Supplemental Material

Online Methods

Human Cardiac Stem Cell Isolation

Cardiac biopsies were obtained from patients undergoing LVAD implantation. NIH guidelines for human subject research are consistent with Institutional Review Board (IRB) exemption based upon the use of tissues that are waste discards from normal and routine clinical procedures of LVAD surgery (45 CFR 46.101). After excision, cardiac tissue remained on ice in cardioplegic solution until processed. Fatty tissue was excised and remaining cardiac tissue was suspended in Basic Buffer (15 mL) and minced into 1 mm^3 pieces. After mincing, tissue and Basic Buffer were collected in 50 mL Falcon tube. Digestive solution containing collagenase, type II 225 U/mg dry weight (Worthington, #LS004174, Bio Corp, Lakewood, NJ) was dissolved in Basic Buffer (2-2.5 mg/mL) and incubated with tissue pieces for 1.5-2 hours at 37°C with continuous shaking. Digestion solution was refreshed at the one-hour time point and resulting suspensions were centrifuged at 350 *g* for 5 minutes and resuspended in CPC media (see Table 1). Final suspension was filtered through a 100-μm filter (Corning, #352360) followed by a 40-μm filter (Corning, #352340) and centrifuged at 150 *g* for 2 minutes to collect CMs. The supernatant was collected and centrifuged at 350 *g* for 5 minutes and resuspended in CPC media and incubated overnight at 37°C in CO_2 incubator.

The following day, cells in suspension were collected in 50 mL Falcon tube. Any cells attached were dissociated using a 1:1 mixture of Cellstripper (Corning, #25-056-CI) and TrypLE Express (1X) (Thermo Fisher Scientific, #12604-013). Resulting suspension was filtered through a 40-μm filter, centrifuged at 350 *g* for 5 minutes, and resuspended in wash buffer (PBS plus 0.5% bovine serum albumin). To isolate c-Kit^+ cells, suspension was incubated with c-Kit–labeled beads (Miltenyi Biotec, #130-091-332) and sorted according to the manufacturer's protocol. The c-Kit^+ fraction was divided as such: half the population was suspended in CPC media (see Table 1) and the other half was suspended in EPC media (see Table 1). The c-Kit^- population was further incubated with CD90/CD105–labeled beads and sorted according to the manufacturer's protocol (Miltenyi Biotec, #130-096-253/130-051-201). Cells positive for CD90/CD105 were suspended in MSC media (see Table 1). To isolate an EPC population, at 1 week the c-Kit^+ population plated in EPC media was further sorted using CD133–labeled beads and sorted according to the manufacturer's protocol (Miltenyi Biotec, #130-097-049). All cells were cultured at 37°C in CO_2 incubator in their respective growth media. CPC and EPC were split 1:2 when they reached 60-70% confluency. MSC were split 1:2 when they reached 90% confluency. Patient information for the five cardiac samples used in this study can be found in Online Table III. All cells used in this study were mid-passage (passages 5–9).

Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR) and Bioinformatics

Total RNA was isolated from cardiac stem cell populations using Quick-RNA MiniPrep kit (Zymo Research, #R1055) according to manufacturer's protocol. 500 ng of RNA were used to generate complementary DNA (cDNA) using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, #170-8891). The amplified cDNA was diluted at a ratio of 1:10 in DNase- and RNase- free water. qRT-PCR were completed using iQ SYBER Green (Bio-Rad Laboratories, #170-8882) on a CFX Real-Time PCR Detection System (Bio-Rad). cDNA was amplified using primers specific to genes of interest (listed in Online Table II). The comparative or ΔΔCt method of qRT-PCR data analysis was used to analyze samples; variability in cDNA concentration was normalized using

18S. Hierarchical clustering and supervised clustering for gene expression profiling were performed using Expander 7.1 software[1].

Immunocytochemistry

Cardiac stem cell populations were plated on 2-well chamber glass slides (10,000 cells/well) in their respective growth media (see Table 1) for a minimum of 24 hours. After incubation, slides were washed with PBS and fixed in 4% paraformaldehyde for 5 minutes at 4°C. Following fixation, the slides were washed twice with PBS and permeabolized in PBS plus 0.1% Triton X-100, 0.1 M Glycine for 3 minutes, then washed once with PBS and blocked with TNB (1X TN (Tris-HCl, NaCl) Buffer, 5 µg/mL blocking reagent (PerkinElmer, #FP1012)) for 30 minutes. Primary antibodies were diluted in TNB (see Online Table I) and incubated overnight at 4°C. The following day slides were washed twice with PBS. Fluorescently conjugated secondary antibodies were diluted in TNB (1:200) and incubated 1.5 hours at room temperature. For c-Kit staining a horseradish peroxidase (HRP)-linked secondary antibody (1:500) was used, followed by tyramide signal amplification (1:50) (PerkinElmer, #NEL753001KT). After washing twice with PBS, DAPI was included in a final wash to fluorescently label the nuclei, and slides were coverslipped with Vectashield® mounting reagent (Vector Laboratories, #H-1000). All slides were imaged using a Leica TCS SP8 confocal microscope. A table of antibodies and dilution ratios is available in Online Table I.

Cell Morphology Measurement

Cardiac stem cell populations were imaged using a Leica DMIL inverted tissue culture phase contrast microscope. Cell morphology was measured by tracing the outline of the cells using Image J software. The three measurements analyzed were Area, Roundness, and Length-to-Width (L/W) ratios. L/W ratios were calculated by dividing Feret/Min Feret measurements. A minimum of 30 cells was measured per cell line.

Cell Proliferation Assay

Cell populations were plated in quadruplicate (1,000 cells/well) in a 96-well black flat bottom plate with 100 µL/well of their respective growth media. Cell proliferation rate was determined using a CyQUANT Direct Cell Proliferation Assay (Thermo Fisher Scientific, #C35011) on days 0, 1, 3 and 5. Doubling times were calculated based on periods of exponential growth using a population doubling time online calculator (http://www.doubling-time.com/compute_more.php).

