

Supplementary experimental procedures:

Quantitative RT-PCR: EBs were harvested every 24 hours during differentiation for total RNA isolation. 2mg of total RNA was reverse transcribed using random decamers, and MMLVRT. Relative RNA expression levels were measured using quantitative RT-PCR. Detection of CPC markers was achieved with FAM/ MGB probes (Applied Biosystems): *Nkx2-5* (cat.# Mm00657783_m1), *Tbx5* (cat.# Mm00803521_m1), *Myh6* (Mm00440354_m1), *Tnnt2* (Mm00441922_m1), *Brachyury* (Mm00436877_m1). The instrument used was the Applied Biosystems ABIPrism 7900HT sequence detection system and the software for data collection and analysis was the SDS2.1. An *18S* RNA probe (Hs99999901_s1) was used in order to normalize the data.

Immunocytochemistry: EBs were harvested at the various indicated timepoints, washed with PBS, and fixed with 4% paraformaldehyde in PBS (overnight at 4°C). The EBs were then dehydrated in 30% sucrose/ PBS (overnight at 4°C) and cryopreserved in O.C.T compound (Sakura, cat.# 4583). The frozen EBs were sectioned and the sections were placed on glass slides for the staining. All solutions used contained 1% donkey serum, and Triton X-100. Primary antibodies used were: mouse anti-Actn1 (Sigma, cat.# EA53, 1:400), mouse anti-Tnni3 (US Biological, cat.# T8665-13F, 1:400), goat anti-Nkx2-5 (Santa Cruz, cat.# sc8697, 1:200), goat anti-Gata5 (R&D, cat.# AF2170, 1:200), goat anti-Bmp2/4 (R&D, cat.# AF355, 1:200), goat anti-Myocardin (Santa Cruz, cat.# sc21559, 1:200), mouse anti-GFP (Molecular Probes, cat.# A11120, 1:400), mouse anti-Ki-67 (Zymed, cat.# 18-0192Z, 1:200), mouse anti-Pcna (Santa Cruz, sc56, 1:200), mouse anti-Cd31 (Abcam, cat# 24590, 1:100), rabbit anti-Von Willebrand factor (Abcam, cat# ab6994, 1:100), rabbit anti-Acta2 (Abcam, cat# ab5694, 1:100). The secondary antibodies used were: donkey anti-mouse Alexa 594 (Molecular Probes, cat.#

A21203, 1:500), donkey anti-goat Alexa 488 (Molecular Probes, cat.# 21202, 1:500), donkey anti-goat Alexa 594 (Molecular Probes, cat.# A11058, 1:500), donkey anti-rabbit Alexa 488 or 594 (Molecular probes, 1:500). F-Actin was detected with the toxin phalloidin which is FITC conjugated (Sigma, P5282, 1:50). Nuclei were detected with DAPI, 4',6-diamidino-2-phenylindole, dilactate (Sigma, cat.# D8417). Antibodies were fluorescently conjugated using either the Zenon mouse IgG labeling kit (Molecular probes, cat.# Z25002), or the Zenon rabbit IgG labeling kit (Molecular probes, cat.# Z25302).

Sorted cells were cultured in suspension for 48 hours and then transferred in a tissue culture dish for another 48 hours. At that point the cells were fixed with 4% paraformaldehyde (1 hour, 25°C) and permeabilized with Triton X-100. The same reagents as above were used for staining.

Images were taken using a Nikon Eclipse E800 upright microscope or a Nikon Eclipse TE200 inverted microscope. The digital cameras used were a Photometrics Coolsnap HQ or a Micromax RTE/CCD-1300-Y/HS. The software used was Metamorph 6.2r2 and Adobe Photoshop 7.0.

Semiquantitative RT-PCR: RNA was isolated from FACS sorted cells (GFP⁺ and GFP⁻ cell populations) between days 5 and 8 of differentiation. 1mg of RNA was reverse transcribed using Superscript III first strand, and the PCR products were amplified using Taq DNA polymerase. A low cycle PCR amplification was used in order to ensure that the signal intensity would stay in the linear range. A Biorad Chemi-Doc XRS was used to visualize the bands on the agarose gels. The intensity level of each band was measured and normalized to that of *Gapdh*. Each PCR reaction was repeated four times in order to ensure a significant result. Genes examined were identified to be uniquely upregulated or downregulated in CPC population during

all four time points of differentiation that were analyzed: *Nkx2-5* (NM_008700), *Myocd* (NM_145136), *Sox18* (NM_009236), *Myl4* (NM_001858), *Myl7* (NM_022879), *Tnnc1* (NM_009393), *Tnnt2* (NM_011619), *Cd34* (NM_133654), *Tfpi* (NM_011576), *Kdr* (NM_010612), *Tek* (NM_013690), *Esam* (NM_027102), *T* (NM_009309), *Pou5f1* (NM_013633), *Sox17* (NM_011441).

Supplementary figure and movie legends:

Supplementary figure 1: Culture and differentiation of mouse embryonic stem cells.

(A) Mouse embryonic stem cells are cultured and maintained undifferentiated on a layer of mitotically inactivated primary mouse embryonic fibroblasts in the presence of leukemia inhibitory factor.

(B) Colonies of mESCs on a layer of mouse primary embryonic fibroblasts. Bar 500µm.

(C) Colonies of mESCs after undergoing a feeder layer subtraction step and cultured on gelatin treated plastic.

(D-J) Differentiation of mESCs through embryoid body formation. (Days 1, 2, 3, 4, 5, 6, 8 respectively).

Supplementary figure 2: Cardiac differentiation of mESCs.

