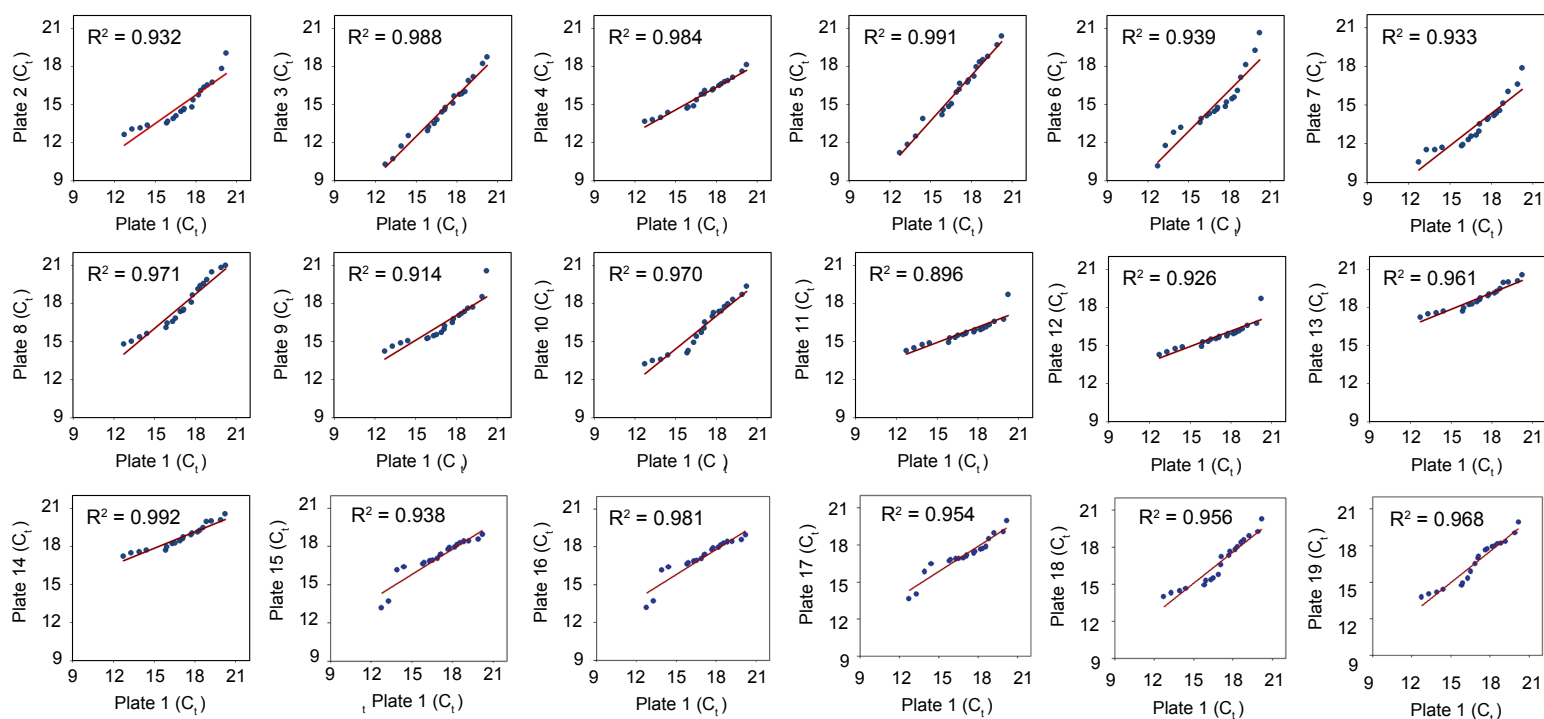


**Figure S1 - Relative expression of lineage markers in single ES cell and cardiomyocyte by real-time quantitative PCR analysis.** Single ES cell (red) and cardiomyocyte (blue) were lysed and reverse transcribed into cDNA and then PCR amplified using primers for the indicated ES cell (Pou5f1) and cardiomyocyte (Nkx2-5, Tnni3) genes. GAPDH expression in each single cell served as a control for cell quality and RNA input. Data represents two independent experiments performed in three replicates.