Flow Cytometry

For live cell analysis, single cells were suspended in 100 µL wash buffer (PBS plus 0.5% bovine serum albumin) and incubated with primary antibody (see Online Table I for dilutions) on ice for 30 minutes. Following, cells were washed with wash buffer and incubated with secondary antibody (1:100) for 20 minutes on ice. For fixed cell analysis, cells were suspended in 4% paraformaldehyde for 5 minutes at room temperature and then washed twice with wash buffer. For c-Kit analysis requiring permeabilization, cells were washed twice and resuspended in PBS plus 0.1% Triton X-100, 0.1 M Glycine for 3 minutes, then washed once. Fixed cells were suspended in 100 µL wash buffer and incubated with primary antibody on ice for 1 hour. Following, cells were washed twice and incubated with secondary antibody (1:100) for 30 minutes on ice. For both fixed and live cells a total of 300 µl wash buffer was added post secondary incubation and the cells were analyzed by flow cytometry with a BD FACS Canto instrument. Unstained and isotype controls were used to establish baseline fluorescence levels. Data was analyzed by Flow Jo software (BD Biosciences). A minimum of 10,000 cell counts was analyzed. Due to low cell count for H13-066 MSC, the c-Kit count was 5,000.

Matrigel Tube Formation
Growth factor reduced matrigel (Corning, #356231) was used to coat a 96-well flat bottom plate (50 µl/well) and incubated for 30 minutes at 37°C. Cardiac stem cell populations were plated in duplicate (5,000 cells/well) suspended in 100 µL/ well of EPC basal medium (see Table 1) and incubated at 37°C in CO_2 incubator. Cell tube formation was imaged using a Leica DMIL inverted tissue culture phase contrast microscope 12-16 hours after plating.

MSC- Colony-forming unit-fibroblast (CFU-F) Assay
Cells were suspended in CFU-F assay medium: DMEM-low glucose (Thermo Fisher Scientific, #11054-020) with 10% FBS, 2 mM L-Glutamine and Gentamicin (10 mg/mL), and plated at 200 cells per 100 mm. Medium changed every 3 days and after 14 days of growth, dishes were washed with PBS and incubated in crystal violet at room temperature for 30 minutes. Solution removed by 4 washes of PBS. After dishes were dry, colonies were enumerated for each plate. A minimum of 3 dishes plated per cell line.

Multilineage Mesenchymal Differentiation Potential
The potential for osteogenesis, adipogenesis, and chondrogenesis differentiation was assessed for the three cardiac stem cell populations using StemPro Differentiation Kits following manufacturer's protocol (Thermo Fisher Scientific, #A1007201, #A1007001, and #A1007101). For osteocyte differentiation cells were stained with Alizarian-Red Staining Solution (Millipore, #TMS-008-C), for adipocyte differentiation cells were stained with Oil Red O (Sigma-Aldrich, #O0625), and for chondrocyte differentiation cells were embedded in optimal cutting temperature (OCT) compound, cryosectioned at 5-10 µm, and stained with Alcian-Blue Staining Solution (Millipore, #TMS-010-C).

Statistical Analysis
Data expressed as mean±SEM. Statistical analyses of multiple groups were assessed by 1-way ANOVA with Bonferroni post hoc test. Multiple groups over time were analyzed by 2-way ANOVA. Statistical analysis was performed using GraphPad Prism version 5.0 software. The Pearson product-moment correlation coefficient was calculated using Microsoft Excel 2010. Experiments were performed in triplicate unless stated otherwise. A p-value of less than 0.05 was considered statistically significant

Online Table I. List of antibodies

Antibody	Vendor	Catalog Number	Dilution Flow	Dilution Slides
C-Kit (CD117)	R&D systems	AF1356	1:33	1:200
Thy-1 (CD90)	Biolegend	328109	1:33	1:200
Endoglin (CD105)	Biolegend	323203	1:33	1:200
Prominin-1 (CD133)	Thermo Fisher Scientific	PA5-38014	1:50	1:600
CD34	Santa Cruz Biotechnology	Sc-9095	1:33	-
PTPRC (CD45)	Biolegend	368507	1:33	-
DAPI	Sigma-Aldrich	D9542	-	1:10,000
Phalloidin	Thermo Fisher Scientific	A12379	-	1:1,000

