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Engrafted Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes Undergo Clonal Expansion In Vivo

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Preclinical studies have suggested that transplanted human pluripotent stem cell–derived cardiomyocyte (hPSC-CM) grafts expand because of proliferation.[1] This knowledge came from cell cycle activity measurements that cannot discriminate between cytokinesis or DNA synthesis associated with hypertrophy. To refine our understanding of hPSC-CM cell therapy, we genetically engineered a cardiomyocyte-specific fluorescent barcoding system into an hPSC line. Because cellular progeny have the same color as parental hPSC-CMs, we could identify subsets of engrafted hPSC-CMs with greater clonal expansion.

hPSC lines were generated by knocking 4 copies of the Cre-dependent Brainbow 3.2 lineage reporter[2] into WTC11 cells (Figure [A]). These rainbow hPSCs were transduced with cTnT (cardiac troponin T)–driven Cre, which restricts expression of the rainbow barcoding system to committed cardiomyocytes (Figure [A]). Rainbow labeling was observed after 7 days of differentiation, and immunostaining confirmed that labeled cells express cTnT (Figure [B]). A sparse labeling strategy was used to avoid expression of the same color code in neighboring cells, hence permitting single cell tracking over time (Figure [C]). Cre-mediated recombination elicited all 18 of the possible hues (Figure [D]).

By day 14, hPSC-CMs had clonally expanded (Figure [E]). On average, the number of cardiomyocytes per clone went from 1.03 to 1.71 (day 7 versus 14, $P<0.03$; Figure [F]). Whereas most rainbow hPSC-CMs had not proliferated, some were highly proliferative and a subset of hPSC-CMs continued to proliferate after replating at day 14 (Figure [F]), including hPSC-CMs that were lactate selected. Repeat imaging of replated hPSC-CMs over days 15 to 28 confirmed that neighboring cells had unique hues and daughter cardiomyocytes inherited the parental fluorescent barcode (Figure [G]), definitively demonstrating that these clusters arise from clonal expansion. We observed that hPSC-CM displayed limited cell migration (Figure [G]) and underwent hypertrophic growth as measured by cell area (Figure [H]). At day 28, clonally expanded hPSC-CMs were 4.07-fold smaller than nondividing hPSC-CMs ($P<0.0001$; Figure [H]). Staining confirmed rainbow labeling demarcated cardiomyocytes and showed multinucleation in nondividing, hypertrophied hPSC-CMs (Figure [I] and [J]). Consistent with the subset of clonally expanded hPSC-CMs, we conducted unbiased graph-based clustering on raw single cell RNA sequencing data[3] from isogenic day 15 and day 30 WTC11 hPSC-CMs and found a unique subset (686 of 18 073 cells) with increased mitosis and cytokinesis gene expression (AURKB, CDK1, CCNB1; 5.54-fold to 6.18-fold increase versus all cells, $P<0.0001$). These results suggest the heterogeneity in hPSC-CM proliferation may be regulated transcriptionally.

For the transplantation studies, sparsely labeled day 14 hPSC-CMs were dissociated, resuspended in Matrigel with prosurvival cocktail, and transplanted into the hearts of immune-compromised athymic rats.[1] All animal experiments were

