Expanded Methods For Mishra et al, "CHARACTERIZATION AND FUNCTIONALITY OF CARDIAC PROGENITOR CELLS IN CONGENITAL HEART PATIENTS

Tissue fixation and histological processing

Heart samples obtained were immersed in ice cold PBS. Thin strips of tissue were pinned slightly stretched and submerged in ice-cold PBS containing 4% paraformaldehyde for 1 hour. Tissues were then cut into 5 mm cubes and incubated in fresh fixative for 1 hour. After several PBS washes, tissues were cryoprotected in PBS containing increasing sucrose concentrations, ranging from 5% to 20%. Tissues were then incubated in 2 parts 20% sucrose, 1 part OCT compound (TissueTek) for 1 hour and embedded in fresh solution by rapid freezing in isopentane cooled in liquid nitrogen. A Leica microtome was used to section each tissue and cryosections 7 to 10 μm thick, were adhered to Fisherbrand Superfrost/Plus microscope slides.

Tissue sections were washed with PBS, and blocked in PBS containing 10% normal goat serum (NGS) or 2% bovine serum albumin (BSA). Sections were then incubated in PBS with 2% NGS or 0.5% BSA and diluted primary antibody overnight at 4°C. Slides were washed with PBS with 2% NGS or 0.5% BSA and then incubated with secondary antibody in PBS with 2% NGS or 0.5% BSA for 1 hour at 4°C. Finally, tissue sections were counterstained with DAPI (Biogenex) and sealed with Cytoseal 60 (Richard-Allen Scientific). An Olympus Fluoview 1000 confocal microscope system was used to visualize and capture images of antibody mediated immunofluorescence. Primary antibodies used were c-kit (Abcam, ab60585 & ab5506), Ki67 (Thermo Scientific, RB-1510-p1), Nkx2.5 (R&D, mab2444), Troponin 1 (TnI) (Abcam, ab47003), Smooth Muscle Actin (Sigma, SAB250), CD31/PECAM (R&D Systems Inc., BBA7), Troponin I (ThermoFisher, MS29500), Human Nucleus Marker (Milipore. MAB1281), Sacromeric alpha actin (Sigma, A2172), Connexin 43 (Sigma, C6219), and von Willebrand (Sigma, HPA00185).

Immunostaining of Cardiospheres

Cardiospheres were collected when they had reached 100 to 1000 cells in size. Cardiospheres were frozen in OCT and sectioned at 5μm slices. Cardiosphere sections were fixed in ice cold acetone for 10 min. the slides were blocked with 10% normal goat serum for 45 min in room temperature. Slides were sequentially incubated with diluted primary and secondary antibody for 1 hour in room temperature. Between each step, slides were washed three times (1-5 min each) with 0.1% BSA/PBS. We used primary antibodies against c-kit (H-300, Santa Cruz Biotechnology), GATA-4 (56-327, BD Biosciences), cardiac tropinin I (ab52862, abscam), Isl-1 (R&D Systems, AF1837) and Nkx2.5 (MAB2444, R&D Systems Inc.). For secondary antibodies conjugated, AlexaFluor fluorochromes

were used (Invitrogen). Slides were counterstained with DAPI, and imaged by epifluorescence micrscopy (Leica DM-IRB inverted microscope).

Immunostaining of CDC

CDC on chamber slides were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. The cells were then permeabilized with 0.01% triton-X 100 for 3 minutes.

Prior to immunostaining, cells were blocked with PBS containing 3% BSA. Cells were incubated with primary antibodies to α-sacromeric actin (Abcam cat#ab7799), collagen I (Millipore, cat#MAB3391), connexin 43 (CS43) (Chemicon ca#MAB3067), Myosin heavy chain (MHC) R&D Systems ca# MAB4470), Sacroplasmic reticulum Ca^{2+} ATPase (SERCA2) (Novus Biologicals cat#NB100-237), CD105 (R&D Systems cat# MAB10972), CD90 (BD Biosciences cat#555593), c-kit (R&D systems cat# AF332), Nkx2.5 R&D Systems cat#MAB2444), SSEA-4 (R&D Systems cat#MAB1435) and Oct3/4 (R&D Systems cat#MAB1759). After subsequent incubation with fluorescence tagged secondary antibodies cells were treated with DAPI (1mg/ml). The slides were mounted in Prolong™ (Invitrogen) and visualized by confocal microscopy (Zeiss 510 META).

Flow Cytometry analysis of CDC

c-kit (BD Biosciences cat#550412) CD90 (R&D Systems cat#FAB2067A & FAB2067P), CD105 (R&D Systems cat#FAB10971P) and a lineage cocktail (Lin1) (BD Biosciences cat#34056) of antibodies against hematopoietic lineage surface markers CD3, CD14, CD16, CD19, CD20, and CD56 were used. Labeled cells were analyzed on a Becton-Dickenson FACS Calibur with 10,000 events collected.

In Vitro Cardiac Lineage Differentiation Assays

For cardiomyocyte differentiation, cells were plated at 30,000 cells/cm^2 and treated with 10umol/L of 5-azacytidine in DMEM containing 10% FBS for 24 hours. The cells were then maintained in 2% FBS without 5-azacytidine for 2 weeks and then stained for cardiac troponin I and sarcomeric actin for assessment of differentiation. For endothelial cell differentiation, cells were plated at 30,000 cells/cm^2 and treated with 10 ng/ml vascular endothelial growth factor 165 (R&D Systems) in DMEM containing 10% FBS for 14 days with fractional medium change every 2 days and stained for von Willebrand factor (vWF) and CD31 for assessment of endothelial differentiation. For smooth muscle cell differentiation, cells were plated at lower density (3000 cells/cm^2) and treated with 5 ng/ml platelet-derived growth factor-BB, 2.5 ng/ml human transforming growth factor β1(R&D Systems) in DMEM containing 10% FBS for 14 days, with fractional medium changes of 50% every 2 days. Cells were stained for smooth muscle actin to determine smooth muscle differentiation.

