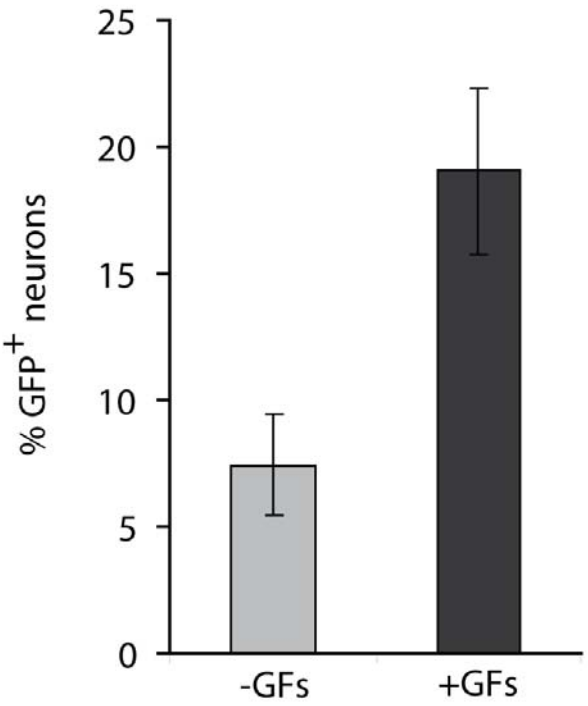
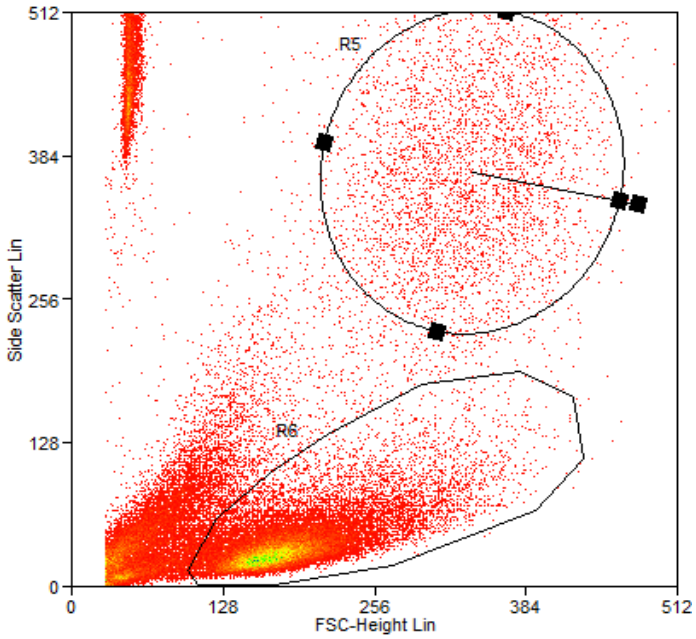
Supplementary Figure 1: *Quantification by FACS analysis of the neurons differentiated within ES-PA6 co-cultures.*



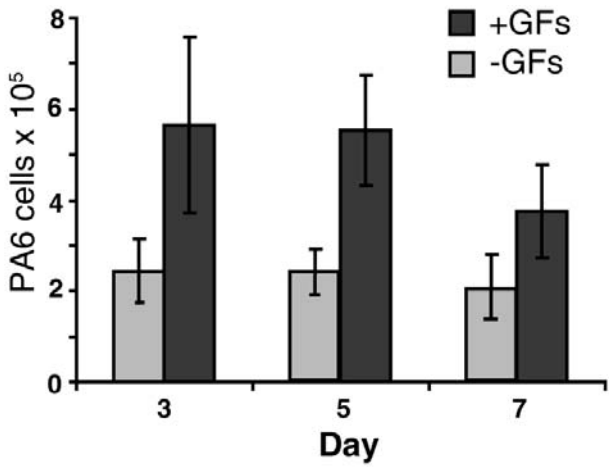
This analysis takes advantage of the fact that the ES cells used in these experiments were engineered by a knock-in strategy (Ying QL et al., Nat Biotechnol., 21(2):183-186, 2003). GFP gene was integrated into the Tau locus and therefore expressed only in neurons.
After 7 days of co-culturing ES cells with PA6 cells in the absence (-GFs) or in the presence of FGF2 and EGF (+GFs), single cells suspensions were prepared and analyzed by flow cytometry to detect GFP^+ cells. As shown in the histogram, the presence of GFs in the medium reinforced neurons differentiation.