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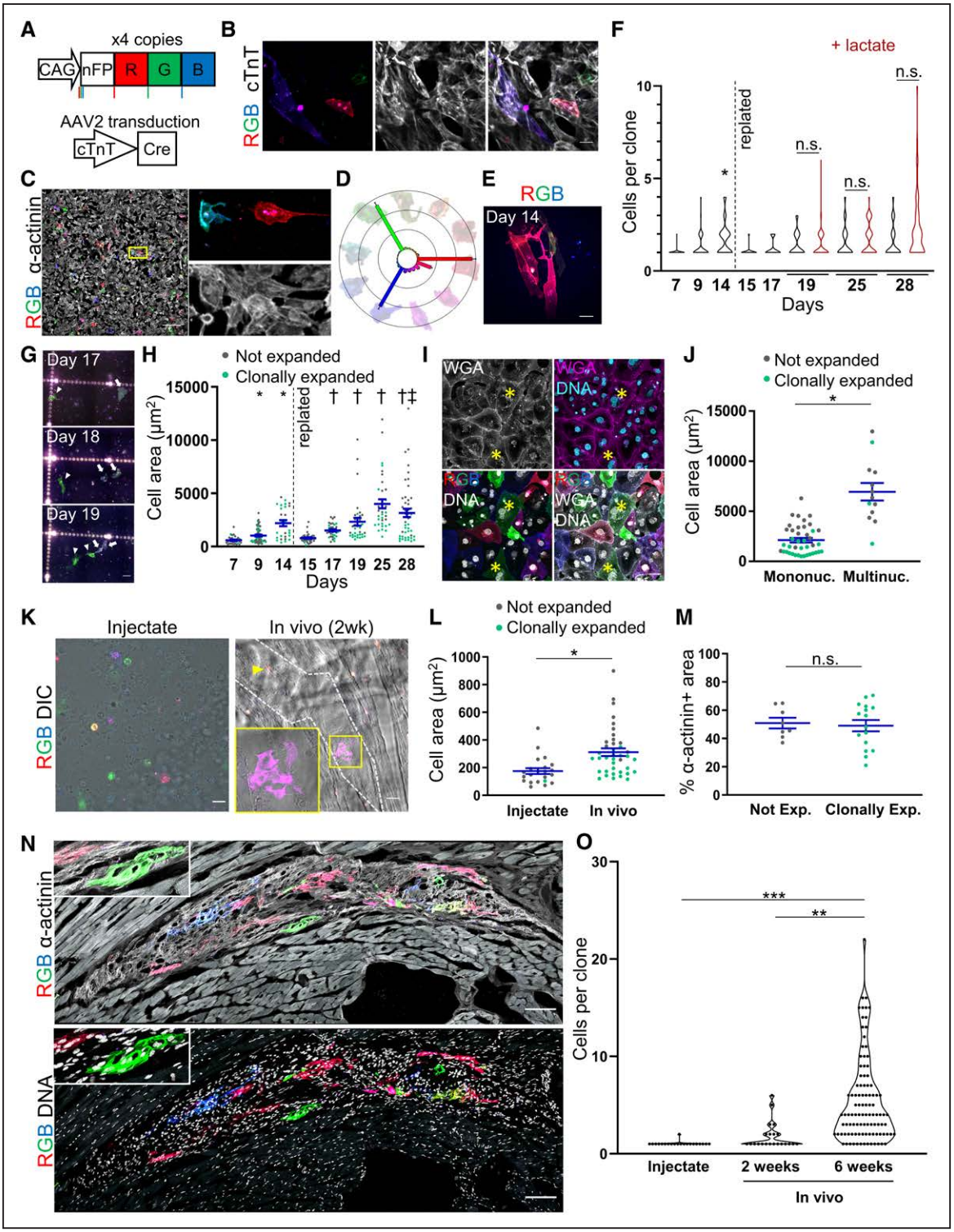

Figure. Select cardiomyocytes clonally expand in vitro and in vivo.

A, Schematic of rainbow human pluripotent stem cell (hPSC) WTC11 line with 4 copies of the rainbow construct and cTnT (cardiac troponin T)–activated labeling. Before expression of Cre recombinase, rainbow hPSCs express a nonfluorescent GFP (green fluorescent protein) mutant (nFP). By means of the Cre–lox recombination system, gene excision may occur randomly at the loxP (red vertical bars), lox2 (green vertical bars), or loxN (blue vertical bars) sites, resulting in expression of 1 of 3 fluorescent proteins: mOrange2 (shown as red), enhanced GFP (shown as green), or mKate2 (shown as blue). Cells were labeled after they committed to the cardiomyocyte lineage by using an adeno-associated virus (AAV) plasmid (Addgene 69916) with inverted terminal repeat sequences flanking a cTnT promoter upstream of the *Cre* and *TdTomato* genes. TdTomato was excised by *Not*I digestion and the resulting construct used for AAV serotype 2 (AAV2) production. AAV2 was added to rainbow hPSCs on differentiation day 0 of a modified directed differentiation protocol.[1] **B**, Rainbow-labeled hPSC–cardiomyocytes (CMs) express cTnT protein (scale bar 20 μm). On day 14, cells were fixed with 4% paraformaldehyde and stained for cTnT (ThermoFisher MA5-12960). Imaging was performed on a Nikon TiE equipped with Yokogawa W1 spinning disk confocal system and high-sensitivity electron multiplying charge-coupled device camera. **C**, Representative image of sparsely labeled rainbow hPSC-CMs on day 9 and stained for α-actinin (Sigma A7811; scale bar, 200 μm). (*Continued*)