Embryoid bodies isolated and stained on differentiation day 21 for cardiac markers Nkx2-5 (A-B), Actn1 (C-D), and Tnni3 (E-F). Scale bar represents 50µm.

Supplementary figure 3: CPC culture of cardiac fibroblasts.

Differentiation day 6 FAC sorted mESC-derived GFP⁺ CPCs were plated on mitotically inactivated primary neonatal cardiac fibroblasts isolated from rat neonates. Cells were plated either in serum free conditions (DMEM/F12, N2, B27) or in 10% fetal bovine serum (DMEM) for 2 weeks. Scale bar represents 100µm.

Supplementary figure 4: Microarray expression analysis of cardiac progenitor cells.

The molecular expression profile of mESC-derived CPCs was examined using microarray expression analysis. EBs were dissociated and FAC sorted between days 5 and 8 of differentiation. RNA was extracted from CPCs (GFP⁺ cells) and GFP⁻ cells. Biotinylated cRNA was hybridized on the Affymetrix murine 430 2.0 array following the two-cycle target labeling sample preparation procedure. Genes with expression levels that are uniquely upregulated or down-regulated 1.5 times in the CPC population when compared to the rest of the cells present in the differentiating embryoid bodies between days 5 and 8 of differentiation.

Supplementary figure 5: Semi-quantitative RT-PCR analysis.

The relative expression levels of genes that were identified to be upregulated or down-regulated uniquely in the CPC population using microarray analysis were further analyzed using semi quantitative RT-PCR analysis. Expression values for each gene were normalized to that of *Gapdh*. Student's t-test was used to calculate whether there was a significant difference between the expression levels of a particular gene in the CPC population and the rest of the cells in the embryoid bodies at the designated time points; **, $p \leq 0.01$.

Supplementary figure 6: *in situ* hybridization

Whole mount *in situ* hybridization analysis for genes identified by microarray expression analysis to be upregulated in the mESC-derived CPCs. Hybridization was done on mouse embryos isolated on embryonic days 7.5, 8.5, and 9.5. Genes analyzed are: *BC054438*, *Dpp4*, and 3110004L20Rik. Black arrowheads indicate the cardiac crescent; H, heart; OFT, outflow tract; UA, umbilical artery.

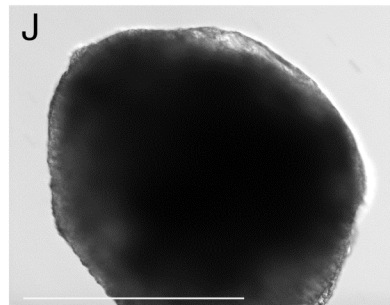
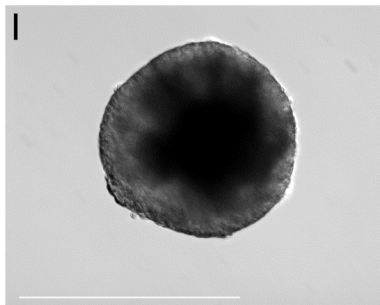
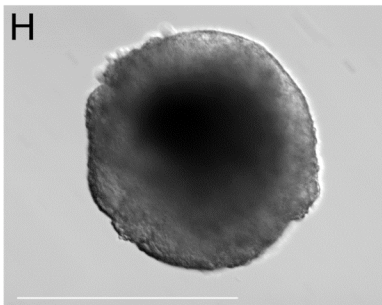
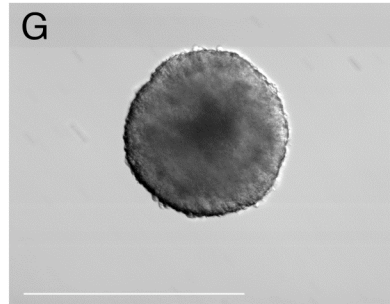
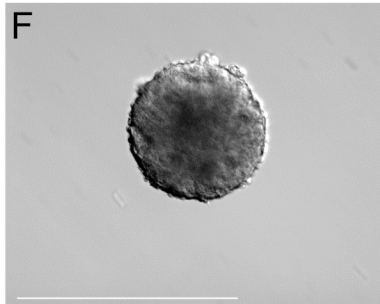
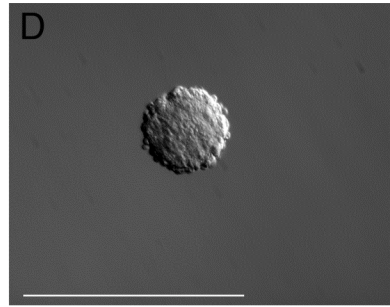
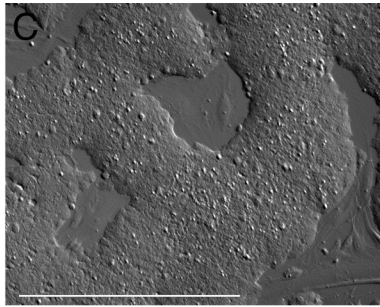
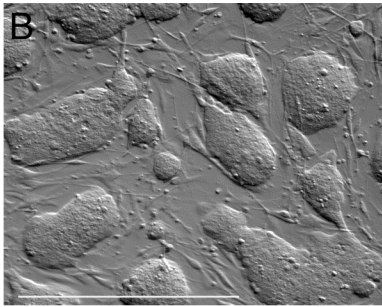
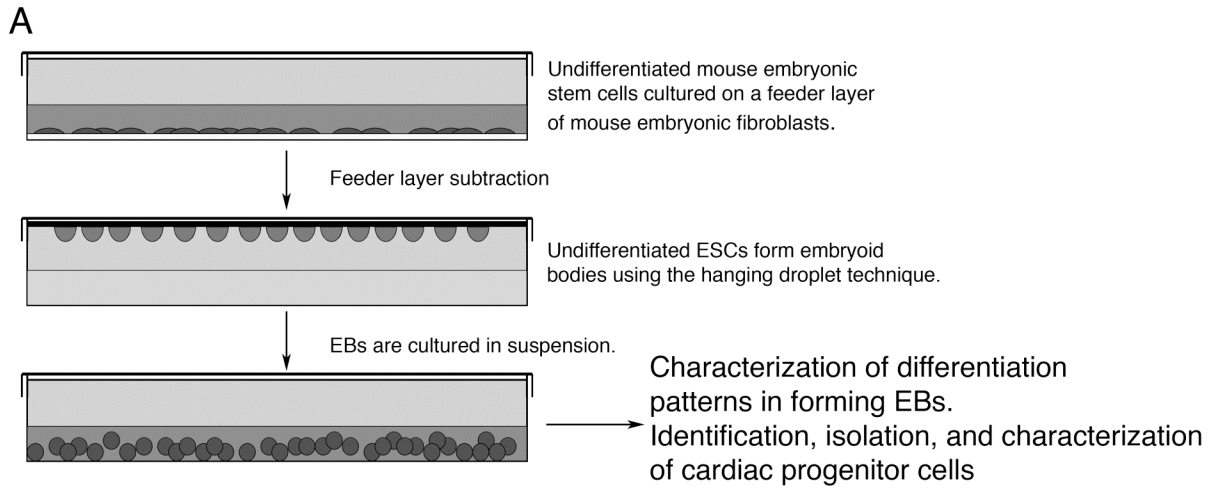
Supplementary movie 1. Spontaneously contracting cardiac areas in a culture of differentiating mESCs.