Supplementary Figure 2: *Cytofluorimetric absolute cells count of ES-derived cells and PA6 cells.*

A

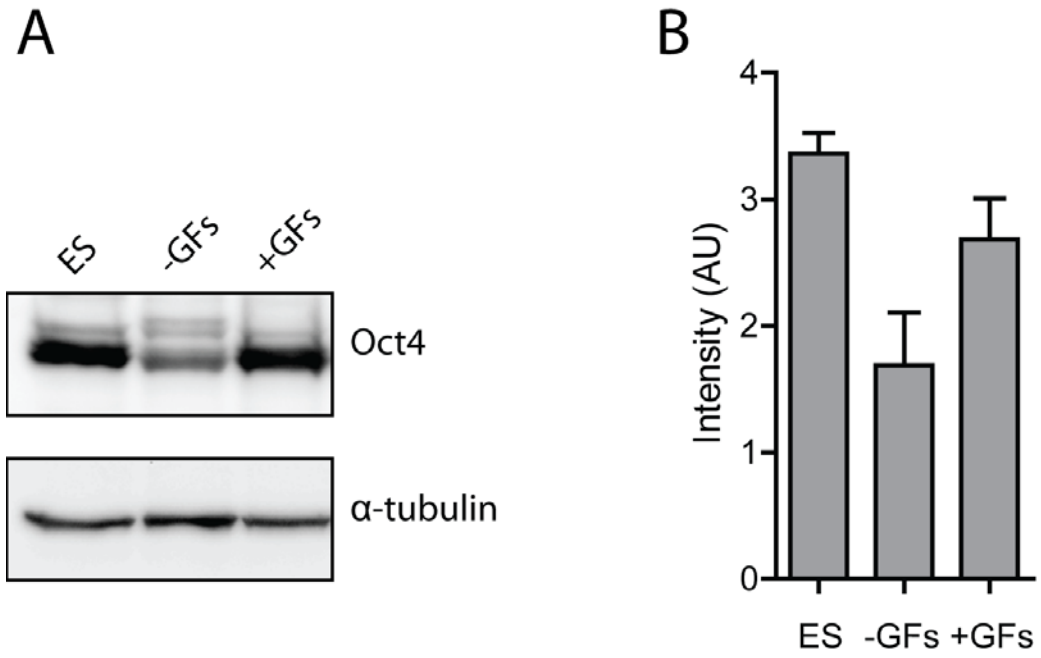


B



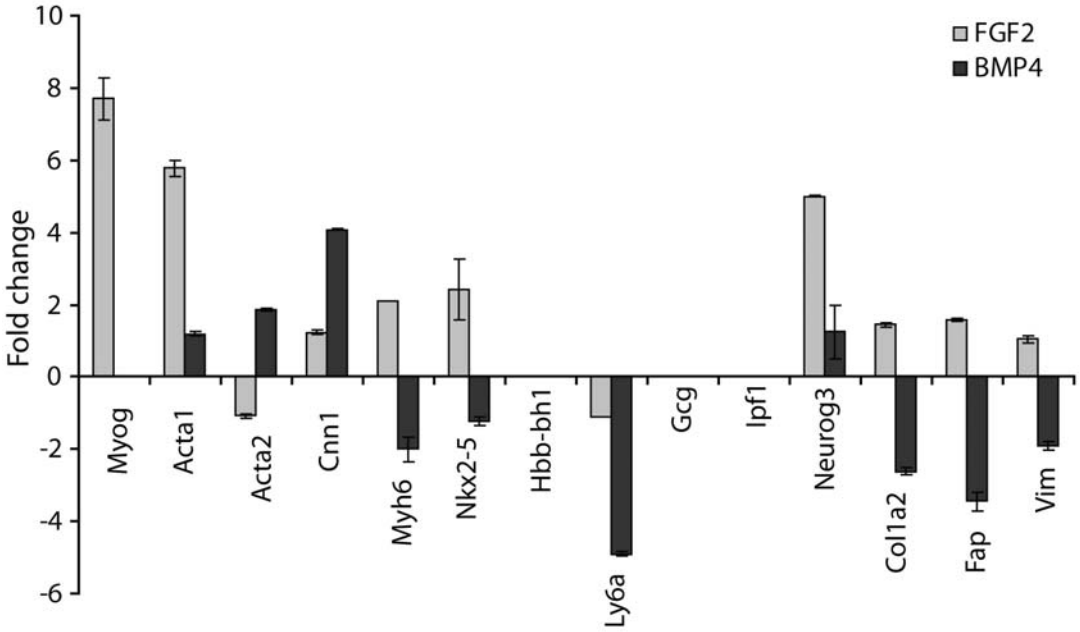
(**A**) ES-derived cells (R6, lower gate) and PA6 cells (R5, upper gate), visualized in the dot plot, can be easily separated thank to their different physical parameters, thus allowing their independent analysis. The fluorescent beads used to perform the count are visible in the upper left side of the plot. (**B**) The number of PA6 cells in the co-cultures was analyzed during time and upon GFs stimulation. Since PA6 cells are growth-inhibited when confluent, their number did not increase during time. However, when GFs were added to the culture medium, PA6 cells proliferated until day three to stop their growth in the following days.

Supplementary Figure 3: *biochemical detection and quantification of Oct4 protein in ES-PA6 co-cultures.*



