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In situ transcriptome characteristics are lost following culture adaptation of adult cardiac stem cells

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Regenerative therapeutic approaches for myocardial diseases often involve delivery of stem cells expanded *ex vivo*. Prior studies indicate that cell culture conditions affect functional and phenotypic characteristics, but relationship(s) of cultured cells derived from freshly isolated populations and the heterogeneity of the cultured population remain poorly defined. Functional and phenotypic characteristics of *ex vivo* expanded cells will determine outcomes of interventional treatment for disease, necessitating characterization of the impact that *ex vivo* expansion has upon isolated stem cell populations. Single-cell RNA-Seq profiling (scRNA-Seq) was performed to determine consequences of culture expansion upon adult cardiac progenitor cells (CPCs) as well as relationships with other cell populations. Bioinformatic analyses demonstrate that identity marker genes expressed in freshly isolated cells become undetectable in cultured CPCs while low level expression emerges for thousands of other genes. Transcriptional profile of CPCs exhibited greater degree of similarity throughout the cultured population relative to freshly isolated cells. Findings were validated by comparative analyses using scRNA-Seq datasets of various cell types generated by multiple scRNA-Seq technology. Increased transcriptome diversity and decreased population heterogeneity in the cultured cell population may help account for reported outcomes associated with experimental and clinical use of CPCs for treatment of myocardial injury.

Stem cell therapy is a promising approach for mitigating pathological diseases such as heart failure, with cell populations derived from diverse origins proposed for autologous as well as allogeneic cell therapy¹⁻³. The presumption that donor cells retain essential characteristics derived from their original identity during *in vitro* expansion important to enhance regeneration has led to isolation of cardiac progenitor cells (CPCs) subjected to culture for expansion prior to reintroduction. Multiple donor cell types have been tested for basic biological characteristics and efficacy, with widely varying isolation and adoptive transfer methods^{4,5}. For example, CPCs used in clinical trials for cardiac repair are isolated and cultured using varying and unstandardized protocols⁶⁻⁹. Transcriptome profiling of cultured CPCs using varying isolation methods showed surprisingly high similarity¹⁰, possibly accounting for consistently modest functional improvement outcomes in the myocardium regardless of cell type³. However, bulk RNA sample profiling of cultured CPCs in prior studies masks population heterogeneity inherent to freshly isolated CPCs¹¹. Therefore, understanding the consequences and impact of culture expansion upon the transcriptome at the single cell level is essential to optimize and advance approaches intended to improve efficacy of stem cell-based cardiac regenerative therapy.

Transcriptome profiling of freshly isolated CPCs is challenging due to low yields of resident adult stem cells, with very limited transcriptome information on primary isolates of other stem cells¹²⁻¹⁵. Implementation of single-cell RNA-Seq (scRNA-Seq) allows for transcriptional profiling of low cell numbers as well as revealing population heterogeneity. Technical aspects of scRNA-Seq tend toward choosing between transcriptome depth with limited number of cells versus massively parallel sequencing using hundreds to thousands of cells with shallower transcriptome coverage. Recent advances in massively parallel scRNA-Seq demonstrate the capability

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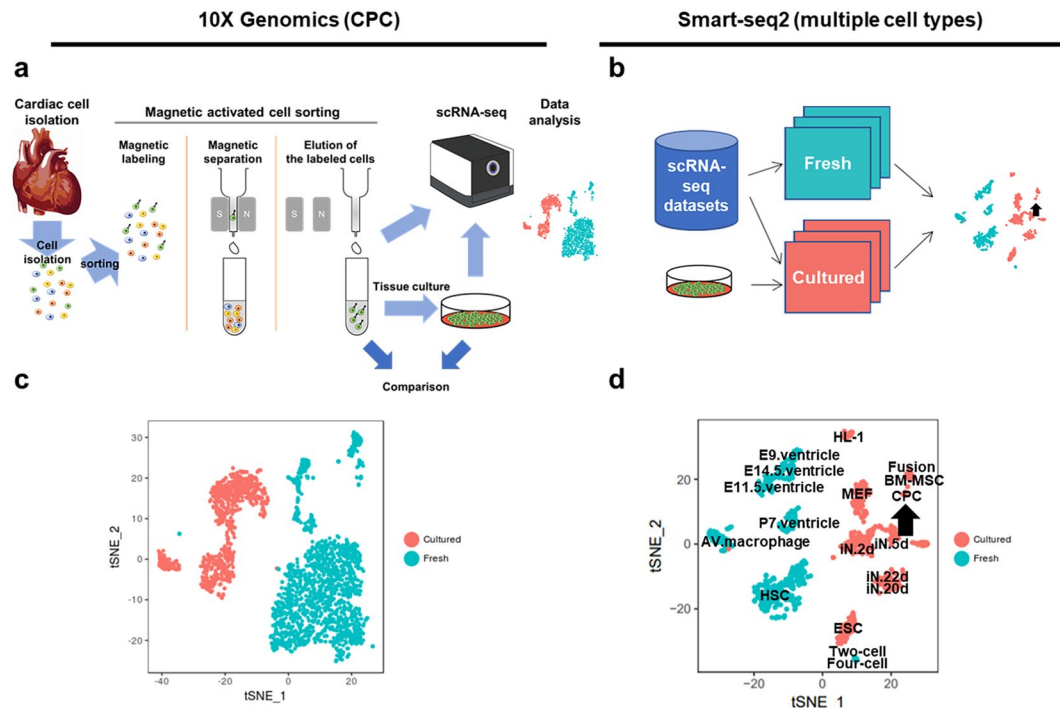


Figure 1. Transcriptomic differences between freshly isolated and cultured cells revealed by scRNA-Seq. **(a,b)** Workflow of scRNA-Seq of CPCs using 10X Genomics **(a)** or Smart-Seq2 technology **(b)**. Datasets for Smart-Seq2 analyses were derived from open access transcriptome repositories for both fresh or cultured samples (scRNA-Seq datasets, blue cylinder; see supplemental table for detail information) or samples prepared in-house from cultured CPCs (represented as dish). **(c,d)** t-SNE plots show freshly isolated cells cluster separately from cultured cells using 10X Genomics dataset (1,615 fresh cells and 850 cultured cells; **c**) or Smart-seq. 2 dataset (576 fresh cells and 550 cultured cells; **d**). Arrow indicates cultured CPCs in **(b,d)**. CPC, cardiac progenitor cell; BM.MSC, bone marrow-derived mesenchymal stromal cell; MEF, mouse embryonic fibroblast; ESC, embryonic stem cell; iN, induced neuronal cell; HL-1, atrial cardiomyocyte cell line; Fusion, Fusion cell of BM-MSC and HL-1; AV macrophage, atrioventricular node macrophage; E9, E11.5, E14.5, embryo 9, 11.5, 14.5 days, respectively; P7, postnatal 7 days; HSC, hematopoietic stem cell; Two-cell, embryonic cells at two-cell stage; Four-cell, embryonic cells at four-cell stage.

