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ETS-related Transcription Factors ETV4 and ETV5 Are Involved in Proliferation and Induction of Differentiation-associated Genes in Embryonic Stem (ES) Cells*[S]

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Tadayuki Akagi[‡1], **Satu Kuure**[§], **Kousuke Uranishi**[‡], **Hiroshi Koide**[‡2], **Frank Costantini**[¶], **and Takashi Yokota**[‡3]

From the [‡]*Department of Stem Cell Biology, Graduate School of Medical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan, the* [§]*Institute of Biotechnology, University of Helsinki, 00790 Helsinki, Finland, and the* [¶]*Department of Genetics and Development, Columbia University Medical Center, New York, New York 10032*

Background: Characteristics of ES cells are controlled by gene regulatory networks, and ETV4 and -5 participate in the network.
Results: Proliferation, induction of ectodermal marker genes, and expression of stem cell-related genes were impaired in *Etv4/5* double KO ES cells.
Conclusion: ETV4 and -5 crucially define properties of ES cells.
Significance: Gene regulatory networks are dysregulated in the double KO ES cells.

The pluripotency and self-renewal capacity of embryonic stem (ES) cells is regulated by several transcription factors. Here, we show that the ETS-related transcription factors *Etv4* and *Etv5* (*Etv4/5*) are specifically expressed in undifferentiated ES cells, and suppression of *Oct3/4* results in down-regulation of *Etv4/5*. Simultaneous deletion of *Etv4* and *Etv5* (*Etv4/5* double knock-out (dKO)) in ES cells resulted in a flat, epithelial cell-like appearance, whereas the morphology changed into compact colonies in a 2i medium (containing two inhibitors for GSK3 and MEK/ERK). Expression levels of self-renewal marker genes, including *Oct3/4* and *Nanog*, were similar between wild-type and dKO ES cells, whereas proliferation of *Etv4/5* dKO ES cells was decreased with overexpression of cyclin-dependent kinase inhibitors (*p16/p19*, *p15*, and *p57*). A differentiation assay revealed that the embryoid bodies derived from *Etv4/5* dKO ES cells were smaller than the control, and expression of ectoderm marker genes, including *Fgf5*, *Sox1*, and *Pax3*, was not induced in dKO-derived embryoid bodies. Microarray analysis demonstrated that stem cell-related genes, including *Tcf15*, *Gbx2*, *Lrh1*, *Zic3*, and *Baf60c*, were significantly repressed in *Etv4/5* dKO ES cells. The artificial expression of *Etv4* and/or *Etv5* in *Etv4*/5 dKO ES cells induced re-expression of *Tcf15* and *Gbx2*. These results indicate that *Etv4* and *Etv5*, potentially through regulation of *Gbx2* and *Tcf15*, are involved in the ES cell proliferation and induction of differentiation-associated genes in ES cells.

The self-renewal capacity and pluripotency of murine embryonic stem (ES)[4] cells are maintained through stimulations of both leukemia inhibitory factor (LIF) and BMP4 (bone morphogenic protein 4) *in vitro* (1–3). LIF stimulation activates JAK-STAT3 and PI3K-AKT pathways and maintains pluripotency of ES cells (4). In fact, artificial activation of either STAT3 or AKT maintains self-renewal of ES cells in the absence of LIF (5, 6). The transcription factors KLF4 and TBX3 are downstream regulators of STAT3 and AKT, respectively, and are involved in the maintenance of pluripotency (4). BMP induces expression of the *Id* (inhibitory of DNA binding) genes via the Smad pathway, and ID proteins suppress differentiation and sustain self-renewal of ES cells in collaboration with STAT3 (1).

In addition to the signal transduction pathways, including JAK-STAT, PI3K-AKT, and BMP-SMAD, several transcription factors, including OCT3/4, SOX2, and NANOG, are known to be major regulators of self-renewal. *Oct3/4*-deficient embryos develop to the blastocyst stage, but their inner cell mass from which ES cells are established loses pluripotency (7). A conditional *Oct3/4*-knock-out ES cell experiment showed that *Oct3/4* deficiency promotes differentiation of ES cells into extraembryonic trophectodermal cells (7, 8). *Sox2*-deficient blastocysts form abnormal inner cell masses and fail to undergo outgrowth, and *Sox2*-null ES cells differentiate into trophoblast cells (9, 10). Although *Nanog*-deficient ES cells retain the capacity for self-renewal (11), deletion of the *Nanog* gene causes early embryonic lethality, whereas forced expression of Nanog in ES cells accelerates their self-renewal in a LIF-independent manner (12, 13). Furthermore, other transcriptional regulators, including ESRRB (14–16), DAX1 (17–19), SALL4 (20–22), ZIC3 (23), KLF4 (24), MYC (25, 26), and MAX (27), have been identified as key regulators of the self-renewal capacity and pluripotency of ES cells.

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[1] To whom correspondence may be addressed. Fax: 81-76-234-4238; E-mail: tadayuki@staff.kanazawa-u.ac.jp.
[2] Present address: Laboratory of Molecular and Biochemical Research, Research Support Center, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan.
[3] To whom correspondence may be addressed. Fax: 81-76-234-4238; E-mail: tyokota@med.kanazawa-u.ac.jp.

[4] The abbreviations used are: ES, embryonic stem; GO, gene ontology; LIF, leukemia inhibitory factor; dKO, double knock-out; Tet, tetracycline; EB, embryoid body; BMP, bone morphogenic protein.

High-throughput analyses revealed that these transcription factors form a complex network of regulatory and/or feed-forward loops in ES cells. For example, chromatin immunoprecipitation experiments showed that OCT3/4, NANOG, SOX2, and other ES cell-specific transcription factors co-occupy target genes in ES cells and participate in regulatory loops that maintain self-renewal and pluripotency (24, 28–33). Protein-protein interaction networks centered on OCT3/4, NANOG, and MYC are thought to be involved in the maintenance of ES cell characteristics (34–37). Recent studies have shown that ES cells and tumor cells often possess similar characteristics, including rapid cell proliferation, self-renewal capacity in the undifferentiated state, and gene expression signatures (38, 39), indicating that genes involved in oncogenesis may also play role(s) in the constitution of ES cell characteristics.

The ETS transcription factors of the PEA3 group, including ETV1 (also called ER81), ETV4 (also called PEA3), and ETV5 (also called ERM), are involved in critical physiological processes, such as early development, organogenesis, and morphogenesis (40). ETV4 and ETV5 often have similar functions during morphogenesis, but ETV1 is thought to be different. A single knockout of either *Etv4* or *Etv5* is not sufficient to cause kidney defects, but *Etv4/5* double knock-out mice do not develop kidneys, suggesting that ETV4 and ETV5 are functionally redundant (41). These transcription factors also function as oncoproteins in several tumor cells and promote cell proliferation (42). Interestingly, the BioGPS Database, as well as several studies, indicates that *Etv4* and *Etv5* are expressed in ES cells (32, 33, 43), indicating that ETV4 and ETV5 could be involved in the self-renewal capacity and/or pluripotency of ES cells. In the present study, we discovered that the expression of *Etv4* and *Etv5* is regulated by OCT3/4, and investigations of *Etv4* and *Etv5* double knock-out ES cells clarified that these two molecules are involved in the proliferation and differentiation of ES cells.

