actin, alpha, cardiac muscle 1</td>
<td>6,699</td>
<td>78</td>
<td>86</td>
</tr>
<tr>
<td>Hs.152531</td>
<td>HAND1</td>
<td>heart and neural crest derivatives expressed 1</td>
<td>8,492</td>
<td>109</td>
<td>78</td>
</tr>
<tr>
<td>Hs.533566</td>
<td>H19</td>
<td>H19, imprinted maternal expressed transcript</td>
<td>11,924</td>
<td>154</td>
<td>78</td>
</tr>
<tr>
<td>Hs.41690</td>
<td>DSC3</td>
<td>desmocollin 3</td>
<td>2,228</td>
<td>29</td>
<td>77</td>
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<tr>
<td>Hs.389669</td>
<td>TMEM88</td>
<td>transmembrane protein 88</td>
<td>8,007</td>
<td>114</td>
<td>70</td>
</tr>
<tr>
<td>Hs.86154</td>
<td>LIN28A</td>
<td>lin-28 homolog A (C. elegans)</td>
<td>5,123</td>
<td>74</td>
<td>69</td>
</tr>
<tr>
<td>Hs.95612</td>
<td>DSC2</td>
<td>desmocollin 2</td>
<td>3,342</td>
<td>49</td>
<td>69</td>
</tr>
<tr>
<td>Hs.610096</td>
<td>CTSV</td>
<td>cathepsin V</td>
<td>2,387</td>
<td>36</td>
<td>66</td>
</tr>
<tr>
<td>Hs.614734</td>
<td>PROM1</td>
<td>prominin 1</td>
<td>1,557</td>
<td>24</td>
<td>65</td>
</tr>
<tr>
<td>Hs.1833114</td>
<td>ARHGAP28</td>
<td>Rho GTPase activating protein 28</td>
<td>3,250</td>
<td>51</td>
<td>64</td>
</tr>
<tr>
<td>Hs.518921</td>
<td>NPNT</td>
<td>nephrocin</td>
<td>3,022</td>
<td>49</td>
<td>62</td>
</tr>
<tr>
<td>Hs.24258</td>
<td>GUCY1A3</td>
<td>guanylate cyclase 1, soluble, alpha 3</td>
<td>2,026</td>
<td>34</td>
<td>59</td>
</tr>
<tr>
<td>Hs.542050</td>
<td>EPCAM</td>
<td>epithelial cell adhesion molecule</td>
<td>2,216</td>
<td>38</td>
<td>58</td>
</tr>
<tr>
<td>Hs.655654</td>
<td>RELN</td>
<td>reelin</td>
<td>3,319</td>
<td>64</td>
<td>52</td>
</tr>
<tr>
<td>Hs.743478</td>
<td>LIF1</td>
<td>lymphoid enhancer-binding factor 1</td>
<td>1,590</td>
<td>33</td>
<td>48</td>
</tr>
<tr>
<td>Hs.296648</td>
<td>BMP5</td>
<td>bone morphogenetic protein 5</td>
<td>1,429</td>
<td>31</td>
<td>45</td>
</tr>
<tr>
<td>Hs.82002</td>
<td>EDNRB</td>
<td>endothelin receptor type B</td>
<td>1,358</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>Hs.644105</td>
<td>CD24</td>
<td>CD24 molecule</td>
<td>4,651</td>
<td>107</td>
<td>43</td>
</tr>
<tr>
<td>Hs.253994</td>
<td>FREM2</td>
<td>fraser-related extracellular matrix protein 2</td>
<td>926</td>
<td>22</td>
<td>41</td>
</tr>
<tr>
<td>Hs.140036</td>
<td>IGF2BP1</td>
<td>insulin-like growth factor 2 mRNA binding protein 1</td>
<td>3,146</td>
<td>83</td>
<td>38</td>
</tr>
<tr>
<td>Hs.191842</td>
<td>CDH3</td>
<td>cadherin 3, type 1, P-cadherin (placental)</td>
<td>2,564</td>
<td>69</td>
<td>37</td>
</tr>
<tr>
<td>Hs.657792</td>
<td>LR2P2</td>
<td>low density lipoprotein receptor-related protein 2</td>
<td>1,210</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Hs.745009</td>
<td>SIPA1L2</td>
<td>signal-induced proliferation-associated 1 like 2</td>
<td>3,323</td>
<td>96</td>
<td>34</td>
</tr>
<tr>
<td>Hs.494538</td>
<td>PTC1</td>
<td>patched 1</td>
<td>3,903</td>
<td>114</td>
<td>34</td>
</tr>
<tr>
<td>Hs.427202</td>
<td>TTR</td>
<td>transthyretin</td>
<td>2,301</td>
<td>67</td>
<td>34</td>
</tr>
</tbody>
</table>