Online Table II. List of primers

	Target	Fwd Primer Sequence	Rev Primer Sequence
Growth Factors and Cytokines	ANGPT2	TCCAAGCAAAATTCCATCATTG	GCCTCCTCCAGCTTCCATGT
	NRG1	GCCAATATCACCATCGTGGAA	CCTTCAGTTGAGGCTGGCATA
	PECAM1	CCAAGCCCGAACTGGAATCT	CACTGTCCGACTTTGAGGCT
	FGF2	CTGGCTATGAAGGAAGATGGA	TGCCCAGTTCGTTTCAGTG
	GATA4	CTCAGAAGGCAGAGAGTGTGTCAA	CACAGATAGTGACCCGTCCCAT
	HBEGF	ACAAGGAGGAGCACGGGAAAAG	CGATGACCAGCAGACAGACAGATG
	HGF	GGCTGGGGCTACACTGGATTG	CCACCATAATCCCCCTCACAT
	IGF2	GACCGCGGCTTCTACTTCAG	AAGAACTTGCCCACGGGGTAT
	SMA	CCCAGCCAAGCACTGTCAGGAATCCT	TCACACACCAAGGCAGTGCTGTCC
	CXCL12	CAGTCAACCTGGGCAAAGCC	AGCTTTGGTCCTGAGAGTCC
	TGFβ1	AAGGACCTCGGCTGGAAGTGC	CCGGGTTATGCTGGTTGTA
ECM Proteins	FN1	GAAGGCTTGAACCAACCTACG	TGATTCAGACATTCGTTCCCAC
	MMP1	CTCAATTTCACTTCTGTTTTCTG	CATCTCTGTCGGCAAATTCGT
	COL1A1	GTCGAGGGCCAAGACGAAG	CAGATCACGTCATCGCACAAC
	COL3A1	GGTGCTCGGGGTAATGACG	TCCAGGGAATCCGGCAGTT
	TIMP1	TGCCGCATCGCCGAGAT	ATGGTGGGTTCTTGGTG
Inflammatory Factors	IL1B	CCCTAAACAGATGAAGTGCTCCTT	GTAGTCGGATGCCGCCAT
	IL6	TCGAGCCCACCGGGAACGAA	GCAGGGAAGGCAGCAGGCAA
	TNF	GCCGCATCGCCGTCTCCTAC	AGCGCTGAGTCGGTCACCCT
	IRF1	TTTGTATCGGCCTGTGTGAATG	AAGCATGGCTGGGACATCA
	MHC Iα	GCCCACTCACAGACTGACC	CTGGATGGTGTGAGAACCGTC
	MHC Iβ	CCTGAGATGGGAGCCGTCTT	CTCCGATGACCACAACTGCT
	MHC II DQα	TGTCTGGCAGTTGCCTATGT	TCAGGAACCTCATTGGTGGC
	MHC II DQβ	CCTCCACCAGCAGGACTC	GCAGCTAGGAATTCTGGGCA
	MHC II Trans	AGAGCACATAGGACCAGATGA	GCTTCCAGTGCTTCAGGTCT
	CD40	ACCCTTGGACAAGCTGTGAG	TAAAGACCAGCACCAAGAGG
	CD80	GCAGGGAACATCACCATCCA	TCACGTGGATAACACCTGAACA
	CD86	GGGACTAGCACAGACACACG	CTGAAGTTAGCAGAGAGCAGGAA
	18S	CGAGCCGCCTGGATACC	CATGGCCTCAGTTCCGAAAA

Online Table III. Clinical Profile of Patients Used for Stem Cell Isolation

Patient ID	Age, y	Sex	EF%	Cardiac Index	Diabetes	Hyper-lipidemia	Smoker	Infarct	Ischemia	Ace Inhibitor	β-blocker	Anticoagulant	NYHA
H13-064	38	M	15-25%	-	X	X	X	✓	✓	X	✓	✓	IV
H13-065	54	M	-	-	-	-	-	-	-	-	-	-	-
H13-066	55	M	15%	1.62	X	-	✓	✓	✓	✓	✓	✓	IV
H13-068	62	M	20%	-	✓	✓	✓	✓	✓	✓	✓	✓	III
H13-070	42	M	20%	1.54	X	-	-	✓	-	✓	✓	-	IV

EF indicates ejection fraction; NYHA, New York Heart Association; patient information is (✓) positive; (x) negative; (-) unavailable

Online Figure Legends

Online Figure I. Cardiac stem cell populations exhibit distinct cell morphology characteristics
Representative phase contrast images of cardiac cell population from three human patient lines. Scale bar, 100 uM.

Online Figure II. Patient data clusters based on stem cell subpopulation
A-C, Scatter plots showing individual patient averages for the morphometric parameters of area **(A)**, roundness **(B)**, and length-to-width (L/W) ratio **(C)** (n=4-5 patients, minimum of 30 cells traced per cell type per patient).

Online Figure III. Cardiac stem cell populations exhibit distinct growth kinetics
A-E, Growth rates for cardiac cell populations displayed by individual patient lines. CyQuant assay measured at day 0, day 1, day 3, and day 5 for patient line H13-064 **(A)**, H13-066 **(B)**, H13-068 **(C)**, H13-065 **(D)**, and H13-070 **(E)**.

Online Figure IV. Flow cytometry analysis of stem cell markers
Representative flow cytometry plots analyze the percent of c-Kit, CD90, CD105, CD133, and CD45 expression in the three cardiac cell populations.

Online Figure V. Analysis of markers used for stem cell isolation
A-C, Flow cytometry data from individual patient lines showing percent c-Kit, CD90, CD105, and CD133 expression in CPC **(A)**, EPC **(B)**, and MSC **(C)** fixed in 4% paraformaldehyde (PFA). **D-F**, Flow cytometry data from the same three patient lines showing percent of CD90, CD105, and CD45 expression of live CPC **(D)**, EPC **(E)**, and MSC **(F)**.

Online Figure VI. C-Kit Internalization occurs more readily in CPC than other cardiac stem cell populations
A-C, Representative flow cytometry plots showing the percent of CPC, EPC, and MSC that express c-Kit when antibody labeling live cells **(A)**, cells fixed in 4% paraformaldehyde **(B)**, or cells that were both fixed and permeabilized **(C)**.

Online Figure VII. Pearson correlation heat map matrix comparing gene expression profiles of LVAD patients
Pearson product-moment correlation coefficient heat map matrix of individual patient lines. Red color signifies high correlation, blue color signifies low correlation.

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A-C, Differentiation potential for osteogenesis, adipogenesis, and chondrogenesis was assessed for the three cardiac stem cell populations relative to bMSC. For osteocyte differentiation cells were stained with Alizarian-Red Staining Solution **(A)** scale bar, 250 uM, for adipocyte differentiation cells were stained with Oil Red O **(B)** scale bar, 50 uM, and for chondrocyte differentiation cells were embedded in optimal cutting temperature (OCT) compound, cryosectioned and stained with Alcian-Blue Staining Solution **(C)** scale bar, 100 uM (n=3 patients).