Figure Continued. AAV2 was serially diluted to determine optimal dose for sparse labeling. Images were collected on a Leica SP8 confocal microscope with fixed laser lines and a spectral detector. **D**, Images were analyzed on a per pixel basis and binned by the 18 possible hues using a custom generated MATLAB code. Examples of rainbow hPSC-CMs expressing different hues are shown around the color wheel. **E**, Clonally expanded rainbow hPSC-CMs at day 14 in the differentiation (scale bar, 10 μm). **F**, Quantification of rainbow hPSC-CM clonal expansion over time and after replating on day 14. Cultures that were lactate selected on days 18 to 22 are in red. At least 24 clones were analyzed per condition. **P*<0.05 vs day 7. **G**, Time lapse imaging of cells that were dissociated into single-cell suspensions and replated rainbow hPSC-CMs showing cell proliferation (scale bar, 40 μm). Arrows point to an hPSC-CM that divides between day 17 and 18; arrowheads point to an hPSC-CM that divides between day 18 and 19. **H**, Cell area in nonexpanded (ie, singlets) and clonally expanded hPSC-CMs (ie, ≥ 2 cells) are color coded gray or green, respectively. **P*<0.05 vs day 7, †*P*<0.05 vs day 15, ‡*P*<0.05 not expanded vs clonally expanded at day 28. **I**, Rainbow hPSC-CMs stained for wheat germ agglutinin (WGA) and DNA show instances of multinucleation, indicated by asterisks (scale bar, 20 μm). **J**, Quantification of cell area as a function of the number of nuclei per cell for rainbow hPSC-CMs on day 28. Nondividing and clonally expanded hPSC-CMs are labeled in gray or green, respectively. **P*<0.05. **K**, Left image from the injectate 2 hours after preparation shows sparse labeling of rainbow hPSC-CMs (scale bar, 20 μm). Right image from host rat heart shows engrafted hPSC-CMs 2 weeks after injection; graft region is marked by dashed lines. Arrowhead points to a singlet rainbow hPSC-CM; box and inset show clonally expanded hPSC-CM (scale bar, 40 μm). On day 14, sparsely labeled rainbow hPSC-CMs were resuspended in Matrigel plus a prosurvival cocktail (50 million cells/mL). Athymic male Sprague-Dawley rats (rnu-rnu, 250–300 g, Harlan) were administered 5 mg/kg cyclosporin for 7 days, starting a day before the engraftment procedure. Animals received 1 mg/kg sustained-release buprenorphine, were induced with inhaled isoflurane, and were mechanically ventilated during lateral thoracotomy to expose the left ventricle, where 3 injections were performed to deliver 5 million cells in total. Hearts were perfused with KB cardioplegia buffer followed by 4% paraformaldehyde, fixed with 4% paraformaldehyde overnight, washed with phosphate-buffered saline, and mounted in optimal cutting temperature (Tissue-Tek) for cryosectioning of 10-μm-thick specimens that were stained and analyzed as described previously. **L**, Quantification of cell area in engrafted rainbow hPSC-CMs 2 weeks after engraftment and in comparison with injectate. **P*<0.05. **M**, Quantification of α-actinin content normalized by cell area for engrafted rainbow hPSC-CMs that did not divide or clonally expand. Images were analyzed in ImageJ to quantify the area of α-actinin and then dividing by the total cell area. **N**, Image of rainbow reporter hPSC-CMs 6 weeks after engraftment costained with α-actinin (top) and DNA (bottom). Inset shows higher magnification. Scale bars, 100 μm. **O**, Quantification of clonal expansion at 2 weeks and 6 weeks after engraftment. ***P*<0.001; ****P*<0.0001. Single-cell RNA sequencing analysis and quantification was performing using 10× Genomics Cell Ranger, Loupe Browser, and Benjamini-Hochberg correction for multiple tests. GraphPad Prism V8 was used for 2-tailed unpaired *t* tests (Figure [**H**; clonally expanded versus not expanded at day 28], [**J**], [**M**], and [**N**]), 1-way analysis of variance with Tukey post hoc tests (Figure [**O**]), and 1-way repeated-measures analysis of variance with Tukey test (Figure [**F**] and [**H**]). A *P* value lower than 0.05 was considered statistically significant. Each quantification was derived from a minimum of 3 independent experiments (different passages for in vitro experiments or different animals in in vivo studies). All average values described in the text and figures are mean values, and error bars represent standard error of the mean. All data and materials are available from the corresponding author on request.

performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the University of Washington institutional animal care and use committee (protocol 4376-01). Terminal deoxynucleotidyl transferase dUTP nick end labeling staining demonstrated that 17.8±2.6% of hPSC-CMs underwent apoptosis 24 hours after injection. Two weeks after engraftment, most hPSC-CMs hypertrophied (1.75-fold increase versus injectate, *P*<0.002), whereas some subsets clonally expanded (Figure [K] and [L]). To ensure that the results were not driven by false positives, the cell injectate was imaged to confirm that neighboring hPSC-CMs did not express the same barcode at the time of transplantation (Figure [K]). Differences in proliferative potential among engrafted hPSC-CMs were not attributable to differences in sarcomere content, because the proportion of α-actinin+ area was the same in both clonally expanding and nonexpanding groups (*P*>0.77; Figure [M]). After 6 weeks of engraftment, cumulative clonal expansion increased further, demonstrating that hPSC-CMs continue to proliferate in vivo at later time points, with the average number of cells per clone being 1.05 (injectate), 1.77 (2 weeks), and 5.41 (6 weeks; *P*<0.001 versus 2 weeks, *P*<0.0001 versus injectate; Figure [N] and [O]). Notably, the heterogenous amount of clonal expansion among engrafted hPSC-CMs would not have been observed without the rainbow single-cell reporter.

hPSC-CM therapy is rapidly approaching clinical use, and it is critical to understand how these cells behave in vivo. Single-cell transcriptomics assays[3] and DNA content analysis[4] have revealed profound molecular heterogeneity among hPSC-CMs. By generating a cTnT lineage rainbow reporter, longitudinal tracking of the hypertrophic and proliferative growth of individual hPSC-CMs is now possible. This approach demonstrates that hPSC-CMs have heterogeneous levels of proliferation in vitro and after engraftment in host myocardium. By examining the generation of newly formed cardiomyocytes, rather than using proxies for cell proliferation, this study distinguished bona fide cardiomyocyte division versus incomplete cell cycle activation. The heterogenous proliferative capacity among hPSC-CMs is consistent with findings that demonstrated that only a few clonally dominant cardiomyocytes generate most of the adult zebrafish heart,[5] suggesting a similar mechanism may underlie hPSC-CM graft expansion. Proliferative hPSC-CMs express normal levels of sarcomere contractile elements, suggesting increased hPSC-CM clonal expansion could efficiently repopulate myocardium lost to injury. This is in line with cardiac cell therapy optimizations, such as cotransplantation of hPSC-CMs with epicardial cells,[1] demonstrating improved outcomes from stimulating grafted hPSC-CM proliferation, although graft cell function and clinical relevance remains uncertain. Thus, controlling engrafted hPSC-CM clonal expansion holds promise for improving cardiac regenerative therapies.

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Disclosures

Drs Murry and El-Nachef are significant equity holders in Sana Biotechnology. Drs Murry and El-Nachef hold patents related to heart regeneration technology. Dr Sniadecki is a significant equity holder in Stasys Medical Corporation and a modest equity holder in Curi Bio. The other authors report no conflicts.

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