The rightmost column indicates fold change between the mean value of the cardiac progenitor cells (CPCs) and the mean value of the epicardium-derived cells (EPDCs).
To investigate the homogeneity in the CPC and EPDC cultures, we performed immunocytochemistry and stained for WT-1 and CD73 in EPDCs, and Nkx2.5 and Islet-1 in CPCs. As shown in Fig. 1C, the EPDC cultures are highly homogenous with regard to positive immunostaining for WT-1 and CD73. Similarly, the CPC cultures consist mainly of cells staining positive for Nkx2.5 and Islet-1.

**Differentially Expressed Genes between CPCs and EPDCs Maintained under Normoxia**

To identify genes that were significantly differentially expressed between the CPCs and the EPDCs, we applied the SAM algorithm, which estimates the FDR, on all samples obtained from cells cultured in normoxia. Significantly up- or downregulated genes in the two groups of samples were identified with FDR \( < 0.05 \) and FC \( > 2 \) as a combined selection criterion. In total 3,289 mRNAs were identified as differentially expressed between these two populations. Of these, 1,910 showed higher expression in CPCs than in EPDCs (Suppl. Table S1), and 1,379 were more highly expressed in EPDCs than in CPCs (Suppl. Table S2). Tables 1 and 2 show the top 30 genes with largest differences in each of the two cell populations.

**GO annotation enrichment.** To map the functional properties of the significantly upregulated genes in the CPCs and the EPDCs, respectively (Suppl. Tables S1 and S2), a GO enrichment analysis was performed using Cytoscape (33) with the ClueGO plugin (6). The results show that the genes that show upregulation in the CPCs are highly involved in processes related to cell division and DNA replication as well as in the organization of cytoskeleton. A large fraction of the genes are involved in cardiac muscle cell action potential and regulation of contraction (Fig. 2A). With regard to the genes that are upregulated in the EPDCs, a large fraction of these are involved in regulation of apoptosis, signal transduction, and kappaB signaling. Other significantly overrepresented annotations are related to vascular development and regulation of cell-cell adhesion (Fig. 2B).

**Pathway analysis.** To investigate which pathways that are upregulated in CPCs and in EPDCs, pathway analyses were performed using each of the lists of differentially expressed genes (Suppl. Tables S1 and S2). Notably, there were many more significantly overrepresented pathways identified in the EPDCs than in CPCs. Using a threshold of FDR \( < 0.05 \), we identified 14 pathways as overrepresented among the 1,910 significantly upregulated genes in the CPCs and 64 pathways as overrepresented among the 1,379 significantly upregulated genes in the EPDCs (Fig. 3, A and B).

**Protein-protein interaction analysis.** To further investigate the interactivity among the upregulated genes in these cell types we performed a protein-protein interaction analysis to identify putative functional modules and hub proteins in CPCs and EPDCs, respectively. For this analysis we used significantly upregulated genes with FDR \( < 0.05 \) and FC \( > 7 \) as input to the STRING software (210 genes for CPCs and 278 genes for EPDCs), applying parameter settings as described in MATERIALS AND METHODS. Results from this analysis show interesting functional modules and hub proteins among the upreg-

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**Table 2. The top 30 differentially expressed genes from Suppl. Table S2 with significantly higher expression in the EPDCs than in the CPCs**

