On-line Only Data Supplement

Methods

Isolation of human bone marrow-derived MSCs and p75MSCs. To obtain MSCs, bone marrow aspirates were taken from the iliac crest of healthy adult donors. Mononuclear cells were isolated with the use of density gradient centrifugation (Ficoll-Paque, Amersham Pharmacia Biotech) and resuspended in complete culture medium consisting of Alpha-MEM (GIBCO/BRL, Grand Island, NY); 17% FBS (Atlanta Biologicals, Norcross, GA); 100 units/ml penicillin (GIBCO/BRL); 100 μg/ml streptomycin (GIBCO/BRL); and 2 mM L-glutamine (GIBCO/BRL). Cells were plated in 20 ml of medium in a 150 cm^2 culture dish and incubated in a humidified incubator (Thermo Electron, Forma Series II, Waltham, MA) with 95% air and 5% CO_2 at 37°C. After 24 h, nonadherent cells were removed. Adherent cells were washed twice with PBS and incubated with fresh medium. The primary adherent cells were cultured and propagated.

To obtain p75MSCs or CD133MSCs, bone marrow stem/progenitor cells were isolated by MACS using antibodies against the p75LNGFR or CD133 (Prominin 1). Freshly isolated bone marrow mononuclear cells from the Ficoll gradient were resuspended in 0.4 ml of PBS containing 0.5% bovine serum albumin and 2 mM EDTA. After adding mouse anti-human antibody conjugated to magnetic beads (CD271, CD133; Miltenyi Biotech, Auburn, CA), the sample was incubated for 30 min at 4°C, and then applied to a magnetic column (LS Column; Miltenyi Biotech). The bound fraction was eluted with 5 ml of MACS buffer and the cells were concentrated by centrifugation at 1000 x g for 8 min. After re-suspension, the entire isolate was

cultured in complete culture medium. MSC-like cells appeared as small colonies after about 1 week, and the cells were expanded.

Isolation and culture of adult rat cardiac fibroblasts. Procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont. Ventricular fibroblasts were isolated from hearts of adult Sprague-Dawley rats. The hearts were minced and enzymatically-dissociated into single cell suspension. Non-myocytes were separated by discontinuous density gradient centrifugation and cultured in DMEM/F-12 supplemented with 10 % FCS. Passage 2 cells were used for experiments.

Cell culture in CdM and evaluation of cell number. Adult rat CPCs and cardiac fibroblasts were plated at 500 cells/cm^2 and cultured in their respective growth mediums. Three days after plating, the medium was removed, the wells were washed twice with PBS, and the cells were then exposed to CdM or to fresh serum-free medium (Alpha-MEM). For time course proliferation studies, CdM and serum-free medium were changed every 2 days. In signal transduction inhibitor studies, we used the following pharmacological inhibitors: AG490, inhibitor of Jak2/STAT3 pathway; Stattic, inhibitor of STAT3; LY294002, inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt pathway; and PD98059, extracellular signal-regulated kinase (ERK) inhibitor. All of the inhibitors were purchased from Calbiochem (Darmstadt, Germany) and were dissolved in dimethyl sulfoxide (DMSO). CPCs were cultured in CdM with the inhibitors or with the equivalent volume of DMSO as a control for 48 hrs. In cell protection studies, 3 days after plating, medium was replaced with either the CdM or serum-free medium and the cells were exposed to hypoxia in a specialized incubator (1% oxygen) for 48 hrs. The

hypoxia incubator was a model that measured both CO_2 and O_2 (Thermo Electron, Forma Series II, model 3130). Oxygen was maintained at 1% by the injection of nitrogen gas and was monitored continuously.

Cell numbers were quantified by the fluorescent labeling of nucleic acids (CyQuant dye; Molecular Probes, Carlsbad, CA) and with a microplate fluorescence reader (FL_X800; Bio-Tek Instruments Inc., Winooski, VT) set to 480 nm excitation and 520 nm emission. Each experiment was repeated a minimum of 3 times.

Immunocytochemistry. CPCs were fixed with 4% paraformaldehyde in 1x PBS. Non-specific binding was limited by 1 hour incubation in PBS containing 5% goat serum and 0.4% triton X-100. Primary antibodies were incubated overnight at 4ºC. After washing 3x 5 min with PBS, secondary antibody that was diluted 1:1000 (Alexa 594, Molecular Probes) was applied for 1 h at room temperature (RT). After 3x 5 min washes, slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Epifluorescence images were taken using a Leica DM6000B microscope equipped with a CCD camera (Leica DFC350Fx) and FW4000 software. The primary antibodies for immunocytochemistry were as follows: phospho-STAT3 (1:50, Tyr705, #9131; Cell signaling, Danvers, MA); α-sarcomeric actin (1: 500, clone 5C5, #A2172; Sigma); α-smooth muscle actin (1: 800, clone 1A4, #A5228; Sigma); and von Willebrand factor (1: 100, clone 21-43, # AB3442; Chemicon, Temecula, CA). For quantification of differentiation, cells positive for α-sarcomeric actin, α-smooth muscle actin and von Willebrand factor and total cells were counted at least in three fields per slide. The percentage of positive cells was calculated for each slide (n=3 in each group).

DNA replication assay. Three days after the plating, CSCs were cultured in growth medium, CdM or serum-free medium for 24 h, and BrdU (BD Biosciences) was added at a final concentration of 10 μM. Immunocytochemistry was carried out with BrdU antibody (1:100 clone BU33, # B8434; Sigma) and BrdU-positive cell numbers were quantified as above.

