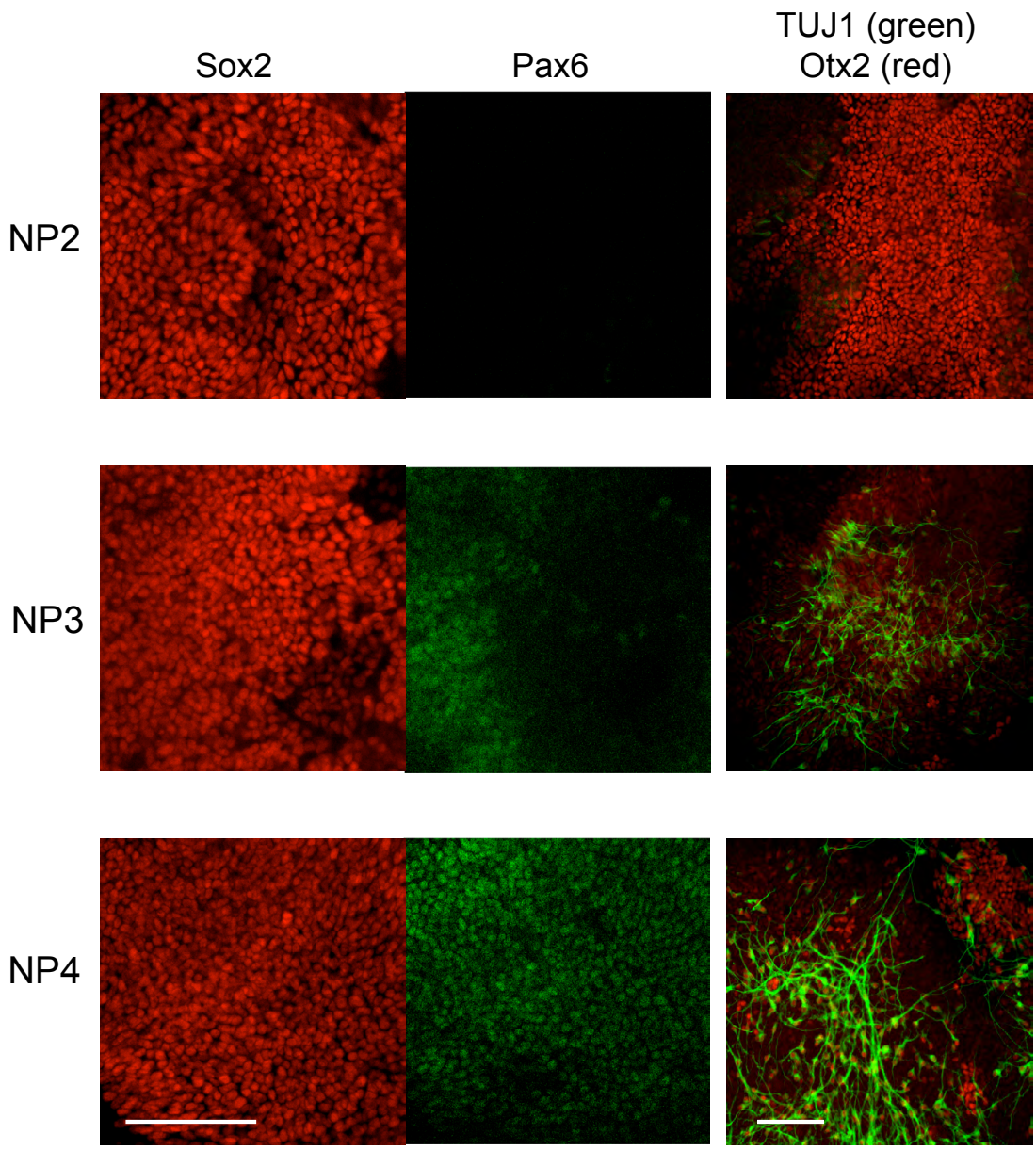

Fig. S1. Immunohistological analysis of NP2 to NP4 cells for expression of Sox2 and Pax6 (double staining) and Otx2 and TuJ1. Sox2 continued to be expressed in the majority of cells, whereas Otx2 began to be downregulated at NP3, in an inverse relationship with the appearance of Pax6-immunopositive and TuJ1-positive NP3 cells and the increase in NP4 cells. The TuJ1 (neuron-specific class III β-tubulin) staining pattern indicated a wider extension of the neurites in NP4 cells compared with NP3 cells. Scale bars: 100 μm.