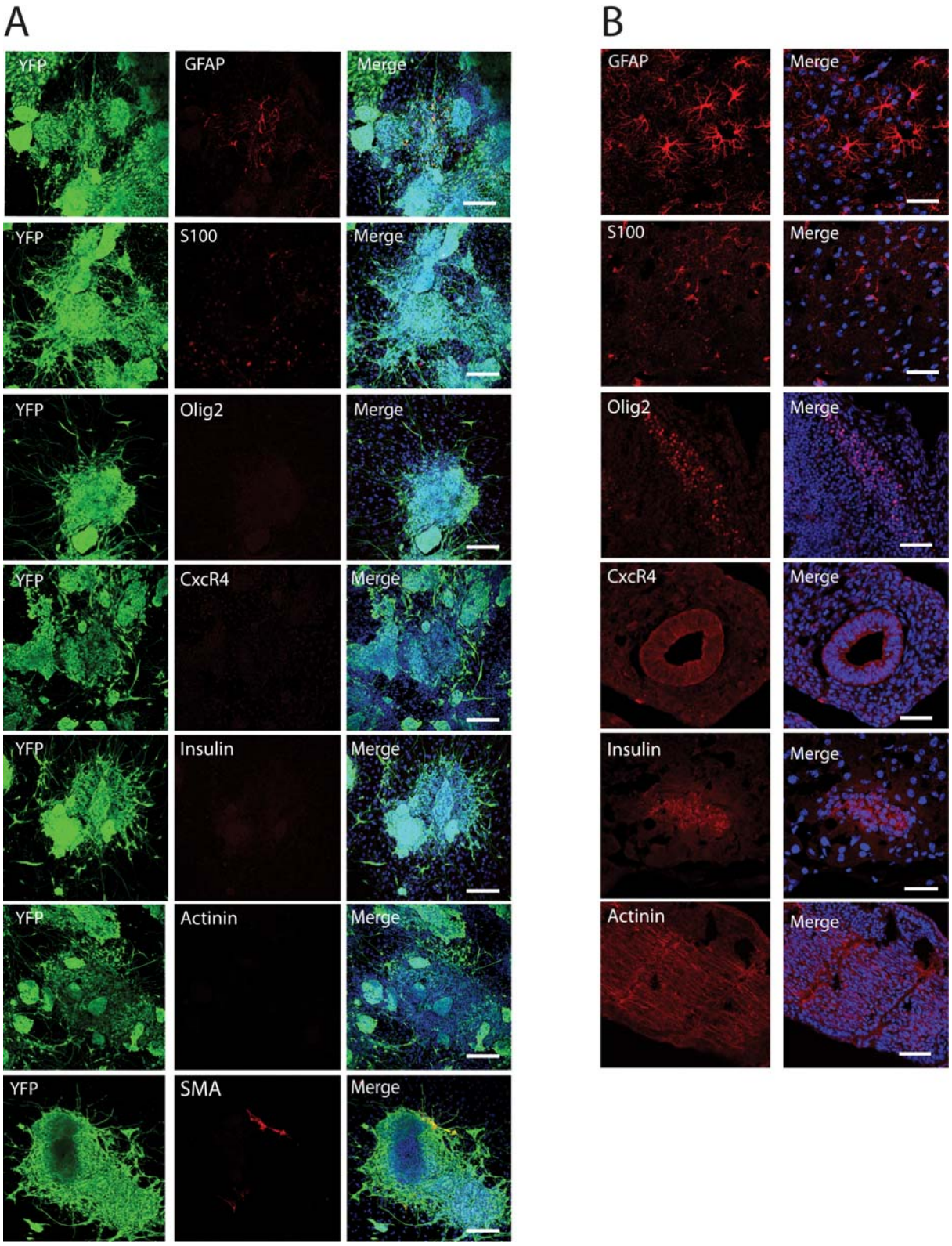
ES cells were co-cultured with PA6 cells for 7 days in N2B27 medium without growth factors (-GFs) or in the presence of FGF2 and EGF (+GFs). (**A**) Oct4 protein and the constitutively expressed α-tubulin protein were detected by western blot in cell lysates purified from ES-PA6 co-cultures and pure cultures of undifferentiated ES cells (ES). (**B**) The immunoreactive bands were quantified by Quantity One software. In absence of GFs the level of Oct4 expression significantly decreased, whereas FGF2 and EGF contributed to maintain a stemness component within the co-cultures.

Supplementary Figure 4: *analysis of gene expression upon FGF2 or BMP4 stimulation.*



Co-cultures of ES and PA6 cells were grown for 6 days in N2B27 medium supplemented with FGF2 or BMP4. Total RNAs were purified and used to analyze the expression of genes representative of skeletal muscle (Myog, Acta1), smooth muscle (Acta2, Cnn1), cardiac muscle (Myh6, Nkx2-5), hematopoietic (Hbb-bh1, Ly6a), pancreatic (Gcg, Ipf1, Neurog3), fibroblasts (Col1a2, Fap, Vim) cells. The analysis was performed by using Taqman low density arrays. Data are normalized against the gene expression level measured in absence of growth factors and any variation of expression below 2 fold change was considered not significant. The experiment was performed in duplicate; error bars indicate SEM. Most of the genes analyzed were not significantly up-regulated and some of them were even down-regulated. For the exceptions Myog, Acta1 (skeletal muscle markers), Neurog3 (pancreatic marker) and Cnn1 (pericytes marker) the presence of the corresponding mature cell types was further investigated by immunofluorescence (see Supplementary Fig. 5 below).