to maximize number of single cells captured per sample while still capturing primary characteristics of transcriptome variation^{11,16,17}. Unfortunately, the relatively recent advent of massively parallel scRNA-Seq has yet to produce the range and depth of scRNA-Seq datasets acquired using Smart-Seq2 technology that is limited by small population samples¹⁸. Therefore, a combination of both scRNA-Seq approaches involving Smart-Seq2 as well as massively parallel transcriptome profiling was used to determine the transcriptome identity and population heterogeneity of CPCs either as freshly isolates versus their cognate cultured counterparts. scRNA-Seq data analysis was performed by Seurat analysis and represented in t-SNE plotting to show transcriptome relationships between single cells. Additionally, consistency of t-SNE plots results were validated by varying perplexity value as well as principal component inclusion values to confirm reproducibility. Based on the scRNA-Seq data analysis comparing freshly isolated cells and cultured cells, we identified common and global transcriptome alterations consequential to *in vitro* expansion. Findings reveal that isolation and *in vitro* expansion of CPCs selects for transcriptional profiles of uniform composition resulting in loss of *in situ* characteristics as well as population heterogeneity. The consequences of this transcriptional drift and homogenization of cellular phenotypes offers fundamental biological insight regarding the basis for consistently modest efficacy of CPC-based cell therapy and prompts reassessment of the rationale for tissue-specific stem cell sources.

Results

Transcriptome drift of freshly isolated CPCs following short term culture. Transcriptional profiling was performed using freshly isolated cells and their *in vitro* derivatives to reveal consequences of short term culture. Population characteristics were revealed by scRNA-Seq using the 10x Chromium platform. Seurat analysis followed by t-SNE plot representation shows the distinct relationship between freshly isolated CPCs ($c\text{-kit}^+/\text{Lin}^-$) versus cultured CPC populations expanded under standard conditions¹⁹ for five passages (Fig. 1a). Both fresh and cultured CPC scRNA-Seq datasets were mapped to mouse genome, aggregated using Cell Ranger v2.0 (10X Genomics), and unsupervised clustering performed using Seurat R package²⁰ (Fig. 1b). Separation between fresh versus cultured CPCs clusters was clearly demonstrated by t-SNE plot²¹, revealing divergence of transcriptome between these two cell populations based upon spatial distance (1,615 fresh CPCs and 850 cultured CPCs; Fig. 1c). Robustness of ‘clear separation’ between fresh cells and cultured cells was tested with multiple different

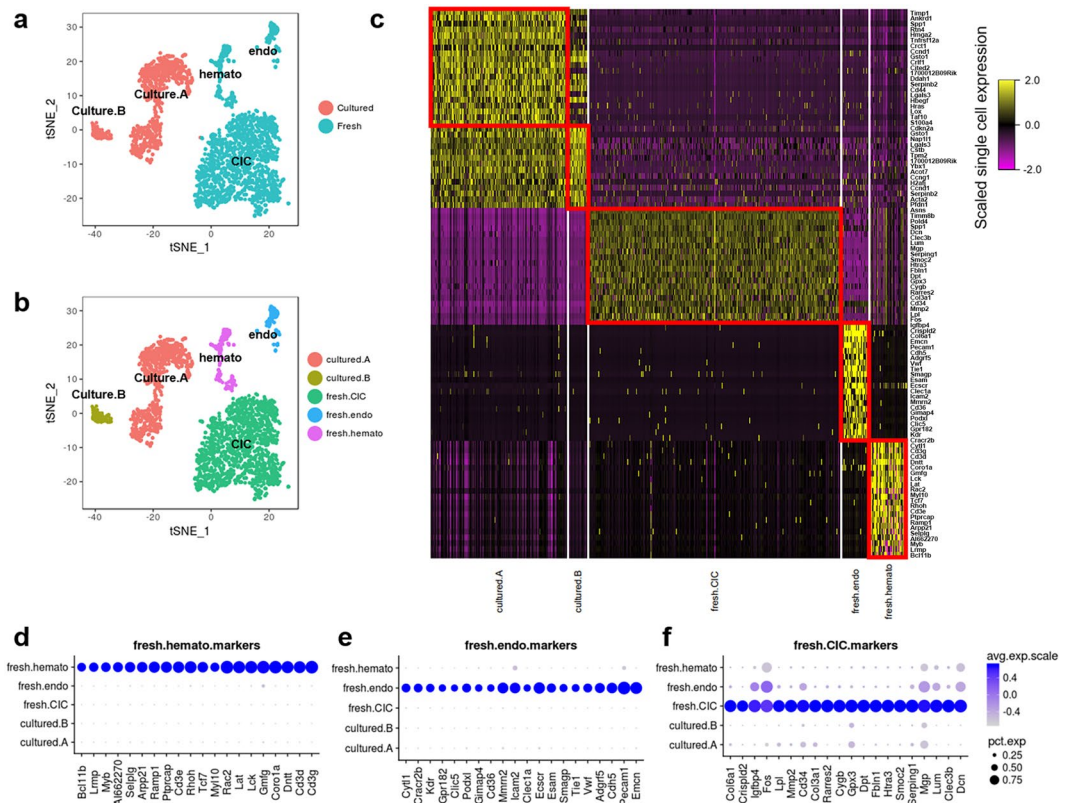


Figure 2. Loss of identity markers following *ex vivo* expansion. **(a,b)** t-SNE plots show single cells labeled by environments **(a)** or cell types **(b)**. Cell clusters are identified by expression level of marker genes and gene set analysis as shown in Figs S4 and S5. **(c)** Heatmap representing top 20 marker genes of each cluster. **(d–f)** Dotplots representing top 20 marker genes of each cluster. Marker genes were identified in an unbiased fashion blind to known cell type markers. Individual dots are sized to reflect the proportion of cells of each type expressing the marker gene and colored to reflect the mean expression of each marker gene across all cells as indicated in the key.

parameter settings as previously reported for fresh murine heart cell isolates¹¹. Clustering is remarkably robust regardless of parameter setting for t-SNE plotting such as perplexity or the number of principal components (Fig. S1). Clustering results reflect differences between fresh and cultured cells and principal component analysis (PCA) also showed distinctive clustering in consistent with t-SNE results (Fig. S2). Collectively, these findings reveal substantial transcriptome divergence between fresh versus cultured CPCs at the single cell level.