Experimental Procedures

Cell Culture—ES cell lines PE9 (control wild-type ES cells), PE15-2 (*Etv4* and *Etv5* double knock-out (*Etv4/5* dKO) ES cells), and ZHBTc4 (conditional *Oct3/4*-knock-out ES cells) are derived from previous investigations (8, 44). These cells were cultured on gelatin-coated dishes with LIF-supplemented Dulbecco's modified Eagle's medium (DMEM) as described previously (5, 45). For the 2i culture, PE9 and PE15-2 ES cells were cultured in a chemically defined medium (DMEM/F-12 with N2B27 supplement plus 2i (2 μM mitogen-activated protein kinase/extracellular signal-regulated kinase (PD0325901) and 3 μM glycogen synthase kinase 3 (CHIR99021) inhibitors)) (Wako Pure Chemical, Osaka, Japan) as described previously (46). For the LIF depletion culture, PE9 and PE15-2 ES cells were cultured for 3 days in the absence of LIF in gelatin-coated dishes. To control *Oct3/4* expression, ZHBTc4 ES cells were cultured with or without 1 μg/ml tetracycline (Tet) (Sigma-Aldrich) for 24 to 48 h. To restore *Oct3/4* expression, the culture medium of Tet-treated cells was changed to a Tet-free medium, and the cells were cultured for another 24 h. For the embryoid body (EB) formation assay, ES cells were cultured by a hanging drop method (1×10^4 cells/20 μl). After 3 days, the EBs were transferred to ultra-low attachment tissue culture plates (Corning, Inc.) and then cultured for 6–9 days in the absence of LIF.

Plasmid Construction and Transfection—Construction of mammalian expression vectors pCAGIP-Myc and pCAGIP-FLAG has been described previously (18, 47). A hygromycin-resistant pCAG vector was constructed by inserting the puromycin resistance gene of pCAGIP-Myc into the hygromycin resistance gene. The plasmid was named pCAGIH-Myc. Coding regions of *Etv4* and *Etv5* were amplified by PCR using specific primers listed in supplemental Table S1. pCAGIP-Myc-*Etv4*, pCAGIP-Myc-*Etv5*, pCAGIP-FLAG-*Etv5*, and pCAGIH-Myc-*Etv4* were constructed by inserting corresponding coding sequences into the expression vectors. For reporter plasmid construction, the promoter region (from −2019 to −2486; the transcription start site is considered as +1) of the *Etv4* gene and the intron 6 region of the *Etv5* gene were amplified by PCR using specific primers listed in supplemental Table S1. To introduce mutation of an OCT3/4-binding sequence of these regions, mutated OCT3/4 binding sequences were constructed by PCR using specific primers. PCR products were cloned into pGL4-promoter (48) and termed pGL4-promoter-*Etv4* (−2486/−2019)-wild type, -*Etv4* (−2486/−2019)-mutant, -*Etv5* intron 6-wild type, and -*Etv5* intron 6-mutant, respectively.

Plasmids were introduced into cultured cells using Lipofectamine 2000 (Life Technologies, Inc.) according to the manufacturer's protocol. ETV4- or ETV5-expressing ES cells were established by introduction of pCAGIP-Myc-*Etv4* or -*Etv5* into *Etv4/5* dKO (PE15-2) ES cells and cultured in the presence of 0.5 μg/ml puromycin (Nacalai Tesque, Kyoto, Japan). ETV4/5-expressing PE15-2 ES cells were established as follows. First, ETV4-expressing PE15-2 ES cells were established by introducing pCAGIH-Myc-*Etv4* into PE15-2 ES cells. They were subsequently cultured in the presence of 300 μg/ml hygromycin (Nacalai Tesque). After that, pCAGIP-FLAG-*Etv5* was introduced into the cells. Cells were selected by puromycin, and the cell line was named PE15-2 (ETV4/ETV5) 8. A control cell line was established by introduction of empty vectors (both pCAGIH-Myc and pCAGIP-FLAG) into PE15-2 ES cells in parallel. The cell line was named PE15-2 (ev/ev) 1.

Biotin-labeled DNA Pull-down Assay—Oligonucleotides containing an OCT3/4-binding site of the *Etv4* gene and ETV4/5-binding site of the *Gbx2* gene were synthesized. The oligonucleotide sequences are shown in supplemental Table S1. For a biotin-labeled DNA pull-down assay, 5 μM 3′-biotinylated double-stranded oligonucleotide was incubated overnight at 4 °C with 1 mg of either Myc-*Oct3/4*-, Myc-*Etv4*-, or Myc-*Etv5*-transfected HEK293 cell extracts in the presence of streptavidin-agarose (EMD Millipore, Darmstadt, Germany). For the competition assays, non-labeled oligonucleotide, either wild-type or mutant, was added in 25-fold molar excess. The beads were washed three times with a washing buffer (50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and 150 mM NaCl). Samples were examined by Western blot analysis.

Immunofluorescent Staining, Western Blot Analysis, and Determination of Alkaline Phosphatase Activity—For immunostaining analysis, ES cells were fixed with 4% paraformaldehyde

at 4 °C for 30 min. After permeabilization with 0.5% Triton X-100 in phosphate-buffered saline (PBS), the cells were preincubated with 1% BSA in PBS. The cells were then incubated with antibodies against NANOG (SC1000, Merck Millipore, Darmstadt, Germany), OCT3/4 (sc-9081, Santa Cruz Biotechnology, Inc.), or ESRRB (PP-H6705-00, Perseus Proteomics, Tokyo, Japan) at 4 °C overnight, followed by incubation with 100-fold diluted goat anti-rabbit or anti-mouse IgG FITC (Santa Cruz Biotechnology). For Western blot analysis, samples were mixed with an SDS sample buffer (5× buffer: 50 mM Tris-HCl (pH 6.8), 30% glycerol, 10% SDS, 250 mM dithiothreitol, 10 mM EDTA, and 0.01% Coomassie Brilliant Blue R250), subjected to SDS-10% PAGE, and transferred to a nitrocellulose membrane. The membrane was incubated with antibodies for NANOG, OCT3/4, ESRRB, α-tubulin (M175-3, MBL, Nagoya, Japan), or c-Myc (sc-40; Santa Cruz Biotechnology), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG (EMD Millipore). The blot was visualized using enhanced chemiluminescence reagents (PerkinElmer Life Sciences) with an LAS-1000 image analyzer (Fuji Film). Activity of alkaline phosphatase was determined using either a quantitative alkaline phosphatase ES characterization kit (Merck Millipore) or a vector blue substrate kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's protocols.

For the reporter assay, ZHBTc4 ES cells were transfected with pGL4 reporter plasmids, and these cells were divided into two dishes 24 h after transfection and cultured with or without 1 μg/ml Tet for 24 h. Cells were lysed in the cell lysis buffer, and luciferase activity in the extracts was measured using a luciferase assay kit (Promega, Madison, WI) with an AB-2200 (ATTO, Tokyo, Japan).

Preparation of Nuclear Extracts and the Electromobility Shift Assay—Nuclear extracts were isolated as described before (16). Briefly, ZHBTc4 ES cells treated with or without 1 μg/ml Tet for 3 days were resuspended in a buffer (20 mM HEPES (pH 7.9), 20% glycerol, 10 mM NaCl, 0.2 mM EDTA (pH 8.0), 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin). Samples were incubated on ice for 15 min and spun down at 1,000 rpm for 10 min. The pellet was resuspended in the same buffer at 2.5×10^7 nuclei/ml, and then 62.5 μl of 5 M NaCl was added to the 1 ml of sample. After incubation at 4 °C for 30 min, the sample was centrifuged at 10,000 rpm, and the supernatant was used as a nuclear extract.