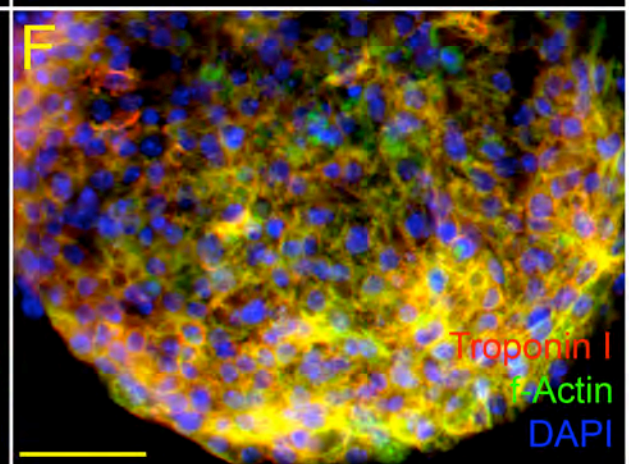
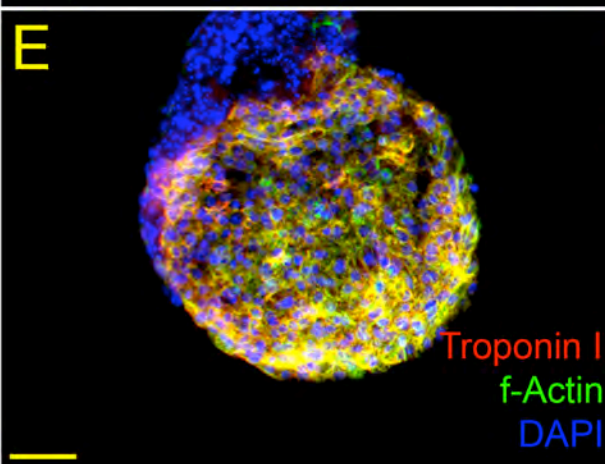
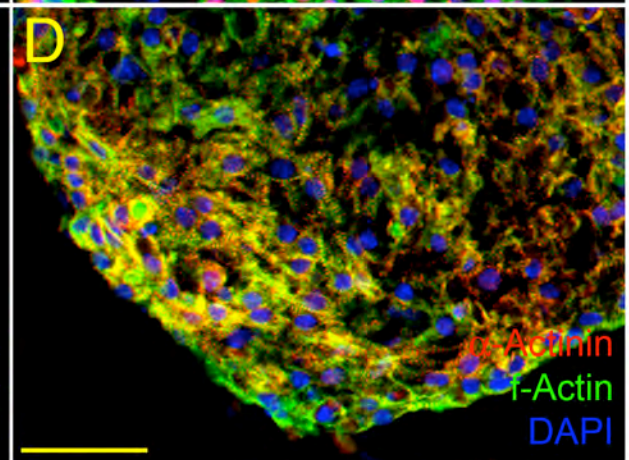
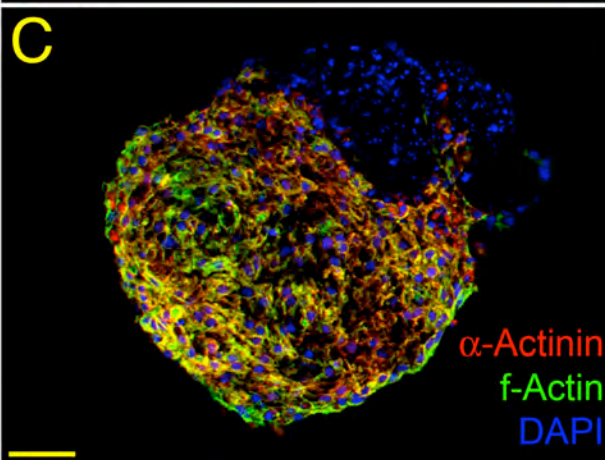
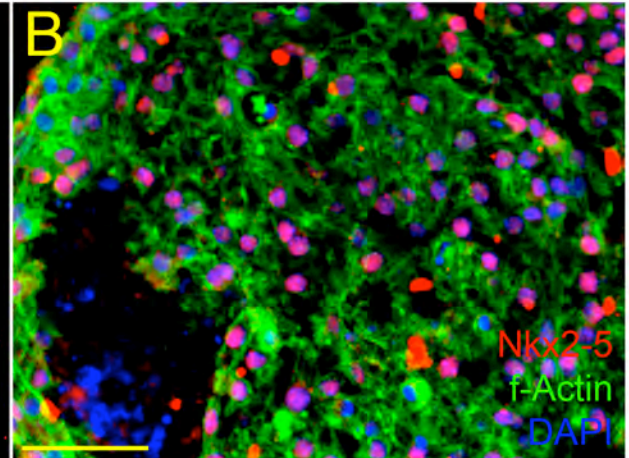
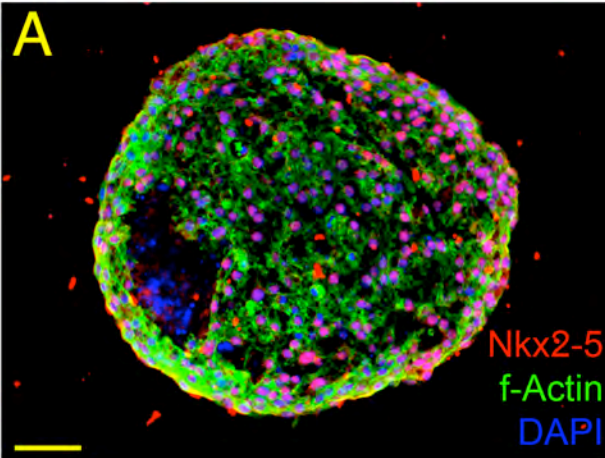
MESCs were differentiated using the hanging droplet technique. The resulting EBs were plated on day 6 of differentiation. The contracting cardiac area shown in this movie was recorded on day 10 of differentiation.