Analyses were broadened to determine transcriptome alterations resulting from culture for additional cell types. Deeper transcriptome coverage for CPCs was achieved using Smart-Seq2 technology as further validation of 10X Genomics results (Fig. 1) that provides wider population-based coverage but possesses limited resolving power for distinguishing marginally different cell types limited due to shallower sequencing depth (50,000–100,000 reads per cell). Meta-analysis of multiple scRNA-Seq datasets including both variety of fresh cells and various cultured cells was performed^{22–28} (Supplementary Table) using single-cell datasets (around 1,500 cells) generated by Smart-Seq2¹⁸. Processing of datasets was performed comparably to initial CPC analyses with mapping to mouse genome and clustering analysis using Seurat R Package. Unsupervised clustering showed similar clear separation between fresh and cultured cells in Smart-Seq2 datasets (576 fresh cells and 550 cultured cells; Fig. 1d) comparable to findings with 10X Genomics (Fig. 1a). Normalization of downloaded Smart-Seq2 scRNA-Seq datasets for sequencing depth prior to comparison to exclude effects of sequencing depth variation was also performed, downsampling to 500,000 reads per cell by random selection of raw reads from fastq files and re-analyzed. Comparable trends of separation between fresh versus cultured cells remained in meta-analysis of downsampled data (Fig. S3), reinforcing the conclusion that transcriptomic alteration by tissue culture is a shared phenomenon regardless of cell type and consistent between the two scRNA-Seq methodologies of 10X Genomics and Smart-Seq2.

Subpopulations revealed by clustering analyses of freshly isolated and cultured CPCs.

Dimensionality reduction followed by unsupervised clustering reveals three distinct sub-clusters within the freshly isolated CPC ($c\text{-kit}^+/\text{Lin}^-$) population (Fig. 2a,b), one of which represents a heterogeneous cell population including smooth muscle cells, pericytes, and two different fibroblast subpopulations (Fig. S4b,c) based upon marker expression of cardiac cells¹¹ was collectively categorized as ‘cardiac interstitial cells’ (CICs). Two other sub-clusters from the fresh isolated CPC ($c\text{-kit}^+/\text{Lin}^-$) population express markers consistent with endothelial or

hematopoietic cells based on gene set analysis (Fig. S4a). Cultured cells were divided into two groups, ‘CultureA’ and ‘CultureB’ through unsupervised clustering (Fig. 2a,b). Gene set analysis using a set of differentially expressed genes (DEGs) between CultureA and CultureB was performed to identify cell type of the two cultured cell groups. Genes enriched in CultureA primarily include cell adhesion-related genes, while genes of CultureB cluster include respiratory energy metabolism-related genes (Fig. S5a–d). Although ClusterA and Cluster B are different through gene set analysis, transcriptomic signature was lacking since no cell type-specific gene set was detected. Additionally, fresh CPC marker expression²⁹ levels were comparatively low in cultured CPCs (Fig. S5f). Each cluster of freshly isolated cell possesses a unique set of DEGs not detected in other clusters, while the two clusters of cultured cell share their DEGs (Fig. 2c). In summary, sub-populations were readily identifiable in freshly isolated CPCs, but not evident in cultured CPCs.

Loss of identity markers and enhanced cell proliferation consequential to culture expansion.

The relationship between fresh versus cultured CPCs was assessed for expression of marker genes between clusters. Identifier marker genes for fresh cell clusters were substantially down-regulated in cultured cells (Figs 2d–f and S5e). Marker transcript identity for cultured cells revealed protein metabolism and cell cycle pathways were enriched in the 10X Genomics cultured dataset assessed by searching for upregulated genes using gene set analysis with gene ontology (GO) terms. Elevated expression of transcripts associated with protein synthesis and cell proliferation is consistent with biological selective pressures of *in vitro* expansion (Fig. 3a). Proliferation-related pathways (such as ‘mesenchymal cell proliferation’) were detected as a common pathway between 10X Genomics and Smart-Seq2 data (Fig. 3b). Expression patterns of all cell cycle (CC) -related genes was assessed in both datasets³⁰ to confirm the result of gene set analysis at individual gene level. Multiple G1/S phase- and G2/M phase-specific genes were commonly upregulated in cultured cells (Fig. 3c–f) including aurora kinases (Aurka and Aurkb), cyclin B2 (Ccnb2), centromere proteins (Cenpa, Cenpe and Cenpf) and cyclin-dependent kinase 1 (Cdk1) in both 10X Genomics and Smart-Seq2 datasets. Based upon expression level of CC genes, cell cycle scores were calculated³⁰ and cell cycle stages were estimated (Fig. 3g–j). A higher G2/M ratio was present in cultured cells indicative of proliferative state (average G2/M ratio: 4.8% and 29.0% in fresh and cultured CPCs, respectively; 9.3% and 43.7% in fresh and cultured cells from meta-analysis, respectively). Collectively, cultured cells are characterized by loss of original identity markers and a transcriptome profile characterized by up-regulated cell proliferation genes.

Increased diversity of transcriptome consequential to *in vitro* expansion. Transcriptome alteration in cultured CPCs and meta-analysis of multiple cell types indicates a global (widespread) alteration of transcriptome rather than limited reprogramming of selected cell type-specific genes. Overall transcriptome diversity reflected by the number of detected genes in 10X Genomics dataset increased two-fold in cultured CPCs, corroborated by Smart-Seq2 dataset meta-analysis (median number of genes detected: 2,065 and 4,312 genes in fresh and cultured cells for 10X Genomics, and 3,490 and 6,528 genes in fresh or cultured cells for Smart-Seq2, respectively; Fig. 4a,b; $p < 0.001$, Wilcoxon rank sum test). Cultured cells still exhibit significantly increased detected genes after normalization by downsampling (median: 4,187 and 5,559 genes in fresh or cultured cells, respectively; Fig. 4c; $p < 0.001$, Wilcoxon rank sum test). These findings support the premise that *ex vivo* expansion of CPCs promotes increased transcriptome diversity despite loss of tissue-specific marker identity (Figs 2 and 3).