<table>
<thead>
<tr>
<th>UniGene ID</th>
<th>Symbol</th>
<th>Gene Description</th>
<th>Mean CPC</th>
<th>Mean EPDC</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs.126256</td>
<td>IL1B</td>
<td>interleukin 1, beta</td>
<td>32</td>
<td>7,976</td>
<td>250</td>
</tr>
<tr>
<td>Hs.164021</td>
<td>CXCL6</td>
<td>chemokine (C-X-C motif) ligand 6</td>
<td>44</td>
<td>8,168</td>
<td>187</td>
</tr>
<tr>
<td>Hs.654458</td>
<td>IL6</td>
<td>interleukin 6</td>
<td>51</td>
<td>8,207</td>
<td>160</td>
</tr>
<tr>
<td>Hs.209558</td>
<td>GLIPR1</td>
<td>GLI pathwayogenesis-related 1</td>
<td>46</td>
<td>6,589</td>
<td>143</td>
</tr>
<tr>
<td>Hs.132314</td>
<td>ELTD1</td>
<td>EGF, latrophilin and seven transmembrane domain containing 1</td>
<td>20</td>
<td>2,711</td>
<td>134</td>
</tr>
<tr>
<td>Hs.83169</td>
<td>MMP1</td>
<td>matrix metallopeptidase 1 (interstitial collagenase)</td>
<td>64</td>
<td>8,434</td>
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<td>Hs.624</td>
<td>CXCL8</td>
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<td>Hs.438231</td>
<td>TFFP2</td>
<td>tissue factor pathway inhibitor 2</td>
<td>61</td>
<td>7,669</td>
<td>125</td>
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<tr>
<td>Hs.654370</td>
<td>FAP</td>
<td>fibroblast activation protein, alpha</td>
<td>20</td>
<td>2,413</td>
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<tr>
<td>Hs.658169</td>
<td>SFRP4</td>
<td>secreted frizzled-related protein 4</td>
<td>18</td>
<td>2,188</td>
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<tr>
<td>Hs.477015</td>
<td>ABIDBP</td>
<td>ABI family, member 3 (NESH) binding protein</td>
<td>51</td>
<td>6,081</td>
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<td>CCL2</td>
<td>chemokine (C-C motif) ligand 2</td>
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<td>10,218</td>
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<td>CFH</td>
<td>complement factor H</td>
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<td>Hs.789</td>
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<tr>
<td>Hs.153952</td>
<td>NTSE</td>
<td>5-nucleotidase, ecto (CD73)</td>
<td>45</td>
<td>4,718</td>
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<tr>
<td>Hs.731660</td>
<td>IL33</td>
<td>interleukin 33</td>
<td>19</td>
<td>1,958</td>
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<td>Hs.1908</td>
<td>SRGN</td>
<td>serglycin</td>
<td>67</td>
<td>5,763</td>
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<td>Hs.591286</td>
<td>PTX3</td>
<td>pentraxin 3, long</td>
<td>41</td>
<td>3,410</td>
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<td>Hs.529053</td>
<td>C3</td>
<td>complement component 3</td>
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<td>Hs.81791</td>
<td>TNFRSF11B</td>
<td>tumor necrosis factor receptor superfamily, member 11b</td>
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<tr>
<td>Hs.613596</td>
<td>SNORD114-3</td>
<td>small nuclear RNA, C/D box 114-3</td>
<td>17</td>
<td>1,212</td>
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<td>Hs.40098</td>
<td>GREM1</td>
<td>gremlin 1, DAN family BMP antagonist</td>
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<td>5,696</td>
<td>69</td>
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<tr>
<td>Hs.457322</td>
<td>TNSAP6</td>
<td>tumor necrosis factor, alpha-induced protein 6</td>
<td>25</td>
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<td>Hs.502328</td>
<td>CD44</td>
<td>CD44 molecule (Indian blood group)</td>
<td>98</td>
<td>5,993</td>
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<tr>
<td>Hs.351316</td>
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<td>serpin peptidase inhibitor, clade E, member 1</td>
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<td>11,642</td>
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<td>Hs.403933</td>
<td>FBXO32</td>
<td>F-box protein 32</td>
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<tr>
<td>Hs.654572</td>
<td>LAMA4</td>
<td>laminin, alpha 4</td>
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<td>1,914</td>
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<td>Hs.1722</td>
<td>IL1A</td>
<td>interleukin 1, alpha</td>
<td>25</td>
<td>2,122</td>
<td>48</td>
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</tbody>
</table>

