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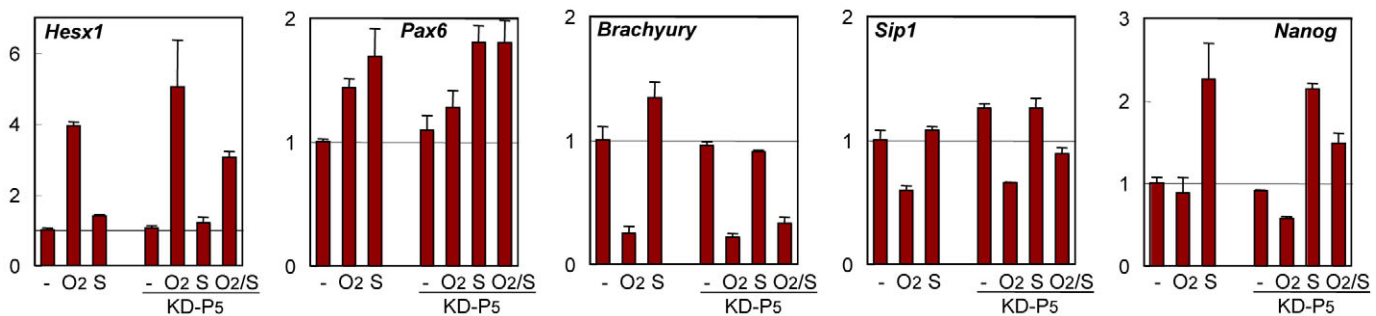
There was an error published in *Development* **139**, 3926-3937.

Fig. S6 was incorrect. The correct Fig. S6, as shown below, now appears in the supplementary data.

We apologise to the authors and readers for this mistake.



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Transcriptional regulatory networks in epiblast cells and during anterior neural plate development as modeled in epiblast stem cells

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SUMMARY

Somatic development initiates from the epiblast in post-implantation mammalian embryos. Recent establishment of epiblast stem cell (EpiSC) lines has opened up new avenues of investigation of the mechanisms that regulate the epiblast state and initiate lineage-specific somatic development. Here, we investigated the role of cell-intrinsic core transcriptional regulation in the epiblast and during derivation of the anterior neural plate (ANP) using a mouse EpiSC model. Cells that developed from EpiSCs in one day in the absence of extrinsic signals were found to represent the ANP of ~E7.5 embryos. We focused on transcription factors that are uniformly expressed in the E6.5 epiblast but in a localized fashion within or external to the ANP at E7.5, as these are likely to regulate the epiblast state and ANP development depending on their balance. Analyses of the effects of knockdown and overexpression of these factors in EpiSCs on the levels of downstream transcription factors identified the following regulatory functions: cross-regulation among Zic, Otx2, Sox2 and Pou factors stabilizes the epiblastic state; Zic, Otx2 and Pou factors in combination repress mesodermal development; Zic and Sox2 factors repress endodermal development; and Otx2 represses posterior neural plate development. All of these factors variably activate genes responsible for neural plate development. The direct interaction of these factors with enhancers of *Otx2*, *Hesx1* and *Sox2* genes was demonstrated. Thus, a combination of regulatory processes that suppresses non-ANP lineages and promotes neural plate development determines the ANP.

KEY WORDS: Epiblast stem cells, Anterior neural plate, Zic factors, Pou factors, Otx2, Sox2

INTRODUCTION

The epiblast serves as the primordium of all somatic lineages in amniotes, where the earliest derivative is the anterior neural plate (ANP). Despite profound interest in the process by which somatic lineages are generated, cell-intrinsic and transcription factor-dependent regulatory mechanisms remain poorly understood, primarily because of the difficulty in accessing the epiblast in post-implantation mammalian embryos. The cell-extrinsic mechanisms that regulate the fate of the epiblast are better understood. It has been shown, for example, that Nodal antagonists secreted from the anterior visceral endoderm disrupt Nodal signaling, which otherwise stabilizes the epiblast state and elicits ANP development (Camus et al., 2006; Perea-Gomez et al., 2002).

The ANP and the posterior neural plate (PNP) are generated via different mechanisms. The ANP is derived directly from the epiblast (Iwafuchi-Doi et al., 2011), whereas the development of the PNP from the epiblast passes through an intermediate state of axial stem cells, which are common precursors for the PNP and paraxial mesoderm (Kondoh and Takemoto, 2012; Takemoto et al., 2011; Tzouanacou et al., 2009).

Recent success in establishing cell lines directly from the egg cylinder epiblast [epiblast stem cells (EpiSCs)] (Brons et al., 2007; Tesar et al., 2007) has opened up new avenues to investigate the cell-intrinsic mechanisms in the epiblast and its derivatives. The epiblast state of EpiSCs is maintained by activin (Nodal substitute) and Fgf2 signaling, and the interruption of these pathways elicits the development of neural plate cells (NPCs), partly mimicking the action of Nodal antagonists during the derivation of the ANP in embryos.

In our present study, we first characterized EpiSCs and their immediately derived NPCs under culture conditions without supply of extrinsic signals. The expression profiles of transcription factor genes under these conditions were compared with those in embryonic tissues. Our findings indicate that the immediate derivatives of EpiSCs under the NPC culture conditions represent the ANP cell state in ~E7.5 embryos. Taking advantage of this observation, we then systematically analyzed the effects on downstream genes of the knockdown and overexpression in EpiSCs of several transcription factors.

These analyses of the transcriptional regulatory networks indicated intricate cross-regulation among the factors to stabilize the epiblast state and to derive the ANP. During the derivation of ANP, inhibitory mechanisms were found to play key roles to suppress the development of mesodermal, endodermal and PNP cells, confining the developmental pathway of epiblast derivatives to ANP. This analysis also highlights the crucial involvement of Zic2/3, Otx2 and Sox2 in these processes. This study thus highlights the advantages of using EpiSCs to examine in detail the regulatory mechanisms that underlie the derivation of somatic lineages.

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MATERIALS AND METHODS

EpiSC cultures

EpiSCs (Tesar et al., 2007) were cultured under two different sets of conditions: one for epiblastic state maintenance involving the supply of 20 ng/ml activin and 10 ng/ml Fgf2 in N2B27 medium on fibronectin-coated dishes; and another for the promotion of NPC development without the addition of growth factors on gelatin-coated dishes (Iwafuchi-Doi et al., 2011).

qRT-PCR analysis

Total RNA was extracted from the epiblastic (inner) layer of egg cylinder stage mouse embryos and EpiSCs, and processed for qRT-PCR analysis as described previously (Iwafuchi-Doi et al., 2011). The mRNA levels relative to *Gapdh* were quantified based on standard curves using cloned cDNA sequences. Primer sequences are listed in supplementary material Table S1.

Microarray analysis

Total RNAs extracted from duplicate epiblastic, NP1 and NP2 cultures were analyzed using an Agilent SurePrint G3 Mouse GE 8×60k Microarray. The normalized data using Agilent Feature Extraction were averaged for duplicate samples, and the data for transcription factor genes, selected by GO filtering and itemized inspection, were analyzed using Excel 2010 (Microsoft) functions. The original data are deposited in the NCBI GEO database with accession number GSE38085.

Knockdown and overexpression of transcription factor genes

shRNA vectors were constructed in pSilencerU6puro (Ambion) using the sequences listed in supplementary material Table S2. For knockdown analysis, 300 ng shRNA vector DNA was complexed with Lipofectamine 2000 (Invitrogen), added to a suspension of 4×10^5 dissociated EpiSCs, and the mixture plated in a well of a 12-well dish (Falcon 3043). Similarly, for overexpression analysis, 50 ng pCAGGS-based expression vector (Sawicki et al., 1998) for each transcription factor was mixed with 300 ng control pSilencerU6puro and used for transfection. After 8 hours, puromycin was added at 5 μg/ml to select transfected cells, and after 24 hours RNAs were extracted for analysis.

Transactivation assays using 10T1/2 fibroblasts

10T1/2 fibroblasts in a 24-well dish were transfected using Lipofectamine 2000 with DNA mixtures containing (per well) enhancer-δ51-Luciferase constructed in pGL4.10 (120 ng; Promega), pRL-Tk-Renilla luciferase (20 ng; Promega), and varying amounts of pCAGGS-based transcription factor expression vector. The following mouse gene enhancers were used: *Otx2* AN (Kurokawa et al., 2004), *Hesx1* ANP (Spieler et al., 2004) and *Sox2* $N2[73bp]_2$ (Iwafuchi-Doi et al., 2011). Luciferase activity was measured using the Dual Luciferase Assay Kit (Promega).