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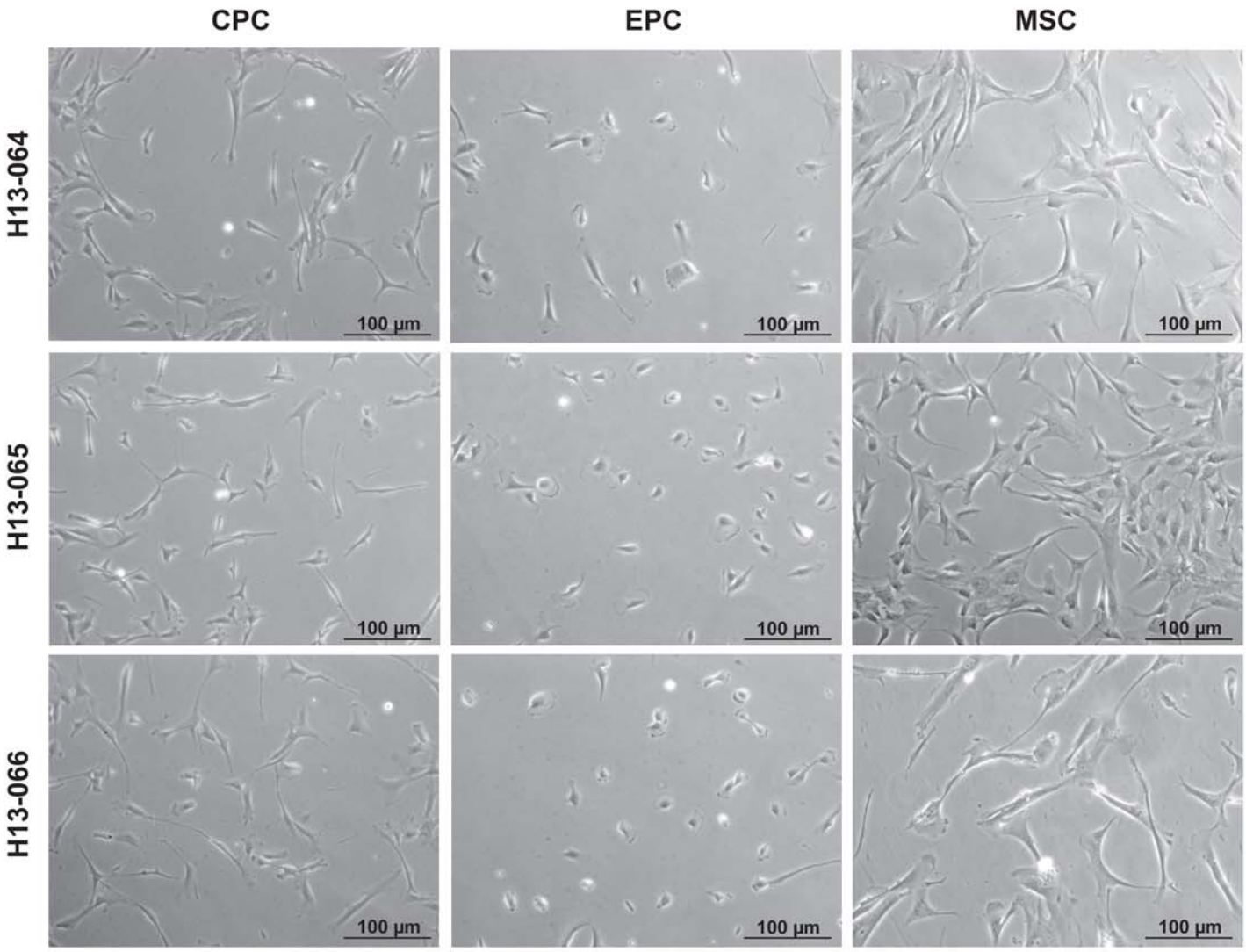
A-H, Relative expression of cardiac derived MSC for co-stimulatory molecules CD40, CD80, and CD86 **(A-C)**, MHC Class II molecules **(D-F)** and MHC Class I molecules **(G and H)** (n=5 patients) compared to control bMSCs. Data are presented



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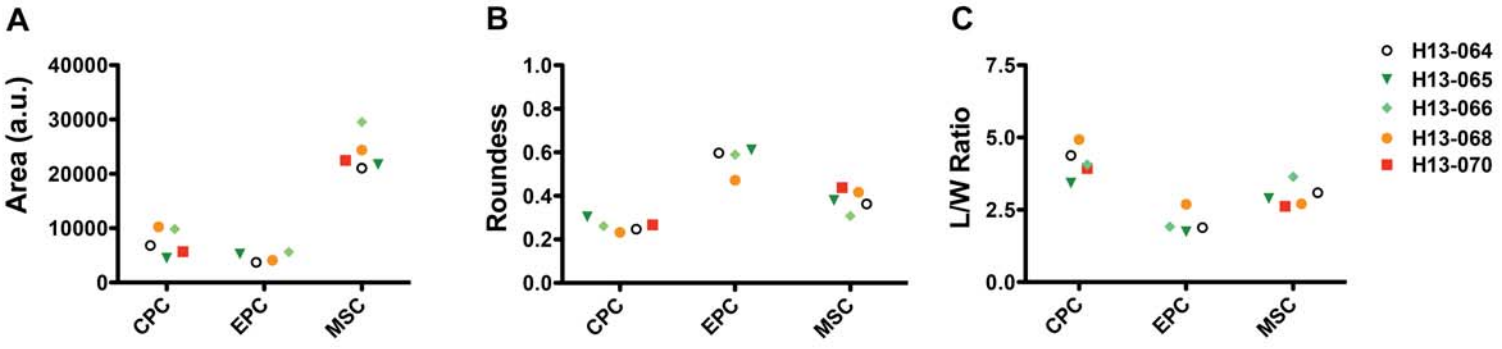