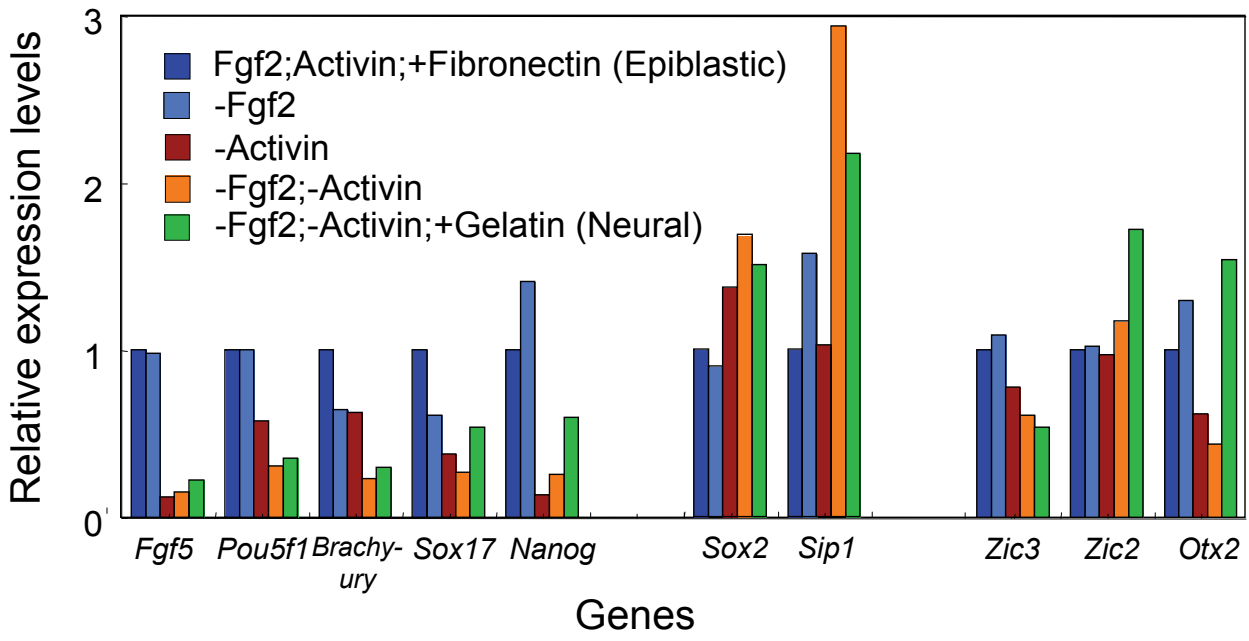

Fig. S2. Synergistic contributions of the removal of growth factors and changing the culture substrate coating to the promotion of ANP cell development from EpiSCs. The complete culture medium contained Fgf2, activin and the culture substrate was coated with fibronectin. The expression levels of genes under various culture conditions, as determined by qRT-PCR, are shown relative to those in EpiSCs grown in complete culture medium. The effects of the removal of Fgf2 on the epiblast state were modest, in contrast to the strong downregulation of *Fgf5*, *Pou5f1*, brachyury (*T*), *Sox17* and *Nanog* after the removal of activin. However, downregulation of *Pou5f1*, *T* and *Sox17* was significantly enhanced by the simultaneous removal of Fgf2 and activin. *Sox2* and *Sip1* were strongly activated by the removal of Fgf2 and activin together. The activation of Otx2 and Zic2, which will be shown to be crucial for ANP development in a later section, was promoted by altering the substrate coating from fibronectin to gelatin.