Cell Transplantation and Echocardiography

Briefly, the rats were anesthetized with 1.5% isoflurane, intubated, and ventilated. A left thoracotomy was performed, the LAD was ligated, and after ten minutes, either cell ($1x10^6$) or media (roughly 300□l) were injected into four to five regions within the infarct and peri-infarct regions. The hCDCs were from patients (n=6) who were between 2 months and 6 months of age. Rats were allowed to recover under close supervision and supplemented with analgesics.

Transthoracic echocardiograms were performed on the rats using a Visual Sonics Vevo 770 series 17.5-MHz transducer. The rats were lightly anesthesized with 1% isoflurane and temperature maintained at 37ºC. Animal heart rate was maintained above 300 beats per
minute for the duration of the examination. Two-dimensional and M-mode echocardiography were used to assess wall motion, chamber dimensions, fractional shortening, and ejection fraction, while color flow Doppler was used to determine valve function. Baseline echocardiograms were acquired before LAD ligation with additional echocardiograms acquired at 7 days and 28 days after infarction. The images were obtained in triplicate by an echocardiographer who was blinded to the treatment group.

Figure Captions

Figure SI-1
Quantification of c-kit expression in the four different chambers of the heart by confocal microscopy. *** P<0.05

Figure S1-2
Confocal microscopy images identify c-kit$^+$tryptase$^+$ co-expression (a-c) which occurred at very low rate (<0.5%). Tryptase expression and c-kit expression are shown with DAPI staining in (a) and (b) repectively while co-expression is shown in (c).

Figure SI-3
Growth profiles for hCDCs are shown (a-c). Graph (a) shows growth over time of 3 different hCDC harvest generations (1, 2, 3) from 2 separate patient RA specimens. Graphs (b) and (c) compare growth of hCDC harvest generations 2 and 3 from multiple patient RA specimens. Patient ages indicated in legend.

Figure SI-4
Confocal microscopy images of CDC at early passage (P0) are shown. CDC expressing SSEA-4 and Oct3/4 are shown (a & b). Both SSEA-4 and Oct3/4 expression were rare and inconsistent between CDC across different patient samples. C-kit expression is shown in (c). The majority of CDC at P0 expressed Nkx2.5 (d).

Figure SI-5
Confocal microscopy images identify no fibroblast cells within the CDCs while the human fibroblasts were used as the positive control. Collagen type I was used as the positive marker for smooth muscle cells and DAPI identified the nuclei in blue.

Table S1

< 30 Days	1 month – ≤ 2 years	> 2 years – ≤ 13 years
*Transposition of the Great Arteries (6)	Ventricular Septal Defect (19)	Anomalous Aortic Origin of Coronary Artery (8)
*Hypoplastic Left Heart Syndrome (4)	*Tetralogy of Fallot (8)	Secundum Atrial Septal Defect (4)
Total Anomalous Pulmonary Venous Connection (4)	Atrioventricular Septal Defect (6)	Aortic Stenosis, Valvar (2)
*Pulmonary Atresia (3)	Aortic Stenosis, Subvalvar (2)	Aortic Stenosis, Subvalvar (2)
Coarctation of Aorta (2)	Secundum Atrial Septal Defect (2)	Atrioventricular Septal Defect (2)
Interrupted Aortic Arch (2)	Vascular Ring (2)	Coarctation of Aorta (2)
*Tetralogy of Fallot (2)	Cor Triatriatum (1)	Aortic Aneurysm (1)
Truncus Arteriosus (2)	*Double Chambered Right Ventricle (1)	Cardiomyopathy (1)
Aortopulmonary Window (1)	Double Outlet Right Ventricle (1)	Double Inlet Left Ventricle (1)
Atrioventricular Septal Defect (1)	Heterotaxy (1)	Double Outlet Right Ventricle (1)
	Partial Anomalous Pulmonary Venous Connection (1)	Congenitally Corrected Transposition of the Great Arteries (1)
	*Pulmonary Atresia (1)	*Pulmonary Atresia (1)
	Tracheal Stenosis (1)	*Pulmonary Venous Stenosis (1)
	*Tricuspid Atresia (1)	Transposition of the Great Arteries (1)
		Ventricular Septal Defect (1)