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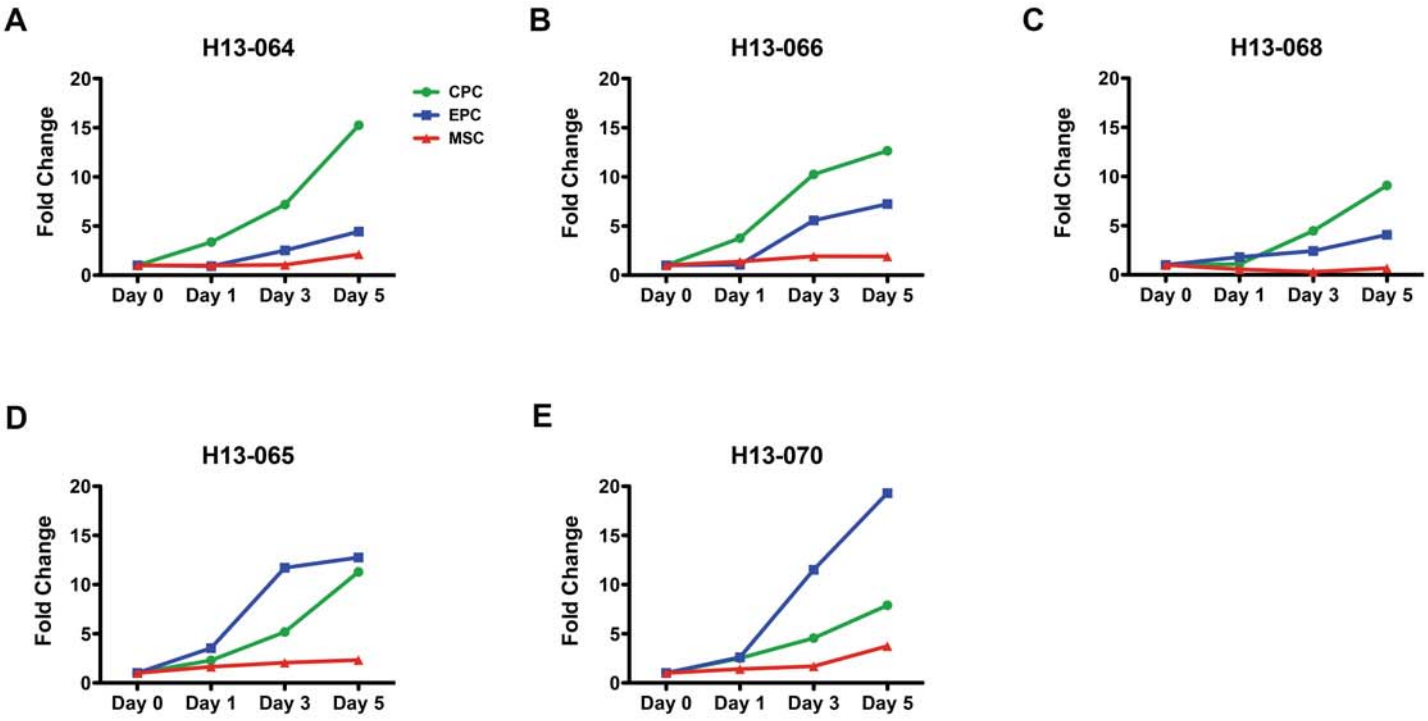

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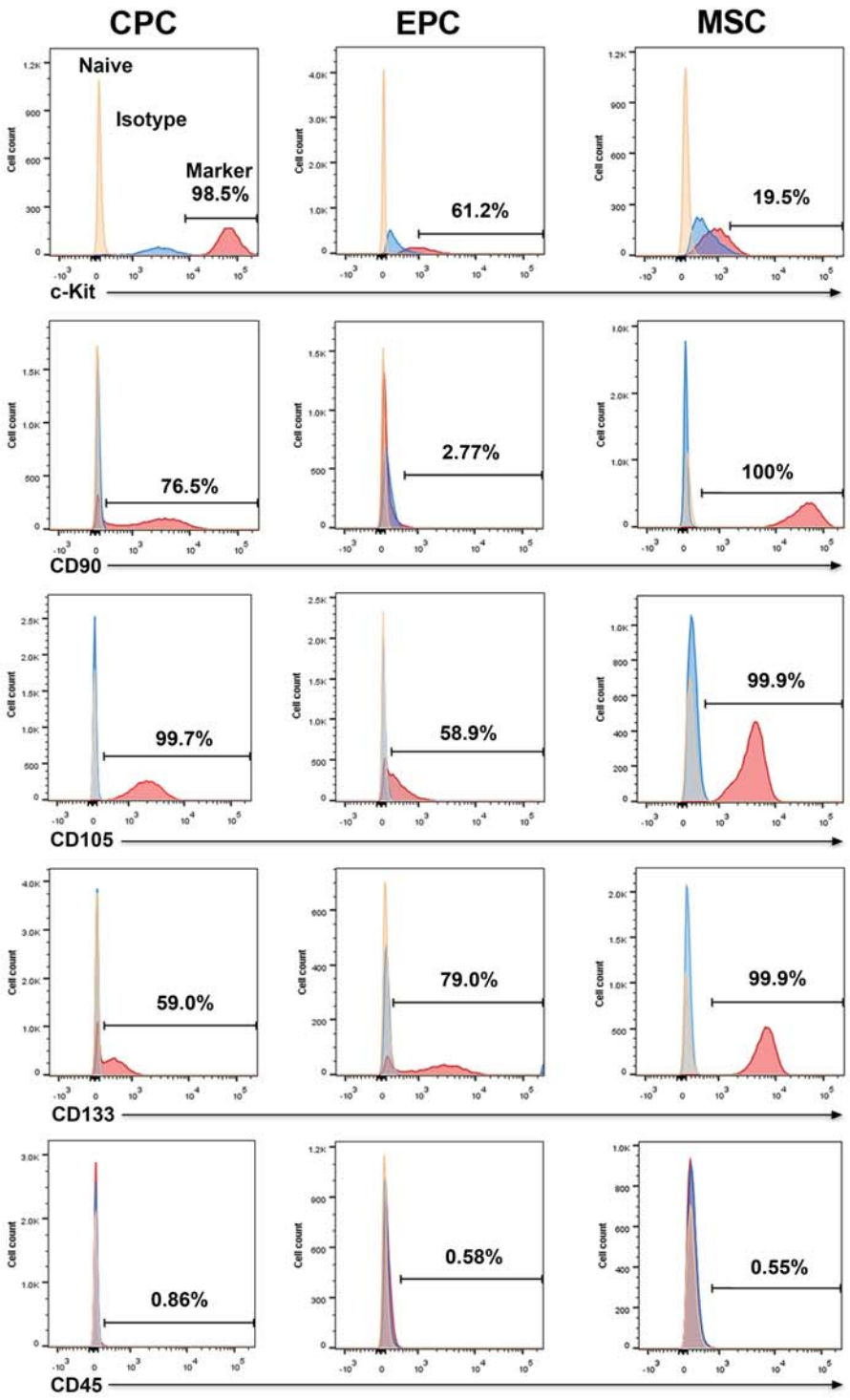