The rightmost column indicates fold change between the mean value of the CPCs and the mean value of the EPDCs.
lated genes in both cell types (Fig. 4, A and B). Interaction networks with three or fewer proteins are not included in the results. Notably, much higher interactivity was observed for the EPDCs than for the CPCs (Fig. 4).

Effect of Hypoxia on Gene Expression in CPCs and EPDCs

To investigate the hypoxic response in CPCs and EPDCs, we maintained the cells in hypoxic conditions in parallel to normoxic conditions for 24 h. The cells were subsequently harvested, and the difference in gene expression was determined. It is interesting to note that culturing the cells under hypoxic conditions had very little effect on gene expression in both CPCs and EPDCs. Overall, the CPC population was more sensitive to hypoxic stimulation than the EPDCs. In total 35 genes demonstrated at least 50% increased gene expression after hypoxia stimulation in CPCs compared with 16 genes in the EPDCs. Five of these genes (AK4, ALDOC, BNIP3P1, PGK1, SLC2A1) were overlapping between CPCs and EPDCs. We also observed two specific genes in each population (no overlap) that were repressed after hypoxia stimulation (Fig. 5A). The complete lists of all genes that were affected by hypoxia are shown in Tables 3 and 4.

Expression of Paracrine Factors in CPCs and EPDCs

Although the mechanisms by which stem cell-based therapy and/or activation may contribute to cardiac regeneration are still unknown, paracrine signaling appears to play a significant role. Thus, we were interested in investigating in detail the expression of genes for paracrine factors in CPCs and EPDCs. In total 92 genes from a predefined panel of biomarker for cardiovascular diseases (http://www.olink.se, Proseek Multiplex CVD I) were investigated. This analysis only included the normoxia conditions, and genes that significantly differed (FDR < 0.05) in expression between CPCs and EPDCs were identified. In total 30 genes were identified as differentially expressed, and the corresponding expression levels of these genes are shown in Fig. 6. Notably, for all except one gene (KITLG), the expression levels were higher in the EPDCs than in the CPCs. Interestingly, the CXCL genes together with CXCL1, MMP1, and IL6 displayed the largest differences (Fig. 6). These data show that EPDCs are highly active cells and that they have the capacity to generate a large panel of biologically active paracrine factors.

DISCUSSION

The present study reports for the first time a transcriptomic comparison of human iPSC-derived CPCs and human EPDCs. In addition, we also assessed the effect on gene expression of culturing these cells under hypoxic versus normoxic conditions, which, could be argued, is a more physiologically rele-
vant situation for cardiac progenitors in vivo, especially during a situation of cardiac injury when the cells become activated. The results presented here provide valuable insights into the molecular machinery operating at the transcriptional level in these progenitor populations. Notably, the impact of hypoxic vs normoxic culture conditions appears to be relatively modest, at least judging by the minor changes in gene expression observed between those experimental conditions.