*Cyanotic lesion

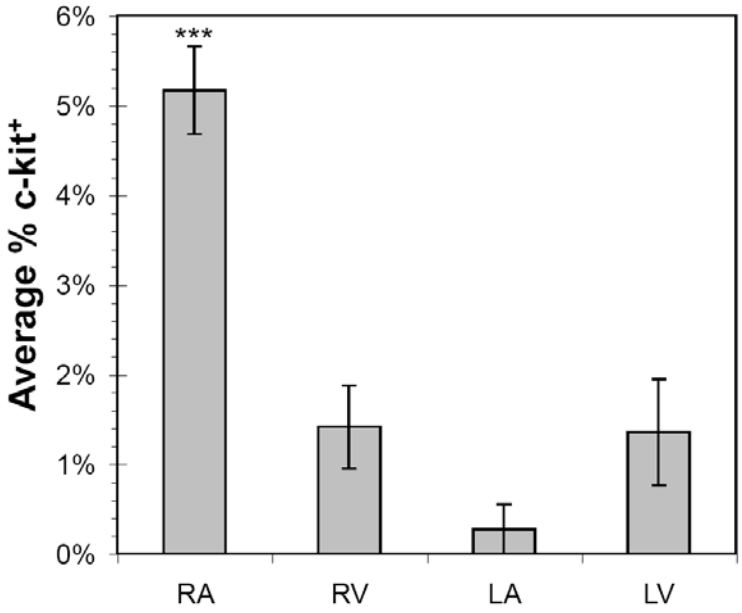

Figure. S1-1

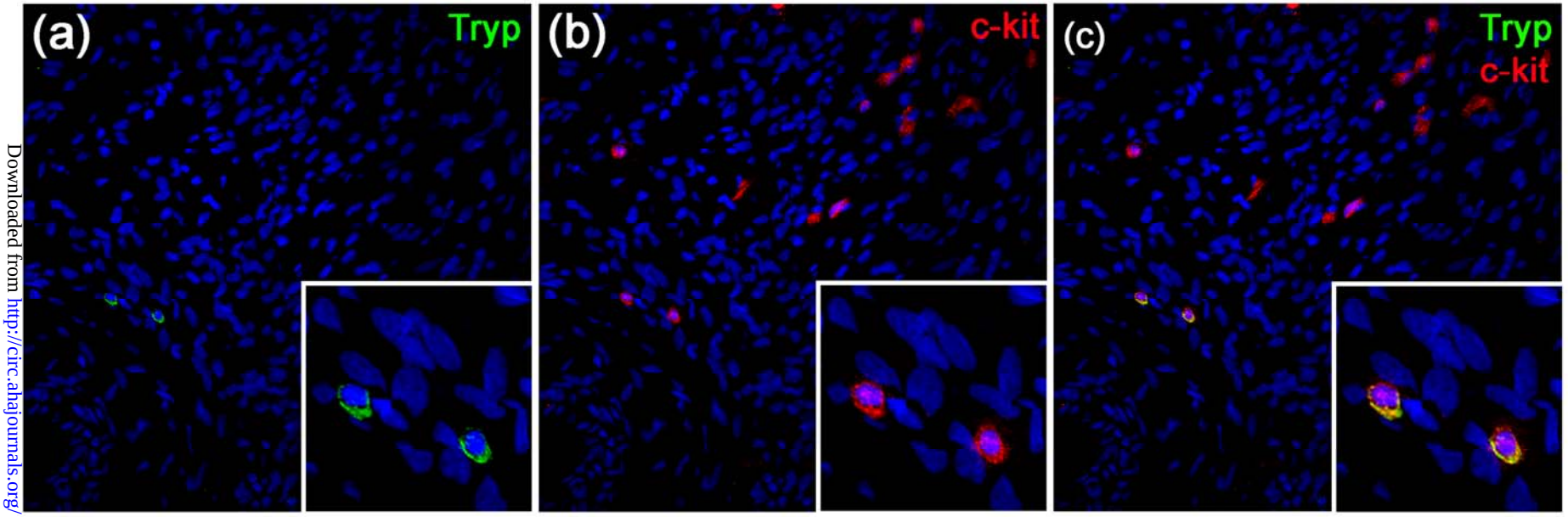

Figure S1-2

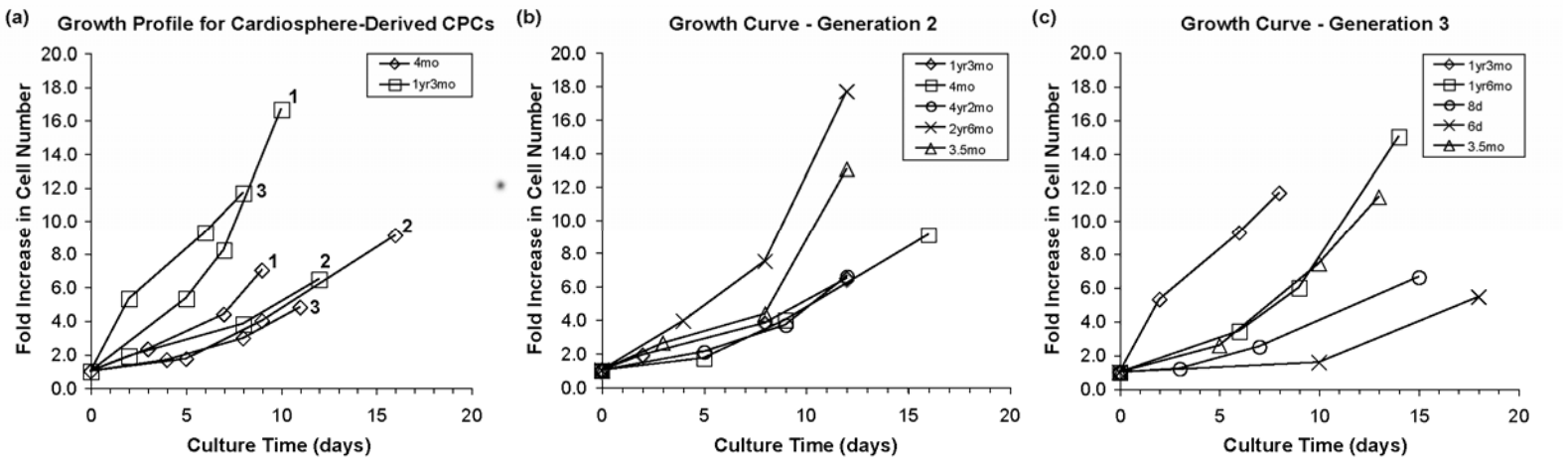

Figure S1-3

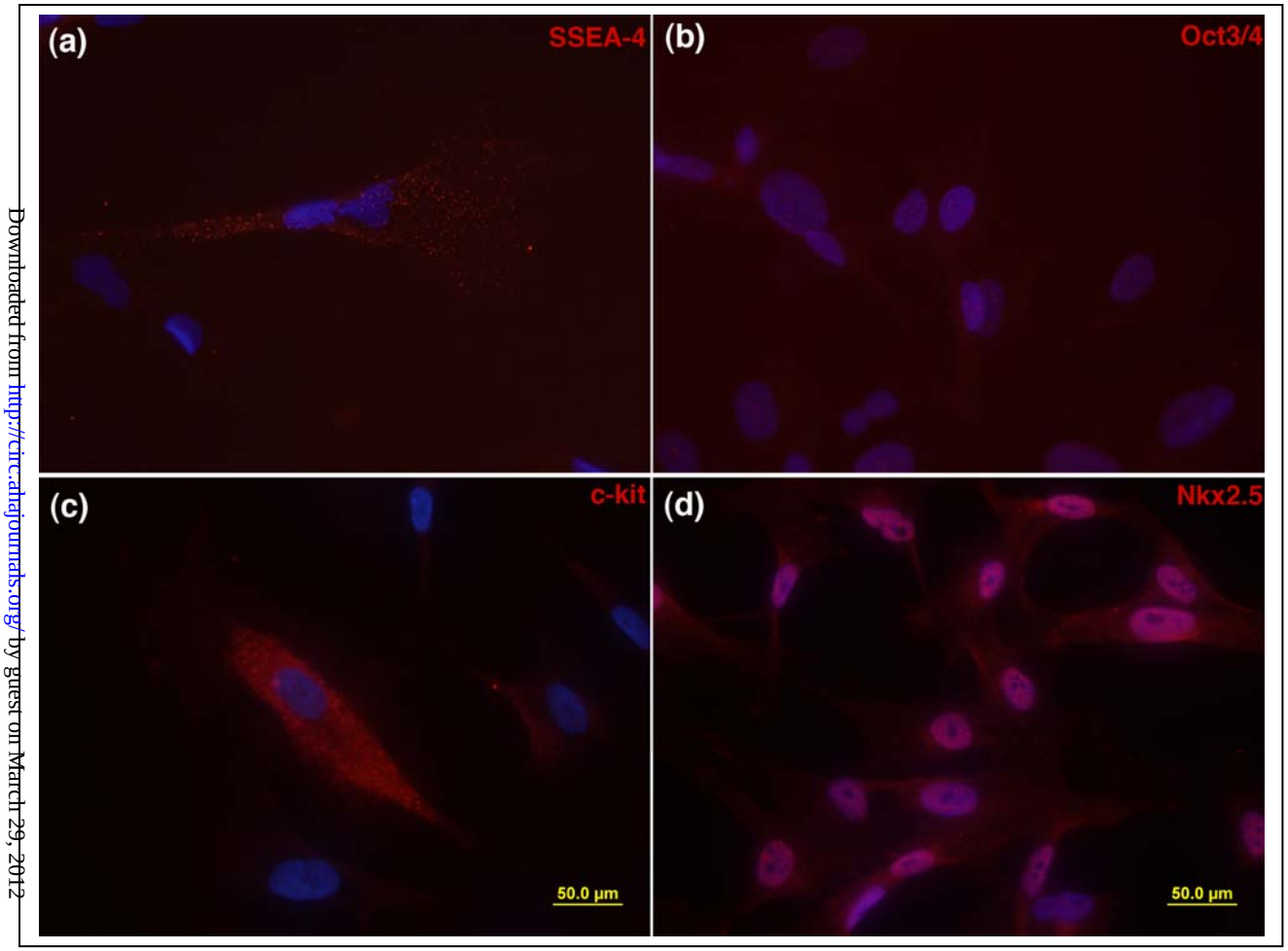

