SUPPLEMENTAL DATA: Cardiomyogenic potential of c-kit$^+$ expressing cells derived from neonatal and adult mouse hearts.

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Expanded Methods Section

Mice. ACT-EGFP (C57BL/6-Tg(ACTB-EGFP)1Osb/J) mice expressing EGFP under control of the β-actin promoter were obtained from the Jackson Laboratory (Bar Harbor, ME). MHC-nLAC mice[1] express a cardiac-restricted, nuclear-localized β-galactosidase reporter and are maintained in a DBA/2J inbred background. All primary cells used in this study (non-transgenic, single transgenic, or ACT-EGFP / MHC-nLAC double transgenic) were in a $[DBA/2J \times C57Bl/6J]_{f1}$ genetic background. Syngeneic $[DBA/2J \times C57Bl/6J]_{f1}$ mice (obtained from the Jackson Laboratory) and immune compromised NOD/SCID-IL2-gamma null mice (obtained from the *In Vivo* Therapeutics Core, Indiana University Simon Cancer Center, Indianapolis, IN) were used as recipients for intra-cardiac engraftment experiments.

Flow cytometry of c-kit$^+$ cells. Crude bone marrow mono-nucleated cells were prepared as described previously.[2] Cardiac cells from neonatal (day 7) or adult (10-12 weeks) mice were isolated by mincing myocardium in 0.1% Collagenase IV (GIBCO, Carlsbad, CA) and incubating for 45 minutes at 37°C.[3] Cells were then filtered through a 40-μm mesh. Alternatively, cardiac cells were isolated by mincing hearts in a digest buffer containing Liberase/Blendzyme (0.28 Wuensch Units/ml, Roche Pharmaceuticals, Indianapolis, IN), 10 mM Hepes, 30 mM Taurin and 50 μg/ml DNAse I (Roche Pharmaceuticals) and incubating for 45 minutes at 37°. Cells were stained with PerCP-conjugated antibody against the hematopoietic lineage marker CD45 (BD Bioscience cat.# 557235) or anti-c-kit-PE antibody (BD Bioscience cat. #553355) for 45 minutes (isotype-matched control antibodies were used to establish negative threshold; all antibodies were from BD Biosciences, San Jose, CA). Cells were analyzed by 3-color flow cytometry using a FACSCalibur flow cytometer and Cell Quest Pro software (BD

Bioscience, San Jose, CA). Each analysis included 50,000 events. Similar results were obtained with cells isolated via collagenase and Liberase/Blendzyme digestion.

MACS-isolation of c-kit$^+$ cells from the heart. Cells from enzymatically-dispersed hearts were reacted with magnetic beads conjugated with mouse anti-c-kit antibody (Miltenyi Biotec, Auburn, CA cat.# 130091224) for 20 min and subsequently separated by two cycles of magnet-activated cell sorting into c-kit$^+$ and c-kit$^-$ fractions.

Co-culture of c-kit$^+$ cells with fetal mouse cardiomyocytes. To generate primary fetal cardiomyocyte cultures, embryonic day 15 mice were sacrificed, and the ventricles were removed, minced, and dissociated with collagenase I (0.2%, Wothington, Lakewood, NJ) for 60 minutes at 37°C. The resulting dispersed cell suspension was plated on gelatin-coated Lab-Tek 2-well culture dishes (Fischer, Rochester, NY) at a density of 25,000 to 50,000 cells per cm^2 and cultured for 24 hours in DMEM (GIBCO) supplemented with 10% FBS (GIBCO) and antibiotics (penicillin 500,000 U/L, streptomycin 50 mg/L, gentamicin 50 mg/L; Sigma, St. Louis, MO). For co-culture, the medium was replaced with DMEM (GIBCO) supplemented with 10% FBS (GIBCO), insulin (10 ug/mL; Sigma), transferrin (10 ug/mL; Sigma), and antibiotics (as above). Freshly isolated c-kit$^+$ cells were then seeded on the primary cardiomyocyte cultures at a density of 2,500 to 5,000 cells per cm^2 and cultured for an additional 7 days. Analogous co-culture protocols have been employed to monitor cardiomyogenesis in a variety of progenitor cell populations.[4-6] The Collagenase IV and Liberase/Blendzyme protocols gave rise to c-kit$^+$ cells with similar levels of cardiomyogenic activity. The rational for the use of the cell culture system was based on our desire to utilize mouse cells so that we could take advantage of our well characterized transgenic models to distinguish differentiation vs. fusion events. Fetal cardiomyocytes were use in preference to neonatal cardiomyocytes as

they give rise to better cultures (this is due to the marked cardiomyocyte cell cycle withdrawal that occurs at birth). The observation that neonatal c-kit$^+$ cells undergo apparent cardiomyogenic differentiation when co-cultured with fetal mouse cardiomyocytes supports the notion that fetal cardiomyocyte cultures can provide an environment suitable to promote/permit cardiomyogenic differentiation. Thus, the age mis-match when using fetal cardiomyocytes and neonatal progenitors does not appear to be problematic.

Myocardial infarction and intra-cardiac grafting. For myocardial infarction, 12 week old mice were endotracheally intubated and ventilated with a rodent ventilator (Harvard Apparatus, Holliston, MA). Anesthesia was maintained with inhalational isoflurane. A thoracotomy was performed and an 8-0 polypropylene ligature was placed around the distal left anterior descending coronary artery as described previously.[7] The chest was closed and the animals were weaned from the ventilator and extubated. For intra-cardiac grafting, donor cells suspended in 3 µl phosphate-buffered saline were injected into the anterior and posterior infarct border zones of the ischemic myocardium after coronary ligation. Animal care and all experimental procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-23, revised 1996).

Immunohistochemistry. For co-culture experiments, cells were fixed with 4% paraformaldehyde for 10 minutes and reacted with the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-GAL) at 37°C for 2 hours. After permeabilization with 0.2% Triton X-100, cells were incubated with mouse anti-α-actinin (Sigma A7811) and rabbit anti-GFP alexa fluor 488 conjugate (Invitrogen A21311) over night at 4°C. A goat-anti-mouse rhodamine-conjugated secondary antibody (Millipore

AP124R, Billerica, MA) was used to visualize α-actinin immune reactivity. For other studies, antibodies recognizing smooth muscle actin (Sigma A2547) and Isolectin B4 (Vector FL-1201) were employed. Nuclei were visualized by staining with Hoechst dye (Molecular Probes, Eugene, Oregon), and cover slips were mounted using Vectashield (Vector Laboratories, Burlingame, CA).