Both CPCs and EPDCs represent cell populations that could be used for drug discovery as shown in our recent report on the use of CPCs in phenotypic screening (9). In recent years, phenotypic screening has been repopularized, and two key reports have shown the importance of phenotypic screening to discover novel, first-in-class drugs (10, 36). In areas of novel biology where there is limited knowledge of potential pathways and targets for disease intervention, screening compounds in an unbiased fashion on disease-relevant cells for the phenotype of interest is particularly attractive (41). This approach permits the discovery of novel biological targets and compounds, as well as compounds showing new mechanisms of action or polypharmacology. The cells most representative of human physiology are human primary or human iPSC-derived cells, and hence, they should be used where possible in phenotypic screening campaigns (11). However, a major chal-

Fig. 3. Pathway enrichment analysis. A: overrepresented pathways among genes that are significantly higher expressed in CPCs than in EPDCs. B: overrepresented pathways among genes that are higher expressed in the EPDCs than in the CPCs. More overrepresented pathways are identified in the set of genes that are upregulated in the EPDCs compared with the CPCs. Lines between pathways indicate a high fraction of common genes.
Fig. 4. A: protein-protein interaction network based on genes that are significantly upregulated in CPCs compared with EPDCs and have a fold change > 7. B: protein-protein interaction network based on genes that are significantly upregulated in EPDCs compared with CPCs and have a fold change > 7. A higher interactivity is shown among gene products that are upregulated in EPDCs compared with gene products that are upregulated in CPCs.
Table 3. Hypoxia responsive genes in the CPCs

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>FC 250</th>
<th>FC 290</th>
<th>FC 242</th>
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<tbody>
<tr>
<td>AK4</td>
<td>adenylate kinase 4</td>
<td>1.7</td>
<td>1.5</td>
<td>2.3</td>
</tr>
<tr>
<td>ALDOC</td>
<td>aldolase C, fructose-bisphosphate</td>
<td>2.0</td>
<td>2.1</td>
<td>2.1</td>
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<tr>
<td>ANK5B</td>
<td>ankyrin repeat and sterile alpha motif domain containing 4B</td>
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<td>1.5</td>
<td>1.6</td>
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<tr>
<td>BNIP3</td>
<td>BCL2/adenovirus E1B 19 kDa interacting protein 3</td>
<td>3.9</td>
<td>3.9</td>
<td>2.5</td>
</tr>
<tr>
<td>BNIP3P1</td>
<td>BCL2/adenovirus E1B 19 kDa interacting protein 3 pseudogene 1</td>
<td>4.2</td>
<td>3.1</td>
<td>2.0</td>
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<tr>
<td>CDC47L</td>
<td>cell division cycle associated 7-like</td>
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<td>1.7</td>
<td>1.6</td>
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<td>CDH10</td>
<td>cadherin 10, type 2 (T2-cadherin)</td>
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<td>1.8</td>
<td>2.0</td>
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<td>DHR33</td>
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<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>GLYATL2</td>
<td>glycine-N-acetyltransferase-like 2</td>
<td>2.4</td>
<td>2.2</td>
<td>1.6</td>
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<td>GPI</td>
<td>glucose-6-phosphate isomerase</td>
<td>1.8</td>
<td>1.7</td>
<td>1.6</td>
</tr>
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<td>H1F0</td>
<td>H1 histone family, member 0</td>
<td>1.7</td>
<td>1.6</td>
<td>1.6</td>
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<td>HAVCR1</td>
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<td>3.8</td>
<td>4.0</td>
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<td>hexokinase 2</td>
<td>2.7</td>
<td>3.8</td>
<td>1.9</td>
</tr>
<tr>
<td>KDM3A</td>
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<td>1.7</td>
<td>1.7</td>
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<tr>
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<td>lactate dehydrogenase A</td>
<td>3.9</td>
<td>4.0</td>
<td>3.4</td>
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<tr>
<td>LOX</td>
<td>lysyl oxidase</td>
<td>2.7</td>
<td>2.5</td>
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<td>1.8</td>
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<td>P4HA1</td>
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<td>2.1</td>
<td>2.1</td>
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<td>PFKFB4</td>
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<td>PGK1</td>
<td>phosphoglycerate kinase 1</td>
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<td>1.8</td>
<td>1.6</td>
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<td>RLF</td>
<td>rearranged L-myc fusion</td>
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<td>1.7</td>
<td>1.6</td>
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<tr>
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<td>1.7</td>
<td>1.8</td>
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<td>1.6</td>
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<td>1.6</td>
<td>1.5</td>
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<tr>
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<td>2.0</td>
<td>1.6</td>
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<td>TCP11L2</td>
<td>t-complex 11, testis-specific-like 2</td>
<td>1.6</td>
<td>1.8</td>
<td>1.9</td>
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<td>TGBF1</td>
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<td>1.5</td>
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<td>UNC13C</td>
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<td>1.9</td>
<td>1.7</td>
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The genes indicated in boldface represent those that are induced in both the CPCs and in the EPDCs after hypoxia stimulation. The columns to the right show the fold change (FC) values across the different CPC batches.