Figure S1-4

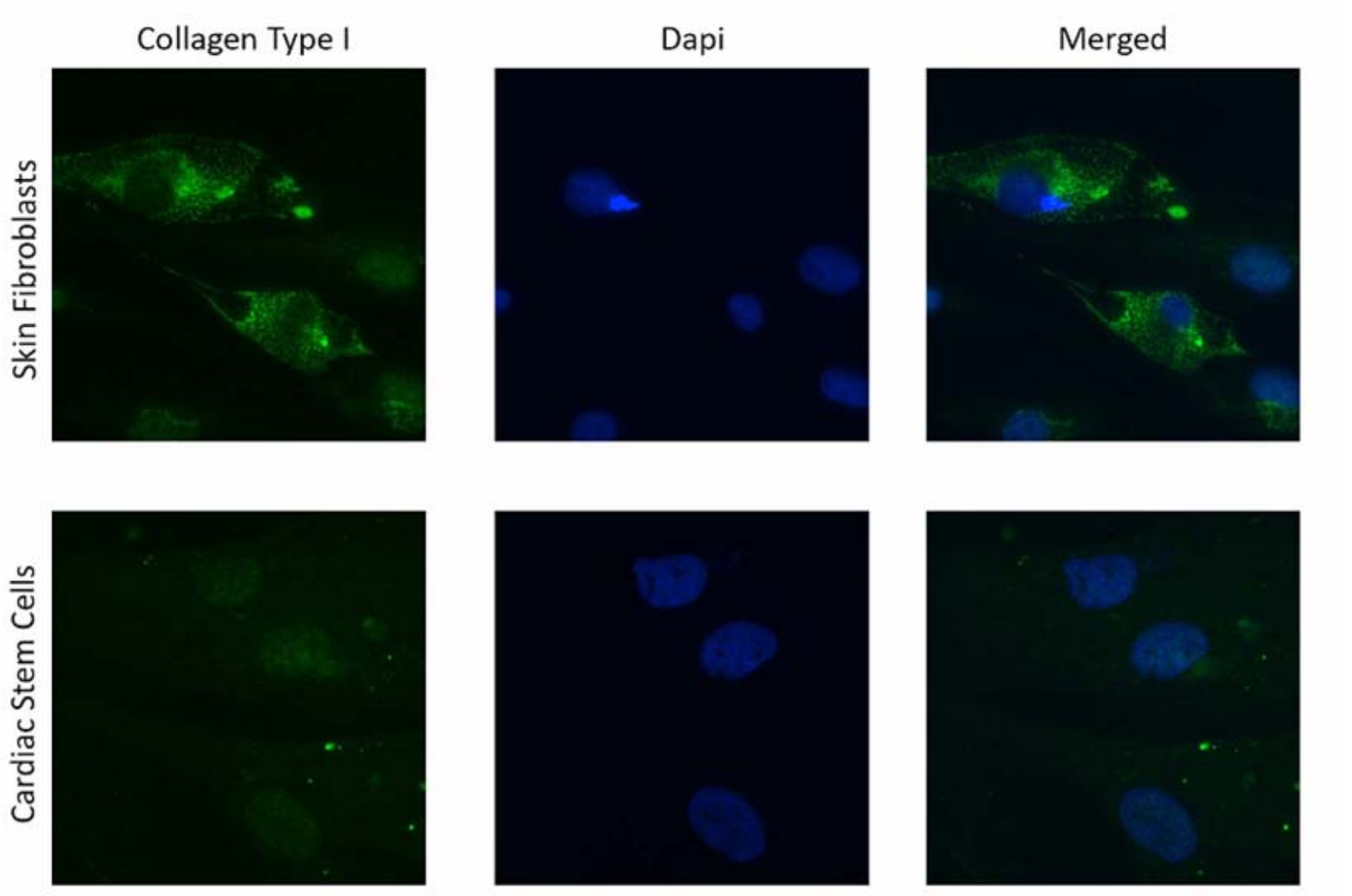

Figure S1-5

Expanded Methods For Mishra et al, "CHARACTERIZATION AND FUNCTIONALITY OF CARDIAC PROGENITOR CELLS IN CONGENITAL HEART PATIENTS