Immunoblotting. Cells were lysed in a buffer that consisted of 0.1% sodium dodecyl sulphate (SDS) and complete protease inhibitor cocktail (Roche, Basel, Switzerland) in PBS. Protein concentration was determined by the DC protein assay (Biorad, Hercules, CA). Twenty μg of protein was separated by SDS-PAGE. After electrophoresis, the gels were electroblotted to polyvinylidene difluoride (PVDF) membranes. All electrophoresis and electroblotting used Novex reagents and systems (Invitrogen, Carlsbad, CA). The blots were blocked for 1 h at RT in 5% nonfat dry milk in PBS with 0.1% Tween 20 (PBST), washed 3 × 5 min in PBST, and incubated in primary antibodies in PBST with 5% BSA overnight at 4ºC. After 3x 5 min washes in PBST, the blots were incubated in secondary antibody conjugated to horseradish peroxidase conjugate (1: 2000, Sigma) in PBST for 1 h at RT. Unbound secondary antibody was removed and positive bands were detected with a chemiluminescent reaction. The primary antibodies for immunoblotting were Ki67 (1: 200, clone SP6, Abcam, Cambridge, MA); p-STAT3 (Tyr705, 1: 1000), total-STAT3 (1: 1000, #4904, Cell Signaling), p-Akt (Ser 473, 1:1000, # 9271, Cell Signaling), total- Akt (1:1000, # 9272, Cell Signaling), Insulin (1:1000, # I2018, Sigma); and β-actin (1: 5000, clone AC-15, # A5441, Sigma).

ELISAs for IGF-1, Insulin, and CTGF. For assay of human IGF-1, we used 1x p75 CdM and commercial ELISA reagents with the manufacturer's protocol (# DY291, R and D Systems). For assay of Insulin and CTGF, high protein-binding plates were incubated with 1 or 10x p75 CdM

overnight at room temperature to capture antigens from CdM. Wells were then washed with mild detergent (0.05% Tween-20 in PBS) followed by blocking with 1% BSA in PBS for 1 hour. After blocking buffer was thoroughly washed off from the wells, samples were incubated with 100 µl of biotin-conjugated polyclonal antibody to CTGF at 5µg/100 µl (#P252Bt, Peprotech) for 2 hours at room temperature. Polyclonal mouse anti-Insulin antibody (Santa Cruz) was incubated for 2 hours at room temperature, followed by 3 washes with wash buffer. The wells were then incubated in anti-mouse biotin conjugated IgG (B7264, Sigma Aldrich) for 2 hours at room temperature. After washing in wash buffer 3 times, samples for CTGF and Insulin ELISA were incubated in Streptravidin conjugated HRP (1:2000) for 2 hrs at room temperature, followed by washing and addition of 100 µl of substrate ABTS (Thermo Scientific; #37615) for 20 minutes. Absorbance was measured (450 nm) on a Synergy HT plate reader.

Myocardial infarction surgery in mice. Male mice at 9-10 weeks of age underwent permanent ligation of the Left Anterior Descending Coronary Artery (LAD) to induce myocardial infarction (C57bl6 mice, Taconic, Hudson, NY). Mice were not included in the study if they did not survive the initial MI surgery, did not achieve a successful MI (blanching observed at time of treatment), or died during treatment application. Following all procedures, mice were given analgesia (buprenorphine, 0.05-0.1 mg/kg i.p.) and monitored for signs of distress until termination of the study. All procedures were done in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont.

For permanent LAD ligation surgery, mice were anesthetized with 2-4 % Isoflurane, shaved, weighed, intubated, and then maintained for the duration of the procedure on a sterile surgical field with the use of a mechanical ventilation system (MiniVent, Harvard Apparatus, Holliston, MA). Throughout the surgery and during the recovery period, body temperatures were

maintained with a heated water pad system (Gaymar T-Pump TP-500, Gaymar Industries, Orchard Park, NY). Viewing the chest through a dissecting microsope (Stemi 2000-C, Carl Zeiss MicroImaging, Thornwood, NY) a dermal incision was made, the underlying fascia were removed, and the thoracic musculature was retracted to expose the left ribcage. Next the intercostal muscles were retracted and the outer (parietal or visceral) pericardium was removed to expose the LAD. The LAD was then ligated (2.0-3.0 mm from left atrial apex) with 8.0 nylon suture (Henry Schein, Melville, NY) and blanching within the myocardium of the left ventricle was noted. The intercostals were rejoined with a 6.0 nylon suture (Henry Schein), the lungs were reinflated, and overlying dermis rejoined with a 6.0 nylon suture. All mice were recovered to an ambulatory state prior to any subsequent treatment procedure. Survival after the MI surgery was > 90%. Sham-operated mice underwent all procedures except that the suture was placed under the LAD but was not ligated.

Infusion of p75MSC CdM after MI in mice. To evaluate p75 CdM treatment in an unbiased manner, all animals were randomized to treatment (after LAD ligation). Following 24 hour recovery of an animal after the first surgery, the mouse was then again anesthetized, intubated, and the chest opened. Once the intact suture and area of blanching were confirmed, 30x p75 CdM (200 uL) or vehicle (Alpha-MEM, 200 uL) warmed to 37°C was delivered to the entire cardiovascular arterial tree by injecting the solution into the lumen of the left ventricle (LV). Injections were performed slowly (over a period of 1 minute) with a 30.5 gauge needle inserted below the great cardiac vein (LV apex) at an angle 45° to the myocardium. Following treatment with either CdM or vehicle, the needle was removed and the intercostals were rejoined using 6.0 chromic gut suture (Ethicon, Johnson and Johnson, Inc., Livingston, UK), lungs then reinflated,

and overlying dermis rejoined with 6.0 nylon suture. All mice were then recovered to an ambulatory state and transferred to the vivarium for the remaining duration of the experiment.

For CK assays, left ventricular tissue was dissected away from the atria and the aorta, further separated into anterior LV and posterior LV/septum, and immediately snap frozen by submersion of cryovials in liquid N2. The LV tissues were maintained at -80°C until the day of the CK assay (see below).

TUNEL Assay. TUNEL was performed as reported previously.[1] Quantification of TUNEL-positive cells within zones of infarction was performed in an unbiased fashion by a viewer that was blinded to slide (sample) identity. Cells were counted with Image Pro Plus Software as reported previously [1].