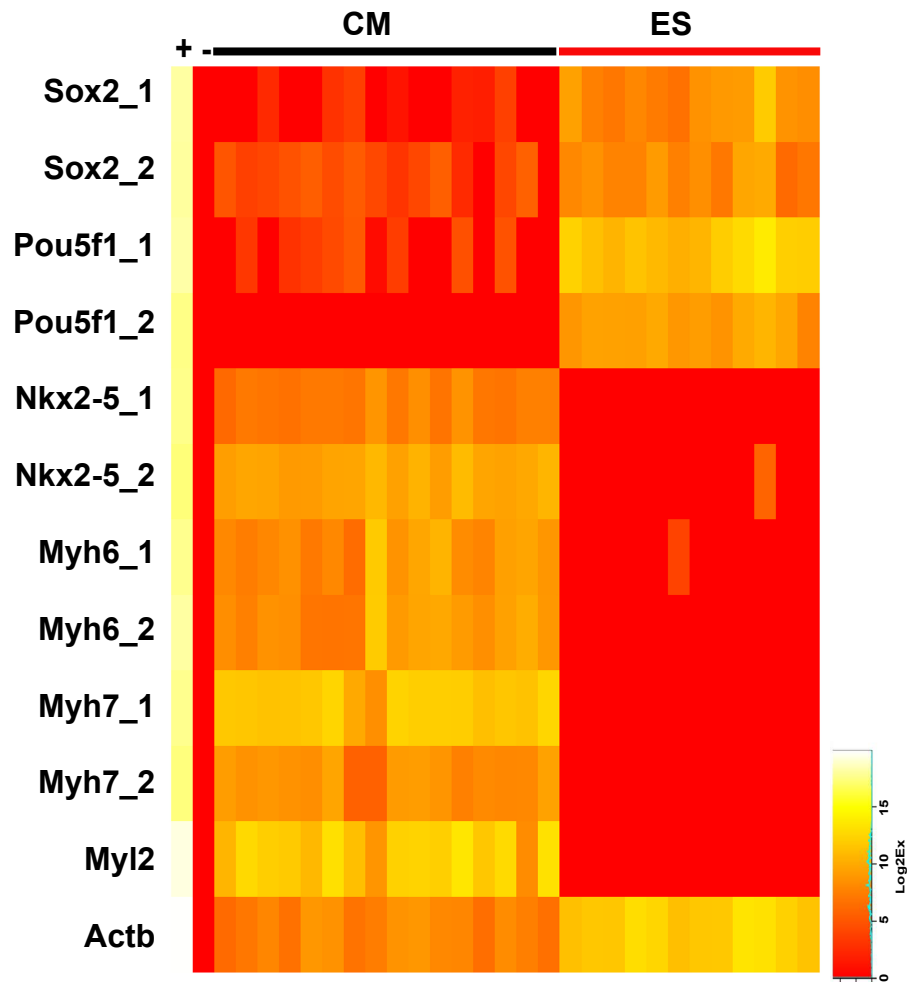
A



B

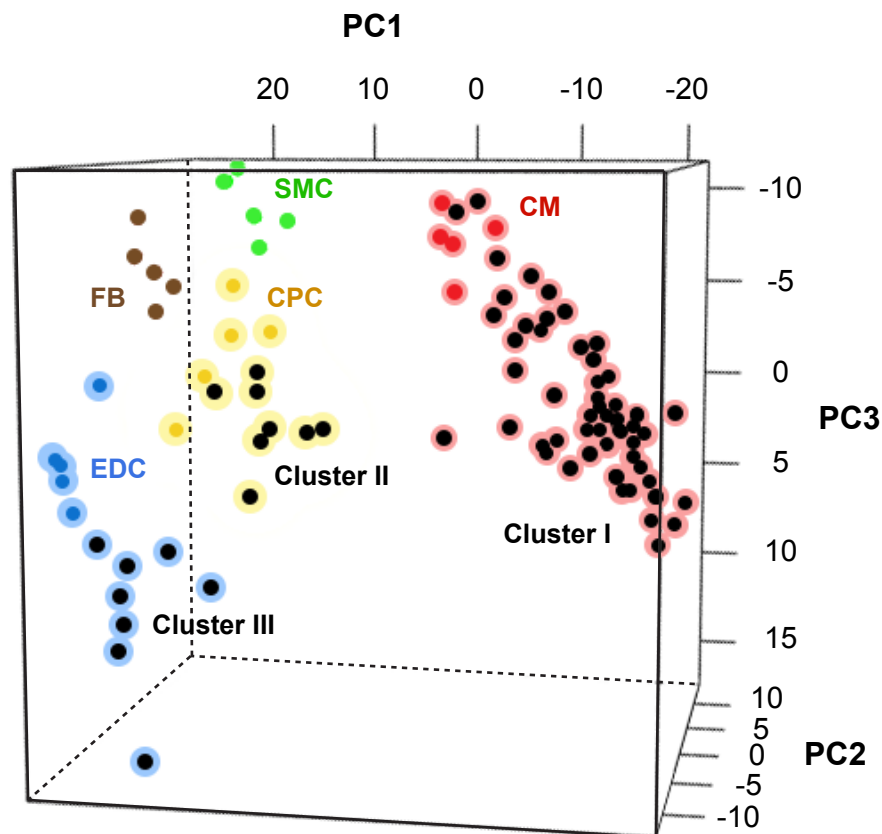
| Plate | 1 | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    | 13    | 14    | 15    | 16    | 17    | 18    | 19    |   |
|-------|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---|
| 1     | 1 | 0.932 | 0.988 | 0.984 | 0.991 | 0.939 | 0.933 | 0.971 | 0.914 | 0.970 | 0.896 | 0.926 | 0.961 | 0.992 | 0.938 | 0.981 | 0.954 | 0.956 | 0.968 |   |
| 2     |   | 1     | 0.962 | 0.966 | 0.953 | 0.967 | 0.990 | 0.972 | 0.990 | 0.959 | 0.958 | 0.953 | 0.979 | 0.951 | 0.82  | 0.941 | 0.9   | 0.973 | 0.95  |   |
| 3     |   |       | 1     | 0.990 | 0.994 | 0.968 | 0.965 | 0.982 | 0.948 | 0.979 | 0.927 | 0.946 | 0.980 | 0.995 | 0.927 | 0.978 | 0.964 | 0.972 | 0.974 |   |
| 4     |   |       |       | 1     | 0.992 | 0.948 | 0.966 | 0.984 | 0.951 | 0.991 | 0.929 | 0.956 | 0.982 | 0.990 | 0.895 | 0.975 | 0.93  | 0.984 | 0.991 |   |
| 5     |   |       |       |       | 1     | 0.950 | 0.953 | 0.979 | 0.937 | 0.980 | 0.914 | 0.946 | 0.976 | 0.994 | 0.93  | 0.983 | 0.954 | 0.974 | 0.978 |   |
| 6     |   |       |       |       |       | 1     | 0.975 | 0.941 | 0.964 | 0.924 | 0.958 | 0.901 | 0.957 | 0.955 | 0.877 | 0.919 | 0.961 | 0.934 | 0.912 |   |
| 7     |   |       |       |       |       |       | 1     | 0.969 | 0.984 | 0.964 | 0.957 | 0.962 | 0.984 | 0.956 | 0.828 | 0.94  | 0.909 | 0.977 | 0.952 |   |
| 8     |   |       |       |       |       |       |       | 1     | 0.945 | 0.986 | 0.901 | 0.975 | 0.988 | 0.981 | 0.876 | 0.988 | 0.914 | 0.986 | 0.974 |   |