Tissue fixation and histological processing

Heart samples obtained were immersed in ice cold PBS. Thin strips of tissue were pinned slightly stretched and submerged in ice-cold PBS containing 4% paraformaldehyde for 1 hour. Tissues were then cut into 5 mm cubes and incubated in fresh fixative for 1 hour. After several PBS washes, tissues were cryoprotected in PBS containing increasing sucrose concentrations, ranging from 5% to 20%. Tissues were then incubated in 2 parts 20% sucrose, 1 part OCT compound (TissueTek) for 1 hour and embedded in fresh solution by rapid freezing in isopentane cooled in liquid nitrogen. A Leica microtome was used to section each tissue and cryosections 7 to 10 μm thick, were adhered to Fisherbrand Superfrost/Plus microscope slides.

Tissue sections were washed with PBS, and blocked in PBS containing 10% normal goat serum (NGS) or 2% bovine serum albumin (BSA). Sections were then incubated in PBS with 2% NGS or 0.5% BSA and diluted primary antibody overnight at 4°C. Slides were washed with PBS with 2% NGS or 0.5% BSA and then incubated with secondary antibody in PBS with 2% NGS or 0.5% BSA for 1 hour at 4°C. Finally, tissue sections were counterstained with DAPI (Biogenex) and sealed with Cytoseal 60 (Richard-Allen Scientific). An Olympus Fluoview 1000 confocal microscope system was used to visualize and capture images of antibody mediated immunofluorescence. Primary antibodies used were c-kit (Abcam, ab60585 & ab5506), Ki67 (Thermo Scientific, RB-1510-p1), Nkx2.5 (R&D, mab2444), Troponin 1 (TnI) (Abcam, ab47003), Smooth Muscle Actin (Sigma, SAB250), CD31/PECAM (R&D Systems Inc., BBA7), Troponin I (ThermoFisher, MS29500), Human Nucleus Marker (Milipore. MAB1281), Sacromeric alpha actin (Sigma, A2172), Connexin 43 (Sigma, C6219), and von Willebrand (Sigma, HPA00185).

Immunostaining of Cardiospheres

Cardiospheres were collected when they had reached 100 to 1000 cells in size. Cardiospheres were frozen in OCT and sectioned at 5μm slices. Cardiosphere sections were fixed in ice cold acetone for 10 min. the slides were blocked with 10% normal goat serum for 45 min in room temperature. Slides were sequentially incubated with diluted primary and secondary antibody for 1 hour in room temperature. Between each step, slides were washed three times (1-5 min each) with 0.1% BSA/PBS. We used primary antibodies against c-kit (H-300, Santa Cruz Biotechnology), GATA-4 (56-327, BD Biosciences), cardiac tropinin I (ab52862, abscam), Isl-1 (R&D Systems, AF1837) and Nkx2.5 (MAB2444, R&D Systems Inc.). For secondary antibodies conjugated, AlexaFluor fluorochromes

were used (Invitrogen). Slides were counterstained with DAPI, and imaged by epifluorescence micrscopy (Leica DM-IRB inverted microscope).

Immunostaining of CDC

CDC on chamber slides were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. The cells were then permeabilized with 0.01% triton-X 100 for 3 minutes.

Prior to immunostaining, cells were blocked with PBS containing 3% BSA. Cells were incubated with primary antibodies to α-sacromeric actin (Abcam cat#ab7799), collagen I (Millipore, cat#MAB3391), connexin 43 (CS43) (Chemicon ca#MAB3067), Myosin heavy chain (MHC) R&D Systems ca# MAB4470), Sacroplasmic reticulum Ca^{2+} ATPase (SERCA2) (Novus Biologicals cat#NB100-237), CD105 (R&D Systems cat# MAB10972), CD90 (BD Biosciences cat#555593), c-kit (R&D systems cat# AF332), Nkx2.5 R&D Systems cat#MAB2444), SSEA-4 (R&D Systems cat#MAB1435) and Oct3/4 (R&D Systems cat#MAB1759). After subsequent incubation with fluorescence tagged secondary antibodies cells were treated with DAPI (1mg/ml). The slides were mounted in Prolong™ (Invitrogen) and visualized by confocal microscopy (Zeiss 510 META).

Flow Cytometry analysis of CDC

c-kit (BD Biosciences cat#550412) CD90 (R&D Systems cat#FAB2067A & FAB2067P), CD105 (R&D Systems cat#FAB10971P) and a lineage cocktail (Lin1) (BD Biosciences cat#34056) of antibodies against hematopoietic lineage surface markers CD3, CD14, CD16, CD19, CD20, and CD56 were used. Labeled cells were analyzed on a Becton-Dickenson FACS Calibur with 10,000 events collected.

In Vitro Cardiac Lineage Differentiation Assays

For cardiomyocyte differentiation, cells were plated at 30,000 cells/cm^2 and treated with 10umol/L of 5-azacytidine in DMEM containing 10% FBS for 24 hours. The cells were then maintained in 2% FBS without 5-azacytidine for 2 weeks and then stained for cardiac troponin I and sarcomeric actin for assessment of differentiation. For endothelial cell differentiation, cells were plated at 30,000 cells/cm^2 and treated with 10 ng/ml vascular endothelial growth factor 165 (R&D Systems) in DMEM containing 10% FBS for 14 days with fractional medium change every 2 days and stained for von Willebrand factor (vWF) and CD31 for assessment of endothelial differentiation. For smooth muscle cell differentiation, cells were plated at lower density (3000 cells/cm^2) and treated with 5 ng/ml platelet-derived growth factor-BB, 2.5 ng/ml human transforming growth factor β1(R&D Systems) in DMEM containing 10% FBS for 14 days, with fractional medium changes of 50% every 2 days. Cells were stained for smooth muscle actin to determine smooth muscle differentiation.