Creatine Kinase Assay. The remaining creatine kinase (CK) activity in left ventricular tissues was assessed to determine the extent of infarction in mouse hearts as reported previously [2,3]. The loss of CK activity directly reflects the loss of viable myocardium after MI. The percentage of left ventricle with infarction was calculated based on observed total LV CK activity (IU/mg protein) in left ventricles of normal hearts without infarction. The percent of MI = 100 x [NL CK-LV CK] / Δ, where NL CK is the amount of CK in tissue from normal LV (IU/mg of soluble protein), LV CK is total remaining CK activity in the LV after MI (IU/mg soluble protein), and Δ is the difference between the amount of CK in normal zones of myocardium and in zones of myocardium with infarction.

Immunohistochemistry. Rats were euthanized under isoflurane, their hearts harvested and washed in PBS to remove remnant blood. Hearts were fixed in 4% paraformaldehyde overnight and equilibrated in 15 and 30% sucrose consecutively for cryoprotection. After mounting in OCT (Tissue-Tek), serial sections were performed from apex to base at 20 microns (Leica CM1800 Cryostat) and sections were mounted on glass slides. Slides were dried at 37°C and washed twice in 1x PBS. Primary antibodies were against Ki67 (clone SP6, 1:100; Abcam), CD31, (1:50, #555026, BD Biosciences), and smooth muscle alpha actin (1:500, Sigma). Primary antibodies were detected with secondary antibodies conjugated to Alexa 574 (1:2000). Slides were mounted in Vectashield with DAPI (Vector Labs). Sections were imaged by epifluoresence deconvolution microscopy (Leica DM6000B; Leica) with Leica FW4000 software.

Statistical analysis. Comparisons of parameters among the three groups were made with one-way analysis of variance (ANOVA) followed by Scheffé's multiple comparison test. Comparisons of parameters between two groups were made by unpaired Student's *t*-test. $P < 0.05$ was considered significant.

References for Supporting Information:

1. French, CJ, Spees, JL, Zaman, AK, Taatjes, DJ, Sobel, BE. The magnitude and temporal dependence of apoptosis early after myocardial ischemia with or without reperfusion. *FASEB J.* 2009;23:1177-1185.
2. Kjekshus, JK, Sobel, BE. Depressed myocardial creatine phosphokinase activity following experimental myocardial infarction in rabbit. *Circ Res.* 1970;27:403-414.
3. Zaman, AK, French, CJ, Spees, JL, Binbrek, AS, Sobel, BE. Vascular rhexis in mice subjected to non-sustained myocardial ischemia and its therapeutic implications. *Exp Biol Med.* 2011;236:598-603.

Supplemental Figure 1. Top two panels: Phase contrast images of CSCs, CPCs in CPC growth medium. Lower four panels: CPCs treated with CdM from MSCs, p75MSCs, or fibroblasts or SFM for 8 days (magnification, 10x). Please note that only the cells in the lower four panels were seeded at the same time and at the same density. CPC growth medium = CSC medium with 2% FBS. Scale bars = 50 μM

Supplemental Figure 2. Proliferation of CPCs. (A) Dose-dependent effect of 10x-concentrated CdM on CPC proliferation. CPC growth in 1x CdM from one MSC donor and one p75MSC donor is shown for reference. Control cell number (60,191 cells) was regarded as 100%. Data are mean ± SEM, *n* = 3. 10x CdM from two different donors was assayed for each cell type. *, *p* [lt] .0001 versus baseline; **, *p* [lt] .0001 versus day 4; †, *p* [lt] .05 versus day 8. CdM, conditioned medium. (B) Growth of CPCs in p75MSC CdM is dependent on signaling through STAT3 and abolished by incubation with the specific STAT3 inhibitor, "Stattic" (10 μM). *, *p* [lt] .001 versus DMSO vehicle on day 2; **, *p* [lt] .001 versus DMSO vehicle on day 4. (C) Inhibitory effects of AG490 (10 μM) and LY294002 (10 μM) on CPCs incubated with 1x CdM for 48 hours. Control cell numbers (99,965 cells in SFM, and 164,614 cells in GM) were regarded as 100%. For C, data are mean ± SEM, *n* = 3 to 6. *, *p* [lt] .0001 versus control; **, *p* [lt] .01 versus AG; †, *p* [lt] .05 versus LY. Con: control, DMSO. AG: AG490, Jak2/STAT3 pathway inhibitor. LY: LY294002, inhibitor of PI3K/Akt pathway. A+L: AG490 + LY294002. CdM, conditioned medium. SFM, serum-free α-MEM (Left). GM, CPC growth medium (CSC medium with 2% FBS) (Right).

Supplemental Figure 3. Time course changes in the numbers of CPCs treated with SFM supplemented with various growth factors (EGF, bFGF, and LIF; 10 ng/ml) and in the absence of Insulin-Transferrin-Selenium. Control cell number (64,026 cells) was regarded as 100%. Data are mean ± SEM, *n* = 3. *, *p* [lt] .01 SFM and SFM+EGF+FGF versus baseline; **, *p* [lt] .001 SFM, SFM+EGF+FGF, and SFM+LIF+EGF+FGF versus baseline. Data for growth in 1x CdM from one MSC donor is shown for reference.