Oligonucleotides containing the OCT3/4-binding site of the *Etv5* gene were synthesized. The oligonucleotide sequences are shown in supplemental Table S1. Double-stranded probes were end-labeled with [γ-^{32}P]ATP (MP Biomedicals, Santa Ana, CA) and purified by Mini Quick Spin Oligo columns (Roche Diagnostics, Basel, Switzerland). For a binding reaction, 5 μg of nuclear extracts were incubated with a radiolabeled probe, 2 μg of poly(dI-dC), and 300 μg/ml BSA either with or without a 200-fold excess of cold probe, either wild-type or mutated oligonucleotides, as a competitor. Also, either 2–4 μg of anti-OCT3/4 antibody (sc-5279; Santa Cruz Biotechnology) or an anti-FLAG antibody (F3165; Sigma-Aldrich) were added to the reaction mixture for a supershift assay. After incubation on ice for 40 min, the samples were subjected to electrophoresis in a nondenaturing 6% acrylamide gel at 100 V for 2 h. The gel was dried, and radiolabeled bands were visualized with an image analyzer (BAS-2000, Fuji Film, Tokyo, Japan).

Reverse Transcription-PCR (RT-PCR), Quantitative RT-PCR, and Oligonucleotide-based DNA Microarray Analysis—Total RNAs were isolated from cells with Sepasol-RNA I Super G (Nacalai Tesque) and converted to cDNAs by ReverTraAce (Toyobo, Osaka, Japan) with oligo(dT)$_{12-18}$ primers (Nippongene, Tokyo, Japan). Gene expression was determined by either regular RT-PCR or quantitative RT-PCR using SYBR Green (Mx3005P; Agilent Technologies, Santa Clara, CA). For quantitative RT-PCR, all samples were tested in triplicate, and the results from each sample were normalized relative to glyceraldehyde-3-phosphate dehydrogenase with the ΔΔ*Ct* method. Primer sequences have been described previously (16, 49) or are listed in supplemental Table S1.

The 3D-Gene Mouse Oligo chip 24k (Toray Industries Inc., Tokyo, Japan) was used for the oligonucleotide-based DNA microarray analysis. Briefly, one pair of RNA samples was collected from control wild-type and *Etv4/5* dKO ES cells. Total RNA was labeled with either Cy3 or Cy5, and Cy3 or Cy5 signals were obtained using the 3D-Gene Scanner (Toray Industries Inc.) and processed by 3D-Gene Extraction (Toray Industries Inc.). Detected signals for each gene were normalized by a global normalization method (Cy3/Cy5 ratio median = 1). Gene ontology term analysis and pathway analysis were done. The raw data has been deposited in the Gene Expression Omnibus (GEO) database, under number GSE47225.

Cell Proliferation Assay—The number of viable cells was measured by a direct cell count and a WST-1 assay. The WST-1 assay was performed according to the manufacturer's protocol (Roche Diagnostics). Briefly, cells were cultured in a 96-well plate for 3 days. Ten microliters of WST-1 reagent were added to each well, followed by incubation at 37 °C. The absorbance of the culture medium was measured at 450 and 630 nm using a microplate reader (Tecan, Männedorf, Switzerland).

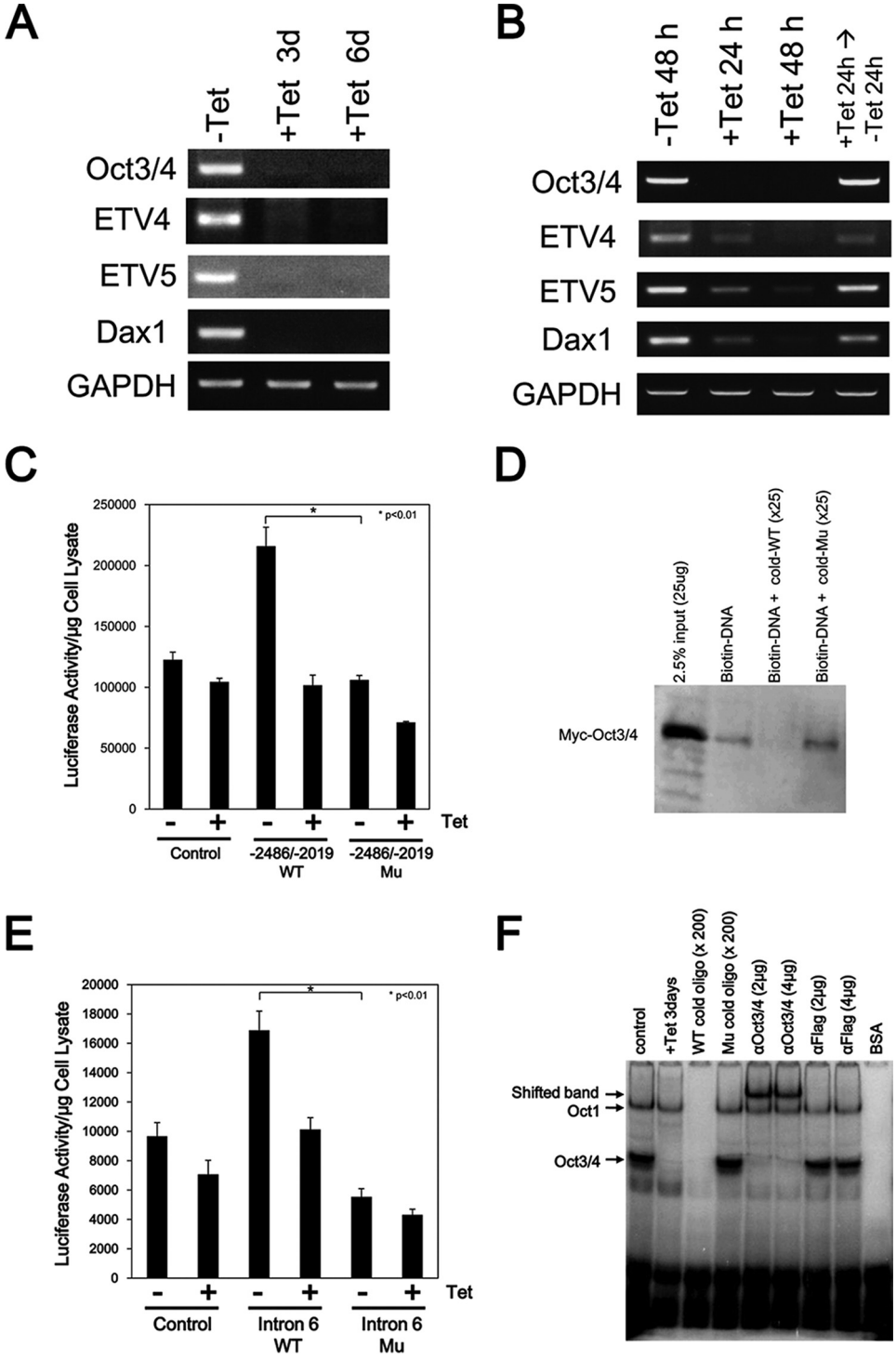
Results

Expression of Etv4 and Etv5 mRNA Correlates with Oct3/4 Expression—The transcription factor OCT3/4 is one of the major regulators of the self-renewal of ES cells. ZHBTc4 ES cells are Tet-inducible *Oct3/4*-conditional knock-out ES cells, which differentiate into trophectoderm after *Oct3/4* depletion (8). Here we examined the expression of *Etv4* and *Etv5* mRNA in ZHBTc4 ES cells. *Oct3/4* was expressed in ZHBTc4 ES cells in the absence of Tet, and the expression was repressed by Tet treatment for 3–6 days (Fig. 1*A*). *Etv4* and *Etv5* as well as *Dax1*, which is a direct downstream target of OCT3/4 (50), were also expressed in control ZHBTc4 ES cells, and their expression was also repressed after the treatment with Tet (Fig. 1*A*), indicating that *Etv4* and *Etv5* are expressed in undifferentiated ES cells and that their expression is repressed in trophectodermal cells.