Fig. 5. A: response from the hypoxic stimulation showed a small fraction of genes with changed expression after the hypoxia treatment. In total 36 genes from the CPCs and 16 genes from the EPDCs showed at least 50% increase in gene expression, in each of the replicated samples. As shown in the Venn diagram, 5 of these genes were upregulated in both the CPCs and in the EPDCs. In total, 4 genes, 2 in the CPCs and 2 in the EPDCs, were identified with at least 50% repression in gene expression values in all of the replicated samples. No overlapping genes between the CPCs and the EPDCs were identified, with repressed expression after hypoxia stimulation. B: gene expression in CPCs and EPDCs under normoxia and hypoxia. Five genes show hypoxic response in both the CPCs and in the EPDCs, and the expression levels for these 5 genes in all replicates, before and after hypoxia stimulation, are shown in the graph. All 5 genes show upregulated expression with at least 50% after hypoxia stimulation. Blue bars represent CPCs, and green bars represent EPDCs.
Hypoxia responsive genes in the EPDCs

<table>
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<th>Gene Description</th>
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<th>FC 9633</th>
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<td>adenylate kinase 4</td>
<td>1.9</td>
<td>2.7</td>
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<td>ALDOC</td>
<td>aldolase C, fructose-bisphosphate</td>
<td>2.1</td>
<td>2.5</td>
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<td>BNIP3P1</td>
<td>BCL2/adenovirus E1B 19 kDa interacting protein 3 pseudogene 1</td>
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<td>2.1</td>
<td>3.0</td>
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<td>enolase 2 (gamma, neuronal)</td>
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<td>2.0</td>
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<td>N-myc downstream regulated 1</td>
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<td>PDK1</td>
<td>pyruvate dehydrogenase kinase, isozyme 1</td>
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<td>2.4</td>
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<td>2.9</td>
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<td>PGK1</td>
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<td>1.7</td>
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<td>2.6</td>
<td>1.7</td>
<td>3.3</td>
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<td>transmembrane protein 45A</td>
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<td>2.3</td>
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<td>1.9</td>
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<tr>
<td>RNY4P13</td>
<td>RNA, Ro-associated Y4 pseudogene 13</td>
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<td>2.0</td>
<td>1.5</td>
<td>2.9</td>
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<tr>
<td>TAF9B</td>
<td>TAF9B RNA polymerase II, TATA box binding protein (TBP)- associated factor, 31 kDa</td>
<td>2.0</td>
<td>1.6</td>
<td>1.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The genes indicated in boldface represent those that are induced in both the EPDCs and in the CPCs after hypoxia stimulation. The columns to the right show the FC values across the EPDC donors.

suggest that hypoxia does not have a dramatic effect on the phenotype of the cells, as only minor changes in gene expression were observed, primarily related to a small number of genes involved in cell survival and glycolysis. These results help to validate that running screens under normoxic conditions should still provide relevant read-outs, at least for these cell populations.

The five genes that were upregulated during hypoxia in both CPCs and EPDCs in our experimental conditions (Fig. 5B) have all been previously identified as hypoxia-responsive genes in other cell types. Notably, the levels of the stress-responsive protein AK4 have been reported to be increased in cultured embryonic kidney cells exposed to hypoxia (22). Furthermore, in response to hypoxia, hypoxia-inducible factor (HIF)-α has been shown to upregulate the transcript level of the glycolytic enzyme ALDOC as well as BNIP3 in cardiomyocytes, which is important in the cardiomyocyte death pathway (13, 42). The enzyme PGK1 and the glucose transporter SLC2A1 (GLUT1) are also directly regulated by HIF-1α (24), and, in our study, these genes are upregulated in both CPCs and EPDCs after the hypoxic stimulation.