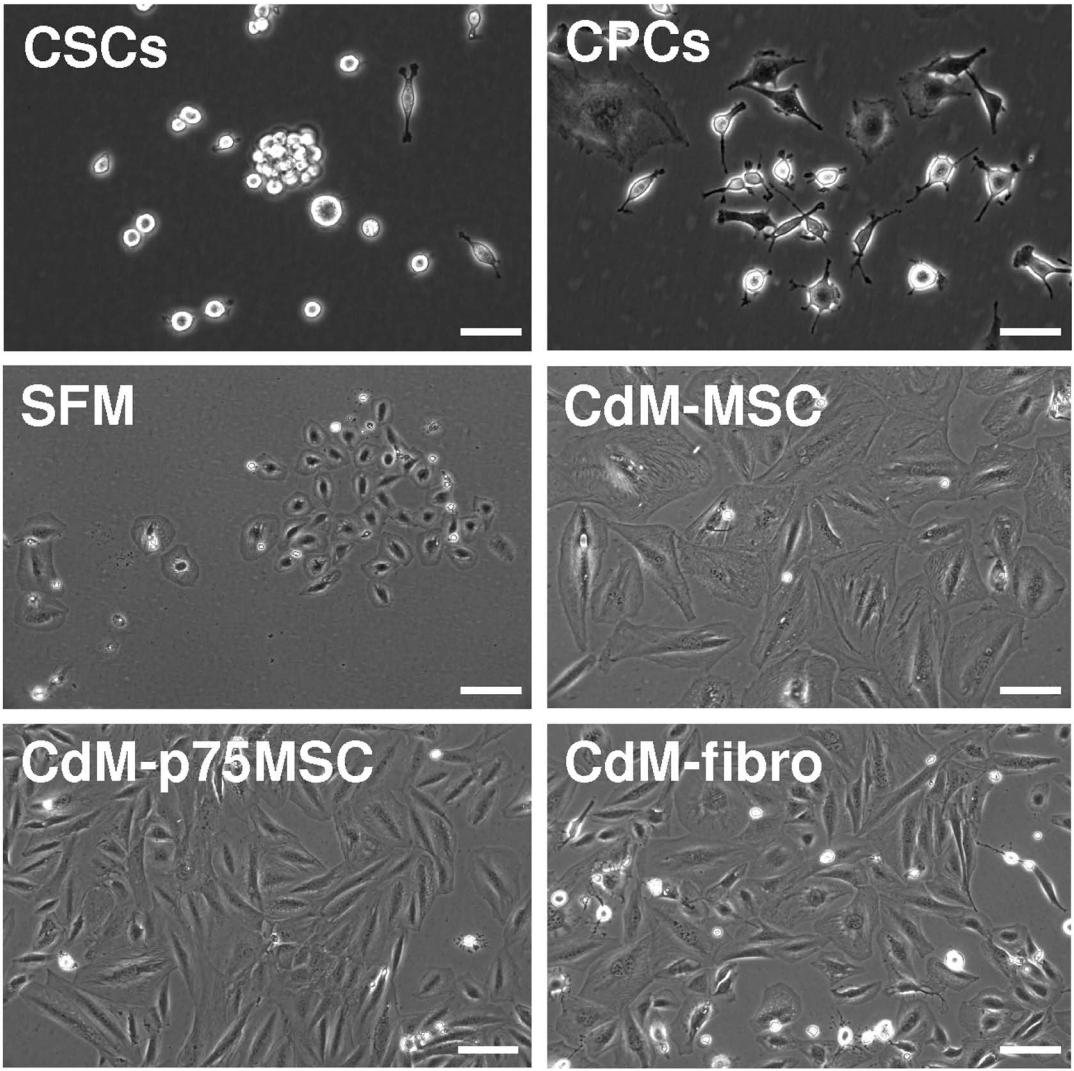
Supplemental Figure 4. Differentiation of CPCs expanded in CdM. (A) Immunofluorescent staining for α –SA, α-sarcomeric actin; SMA, α-smooth muscle actin; and vWF, von Willebrand factor. Left panels (Baseline) show the CPCs in growth medium 3 days after plating, and the right panels show CPCs expanded in CdM for 4 days. (B) Quantification of % positive cells for α –SA, SMA, and vWF at for cells incubated in growth medium, CdM and CTGF-D4 (3 ng/ml)/Insulin (30 ng/ml). Data are mean ± SEM, *n* = 3. CdM, conditioned medium, Scale bars = 100 μM. Note; the total count

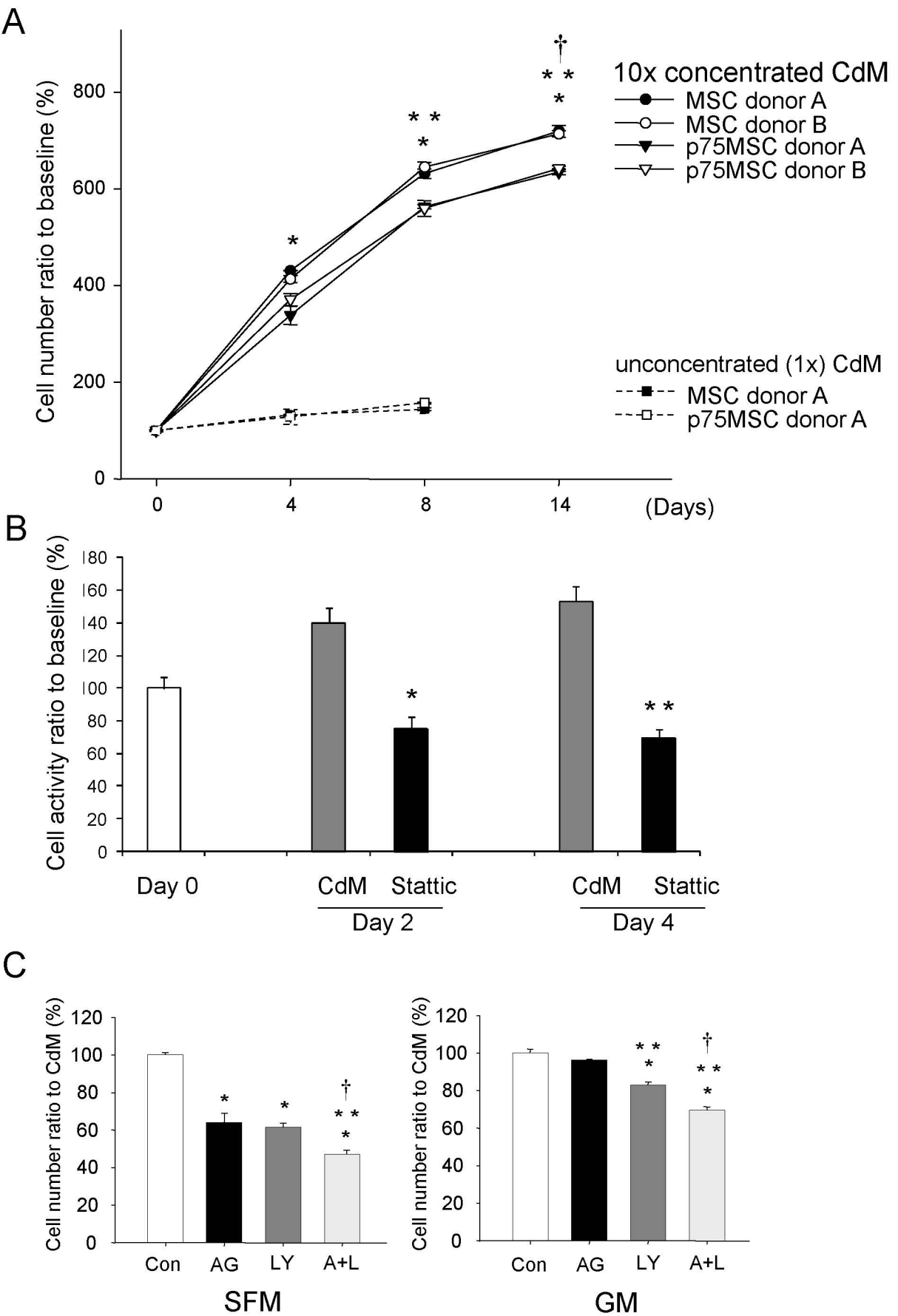
for three lineages exceeds a 100% as s portion of CPC-derivatives in culture stain positive for both SMA and α –SA after 4 days of differentiation under the above conditions.