For cell transplantation experiments, recipient hearts were harvested, washed in phosphate-buffered saline, and immersion fixed in 1% paraformaldehyde, 1% cacodylic acid, phosphate-buffered saline for 24 hours at 4°C, cryoprotected in 30% sucrose, embedded in OCT (Sakura Finetek, Torrance, CA) and sectioned at 10 μM using standard protocols.[8] Sections were reacted with X-GAL, and processed for α-actinin and EGFP immune reactivity as described above.

Statistical analyses. Results were expressed as mean ± standard error of the mean (SEM). Multiple group comparisons were performed by one-way analyses of variance (ANOVA) followed by the Bonferroni procedure for comparison of means. Comparisons between two groups were performed using the unpaired Student's t-test. Data were considered statistically significant at a value of $p \leq 0.05$.

Methods References

1. Soonpaa MH, Koh GY, Klug MG, Field LJ. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. *Science.* 1994;264(5155):98-101.

2. Deindl E, Zaruba MM, Brunner S, Huber B, Mehl U, Assmann G, Hoefer IE, Mueller-Hoecker J, Franz WM. G-CSF administration after myocardial infarction in mice attenuates late ischemic cardiomyopathy by enhanced arteriogenesis. *Faseb J.* 2006;20(7):956-958.

3. Zaruba MM, Theiss HD, Vallaster M, Mehl U, Brunner S, David R, Fischer R, Krieg L, Hirsch E, Huber B, Nathan P, Israel L, Imhof A, Herbach N, Assmann G, Wanke R, Mueller-Hoecker J, Steinbeck G, Franz WM. Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction. *Cell Stem Cell.* 2009;4(4):313-323.

4. Kubo H, Jaleel N, Kumarapeli A, Berretta RM, Bratinov G, Shan X, Wang H,

Houser SR, Margulies KB. Increased Cardiac Myocyte Progenitors in Failing Human Hearts. *Circulation.* 2008;118:649-657.

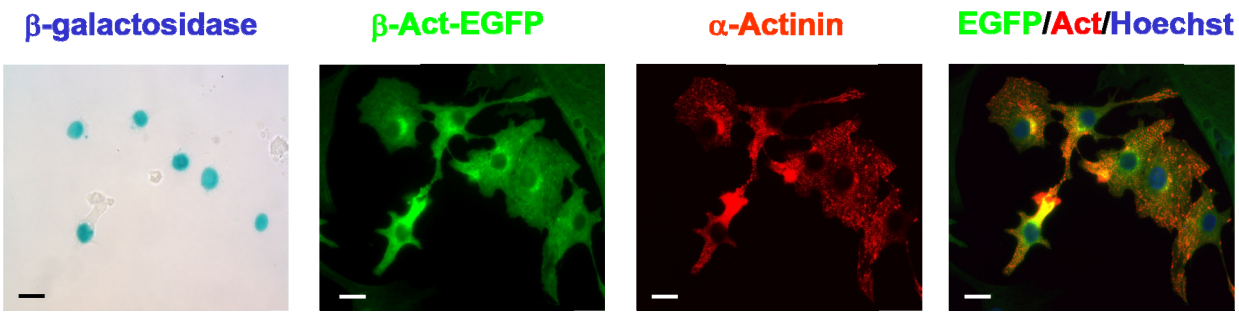
5. Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, Aicher A, Fleming I, Busse R, Zeiher AM, Dimmeler S. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. *Circulation.* 2003;107(7):1024-1032.

6. Pfister O, Mouquet F, Jain M, Summer R, Helmes M, Fine A, Colucci WS, Liao R. CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circ Res.* 2005;97(1):52-61.

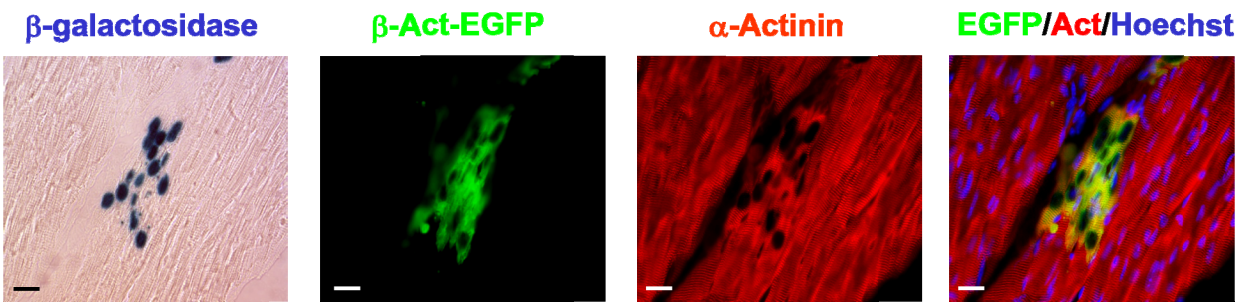
7. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature.* 2004;428(6983):664-668.

8. Junqueira, ed. *Basic Histology.* 5Rev Ed ed: Appleton & Lange; 1986.

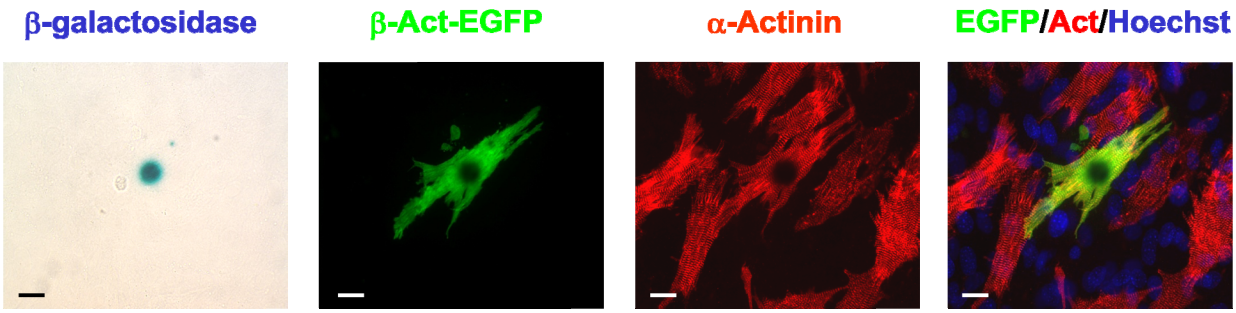
Supplemental Figure 1. Analysis of fetal cardiomyocyte cultures from ACT-EGFP / MHC-nLAC double transgenic mice. Cells were fixed, reacted with X-GAL, and processed for anti-EGFP (green) and anti-α actinin (red) immune reactivity. Bright field, single color fluorescence and merged images are shown. Bar = 20 microns.