Supplementary movie 2. Spontaneously contracting cardiac areas in a culture of differentiating mESCs express GFP under the control of the *Nkx2-5* enhancer.

Supplementary movie 3. Spontaneously contracting cardiac areas in a culture of differentiating mESCs express GFP under the control of the *Nkx2-5* enhancer.

Supplementary movie 4. FAC sorted GFP⁺ cells continue to express the transgene while spontaneously contracting.

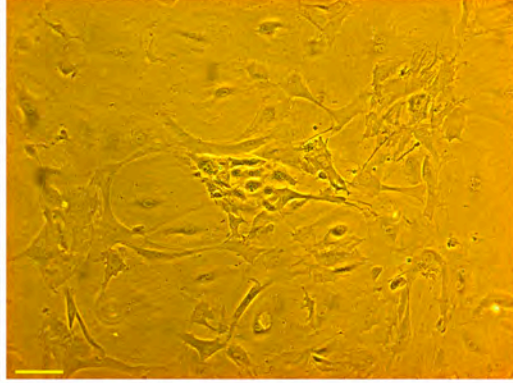
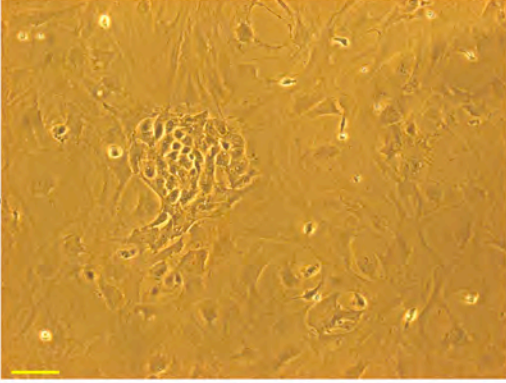




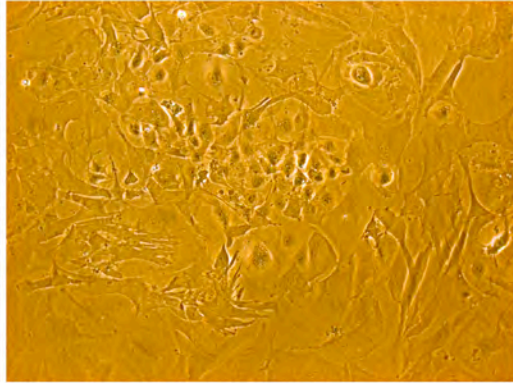
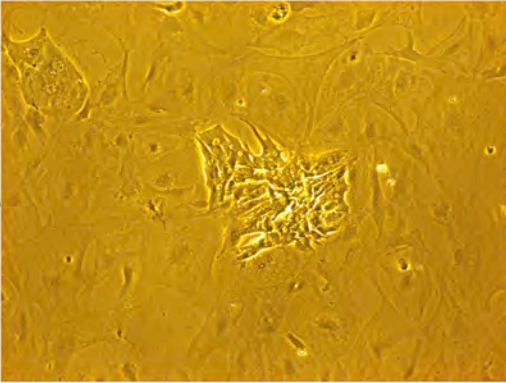
Serum free culture