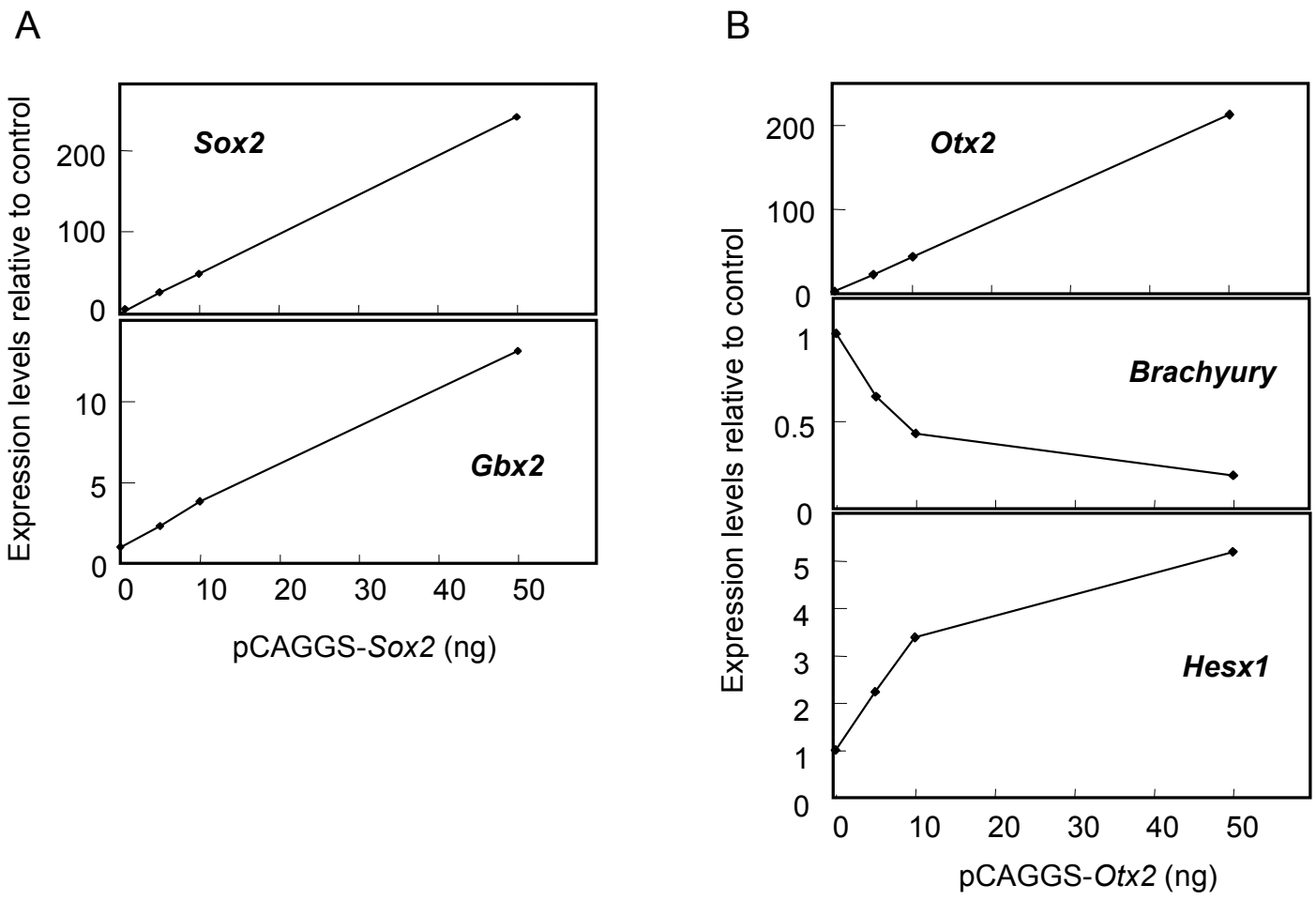

Fig. S3. Responses of the indicated genes to varying amounts of transfected Sox2 and Otx2 expression vectors. Expression levels were determined by qRT-PCR and are shown relative to the endogenous levels in EpiSCs. Exogenous *Sox2* and *Otx2* increased linearly with the vector input over the range of 0-50 ng per well. (**A**) Sox2 transfection. Endogenous *Gbx2* was activated linearly with the exogenous Sox2 level. (**B**) Otx2 transfection. The repression of brachyury (*T*) and activation of *Hesx1* by Otx2 reached a plateau with ~10 ng of expression vector.