| 9     |   |       |       |       |       |       |       |       | 1     | 0.942 | 0.982 | 0.937 | 0.962 | 0.932 | 0.817 | 0.918 | 0.9   | 0.962 | 0.939 |   |
| 10    |   |       |       |       |       |       |       |       |       | 1     | 0.907 | 0.977 | 0.986 | 0.980 | 0.873 | 0.979 | 0.899 | 0.991 | 0.995 |   |
| 11    |   |       |       |       |       |       |       |       |       |       | 1     | 0.877 | 0.932 | 0.914 | 0.812 | 0.874 | 0.906 | 0.923 | 0.909 |   |
| 12    |   |       |       |       |       |       |       |       |       |       |       | 1     | 0.970 | 0.940 | 0.83  | 0.968 | 0.86  | 0.987 | 0.963 |   |
| 13    |   |       |       |       |       |       |       |       |       |       |       |       | 1     | 0.978 | 0.862 | 0.973 | 0.917 | 0.989 | 0.973 |   |
| 14    |   |       |       |       |       |       |       |       |       |       |       |       |       | 1     | 0.921 | 0.98  | 0.956 | 0.97  | 0.973 |   |
| 15    |   |       |       |       |       |       |       |       |       |       |       |       |       |       | 1     | 0.914 | 0.965 | 0.858 | 0.874 |   |
| 16    |   |       |       |       |       |       |       |       |       |       |       |       |       |       |       | 1     | 0.926 | 0.976 | 0.968 |   |
| 17    |   |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       | 1     | 0.898 | 0.892 |   |
| 18    |   |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       | 1     | 0.984 |   |
| 19    |   |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       | 1 |