Interestingly, transcriptome alterations prompted by *ex vivo* expansion increase population homogeneity compared to freshly isolated CPCs extracted from their native niche *in vivo* microenvironment (Fig. 4d). The postulate that cultured cells exhibit greater transcriptome similarity than freshly isolated cells was confirmed by Smart-Seq2 datasets (Fig. 4e). The environmental influence of cell culture promotes transcriptome migration toward a common shared profile throughout the population with minimal unique signatures. In summary, environment-dependent influences play a major role in determination of cellular transcriptome and *ex vivo* culture expansion of isolated CPCs dramatically alters their transcriptional profile.

Discussion

Every cell type is characterized by a unique set of expressed genes known as identity markers together with commonly shared gene profiles. Retaining cellular identity is important to maintain functional properties that presumably are inextricably associated with biological activity. In the context of cardiac stem cell therapy, multiple cell types have been used for therapeutic intervention with standard protocols involving *in vitro* cell expansion to provide sufficient quantities for introduction into the damaged myocardium. However, drift in transcriptional identity of freshly isolated CPCs used for stem cell treatment consequential to *in vitro* expansion has not been addressed on the single cell level. Findings from this study derived from massively parallel digital transcriptomic profiling and meta-analysis of multiple scRNA-Seq datasets reveal that *in vitro* CPC expansion is associated with increased transcriptome diversity by acquiring expression of thousands of genes, up-regulation of cell cycle and metabolism genes, and loss of identity marker gene expression that increased transcriptional similarity among multiple types of cultured cells (Fig. 5). Transcriptional profiles in response to environment are largely conserved among various cell types, which has important implications for implementation and expectations of cardiac stem cell therapeutic implementation as discussed below.

Consequences and desirability of phenotypic and functional alterations to *ex vivo* expanded CPCs are largely unrecognized and uncharacterized, but there is ample evidence that culture conditions exert profound influence upon cellular biological properties^{31–34}. For example, low oxygen tension improved self-renewal and maintained differentiation potential³⁵, whereas microcarrier and stirrer systems increased CPC expansion while retaining cellular phenotype and omics profiles³¹. Stem cell immaturity and inhibition of gene methylation was promoted by replacement of serum with human supplements^{34,36,37}. Collectively, the intent of such studies is to improve cell expansion efficiency or the quality of the expanded cells through the manipulation of culture conditions, but the