Production of *Otx2*$^{-/-}$ embryos transgenic for enhancer N2-*lacZ*

The enhancer N2-*lacZ* transgene (Iwafuchi-Doi et al., 2011) was injected into fertilized mouse eggs derived from *Otx2*$^{+/-}$ parents, and the genotypes of the implants were determined by PCR analysis of yolk sac DNA.

Other procedures

Immunofluorescence staining, the enhancer activity assay in EpiSCs and NPCs, and electrophoretic mobility shift assay (EMSA) were performed as described previously (Iwafuchi-Doi et al., 2011).

RESULTS

Dynamic changes in transcription factor gene expression during derivation of NPCs from the epiblast in embryos and from EpiSCs in culture

To derive NPCs from EpiSCs, we adopted a culture condition without the supply of exogenous signaling factors, which was employed for neural tissue derivation from embryonic stem (ES) cells in monolayer culturing (Ying et al., 2003). Under this condition, Pax6-immunopositive cells and TuJ1 (Tubb3)-positive cells appeared at NP3 (3 days in the NPC culture condition), and the proportion of these cells increased at NP4 (supplementary material Fig. S1), indicating progression of neural development. To characterize the initial process by which the NPCs are derived from EpiSCs, we investigated the expression profiles of a panel of transcription factor genes expressed in EpiSCs under the epiblast-maintenance condition and under the NPC condition for 1 (NP1) and 2 (NP2) days using qRT-PCR. In parallel, epiblasts from E6.5 egg cylinders and from the inner cell layer of the anterior and posterior halves of E7.5 embryos were collected (Fig. 1A) and analyzed for the purposes of comparison. The genes included in the panels characterize the epiblast and specific domains of the neural plate (Fig. 1B); references for the expression patterns of these genes in mouse embryos from E6.5 to E8.5 are listed in supplementary material Table S3. *Fgf5* was included in the analysis as a hallmark of the epiblast state, although it is not a transcription factor gene. Transcript levels of the genes were quantified using a standard curve method and are shown relative to 10^{-3} of the *Gapdh* level.

The expression levels of *Fgf5* and *Pou5f1*, which both characterize the epiblast state, are similar between E6.5 epiblast and EpiSC and decrease similarly in the E7.5 inner layers and NP1 cells (Fig. 1C,E). This indicated that EpiSCs in the epiblast maintenance condition share major characteristics of the E6.5 epiblast, and that E7.5 inner layer and NP1 cells have similarly departed the epiblast state. The decrease in the expression of other genes characteristic of epiblast, *Eomes* and *Nanog*, in E7.5 inner layers and NP1 cells (Fig. 1C) also supports this notion, although some differences in the net expression levels might partly reflect differences between the states of cells in embryos and in culture. The NP1 cells were also characterized by activation of *Sip1*, which is characteristic of the neural plate (Chng et al., 2010; Miyoshi et al., 2006). In addition, transcription factor genes that are expressed at high levels exclusively in the ANP (as represented by anterior inner layer), i.e. *Otx2*, *Sox2* and *Pou3f1* (see also Fig. 2, below), were strongly expressed in NP1 cells (Fig. 1D,E). These observations indicate that epiblastic EpiSCs and NP1 cells to a large extent represent the epiblast and ANP states in embryos, respectively. The strong expression of *Sox1* (Uchikawa et al., 2011; Wood and Episkopou, 1999), *Pou3f2* (Bouchard et al., 2005) and *Otx1* (Suda et al., 1999) in the neural plate is known to occur in embryos after E8.0. Here, these genes were activated only in NP2 cells, indicating that NP1 cells represent the neural plate in embryos prior to E8.0 (Fig. 1D-F).

The gradual decrease of *Pou5f1* expression in NP1 and NP2 cells and the steep decrease in *Pou3f1* expression in NP2 cells are consistent with the expression profiles documented for embryonic ANP (Perea-Gómez et al., 1999; Zwart et al., 1996). Genes characteristic of the ANP (*Hesx1*, *Otx1* and *Pax6*) were activated in the NP1/2 cells, whereas expression of genes characteristic of the PNP (*Gbx2* and *Nkx2.1*) remained very low (Fig. 1F,G). Moreover, the expression of brachyury (*T*) and *Sox17*, which mark mesodermal and endodermal precursors, respectively, was strongly inhibited (Fig. 1G). These observations indicate that EpiSCs develop mostly into the ANP under the NPC culture condition.

The NPC culture condition differed from that used for epiblast maintenance by the absence of activin and Fgf2 signaling and by the coating of the culture substrate with gelatin rather than fibronectin. Evaluation of the contribution of these differences to the promotion of ANP development (supplementary material Fig. S2) indicated that the effects of the culture conditions are synergistic, without a simple relationship with a single signaling system.

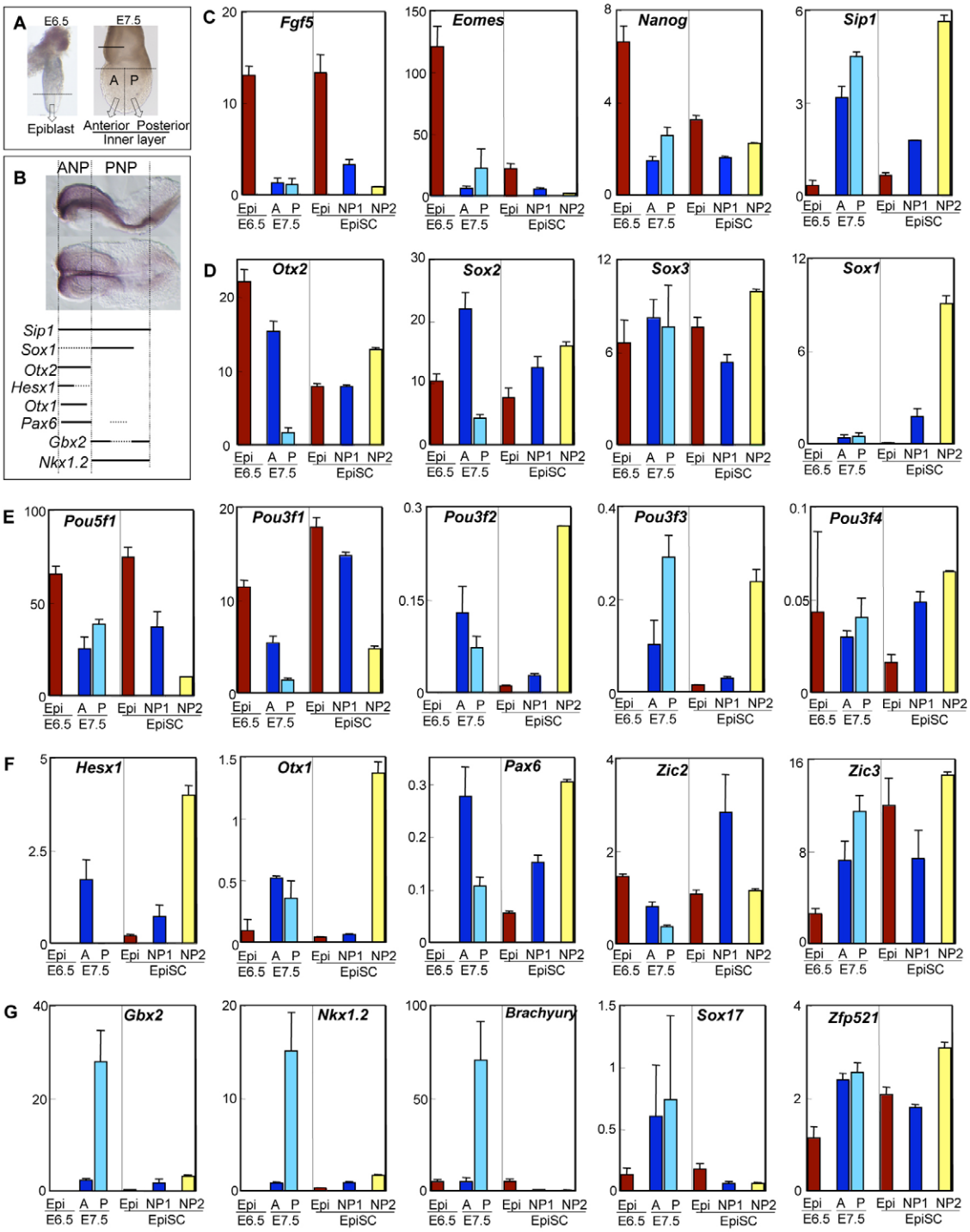


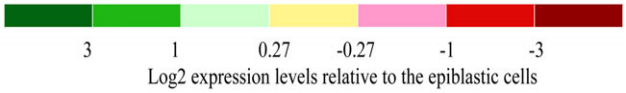
Fig. 1. qRT-PCR analysis of transcription factor gene expression in the epiblast and during neural plate derivation. (**A**) The regions of the mouse embryo isolated after removal of the endodermal layer and used in the analysis. Scale bar: 200 μm. (**B**) The axial expression domains of transcription factors in the ANP and PNP after E8.0, aligned to E8.0 mouse embryos hybridized for *Sox2* transcripts as shown in lateral (top) and dorsal (bottom) views. (**C-G**) Gene expression levels (relative to 10^{-3} *Gapdh*) determined by qRT-PCR are indicated for the embryonic regions indicated in A, epiblastic EpiSCs (Epi) and NP1 and NP2 cells ($n \geq 3$). The genes are loosely grouped according to their expression specificity or gene family. Error bars indicate s.e. ANP, anterior neural plate; PNP, posterior neural plate.

