Supplemental Information

INVENTORY OF SUPPLEMENTARY INFORMATION

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SUPPLEMENTARY EXPERIMENTAL PROCEDURES

DNA Construction

Pax6 and its mutants were constructed into pLenti vector with a FUGW backbone and an inducible lentiviral vector (Clontech) (Xia et al., 2008; Xia and Zhang, 2007; Xia et al., 2007). Pimers for amplifying Pax6a (1-422) and Pax6b (1-436) are as follows: Forward, CATATTCGAGCCCCGTGGAATCC; Reverse, TTACTGTAATCTTGGCCAGTATTG. Forward primer for amplifying Pax6 ΔPAI (77-422) is ATGAGAGTAGCGACTCCAGAAGTTG and forward primer for Pax6 ΔPD (202-422) is ATGCGACTTCAGCTGAAGCGG. For deleting HD in Pax6 (delete 210-269 in Pax6a and 224-283 in Pax6b) two step PCR is used with two additional primers: Forward, CGACTTCAGCTGAAGCGGAAGAAACTGAGGAATCAGAGA; Reverse, GTCTTCTCTGATTCCTCAGTTTCTTCCGCTTCAGCTGAAGTCG. For subcloning Pax6 dominant negative mutants (D/N), the last 78 amino acids of the PST transactivation domain are removed by the reverse primer, TTGCATAGGCAGGTTATTTGC. All the constructs have been verified by DNA sequencing.

Lentivirus production and transduction of ESCs.

The constructs of lentiviral vectors for knockdown of Pax6 are shown in supplementary Figure 2 and lentivirus production was described previously (Xia et al., 2008). For transduction of ESCs, human H9 ESCs or mouse ESCs (D3 and 46C) were collected by brief centrifugation. Cell pellets were then incubated with 100μl of concentrated virus (10^6 transducing units/ml) at 37^{o}C for 30 min. The virus and cell mixture was then transferred to the MEF feeder layer overnight before changing medium on the next day. Forty-eight hours after infection, blasticidin or puromycin was added to the cells for

selecting drug-resistant clones. The final concentration of blasticidin or puromycin was 5μg/ml for elongation factor-1 α (EF1α) promoter and 2μg/ml for phosphoglycerate kinase (PGK) promoter. To make stable transduced monoclonal RNAi lines, ESCs were pretreated with ROCK inhibitor and then typsinized to single cells before plating on the MEF feeder (Watanabe et al., 2007).

The inducible lentivirus system, purchased from Clontech (Mountain View, CA), was modified by replacing the CMV promoter driving rtTA-Advanced in the pLVX-Tet-On Advanced vector with the EF1α promoter to optimize transgene expression in human ESCs.

Western blotting

Cell pellets were lysed in a lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, with 10 mM NaF, 10 mm disodium pyrophosphate, and 1X protease inhibitor cocktail, Sigma) and passed through a $28^{1/2}$ gauge needle several times. The particulate fraction was removed by centrifugation, and 30 μg of proteins in the supernatant were boiled in SDS–PAGE sample buffer and separated by SDS–PAGE.

Microarray analysis

Luc RNAi and Pax6 RNAi human ESC lines were differentiated to NE cells for 6 days. Total RNA was extracted using Trizol (Invitrogen) and mRNA pooled from two individual lines of each group was hybridized on Affymetrix GeneChip Human Genome HG-U133 Plus 2.0 arrays according to the manufacturer's instructions. The data were deposited in the ArrayExpress database (accession number E-MEXP-2668).

mRNA extraction and RT-PCR

Total RNA was isolated using the Trizol kit (Invitrogen). 1 μg of total RNA from each sample was reverse transcribed into cDNA and subjected to real-time PCR using the Power SYBR Green kit (Applied Biosystems, UK). Primer oligonucleotides used for real-time PCR were as follows (most primers target both human and mouse genes except when they are specifically labeled):

Gene	Forward Primer	Reverse Primer
Pax6	TCTTTGCTTGGGAAATCCG	CTGCCCGTTCAACATCCTTAG
Oct4(human)	ACATCAAAGCTCTGCAGAAAGAACT	CTGAATACCTTCCCAAATAGAACCC
Oct4 (mouse)	ACATGAAAGCCCTGCAGAAGGAGCT	GAGAACGCCCAGGGTGAGCC
Nanog	ATTCTTCCACCAGTCCCAAA	ATCTGCTGGAGGCTGAGGTA
Sox2	GCCCTGCAGTACAACTCCAT	TGGAGTGGGAGGAAGAGGTA
Fabp7	TGTGACCAAACCAACGGTAAT	CTTTGCCATCCCATTTCTGTA
Lhx2	TTACGGCAGGAAAACACGG	TGCCAGGCACAGAAGTTAAG
Six3	ACTACCAGGAGGCCGAGAAG	CAGTTCGCGTTTCTTGCTG
Six6	AACAAGAATGAGTCGGTGCT	CAGCGGGAACTTCTTCCTTA
Map2	GGTCACAGGGCACCTATTCA	TGTTCACCTTTCAGGACTGC
Lmo3	AAGGCACTGGACAAATACTGG	CACGCATCACCATCTCAAAG
Lix1	GGAATTTTGGGAAAGCAAGC	CAGCACTGAAAGTTGCCAAA

Dlk1	TCCTGAAGGTGTCCATGAAAG	GTGGTTGTAGCGCAGGTTG
Meis2	CCAGGGGACTACGTTTCTCA	TAACATTGTGGGGCTCTGTG
Dach1	GGTGGTGTGCAATGTGGA	ATGCGGCATGATGTGAGAG
N-Cad	TCCTGATATATGCCCAAGACAA	TGACCCAGTCTCTCTTCTGC
Sox1	GTTTTTTGTAGTTGTTACCGC	GCATTTACAAGAAATAATAC
Nedd9	CCCATCCAGATACCAAAAGG	TCTCTCCCACTGGAACTGAA
Nr2f2	AAGCACTACGGCCAGTTCAC	GTCTCATGCCCACTTTGAGG
Fezf2	CGGCGAGAAGCAGTACAAAT	GTTTGCGCACATGTTTCTTT
Zic1	AGCCACGATGCTCCTGGACGC	TGGCCCAGGGCCGCAGCAGC
Meis1	GATGATTCAAGCCATACAAG	GGGGTTCCTCCTGAACGAGT
Mash1	AACGAGCGCGAGCGCAACCG	TTGGAGTAGTTGGGGGAGATG
Pax3	GCTGTGCCCAGGATGATGC	CTGGTACCTGCACAGGATCT
Lmo1	ACGGAGCGCCCGAGATGATG	GGCACAGGATGAGGTTGGCC
Pou3f2	CCGCAGCGTCTAACCACTAC	GTGGGACAGCGCGGTGATCC
Crx(human)	TATTCTGTCAACGCCTTGGCCCTA	TGCATTTAGCCCTCCGGTTCTTGA
RPE65(human)	GCCCTCCTGCACAAGTTTGACTTT	AGTTGGTCTCTGTGCAAGCGTAGT
Chx10(human)	ATTCAACGAAGCCCACTACCCAGA	ATCCTTGGCTGACTTGAGGATGGA
FoxG1(human)	AGAAGAACGGCAAGTACGAGA	TGTTGAGGGACAGATTGTGGC
En1(human)	GGACAATGACGTTGAAACGCAGCA	AAGGTCGTAAGCGGTTTGGCTAGA
Hoxb4	AAAGAGCCCGTCGTCTACC	GTGTAGGCGGTCCGAGAG
Nkx2.1(human)	AACCAAGCGCATCCAATCTCAAGG	TGTGCCCAGAGTGAAGTTTGGTCT
Cdx2	TGGAGCTGGAGAAGGAGTTT	CTGCTGCTGCTGTTGCTG
Gata6	GTGAACTGCGGCTCCATC	GTGTGACAGTTGGCACAGGA
K18	ATGCGCCAGTCTGTGGAG	CCTGAGATTTGGGGGCATC
Lama3	TGTTAATCGGGCAACACAAA	GGTGCTTTCCAAAGTTCCTG
Brachyury (human)	ACAGCCAGCAACCTGGGTA	CATGCAGGTGAGTTGTCAGAA
Sox17	ATACGCCAGTGACGACCAG	GCGGCCGGTACTTGTAGTT
Hnf1b	AGAGGGAGGTGGTCGATGTC	AGCTGATCCTGACTGCTTTTG
Pdx1	CAAAGCTCACGCGTGGAAAG	TGATGTGTCTCTCGGTCAAG
Vegfr2	TAGAAGGTGCCCAGGAAAAG	CAAGTAGCCTGTCTTCAGTTC
Gapdh	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