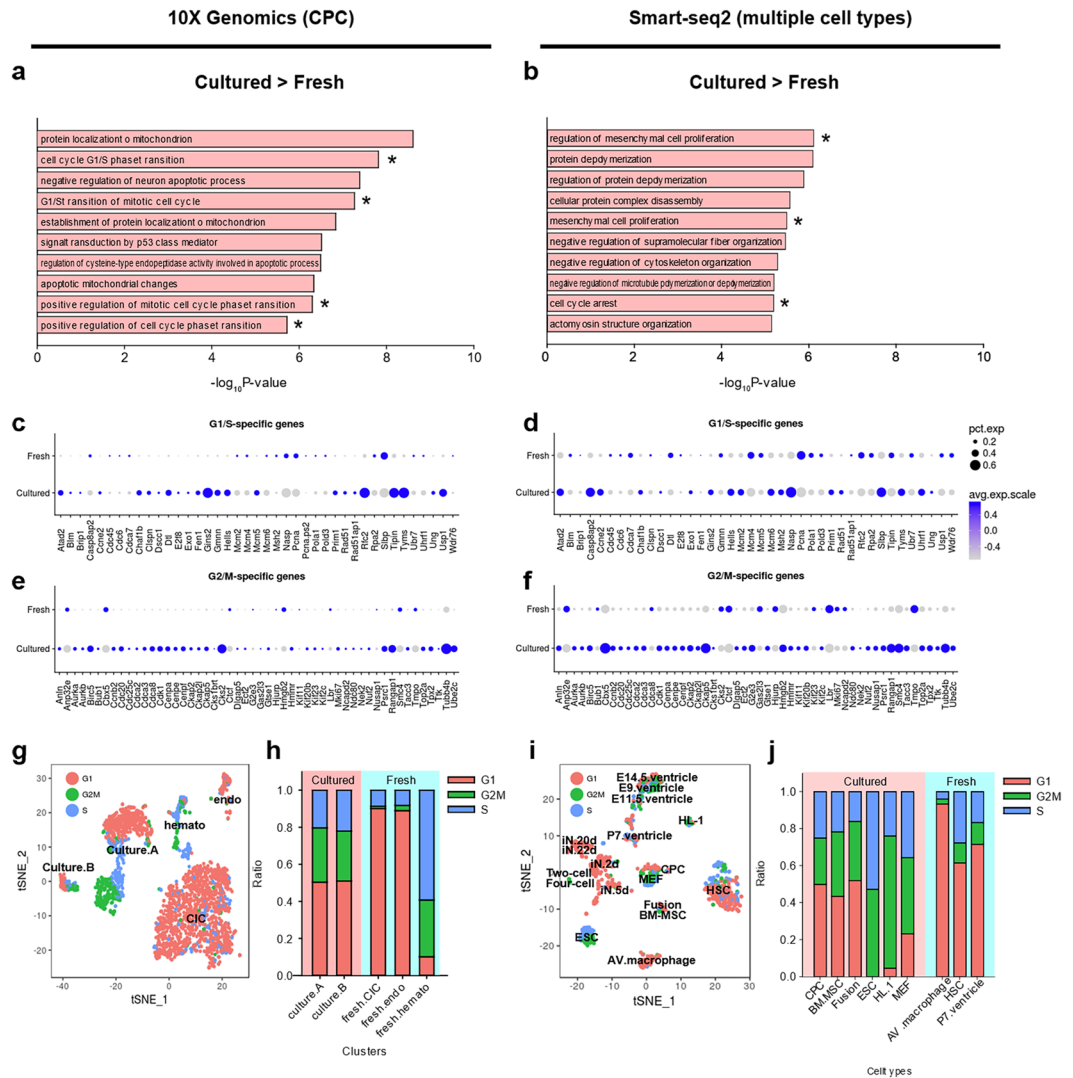


Figure 3. Culture expansion increases expression of cell proliferation-related genes. **(a,b)** Bar graph represents top 10 GO terms up-regulated in cultured cells revealed by gene set enrichment analysis from 10X Genomics **(a)** or Smart-Seq2 data analysis **(b)**. Asterisks represent cell proliferation-related GO terms commonly observed in both 10X Genomics and Smart-Seq2. **(c-f)** Dotplots representing expression level of cell cycle genes. The size of dots represents the percentage of cells with sequencing reads for each gene and color code represents average expression level. Expression level of G1/S and G2/M-specific genes are shown in **(c,e)**, respectively, for 10X Genomics dataset with identical genes shown in **(d,f)** for Smart-Seq2 dataset. Individual dots are sized to reflect the proportion of cells of each type expressing the marker gene and colored to reflect the mean expression of each marker gene across all cells, as indicated in the key. **(g,i)** t-SNE plots labeled by estimated cell cycle status for 10X Genomics data **(g)** or Smart-Seq2 data **(i)**. **(h,j)** Stacked bar graph representing ratio of cell cycle stages in each cluster for 10X Genomics data **(h)** or Smart-Seq2 data **(j)**. Embryonic cells were excluded from fresh cells and experimentally differentiated neurons were excluded from cultured cells in panel **(j)** because embryonic cells are highly proliferative unlike other fresh cells and differentiated neurons are post-mitotic cells unlike other cultured cells.

referential point of how such manipulations drift from ancestral fresh cell origins was not a factor in assessments. Consequences of *in vitro* expansion and biological drift upon cellular properties is an important consideration, especially if the intended outcome is to capitalize upon the identity and functionality of forerunners by creating an expanded derivative population with traits conserved from their origins.

Transcriptome profiles may be more profoundly altered by culture conditions depending upon cellular differentiation status. Culture conditions provoked relatively minor differences in mesenchymal stem cell (MSC) from uncultured predecessors at transcriptome level¹², whereas terminally differentiated cells such as brain macrophages and endometrial cells are all altered substantially by *in vitro* conditions^{14,15}. Previous studies suggest CPC multipotentiality for lineage commitment³⁸ that could lead to prediction of modest transcriptomic changes resulting from *in vitro* expansion as noted for MSC¹². However, results with CPCs presented in this report indicate profound changes in transcriptome with coordinated up-regulation of majority of cell cycle genes (Fig. 3),

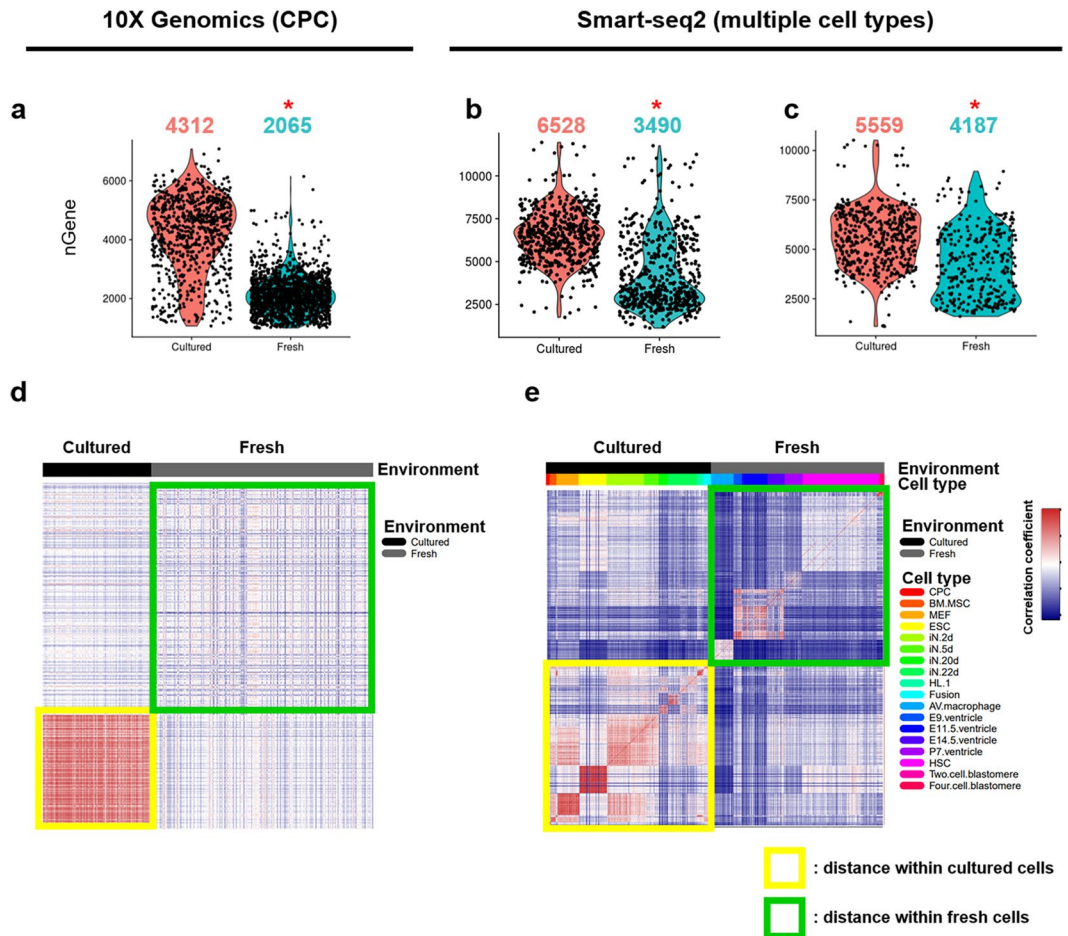


Figure 4. Cells acquire thousands of genes expressed during tissue culture while losing heterogeneity. (a–c) Violin plots represent the number of detected genes in freshly isolated cells or cultured cells in 10X Genomics (a) or in Smart-Seq2 (b,c) datasets. The number above the violin plots represent the median value of the number of detected genes. nGene, the number of detected genes; *p-value < 2.2e-16, Wilcoxon rank sum test. (d,e) Heatmaps depicting similarity between single-cells, indicating cultured cells have higher similarity compared with the similarity between fresh cells in 10X Genomics (d) or in Smart-Seq2 datasets (e). CPC, cardiac progenitor cell; BM.MSC, bone marrow-derived mesenchymal stromal cell; MEF, mouse embryonic fibroblast; ESC, embryonic stem cell; iN, induced neuronal cell; HL-1, atrial cardiomyocyte cell line; Fusion, Fusion cell of BM-MSC and HL-1; AV macrophage, atrioventricular node macrophage; E9, E11.5, E14.5, embryo 9, 11.5, 14.5 days, respectively; P7, postnatal 7 days; HSC, hematopoietic stem cell; nGene, the number of genes detected.