Microarray analysis of transcription factor expression profiles during NPC derivation

Using the same RNAs as used for the qRT-PCR analysis shown in Fig. 1, the expression profiles of a broader range of transcription factor genes were investigated using microarrays. After exclusion of genes that gave only background signals, including most Hox genes, the expression profiles of 994 transcription factor genes were analyzed during the progression from epiblastic to NP1 and NP2 cells. The results, as summarized in Table 1 and detailed in supplementary material Table S4, were generally consistent with

Table 1. Classification of transcription factor genes according to microarray expression profiles in epiblastic, NP1 and NP2 cells

Profile class	Expression profiles			No. assigned among 994 genes	Representative genes
	Epiblastic	NP1	NP2		
A				35	*Hesx1*, *Sip1*, *Irx3*
B				42	*Zic2*, *Gli2*
C				74	*Nr6a1*, *Sox1*, *Sox2*, *Hes5*, *Gbx2*
D				23	*Dmbx1*, *Sall1*
E				290	*Otx2*, *Nanog*, *Pou3f2*, *Pbx1*
F				265	*Sox3*, *Pou3f4*, *Zfp521*, *Pbx2*
G				126	*Snai2*, *Gli1*, *Hes1*
H				18	*Pou5f1*, *Mycn*, *Myc*
I				40	*Eomes*, *Klf5*
J				31	*Sox17*, *Foxa1*
K				18	*T*, *Foxa2*, *Gata6*, *Gsc*
L				13	*Sox15*, *Pou3f1*
M				19	*Foxb1*, *Zfxh2*



the qRT-PCR data for the genes presented in Fig. 1. Two-thirds (681) of the genes maintained their expression levels, i.e. any changes did not exceed 2-fold, as compared with the epiblastic state (Table 1, classes E-G), whereas the remaining genes were either activated or downregulated to greater extents. The majority of genes that were strongly activated in NP1 and/or NP2 cells (classes A-D) were associated with neural plate development. The genes that were activated strongly in NP1 cells included *Irx3*, which is activated at E7.5 in the ANP (Houweling et al., 2001), and also those indicated in the qRT-PCR analyses (*Hesx1*, *Sip1*, *Zic2*; Fig. 1). A significant proportion of the genes that were strongly downregulated in NP1 and NP2 cells (classes I-K) were representative of genes involved in endodermal (*Eomes*, *Sox17*, *Foxa1*, *Foxa2*, *Gata6*) or primitive streak-dependent mesodermal (*T*, *Gata6*, *Gsc*) development.

Nr6a1 (*Gcnf*) plays a major role in the repression of *Pou5f1* during the epiblast-neural plate stages of embryo development (Fuhrmann et al., 2001) and in the retinoic acid-induced differentiation of ES cells (Gu et al., 2005). *Nr6a1* was upregulated during the development of EpiSCs to NP2 cells (class C), accounting for the parallel decrease in *Pou5f1* expression (Fig. 1).

Class L genes, as exemplified by *Sox15*, were sharply repressed in NP2 cells, whereas class M genes, as exemplified by *Foxb1*, were maintained at low expression levels in NP1 and strongly activated in NP2 cells. Strong downregulation of the major Myc genes *Mycn* and *Myc* (class H) was also observed in NP2 cells. These observations indicate the progression of neural plate development from NP1 (<E8.0 ANP) to the less proliferative NP2 (>E8.0 ANP) cells.

Regional expression of transcription factors in embryos and EpiSC cultures

We noted that *Sox2*, *Pou3f1* and *Otx2* expressed from the E6.5 epiblast stage developed a clear anterior dominance at E7.5 (Fig. 1D,E), consistent with published histological data (Ang et al., 1994; Iwafuchi-Doi et al., 2011; Wood and Episkopou, 1999; Zwart et al., 1996). It has been reported that *Pou5f1* is downregulated in an inverse spatial relationship with the above three genes (Perea-Gómez et al., 1999). To confirm the expression profiles at the individual cell level, embryos at E6.5 and ~E7.5, as well as EpiSCs under both epiblast and NPC culturing conditions, were immunostained for these transcription factors.

In E6.5 embryos, Sox2, Pou5f1, Pou3f1 and Otx2 proteins were expressed uniformly throughout the epiblast nuclei (Fig. 2A). However, at ~E7.5, a high level of Sox2 was observed in the nuclei of the developing ANP (Fig. 2Ba), paralleled by a reduction in Pou5f1 (Fig. 2Bb) and by high expression of Pou3f1 (Fig. 2Bc) and Otx2 (Fig. 2Bd) in the same ANP domain.

In the epiblast state, the majority of EpiSC nuclei expressed Sox2, Pou3f1 and Otx2 with Pou5f1, analogous to the E6.5 epiblast (Fig. 2C). However, NP1 cells strongly expressed Pou3f1 and Otx2 at the periphery of their colonies (Fig. 2Dbe). Conversely, Pou5f1 was downregulated at the periphery of colonies but remained highly expressed in the medial portion, with an inverse spatial relationship with Pou3f1 and Otx2 (Fig. 2Dcd). This medial high Pou5f1 expression was reduced in NP2 cells (data not shown). Sox2 expression also showed a moderate peripheral enhancement (Fig. 2Da). Thus, the development of NPCs from EpiSCs, which initiates at the periphery of the colonies, shares many features with the development of the ANP in embryos.

Experimental design for transcription factor manipulation in EpiSCs

We reasoned that the transcription factors that are initially expressed throughout the epiblast but shift their expression sites at the beginning of ANP development might play crucial roles in the maintenance of the epiblastic state and in the initiation of ANP development, subject to their balance. We selected Sox2, Pou5f1, Pou3f1 and Otx2 (Fig. 2), as well as Nanog and Zic2/3, as candidate transcription factors with such functions. Nanog is known to stabilize the epiblastic state (Mitsui et al., 2003; Vallier et al., 2009) and to be downregulated in the forming ANP (Hart et