Cell Transplantation and Echocardiography

Briefly, the rats were anesthetized with 1.5% isoflurane, intubated, and ventilated. A left thoracotomy was performed, the LAD was ligated, and after ten minutes, either cell ($1x10^6$) or media (roughly 300□l) were injected into four to five regions within the infarct and peri-infarct regions. The hCDCs were from patients (n=6) who were between 2 months and 6 months of age. Rats were allowed to recover under close supervision and supplemented with analgesics.

Transthoracic echocardiograms were performed on the rats using a Visual Sonics Vevo 770 series 17.5-MHz transducer. The rats were lightly anesthesized with 1% isoflurane and temperature maintained at 37ºC. Animal heart rate was maintained above 300 beats per
minute for the duration of the examination. Two-dimensional and M-mode echocardiography were used to assess wall motion, chamber dimensions, fractional shortening, and ejection fraction, while color flow Doppler was used to determine valve function. Baseline echocardiograms were acquired before LAD ligation with additional echocardiograms acquired at 7 days and 28 days after infarction. The images were obtained in triplicate by an echocardiographer who was blinded to the treatment group.

Figure Captions

Figure SI-1
Quantification of c-kit expression in the four different chambers of the heart by confocal microscopy. *** P<0.05

Figure S1-2
Confocal microscopy images identify c-kit$^+$tryptase$^+$ co-expression (a-c) which occurred at very low rate (<0.5%). Tryptase expression and c-kit expression are shown with DAPI staining in (a) and (b) repectively while co-expression is shown in (c).

Figure SI-3
Growth profiles for hCDCs are shown (a-c). Graph (a) shows growth over time of 3 different hCDC harvest generations (1, 2, 3) from 2 separate patient RA specimens. Graphs (b) and (c) compare growth of hCDC harvest generations 2 and 3 from multiple patient RA specimens. Patient ages indicated in legend.

Figure SI-4
Confocal microscopy images of CDC at early passage (P0) are shown. CDC expressing SSEA-4 and Oct3/4 are shown (a & b). Both SSEA-4 and Oct3/4 expression were rare and inconsistent between CDC across different patient samples. C-kit expression is shown in (c). The majority of CDC at P0 expressed Nkx2.5 (d).

Figure SI-5
Confocal microscopy images identify no fibroblast cells within the CDCs while the human fibroblasts were used as the positive control. Collagen type I was used as the positive marker for smooth muscle cells and DAPI identified the nuclei in blue.

Table S1

< 30 Days	1 month – ≤ 2 years	> 2 years – ≤ 13 years
*Transposition of the Great Arteries (6)	Ventricular Septal Defect (19)	Anomalous Aortic Origin of Coronary Artery (8)
*Hypoplastic Left Heart Syndrome (4)	*Tetralogy of Fallot (8)	Secundum Atrial Septal Defect (4)
Total Anomalous Pulmonary Venous Connection (4)	Atrioventricular Septal Defect (6)	Aortic Stenosis, Valvar (2)
*Pulmonary Atresia (3)	Aortic Stenosis, Subvalvar (2)	Aortic Stenosis, Subvalvar (2)
Coarctation of Aorta (2)	Secundum Atrial Septal Defect (2)	Atrioventricular Septal Defect (2)
Interrupted Aortic Arch (2)	Vascular Ring (2)	Coarctation of Aorta (2)
*Tetralogy of Fallot (2)	Cor Triatriatum (1)	Aortic Aneurysm (1)
Truncus Arteriosus (2)	*Double Chambered Right Ventricle (1)	Cardiomyopathy (1)
Aortopulmonary Window (1)	Double Outlet Right Ventricle (1)	Double Inlet Left Ventricle (1)
Atrioventricular Septal Defect (1)	Heterotaxy (1)	Double Outlet Right Ventricle (1)
	Partial Anomalous Pulmonary Venous Connection (1)	Congenitally Corrected Transposition of the Great Arteries (1)
	*Pulmonary Atresia (1)	*Pulmonary Atresia (1)
	Tracheal Stenosis (1)	*Pulmonary Venous Stenosis (1)
	*Tricuspid Atresia (1)	Transposition of the Great Arteries (1)
		Ventricular Septal Defect (1)