For separating Pax6a and Pax6b, primer sets spanning exon5a (Forward: CGGAGTGAATCAGCTCGGTG; Reverse: CCGCTTATACTGGGCTATTTTGC) were used for regular PCR and analyzed by 2.5% gel.

Chromatin immunoprecipitation (ChIP)

Inducible GFP, Pax6a-GFP and Pax6b-GFP human ESC lines were treated with 2μg/ml doxycycline for 1 or 3 days to induce transgene expression. After cross-linking with 1% formaldehyde at 37^{o}C for 10 min, the cells were harvested by scraping. The fixed cells were then washed and prepared with the EZ-ChIP^{TM} kit according to the manufacturer's suggestions (Millipore). The chromatin was sheared by sonication and incubated with GFP antibody (Chemicon, rabbit IgG). The immunoprecipitates were then washed 5 times, crosslinks were reversed and immunoprecipitated DNA was subjected to qRT-PCR

analysis. Primer pairs against promoter regions of the pluripotent and NE genes were as follows:

Targets	Forward Primer	Reverse Primer
Oct4	ACCAGGCCCCATAATCTACC	TTCCCCCACTCTTATGTTGC
Nanog	GGGGGATACTCGGGATACTC	GGAAAAGCAGGGTGACATTC
Fabp7	CGGACATACTTCTGACTTTTTGG	GATGCTCTGTGGCAAGATGA
Six3	ACGGCTGTCTCTGGCTAAGT	GGGAAACCTAACGTGACTGG
Lmo3	CCAGCGAGGGGTAACAGAT	CAGCCAATGCACTGAGAAGA
Meis2	GCCAAACTGAGGCTCTTCAA	CCCCCTTTCCTGGTAGGTAT
Dach1	GTGGAAAACACCCCTCAGAA	CTTGTTCCACATTGCACACC
N-Cad	AAAAGCCTAGCCAGCAACAG	GCTTTTCTGCTTTGGGTGAC

Immnuostaining

Antibodies used in this study for immunostaining were Pax6 (1:5,000, mouse IgG, Developmental Studies Hybridoma Bank), Sox1 (1:1,000, goat IgG, R&D), Otx2 (1:2,000, goat IgG, R&D), FoxG1(1:1,500; gift from Dr. Y. Sasai), Sox2 (1:1,000, goat IgG, R&D), Fabp7 (1:1,000, rabbit IgG, Chemicon), N-cadherin (1:1,000, mouse IgG, Santa Cruz Biotechnology), Brachyury (1:50, goat IgG, R&D), AFP (1:500, rabbit IgG, NeoMarkers) and Gata6 (1:500, rabbit IgG, Santa Cruz Biotechnology).

Proliferation Analysis

Proliferation of Pax6 knockdown lines or Pax6 overexperssion lines was assessed using a "Click-iT EdU" kit purchased from Invitrogen according to the manufacturer's instructions (Weick et al., 2009). For Luc RNAi and Pax6 RNAi lines, cells were differentiated for 8 days and 10μM EdU was added to the cells and allowed for 6 hours of incorporation before fixation and EdU detection. For inducible overexpression lines, cells were treated with doxycycline for 1, 2 and 3 days. The cells were then labeled with EdU for another 6 hours in the presence of doxycycline.

Cell Cycle Analysis

Cells were trypsinized into single cells and fixed in 75% ethanol/PBS overnight. Cells were then washed with PBS and stained with propidium iodide solution (3.8 mM sodium citrate, 50 μg/ml propidium iodide, 0.5 μg/ml RNase A) for 3 hours before analyzed by flow cytometry.