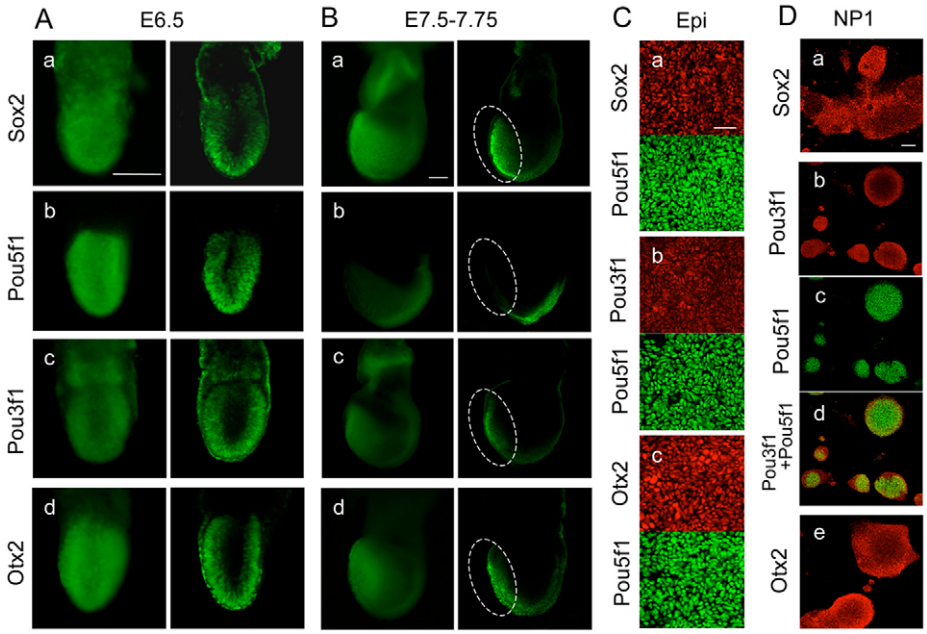


Fig. 2. Immunofluorescent detection of transcription factors in egg cylinder embryos and EpiSCs. (**A**,**B**) Mouse embryos at (A) E6.5 and (B) E7.5-7.75 were stained for (**a**) Sox2, (**b**) Pou5f1, (**c**) Pou3f1 and (**d**) Otx2. Whole-mount view (left) and optical sections through the medial plane (right). The ANP regions are outlined in the optical sections. (**C**) Epiblastic EpiSCs doubly stained for Pou5f1 and (**a**) Sox2, (**b**) Pou3f1 or (**c**) Otx2. (**D**) Immunofluorescence images of NP1 cells stained for (**a**) Sox2, (**b**) Pou3f1, (**c**) Pou5f1 (the same field as b), (**d**) a merge of b and c, and (**e**) Otx2. Scale bars: 100 μm.

al., 2004). The involvement of Zic factors in the early stages of neural development was first indicated in *Xenopus* (Mizuseki et al., 1998; Nakata et al., 1997) and supported by *Zic3* mutant mouse phenotypes (Inoue et al., 2007; Ware et al., 2006). We expected that knockdown and overexpression of these transcription factors in the EpiSCs would strongly affect the expression of immediate downstream genes, and that analysis of these effects, with the rationale shown in supplementary material Fig. S4, would reveal the transcriptional regulatory networks that regulate epiblast and ANP states.

Expression vectors for shRNAs (supplementary material Table S2) or pCAGGS-based expression vectors for the transcription factor genes were transfected into epiblastic cells, and transfected cells selected by puromycin (supplementary material Table S5). The effects on other genes of manipulating transcription factor levels were evaluated by qRT-PCR after 24 hours. The knockdown of transcription factor genes typically reduced their expression to ~30-40% of normal levels (supplementary material Table S6). Overexpression using pCAGGS-based vectors was very efficient, often reaching levels 100-fold greater than endogenous expression (supplementary material Table S6). However, lower expression levels were sufficient to affect downstream genes (see below; supplementary material Fig. S3).

The impact of single transcription factor manipulations in epiblastic cells

The results of the transcription factor knockdown and overexpression under the epiblast stabilized culture condition are shown in Fig. 3 and supplementary material Fig. S5. In cases indicative of activation or repression of a gene according to the criteria given in supplementary material Fig. S4, areas are highlighted in yellow or pink, respectively.

Genes characteristic of epiblasts (*Fgf5*, *Eomes* and *Nanog*) were activated by Zic2/3. *Fgf5* and *Eomes* were also activated by Otx2, indicating analogous regulation. *Fgf5* was also activated by Pou3f1, and *Nanog* was activated by Zic2/3 and Sox2 rather than by Otx2.

The regulation of *Sox2*, *Otx2* and *Pou5f1* suggested cross-regulation in the maintenance of the epiblast state as well as in the promotion of ANP development. *Otx2* and *Sox2* were activated by Zic2/3, and *Otx2* was also activated by Sox2. These regulations might maintain the epiblast state in cooperation with Pou5f1 and promote ANP development once Pou5f1 is downregulated. It is interesting to note that *Otx2* was activated by Pou5f1, whereas *Pou5f1* expression was repressed by overexpression of Otx2. This negative-feedback loop might prevent an excess of Pou5f1 in the epiblast that otherwise would promote neural development through the activation of a set of neural plate genes (*Hesx1*, *Pax6*, *Gbx2* and *Pou3f2/3*). The repression of *Pou5f1* by Otx2 and Sox2 might also reinforce the Nr6a1-dependent repression of *Pou5f1* (Fuhrmann et al., 2001) during ANP development (Table 1).

The regulation of *Sox1*, *Sip1* and *Otx1*, which are all strongly activated in the neural plate after E8.0 and in NP2 cells (Fig. 1), was similar and involved moderate activation by Zic2/3 and Pou5f1/3f1, and also by Otx2 in the case of *Sip1* and *Otx1*. Among the Pou3f factor genes, *Pou3f1* was uniquely activated by Zic2/3, Sox2 and Nanog, which is likely to reflect its strong expression in NP1 cells and downregulation in NP2 cells. The other three Pou3f genes responded variably (supplementary material Fig. S5). The regulation of *Zic3* and *Zic2* differed: *Zic3* was activated by Sox2, Pou5f1 and Nanog, whereas *Zic2* was activated only by Pou5f1.

Regulation of the ANP genes *Hesx1* and *Pax6* was characterized by their activation by Otx2 and Pou5f1/3f1. *Pax6* was also activated by Sox2 and Nanog. This suggested that Otx2 function is crucial for the neural plate to gain anterior characteristics. The regulation of two genes characteristic of PNP, *Gbx2* and *Nkx1.2*, showed interesting differences. *Gbx2* was strongly activated by Sox2 and to lesser extent by Pou5f1/3f1. By contrast, *Nkx1.2* was activated by Zic2/3 and Pou5f1 and repressed by Otx2 and Nanog. The Otx2-dependent repression of PNP-specific genes will be elaborated below.

Interestingly, the *T* gene, which represents mesoderm precursors, was strongly repressed by Otx2 and also inhibited by Zic2/3 and Pou5f1/3f1, showing opposite responses to many of the genes characteristic of neural plate described above. A low level of exogenous Otx2 expression was sufficient to exert efficient repression of *T* (supplementary material Fig. S3). *Sox17*, which is characteristic of endoderm precursors, was activated by Otx2 and