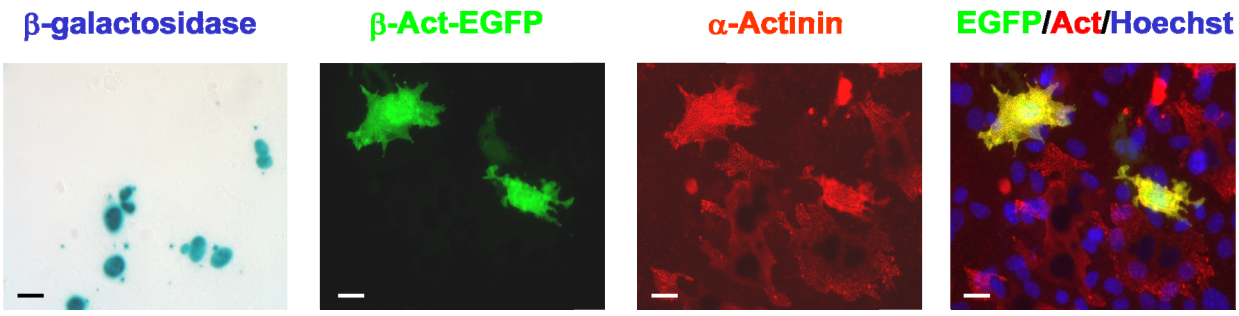
Supplemental Figure 2. Analysis of intra-cardiac grafts using donor fetal cardiomyocytes from ACT-EGFP / MHC-nLAC double transgenic mice and a normal, syngeneic recipient. Hearts were harvested, rinsed with PBS, fixed, reacted with X-GAL, and processed for anti-EGFP (green) and anti-α actinin (red) immune reactivity. Bright field, single color fluorescence and merged images are shown. Bar = 20 microns.



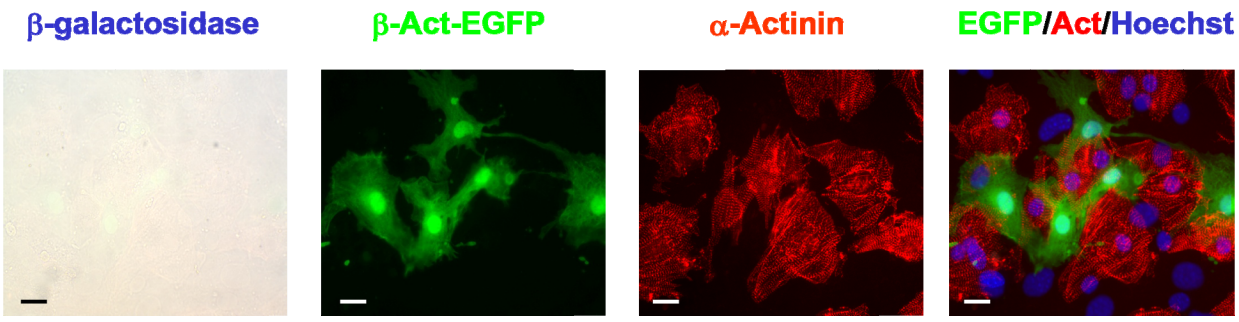
Supplemental Figure 3. Analysis of co-cultures containing cardiac resident c-kit^{+} cells from neonatal ACT-EGFP / MHC-nLAC double transgenic mice and fetal cardiomyocytes from non-transgenic mice. After 7 days of co-culture, the cells were fixed, reacted with X-GAL, and processed for anti-EGFP (green) and anti-α actinin (red) immune reactivity. Bright field, single color fluorescence and merged images are shown. Bar = 20 microns.



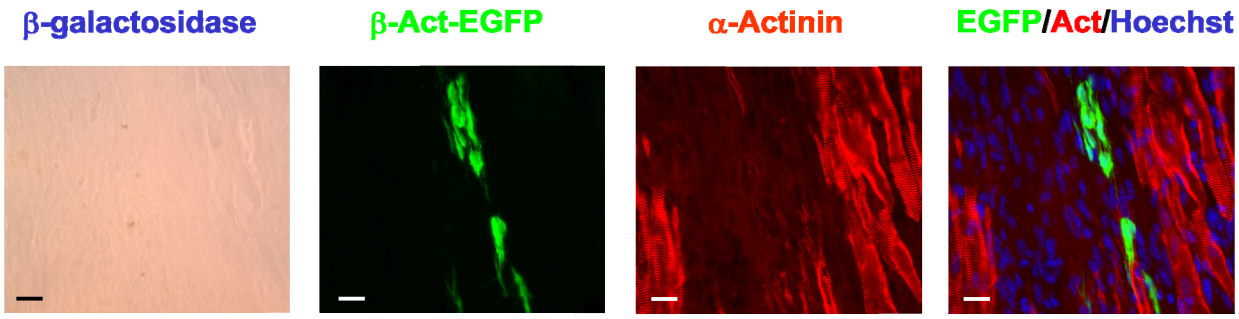
Supplemental Figure 4. Analysis of co-cultures containing cardiac resident c-kit^{+} cells from neonatal ACT-EGFP transgenic mice and fetal cardiomyocytes from MHC-nLAC transgenic mice. After 7 days of co-culture, the cells were fixed, reacted with X-GAL, and processed for anti-EGFP (green) and anti-α actinin (red) immune reactivity. Bright field, single color fluorescence and merged images are shown. Bar = 20 microns.



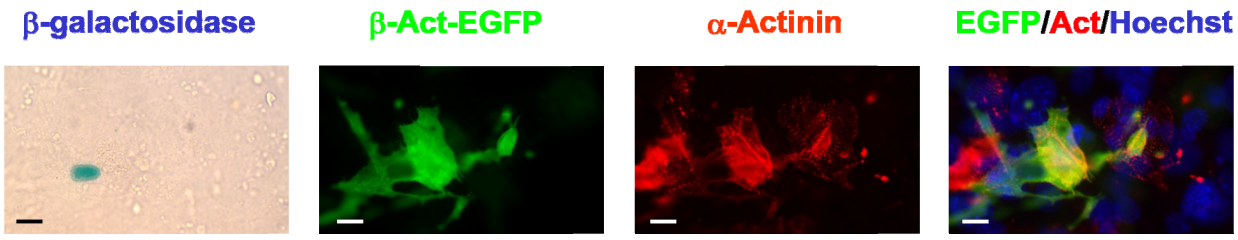
Supplemental Figure 5. Analysis of co-cultures containing cardiac resident c-kit^{+} cells from normal adult ACT-EGFP / MHC-nLAC mice and fetal cardiomyocytes from non-transgenic mice. After 7 days of co-culture, the cells were fixed, reacted with X-GAL, and processed for anti-EGFP (green) and anti-α actinin (red) immune reactivity. Bright field, single color fluorescence and merged images are shown. Bar = 20 microns.