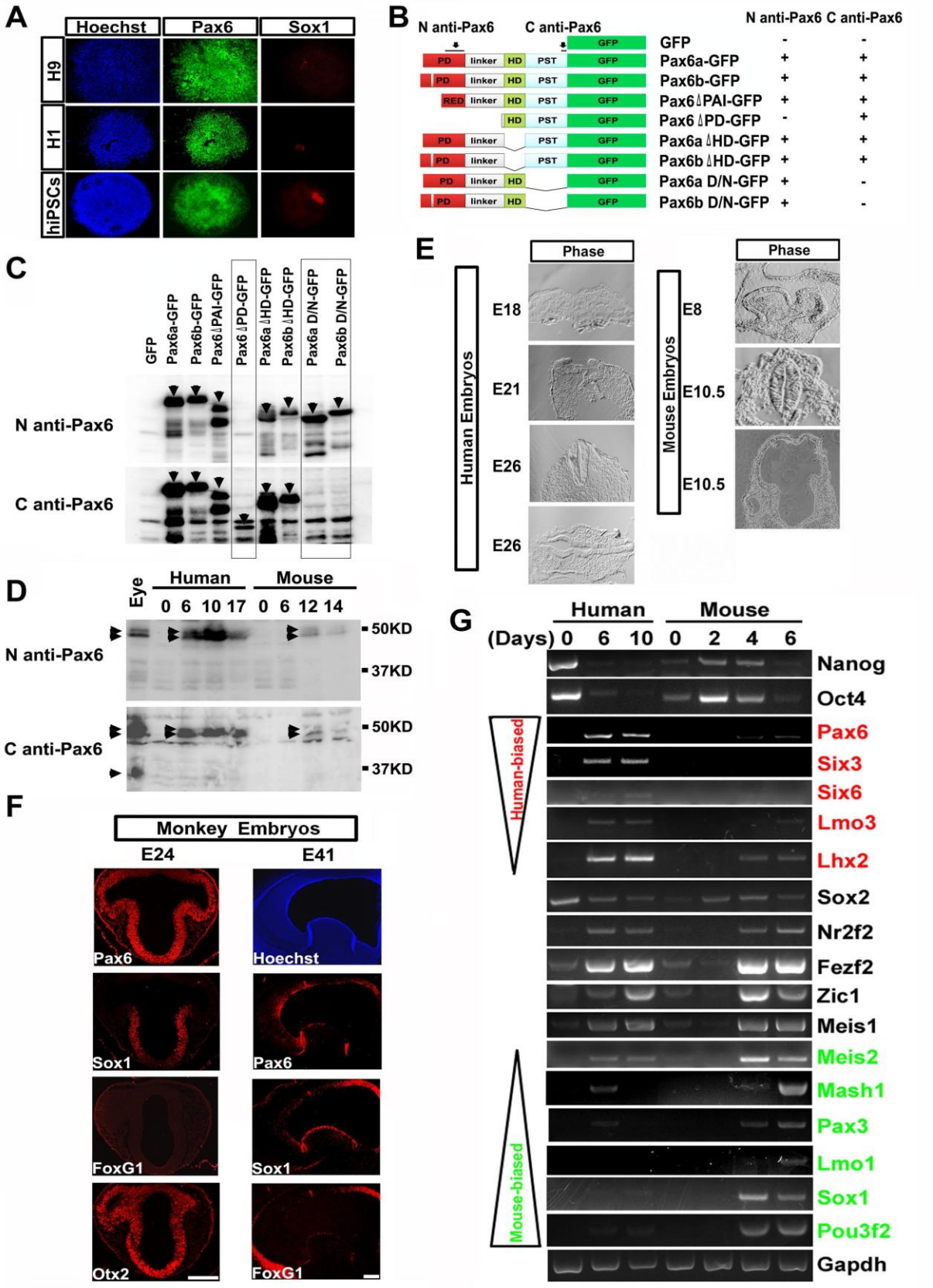

A
Hoechst
Pax6
Sox1
H9
H1
hiPSCs
B
N anti-Pax6
C anti-Pax6
N anti-Pax6 C anti-Pax6
PD linker HD PST GFP
GFP - -
Pax6a-GFP + +
Pax6b-GFP + +
Pax6ΔPAI-GFP + +
Pax6 ΔPD-GFP - +
Pax6a ΔHD-GFP + +
Pax6b ΔHD-GFP + +
Pax6a D/N-GFP + -
Pax6b D/N-GFP + -
C
GFP
Pax6a-GFP
Pax6b-GFP
Pax6ΔPAI-GFP
Pax6 ΔPD-GFP
Pax6a ΔHD-GFP
Pax6b ΔHD-GFP
Pax6a D/N-GFP
Pax6b D/N-GFP
N anti-Pax6
C anti-Pax6
D
Eye
Human
Mouse
0 6 10 17 0 6 12 14
N anti-Pax6
C anti-Pax6
50KD
37KD
50KD
37KD
E
Phase
Phase
Human Embryos
E18
E21
E26
E26
Mouse Embryos
E8
E10.5
E10.5
F
Monkey Embryos
E24
E41
Pax6
Hoechst
Sox1
Pax6
FoxG1
Sox1
Otx2
FoxG1
G
Human
Mouse
(Days) 0 6 10 0 2 4 6
Nanog
Oct4
Pax6
Six3
Six6
Lmo3
Lhx2
Sox2
Nr2f2
Fezf2
Zic1
Meis1
Meis2
Mash1
Pax3
Lmo1
Sox1
Pou3f2
Gapdh
Human-biased
Mouse-biased

Figure S1. Pax6a and Pax6b, but not Pax6ΔPD, are expressed in early human NE, Related to Figure 1.

(A) H9 hESCs, H1 hESCs and human induced pluripotent stem cells are differentiated to primitive NE cells for 8 days, which are Pax6+/Sox1-.

(B**)** Schematic representation of Pax6 isoforms and mutant constructs for the present study. Pax6a has two DNA binding domains, the paired domain (PD) and homeodomain (HD), and a proline-serine-threonine (PST)-rich transactivation domain. Pax6b has an extra exon5a inserted within the PD and is 14 amino acids longer than Pax6a. The third isoform of Pax6 (Pax6ΔPD) lacks the entire paired domain. Monoclonal mouse anti-Pax6 (DSHB) recognizes RED subunit of the paired domain and rabbit anti-Pax6 (Covance) recognizes the most C-terminus of Pax6.

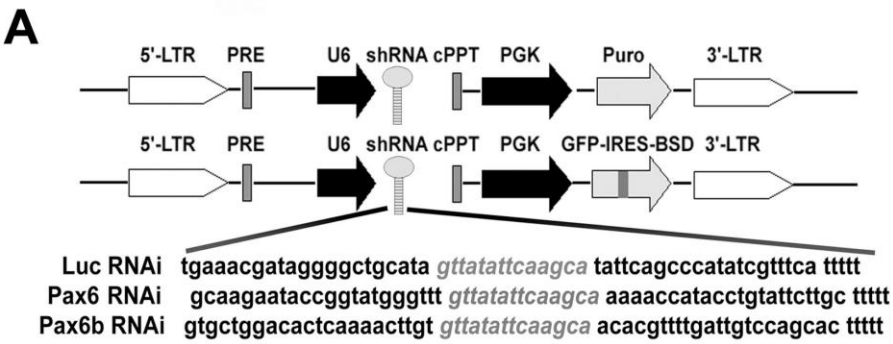
(C**)** The expression of Pax6 isoforms and mutants are verified using the above antibodies after transfection of these constructs into HEK293 cells. The rabbit C-terminal antibody can distinguish all three isoforms of Pax6.

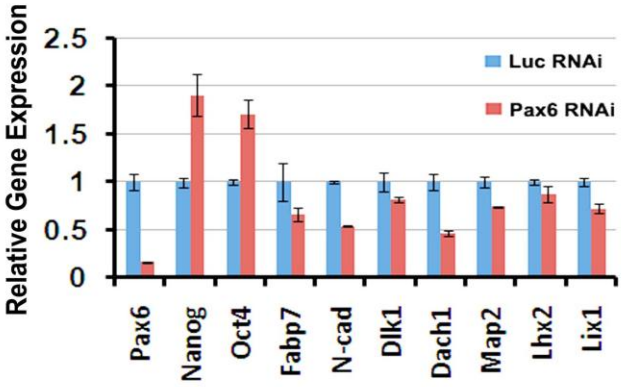
(D**)** While eye tissues from newborn mice have all the three Pax6 isoforms (arrowheads), NE differentiated from human and mouse ESCs mainly express Pax6a and Pax6b but not Pax6ΔPD.

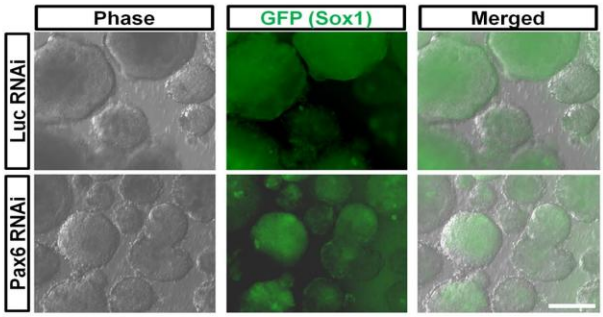
(E) Phase contrast images of human and mouse fetal tissues presented in Figure 1.