**Figure S2 - Quality control of single cell expression data among different Fluidigm® arrays.** (A) Quantile-Quantile plots of the housekeeping gene Beta-Actin between plate 1 and the other 18 plates. (B) A table of Pearson correlation coefficient for Beta-actin expression among the nineteen plates.



**Figure S3 - Confirmation of single ES cell and cardiomyocyte gene expression using independent primers.** Single ES cells (ES) or embryo derived cardiomyocytes (CM) were isolated and reverse transcribed into cDNA and amplified on the Fluidigm platform using primers targeting ES cell genes (Sox2 and Pou5f1) and cardiomyocytes genes (Nkx2-5, Myh6, Myh7). Note the selective expression of Sox2 and Pou5f1 in single ES cells and Nkx2-5, Myh6, Myh7 in cardiomyocytes. Data represents the results from two independent experiments.

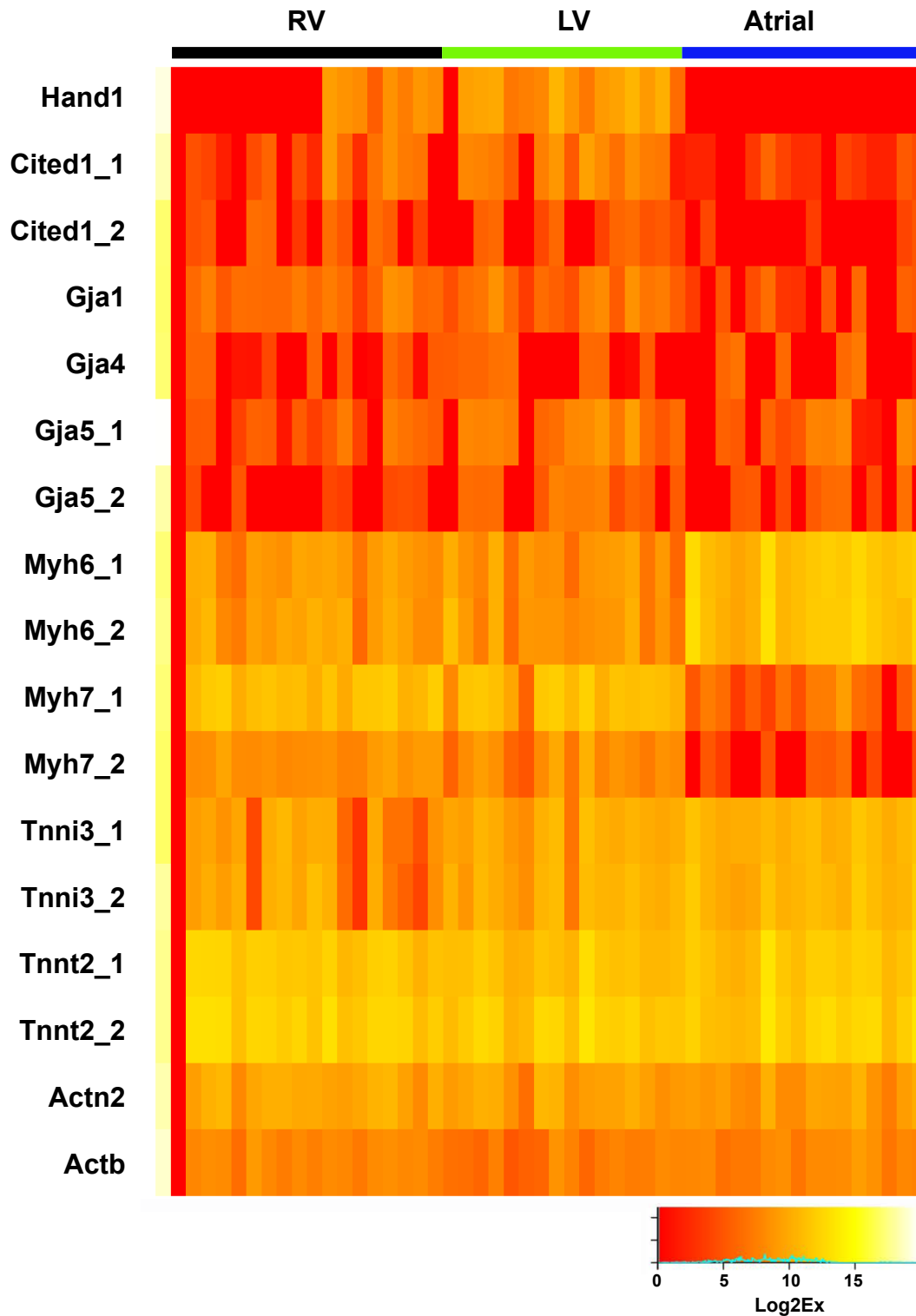
A



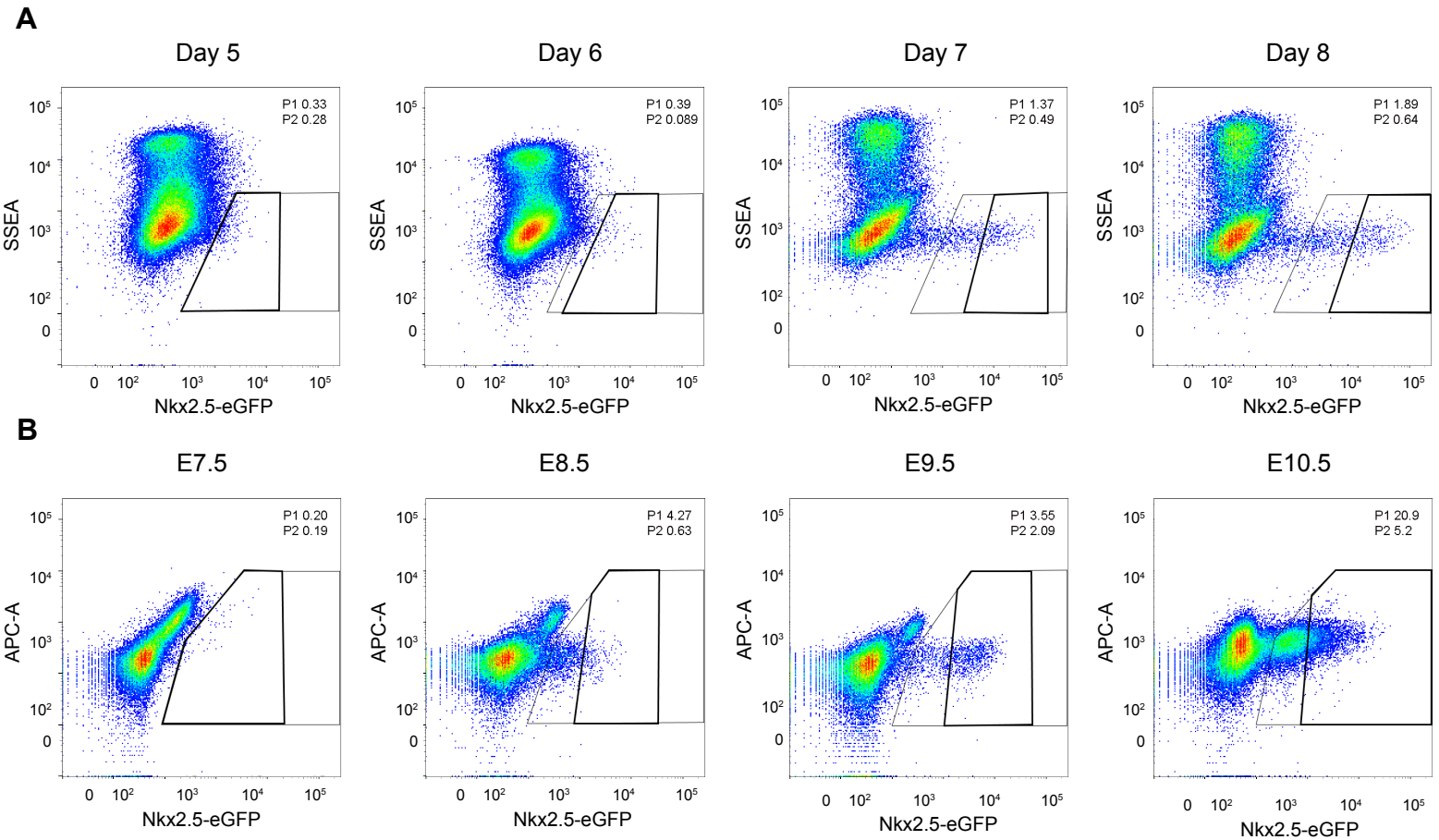
B

|             | Cluster I | CM    | Cluster II | CPC    | SMC    | Cluster III | EDC    | FB    |
|-------------|-----------|-------|------------|--------|--------|-------------|--------|-------|
| Cluster I   | 0.839     | 0.737 | 0.297      | 0.024  | 0.147  | -0.028      | -0.119 | 0.156 |
| CM          |           | 0.928 | 0.098      | -0.109 | -0.116 | -0.127      | -0.181 | 0.016 |