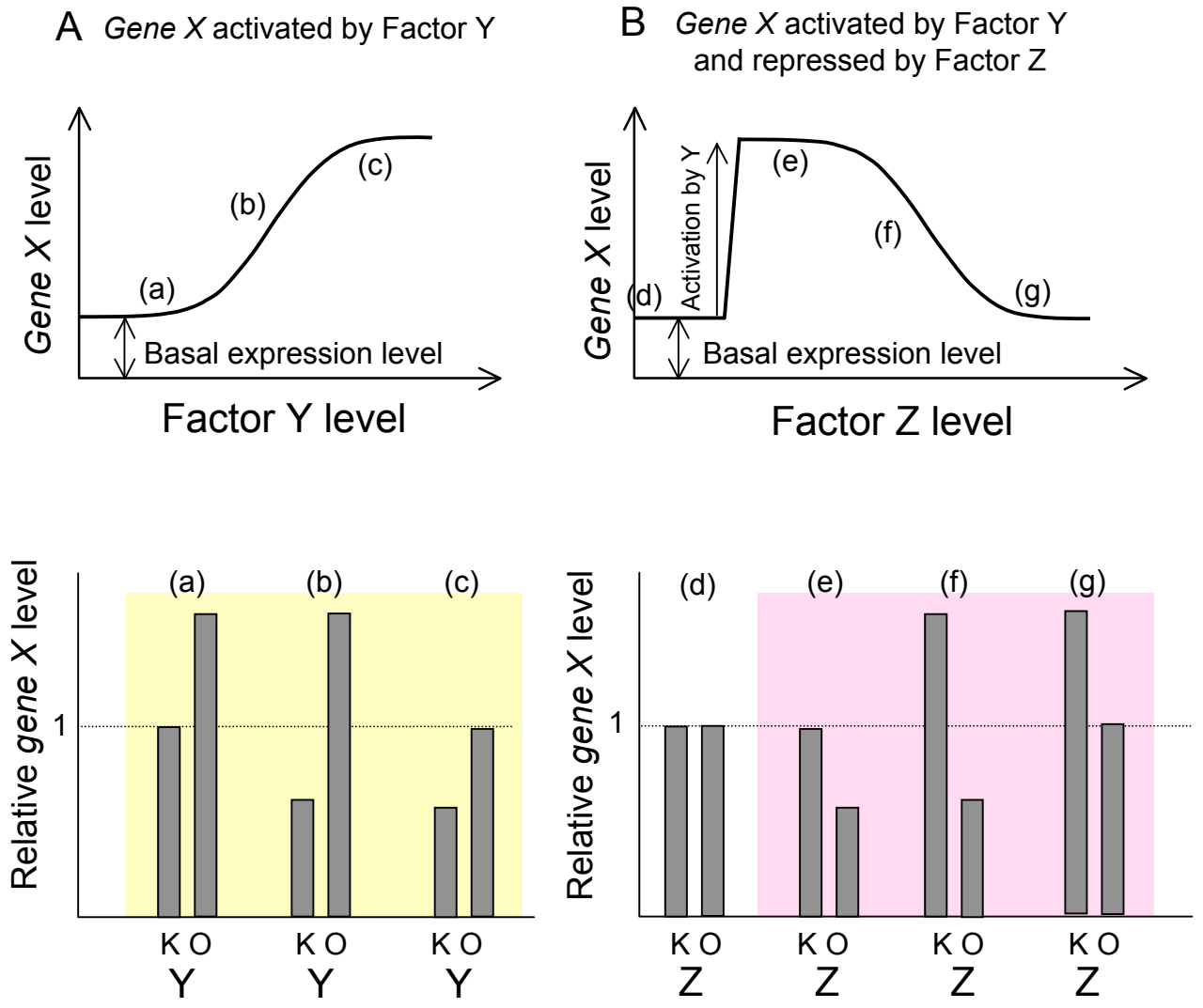

Fig. S4. Responses of genes to transcription factor manipulations according to their activation and/or repression status. The expected responses are shown schematically in the case of activation (A) and repression (B). The gene *X* level without knockdown or overexpression of factor Y (A) or factor Z (B) under a given condition is indicated as 1 for bar graphs. (**A**) In the case where gene *X* is activated by transcription factor Y, the effects of knockdown (K) and overexpression (O) of factor Y depend on the status of activation: (a) if gene *X* is not yet activated by Y, only the overexpression of Y increases gene *X* expression above basal levels; (b) when gene *X* has already been activated but not to the level of saturation, the knockdown of Y reduces, whereas the overexpression of Y augments, gene *X* expression; (c) if gene *X* is already activated by Y to saturation levels, only a knockdown of Y downregulates gene *X*. (**B**) Cases in which gene *X* is activated by transcription factor Y and repressed by Z: (d) if gene *X* is not yet activated, neither knockdown nor overexpression of Z affects its basal expression level; (e) under conditions where gene *X* is activated by a transcription factor (Y), but is not repressed by Z, then only the overexpression of Z will reduce gene *X* expression; (f) if gene *X* is in the middle of repression by Z, then knockdown of Z augments, and its overexpression reduces, gene *X* expression; (g) when gene *X* is completely repressed by Z, only knockdown of Z augments its expression.

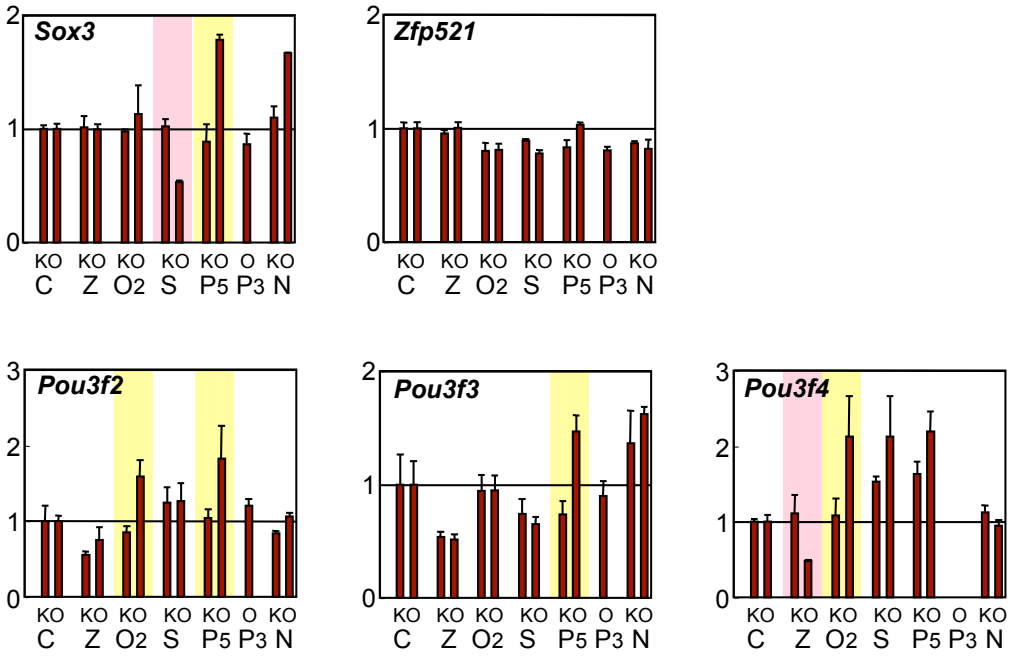

Fig. S5. Continuation of the analysis shown in Fig. 3, representing cases with miscellaneous responses to exogenous transcription factor manipulations.

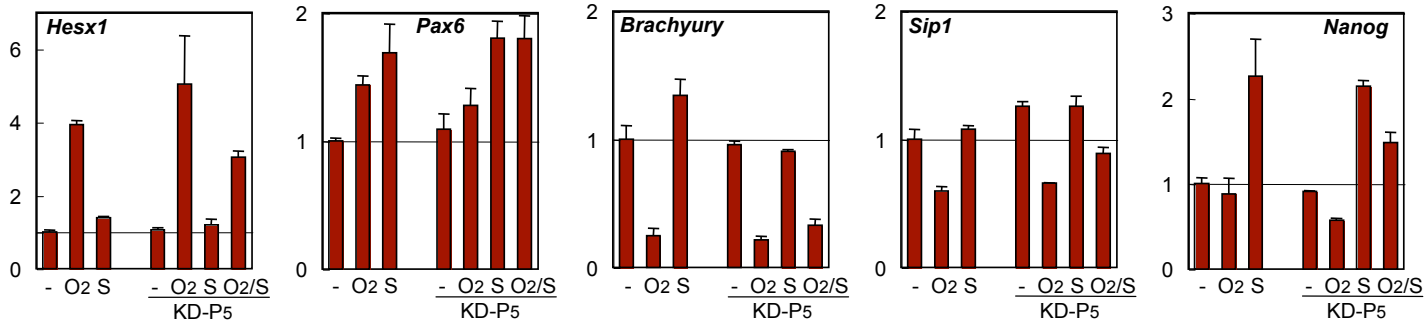

Fig. S6. The effects of overexpressing Otx2 or Sox2 and of Pou5f1 knockdown on the expression of five representative transcription factor genes in epiblastic state EpiSCs. Analysis was in parallel with those described in Fig. 4A. O2, Otx2; S, Sox2; KD-P5, Pou5f1 knockdown.