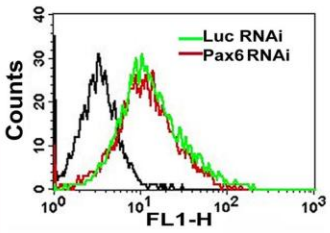
(F) Expression of Otx2, Pax6, Sox1 and FoxG1 in early monkey embryos. At E24, Pax6 and Otx2 are expressed throughout the neural tube (cross section), whereas Sox1 is restricted to part of the neuroepithelia and FoxG1 is not expressed. At E41, Pax6, Sox1, and FoxG1 are expressed in the ventricular zone of the forebrain (sagital section). Scale bars, 100 μm.

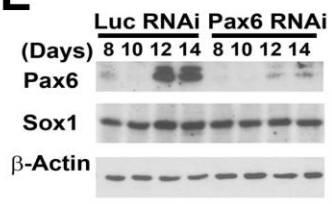
(G) Comparison of gene expression profiles between human and mouse NE. Gene profiles were compared between our human NE microarray data (Lavaute et al., 2009; Li et al., 2009; Pankratz et al., 2007) and previously reported mouse NE microarray data (Aiba et al., 2006). These two sets of databases were chosen because the culture condition and time course are comparable. We then confirmed the expression of the most highly expressed genes using primer sets that target both human and mouse genes. *Pax6*, *Six3*, *Six6* and *Lmo3* are mainly expressed in human NE, while *Pax3*, *Lmo1*, *Sox1* and *Pou3f2* are predominant in mouse NE, suggesting differential use of transcriptional networks underlying NE specification in these two species.


A
5'-LTR
PRE
U6
shRNA
cPPT
PGK
Puro
3'-LTR
5'-LTR
PRE
U6
shRNA
cPPT
PGK
GFP-IRES-BSD
3'-LTR
Luc RNAi tgaaacgataggggctgcata gttatattcaagca tattcagcccatatcgtttca ttttt
Pax6 RNAi gcaagaataccggtatgggttt gttatattcaagca aaaaccatacctgtattcttgc ttttt
Pax6b RNAi gtgctggacactcaaaacttgt gttatattcaagca acacgttttgattgtccagcac ttttt


B
Relative Gene Expression
2.5
2
1.5
1
0.5
0
Luc RNAi
Pax6 RNAi
Pax6
Nanog
Oct4
Fabp7
N-cad
Dlk1
Dach1
Map2
Lhx2
Lix1


C
Phase
GFP (Sox1)
Merged
Luc RNAi
Pax6 RNAi


D
Counts
Luc RNAi
Pax6 RNAi
FL1-H


E
Luc RNAi
Pax6 RNAi
(Days) 8 10 12 14 8 10 12 14
Pax6
Sox1
β-Actin

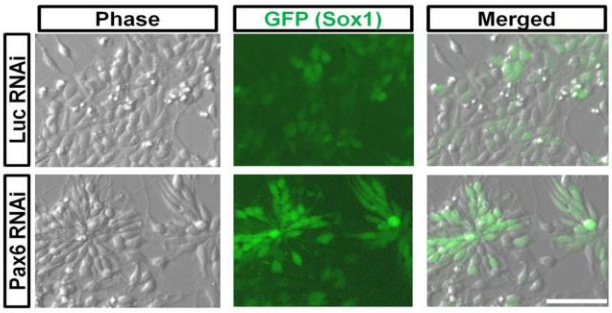

F
Phase
GFP (Sox1)
Merged
Luc RNAi
Pax6 RNAi

Figure S2. Knockdown of Pax6 blocks NE specification in human but not mouse ESCs, Related to Figure 2.

(A) Schematic representation of lentiviral backbone of the vectors for RNAi knockdown of all isoforms of Pax6 or Pax6b in human and mouse ESCs. RNAi is driven by the U6 promoter, whereas the antibiotics-resistant genes and GFP are driven by the PGK promoter.

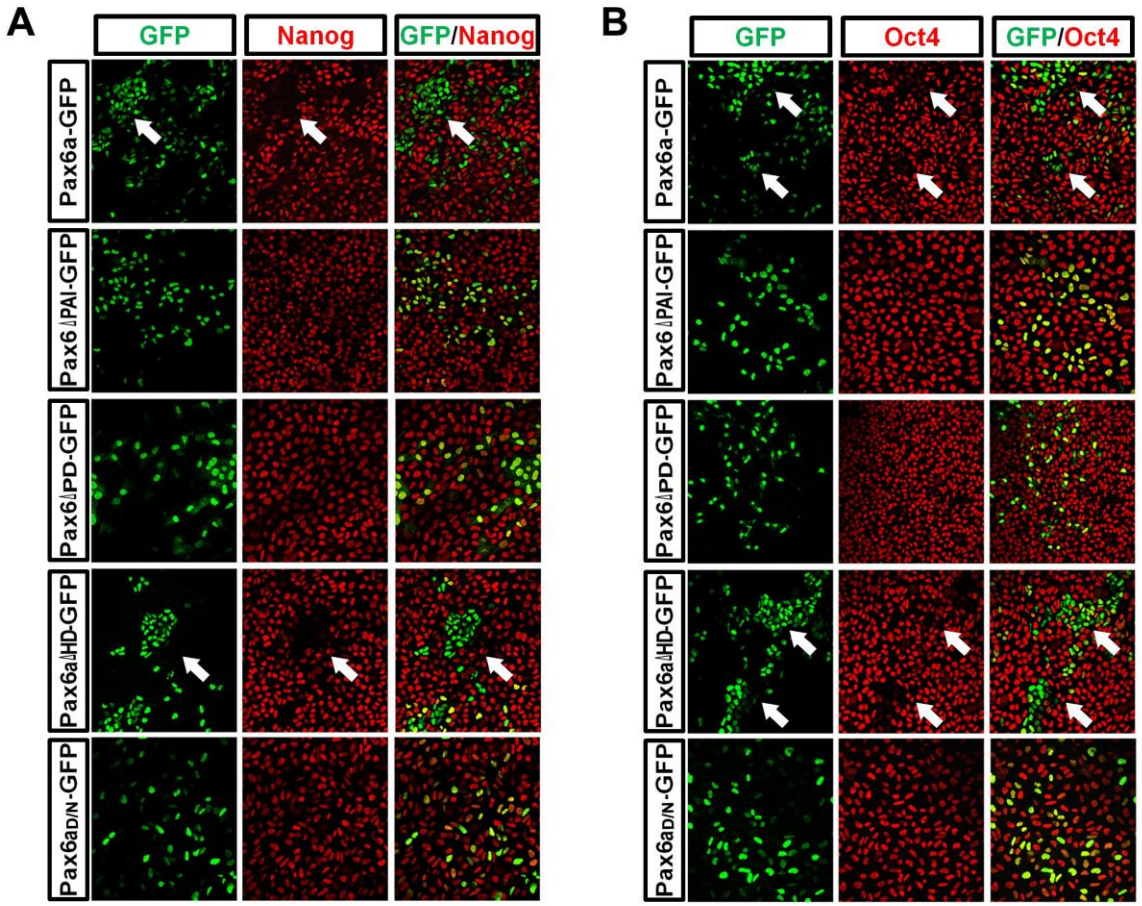
(B) Pax6 is also required for dual SMAD inhibition-mediated NE specification. Luc RNAi and Pax6 RNAi lines were grown on matrigel. Noggin (500ng/ml) and SB431542 (10μM) were added in the culture medium for 4 days to induce a neural fate (Chambers et al., 2009). Pluripotent genes and NE genes were analyzed by qRT-PCR.