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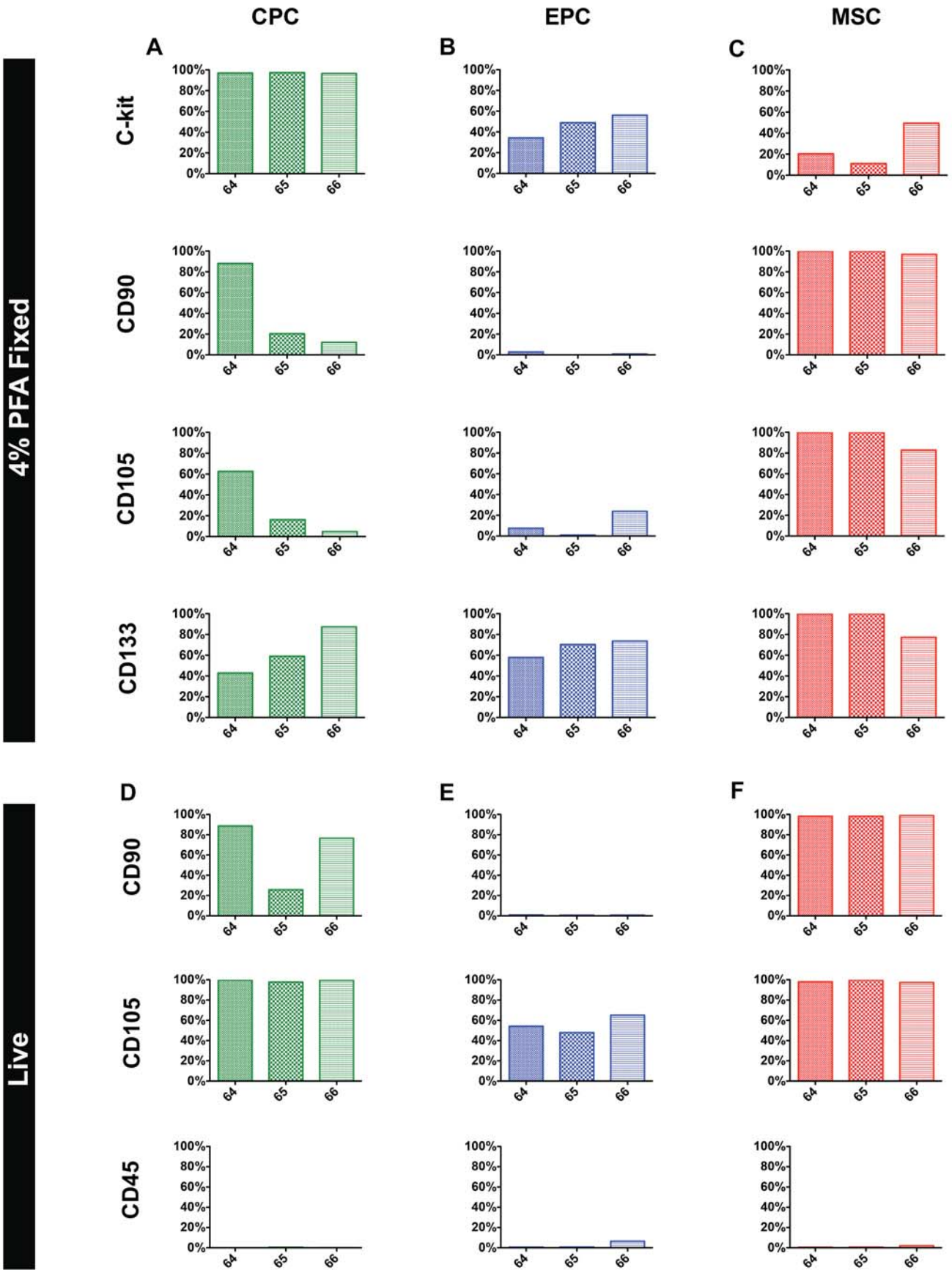