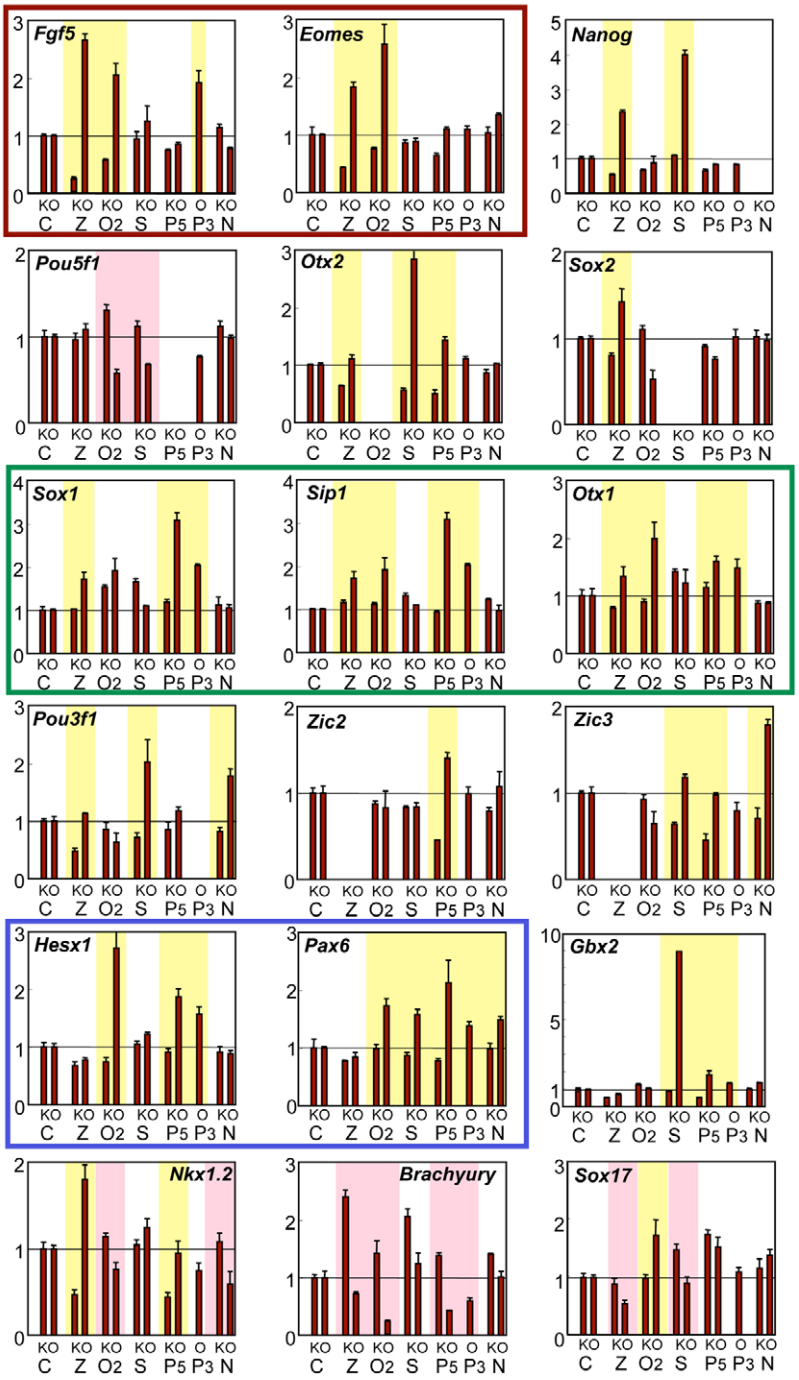


Fig. 3. Effects of knockdown and overexpression of transcription factors on their target genes under the epiblast maintenance culture condition. Impact of knockdown (K) or overexpression (O) of Zic2/3 (Z), Otx2 (O2), Sox2 (S), Pou5f1 (P5), Pou3f1 (P3) and Nanog (N) on the expression of the indicated genes as determined by qRT-PCR, compared with control (C) cultures. These effects were judged to have activated (yellow) or repressed (pink) a gene in accordance with the criteria shown in supplementary material Fig. S4. As the effects of Pou3f1 knockdown on downstream genes were minimal in the initial survey, only overexpression data are shown for Pou3f1 in order to distinguish between the common and unique functions of Pou5f1 and Pou3f. Data for genes found to be under analogous regulation are bound by a rectangle. Error bars indicate s.e.

repressed by Sox2, and possibly by a high level of Zic2/3. The expression of *Zfp521* (Kamiya et al., 2011) was unaffected by these transcription factor manipulations (supplementary material Fig. S5).

Thus, in many cases, Zic2/3 activity contributed crucially to the activation of epiblast- and neural plate-specific genes and to the repression of the mesodermal gene *T* and endodermal gene *Sox17*. Sox2 activity was strongly antagonistic to the endodermal genes. A strong association between Otx2 activity and the activation of ANP genes was also noted. Overexpression of Pou3f1 elicited analogous effects to that of Pou5f1. These and further regulatory interactions are compiled in Table 2 and are classified into lineage regulation and neural plate development categories (see below, Fig. 5).

Multiple transcription factor manipulations in the epiblastic state

To investigate the possible synergistic effects of altering transcription factor levels, we combined Pou5f1 knockdown with overexpression of Otx2 and Sox2 in the epiblast maintenance culture condition (Fig. 4A) as a means of mimicking the transcription factor changes that take place at the anteriormost part of the E7.0-7.5 embryonic inner layer (Fig. 2A). In most cases, the effects of these combined changes were not synergistic, displaying either one of the effects or their averages (supplementary material Fig. S6). However, in several interesting cases, synergistic or antagonistic effects of changing transcription factor levels were observed.

Table 2. The regulatory targets of individual transcription factors in cell lineage regulation and neural plate development as deduced from knockdown and overexpression effects

Transcription factor	Cell lineage regulation	Target genes	Neural plate development	Target genes
Zic2/3	Supporting epiblastic state	*Fgf5*, *Eomes*, *Nanog*, *Otx2*, *Sox2*	Promotion of ANP	*Otx2*, *Pou3f1*, *Sox2*
	Inhibition of mesodermal state	***T***	Promotion of >E8 neural plate	*Sox1*, *Sip1*, *Otx1*, *Gbx2**, *Nkx1.2**
'High expression'	**Inhibition of endodermal state**	***Sox17***		
Otx2	Supporting epiblastic state	*Fgf5*, *Eomes*	Promotion of ANP	*Hesx1*, *Pax6*, *Otx1*, *Sox2*‡
	Promotion of endodermal state	*Sox17*	Promotion of >E8 neural plate	*Sip1*
	Inhibition of mesodermal state	***T***	**Inhibition of PNP**	***Gbx2***, ***Nkx1.2***, ***Sox1****
'High expression'	**Preventing excess Pou5f1**	***Pou5f1***	Destabilizing epiblastic state	*Pou5f1*
Sox2	Supporting epiblastic state	*Nanog*, *Zic3*, *Otx2*	Promotion of ANP	*Otx2*, *Pou3f1*, *Pax6*
	Inhibition of endodermal state	***Sox17***, ***Eomes***	Promotion of PNP	*Gbx2*
'High expression'	**Preventing excess Pou5f1**	***Pou5f1***	Destabilizing epiblastic state	*Pou5f1*
Pou5f1/3f1	**Inhibition of mesodermal state**	***T***	Enhancement of some neural plate traits	*Hesx1*, *Pax6*, *Gbx2*
Pou5f1	Supporting epiblastic state	*Otx2*, *Zic2*, *Zic3*, *Sox2*‡	Inhibition of ANP	*Otx1*
			Enhancement of some neural plate traits	*Pou3f2*, *Pou3f3*
Nanog	Supporting epiblastic state	*Zic3*, *Sox3*, *Pou3f1*	Enhancement of some neural plate traits	*Pou3f1*, *Pax6*

Inhibitory effects contributing to the ANP state are indicated in bold.
*Target genes identified by knockdown experiments using NP1 cells (Fig. 4B).
‡Regulatory interactions indicated by enhancer analysis (Fig. 6).

Otx1 expression was augmented by knockdown of Pou5f1 alone, and further activated by combined overexpression of Otx2 and Sox2 (Fig. 4A). *Sox17* was activated by Otx2 overexpression (Fig. 3), and this was enhanced by combination with Pou5f1 knockdown. However, this activation of *Sox17* was completely repressed by exogenous *Sox2* expression (Fig. 4A). Similarly, *Eomes*, which is strongly expressed in epiblast cells (Fig. 1) and is implicated in endodermal development (Teo et al., 2011), was activated further by exogenous Otx2, but this Otx2-dependent activation was cancelled by the coexpression of exogenous Sox2 (Fig. 5A). These observations indicate that, whereas Otx2 promotes endodermal development by activating relevant transcription factor genes, Sox2 strongly antagonizes and overrides this Otx2 effect.

As indicated above (Fig. 3, supplementary material Fig. S5), *Gbx2*, which is characteristic of PNP, was strongly activated by Sox2. However, this Sox2-dependent activation was totally suppressed by coexpression of Otx2 (Fig. 4A). Another PNP gene, *Nkx1.2*, was moderately repressed by Otx2, and this effect was enhanced by a combined Pou5f1 knockdown. These results indicate that Sox2 expression generally supports neural plate development, regardless of whether it is the ANP or PNP. However, Otx2 inhibits the posterior characteristics and allows only the development of the ANP while also promoting anterior characteristics such as *Hesx1* expression.

Transcription factor manipulations that promote ANP development

In the above experiments, specific transcription factor levels were manipulated under epiblast maintenance culture conditions. If the observed regulatory interactions participate in ANP derivation, their proper combination would be expected to accelerate ANP development under the NPC culture condition. To characterize the gene regulatory network in NP1 cells, we individually knocked down Zic2/3 and Otx2 in these cells. The knockdown effects of these factors were similar to those in the epiblastic state for many genes, as exemplified by *Fgf5*, *Eomes* and *T* (Fig. 4B), indicating a similarity of the core gene regulatory network between the epiblastic and ANP states. However, *Sox1*, *Nkx1.2* and *Gbx2*, which are activated in the PNP of embryos after E8.0, responded more sharply to Zic2/3 knockdown by reducing their expression and to Otx2 knockdown by enhancing their expression (Fig. 4B). These observations confirmed the involvement of Zic2/3 in the activation of neural plate genes and the activity of Otx2 as an antagonist of PNP-dedicated genes.

We next tested the effects of Otx2 overexpression, Sox2 overexpression, Pou5f1 knockdown and combinations of these conditions in NP1 cells. The impact of Sox2 overexpression alone or in combination with a Pou5f1 knockdown was no greater than that of single factor manipulations (data not shown). However, the combination of Otx2 overexpression and Pou5f1 knockdown synergized their effects, either promoting ANP-specific gene expression or inhibiting PNP-specific genes (Fig. 4C). Expression of *Hesx1* and *Pax6*, which was moderately activated in control NP1 cells, and that of *Otx1*, which was activated only at the NP2 stage (Fig. 1F), was significantly augmented by the combination of Otx2 overexpression and Pou5f1 knockdown, in an additive fashion for *Hesx1* and *Otx1* and cooperatively for *Pax6* (Fig. 4C). By contrast, the PNP gene *Nkx1.2* was downregulated by Otx2 overexpression and Pou5f1 knockdown. The Otx2-dependent repression of *Gbx2* was also confirmed (Fig. 4C).