We also examined the effects of short term exposure to Tet on the expression of *Etv4* and *Etv5* in ZHBTc4 ES cells. *Oct3/4* expression was repressed by Tet treatment within 24 h, and its expression was recovered by the removal of Tet (Fig. 1*B*). Expression of *Etv4* and *Etv5*, as well as *Dax1*, was reduced after

treatment with Tet for 24–48 h and recovered by the removal of Tet (Fig. 1*B*), showing that expression of *Etv4* and *Etv5* mRNA correlates with *Oct3/4* expression in ES cells.

Next, we investigated whether expression of *Etv4* and *Etv5* is regulated by OCT3/4 in ES cells. The consensus sequence of an OCT3/4-binding site is known to be ATGCAAAT. The *Etv4* gene contains several consensus OCT3/4-binding sequences, and the promoter region (from −2486 to −2019, transcription start site considered as +1) of the gene contains the consensus sequences and had enhancer activity in ES cells (Fig. 1*C*). The enhanced activity was repressed after Tet stimulation (*Oct3/4*-depleted condition) in ZHBTc4 ES cells, and no enhancer activity was detected when the consensus sequences were mutated (from ATTTGCAT to GACGTGGG). An association of the consensus sequences and OCT3/4 was observed by a biotin-labeled DNA pull-down assay. Myc-tagged OCT3/4 was precipitated with a biotin-labeled oligonucleotide contacting the OCT3/4-binding site of the *Etv4* gene, and the association was competed by an excess of unlabeled oligonucleotide, but not by unlabeled mutated oligonucleotide (Fig. 1*D*).



Because intron 5 and intron 6 of the *Etv5* gene contain the consensus sequences for OCT3/4, we also performed a luciferase reporter assay and found that the intron 6 region, but not the intron 5 region, had an enhancer activity in ES cells (Fig. 1*E*) (data not shown). Similar to the result with the *Etv4* gene, the enhanced activity was reduced after Tet stimulation in ZHBTc4 ES cells. No enhancer activity was detected when the consensus sequences were mutated from ATGCAAAT to GTGCCGCT. An electrophoretic mobility shift assay (EMSA) revealed that the 25-bp oligonucleotide containing the OCT3/4 binding site of intron 6 formed a complex with OCT3/4 *in vitro* (Fig. 1*F*). The complex was not detected when a nuclear extract from Tet-treated ZHBTc4 ES cells was examined. The unlabeled competitor (200-fold excess) inhibited the formation of the complex, but it was not competed by an excess of unlabeled mutated oligonucleotide. Also, the band was supershifted by the addition of an anti-OCT3/4 antibody but not an anti-FLAG antibody as a negative control. These results indicate that OCT3/4 binds to the *Etv4* and *Etv5* genes and regulates their expression.

Etv4 and Etv5 dKO ES Cells Maintain an Undifferentiated State—ETV4 and ETV5 have similar molecular structures and exhibit overlapping expression, and redundant activities of ETV4 and ETV5 have been noted in kidney development (41). Also, RNAi depletion of *Etv5* in ES cells did not result in obvious phenotypes (51). Thus, we analyzed *Etv4/5* dKO ES cells to explore the functions of ETV4 and ETV5 in ES cells.

One major characteristic of ES cells is the capacity for self-renewal; they have the potential to proliferate in an undifferentiated state. First, we examined whether *Etv4/5* dKO ES cells maintain an undifferentiated state or not. Control wild-type ES cells (PE9 ES cells) formed a compact colony, which is typical of undifferentiated ES cells, in the presence of LIF. In contrast, *Etv4/5* dKO ES cells (PE15-2 ES cells) failed to form a compact colony but showed an epithelial cell-like morphology even in the presence of LIF (Fig. 2*A*). After removing LIF from the culture medium, both control and the dKO ES cells showed epithelial cell-like morphology. Expression analysis showed that *Nanog*, *Oct3/4*, *Esrrb*, and *Dax1* were expressed in *Etv4/5* dKO ES cells as well as control cells and down-regulated after depletion of LIF from culture medium (Fig. 2*B*). We also examined protein expression of self-renewal markers, including NANOG, OCT3/4, and ESRRB, in *Etv4/5* dKO ES cells. Immunofluorescent staining and Western blot analysis revealed that NANOG, OCT3/4, and ESRRB were expressed in *Etv4/5* dKO ES cells (Fig. 2, *C–F*). In addition, relative alkaline phosphatase activity, which is a marker of undifferentiated pluripotent stem cells, was comparable between wild-type and *Etv4/5* dKO ES cells (Fig. 2, *G* and *H*). Self-renewal capacity is maintained by LIF stimulation followed by STAT3 activation. A recent study showed that inhibition of GSK3 and MEK/ERK pathways by small molecule inhibitors (2i) achieves LIF/STAT3-independent self-renewal of ES cells without serum (46), and naive ES cells can be cultured in the 2i medium, whereas primed epiblast stem cells cannot (52). Here, we examined whether *Etv4/5* dKO ES cells can be cultured in the 2i medium. As shown in Fig. 3*A*, PE9 wild-type and PE15-2 *Etv4/5* dKO ES cells grew in the 2i medium, and the epithelial-like morphology of PE15-2 ES cells was changed into a compact colony in the 2i medium. In PE9 wild-type ES cells, expression of *Etv5* mRNA was maintained in the 2i medium; however, *Etv4* mRNA was drastically repressed, suggesting that ETV4 is dispensable for the native status of ES cells. Quantitative RT-PCR analysis demonstrated that undifferentiated marker genes (*Oct3/4*, *Nanog*, and *Esrrb*), as well as naive marker genes (*Dax1* and *Rex1*) were expressed in PE9 and PE15-2 ES cells in the 2i medium (Fig. 3*C*), suggesting that *Etv4/5* dKO ES cells are able to maintain their naive status. Taken together, these results suggest that ETV4 and ETV5 are dispensable for the maintenance of the undifferentiated state of ES cells.