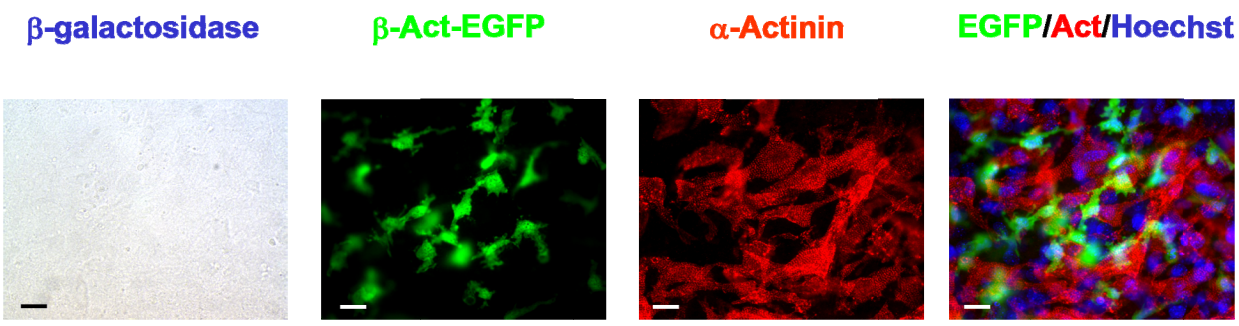
Supplemental Figure 6. Analysis of intra-cardiac grafts using donor cardiac-resident c-kit^{+} cells from normal adult ACT-EGFP / MHC-nLAC double transgenic mice and an infarcted NOD-SCID-IL2 recipient. Hearts were harvested, rinsed with PBS, fixed, reacted with X-GAL, and processed for anti-EGFP (green) and anti-α actinin (red) immune reactivity. Bright field, single color fluorescence and merged images are shown. Bar = 20 microns.



Supplemental Figure 7. Analysis of co-cultures containing adult cardiac resident c-kit^{+} cells from infarcted adult ACT-EGFP / MHC-nLAC mice and fetal cardiomyocytes from non-transgenic mice. After 7 days of co-culture, the cells were fixed, reacted with X-GAL, and processed for anti-EGFP (green) and anti-α actinin (red) immune reactivity. Bright field, single color fluorescence and merged images are shown. Bar = 20 microns.

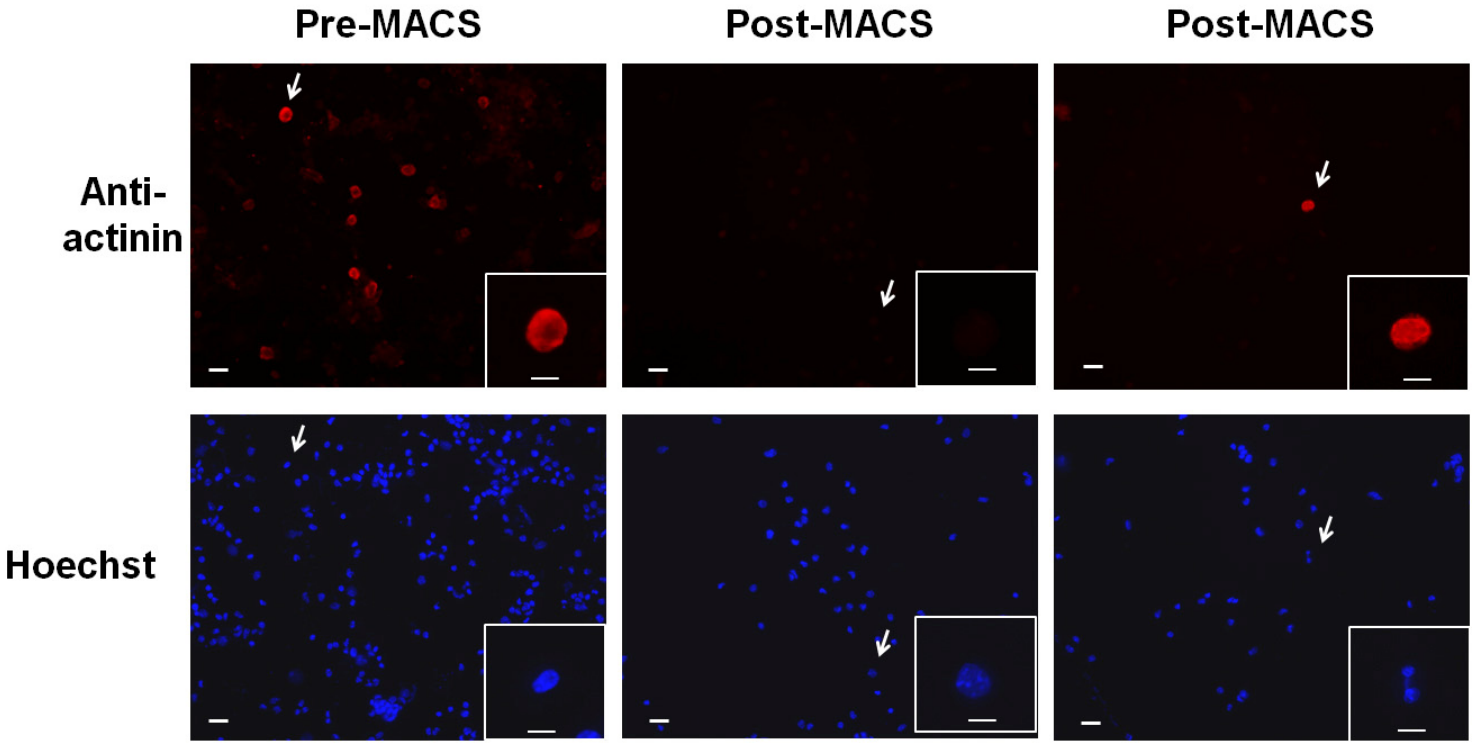


Supplemental Figure 8. Analysis of co-cultures containing adult cardiac resident c-kit^{+} cells from infarcted adult ACT-EGFP / MHC-nLAC mice and fetal cardiomyocytes from non-transgenic mice. After 7 days of co-culture, the cells were fixed, reacted with X-GAL, and processed for anti-EGFP (green) and anti-α actinin (red) immune reactivity. Bright field, single color fluorescence and merged images are shown. Bar = 20 microns.