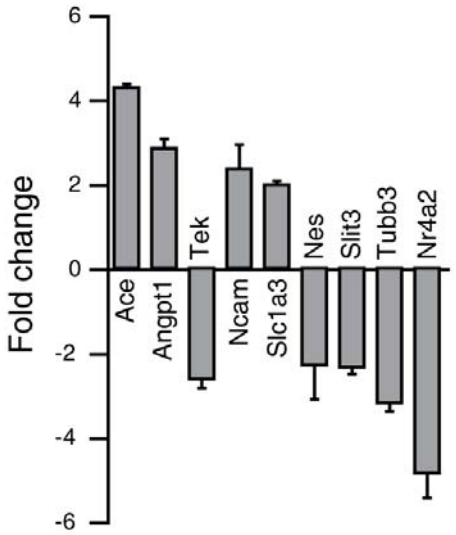
Supplementary Figure 5: *characterization by immunofluorescence analysis of ES-PA6 co-cultures for the presence of glia, endoderm, pancreatic and smooth muscle cells.*



(**A**) After 7 days of co-culturing YFP-ES with PA6 cells in presence of FGF2 and EGF, cells were fixed and analyzed by immunofluorescence to detect astrocytes (with anti-GFAP and

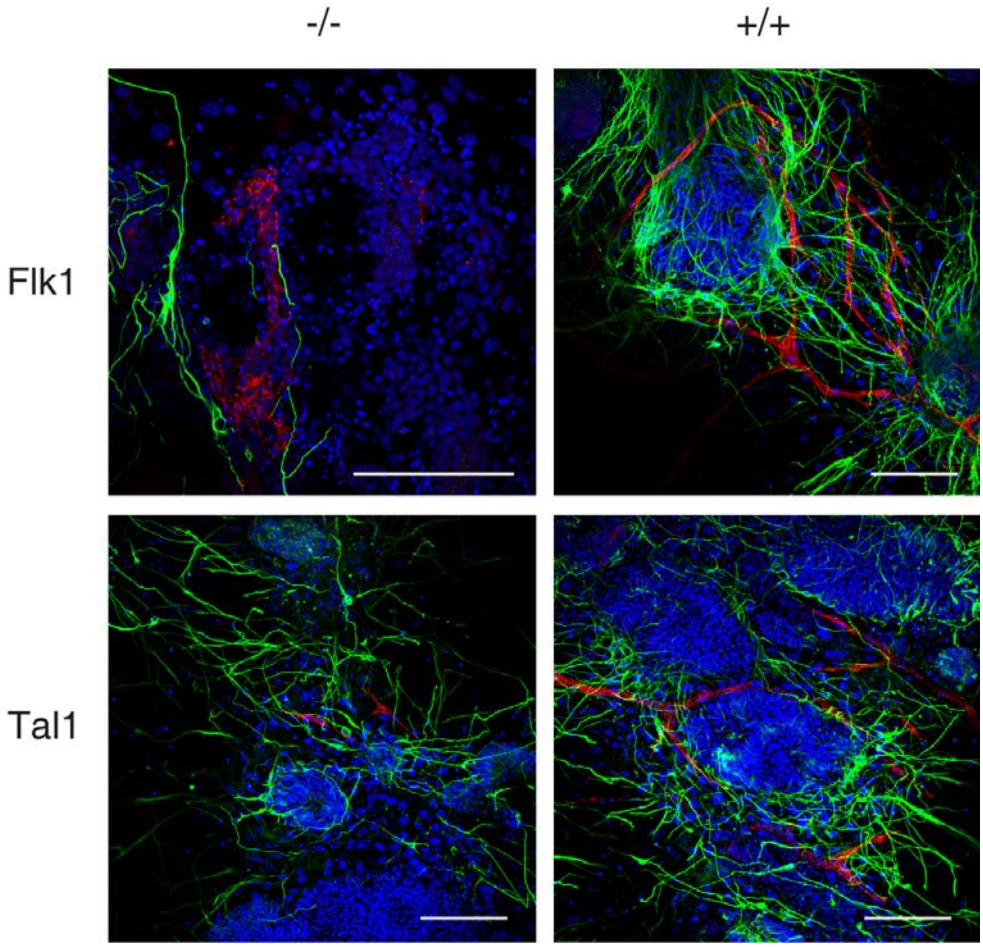
S100 antibodies), oligodendrocytes (with anti-olig2 antibody), mesoderm (with anti-CxcR4 antibody), skeletal muscle (with anti-Actinin antibody) and smooth muscle cells (with anti-SMA antibody). Other than few $GFAP^+$ and SMA^+ cells no oligodendrocytes, mesoderm or pancreatic cells were detected. Scale bars: 150 μm (**B**) Sections of brain and pancreas from adult mouse were used as positive controls for the GFAP, Olig2, S100 and Insulin immunostainings, whereas the lung and tail sections used for CxcR4 and Actinin immunostainings were obtained from day 13.5 mouse embryos. Scale bars 40 μm except for actinin and olig2 immunostainings that are 75 and 50 μm respectively.