(C) Knockdown of Pax6 in mouse ESCs does not affect neural induction. The Sox1/GFP reporter line (46C) infected with luciferase or Pax6 RNAi lentivirus (no GFP) is differentiated toward the neural lineage for 8 days. GFP expression, indicative of neural differentiation, is observed under the fluorescent microscope.

(D) GFP expression in mouse Sox1/GFP reporter lines is quantified by flow cytometry.

(E) Western blotting analysis verifies the knockdown of Pax6 and no change of endogenous Sox1 expression during mouse D3 line neural differentiation.

(F) Healthy neurons are generated from both the control and Pax6 knockdown mouse ESC groups at day 14. Scale bars, 50 μm.


A
GFP
Nanog
GFP/Nanog
Pax6a-GFP
Pax6 ΔPAI-GFP
Pax6ΔPD-GFP
Pax6aΔHD-GFP
Pax6aD/N-GFP
B
GFP
Oct4
GFP/Oct4
Pax6a-GFP
Pax6 ΔPAI-GFP
Pax6ΔPD-GFP
Pax6aΔHD-GFP
Pax6aD/N-GFP

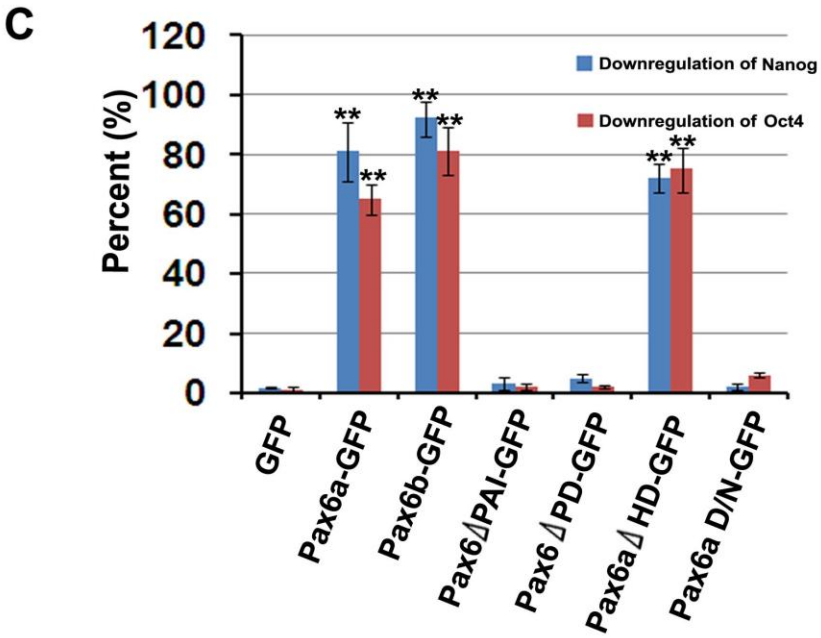

C
Percent (%)
120
100
80
60
40
20
0
Downregulation of Nanog
Downregulation of Oct4
**
GFP
Pax6a-GFP
Pax6b-GFP
Pax6ΔPAI-GFP
Pax6ΔPD-GFP
Pax6aΔHD-GFP
Pax6a D/N-GFP

Figure S3. PD and PST transactivation domains, but not HD, are required for Pax6 NE inducing activity, Related to Figure 4.
(A) Nanog expression in hESCs with overexpression of wild type and mutant Pax6.
(B) Oct4 expression in hESCs with overexpression of wild type and mutant Pax6.
(C) Percentage of cells with Nanog or Oct4 downregulation among overall Pax6 overexpressing cells (GFP positive). Removal of PAI, PD or PST transactivation domain all abrogates the ability of Pax6a to downregulate Nanog and Oct4. Deletion of the entire HD within Pax6a does not affect Pax6a in differentiating hESCs and Pax6aΔHD-expressing cells also aggregate to form neural rosettes. **, $p<0.01$ vs GFP control.

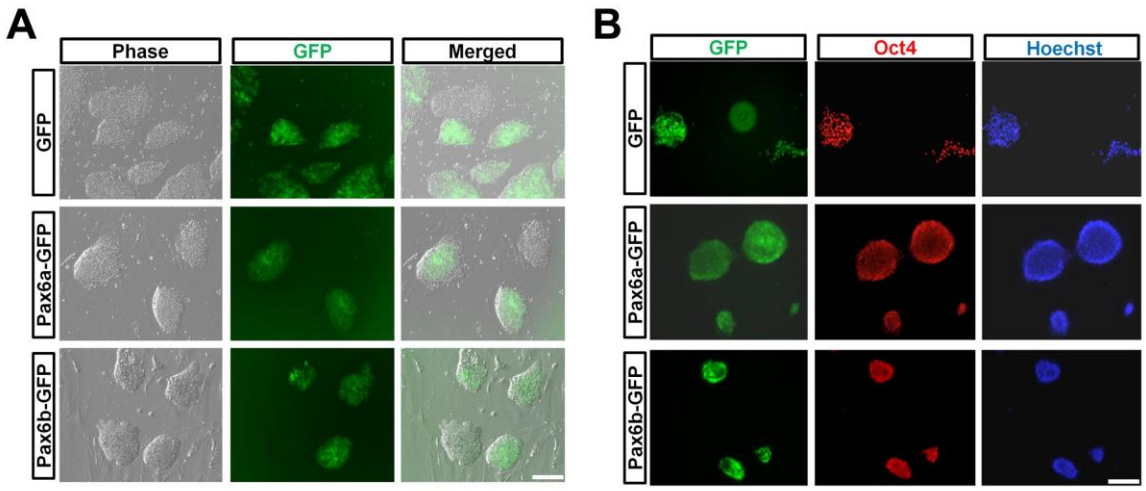


Figure S4. Overexpression of Pax6 in mouse ESCs does not cause differentiation, Related to Figure 5.
(A) Mouse ESCs express GFP, Pax6a-GFP, or Pax6b-GFP retain the typical ESC morphology and the same growth rate.
(B) Overexpression of GFP, Pax6a-GFP, or Pax6b-GFP in mouse ESCs does not alter Oct4 expression in the ESC colonies. Scale bars, 50 μm.