Immunostaining of NP1 cells with Pou5f1 knockdown and Otx2 overexpression confirmed augmented Pax6 expression in the majority of cells (Fig. 4D), which otherwise occurs only in NP3 cells in control cultures (supplementary material Fig. S1). As the strong expression of *Otx1* and *Pax6* in the ANP occurs only in mouse embryos after E8.0 (Fig. 1), the above observations indicated that the combination of Pou5f1 knockdown and Otx2 overexpression accelerated the development of the EpiSC-derived ANP cells to reach stages beyond E8.0 in 1 day of culturing.

Direct activation of ANP-associated transcription factor gene enhancers by core transcription factors

We sought to establish mechanistic links between the effects of the transcription factor manipulations and the action of the transcription factors themselves on their target genes. The enhancer

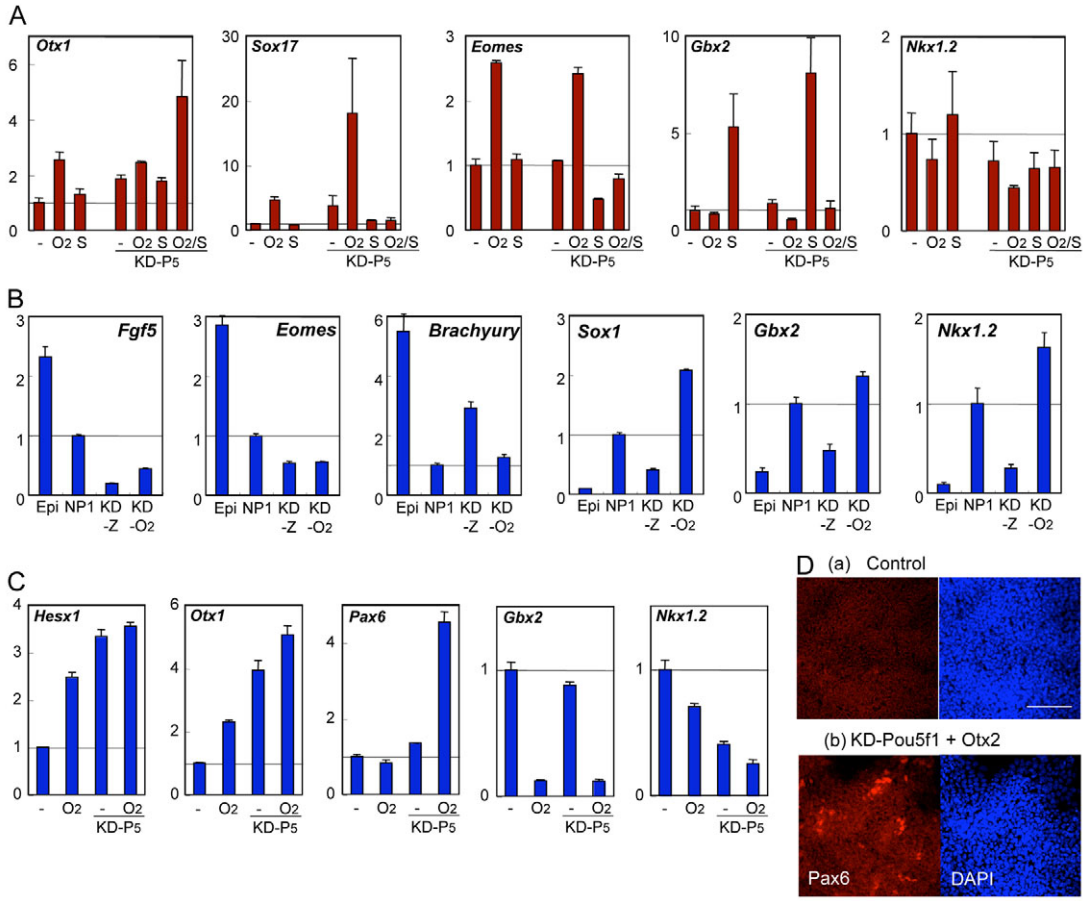


Fig. 4. Effects of combined manipulations of transcription factor levels in epiblastic and NP1 cells. (**A**) Effects of overexpression of Otx2 (O2), Sox2 (S) or both (O2/S) or in combination with Pou5f knockdown (KD-P5) in epiblastic cells, in comparison with levels in control (–) cells. (**B**) The effects of Zic2/3 knockdown (KD-Z) and Otx2 knockdown (KD-O2) on the expression of six representative transcription factor genes in NP1 cells. The effects on *Fgf5*, *Eomes* and brachyury (*T*) were similar to those in the epiblastic state (Fig. 3). However, genes expressed in the PNP of embryos after E8 (*Sox1*, *Gbx2* and *Nkx1.2*) clearly responded to these transcription factor manipulations in NP1 cells, which was not observed in the epiblastic state. (**C**) Effects of a combination of Otx2 overexpression and Pou5f1 knockdown in NP1 cells. (**D**) Pax6 immunostaining of NP1 control cultures (**a**) and cultures with Pou5f1 knockdown and Otx2 overexpression (**b**). Nuclei were counterstained with DAPI. Error bars indicate s.e. Scale bar: 100 μm.

sequences of the *Otx2*, *Hesx1* and *Sox2* genes that have been shown to be active in the embryonic ANP were cloned in a luciferase reporter vector for transactivation assays. 10T1/2 mouse embryo fibroblasts were chosen as the host cells, as the endogenous expression levels are negligible for those transcription factors to be expressed exogenously (supplementary material Table S7).

The *Otx2* AN enhancer (Kurokawa et al., 2004) containing a Pou binding site and a putative Zic binding site (supplementary material Fig. S7A) was activated by Pou3f1 and more strongly by Pou5f1, and this activation was augmented further by the coexpression of Zic2 (supplementary material Fig. S7A). This is consistent with the effects of Zic2/3 knockdown and Pou factor overexpression on Otx2 (Fig. 3). The *Hesx1* ANP enhancer, which is located immediately upstream of the promoter and contains three essential Otx2 binding sites (Chou et al., 2006; Spieler et al., 2004), was activated by exogenous Otx2 (supplementary material Fig. S7B).

Activation of the N2 core enhancer of the *Sox2* gene depends on the binding of Pou factors to their bipartite binding sites in both epiblast and ANP (Iwafuchi-Doi et al., 2011). However, neither Pou5f1 nor Pou3f1 alone activated this enhancer (see below, Fig. 6C). We identified additional binding sites for Zic and Otx factors in the N2 core sequence (Fig. 6A, supplementary material Fig. S8). Mutations of the Zic site (Mut-ZIC) or Pou sites (Mut-POU) inactivated N2 core enhancer activity in both epiblastic and NP1 cells (Fig. 6A). By contrast, mutation of the Otx factor binding site (Mut-OTX) attenuated N2 enhancer activity only in NP1 cells (Fig. 6Ac) and not in epiblastic cells (Fig. 6Ab), suggesting that Otx2 activity is essential for N2 enhancer activation in ANP cells only.

That Otx2 is not required for N2 enhancer activation and N2-dependent *Sox2* expression in the epiblast (E6.75) was confirmed using an *Otx2*$^{-/-}$ mutant (Matsuo et al., 1995). When an enhancer N2-*lacZ* transgene was introduced into *Otx2*$^{-/-}$ embryos, comparable expression of N2-*lacZ* and *Sox2* at levels equivalent to those in wild-type embryos was observed in the epiblast, whereas N2 activation was totally lost in E7.75 *Otx2*$^{-/-}$ embryos, as was *Sox2* expression (Fig. 6B).