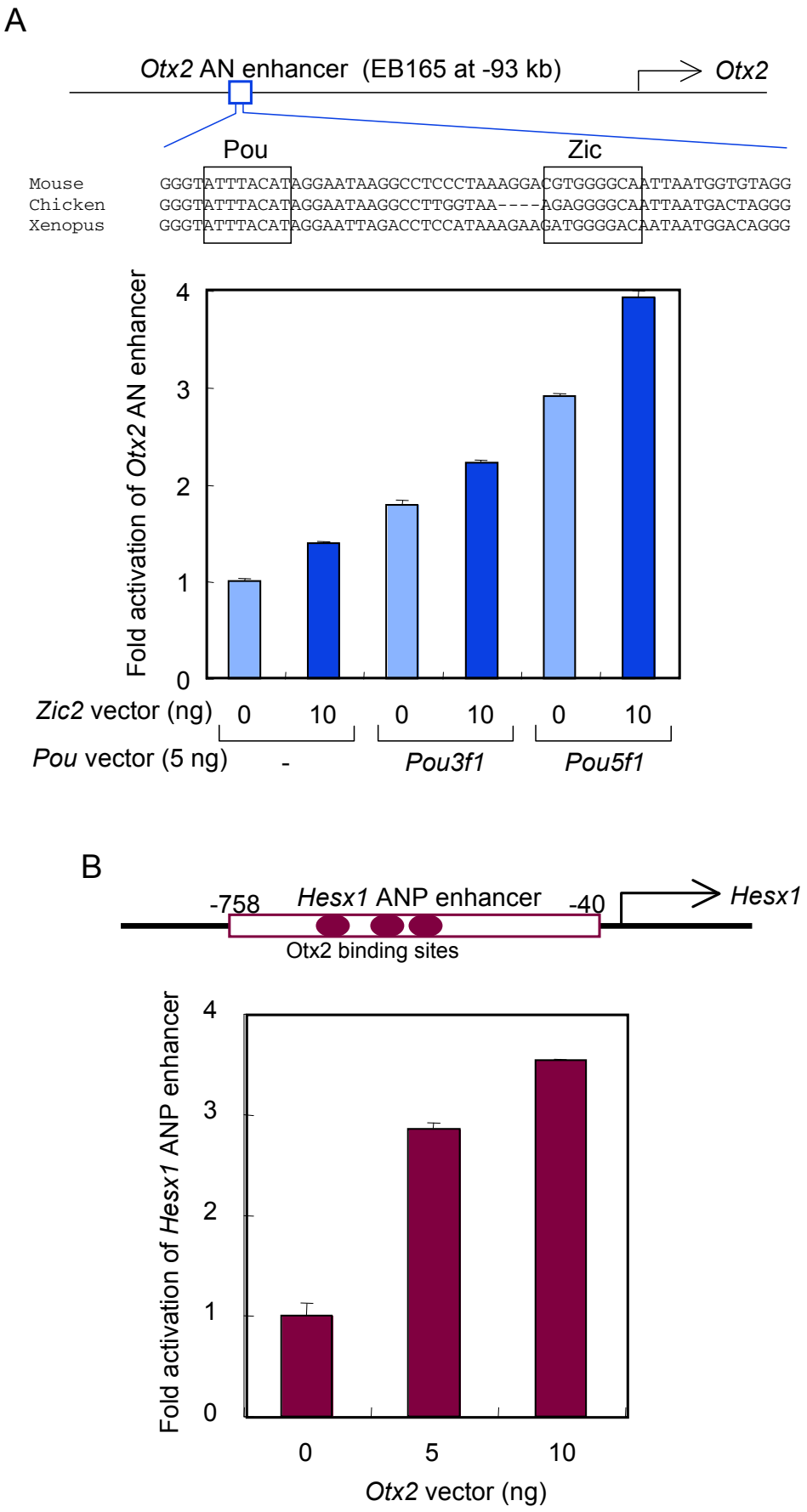

Fig. S7. Activation of *Otx2* AN and *Hesx1* ANP enhancers. (**A**) *Otx2* AN enhancer (Kurokawa et al., 2004) (165 bp EB fragment) containing a Pou binding site and a putative Zic binding site (top) was joined to the luciferase reporter, and its activation by Zic2 and Pou factors was measured in 10T1/2 fibroblasts. (**B**) The *Hesx1* ANP enhancer used in this study (top) contained three previously characterized Otx2 binding sites (Spieler et al., 2004). Its activation by Otx2 was measured by luciferase reporter assay.