Supplementary Figure 6: *Gene expression analysis in pure PA6 cell cultures stimulated with FGF2.*



Confluent PA6 cells were stimulated with 20 ng/ml of FGF2 for three days in N2B27 medium and mRNAs were purified after 6 days. Gene expression was analyzed with a Taqman Low Density arrays by comparing mRNA levels in stimulated and not stimulated cells. Genes with a fold change >2 were considered differentially expressed and are shown in the plot. The experiment was performed in duplicate; error bars indicate SEM.

Supplementary Figure 7: *2D in vitro differentiation of Flk1 $^{-/-}$ and Tal1 $^{-/-}$ ES cell lines.*



Flk1 $^{-/-}$ and Tal1 $^{-/-}$ ES cells, along with their parental wild type cell lines, were differentiated by co-culturing them with PA6 cells in N2B27 medium in presence of GFs. After seven days, cells were fixed and immunostained with anti-VE-Cadherin and anti-βIII-tubulin antibodies in order to detect endothelial cells (red) and neurons (green) respectively. Scale bars: 150 μm.

Supplementary Table 1: *List of shRNA MISSION clones used to stably knock-down the expression of Flk1, Ace, Tal1 and T.*

shRNA targeting Flk1
TRCN0000055068
TRCN0000055069
TRCN0000055070
TRCN0000055071
TRCN0000055072

shRNA targeting Ace
TRCN0000031143
TRCN0000031142
TRCN0000031141
TRCN0000031140
TRCN0000031139

shRNA targeting Tal1
TRCN0000042576
TRCN0000042574
TRCN0000042573
TRCN0000042575
TRCN0000042577

shRNA targeting T
TRCN0000082003
TRCN0000082004
TRCN0000082006
TRCN0000082005
TRCN0000082007

Supplementary Table 2: *list of the genes used to customize the Taqman Low Density Array.*

Gene Name	Taqman Probe
Angiotensin I converting enzyme	Ace-Mm00802048_m1
Alpha skeletal actin	Acta1-Mm00808218_g1
Alpha-smooth muscle actin	Acta2-Mm00725412_s1
Actin beta	Actb-Mm00607939_s1
Alk4	Acvr1b-Mm00475708_m1
ActRII	Acvr2a-Mm00431657_m1
Angiopoietin1	Angpt1-Mm00456503_m1
Angiopoietin2 (antagonista di Angio1)	Angpt2-Mm00545822_m1
Cadherin2 (N-cadherin)	Cdh2-Mm00483213_m1
VE-Cadherin	Cdh5-Mm00486938_m1
Choline acetyltransferase	Chat-Mm01221880_m1
Chordin	Chrd-Mm00438203_m1
Calponin1	Cnn1-Mm00487032_m1
Pro-collagen 1 alpha2	Col1a2-Mm00483888_m1
Cxcr4	Cxcr4-Mm99999055_m1
Doublecortin	Dcx-Mm00438401_m1
Desmin	Des-Mm00802455_m1
EGFreceptor	Egfr-Mm00433023_m1
Endoglin	Eng-Mm00468256_m1
BLBP	Fabp7-Mm00445225_m1
Fap (fibroblast activation protein)	Fap-Mm00484254_m1
FGF2	Fgf2-Mm00433287_m1
FGF5	Fgf5-Mm00438919_m1
FGFR1	Fgfr1-Mm00438923_m1
FGFR2	Fgfr2-Mm00438941_m1
Flt-1 (VEGFR-1)	Flt1-Mm00438980_m1
HNF3beta	Foxa2-Mm00839704_mH
SSEA-1	Fut4-Mm00487448_s1
GAD	Gad2-Mm00484623_m1
Galactosylceramidase (Galactocerebrosidase)	Galc-Mm00484646_m1
GATA4	Gata4-Mm00484689_m1
Glucagon	Gcg-Mm00801712_m1
Glial fibrillar acidic protein	Gfap-Mm00546086_m1
Goosecoid	Gsc-Mm00650681_g1
Hemoglobin Z, beta-like embryonic chain	Hbb-bh1-Mm00433932_g1
CD102 (ICAM2)	Icam2-Mm00494862_m1
ActivinbA (inhibin)	Inhba-Mm00434338_m1
Insulin2	Ins2-Mm00731595_gH
Pdx1	Ipf1-Mm00435565_m1
Flk-1 (VEGFR-2)	Kdr-Mm00440099_m1
Keratin14	Krt14-Mm00516876_m1
Sca-1 (lymphocyte antigen 6 complex)	Ly6a-Mm00726565_s1
Mapt	Mapt-Mm00521988_m1
CD146	Mcam-Mm00522397_m1
Alpha cardiac MHC	Myh6-Mm00440354_m1
Cardiac myosin ventricular light chain	Myl2-Mm00440384_m1
MyoD	Myod1-Mm00440387_m1
Myogenin	Myog-Mm00446194_m1