in contrast to down-regulated cell cycle inhibitors promoting increased cell cycle activity in MSC¹². Technical differences in transcriptome analyses could also play a role, as the microarray used for comparing fresh MSC with cultured MSC might not be sensitive as RNA-sequencing used for other cell types presented herein. Changes in transcriptome resultant to culture conditions for CPC may also reflect capacity of the *in vivo* niche to inhibit cellular activation and proliferation. Considerations of bulk versus scRNA-Seq and microarray versus whole transcriptome profiling will always present challenges when performing cross-platform comparisons of transcriptomic datasets and needs further attention to identify potential limitations and caveats.

The advent of scRNA-Seq technology has enabled a powerful advance in delineation of population heterogeneity of freshly isolated cells^{17,39–41}. In comparison, relatively little attention has been focused upon characterization of population heterogeneity for donor cells prepared for cell therapy, particularly at the single cell level. The overarching findings presented herein show that CPC population heterogeneity decreases following *in vitro* expansion resulting in a more homogeneous transcriptomic profile. Future studies will need to determine the general applicability of these observations to additional cardiac-derived stem cell types as recently characterized by our group²⁵. Implications of donor cell population homogeneity for therapeutic stem cell treatment are significant, as the capacity of *ex vivo* expanded effector cells to maintain their acquired *in vitro* transcriptomic profile or adapt to *in vivo* environmental conditions upon reintroduction to damaged myocardium remain wholly unexplored.

The substantial alteration of transcriptome as well as loss of original identity markers suggests a marked change in functional characteristics of *in vitro* expanded cells from their freshly isolated ancestors. A key question to be resolved remains as to whether alterations prompted by culture conditions exhibit plasticity upon

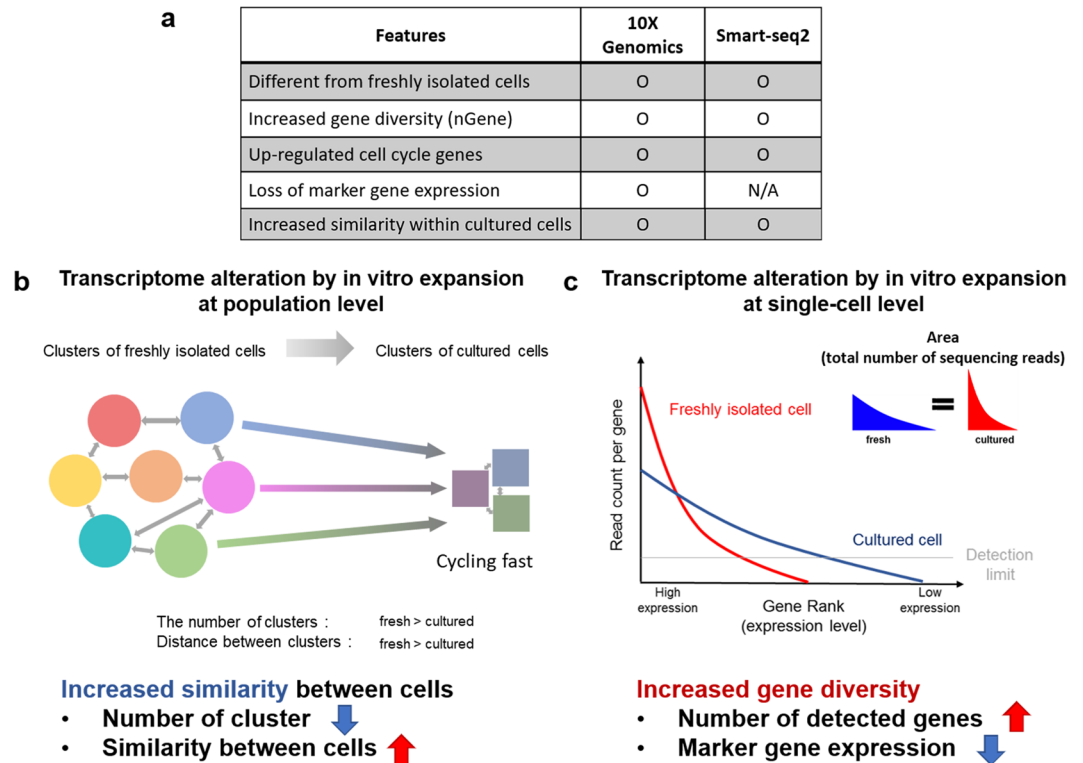


Figure 5. Transcriptome alteration by *in vitro* expansion. (a) Five key features of cells acquired during tissue culture. (b,c) Proposed model at the aspect of population (b) or single-cell (c) level. *Ex vivo* expansion in culture prompts loss of transcriptomic heterogeneity, leading to high similarity between cultured cells regardless of cell type. Expression of identity markers is suppressed with concomitant acquisition of expression for thousands of genes following culture characterized by increased transcriptome diversity.

introduction to intact tissue. Limitations of our conclusions include the possibility that murine cells may be less stable in culture compared to human cells and that cell products prepared for clinical trials may be used at lower passages. Tissue culture influence upon efficacy of cell therapy and mitigation of undesirable transcriptional reprogramming requires systematic analyses using the multiple cell types currently being advances for clinical interventional approaches. Findings of considerable transcriptional drift and decreased population heterogeneity for *in vitro* expanded cells revealed in this study could well account for consistently modest outcomes of cardiovascular cell therapy regardless of chosen cell type⁴². Greater appreciation of the impact, permanence, and functional benefits or impairments yielded by *in vitro* expansion of stem cells will contribute significantly toward development of improved protocols and cell preparations to enhance the reparative potential of adoptively transferred regeneration-associated cellular effectors.