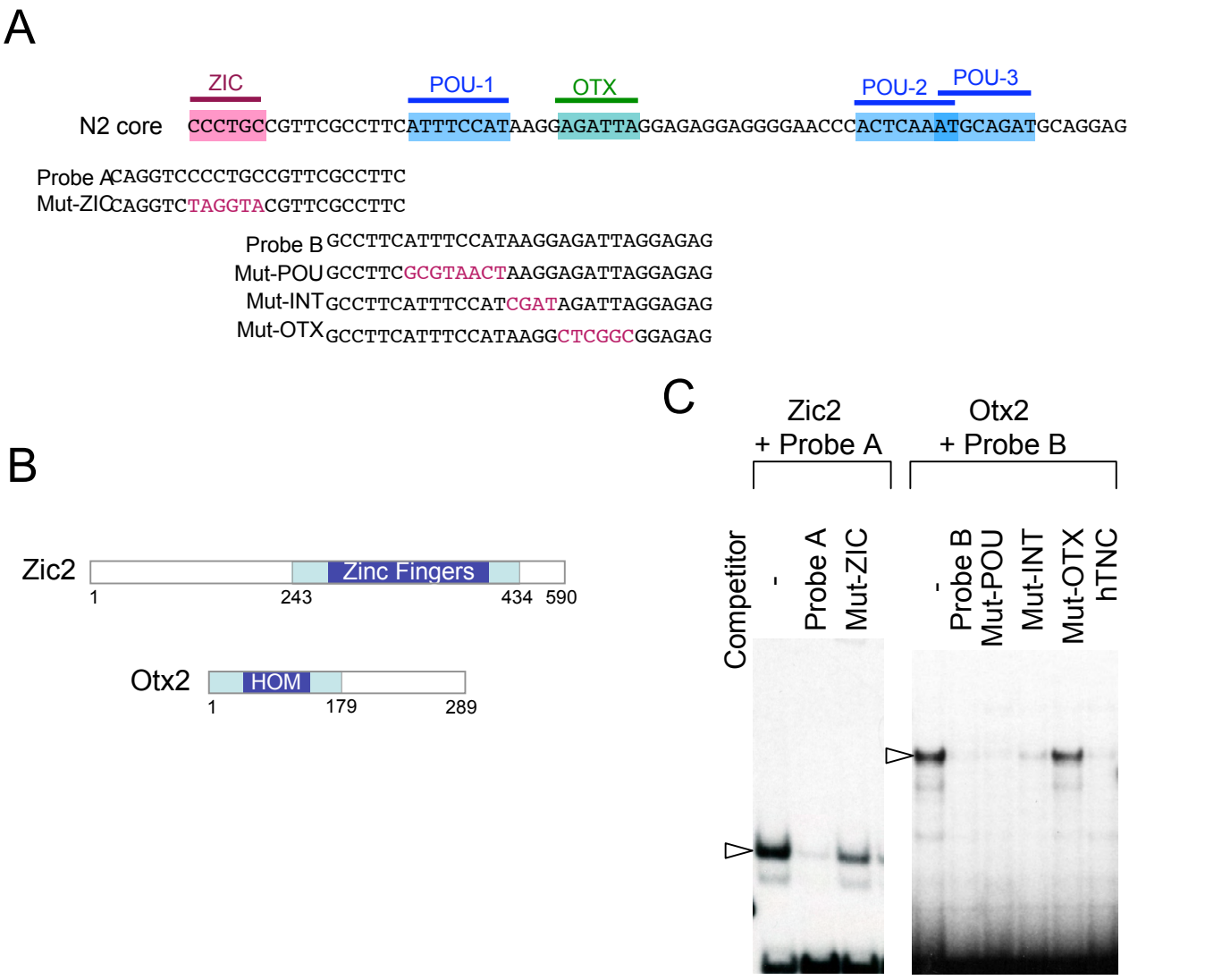

Fig. S8. EMSA analysis of the binding of Zic2 and Otx2 to the N2 enhancer core sequence. (**A**) Probes and mutated competitor sequences used in the EMSA assay. (**B**) In vitro synthesized transcription factors used in the EMSA assay. The domains of these proteins, indicated by light-blue shading, were synthesized in vitro. HOM, homeodomain. (**C**) Competitive inhibition of factor binding to radiolabeled probes using various mutated competitor sequences. The *hTNC* (human tenascin C) competitor contains an OTX binding sequence (Briata et al., 1999). The band positions of Zic2 plus Probe A and Otx2 plus Probe B are indicated by open arrowheads. The formation of the former complex was inhibited by the probe sequence, but not by the Mut-ZIC sequence. The latter complex was inhibited by competitors carrying intact Otx2 binding sequences, but not by the Mut-OTX sequence. Zic3 gave identical results to Zic2 (data not shown).