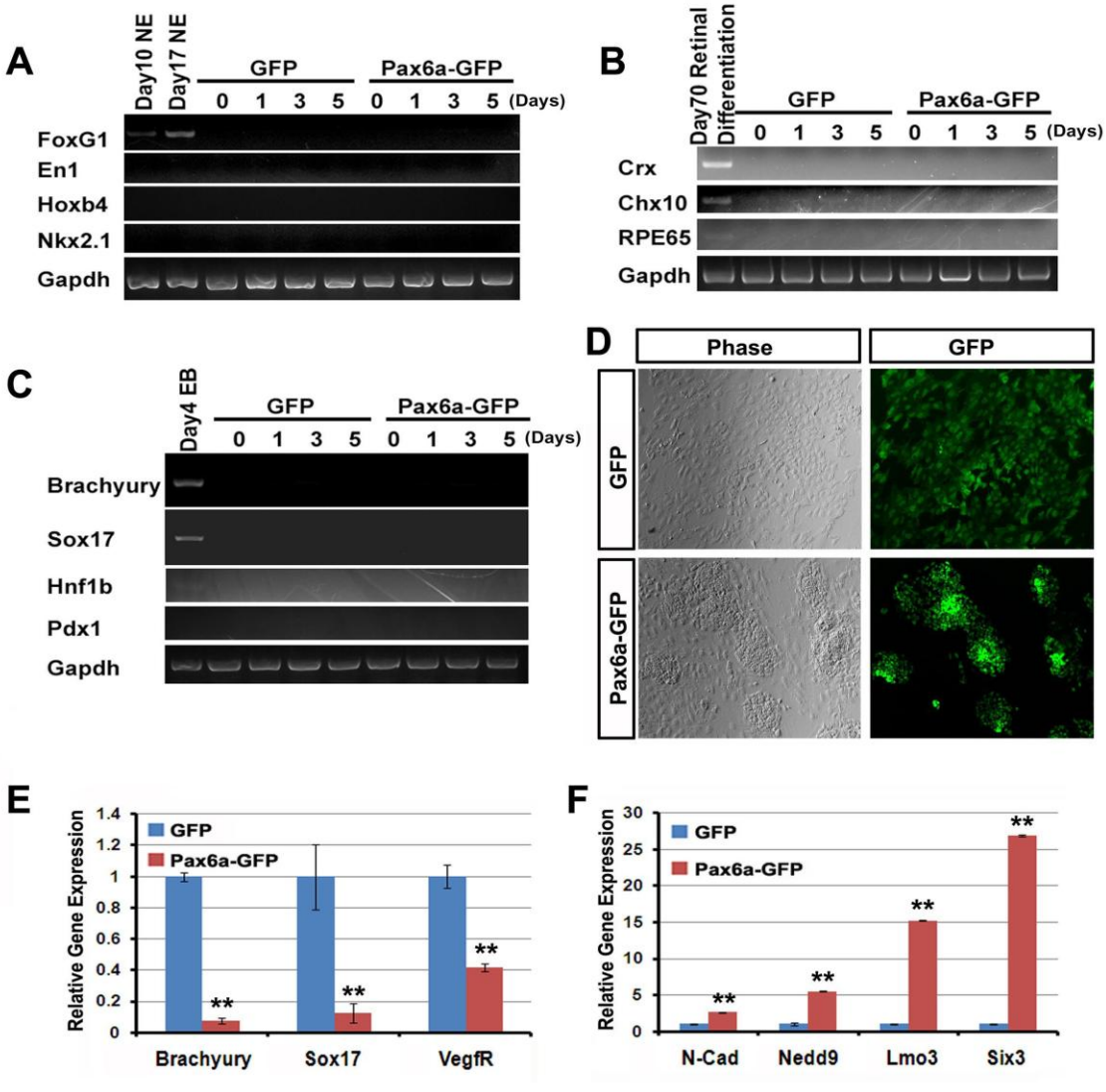

A
Day10 NE
Day17 NE
GFP
Pax6a-GFP
0 1 3 5 0 1 3 5 (Days)
FoxG1
En1
Hoxb4
Nkx2.1
Gapdh
B
Day70 Retinal Differentiation
GFP
Pax6a-GFP
0 1 3 5 0 1 3 5 (Days)
Crx
Chx10
RPE65
Gapdh
C
Day4 EB
GFP
Pax6a-GFP
0 1 3 5 0 1 3 5 (Days)
Brachyury
Sox17
Hnf1b
Pdx1
Gapdh
D
Phase
GFP
GFP
Pax6a-GFP
E
Relative Gene Expression
GFP
Pax6a-GFP
Brachyury
Sox17
VegfR
F
Relative Gene Expression
GFP
Pax6a-GFP
N-Cad
Nedd9
Lmo3
Six3

Figure S5. Overexpression of Pax6a converts human ESCs to NE, Related to Figure 6.
(A) Overexpression of Pax6a-GFP in hESCs does not induce expression of *FoxG1*, *En1*, *Hoxb4* or *Nkx2.1*, representative genes for forebrain, midbrain, spinal cord or ventral telencephalon, respectively. This suggests that Pax6a converts hESCs to primitive NE but not regional progenitors.
(B) Overexpression of Pax6a does not induce retinal gene expression, however, hESCs, after 70 days of differentiation (Meyer et al., 2009), now expressed retinal genes.
(C) Pax6a overexpressing cells do not express mesodermal, endodermal and pancreatic makers.
(D) Pax6a drives hESCs into NE even under mesoendodermal differentiation conditions. Inducible Pax6a-GFP hESCs were plated on matrigel and treated with doxycycline overnight to induce Pax6a-GFP expression. Cells were then grown in RPMI medium containing 100ng/ml activin A and 5μM Bio (GSK3 inhibitor to mimic Wnt signaling) for 24 hours followed by culturing in 2% FBS/RPMI to initiate mesoendodermal differentiation (Kroon et al., 2008). Under a fluorescent microscope, Pax6a-GFP cells still aggregate and form rosettes.
(E) qRT-PCR analysis revealed that Pax6a-GFP expression inhibits mesoendodermal gene expression induced by activin A and Bio. **, $p<0.01$ vs GFP control.
(F) Pax6a-GFP overexpression increases NE genes even under the conditions that favor mesoendoderm differentiation. **, $p<0.01$ vs GFP control.