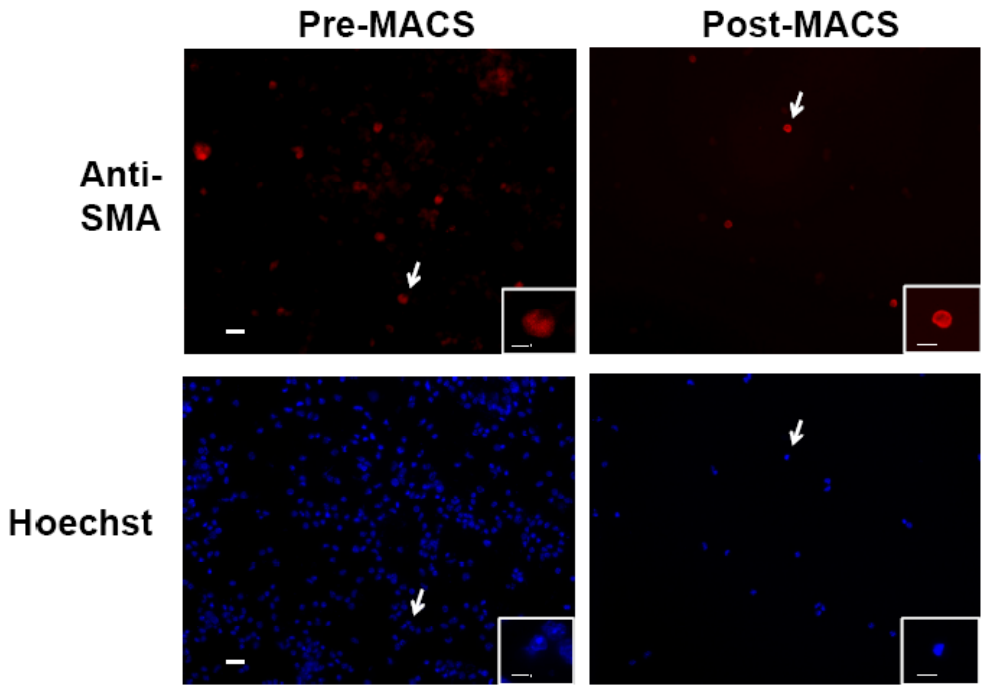


Supplemental Figure 9. Immune cytology analyses for (A) alpha-actinin, (B) smooth muscle actin, and (C) isolectin B4 expression in cells from collagenase-dispersed day 2 post-natal hearts prior to (Pre-MACS) and immediately after (Post-MACS) enrichment for c-kit+ cell populations. Bars = 20 microns (5 microns in inset).

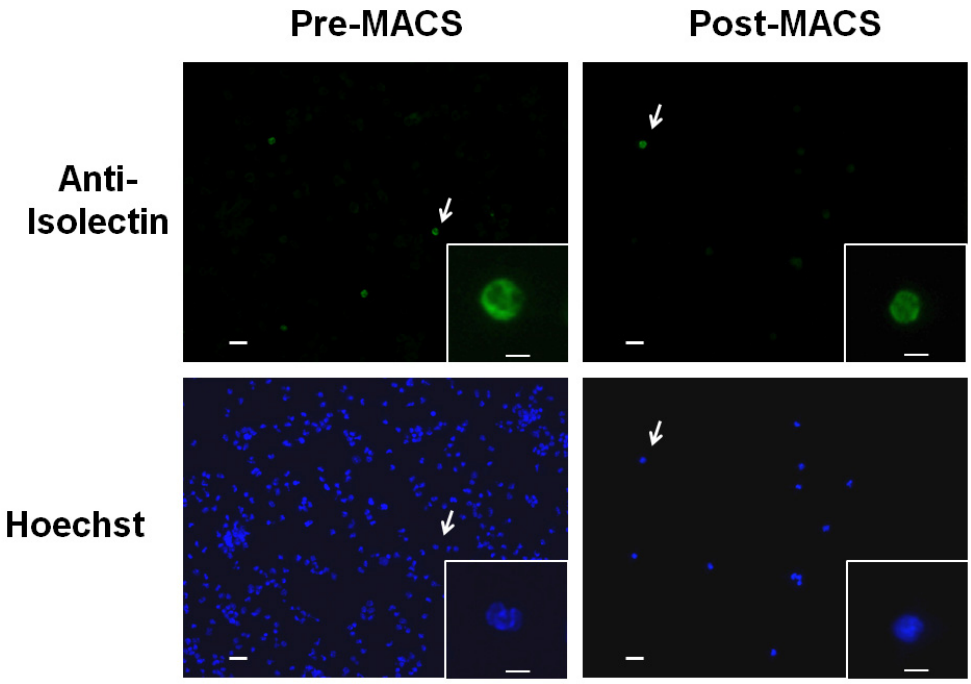
A. Alpha-actinin immune reactivity (rhodamine-conjugated secondary antibody; note the preponderance of Post-MACS cells lack immune reactivity in the two representative fields shown):