CPCs and EPDCs are two distinct cell populations. Our results revealed a differential pattern of gene expression in these two cell types. In CPCs, the majority of enriched genes are involved in DNA synthesis and nuclear and cell division, suggesting CPCs are highly proliferative. Genes related to regulation of cardiomyocyte action potential and contraction are also enriched in CPCs, indicating that these cells are at least partially committed to the cardiomyocyte lineage. In contrast, in EPDCs a large fraction of the enriched genes include those involved in the apoptotic pathway, regulation of inflammatory signaling, and vascular development. The EPDCs have been extensively characterized EPDCs up to passage 12 and are stable in culture. Thus, the appearance of the upregulated apoptotic genes is unlikely to be due to culture conditions, but, rather, we suggest that these cells are prone to apoptosis. Alternatively, the relatively higher number of genes related to apoptosis could also be an artifact of comparing primary cells to ones that have been reprogrammed to a pluripotent state. After acute MI, there is marked increase in the number of EPDCs, based on the expression of WT-1, which rapidly declines in the following days, suggesting that in the in vivo situation EPDCs disappear at a high rate, either from apoptosis or their differentiation into myofibroblasts with downregulated expression of WT-1. As shown in our results, EPDCs produce a lot of proinflammatory factors that may promote apoptosis of these cells. It is interesting to note that several of the genes that are expressed at significantly higher levels in EPDCs compared with CPCs belong to the chemokine family (e.g., IL1B, CXCL6, IL6, CXCL8, CCL2, and IL33). This prompted us to perform a more focused analysis of some of these factors that may influence the growth and functional activities of nearby cells expressing the receptors for these factors and may also play a role in the inflammatory process post-MI. These results are in line with the notion that the epicardium is providing key factors to nurture growth of the developing heart as well as during injury-induced cardiac regeneration (25).

The development of novel strategies to increase cardiomyocyte renewal and repair is of great importance for the treatment of cardiac diseases. It is intriguing to speculate about the possible use of EPDCs and CPCs for future cardiac regenerative medicine, and two main avenues have been envisioned. One is the opportunity to isolate and expand ex vivo large quantities of progenitor cells that subsequently can be transplanted into the injured myocardium to aid repair, acting through various mechanisms to replenish new cardiomyocytes and blood vessels. There are, however, a number of challenges that need to be addressed before this becomes a clinical reality (32). The other alternative is to take...
advantage of progenitor cells residing in the adult heart and through pharmacological means activate those cells to promote cardiac repair. Recently, Zangi et al. (44) reported that intramyocardial injection of modified RNA encoding human VEGF-A increased the proliferation and differentiation of EPDCs toward endothelial cells, resulting in improved cardiac function and myocardial outcome after MI in mice. A recent study also suggests the intriguing possibility of directly reprogramming fibroblasts into CPCs using a set of defined factors (20). Although these data are promising, further studies are needed to determine if these results are translatable to the human system, and also the therapeutic opportunity in the clinical setting must be carefully evaluated from efficacy and safety perspectives.

Taken together, the data presented here provide further understanding of the characteristics of human iPSC-derived CPCs and human EPDCs. The differential gene expression profile of CPCs and EPDCs suggests that they may play different roles in cardiac homeostasis and post-MI repair, and an additive or synergistic effect may be achieved by activating both CPCs and EPDCs. Importantly, the gene expression profiles are affected only to a very minor degree by culturing the cells in hypoxic conditions, which, from a practical perspective, greatly simplifies the handling of the cells during experimental interrogations. Finally, it is also interesting to note that under the experimental conditions used here, the EPDCs appear to have the capacity to generate more paracrine factors compared with CPCs. Representative model systems, based on human cells, will pave the way for further advancements toward the development of novel interventions for cardiac regeneration.

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