| Cluster II  |           |       | 0.844      | 0.576  | 0.512  | 0.531       | 0.428  | 0.676 |
| CPC         |           |       |            | 0.868  | 0.544  | 0.436       | 0.435  | 0.632 |
| SMC         |           |       |            |        | 0.913  | 0.325       | 0.372  | 0.637 |
| Cluster III |           |       |            |        |        | 0.785       | 0.786  | 0.584 |
| EDC         |           |       |            |        |        |             | 0.941  | 0.617 |
| FB          |           |       |            |        |        |             |        | 0.895 |

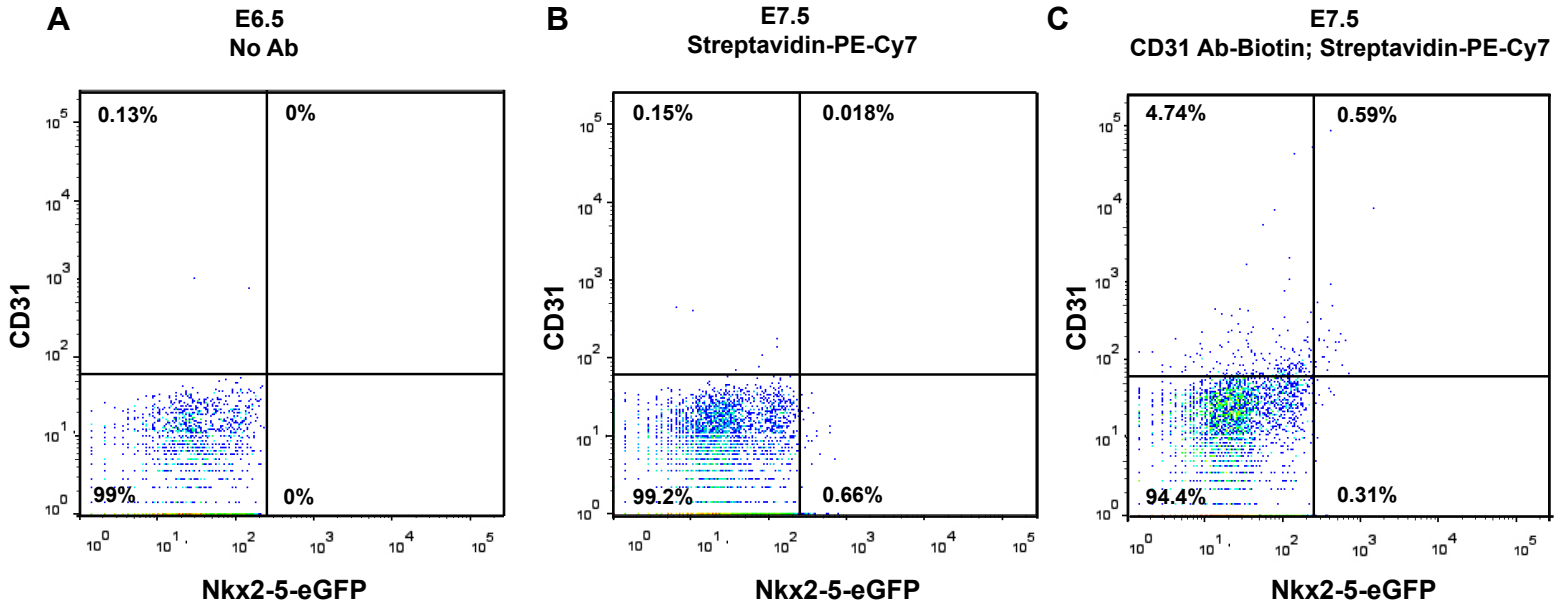
**Figure S4 - Bioinformatic analysis of single cells from day 10.5 embryonic mouse heart.** (A) A three-dimensional PCA plot of all single cells derived from a day 10.5 embryonic mouse heart (black dot) along with standard cardiomyocytes (CM) (red dot), smooth muscle cells (SMC) (green dot), cardiac progenitor cells (CPC) (yellow dot), fibroblasts (FB) (brown dot), and endothelial cells (EDC) (blue dot). Note that cells in Cluster I associates closest with CM and Cluster III with EDC. Cluster II appears to sit between CPC and CM. (B) A table of Pearson correlation coefficients of gene expression between each cell cluster in day 10.5 embryonic heart and standard cells. Data represents combined results from two independent experiments.