ETV4 and ETV5 Regulate Proliferation of ES Cells—After finding that *Etv4/5* dKO ES cells expressed self-renewal marker genes, next we compared the proliferation of control wild-type and *Etv4/5* dKO ES cells. A WST-1 assay demonstrated that cell proliferation was reduced in *Etv4/5* dKO ES cells (Fig. 4*A*). Direct cell counting also showed that proliferation of *Etv4/5* dKO ES cells was significantly decreased compared with wild-type ES cells (Fig. 4*B*). We also compared cell counts between wild-type and *Etv4/5* dKO ES cells cultured in the 2i medium. As shown in Fig. 4*C*, proliferation of *Etv4/5* dKO ES cells is slower than wild-type ES cells, indicating that proliferation of the dKO ES cells is not recovered even in the 2i condition. Of

FIGURE 1. **Expression of *Etv4* and *Etv5* mRNA in ZHBTc4 ES cells.** *A*, reduction of *Etv4* and *Etv5* mRNA expression after repression of *Oct3/4* expression. ZHBTc4 ES cells were cultured with or without 1 μg/ml Tet for 3 or 6 days. Expression of the indicated genes was examined by RT-PCR. *B*, induction of *Etv4* and *Etv5* mRNA expression by *Oct3/4* expression. ZHBTc4 ES cells were cultured with or without 1 μg/ml Tet for 24–48 h. To restore *Oct3/4* expression, the culture medium of Tet-treated cells was changed to a Tet-free medium, and the cells were cultured for another 24 h. *Dax1* was used for a positive control as an OCT3/4 target gene. *Gapdh* was used as an internal control. All results are representative of three independent experiments. *C*, determination of an OCT3/4-responsive element in the *Etv4* gene by a luciferase assay. The promoter region of the *Etv4* gene contains a consensus sequence for an OCT3/4 binding site (ATTTGCAT). ZHBTc4 ES cells were transfected with either pGL4-promoter (control), pGL4-promoter-*Etv4* (−2486/−2019)-wild type (−2486/−2019 WT), or pGL4-promoter-*Etv4* (−2486/−2019)-mutant (−2486/−2019 Mu). ZHBTc4 ES cells were divided into two dishes 24 h after transfection and cultured with (+) or without (−) 1 μg/ml Tet for 24 h, and luciferase activity was measured. The sequence of the OCT3/4-responsive element was changed from ATTTGCAT to GACGTGGG. Shown are means (*bars*) and S.D. (*error bars*) of triplicate assays. *D*, OCT3/4 directly binds to the putative binding sequences. The association of DNA and OCT3/4 was examined by a biotin-labeled DNA pull-down assay. Biotin-labeled oligonucleotide containing the putative binding site was incubated with cell extracts from Myc-*Oct3/4*-transfected HEK293 cells either with or without 25-fold non-labeled WT or mutated (*Mu*) oligonucleotide. The result shown is representative of three separate experiments. *E*, determination of an OCT3/4-responsive element in the *Etv5* gene by a luciferase assay. The intron 6 region of the *Etv5* gene contains the consensus sequence of an OCT3/4 binding site (ATGCAAAT). ZHBTc4 ES cells were transfected with either pGL4-promoter (control), pGL4-promoter-*Etv5* intron 6-wild type (*Intron 6 WT*), or pGL4-promoter-ETV5 intron 6-mutant (*Intron 6 Mu*). ZHBTc4 ES cells were divided into two dishes 24 h after transfection and cultured with (+) or without (−) 1 μg/ml Tet for 24 h, and luciferase activity was measured. The sequence of the OCT3/4-responsive element was changed from ATGCAAAT to GTGCCGCT. Shown are the means (*bars*) and S.D. (error bars) of triplicate assays. *F*, OCT3/4 directly binds to the putative binding sequences. An association of DNA and OCT3/4 was examined by an EMSA. Radiolabeled oligonucleotide containing the putative binding site was incubated with nuclear extracts from ZHBTc4 ES cells either with or without 200-fold cold WT or cold mutated (*Mu*) oligonucleotides, an anti-OCT3/4 antibody, or an anti-FLAG antibody. The result shown is representative of three separate experiments. Note that a band of OCT1-DNA complex can be detected in this assay as described before (66).