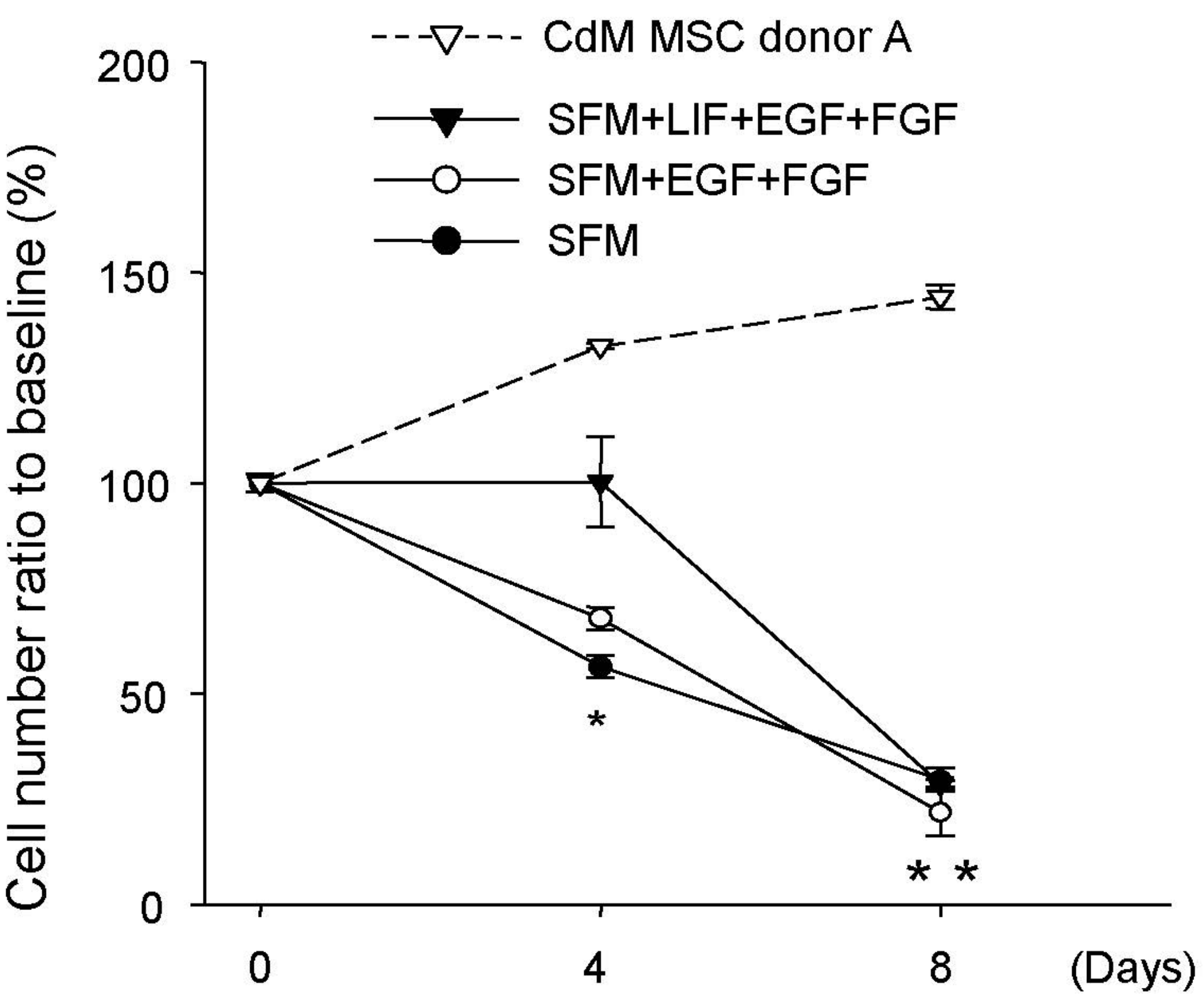
Supplemental Figure 5. Intra-arterial infusion of p75MSC CdM 24 hours after MI significantly reduces the level of cardiac apoptosis/necrosis at 48 hours after MI in adult mice. (A) TUNEL stains of heart sections from vehicle (SFM-treated) and CdM-treated C57bl6 mice. At 24 hours after LAD ligation, 200 μl of SFM or 30x p75MSC CdM was slowly infused into the left ventricle lumen (intra-arterial delivery). (B) Quantification of TUNEL-$^{+}$ cells in heart sections of animals that received intra-arterial infusion of SFM or 30x p75MSC CdM 24 hours after MI. Scale bars = 100 μM.

Supplemental Figure 6. CSCs primed with 30x p75MSC CdM engraft into sub-epicardial tissues after MI and migrate into specific zones with infarction. Image shows the localization of CSC-derived GFP^{+} cells, 1 week after MI and CSC/CdM injections. The dashed white arrow indicates the direction of the tangential sub-epicardial injection. The small yellow arrows indicate the direction of cell migration into the myocardium from the sub-epicardial space. Scale bars = 50 μM.