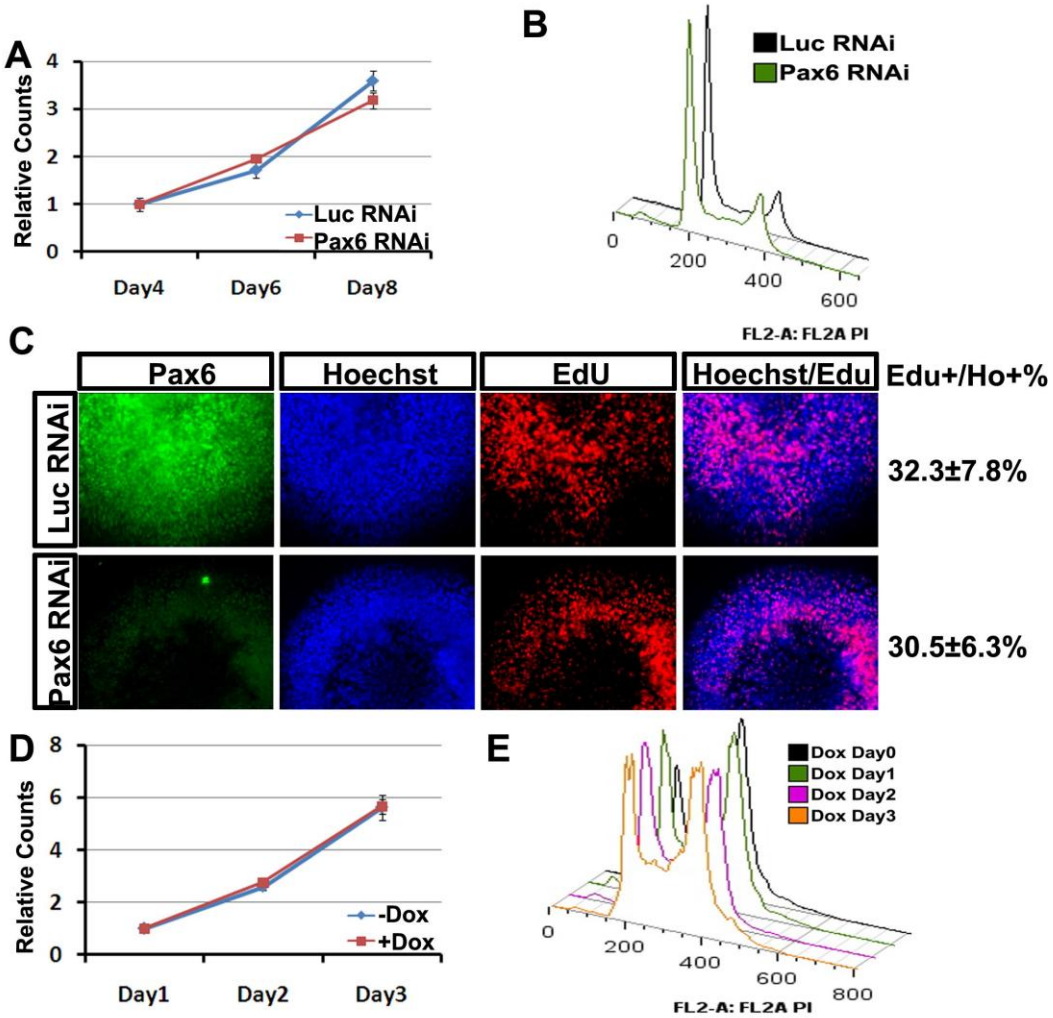

A
Relative Counts
4
3
2
1
0
Luc RNAi
Pax6 RNAi
Day4
Day6
Day8
B
Luc RNAi
Pax6 RNAi
0
200
400
600
FL2-A: FL2A PI
C
Pax6
Hoechst
EdU
Hoechst/Edu
Edu+/Ho+%
Luc RNAi
32.3±7.8%
Pax6 RNAi
30.5±6.3%
D
Relative Counts
8
6
4
2
0
-Dox
+Dox
Day1
Day2
Day3
E
Dox Day0
Dox Day1
Dox Day2
Dox Day3
0
200
400
600
800
FL2-A: FL2A PI
F
Pax6
Hoechst
EdU
Hoechst/Edu
Edu+/Ho+%
Day 0
47.3±5.8%
Day 1
46.6±8.9%
Day 2
48.5±7.2%
Day 3
48.2±4.0%

Figure S6. Overexpression or knockdown of Pax6 does not have biased proliferation or cell death, Related to Figure 7.
(A) Growth curves for Luc RNAi or Pax6 RNAi human ESCs during NE differentiation.
(B) Cell cycle comparison between Luc RNAi and Pax6 RNAi human ESCs after 8 days of differentiation. Knockdown of Pax6 does not obviously cause cell death. Left peak, G1 phase; Right peak, G2 phase.
(C) Luc RNAi and Pax6 RNAi human ESCs show similar proliferation rate after 8 days of differentiation as analyzed by EdU incorporation.
(D) Growth curves for inducible Pax6a-GFP hESC line in the presence or absence of doxycycline.
(E) Doxycycline induced Pax6a-GFP overexpression does not obviously alter cell cycle parameters.
(F) Doxycycline induced Pax6a-GFP overexpression does not show accelerated cell proliferation.

Movie S1: Time lapse video recording of GFP inducible hESC line during doxycycline treatment, Related to Figure 6.
Movie S2: Time lapse video recording of Pax6a-GFP inducible hESC line during doxycycline treatment, Related to Figure 6.

Zhang et al., Supplementary Table. 1, Related to Figure 2.
List of most upregulated genes in control and Pax6-knockdown cells after 6 days of differentiation

Gene Name	Gene ID	Affimetrix ID	Gene Identifier	UG Cluster	Upregulated Ratio in Luc RNAi	Upregulated Ratio in Pax6 RNAi
Fibroblast growth factor 9	FGF9	206404_at	NM_002010	Hs.111	398.7	178
Calpain 6	CAPN6	202966_at	NM_014289	Hs.496593	201.6	58.3
Sp8 transcription factor	SP8	237449_at	BF447038	Hs.195922	105.5	29.1
Nuclear receptor subfamily 2, group F, member 1	NR2F1	209505_at	AI951185	Hs.519445	82.46	30.3
Dachshund homolog 1	DACH1	228915_at	AI650353	Hs.129452	73.95	15.4
Zinc finger protein 521	ZNF521	226676_at	AK021452	Hs.116935	71.52	11.6
V-maf musculoaponeurotic fibrosarcoma oncogene homolog	MAF	209348_s_at	AF055376	Hs.134859	69.13	35.9
Mitogen-activated protein kinase 10	MAPK10	204813_at	NM_002753	Hs.125503	68.72	61.4
Paired box 6	PAX6	235795_at	AW088232	Hs.591993	63.51	1.94
GABA receptor	GABRP	205044_at	NM_014211	Hs.26225	54.1	26.4
Dual specificity phosphatase 6	DUSP6	208891_at	BC003143	Hs.298654	47.71	13.6
Fibronectin leucine rich transmembrane protein 3	FLRT3	219250_s_at	NM_013281	Hs.41296	44.3	12.4
Four jointed box 1	FJX1	219522_at	NM_014344	Hs.39384	44.19	22.9
EPH receptor A4	EPHA4	228948_at	T15545	Hs.371218	42.26	16.5
Cadherin 2, type 1, N-cadherin (neuronal)	N-CAD	203441_s_at	NM_001792	Hs.464829	41.87	9.22
Growth arrest-specific 1	GAS1	204457_s_at	NM_002048	Hs.65029	35.7	19.7
Microtubule-associated protein 2	MAP2	225540_at	BF342661	Hs.368281	35.07	15.6
Frizzled-related protein	FRZB	203698_s_at	NM_001463	Hs.128453	31.09	7.54
Ectodermal-neural cortex	ENC1	201341_at	NM_003633	Hs.104925	28.88	2.5
Gremlin 1, cysteine knot superfamily, homolog	GREM1	218469_at	NM_013372	Hs.40098	28.28	6.59
Forkhead box G1	FOXG1	206018_at	NM_005249	Hs.695962	27.46	3.52
GATA binding 3	GATA3	209604_s_at	BC003070	Hs.524134	21.92	9.31