10% FBS

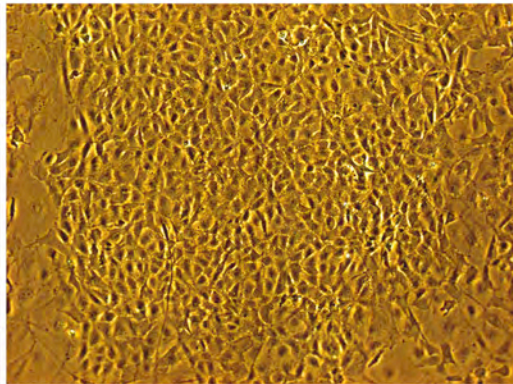
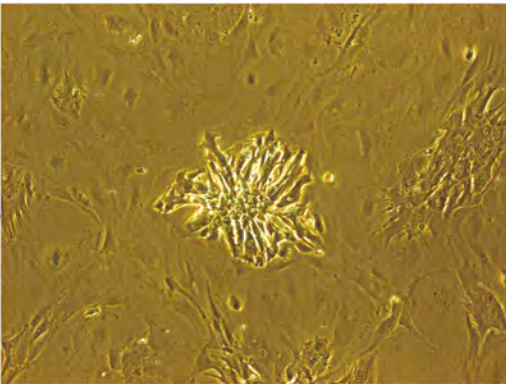
Day 6+3



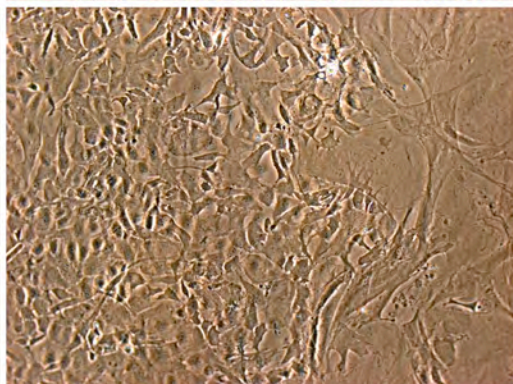
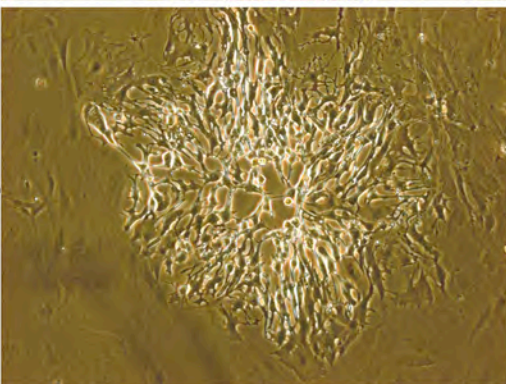
Day 6+7



Day 6+11

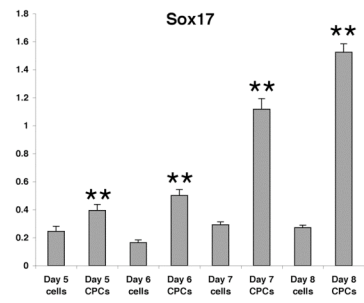
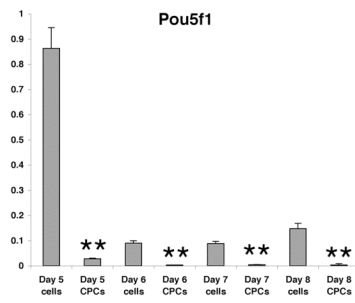
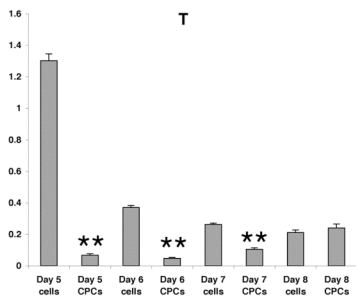
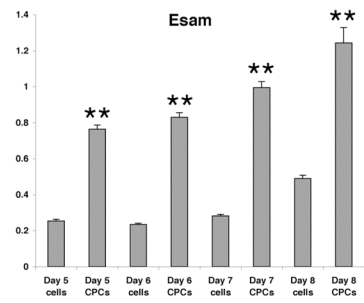
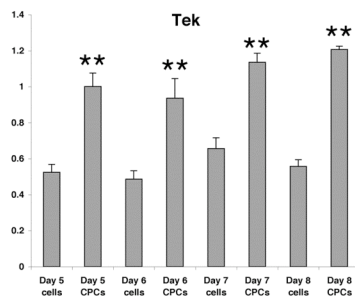
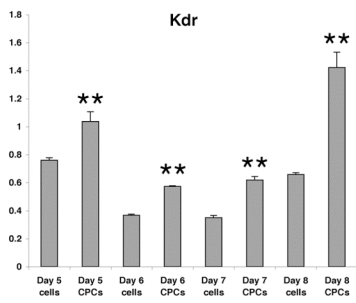
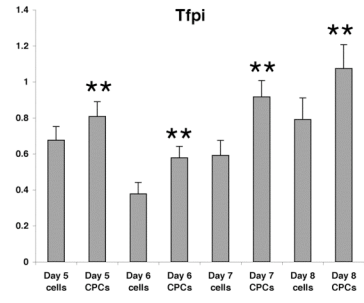
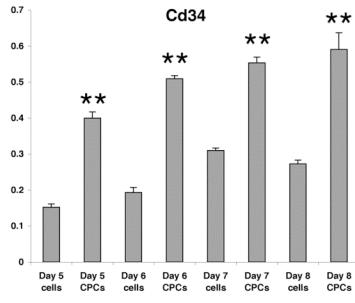
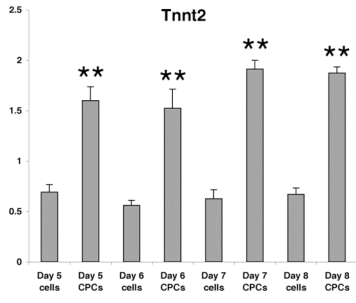
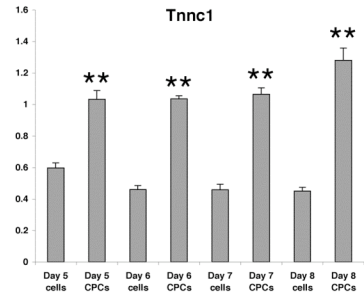
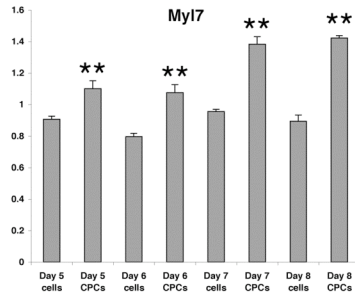
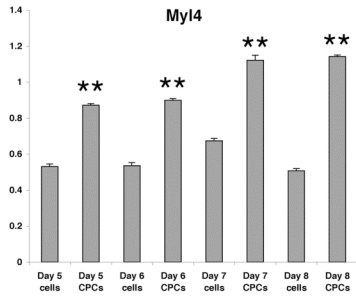
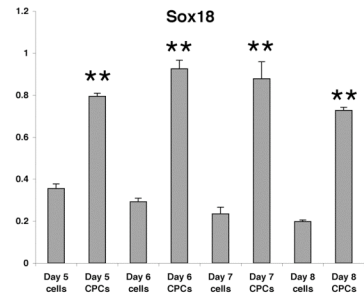
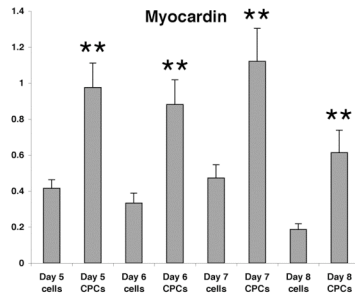
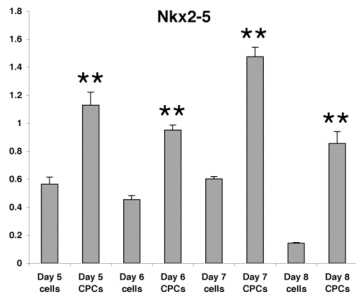