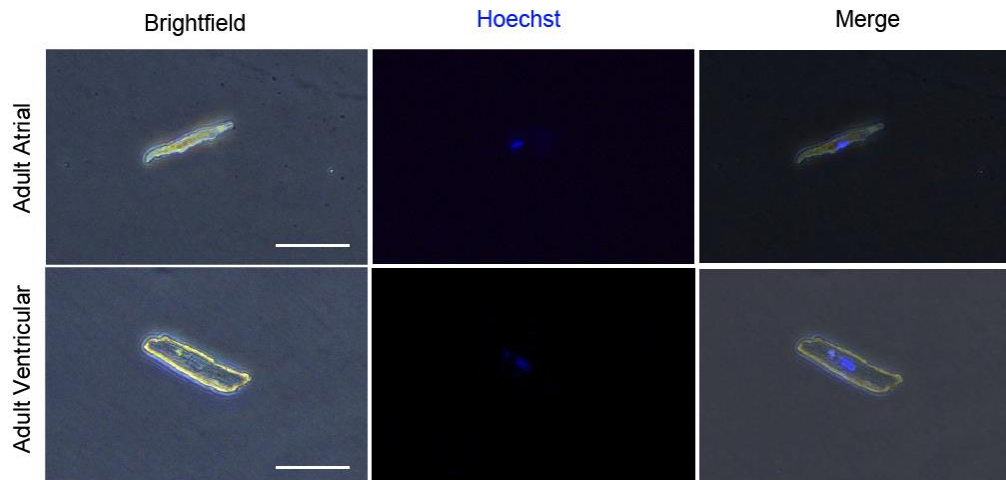
**Figure S5 - Analysis of chamber-specific cardiomyocyte gene expression by single cell Fluidigm assays.** Atrial, right, and left ventricular cardiomyocytes from e10.5 embryonic hearts were isolated as single cells and the mRNA in cell lysate was reverse transcribed using primers targeting the indicated genes. The heatmap shown represents expression of each indicated gene following amplification on the Fluidigm platform. Data represents the results from two independent experiments.



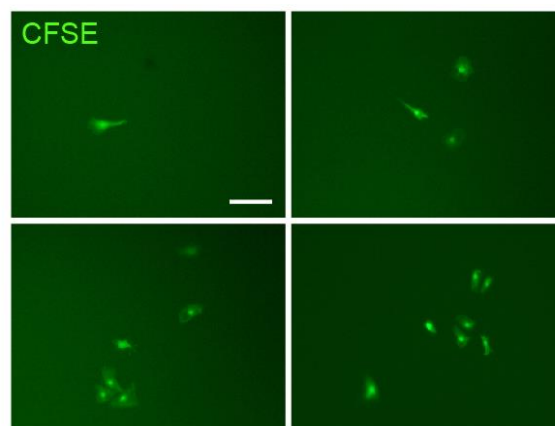
**Figure S6 - Representative FACS plots used to isolate single eGFP+ cells from in vitro differentiated Nkx2-5-eGFP ES cells and transgenic embryos.** Gate 1 (P1) represents fixed gates for the analysis of the percentage of all eGFP+ cells and Gate 2 (P2) represents dynamic gates for sorting the most mature eGFP+ cells at the indicated time point of ES cell in vitro differentiation (A) or transgenic embryo development (B).



**Figure S7 - Flow cytometric analysis of endothelial marker PECAM/CD31 expression in Nkx2-5-eGFP transgenic mouse embryos.** (A) Cells from day 6.5 Nkx2-5-eGFP transgenic mouse embryos without antibody staining. Note the absence of eGFP<sup>+</sup> cells. (B) Cells from day 7.5 Nkx2-5-eGFP transgenic mouse embryos stained with Streptavidin conjugated with PE-Cy7. (C) Same cells as (B) except that biotin conjugated anti-CD31 antibody was used. Note the expression of CD31 in a significant proportion of eGFP<sup>+</sup> cells at embryonic day 7.5. Data represents the results of two independent experiments.