B. Smooth Muscle Actin (SMA) immune reactivity (rhodamine-conjugated secondary antibody):

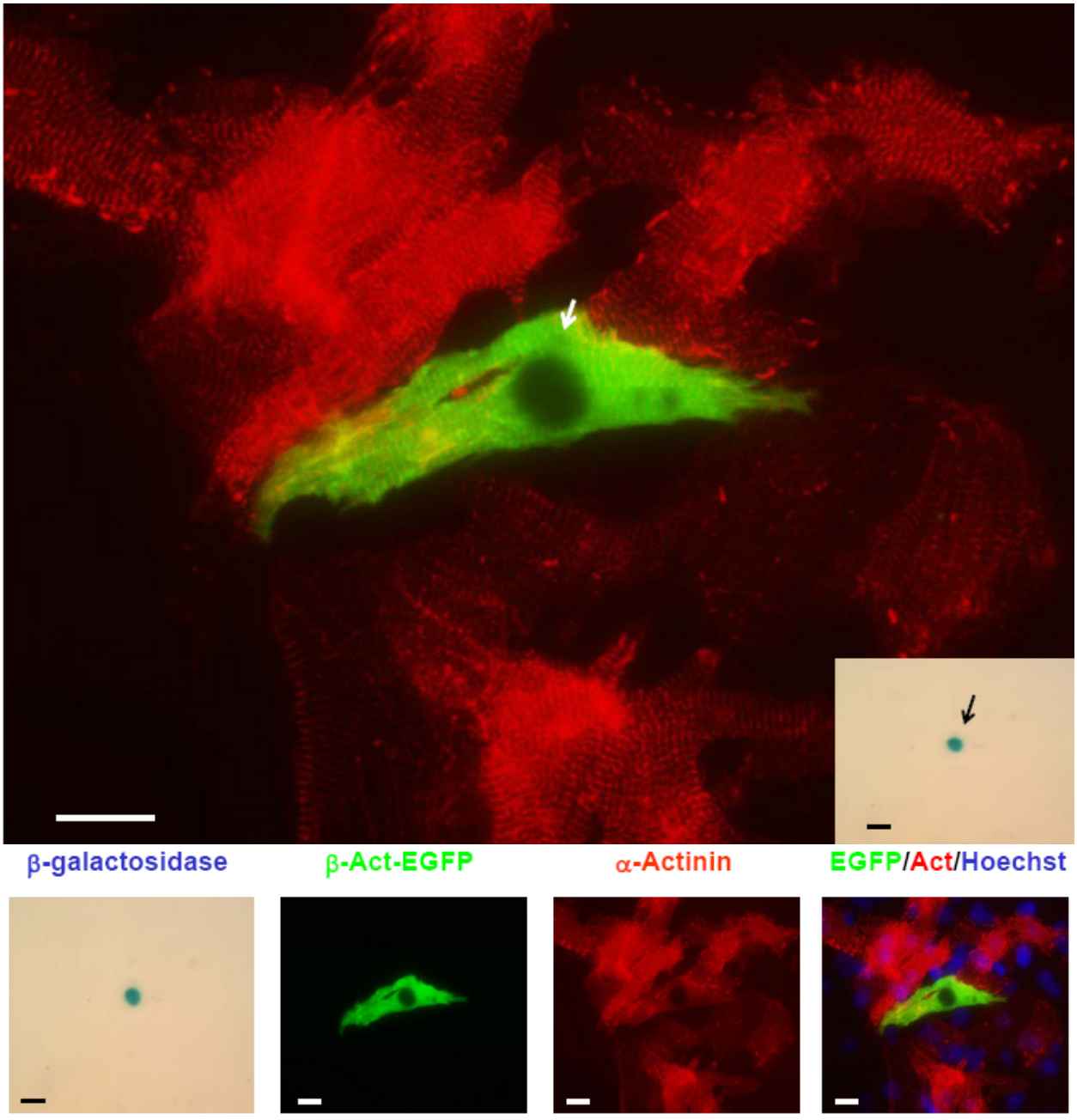


C. Isolectin B4 immune reactivity (FITC-conjugated secondary antibody):

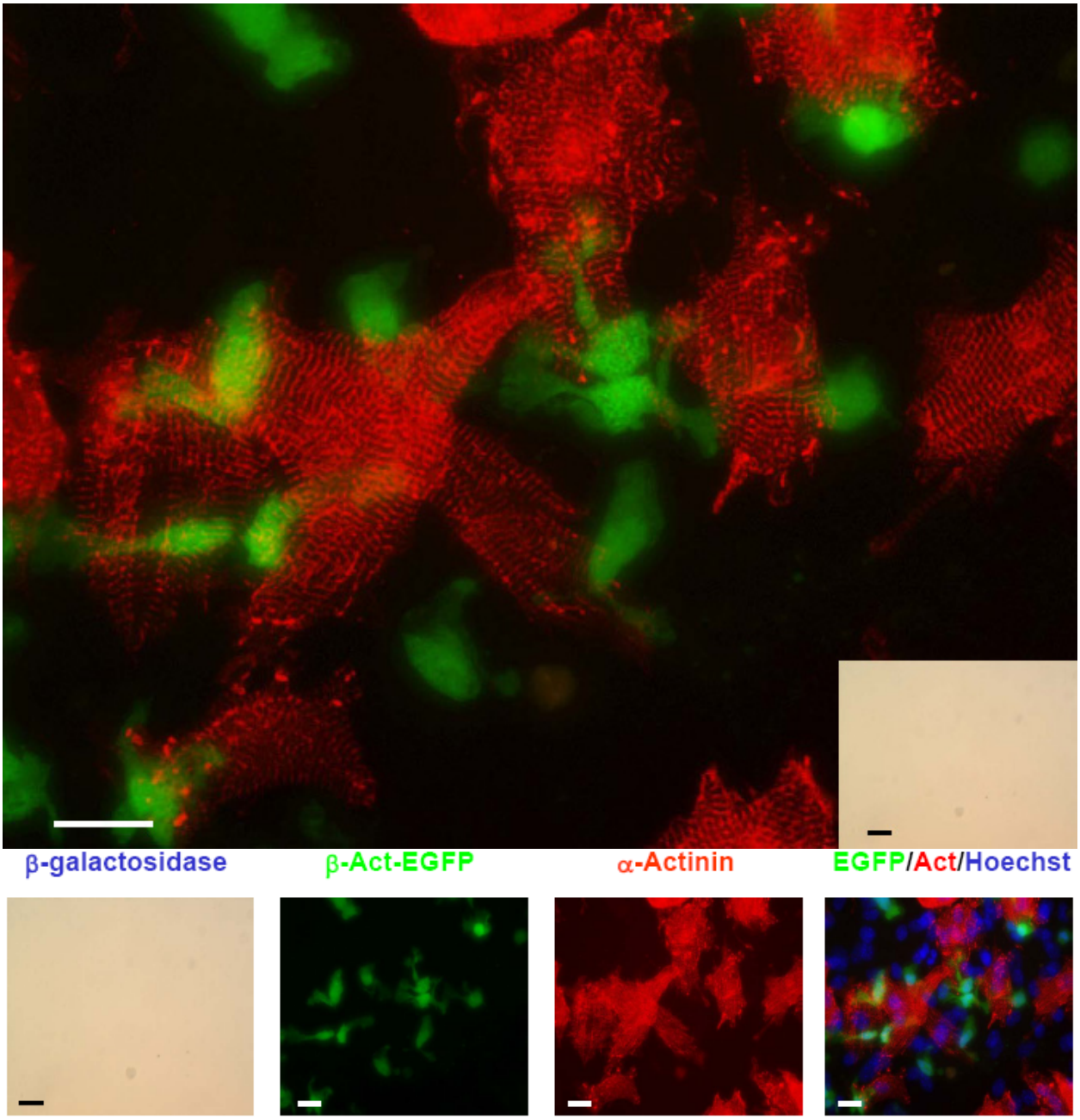


Supplemental Figure 10. Cardiomyogenic potential of c-kit^{+}/CD45^{-} and c-kit^{+}/CD45^{+} sub-populations in the fetal cardiomyocyte co-culture assay. Fetal cardiomyocytes from non-transgenic mice were plated. 24 hours later, FACS was employed to isolate c-kit^{+}/CD45^{-} and c-kit^{+}/CD45^{+} cells from neonatal day 2 ACT-EGFP / MHC-nLAC double transgenic hearts. Alpha actinin immune reactivity and nuclear β-galactosidase activity was used to score cardiomyogenic events after 7 days of co-culture. Bars = 20 microns.