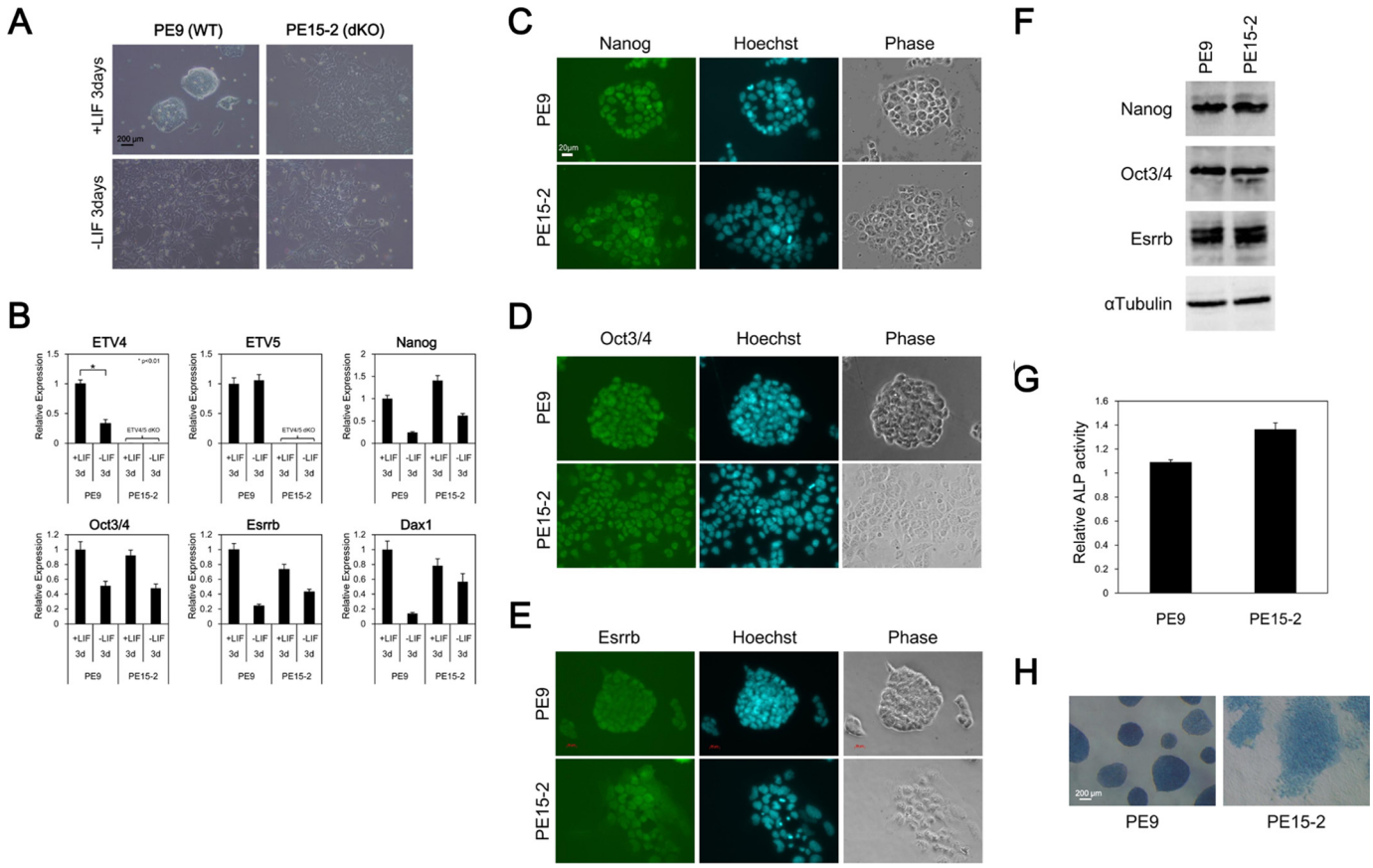


FIGURE 2. ***Etv4/5* dKO ES cells express self-renewal marker genes.** *A*, morphological signatures of PE9 (WT) and PE15-2 (*Etv4/5* dKO) ES cells. PE9 and PE15-2 ES cells were cultured in the presence or absence of LIF for 3 days. Morphological signatures were observed. *B*, expression of self-renewal marker genes in PE9 and PE15-2 ES cells. Expression of the indicated self-renewal marker genes in the conditions of *A* in PE9 and PE15-2 ES cells was examined by quantitative RT-PCR. Expression of *Etv4* and *Etv5* mRNA was only shown in PE9 ES cells. All samples were analyzed in triplicate, and the data were normalized to *Gapdh* expression. *C–E*, immunofluorescent stain in PE9 and PE15-2 ES cells. Protein expression of the self-renewal marker genes, including NANOG (*C*), OCT3/4 (*D*), and ESRRB (*E*), in PE9 and PE15-2 ES cells was examined by immunofluorescent stain. Nuclei were stained with the Hoechst stain. *F*, Western blot analysis in PE9 and PE15-2 ES cells. Protein expression of the self-renewal marker genes (NANOG, OCT3/4, and ESRRB) was examined by Western blot analysis. α-Tubulin was used as a loading control. *G*, determination of alkaline phosphatase activity. Relative activity of alkaline phosphatase was determined in PE9 and PE15-2 ES cells. Shown are the means (*bars*) and S.D. (*error bars*) of triplicate plates. *H*, alkaline phosphatase stain in PE9 and PE15-2 ES cells. Alkaline phosphatase of ES cells was detected by the VECTOR Blue alkaline phosphatase substrate kit. Morphological signatures were observed. All results are representative of several independent experiments.

note, expression of cyclin-dependent kinase inhibitors, including *p16-INK4/p19-ARF*, *p15*, and *p57*, was completely repressed in wild-type ES cells. By contrast, their expression was enhanced in *Etv4/5* dKO ES cells (Fig. 4*D*). These results demonstrate that ETV4 and ETV5 regulate the proliferation of ES cells by controlling cyclin-dependent kinase inhibitors.

Etv4/5 dKO ES Cells Are Impaired in Ectoderm Marker Gene Induction—Next, we assessed differentiation ability, the second characteristic of ES cells, in *Etv4/5* dKO ES cells within a system of EBs in suspension culture, which mimics early embryogenesis *in vitro*. Control wild-type ES cells formed EBs normally, whereas the EBs derived from *Etv4/5* dKO ES cells were much smaller than control cells (Fig. 5*A*). Quantitative RT-PCR analysis revealed that expression of endoderm (*Gata4* and *Gata6*) and mesoderm (*Flk1*, *T*, and *Tbx5*) markers was very similar between PE9 and PE15-2 cells (Fig. 5*B*). In contrast, expression patterns of ectoderm markers (*Fgf5*, *Sox1*, and *Pax3*) differed. *Fgf5* expression was induced in an EB sample that had been incubated for 3 days, and it was repressed immediately in PE9 ES cells, whereas its expression was not induced in PE15-2 ES cells. Strong expression of *Sox1* and *Pax3* was found in an EB sample of PE9 ES cells that had been incubated for 9 days, but they were not induced in PE15-2 cells. These results suggest that *Etv4/5* dKO ES cells have an impaired ability to induce ectoderm marker gene expression *in vitro*.

Gene Expression Signatures of Etv4/5 dKO ES Cells—Recent studies indicate that gene regulatory and/or transcription factor networks are involved in determining the characteristics of ES cells. Because *Etv4/5* dKO ES cells showed reduced proliferation and an impaired ability to induce an ectoderm marker, it is possible that the networks are changed in part. We compared the transcriptional profiles of wild-type and *Etv4/5* dKO ES cells using oligonucleotide-based DNA microarray analysis. Pathway analysis showed that several pathways were significantly changed (supplemental Table S2). Furthermore, gene ontology (GO) term analysis revealed that genes involved in metabolic processes; regulation of transcription, DNA-dependent; translation elongation; transcription, DNA-dependent; and lipid biosynthetic were down-regulated in *Etv4/5* dKO ES cells (Fig. 6*A*). Of note, gene expression-related GO terms (regulation of transcription, DNA-dependent; translation elongation; and transcription, DNA-dependent) were enriched.