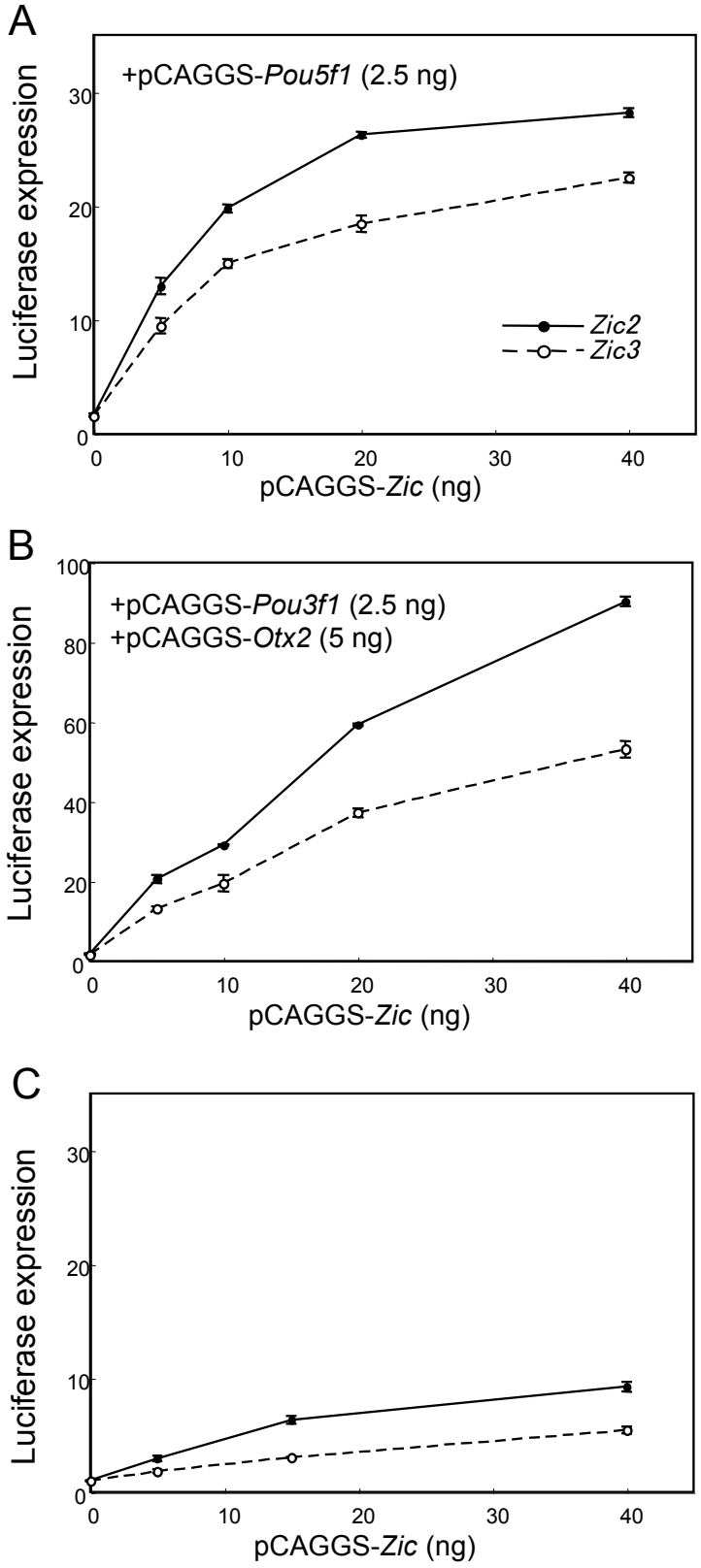

Fig. S9. Comparison of the activity of Zic2 and Zic3 in the activation of the N2 core enhancer. Under the same conditions used for the transfection of 10T1/2 cells with N2[73bp]$_2$-luciferase as described in Fig. 8, varying amounts of expression vectors for *Zic2* (solid circle and solid line) or *Zic3* (open circle and broken line) were co-transfected with (**A**) pCAGGS-*Pou5f1* (2.5 ng), (**B**) pCAGGS-*Pou3f1* (2.5 ng) and pCAGGS-*Otx2* (5 ng), or (**C**) alone. *Zic3* activated the N2 core enhancer in a manner analogous to that of *Zic2* under the conditions given in A and B.

Table S1. Primers used in qRT-PCR analyses

Target gene	Forward primer	Tm (C)	Reverse primer	Tm (C)	Reference sequence (the range of coding sequence)	Target position	of PCR product (bp)	Reference
Gapdh	CATGGCCTTCCGTGTTCCTA	60	GCGGCACGTCAGATCCA	59	NM_008084.2 (51-1052)	734-788	55	This study
Fgf5	AGAGTGGGCATCGGTTTCC	59	TGGGAGCCATTGACTTTGC	58	NM_010203.4 (229-1023)	502-560	59	This study
Nanog	AGGCCTGGACCGCTCAGT	59	AGTTATGGAGCGGAGCAGCAT	60	NM_028016.2 (216-1133)	907-996	60	This study
Eomes	GCCTTCCACCTTTGATGTATCC	58	AAAGCTTTGGCGCCTTCTCT	59	NM_010136.3 (472-2595)	2680-2740	61	This study
Sox2	CCATGGGCTCTGTGGTCAAG	60	CCCTGGAGTGGGAGGAAGAG	60	NM_011443 (412-1371)	1133-1204	72	This study
Sox3	CTGGGACCGTTGCCTTGTA	58	CCGACAGTTACGGCCAAACT	59	NM_009237.2 (1-1353)	1439-1498	60	This study
Sox1	CGGAGGACAAAAGACAAAAACC	59	AAGTTACAGAGCCGGCAGTCA	59	NM_009233.3 (843-2018)	2310-2377	80	This study
Pou5f1	TTCCCTCTGTTCCCGTCACT	58	TGGTGCCTCAGTTTGAATGC	58	NM_013633.2 (62-1120)	1071-1127	57	This study
Pou3f1	AGACCACCATCTGCCGTTTC	59	TCCAGCCACTTGTTGAGCAG	59	NM_011141 (62-1411)	924-1008	85	This study
Pou3f2	CGGATTTACTCAAGCAGACGTG	59	CAACAAAGGCTTCAGCTTGC	58	NM_008899.1 (1-1338)	858-990	133	This study
Pou3f3	CCCTTGACTTCTGCCCTCAA	59	CATCCGCAGCACCCATTC	60	NM_008900.2 (1-1494)	2639-2695	57	This study
Pou3f4	GCCACAGCTGCCTCGAAT	59	CATGGACAAGGGAGCTGGAA	60	NM_008901 (1-1086)	4-58	55	This study
Otx2	GAAAATCAACTTGCCAGAATCCA	59	GCGGCACTTAGCTCTTCGAT	58	NM_144841.3 (333-1202)	551-614	64	This study
Zic2	GCAGGGCCACCTTCTTTTC	57	GCCCATTGAGCACGTTCTG	57	NM_009574.3 (293-1882)	751-824	74	This study
Zic3	TTTTAGGGTGCTGTTGGTTATTGA	59	ACAATTCCTTATCTCCACTTTTCTGTTA	58	NM_009575.2 (554-1954)	3729-3800	72	This study
Sip1	ACACTTTCCTTTCGCTATTCATGA	58	TGAGGCCTAAAAGTGTGTGGTTAC	58	NM_015753.3 (527-4174)	4650-4721	72	This study
Zfp521	TCCCCCGCCAAACTTCA	60	GTACCACCCATCCCTTCGAA	58	NM_145492.4 (235-4170)	762-820	59	This study
Hesx1	TCAGCTCCGGGAAAGCAA	59	CCAGTCCTAAAATGCTCTCAATTG	58	MN_010420.2 (359-916)	385-446	62	This study
Otx1	GCGTCACCCCTTCAAGTCTTT	59	AACAGAGGGTCAGAGCGAAGAG	59	NM_011023.3 (282-1349)	1635-1712	78	This study
Pax6	ATGGGCATTGGTATGTTATAATGAAG	58	AACACAGATCCGCGATCCA	59	NM_013627.4 (526-1836)	2094-2158	65	This study
Gbx2	CAGCGACCACCTTCCCATAC	59	CGCAGTGTTTGTCCTTGTGTCT	59	NM_010262.3 (422-1468)	1793-1853	61	This study
Nkx1.2	CCAGAGGGCGAGGAGAAGT	58	GACCCCTCAGTGGCTTGTGT	59	NM_009123.2 (112-1029)	1156-1212	57	This study
T	TTGAACTTTCCTCCATGTGCTGA	61	TCCCAAGAGCCTGCCACTTT	61	NM_009309.2 (109-1419)	1421-1502	82	Greber et al., 2010
Sox17	ATAAGCCCGAGATGGGTCTTC	58	CCGTGGCTGTCTGAGAGGTT	59	NM_011441.4 (1083-2342)	2209-2275	67	This study