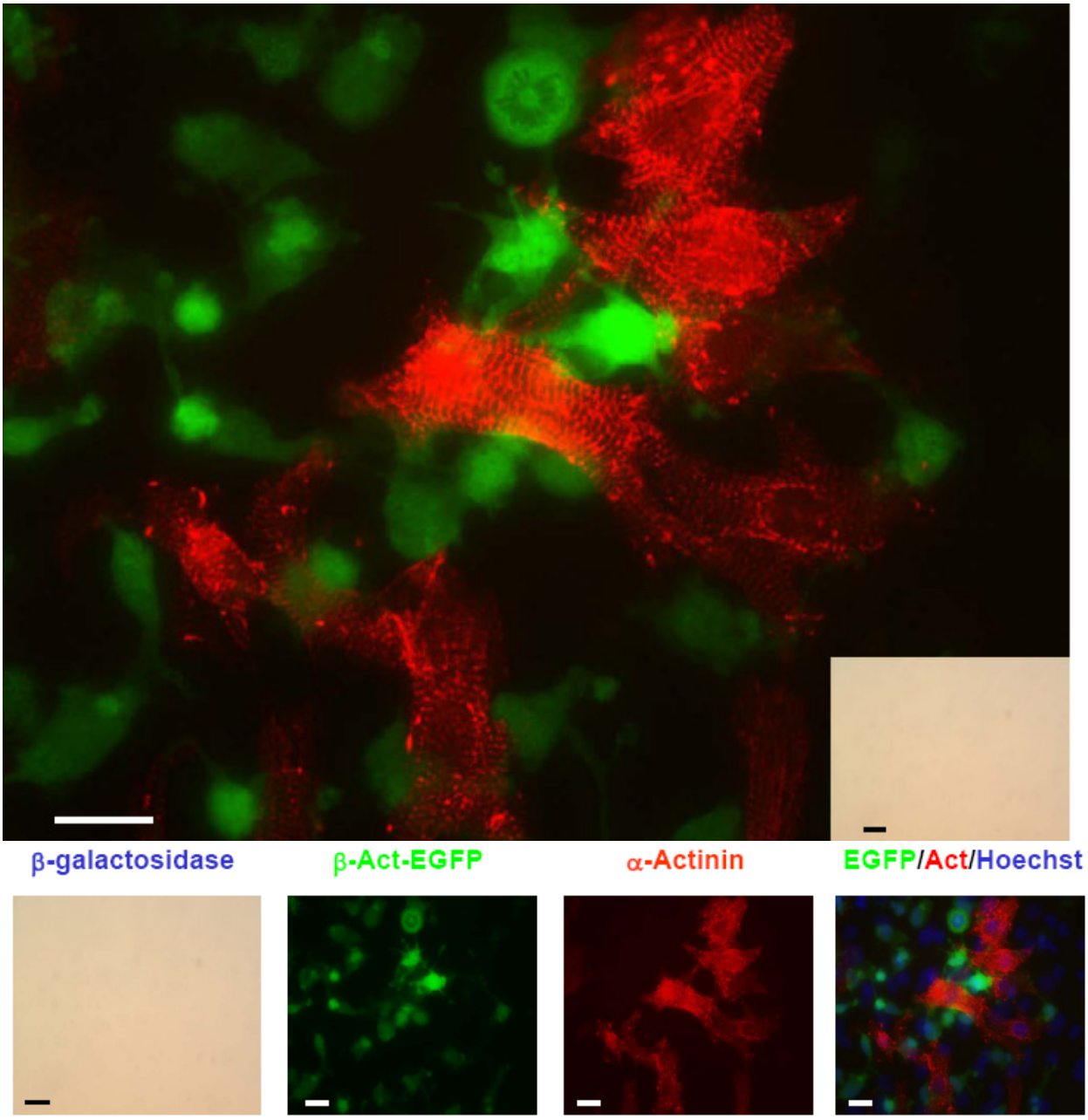
A) Cardiomyogenic potential of the c-kit^{+}/CD45^{-} sub-population. Upper panel shows a merged image, lower panels show the individual channels.



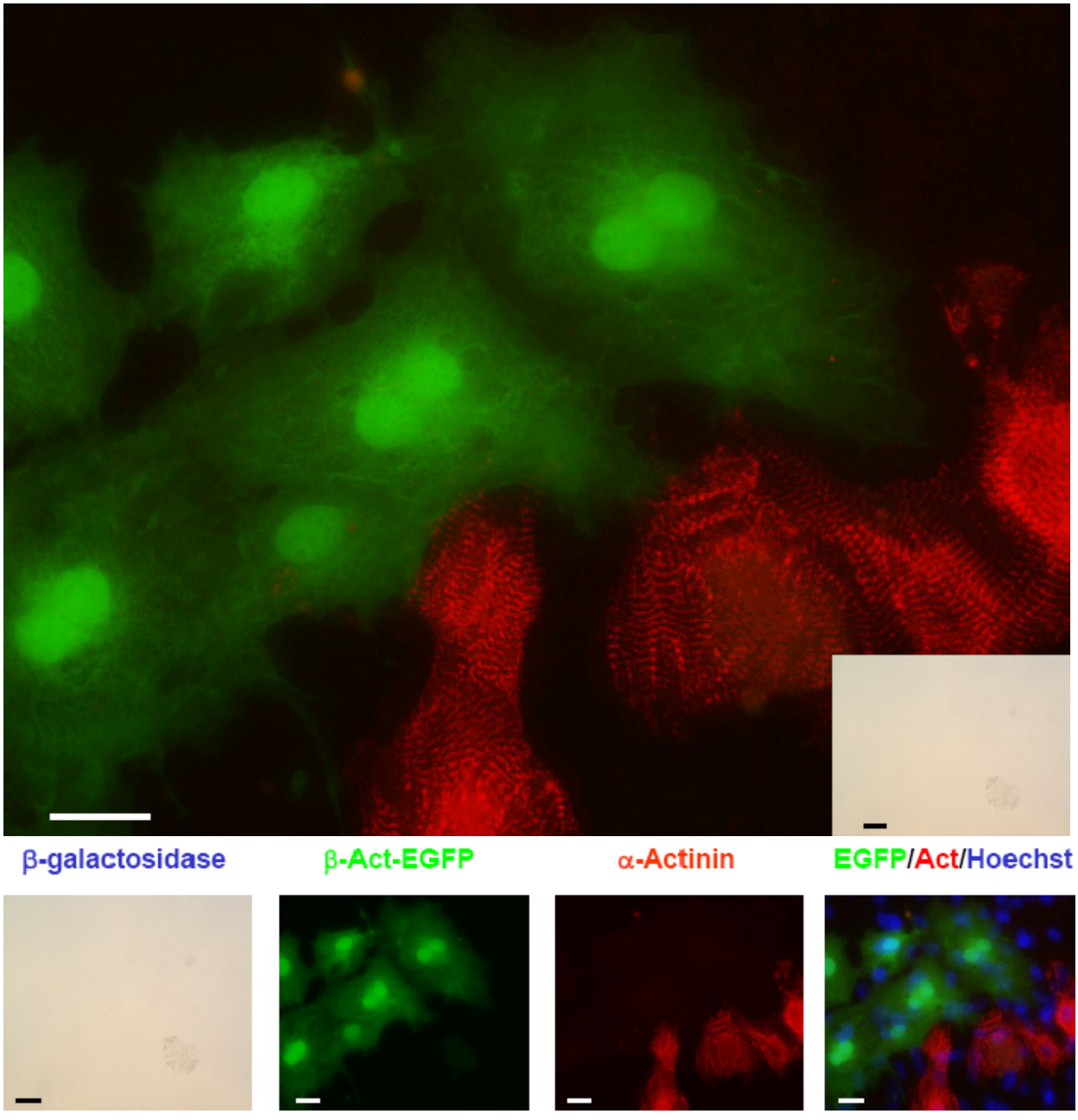
B) Cardiomyogenic potential of the c-kit^{+}/CD45^{+} sub-population. Upper panel shows a merged image, lower panels show the individual channels.