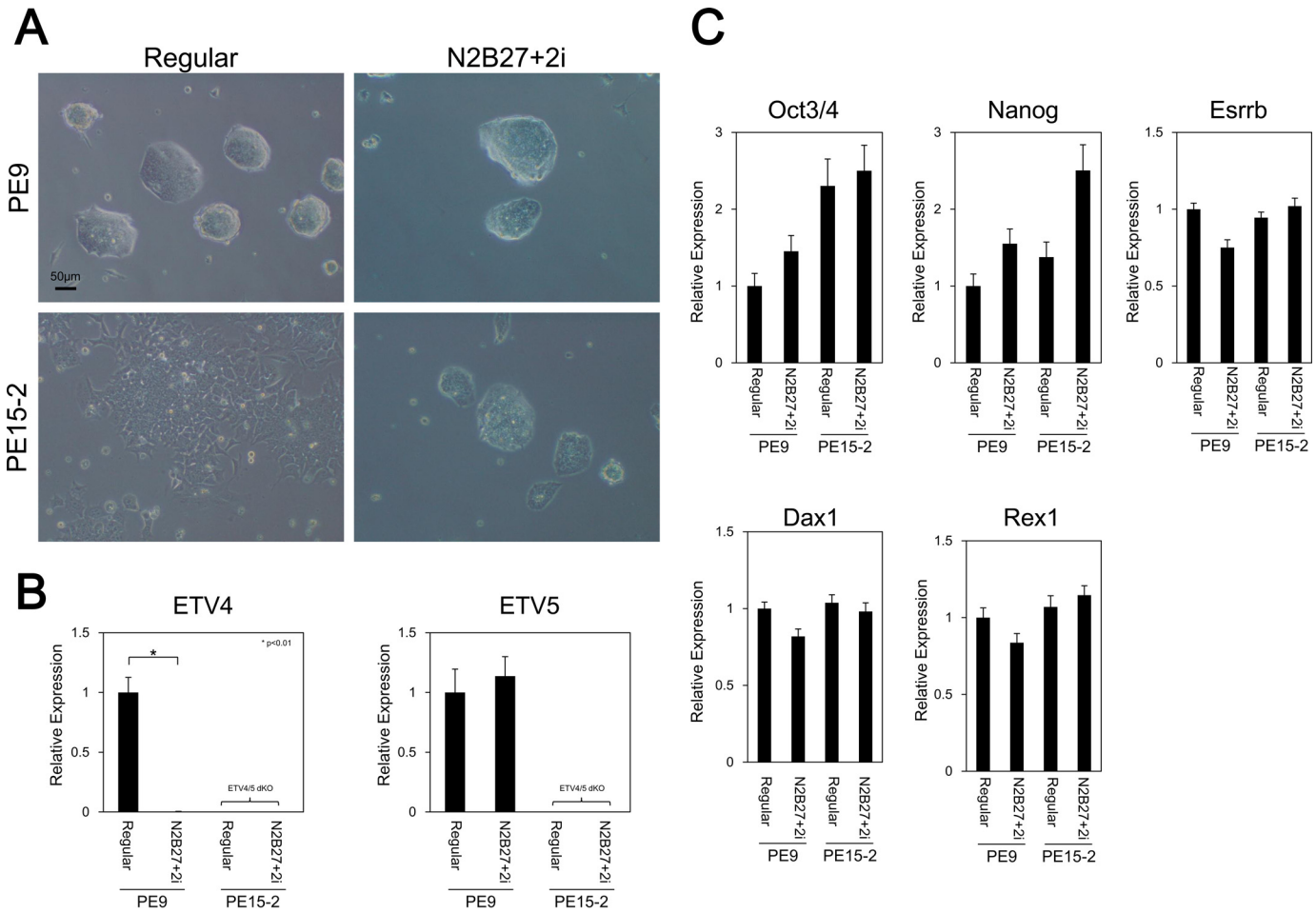


FIGURE 3. ***Etv4/5* dKO ES cells maintain the undifferentiated state in N2B27 serum-free medium plus two inhibitors.** *A*, morphological signatures of PE9 (WT) and PE15-2 (*Etv4/5* dKO) ES cells. PE9 and PE15-2 ES cells were cultured in either a regular serum- and LIF-containing medium or a chemically defined medium (DMEM/F-12 with N2B27 supplement plus 2i (mitogen-activated protein kinase/extracellular signal-regulated kinase (PD0325901) and glycogen synthase kinase 3 (CHIR99021) inhibitors) (2i condition)). Morphological signatures were observed. *B*, expression of *Etv4* and *Etv5* in the 2i condition. Expression of *Etv4* and *Etv5* in the conditions of *A* in PE9 and PE15-2 ES cells was examined by quantitative RT-PCR. *C*, expression of self-renewal marker genes in PE9 and PE15-2 ES cells. Expression of the indicated self-renewal marker genes in the conditions of *A* in PE9 and PE15-2 ES cells was examined by quantitative RT-PCR. All samples were analyzed in triplicate, and the data were normalized to *Gapdh* expression. *Error bars*, S.D.

Genes with the GO term, "regulation of transcription, DNA-dependent," are listed in Table 1. Several stem cell-related transcription factors, including *Tcf15*, *Lrh1/Nr5a2*, *Gbx2*, *Baf60c/Smarcd3*, and *Zic3*, were repressed in *Etv4/5* dKO ES cells. Reduced expression of these genes in *Etv4/5* dKO ES cells was validated by quantitative RT-PCR (Fig. 6*B*), indicating that the transcriptional network is impaired in *Etv4/5* dKO ES cells. We also examined the expression of these genes in the 2i medium. In wild-type ES cells, expression of *Tcf15*, *Gbx2*, and *Baf60c* was repressed, whereas *Zic3* and *Lrh1* were unchanged, indicating that *Tcf15*, *Gbx2*, and *Baf60c* are dispensable for the native status of ES cells (Fig. 6*C*). In *Etv4/5* dKO ES cells, the expression of these genes was still repressed compared with wild-type ES cells, suggesting that repressed genes in the dKO ES cells are not recovered by 2i medium.

Both ETV4 and ETV5 Are Able to Rescue the Epithelial-like Morphology and Expression of Tcf15 and Gbx2 in dKO ES Cells—Next, we established ETV4/5-expressing PE15-2 ES cells, where exogenous ETV4 and ETV5 were stably expressed in *Etv4/5* dKO ES cells. As shown in Fig. 7*A*, artificial expression of ETV4 and ETV5 in the dKO ES cells resulted in compact colony formation, in contrast to the control cells, which remained flat in shape. When we examined gene expression in these cells, expression of *Tcf15* and *Gbx2*, which are known to regulate ES cell characteristics (see "Discussion"), was recovered in the ETV4/5-expressing PE15-2 ES cells (Fig. 7*B*).

Because redundant activities of ETV4 and ETV5 have been reported, we examined whether single overexpression of either ETV4 or ETV5 in PE15-2 ES cells is enough to recover the morphology and gene expression of *Tcf15* and *Gbx2*. *Etv4/5* dKO ES cells were transfected with either an ETV4 or ETV5 expression vector, and the expression of the dysregulated genes in *Etv4/5* dKO ES cells was examined. As shown in Fig. 7*C*, single overexpression of either ETV4 or ETV5 in dKO ES cells resulted in the cells forming a compact colony. Also, expression of *Tcf15* and *Gbx2* was recovered in these cells (Fig. 7*D*). The *Gbx2* promoter contains the ETV4/5 binding site (from −1283 to −1266; transcription start site considered as +1). An association of the consensus sequences and either ETV4 or ETV5 was observed by a biotin-labeled DNA pull-down assay. Either Myc-tagged ETV4 or Myc-tagged ETV5 was precipitated with a biotin-labeled oligonucleotide contacting the ETV4/5-binding