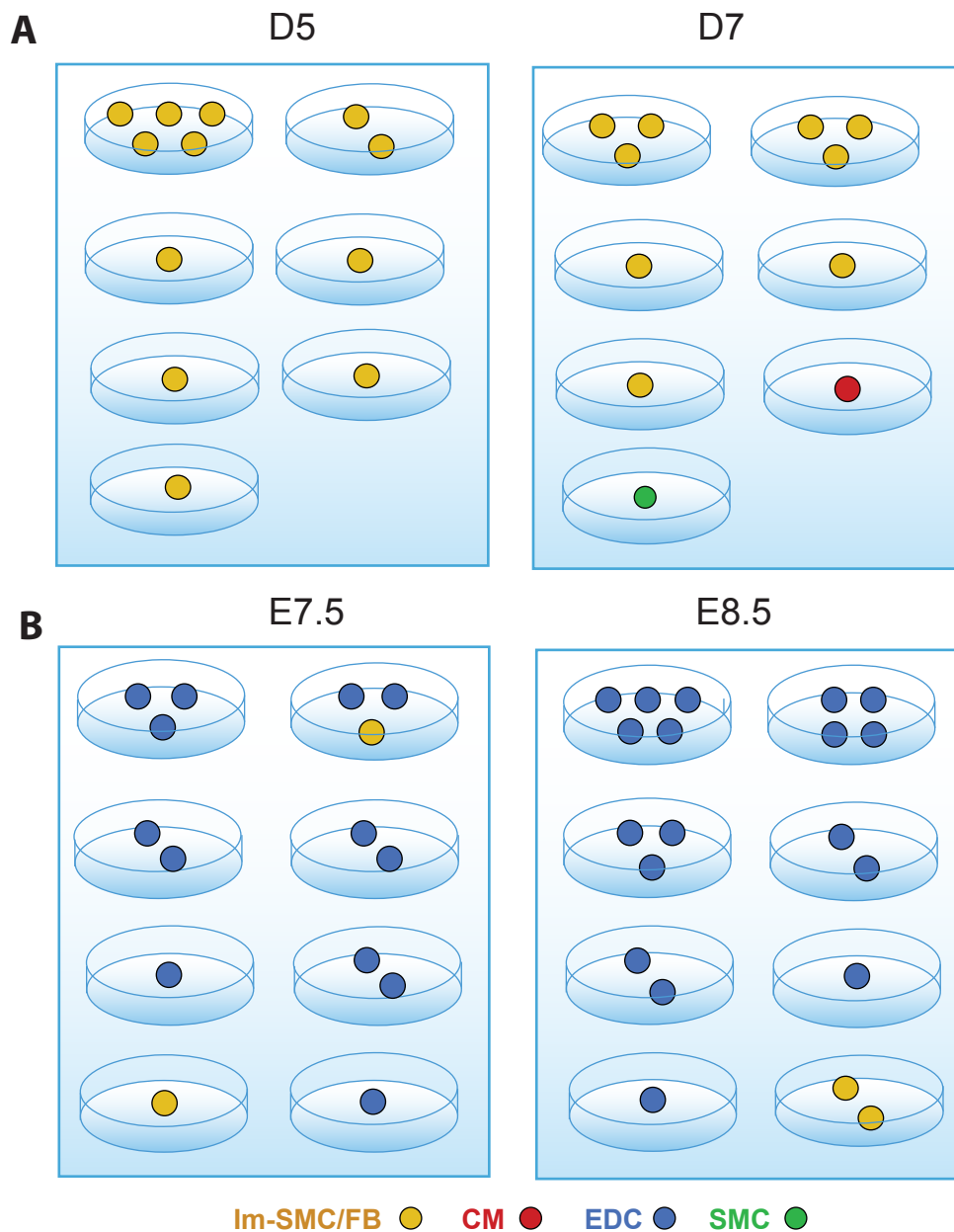


**Figure S8 - Single adult atrial and ventricular cardiomyocytes derived from 3-months old mouse hearts.** Representative bright field (left panel), fluorescence (middle panel), and merged (right panel) images of single cardiomyocytes from atria (upper panels) and ventricles (lower panels) stained with Hoechst 33342 are shown. Bar =200  $\mu$ M.



**Figure S9 - CFSE-stained progenies derived from single CPC after 5 days of in vitro culturing.** Cells in each well of a 96-well plate were treated with CFSE and imaged in 510 nm bandpass filter to highlight the location of the progenies from each single CPC after 5 days of in vitro culturing. Bar=200  $\mu$ m.





**Figure S10 - Determination of lineage decisions by single CPCs during in vitro differentiation in endothelial cell (EDC) medium.** Single eGFP<sup>+</sup> cell isolated by FACS from in vitro differentiated Nkx2.5-eGFP ES cells (A) or transgenic embryo (B) at the indicated day of differentiation or development is cultured in each well of 96-well plate in EDC medium for 5 days. After culturing, cell progenies are isolated and profiled on the Fluidigm assay to determine the exact cell identity. The color of each circle presents the identity of each progeny cell and number of circles represents the number of progeny cells from each starting single cell. The data shown represents all surviving cells from two independent experiments.

**Table S1.** TaqMan primers used for single-cell real-time qPCR

| Gene     | TaqMan primer |
|----------|---------------|
| Gapdh    | Mm99999915_g1 |
| Pou5f1   | Mm03053917_g1 |
| Nkx2.5_2 | Mm00657783_m1 |
| Tnni3_1  | Mm00437164_m1 |