Ncam1	Ncam1-Mm00456815_m1
NeurofilamentH	Nefh-Mm01191456_m1
Nestin	Nes-Mm00450205_m1
neurogenin-3	Neurog3-Mm00437606_s1
NGFreceptor/p75	Ngfr-Mm00446294_m1
Nkx2.2	Nkx2-2-Mm00839794_m1
Nkx2.5	Nkx2-5-Mm00657783_m1
Nurr1	Nr4a2-Mm00443056_m1
Neuropilin-1	Nrp1-Mm00435372_m1
Neuropilin-2	Nrp2-Mm00803101_m1
Olig2	Olig2-Mm01210556_m1
Pax6	Pax6-Mm00443072_m1
CD31 (Pecam1)	Pecam1-Mm00476702_m1
Oct4 (Oct3, Oct3/4)	Pou5f1-Mm00658129_gH
Protein C receptor	Procr-Mm00440992_m1
CD133 (Prominin1)	Prom1-Mm00477115_m1
Roundabout1	Robo1-Mm00803879_m1
Roundabout2	Robo2-Mm00620713_m1
Roundabout3	Robo3-Mm00487934_m1
Roundabout4	Robo4-Mm00452963_m1
Semaphorin 3A	Sema3a-Mm00436469_m1
Vescicular glutamate transporter 1 (Vglut1)	Slc17a7-Mm00812886_m1
GLAST (glial high affinity glutamate transporter)	Slc1a3-Mm00600697_m1
Slit1	Slit1-Mm01198620_m1
Slit2	Slit2-Mm00662153_m1
Slit3	Slit3-Mm01326974_m1
Sox2	Sox2-Mm00488369_s1
Synaptophysin	Syp-Mm00436850_m1
Brachyury	T-Mm00436877_m1
SCL	Tal1-Mm00441665_m1
TATA box binding protein	Tbp-Mm00446973_m1
Tie-2	Tek-Mm00443242_m1
Tyrosine Hydroxilase	Th-Mm00447546_m1
Thrombomodulin	Thbd-Mm00437014_s1
Thrombospondin-1	Thbs1-Mm01335418_m1
Tie-1	Tie1-Mm00441786_m1
Tryptophan Hydroxylase	Tph2-Mm00557717_m1
Beta-III tubulin	Tubb3-Mm00727586_s1
VEGF A	Vegfa-Mm00437304_m1
Vimentin	Vim-Mm00449201_m1
Von Willebrand factor homolog	Vwf-Mm00550376_m1
Rex1	Zfp42-Mm01194090_g1