Table S2. Sequences inserted in pSilencerU6puro (Ambion) to produce shRNAs

Target genes	Sequences inserted (SENSE-loop-ANTISENSE)	References
Control	AGACAGCGAAACTGTTCTC-ttcaagaga-GAGAACAGTTTCGCTGTCT	Zeineddine et al., 2006
*Zic2/3**	GAGAACCTCAAGATCCACA-ttcaagaga-TGTGGATCTTGAGGTTCTC	This study
Otx2	GGCTTCAGGTTATAGTCAAGG-ttcaagaga-CCTTGACTATAACCTGAAGCC	This study
Sox2	GGTTGATATCGTTGGTAAT-ttcaagaga-ATTACCAACGATATCAACC	Ivanova et al., 2006
Pou5f1	AGGTGTTCAGCCAGACCAC-ttcaagaga-GTGGTCTGGCTGAACACCT	Zeineddine et al., 2006
Pou3f1	GCCCATGGACGACGTTTATGC-ttcaagaga-GCATAAACGTCGTCCATGGGC	Iwafuchi-Doi et al., 2011
Nanog	GAGACAGTGAGGTGCATAT-ttcaagaga-ATATGCACCTCACTGTCTC	Wang et al., 2007

*The shRNA vector for *Zic2/3* was designed to target both Zic factor genes because of their functional similarities.

Table S3. References for expression patterns in embryos from E6.5 to E8.5

Genes	References
Fgf5	Hebert et al., 1991
Nanog	Hart et al., 2004
Eomes	Ciruna and Rossant, 1999; Hancock et al., 1999
Sox2	Uchikawa et al., 2011; Wood and Episkopou, 1999
Sox3	Uchikawa et al., 2011; Wood and Episkopou, 1999
Sox1	Uchikawa et al., 2011; Wood and Episkopou, 1999
Pou5f1	Perea-Gomez et al., 1999
Pou3f1	Zwart et al., 1996
Pou3f2	Bouchard et al., 2005
Pou3f3	Bouchard et al., 2005
Pou3f4	Bouchard et al., 2005
Otx2	Ang et al., 1994; Martinez-Barbera et al., 2001
Zic2	Elms et al., 2004; Inoue et al., 2007
Zic3	Elms et al., 2004; Inoue et al., 2007
Sip1	Miyoshi et al., 2006
Zfp521	Kamiya et al., 2011
Hesx1	Yang and Klingensmith, 2006
Otx1	Sakurai et al., 2010; Suda et al., 1999
Pax6	Inoue et al., 2000
Gbx2	Waters et al., 2003
Nkx1.2	Tamplin et al., 2008
T	Wilkinson et al., 1990
Sox17	Kanai-Azuma et al., 2002

Link to Table 4.

http://dev.biologists.org/content/vol0/issue2012/images/data/dev.085936/DC1/DEV085936TableS4.xls

Table S5. Transfection efficiency and selection of transfected cells using puromycin (Pur), as estimated from CAGGS-EGFP expression

Cells	EGFP-expressing cell fraction (%)	
	Without Pur selection	With Pur selection
Epiblastic EpiSCs	77	93
NP1 cells	56	95

Table S6. Knockdown and overexpression efficiencies of transcription factors

Transcription factor genes	Knockdown in epiblastic cells	Knockdown in NP1 cells	Overexpression in epiblastic cells
Zic2	0.59	0.73	256*
Zic3	0.19	0.34	–
Otx2	0.36	0.55	211
Sox2	0.33		257
Pou5f1	0.37	0.14	100
Pou3f1	0.63		146
Nanog	0.18		14

The values indicate the transcript expression levels determined by qRT-PCR after transfection of knockdown or overexpression vectors relative to untreated cells, which was taken as 1.
*pCAGGS-Zic2 was used to overexpress Zic2/3 functions.

Table S7. Endogenous expression of transcription factor genes in 10T1/2 fibroblasts used for enhancer transactivation analysis, as compared with EpiSCs

Gene	10T1/2	EpiSC
Sox2	0.0445±0.0022	7.62±1.51
Zic2	0.0481±0.0149	1.07±0.09
Zic3	0.00872±0.00155	12.0±2.36
Otx2	0.0185±0.0039	7.82±0.45
Pou5f1	0.0161±0.0014	74.4±5.5
Pou3f1	Undetectable	1.78±1.03
Pou3f2	0.00761±0.00154	0.0101±0.0012
Pou3f4	Undetectable	0.0158±0.0043

Expression levels are relative to 10^{-3} *Gapdh* and indicated with standard errors.