**Table S2.** Fluidigm array TaqMan primers

| Gene       | TaqMan primer | Gene       | TaqMan primer |
|------------|---------------|------------|---------------|
| Actb       | Mm01205647_g1 | Myh6_1     | Mm00440354_m1 |
| Actn2      | Mm00473657_m1 | Myh7_2     | Mm01319006_g1 |
| Cdh5       | Mm00486938_m1 | Myh7_1     | Mm00600555_m1 |
| Calponin-1 | Mm00487032_m1 | Myl2       | Mm00440384_m1 |
| Calponin-2 | Mm01169510_m1 | Nkx2.5_2   | Mm00657783_m1 |
| Fn1        | Mm01256744_m1 | Nkx2.5_1   | Mm01309813_s1 |
| Gata4_2    | Mm00484689_m1 | Pdgfra     | Mm00440701_m1 |
| Gata4_1    | Mm01310447_m1 | Pecam1     | Mm00476702_m1 |
| Hand1      | Mm00433931_m1 | Pou5f1     | Mm03053917_g1 |
| Hand2      | Mm00439247_m1 | Sarcolipin | Mm00481536_m1 |
| Hcn4       | Mm01176086_m1 | Sox2       | Mm03053810_s1 |
| Isl1_1     | Mm00627860_m1 | Sm22a      | Mm00441661_g1 |
| Isl1_2     | Mm00517585_m1 | Tbx1       | Mm00448948_m1 |
| Flk1       | Mm01222421_m1 | Tbx5       | Mm00803518_m1 |
| Kit_2      | Mm00442972_m1 | Tnni3_1    | Mm00437164_m1 |
| Kit_1      | Mm00445212_m1 | Tnni3_2    | Mm01330976_m1 |
| Mef2C_2    | Mm01340839_m1 | Tnnt2_2    | Mm01290256_m1 |
| Mef2C_1    | Mm01340842_m1 | Tnnt2_1    | Mm00441920_m1 |
| Mesp1      | Mm00801883_g1 | Vcam1      | Mm01320970_m1 |
| Myh11      | Mm00443013_m1 | Vim        | Mm01333430_m1 |
| Myh6_2     | Mm00440359_m1 | Vwf        | Mm00550376_m1 |

**Table S3.** Independent TaqMan primers used for confirmation of single ESC gene expression.

| Gene     | TaqMan primer | Gene   | TaqMan primer |
|----------|---------------|--------|---------------|
| Pou5f1_1 | Mm03053917_g1 | Sox2_1 | Mm03053810_s1 |
| Pou5f1_1 | Mm00658129_gH | Sox2_2 | Mm00488369_s1 |

**Table S4.** Pearson correlation values for standard cells in reference panel (Figure 1)

|     | CPC   | CM     | SMC    | FB    | EDC    | ES     |
|-----|-------|--------|--------|-------|--------|--------|
| CPC | 0.868 | -0.109 | 0.544  | 0.632 | 0.435  | 0.511  |
| CM  |       | 0.928  | -0.116 | 0.016 | -0.181 | -0.053 |
| SMC |       |        | 0.913  | 0.637 | 0.372  | 0.435  |
| FB  |       |        |        | 0.895 | 0.617  | 0.718  |
| EDC |       |        |        |       | 0.941  | 0.579  |
| ES  |       |        |        |       |        | 0.925  |

**Table S5.** TaqMan primers used for characterizing specific chamber derived cardiomyocytes

| Gene     | TaqMan primer | Gene   | TaqMan primer |
|----------|---------------|--------|---------------|
| Cited1_1 | Mm01235642_g1 | Gja4   | Mm00433610_s1 |
| Cited1_2 | Mm04207352_m1 | Gja5_1 | Mm00433619_s1 |
| Gja1     | Mm01179639_s1 | Gja5_2 | Mm01265686_m1 |

**Table S6.** TaqMan primers used for characterizing cardiomyocyte populations

| Gene       | TaqMan primer | Gene    | TaqMan primer |
|------------|---------------|---------|---------------|
| Hand2      | Mm00439247_m1 | Tnni3_2 | Mm01330976_m1 |
| Mef2C_1    | Mm01340842_m1 | Tnnt2_2 | Mm01290256_m1 |
| Myh6_1     | Mm00440354_m1 | Gata4_1 | Mm01310447_m1 |
| Myh7_1     | Mm00600555_m1 | Myl2    | Mm00440384_m1 |
| Sarcolipin | Mm00481536_m1 |         |               |

**Table S7.** Number of cells analyzed in each single cell experiment

**A.** Standard cells reference panel (Figure 1)

| Cell Type | Count |
|-----------|-------|
| CPC       | 26    |
| CM        | 23    |
| SMC       | 25    |
| FB        | 25    |
| EDC       | 25    |
| ES        | 27    |

**B.** Comparison of mESC and embryo-derived eGFP<sup>+</sup> cells (Figure 3)

| Source | Time Point | Count |
|--------|------------|-------|
| EB     | D5         | 26    |
|        | D6         | 30    |
|        | D7         | 21    |
|        | D8         | 22    |
| Embryo | E7.5       | 27    |
|        | E8.5       | 15    |
|        | E9.5       | 12    |
|        | E10.5      | 31    |

**C.** Comparison of *in vivo* neonatal and adult CMs and *in vitro* mESC-derived CMs (Figure 4)

| Source      | Time Point           | Count |
|-------------|----------------------|-------|
| mESC        | D23                  | 20    |
|             | D29                  | 21    |
| Mouse heart | Neonatal Atrial      | 16    |
|             | Neonatal Ventricular | 17    |
|             | Adult Atrial         | 20    |
|             | Adult Ventricular    | 19    |



**Supplementary Movie 1** - Beating single atrial cardiomyocyte from 3 month old adult mouse.



**Supplementary Movie 2** - Beating single ventricular cardiomyocyte from 3 month old adult mouse.



**Supplementary Movie 3** – Time-lapse video microscopy of Nkx2-5-eGFP+ CPCs from day 6 in vitro differentiated mESCs. FACS-purified eGFP+ cells were imaged every hour with incuCyte system (Essen BioScience) for 5 days.