*Cyanotic lesion

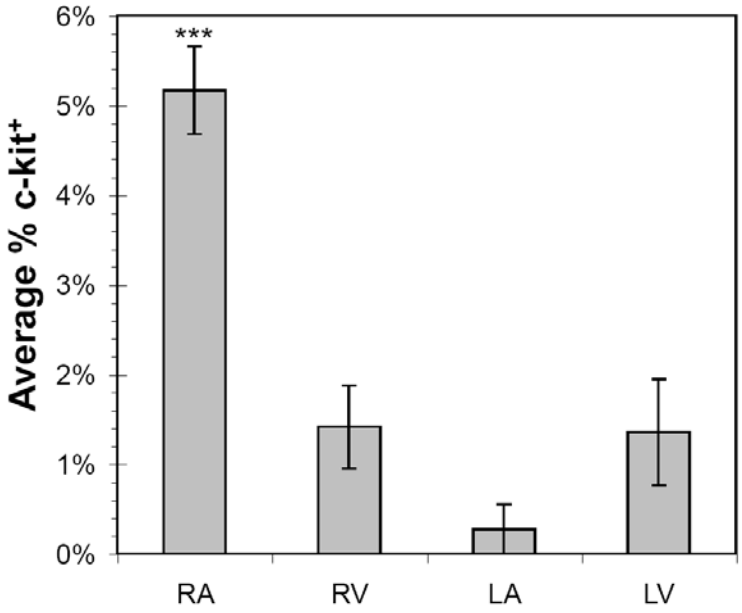

Figure. S1-1

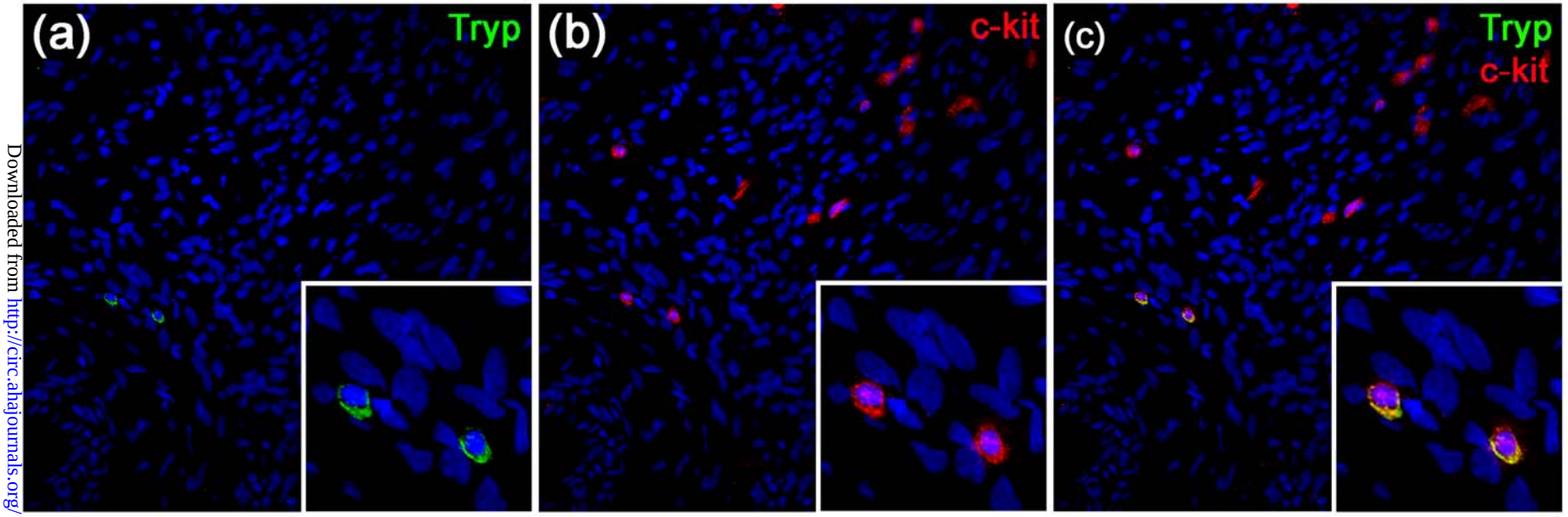

Figure S1-2