We hypothesized that a change in the major Pou factors from Pou5f1 to the Pou3fs during the derivation of ANP from the epiblast caused the emergence of Otx2 dependence. To test this model, we transfected 10T1/2 fibroblasts with a luciferase reporter vector carrying the N2 core enhancer (N2[73bp]$_2$) together with expression vectors for the transcription factors Zic2, Otx2 and one

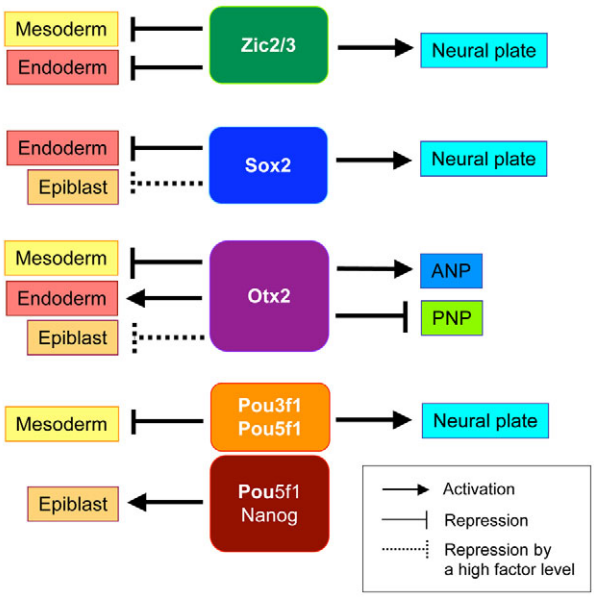


Fig. 5. Regulation of developmental pathways by core transcription factors via their target genes in the EpiSC model. Some of these regulatory pathways have also been indicated by studies using ES cells: inhibition of *Sox17* by Zic2/3 and activation of *Zic3* by Sox2, Pou5f1 and Nanog (Lim et al., 2007), and the promotion of neural development by sustained high-level expression of Pou5f1 (Shimozaki et al., 2003). The function of Pou5f1 and Nanog in stabilizing the epiblastic state has been more clearly indicated in earlier studies (Mitsui et al., 2003; Vallier et al., 2009).

of the Pou factors (Pou5f1, Pou3f1 or Pou3f4). None of the Pou factors activated the enhancer (Fig. 6Ca), nor did coexpression of Otx2 with a Pou factor elicit a substantial level of enhancer activation (Fig. 6Cb). However, when Zic2 was coexpressed, Pou5f1 significantly activated the N2 core enhancer, whereas this was not observed with Pou3f1/4 (Fig. 6Cc). When Zic2 and Otx2 were coexpressed, all Pou factors activated the N2 core enhancer efficiently, even at low Pou expression levels (Fig. 6Cd). The activation by Pou5f1 plus Zic2 was further augmented by coexpression of Otx2. *Zic3* displayed similar effects on N2 enhancer activation (supplementary material Fig. S9).

These results, taken together with our observations of mutant enhancers (Fig. 6A), indicated a model in which (1) the cooperative action of Zic2/3 is essential for Pou factor-dependent activation of the N2 core enhancer and (2) Otx2 action is also essential for Pou3f-dependent N2 core enhancer activation in the ANP, but may be dispensable for Pou5f1-dependent activation in the epiblast (Fig. 6Ce). This mechanism of N2 enhancer regulation sustains *Sox2* expression during the developmental stages at which the major Pou factor function is shifted from Pou5f1 to the Pou3fs.

DISCUSSION

The core transcriptional networks in the epiblast and during ANP derivation

The establishment of EpiSCs from embryonic epiblast has opened up new avenues for the study of core transcriptional networks that maintain the epiblast state and/or give rise to various somatic lineages. Because the expression profiles of transcription factor genes (Fig. 1, Table 1) indicated that epiblastic and NP1 states of EpiSCs in our culture conditions mimicked ~E6.5 epiblast and ~E7.5 ANP, respectively, we focused our analysis on the epiblast and ANP derivation.

We systematically knocked down or overexpressed several transcription factor genes in the epiblastic or ANP derivation states of EpiSCs and quantitatively analyzed the immediate impact on the expression of downstream transcription factor genes. The genes subjected to manipulation were those that are expressed uniformly in the E6.5 epiblast but shift their expression sites into ANP (*Sox2*, *Otx2* and *Pou3f1*) or that avoid ANP (*Pou5f1* and *Nanog*). *Zic2/3* were also included in the analysis because of their involvement in the early phase of neural plate development (Mizuseki et al., 1998; Nakata et al., 1997). This analysis of short-term effects of altered transcription factor levels should be distinguished from the long-term effects of stably overexpressing exogenous genes using human ES cells and mouse EpiSCs (e.g. Chng et al., 2010; Vallier et al., 2009).

The data shown in Figs 3 and 4 and in supplementary material Figs S6 and S7 and as summarized in Table 2 and Fig. 5 highlight the pivotal roles of Zic2/3, Otx2 and Sox2 in the epiblast state and in ANP derivation. The data also suggest intricate interactions and cross-regulation among these factors. For instance, Zic2/3 and Otx2 support the expression of genes characteristic of epiblast such as *Fgf5* and *Eomes*, whereas Otx2 and Sox2 overexpression inhibits *Pou5f1* expression. The function of Otx2 and Sox2 in repressing *Pou5f1* expression might be to accelerate the reduction of *Pou5f1* expression triggered by Nr6a1 activation (Fuhrmann et al., 2001), resulting in the clearance of *Pou5f1* expression from the embryonic ANP (Fig. 2). However, overexpression of Pou5f1 elicited activation of neural plate-associated genes despite the fact that the normal function of Pou5f1 is to stabilize the epiblast state. It is thus possible that an appropriate level of Pou5f1 for the epiblast state is maintained by negative feedback through Otx2 and Sox2.

A remarkable finding is that both the repressive and activating mechanisms function together to generate only ANP from the epiblast (Fig. 5). The repressive mechanisms inhibited mesodermal, endodermal and PNP development to confine the developmental pathway to ANP. Zic2/3, Otx2 and Pou5f1/3f1 inhibited mesodermal development by repressing *T*. Zic2/3 and Sox2 inhibited endodermal development by repressing *Sox17* and *Eomes*. These anti-endodermal effects of Sox2 dominated over the endoderm-promoting effects of Otx2 on *Sox17* and *Eomes* (Fig. 4A). Moreover, Otx2 activity strongly inhibited the development of the PNP transcription factor genes *Nkx1.2*, *Gbx2* and *Sox1* (Fig. 3, Fig. 4A,B).

Along with participating in the repressive mechanisms as described above, Zic2/3, Otx2, Sox2 and Pou3f1 also participate in the activation of various genes characteristic of the neural plate, as exemplified by *Sip1* (Chng et al., 2010; Miyoshi et al., 2006). Among these factors, Otx2 was found to have a prominent role in the promotion of ANP development. *Hesx1*, which determines the anteriormost ANP subdomain (Andoniadou et al., 2007), is directly activated by Otx2 through its interaction with the ANP enhancer (Spieler et al., 2004) (supplementary material Fig. S7B). Moreover, Otx2 overexpression in combination with Pou5f1 knockdown elicited the accelerated development of a fully Pax6-positive ANP cell population in a 1-day period under the NPC culture condition (Fig. 4D), demonstrating the lead function of Otx2 in ANP development.

In addition, Otx2 plays a unique role in sustaining *Sox2* expression during the transitory stages from the epiblast to ANP, when the major Pou function switches from Pou5f1 to Pou3fs (Fig.