Methods

Isolation of c-Kit⁺/Lin⁻ CPC populations. Adult c-Kit⁺ CPCs were isolated and expanded as previously described⁴³. Briefly, for each sample preparation, two FVB female mice hearts were perfused on a Langendorff system for blood removal, and tissue was subsequently digested for 10–15 minutes with Liberase DH digestion buffer (Roche 05401089001, 5 mg/mL in perfusion buffer) and dissociated through pipetting. After removing cardiomyocytes by cell strainers, c-Kit⁺/Lin⁻ CPCs were obtained by immunomagnetic sorting with Lineage depletion kit and CD117-conjugated Microbeads (Miltenyi Biotec 130-048-102). Fresh isolated CPCs were subjected to immediate single-cell RNA-Seq analysis or cultured for five passages and then used for single-cell RNA-seq. All experiments involving mice and use of vertebrate animals were carried out according to Institutional Review Boards (IRB) policy and approved by the Institutional Animal Care and Use Committee (IACUC) at San Diego State University.

Single-cell RNA-seq. *10X Genomics platform.* Freshly isolated and cultured CPCs suspensions were loaded on a Chromium™ Controller (10x Genomics) and single-cell RNA-Seq libraries were prepared using Chromium™ Single Cell 3' Library & Gel Bead Kit v2 (10x Genomics) following manufacturer's protocol. Each library was tested with Bioanalyzer (average library size: 450–490 bp). The sequencing libraries were quantified by quantitative PCR (KAPA Biosystems Library Quantification Kit for Illumina platforms P/N KK4824) and Qubit 3.0 with dsDNA HS Assay Kit (Thermo Fisher Scientific). Sequencing libraries were loaded at 2 pM on an Illumina HiSeq2500 with 2 × 75 paired-end kits using the following read length: 98 bp Read1, 8 bp i7 Index, and 26 bp Read2.

Smart-Seq2 platform. After trypsinizing cultured CPCs, single cells were captured under stereomicroscope by mouth pipetting with a ~0.2 mm diameter flame-pulled glass Pasteur pipet attached to aspirator tube (Sigma-Aldrich, A5177). Selected cells were dispensed into Eppendorf tube containing 10 μ L cell lysis buffer provided by Smart-Seq v4 kit. cDNA was synthesized following manufacturer's protocol (Smart-Seq v4 ultra low amount cDNA kit Clontech, 634888) and illumina sequencing libraries were then constructed using the Nextera XT DNA Sample Preparation kit (Illumina, FC-131-1024). The sequencing libraries were quantified by quantitative PCR (KAPA Biosystems Library Quantification Kit for Illumina platforms P/N KK4824) and Qubit 3.0 with dsDNA HS Assay Kit (Thermo Fisher Scientific). The pooled libraries were sequenced as paired-end 75 \times 75 base reads on a NextSeq500 with mid-output kit.

Data Analysis. *10X Genomics platform.* The raw data was processed with the Cell Ranger pipeline (10X Genomics; version 2.0). Sequencing reads were aligned to the mouse genome mm10. Preparations derived from two mouse hearts per sample were used to produce 1,615 freshly isolated cells or 850 cultured cells for analysis. Cells with fewer than 1,000 genes or more than 10% of mitochondrial gene UMI count were filtered out and genes detected fewer than in three cells were filtered out⁴⁴. Altogether, 2,383 cells and 15,786 genes were kept for downstream analysis using Seurat R Package (v2.3.0). Approximately 2,000 variable genes were selected based on their expression and dispersion. The first 15 principal components were used for the t-SNE projection²¹ and unsupervised clustering⁴⁴. Gene expression pathway analysis was performed using clusterProfiler⁴⁵.

Smart-Seq2 platform. Smart-Seq2 scRNA-Seq datasets were obtained from public databases (Supplementary Table) with exception of the CPC dataset generated for this study. Sequencing reads were mapped to UCSC mouse genome mm10 using STAR v2.5.2b⁴⁶ with default parameters and only uniquely mapped reads were kept. Read counts table was used as an input for generating Seurat object. Cells with fewer than 1,000 genes or more than 10% of mitochondrial gene count were filtered out and genes detected fewer than in three cells were filtered out. To exclude effects of sequencing depth variation, scRNA-Seq raw data were downsampled to 500,000 reads per cell by random selection of raw read from fastq files and re-analyzed. 18,698 genes and 1,126 cells were kept for further analysis. Clustering analysis and downstream analysis were performed as outlined in the 10X Genomics platform section.

Statistics. Significant differences in the number of genes detected between fresh and cultured datasets from both platforms were analyzed with Wilcoxon matched-pairs rank sum test, meeting distribution assumption with statistical significance accepted when $p < 0.05$.

Data availability. scRNA-Seq data generated in this study has been uploaded to the Gene Expression Omnibus (GEO) database (GSE114280). Seven public scRNA-Seq data sets were used in this study (see details in Supplementary Table).