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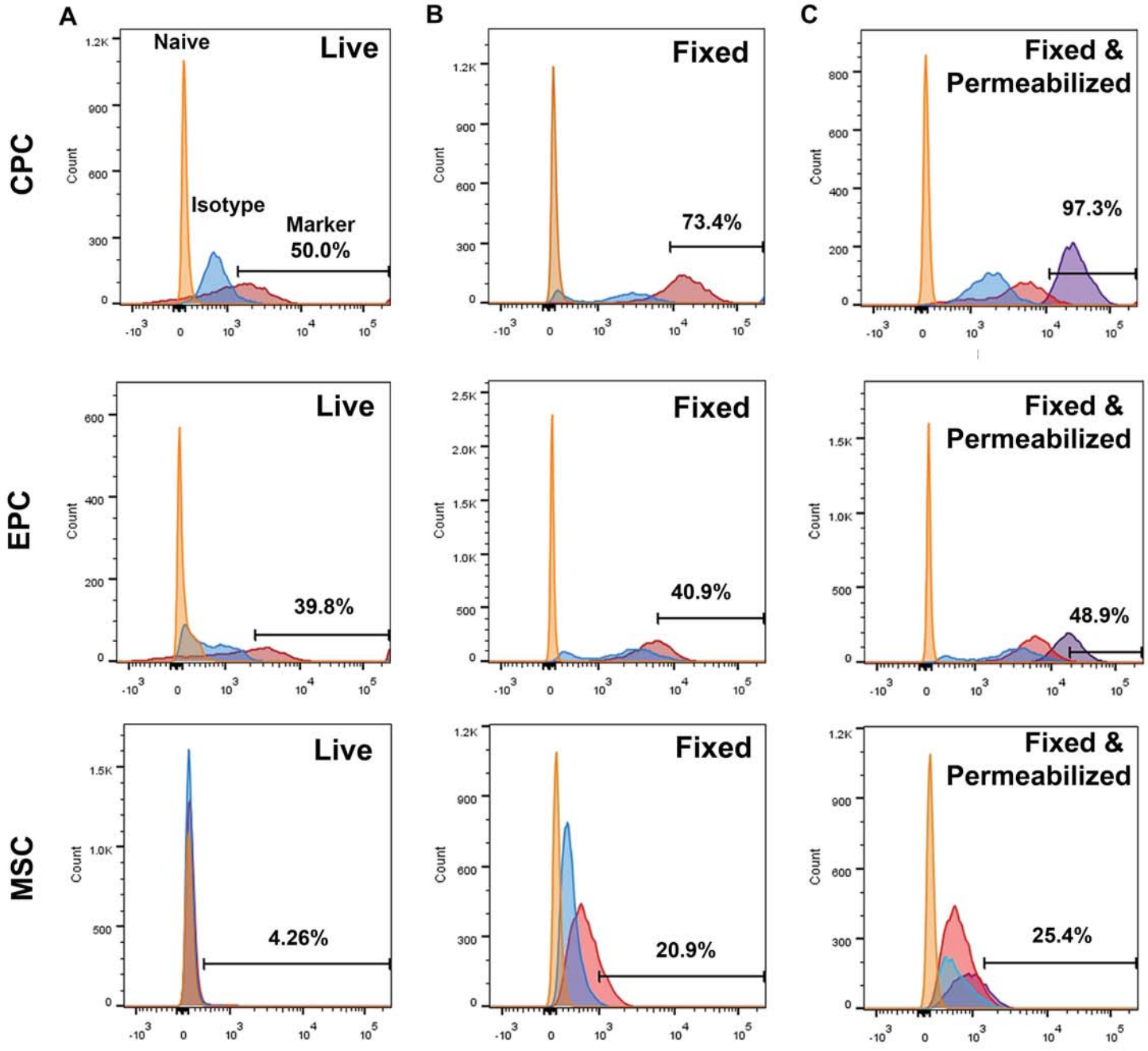

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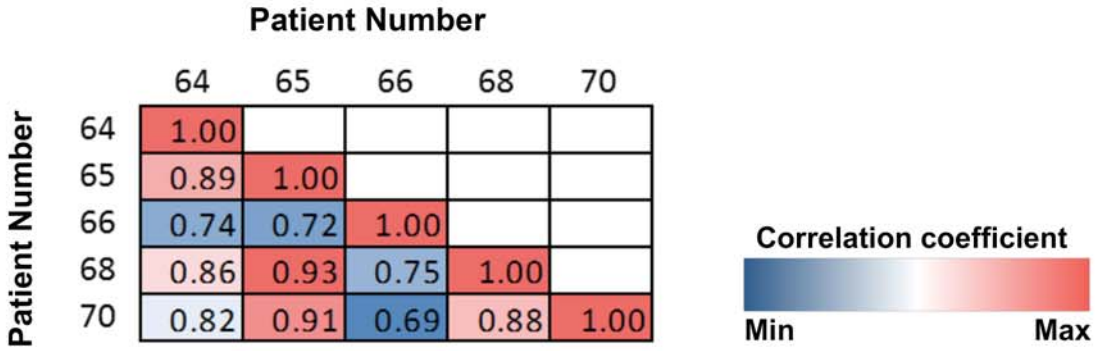