Supplemental Figure 7. CSCs primed with 30x p75MSC CdM survive and differentiate in the heart 1-month following MI and transplantation. (A) Few cells survive after vehicle control injection (1% BSA in alpha MEM) 1-month post MI. (B, B') Two different representative animals grafted with GFP-positive CSCs. Cells were primed with 30x p75MSC CdM and populate the majority of the infarct after 1-month. (B[dprime]) High magnification view, highlighting GFP-positive cells in the core of an infarct. (C, C', C[dprime]) Engrafted CSCs differentiated into CD31-positive endothelial cells and contributed to large blood vessels. (D,D') CSCs and their derivatives, engrafted with CTGF-D4/Insulin, 1-month after injury. (E-E') Engrafted CSCs differentiated into smooth muscle actin (SMA)-positive myofibroblasts (E) and CD31-positive endothelial cells (E') and also contributed to large cardiac blood vessels. Scale bars = 100 μM.


CSCs
CPCs
SFM
CdM-MSC
CdM-p75MSC
CdM-fibro


A
Cell number ratio to baseline (%)
800
600
400
200
0
0
4
8
14
(Days)
10x concentrated CdM
MSC donor A
MSC donor B
p75MSC donor A
p75MSC donor B
unconcentrated (1x) CdM
MSC donor A
p75MSC donor A
B
Cell activity ratio to baseline (%)
180
160
140
120
100
80
60
40
20
0
Day 0
CdM
Stattic
Day 2
CdM
Stattic
Day 4
C
Cell number ratio to CdM (%)
120
100
80
60
40
20
0
Con
AG
LY
A+L
SFM
Cell number ratio to CdM (%)
120
100
80
60
40
20
0
Con
AG
LY
A+L
GM


CdM MSC donor A
SFM+LIF+EGF+FGF
SFM+EGF+FGF
SFM
Cell number ratio to baseline (%)
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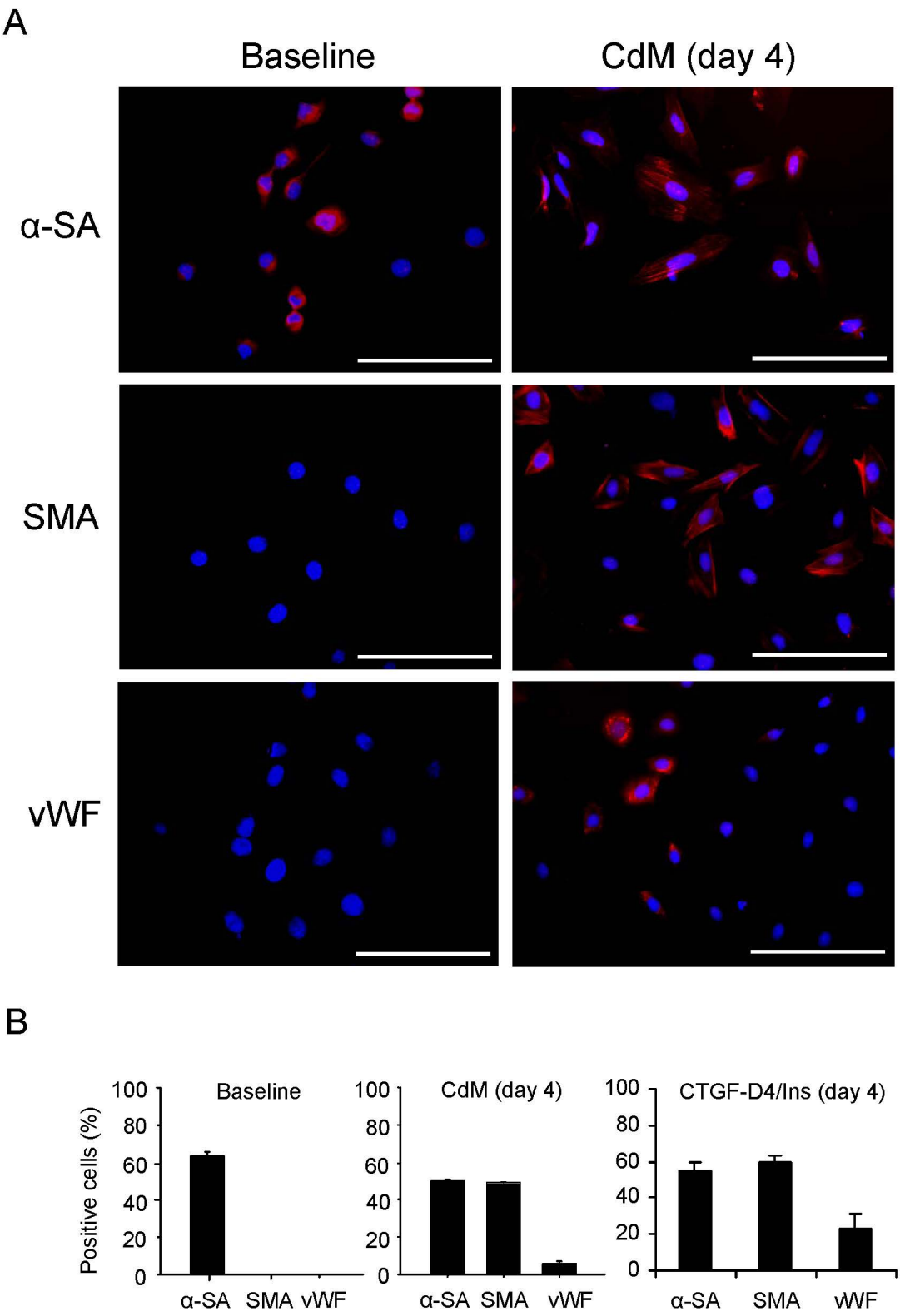
150
100
50
0
0
4
8
(Days)
*
* *