References

1. Caplan, A. I. & Correa, D. The MSC: an injury drugstore. *Cell Stem Cell* **9**, 11–15 (2011).
2. Ellison, G. M. *et al.* Adult c-kit(pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. *Cell* **154**, 827–842 (2013).
3. Nguyen, P. K., Rhee, J. & Wu, J. C. Adult stem cell therapy and heart failure, 2000 to 2016: A systematic review. *JAMA Cardiology* **1**, 831–841 (2016).
4. Eschenhagen, T. *et al.* Cardiomyocyte Regeneration: A Consensus Statement. *Circulation* (2017).
5. Hatzistergos, K. E. & Vedenko, A. Cardiac Cell Therapy 3.0: The Beginning of the End or the End of the Beginning? *Circ. Res.* **121**, 95–97 (2017).
6. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ. Res.* **95**, 911–921 (2004).
7. Mohsin, S. *et al.* Human cardiac progenitor cells engineered with Pim-I kinase enhance myocardial repair. *J. Am. Coll. Cardiol.* **60**, 1278–1287 (2012).
8. Smith, R. R. *et al.* Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation* **115**, 896–908 (2007).
9. Smits, A. M. *et al.* Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an *in vitro* model for studying human cardiac physiology and pathophysiology. *Nat. Protoc.* **4**, 232–243 (2009).
10. Gaetani, R. *et al.* Different types of cultured human adult cardiac progenitor cells have a high degree of transcriptome similarity. *J. Cell. Mol. Med.* **18**, 2147–2151 (2014).
11. Skelly, D. A. *et al.* Single-Cell Transcriptional Profiling Reveals Cellular Diversity and Intercommunication in the Mouse Heart. *Cell Rep.* **22**, 600–610 (2018).
12. Boquest, A. C. *et al.* Isolation and transcription profiling of purified uncultured human stromal stem cells: alteration of gene expression after *in vitro* cell culture. *Mol. Biol. Cell.* **16**, 1131–1141 (2005).
13. Aviles-Vazquez, S. *et al.* Global gene expression profiles of hematopoietic stem and progenitor cells from patients with chronic myeloid leukemia: the effect of *in vitro* culture with or without imatinib. *Cancer Med.* **6**, 2942–2956 (2017).
14. Krjutskov, K. *et al.* Single-cell transcriptome analysis of endometrial tissue. *Hum. Reprod.* **31**, 844–853 (2016).
15. Gosselin, D. *et al.* An environment-dependent transcriptional network specifies human microglia identity. *Science* **356** (2017).
16. Mayer, C. *et al.* Developmental diversification of cortical inhibitory interneurons. *Nature* **555**, 457–462 (2018).
17. Zheng, G. X. *et al.* Massively parallel digital transcriptional profiling of single cells. *Nat. Commun.* **8**, 14049 (2017).
18. Picelli, S. *et al.* Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* **9**, 171–181 (2014).
19. Khan, M. *et al.* beta-Adrenergic regulation of cardiac progenitor cell death versus survival and proliferation. *Circ. Res.* **112**, 476–486 (2013).
20. Satija, R., Farrell, J. A., Gennert, D., Schier, A. F. & Regev, A. Spatial reconstruction of single-cell gene expression data. *Nat. Biotechnol.* **33**, 495–502 (2015).
21. van der Maaten, L. & Hinton, G. Visualizing Data using t-SNE. *J. Mach. Learn. Res.* **9**, 2579–2605 (2008).
22. Biase, F. H., Cao, X. & Zhong, S. Cell fate inclination within 2-cell and 4-cell mouse embryos revealed by single-cell RNA sequencing. *Genome Res.* **24**, 1787–1796 (2014).

23. DeLaughter, D. M. *et al.* Single-Cell Resolution of Temporal Gene Expression during Heart Development. *Dev. Cell* **39**, 480–490 (2016).
24. Freeman, B. T., Jung, J. P. & Ogle, B. M. Single-cell RNA-seq reveals activation of unique gene groups as a consequence of stem cell-parenchymal cell fusion. *Sci. Rep.* **6**, 23270 (2016).
25. Hulsmans, M. *et al.* Macrophages Facilitate Electrical Conduction in the Heart. *Cell* **169**, 510–522 e520 (2017).
26. Ilicic, T. *et al.* Classification of low quality cells from single-cell RNA-seq data. *Genome Biol.* **17**, 29 (2016).
27. Treutlein, B. *et al.* Dissecting direct reprogramming from fibroblast to neuron using single-cell RNA-seq. *Nature* **534**, 391–395 (2016).
28. Yang, J. *et al.* Single cell transcriptomics reveals unanticipated features of early hematopoietic precursors. *Nucleic Acids Res.* **45**, 1281–1296 (2017).
29. Smith, A. J. *et al.* Isolation and characterization of resident endogenous c-Kit⁺ cardiac stem cells from the adult mouse and rat heart. *Nat. Protoc.* **9**, 1662–1681 (2014).
30. Tirosh, I. *et al.* Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* **352**, 189–196 (2016).
31. Gomes-Alves, P. *et al.* *In vitro* expansion of human cardiac progenitor cells: exploring 'omics tools for characterization of cell-based allogeneic products. *Transl. Res.* **171**(96–110), e111–113 (2016).
32. Roobrouck, V. D., Vanuytsel, K. & Verfaillie, C. M. Concise review: culture mediated changes in fate and/or potency of stem cells. *Stem Cells* **29**, 583–589 (2011).
33. Silva, M. M. *et al.* Robust Expansion of Human Pluripotent Stem Cells: Integration of Bioprocess Design With Transcriptomic and Metabolomic Characterization. *Stem Cells Transl. Med.* **4**, 731–742 (2015).
34. Bieback, K. *et al.* Altered gene expression in human adipose stem cells cultured with fetal bovine serum compared to human supplements. *Tissue Eng. Part A* **16**, 3467–3484 (2010).
35. Forsyth, N. R. *et al.* Transcriptome alterations due to physiological normoxic (2% O₂) culture of human embryonic stem cells. *Regen. Med.* **3**, 817–833 (2008).
36. Shahdadfar, A., Fronsdal, K., Haug, T., Reinholt, F. P. & Brinckmann, J. E. *In vitro* expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. *Stem Cells* **23**, 1357–1366 (2005).
37. Dahl, J. A. *et al.* Genetic and epigenetic instability of human bone marrow mesenchymal stem cells expanded in autologous serum or fetal bovine serum. *Int. J. Dev. Biol.* **52**, 1033–1042 (2008).
38. Beltrami, A. P. *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114**, 763–776 (2003).
39. Grun, D. *et al.* Single-cell messenger RNA sequencing reveals rare intestinal cell types. *Nature* **525**, 251–255 (2015).
40. Pollen, A. A. *et al.* Low-coverage single-cell mRNA sequencing reveals cellular heterogeneity and activated signaling pathways in developing cerebral cortex. *Nat. Biotechnol.* **32**, 1053–1058 (2014).
41. Yan, K. S. *et al.* Intestinal Enteroendocrine Lineage Cells Possess Homeostatic and Injury-Inducible Stem Cell Activity. *Cell Stem Cell* **21**, 78–90 e76 (2017).
42. A futile cycle in cell therapy. *Nat. Biotechnol.* **35**, 291 (2017).
43. Konstandin, M. H. *et al.* Fibronectin is essential for reparative cardiac progenitor cell response after myocardial infarction. *Circ. Res.* **113**, 115–125 (2013).
44. Macosko, E. Z. *et al.* Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell* **161**, 1202–1214 (2015).
45. Yu, G., Wang, L. G., Han, Y. & He, Q. Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* **16**, 284–287 (2012).
46. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).

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Author Contributions

T.K. and M.A.S. designed the study. A.C. and K.M.B. isolated and cultured CPCs and B.J.W. captured single-CPCs for Smart-seq2. T.K. performed scRNA-Seq experiments and most of data analysis. B.-H.K. analyzed data. T.K. and M.A.S. wrote the article. O.H.E. edited the article. All authors read and approved the final article.

Additional Information

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