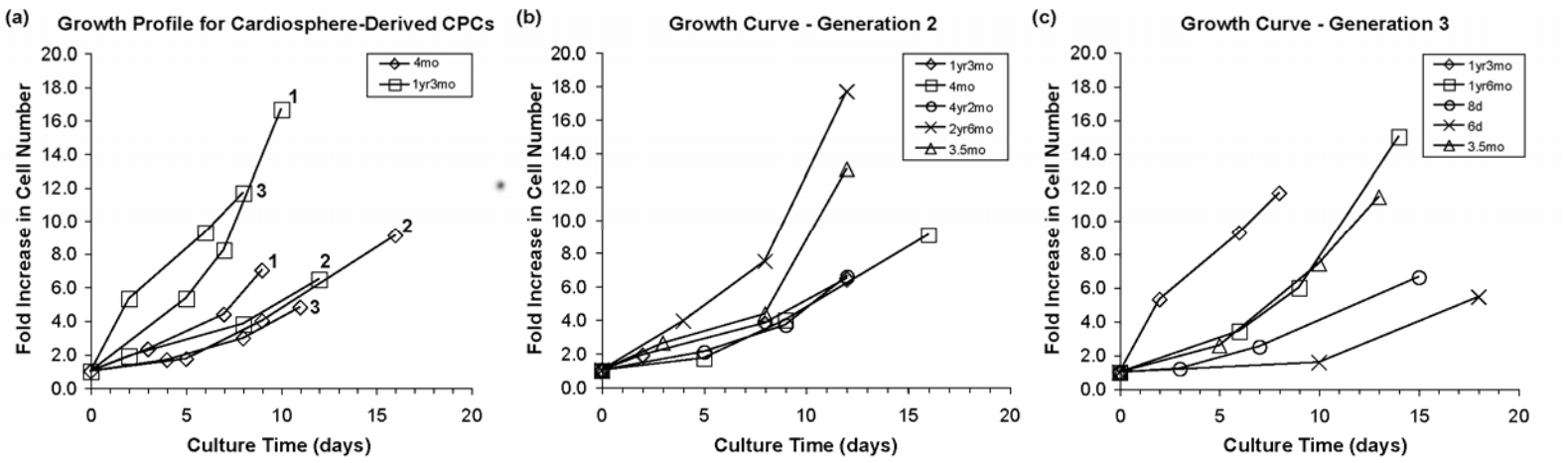

Figure S1-3

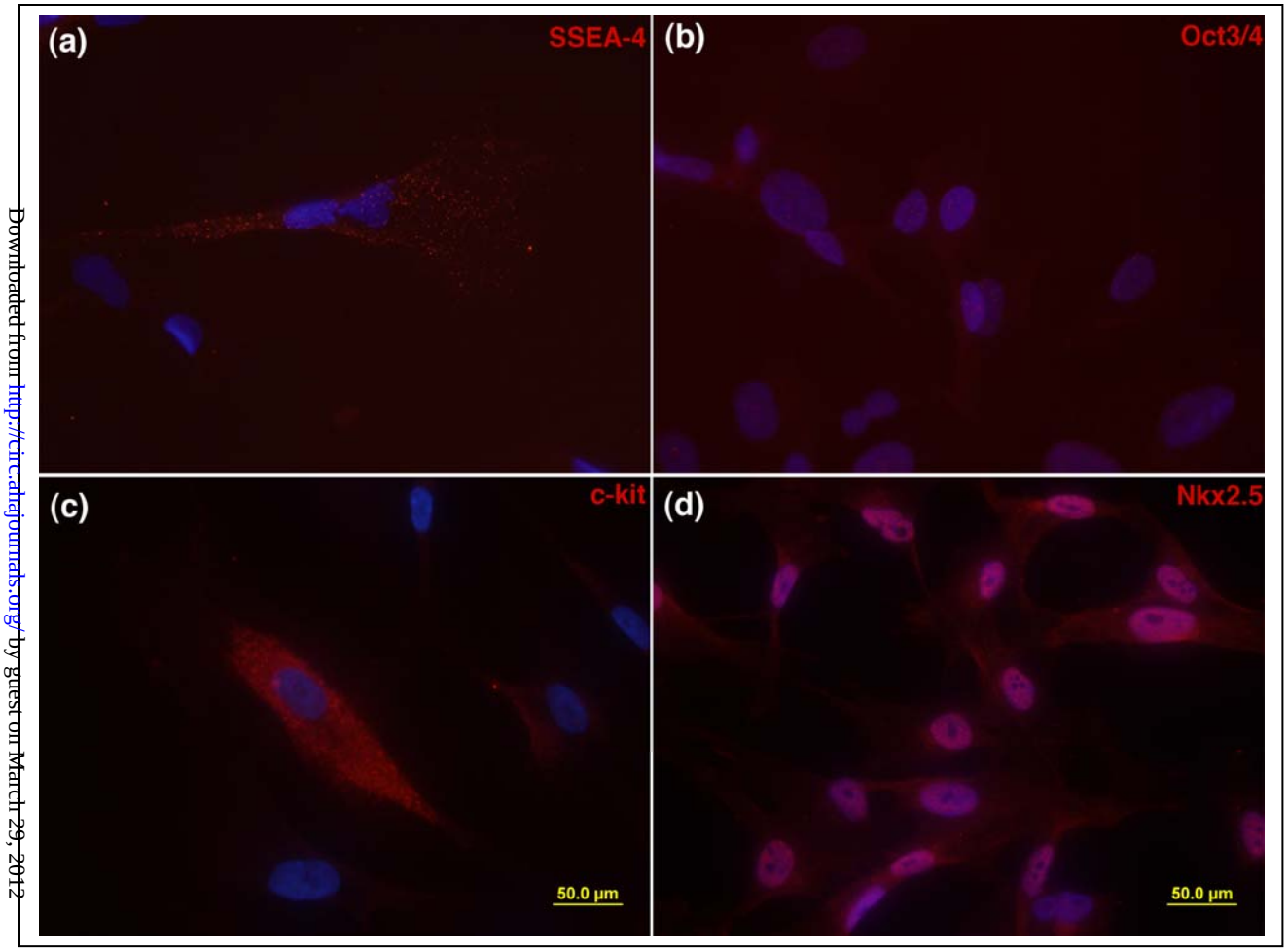

Figure S1-4

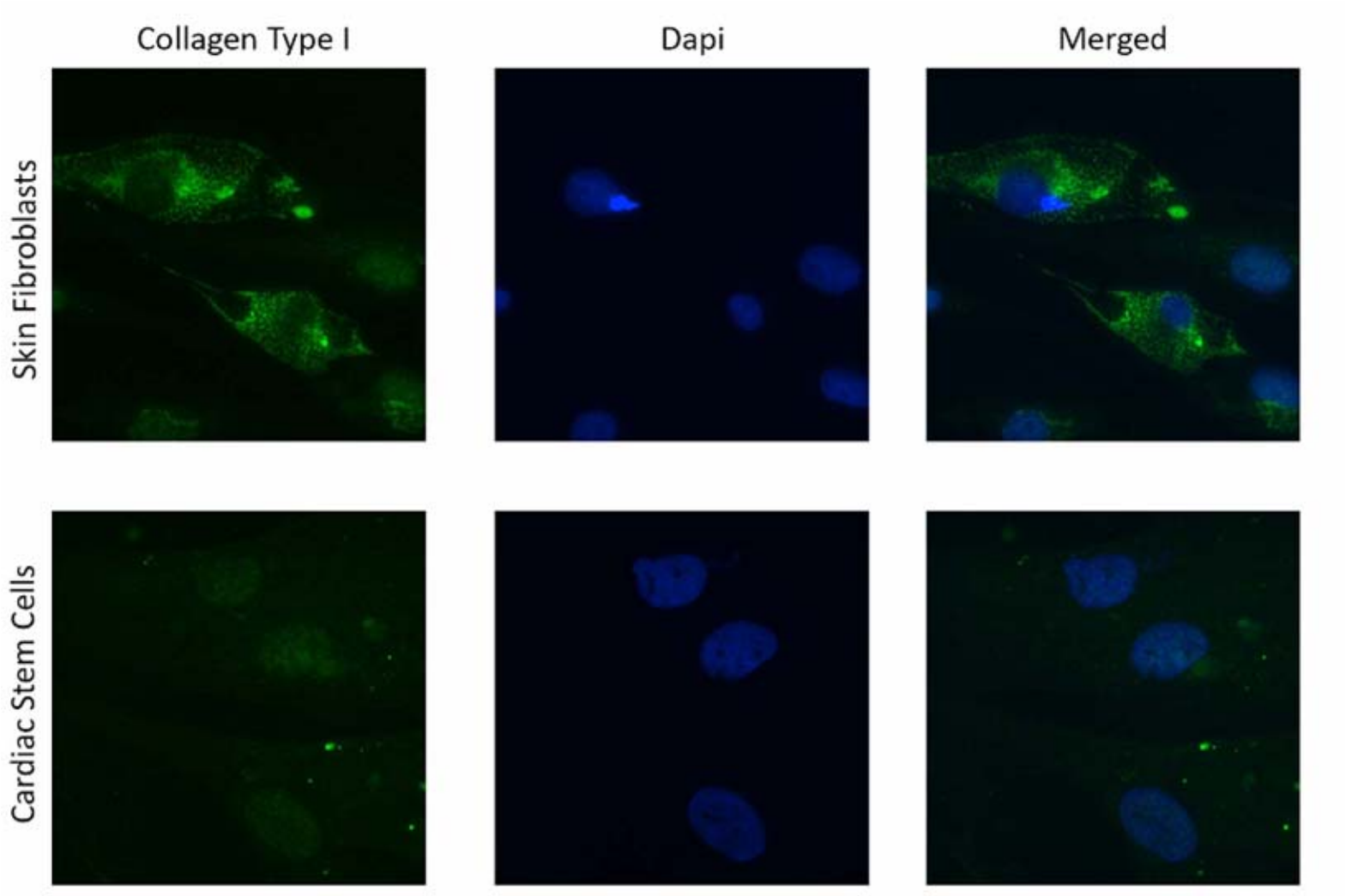

Figure S1-5