Supplemental Figure 11. Cardiomyogenic potential of c-kit$^+$ cells from neonatal bone marrow. Fetal cardiomyocytes from non-transgenic mice were isolated and plated. 24 hours later, c-kit$^+$ cells isolated from the bone marrow of neonatal day 2 ACT-EGFP / MHC-nLAC double transgenic mice were seeded onto the non-transgenic fetal cardiomyocyte cultures. Alpha actinin immune reactivity and nuclear β-galactosidase activity were used to score cardiomyogenic events after 7 days of co-culture. Upper panel shows a merged image, lower panels show the individual channels. Bars = 20 microns.



Supplemental Figure 12. Cardiomyogenic potential of adult heart-derived c-kit^{+} cells following co-culture with neonatal mouse cardiomyocytes. Neonatal cardiomyocytes from non-transgenic mice were isolated and plated. 24 hours later, c-kit^{+} cells isolated from the heart of adult ACT-EGFP / MHC-nLAC double transgenic mice were seeded onto the non-transgenic neonatal cardiomyocyte cultures. Alpha actinin immune reactivity and nuclear β-galactosidase activity were used to score cardiomyogenic events after 7 days of co-culture. Upper panel shows a merged image, lower panels show the individual channels. Bars = 20 microns. No cardiomyogenic events were observed when more than 3,000 c-kit+ cells were screened.



Supplemental Table 1. Summary of the intra-cardiac transplantation experiments.

	no	days post Tx	MI	cell number	graft	recipient strain
	1	11d	-	10,000	+	[DBA/2J x C57Bl/6J]f1
	2	11d	-	10,000	+	[DBA/2J x C57Bl/6J]f1
	3	21d	-	20,000	-	[DBA/2J x C57Bl/6J]f1
	4	21d	-	20,000	+	[DBA/2J x C57Bl/6J]f1
	5	21d	-	15,000	+	[DBA/2J x C57Bl/6J]f1
	6	21d	-	15,000	-	[DBA/2J x C57Bl/6J]f1
	7	13d	-	20,000	+	[DBA/2J x C57Bl/6J]f1
	8	13d	-	20,000	+	[DBA/2J x C57Bl/6J]f1
	9	10d	-	20,000	-	[DBA/2J x C57Bl/6J]f1
	10	10d	-	20,000	-	[DBA/2J x C57Bl/6J]f1
	11	10d	-	20,000	+	[DBA/2J x C57Bl/6J]f1
	12	10d	-	20,000	+	[DBA/2J x C57Bl/6J]f1
	13	10d	-	10,000	+	NOD/SCID-IL2
	14	10d	-	10,000	+	NOD/SCID-IL2
	15	10d	+	10,000	+	NOD/SCID-IL2
	16	10d	+	10,000	+	NOD/SCID-IL2
	17	10d	+	10,000	+	NOD/SCID-IL2
	18	10d	+	10,000	+	NOD/SCID-IL2
	20	10d	-	10,000	-	NOD/SCID-IL2
sum	20			280,000	**14/20**	

Tx: cell transplantation; MI: myocardial infarction

Supplemental Table 2. Quantification of cells with alpha-actinin, smooth muscle actin (SMA) and isolectin B4 immune reactivity following MACS-enrichment of collagenase-dispersed neonatal day 2 hearts cells for c-kit$^+$ expressing populations

c-kit+		
# Hoechst$^+$	# actinin$^+$	% actinin$^+$
10,012	11	0.11
# Hoechst$^+$	# SMA$^+$	% SMA$^+$
6,494	638	9.82
# Hoechst$^+$	# Isolectin$^+$	% Isolectin$^+$
5,359	11	0.24

Supplemental Table 3: Cardiomyogenic potential of (a) neonatal heart c-kit$^+$/CD45$^-$, (b) neonatal heart c-kit$^+$/CD45$^+$, and (c) neonatal bone marrow c-kit$^+$ cells. Neonatal cardiomyocytes from non-transgenic mice were isolated and plated. 24 hours later, the various c-kit$^+$ cell populations isolated from neonatal ACT-EGFP / MHC-nLAC double transgenic mice were seeded onto the non-transgenic fetal cardiomyocyte cultures. Alpha actinin immune reactivity and nuclear β-galactosidase (nLAC) activity were used to score cardiomyogenic events after 7 days of co-culture.

Neonatal c-kit$^+$ Cell	# EGFP$^+$	# act.$^+$	# nLAC$^+$	% CM-genic
(A) CD45$^-$/c-kit$^+$ Heart	7,790	163	163	2.2
(B) CD45$^+$/c-kit$^+$ Heart	3,234	2	2	0.08
(C) c-kit$^+$ Bone Marrow	>50,000	0	0	0