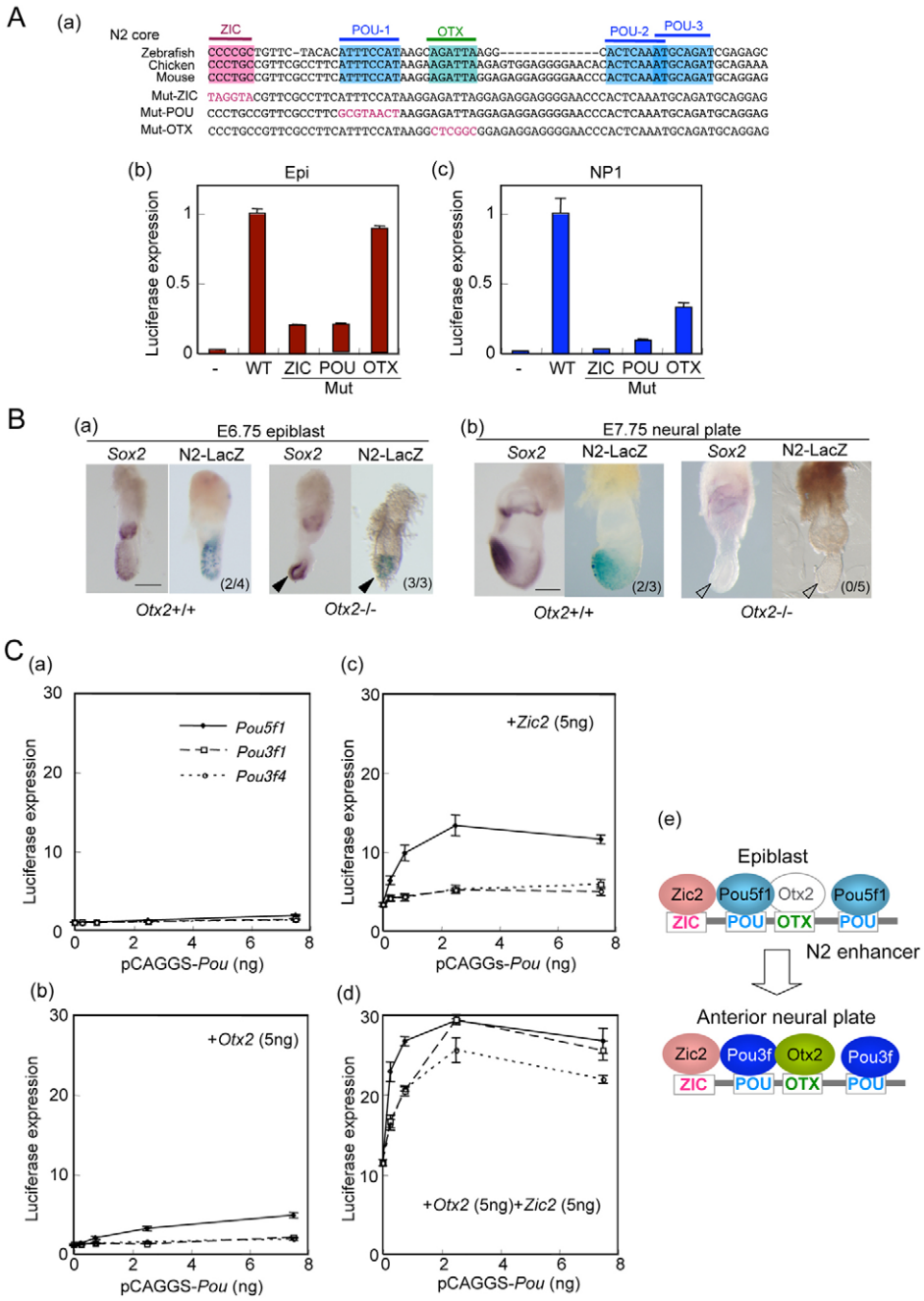


Fig. 6. Differential regulation of the *Sox2* N2 enhancer in the epiblast and ANP. (**A**) (**a**) Transcription factor binding sites in the *Sox2* N2 enhancer core sequence as confirmed by EMSA (Iwafuchi-Doi et al., 2011) (supplementary material Fig. S8) and mutation of these binding sites. (**b**,**c**) Effects of the binding site mutations on N2 activity as assessed in a luciferase reporter assay in transfected epiblastic (b) and NP1 (c) cells. –, luciferase gene without an enhancer; WT, 176 bp N2 enhancer. Mutations of the POU-2/POU-3 sites also inactivated the enhancer in a manner analogous to the POU-1 site mutation investigated here (Iwafuchi-Doi et al., 2011). (**B**) Expression of *Sox2* (in situ hybridization) and N2-*lacZ* activity (positive cases out of total indicated in parentheses) in the epiblast at E6.75 were similar in *Otx2*$^{-/-}$ and wild-type (*Otx2*$^{+/+}$) embryos (**a**) but absent from *Otx2*$^{-/-}$ at E7.75 (**b**) (compare black and open arrowheads). Scale bars: 200 μm. (**C**) Transactivation of the N2 core enhancer by the combined action of Zic2, Otx2 and Pou factors in 10T1/2 fibroblasts. N2[73bp]$_2$-luciferase expression levels relative to those without exogenous factors are shown. (**a**) Pou factors only; (**b**) with Otx2 expression; (**c**) with Zic2 expression; and (**d**) with coexpression of Otx2 and Zic2. (**e**) Model for the differential requirement of Otx2 in the activation of the *Sox2* N2 enhancer. Error bars indicate s.e.

6C). In contrast to Pou5f1, Pou3f factors plus Zic2/3 cannot activate the N2 enhancer of *Sox2* without the cooperation of Otx2.

In summary, the following regulatory functions were identified: cross-regulation among Zic, Otx2, Sox2 and Pou factors stabilizes the epiblastic state; Zic, Otx2 and Pou factors in combination repress mesodermal development; Zic and Sox2 factors repress endodermal development; and Otx2 represses PNP development. All of these factors variably activate genes responsible for neural plate development, along with participating in the above-mentioned repression mechanisms.

In vivo evidence for the deduced functions of Otx2, Zic2/3 and Sox2

The regulatory networks identified in this study using EpiSCs account for some earlier observations in post-implantation embryos. The repression of *T* by Otx2 is novel but accounts for the previous observation that *T* is upregulated in *Otx2*$^{-/-}$ embryos (Kimura et al., 2000). In *Otx2*$^{-/-}$ ↔ wild-type chimeras, where the visceral endoderm defects of *Otx2*$^{-/-}$ embryos were rescued (Rhinn et al., 1999; Rhinn et al., 1998), the ANP tissue that consisted of *Otx2*$^{-/-}$ cells somehow acquired morphological features of anterior neural fold at E7.5 but expressed PNP genes after E8, never activating *Hesx1*. This observation is accounted for by the simultaneous loss of PNP inhibition and ANP activation, which are both attributed to Otx2 (Fig. 5).

Consistent with the repression of *Gbx2* by Otx2, the competitive interaction between *Otx2* and *Gbx2* in the determination of ANP and PNP territories has been demonstrated by chicken embryo electroporation (Katahira et al., 2000) and in compound knockout mouse embryos (Martinez-Barbera et al., 2001).

The contribution of Zic2/3 functions to neural plate development has not been fully established using mouse embryos owing to their functional overlap and to the lack of a *Zic2* null allele. However, among the variable phenotypes previously reported for *Zic3* null embryos, the most severe phenotype was in fact characterized by serious neural plate defects (Inoue et al., 2007; Ware et al., 2006).

In contrast to its anti-endodermal activity, Sox2 did not interfere with the expression of the hallmark mesodermal gene *T* (Fig. 3, supplementary material Fig. S6). This observation is likely to reflect the fact that the majority of PNP cells are not directly derived from the epiblast but through neural/mesodermal bipotential intermediates known as axial stem cells, which reside in the caudal lateral epiblast abutting the primitive streak (Kondoh and Takemoto, 2012; Takemoto et al., 2011). The caudal lateral epiblast in chicken embryos and in late streak stage mouse embryos coexpresses low levels of *Sox2* and *T*, consistent with the bipotential nature of axial stem cells (Delfino-Machín et al., 2005; Kondoh and Takemoto, 2012; Perantoni et al., 2005).

Thus, the model summarized in Fig. 5 should provide a new guide for understanding various in vivo phenomena associated with cell lineage selection in post-gastrulation embryos.

Advantages of EpiSCs in the study of somatic lineage derivation

ES cells are frequently employed as a model system for studying somatic cell derivation. However, to derive a somatic cell lineage with ES cells, the cells must go through the epiblast state, as evidenced by the transient expression of *Fgf5* prior to the activation of genes indicative of somatic development (Kamiya et al., 2011; Thomson et al., 2011). Therefore, the effects of gene manipulations in ES cells can be indirect or ambiguous in terms of determining active stages.

Our present study poses EpiSCs as a more suitable alternative. As EpiSCs serve as the immediate precursor for somatic cells of various lineages, investigations of the short-term effects of manipulating genes will reveal the first-step mechanisms in the derivation of the somatic lineages. Not confined to the epiblast or ANP, as reported in this paper, EpiSCs are useful in the study of various cell lineages. Indeed, it has been demonstrated that EpiSCs can develop into virtually all somatic lineages with appropriate manipulation of culture conditions (Brons et al., 2007; Tesar et al., 2007). In the context of PNP development, it would be interesting if neural/mesodermal bipotential axial stem cells could be produced from EpiSCs.

EpiSCs under various culture conditions can give rise to a critical mass of intermediate state cells during cell lineage derivation. These EpiSC-derived cells can be subjected to a comprehensive analysis of gene regulatory networks, which is not an easy task to undertake in embryonic tissues. In the same way that ES cells have contributed greatly to elucidating transcriptional regulatory networks in pre-implantation embryos (e.g. Masui et al., 2007; Shimosato et al., 2007), EpiSCs will prove to be an excellent tool with which to investigate the regulation of somatic lineage derivation in post-implantation embryos.

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Competing interests statement
The authors declare no competing financial interests.

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