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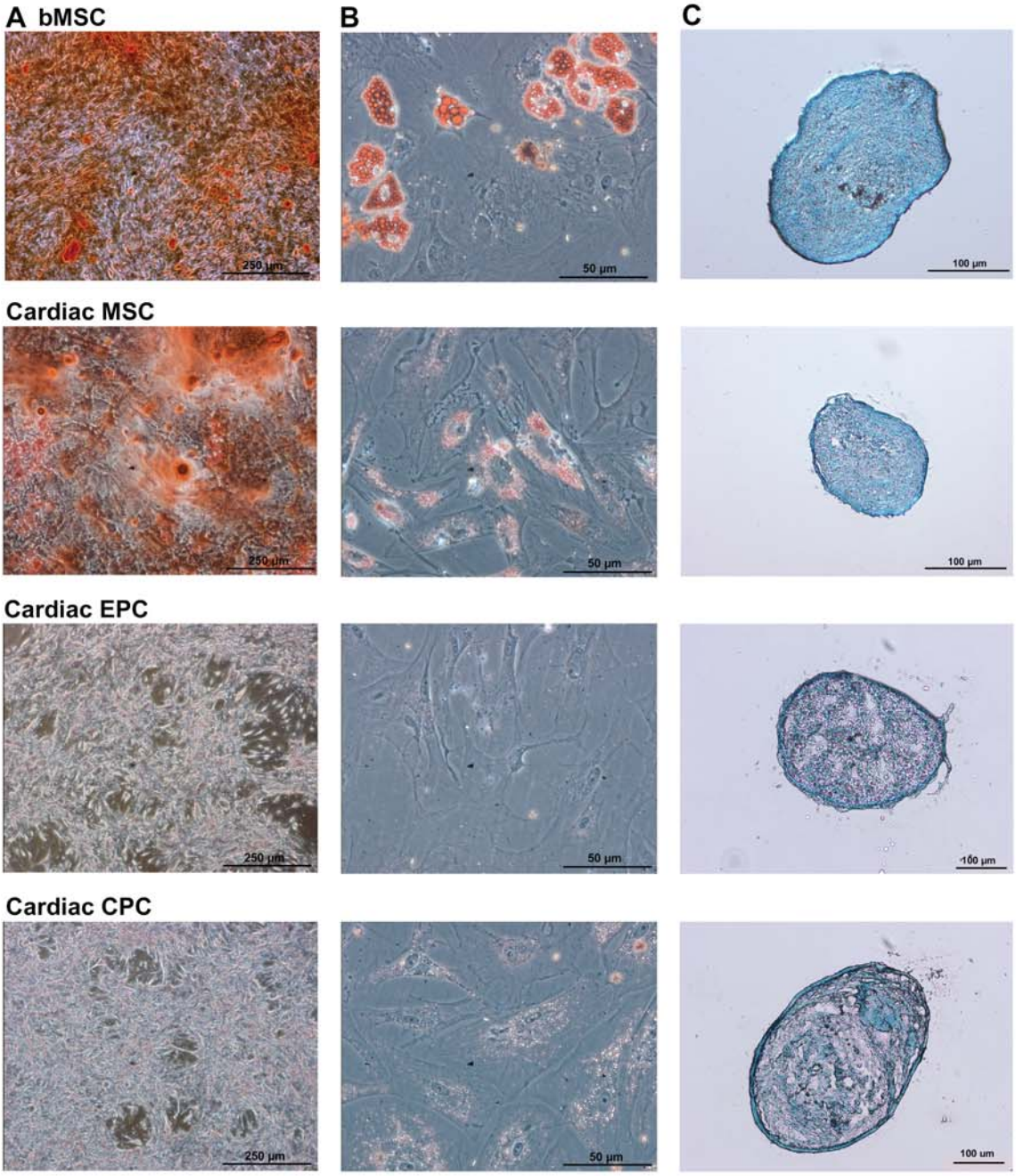

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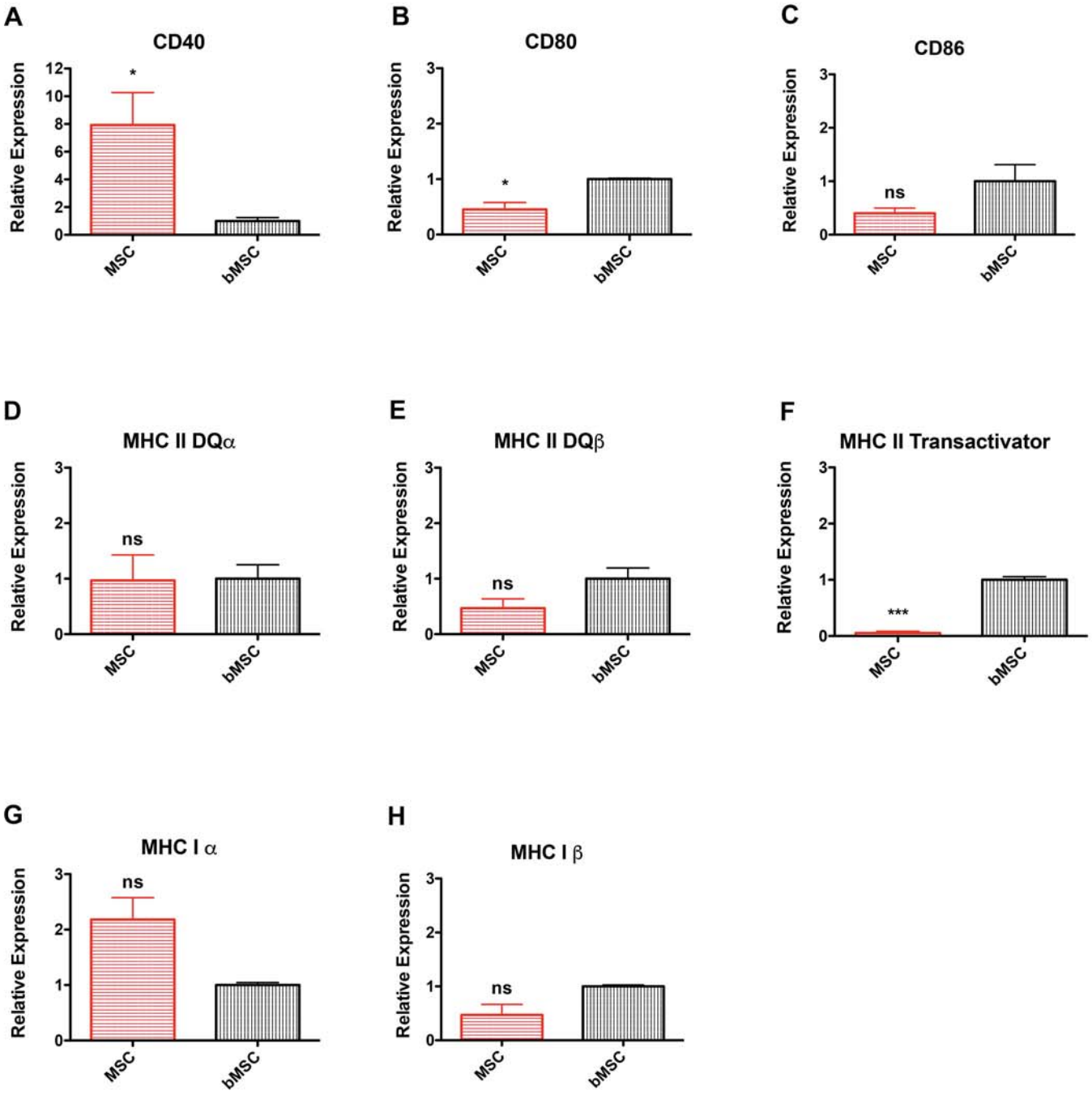

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