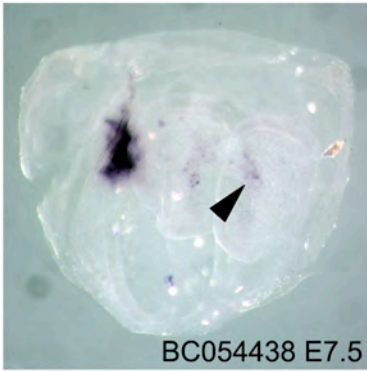
Day 6+14



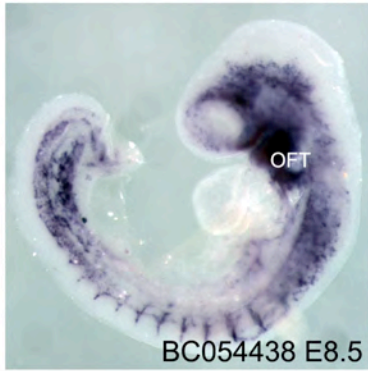
Upregulated genes

UniGene ID	Gene Symbol	Day 5	Day 6	Day 7	Day 8	UniGene ID	Gene Symbol	Day 5	Day 6	Day 7	Day 8	UniGene ID	Gene Symbol	Day 5	Day 6	Day 7	Day 8	UniGene ID	Gene Symbol	Day 5	Day 6	Day 7	Day 8
Mm.277376	Abca1	1.91	2.64	2.28	1.58	Mm.40228	Exoc3l	2.17	2.17	2.78	4.08	Mm.260768	Plvap	3.21	3.02	4.12	6.12						
Mm.686	Actc1	2.41	2.29	1.74	2.97	Mm.271644	Fbn1	3.03	1.88	2.91	5.63	Mm.256712	Ptxnc1	2.74	2.62	2.56	2.31						
Mm.37638	Actn2	2.22	2.93	2.76	3.77	Mm.295740	Fgd5	2.03	2.70	2.68	3.15	Mm.3085	Ptxnc1	2.57	2.51	2.46	3.72						
Mm.24005	Amigo2	1.57	2.31	2.06	2.68	Mm.258908	Flil1	2.13	1.51	2.07	1.91	Mm.259072	Pparg1a	1.59	1.59	2.05	1.77						
Mm.10279	Ankrd1	1.52	1.58	1.86	2.56	Mm.3464	Flt1	2.09	2.86	3.11	5.81	Mm.308126	Ppp1r14c	1.91	2.04	2.71	2.35						
Mm.196330	Ankrd47	1.55	1.68	2.01	2.43	Mm.3291	Flt4	2.10	2.37	2.25	3.47	Mm.3243	Procr	1.77	2.35	3.06	4.34						
Mm.146736	Ap1s2	1.89	2.39	1.78	2.00	Mm.976	Fmo1	1.99	2.05	2.40	2.80	Mm.37213	Ptprb	2.45	3.67	5.02	7.36						
Mm.26743	Apoa1	1.53	3.26	10.43	15.46	Mm.1870	Fmyd5	1.96	1.64	1.52	2.39	Mm.311809	Ptprm	2.28	2.67	3.62	5.14						
Mm.221239	Apob	3.10	2.69	11.21	14.69	Mm.323322	Gaintl4	1.99	1.56	1.79	2.46	Mm.262294	Qk	1.66	2.24	2.03	1.52						
Mm.2161	Apom	1.60	3.74	10.24	11.60	Mm.1222	Gap43	2.12	2.93	3.45	4.39	Mm.358667	Ramp2	2.25	2.91	3.60	3.80						
Mm.361895	Arhgef15	2.03	2.47	2.52	4.74	Mm.252599	Gimap1	2.24	2.70	2.21	3.01	Mm.384029	Rappgef3	1.97	2.52	2.59	7.82						
Mm.104900	Asah2	1.55	1.91	1.90	1.74	Mm.333048	Gimap4	2.55	3.56	4.78	9.37	Mm.227642	Rappgef5	1.95	2.39	2.58	3.97						
Mm.45019	Asah31	2.20	3.30	4.14	7.96	Mm.321047	Gimap6	1.60	1.90	3.35	4.03	Mm.329203	Rasgrp3	1.99	3.20	3.33	3.41						
Mm.27159	Asb2	3.00	3.42	2.76	2.71	Mm.86514	Gimap8	2.66	3.24	3.38	4.63	Mm.181396	Rasip1	2.81	3.04	2.87	4.84						
Mm.4550	At1p1b1	1.50	2.04	3.29	2.67	Mm.24615	Gja4	2.61	2.90	4.22	6.49	Mm.248291	Rassf5	2.70	1.86	1.93	2.54						
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Mm.31915	Cav2	1.92	2.43	4.26	8.13	Mm.275683	Ica1	1.56	1.68	1.82	2.82	Mm.225	She	2.60	3.38	4.42	5.96						
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Mm.29798	Cd34	3.03	1.85	1.98	3.66	Mm.268521	Igf1	2.33	2.60	2.28	2.56	Mm.7444	Sic32a1	2.06	1.82	2.32	2.71						
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Mm.307488	Cdc42ep1	1.87	1.60	1.66	3.27	Mm.282386	Kcnc3	1.88	2.89	6.12	7.17	Mm.42162	Sox7	2.84	3.97	5.18	9.98						
Mm.268397	Cdgap	1.97	1.88	1.69	1.60	Mm.378947	Kdr	1.97	2.17	2.59	1.94	Mm.123110	Spnb2	1.52	1.58	1.67	1.57						
Mm.21767	Cdn5	3.72	3.57	4.59	7.73	Mm.26938	Klf2	1.88	2.52	2.30	2.02	Mm.260838	Siat7a1	2.27	2.32	2.12	1.61						
Mm.247642	Centd3	1.75	2.09	3.59	3.51	Mm.132583	Klf4	1.94	4.14	9.77	14.42	Mm.220821	Stab1	2.05	2.81	3.72	2.22						
Mm.22768	Cldn5	2.57	2.46	2.34	3.78	Mm.25785	Ldb2	1.60	3.22	2.79	2.82	Mm.111904	Stk23	2.31	1.89	2.51	2.15						