Hyaluronan and proteoglycan link protein 1	HAPLN1	205523_at	U43328	Hs.591758	18.74	3.68
LIM homeobox 2	LHX2	206140_at	NM_004789	Hs.696425	17.89	2.74
Dickkopf homolog 1 (Xenopus laevis)	DKK1	204602_at	NM_012242	Hs.40499	15.95	4.54
Lix1 homolog	LIX1	230865_at	N29837	Hs.656702	14.36	9.91
Nuclear receptor subfamily 2, group F, member 2	NR2F2	215073_s_at	AL554245	Hs.701977	14.33	1.39
LIM homeobox 5	LHX5	208333_at	NM_022363	Hs.302029	14.27	3.65
Spondin 1, extracellular matrix protein	SPON1	209436_at	AB018305	Hs.705394	11.8	2.35
Lumican	LUM	201744_s_at	NM_002345	Hs.406475	11.41	4.15
SIX homeobox 3	SIX3	206634_at	NM_005413	Hs.658847	11.24	2.95
Wingless-type MMTV integration site family, member 5B	WNT5B	221029_s_at	NM_030775	Hs.306051	10.82	4.73
IGF binding protein 5	IGFBP5	211959_at	AW007532	Hs.635441	10.56	6.4
Retina and anterior neural fold homeobox	RAX	208242_at	NM_013435	Hs.278957	10.28	5.76
Zinc finger E-box binding homeobox 2	ZEB2	203603_s_at	NM_014795	Hs.34871	9.825	5.9
LIM domain only 3 (rhombotin-like 2)	LMO3	204424_s_at	AL050152	Hs.504908	9.038	2.67
SIX homeobox 6	SIX6	207250_at	NM_007374	Hs.194756	9.02	4.42
Transcription factor AP-2 alpha	TFAP2A	204653_at	BF343007	Hs.519880	8.167	3.9
Meis homeobox 1	MEIS1	204069_at	NM_002398	Hs.526754	7.353	1.91
Cell adhesion molecule 1	CADM1	209032_s_at	AF132811	Hs.370510	6.953	1.53
Collagen, type IV, alpha 6	COL4A6	211473_s_at	U04845	Hs.145586	5.92	0.05
Delta-like 1 homolog	DLK1	209560_s_at	U15979	Hs.533717	5.708	2.47
Transmembrane protein 46	TMEM46	230493_at	AW664964	Hs.433791	5.173	1.9
Cadherin 6, type 2, K-cadherin	CDH6	205532_s_at	AU151483	Hs.171054	5.065	1.2
Fibroblast growth factor 8	FGF8	208449_s_at	NM_006119	Hs.57710	4.385	1.25
Endothelial PAS domain protein 1	EPAS1	200878_at	AF052094	Hs.468410	3.892	0.13
Fatty acid binding protein 7, brain	FABP7	205030_at	NM_001446	Hs.26770	3.761	1.72
Meis homeobox 2	MEIS2	207480_s_at	NM_020149	Hs.510989	3.293	0.31

Zhang et al., Supplementary Table. 2, Related to Figure 2.
List of most downregulated genes in control and Pax6-knockdown cells after 6 days of differentiation

Gene Name	Gene ID	Affimetrix ID	Gene Identifier	UG Cluster	Down-regulated Ratio in Luc RNAi	Down-regulated Ratio in Pax6 RNAi
HL14 gene encoding beta-galactoside-binding lectin, 3 end, clone 2	-	216405_at	M14087	Hs.701974	95.72837	68.22603
Left-right determination factor 1	LEFTY1	206268_at	NM_020997	Hs.656214	89.90803	31.57958
CDNA FLJ12624 fis, clone NT2RM4001754	-	216319_at	AK022686	Hs.636868	70.86941	30.67316
Nanog homeobox	NANOG	220184_at	NM_024865	Hs.661360	41.71951	7.847929
Chemokine (C-X-C motif) ligand 5	CXCL5	214974_x_at	AK026546	Hs.89714	24.46746	11.28631
Left-right determination factor 2	LEFTY2	206012_at	NM_003240	Hs.520187	22.41169	10.57038
Metallothionein 1H	MT1H	206461_x_at	NM_005951	Hs.438462	21.42105	3.860621
Hairy/enhancer-of-split related with YRPW motif 2	HEY2	219743_at	NM_012259	Hs.144287	21.24448	3.07047
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3	GALNT3	203397_s_at	BF063271	Hs.170986	19.08385	2.537366
Teratocarcinoma-derived growth factor 1	TDGF1	206286_s_at	NM_003212	Hs.385870	19.01562	4.435166
Metallothionein 1 pseudogene 2	MT1P2	211456_x_at	AF333388	Hs.632513	15.04931	3.652626
integral membrane protein 2A	-	202746_at	AL021786	-	14.72373	8.572848
Chromosome 9 open reading frame 135	C9orf135	243610_at	AI768674	Hs.444459	14.09766	1.880157
Metallothionein 1X	MT1X	208581_x_at	NM_005952	Hs.374950	13.19	4.166887
Galanin prepropeptide	GAL	214240_at	AL556409	Hs.278959	13.13642	6.820273
CDNA FLJ35259 fis, clone PROST2004251	-	1559280_a_at	AA483467	Hs.105196	12.98873	2.594648

CDNA FLJ35153 fis, clone PLACE6010765	-	226498_at	AA149648	Hs.700704	12.6494	5.031425
B-cell CLL/lymphoma 11A	BCL11A	219497_s_at	NM_022893	Hs.370549	12.07195	1.922503
Phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	204286_s_at	NM_021127	Hs.96	11.94802	3.375307
Metallothionein 1G	MT1G	204745_x_at	NM_005950	Hs.433391	11.27146	4.283678
Homo sapiens cDNA FLJ10768 fis, clone NT2RP4000150	-	224833_at	BE218980	-	11.23587	4.354258
Chromosome 6 open reading frame 117	C6orf117	227226_at	AA418816	Hs.370055	10.49588	4.005213
Indoleamine-pyrrole 2,3 dioxygenase	INDO	210029_at	M34455	Hs.840	10.42948	2.55164
Homo sapiens metallothionein 1L (MT1L)	-	204326_x_at	NM_002450	-	10.2986	5.527771
peptidylprolyl isomerase C	-	204517_at	BE962749	-	9.94312	3.302816
Hypothetical protein (ORF1), clone 00275	-	230195_at	BF672169	Hs.152129	9.09496	4.921882
CD9 molecule	CD9	201005_at	NM_001769	Hs.114286	8.408244	4.141272
Actin, alpha 1, skeletal muscle	ACTA1	203872_at	NM_001100	Hs.1288	8.40739	5.517276
Metallothionein 2A	MT2A	212185_x_at	NM_005953	Hs.647371	8.399238	3.941177
Metallothionein 1E	MT1E	212859_x_at	BF217861	Hs.534330	7.607666	3.152202
Transgelin	TAGLN	205547_s_at	NM_003186	Hs.632099	7.446828	4.015787
Serpin peptidase inhibitor, clade B, member 9	SERPIN B9	209723_at	BC002538	Hs.104879	6.975821	2.159885
POU class 5 homeobox 1	POU5F1	208286_x_at	NM_002701	Hs.249184	6.339634	2.893297
POU class 6 homeobox 1	POU6F1	210265_x_at	AF268617	Hs.646545	6.138216	3.016898
Deleted in bladder cancer 1	DBC1	205818_at	NM_014618	Hs.532316	5.560314	2.455385
BCL2-associated athanogene 2	BAG2	209406_at	AF095192	Hs.55220	5.147716	3.618565
Secretoglobin, family 3A, member 2	SCGB3A 2	228782_at	BG540454	Hs.483765	5.081696	1.550533
Homeobox C4	HOXC4	206858_s_at	NM_004503	Hs.549040	4.105078	1.875036
Metallothionein 1F	MT1F	213629_x_at	BF246115	Hs.513626	4.060011	2.262267
Embryonic stem cell related protein	HESRG	231381_at	BF223023	Hs.652553	4.050482	2.438316
Telomeric repeat binding factor 1	TERF1	203449_s_at	NM_017489	Hs.442707	4.038736	2.252147
Cell division cycle associated 7-like	CDCA7L	225081_s_at	AK022955	Hs.520245	3.507665	2.403946
V-myc	MYC	202431_s_at	NM_002467	Hs.202453	2.764843	1.955703

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