A
Baseline
CdM (day 4)
α-SA
SMA
vWF
B
Positive cells (%)
Baseline
CdM (day 4)
CTGF-D4/Ins (day 4)
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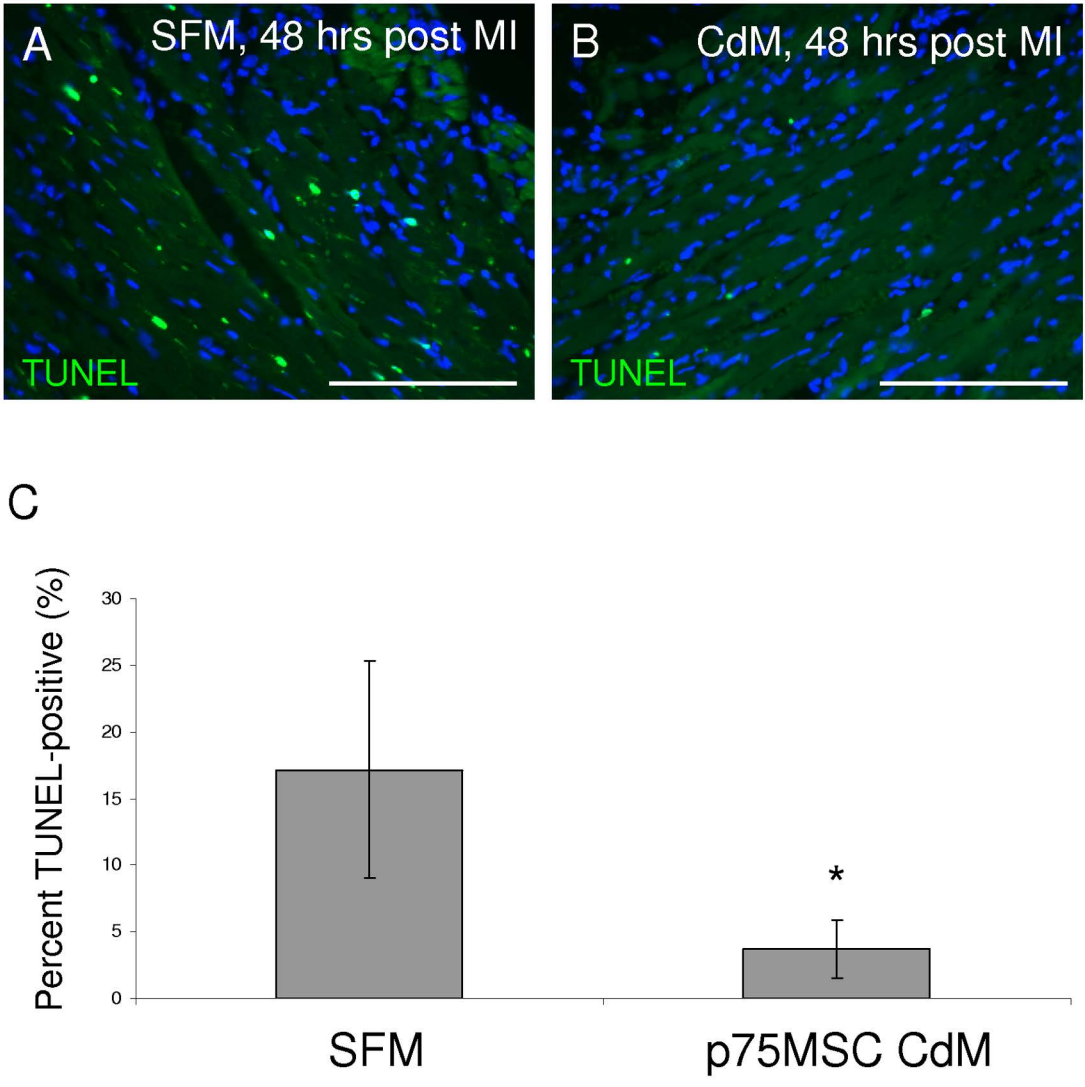
80
60
40
20
0
α-SA
SMA
vWF