Mm.280563	Clec14a	2.12	2.10	2.14	6.78	Mm.29733	Ldb3	3.48	2.76	3.33	5.78	Mm.35789	Synpo2l	2.63	3.08	4.19	4.47						
Mm.295205	Cmya4	2.08	2.57	3.18	3.79	Mm.17185	Lgmn	1.66	2.52	4.75	2.70	Mm.14313	Tek	3.15	2.72	2.79	3.79						
Mm.4352	Col18a1	1.76	1.82	2.10	1.83	Mm.206759	Lrp11	1.93	2.87	2.67	3.33	Mm.124316	Tfpi	2.04	1.95	2.08	1.88						
Mm.738	Col4a1	1.69	2.37	3.19	3.68	Mm.149235	Mfn	1.82	2.33	1.87	2.83	Mm.172346	Tgfbr2	1.60	1.77	2.83	2.77						
Mm.169929	Creb3l2	2.23	1.51	2.15	4.15	Mm.2019	Mirc1	1.63	2.07	4.69	3.84	Mm.330731	Tgm2	1.66	1.57	1.81	1.98						
Mm.2277	Ctsh	1.55	2.90	8.00	9.75	Mm.2941	Mittp	1.54	1.89	5.68	5.81	Mm.24096	Thbd	1.88	2.39	2.82	3.17						
Mm.313915	Cubn	1.87	1.72	2.91	4.82	Mm.149438	Mtus1	1.51	2.82	2.09	2.42	Mm.32067	Thsd11	2.76	2.03	2.26	3.22						
Mm.240830	Dab2	1.58	2.71	4.17	3.29	Mm.10728	Mybpc3	2.73	2.45	3.67	4.88	Mm.124100	Tiam1	1.89	2.26	2.14	3.17						
Mm.62982	Dcblid1	1.52	1.58	2.70	4.04	Mm.290003	Myh6	3.83	3.28	4.24	6.24	Mm.4345	Tie1	3.25	2.39	2.79	4.00						
Mm.29362	Diras2	1.95	4.28	6.63	10.16	Mm.319176	Myh7	3.29	4.05	5.16	6.71	Mm.212428	Tmem100	1.61	2.53	5.12	7.44						
Mm.1151	Dpp4	1.94	4.27	10.14	14.14	Mm.1529	Myi2	3.22	5.46	7.02	9.51	Mm.329776	Tmem2	1.69	2.11	1.99	2.88						
Mm.4729	Dusp2	1.77	1.75	1.72	3.55	Mm.7353	Myi3	2.49	2.63	3.43	3.91	Mm.255332	Tnfrap2	2.26	1.87	2.22	3.50						
Mm.220982	Dysf	2.29	2.46	2.42	3.62	Mm.247636	Myi4	2.20	2.45	2.60	3.25	Mm.712	Tnnc1	1.88	2.37	3.04	4.24						
Mm.215971	Ebf1	1.65	2.08	2.24	2.18	Mm.46514	Myi7	2.09	2.02	2.32	3.63	Mm.247470	Tnnt2	2.57	3.69	3.70	3.38						
Mm.125580	Edi13	2.46	2.02	2.29	3.63	Mm.261329	Myc2b	1.58	2.54	2.46	2.01	Mm.274159	Tspan18	1.80	1.78	1.67	1.64						
Mm.229532	Ednrbb	1.76	1.73	1.92	1.81	Mm.32257	Myocd	2.38	2.25	3.03	4.15	Mm.373672	Ttn	4.07	3.61	6.02	8.78						
Mm.268933	Egfr7	1.76	1.87	1.51	3.20	Mm.120298	Nebl	2.55	2.60	2.91	4.69	Mm.2108	Trt	1.54	3.74	5.61	6.48						
Mm.4454	Elk3	2.50	2.73	2.17	2.24	Mm.329560	Nfatc1	2.05	1.82	2.38	3.37	Mm.34874	Ushbp1	1.96	1.93	2.87	5.56						
Mm.317261	Eltf1	3.01	2.51	2.88	7.07	Mm.57043	Nfkbe1	1.55	1.60	1.51	2.06	Mm.46628	Veze1	2.13	1.84	2.87	3.83						
Mm.27343	Emcn	1.99	2.14	3.68	6.27	Mm.4691	Nid1	1.82	2.30	2.16	1.58	Mm.3929	Zfx1a	1.67	1.67	2.21	1.68						
Mm.286375	Emilin1	2.23	1.59	1.64	1.86	Mm.41974	Nkx2-5	1.90	1.67	2.33	3.42	Mm.331840	Zfp69	1.66	2.00	2.37	4.44						
Mm.241073	Enc1	1.88	1.55	1.59	1.94	Mm.258415	Nos3	2.40	3.70	4.41	6.47	Mm.331088	111003pE23Rik	2.45	2.20	2.68	4.07						
Mm.225297	Eng	2.39	2.16	2.36	2.77	Mm.290610	Notch1	2.29	2.52	3.01	3.70	Mm.23932	3110004L20Rik	1.54	1.69	2.30	3.33						
Mm.164531	Erg	3.00	3.23	3.91	6.41	Mm.24887	Npl	2.31	2.52	2.32	1.52	Mm.318710	3632451O06Rik	1.78	2.18	2.78	2.12						
Mm.41751	Esam1	3.24	3.05	3.58	3.40	Mm.12862	Pcdh12	2.71	3.60	4.29	7.31	Mm.68617	4631423F02Rik	1.84	1.75	2.02	2.52						
Mm.89989	Esrng	1.59	2.20	2.66	3.21	Mm.348053	Pcdhb21	1.99	2.01	1.62	1.89	Mm.334569	4732435N03Rik	1.69	2.04	1.75	1.73						
Mm.292415	Ets1	2.43	1.83	1.57	2.67	Mm.247564	Pde2a	1.59	2.14														