A
SFM, 48 hrs post MI
TUNEL
B
CdM, 48 hrs post MI
TUNEL
C
Percent TUNEL-positive (%)
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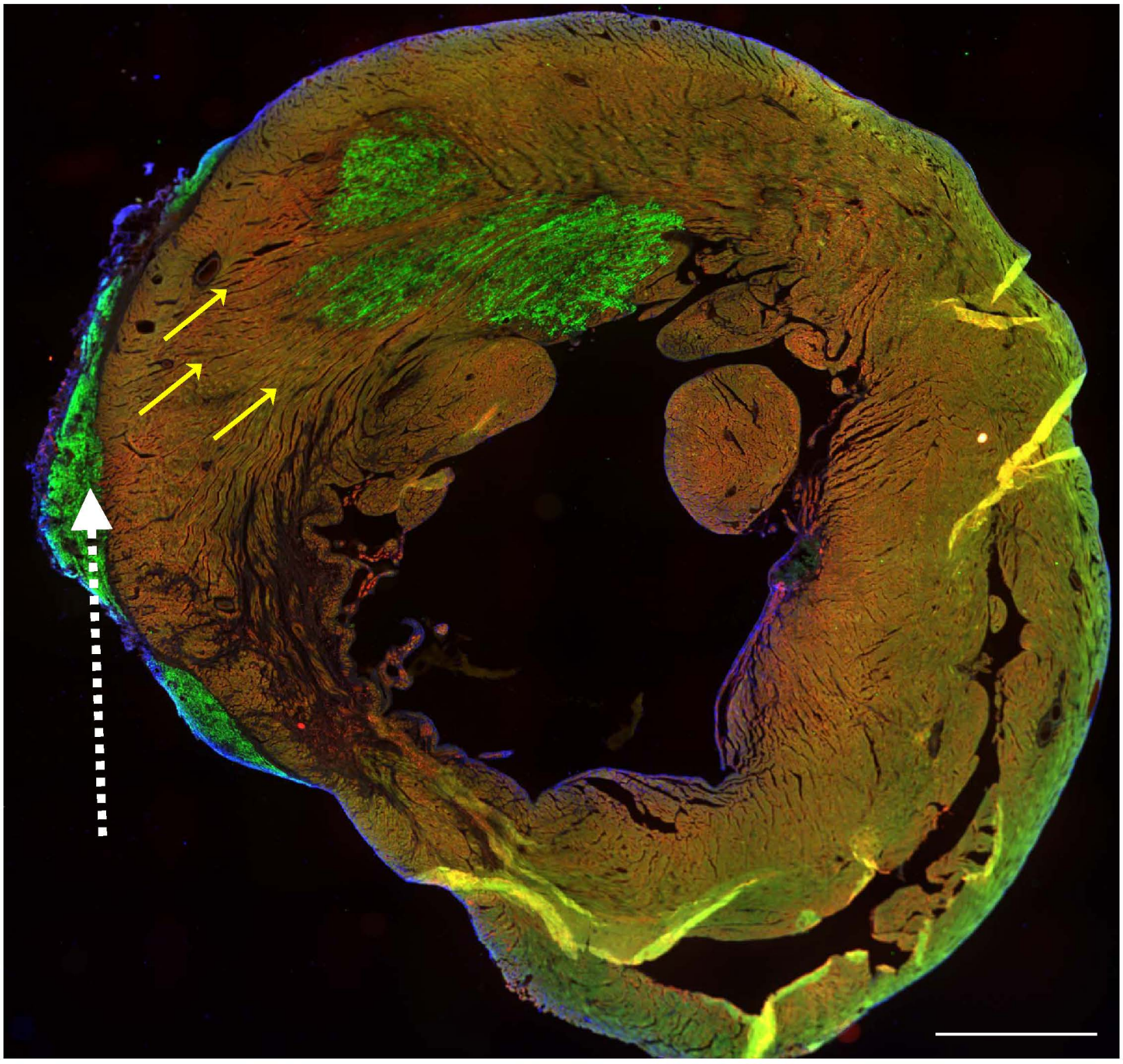
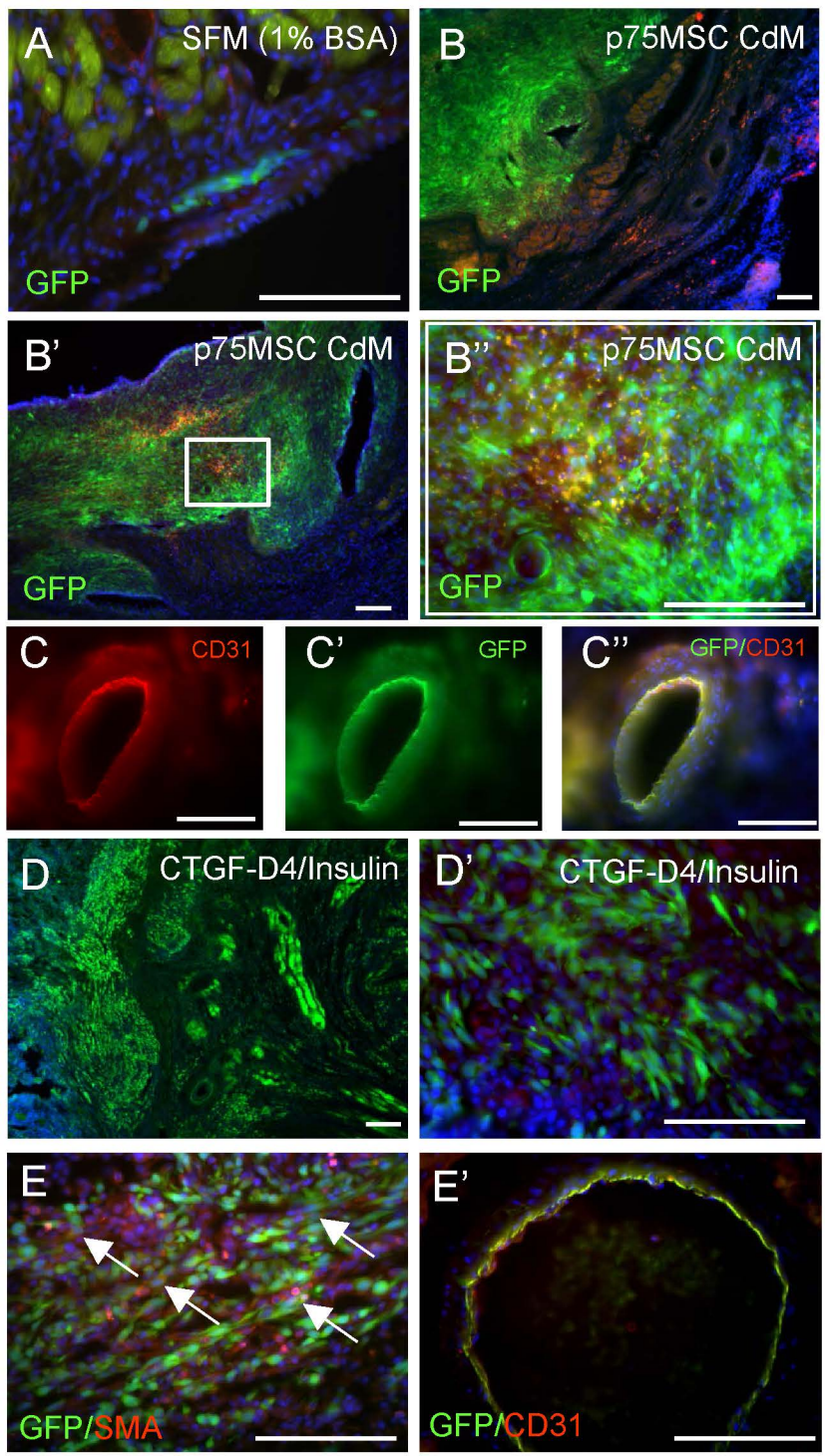
25
20
15
10
5
0
*
SFM
p75MSC CdM




A
SFM (1% BSA)
GFP
B
p75MSC CdM
GFP
B'
p75MSC CdM
GFP
B''
p75MSC CdM
GFP
C
CD31
C'
GFP
C''
GFP/CD31
D
CTGF-D4/Insulin
D'
CTGF-D4/Insulin
E
GFP/SMA
E'
GFP/CD31