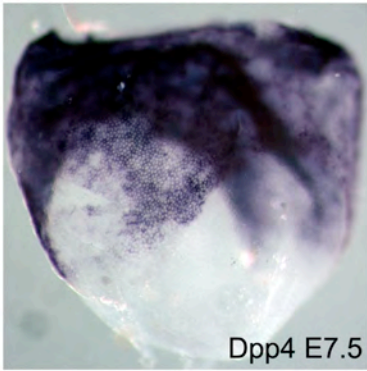
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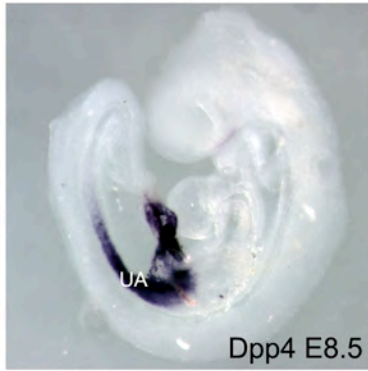
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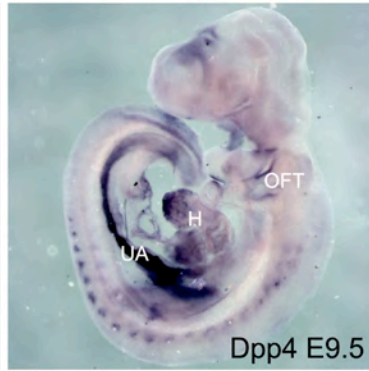
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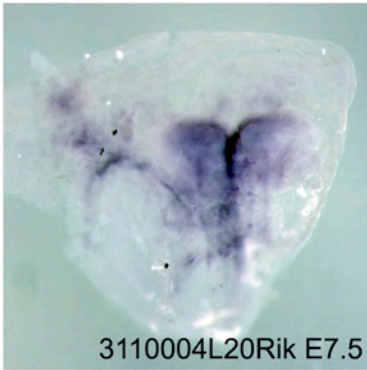
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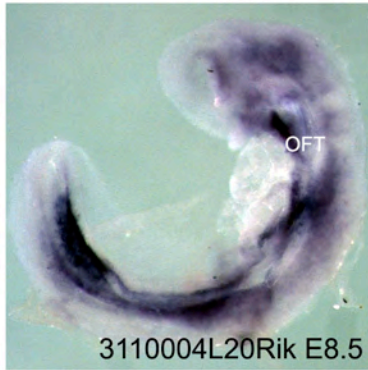
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