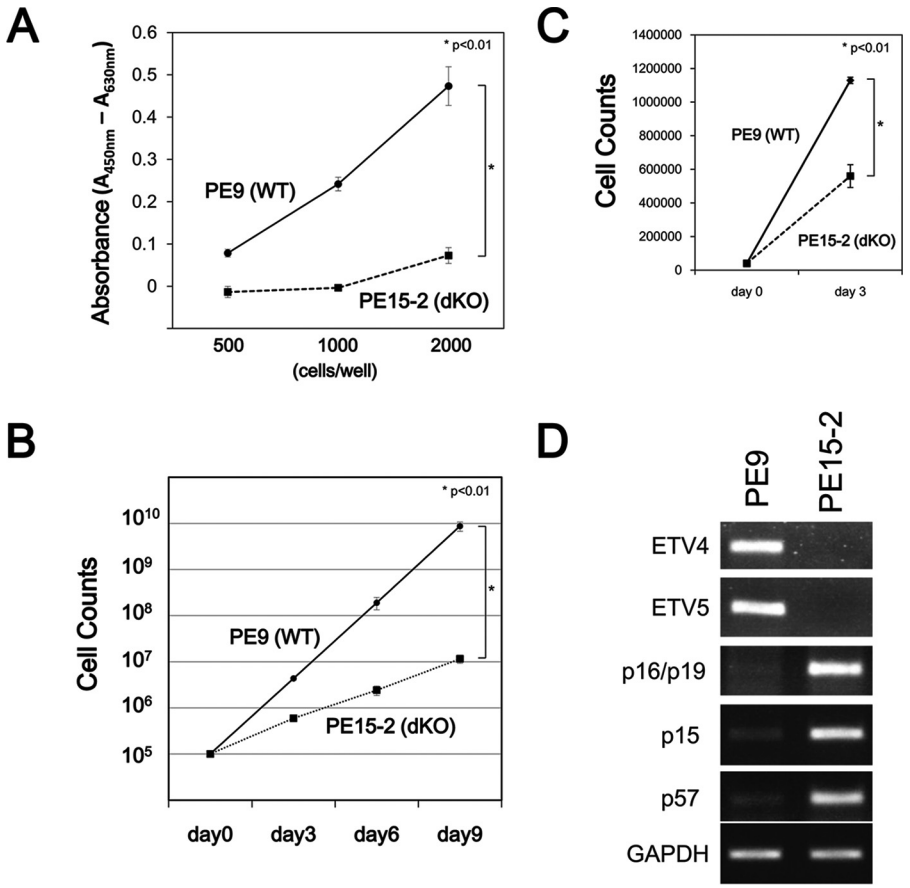


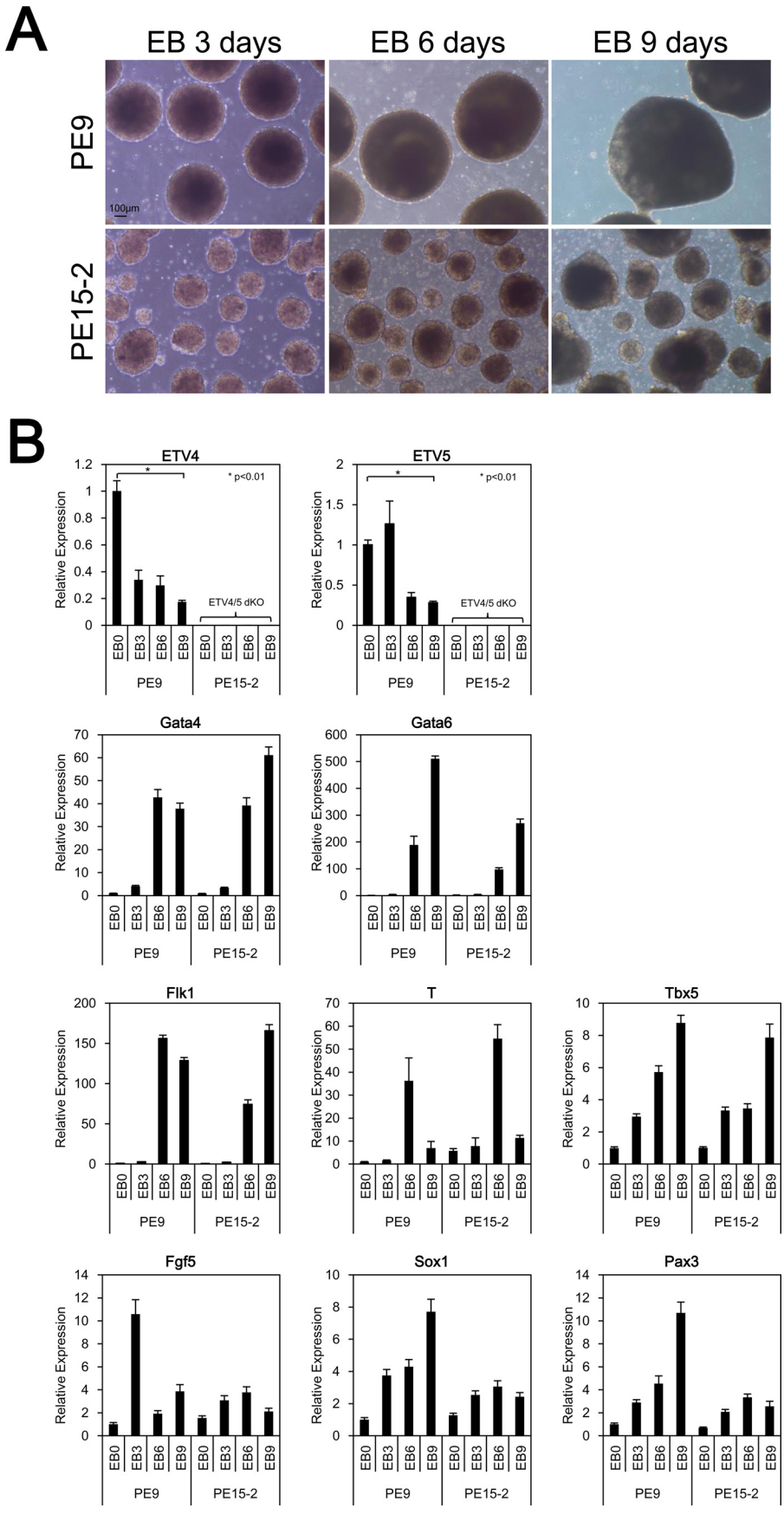
FIGURE 4. **ETV4 and ETV5 are involved in the proliferation of ES cells.** *A*, proliferation of PE9 (WT) and PE15-2 (*Etv4/5* dKO) ES cells. Either PE9 or PE15-2 ES cells were seeded at 500, 1000, or 2000 cells/well in a 96-well dish and subjected to a WST-1 assay. The results represent the means and S.D. (*error bars*) of triplicate assays. *B*, growth curves of PE9 and PE15-2 ES cells. PE9 and PE15-2 ES cells were cultured in the presence of LIF, and the cells were counted at the indicated days. The results represent the means and S.D. of four independent assays. *C*, direct cell counts of PE9 and PE15-2 ES cells in the 2i condition. PE9 and PE15-2 ES cells were cultured in the 2i condition, and the cells were counted at the indicated days. The results represent the means and S.D. of triplicate experiments. *D*, enhanced expression of cyclin-dependent kinase inhibitors in *Etv4/5* dKO ES cells. Expression of cyclin-dependent kinase inhibitors, *p16/p19*, *p15*, and *p57* mRNA in PE9 and PE15-2 ES cells was examined by RT-PCR. *Gapdh* was used as an internal control. The results are representative of three independent experiments.

site of the *Gbx2* gene, and the association was competed by an excess of unlabeled oligonucleotide but not by unlabeled mutated oligonucleotide (Fig. 7*E*). Taken together, these results suggest that the morphological phenotype of *Etv4/5* dKO ES cells is rescued by either ETV4 or ETV5, and *Gbx2* could be a downstream target of ETV4 and ETV5 in ES cells.

Discussion

ETV4 and ETV5 play crucial roles during organogenesis and morphogenesis. ETV4 is important for neuronal development, evidenced by the fact that *Etv4*-deficient mice have defects in neuromuscular innervation (53). Also, *Etv4*-deficient male mice fail to reproduce, probably because of dysfunctions in erection or ejaculation (54). Phenotypes of *Etv5*-deficient mice vary depending on the gene targeting constructions. Mice homozygous for a deletion of exons 2–5 of the *Etv5* gene are viable but display disruptions of spermatogenesis in males and functional disorders of the ovary in females (55, 56). A much more severe phenotype occurs when exons 11 and 12 of the *Etv5* gene are deleted (the same as our *Etv4/5* dKO ES cell construct). In that case, the homozygotes are embryonic lethal at embryonic day 8.5 (41). In addition, ETV4 and ETV5 have essential functions for kidney development. A single knockout of either the *Etv4* or *Etv5* gene had little effect on mouse kidney development, whereas kidney development failed completely in *Etv4/5* dKO mice (41, 44). Because our results show that expression patterns of ectoderm markers differ between wild-type and dKO ES cells in an EB assay, ETV4 and ETV5 are probably involved in early embryogenesis as well as organogenesis.

Etv4 and *Etv5* are downstream targets of GDNF/Ret signaling in kidney development (41, 44) and FGF signaling in limb development (57). Here, we show that *Etv4* and *Etv5* are expressed in undifferentiated ES cells and that their expression is down-regulated after repression of *Oct3/4*. Because OCT3/4 is the major transcription factor in ES cells, it seems reasonable to suggest that ETV4/5 is involved in the stemness of ES cells. In fact, although *Etv4/5* dKO ES cells expressed self-renewal marker genes, including *Oct3/4* and *Nanog*, the cells failed to form a compact colony, which is typical of undifferentiated ES cells. Furthermore, reduction of *Nanog*, *Esrrb*, and *Dax1* in *Etv4/5* dKO was mild at −LIF 3 days (Fig. 2*B*), and expression of *Oct3/4* and *Nanog* increased in *Etv4/5* dKO ES cells cultured with the 2i condition (Fig. 3*C*). Molecular mechanisms of the


A
EB 3 days
EB 6 days
EB 9 days
PE9
PE15-2
100μm
B
ETV4
ETV5
Gata4
Gata6
Flk1
T
Tbx5
Fgf5
Sox1
Pax3
Relative Expression
* p<0.01
ETV4/5 dKO
EB0
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PE15-2

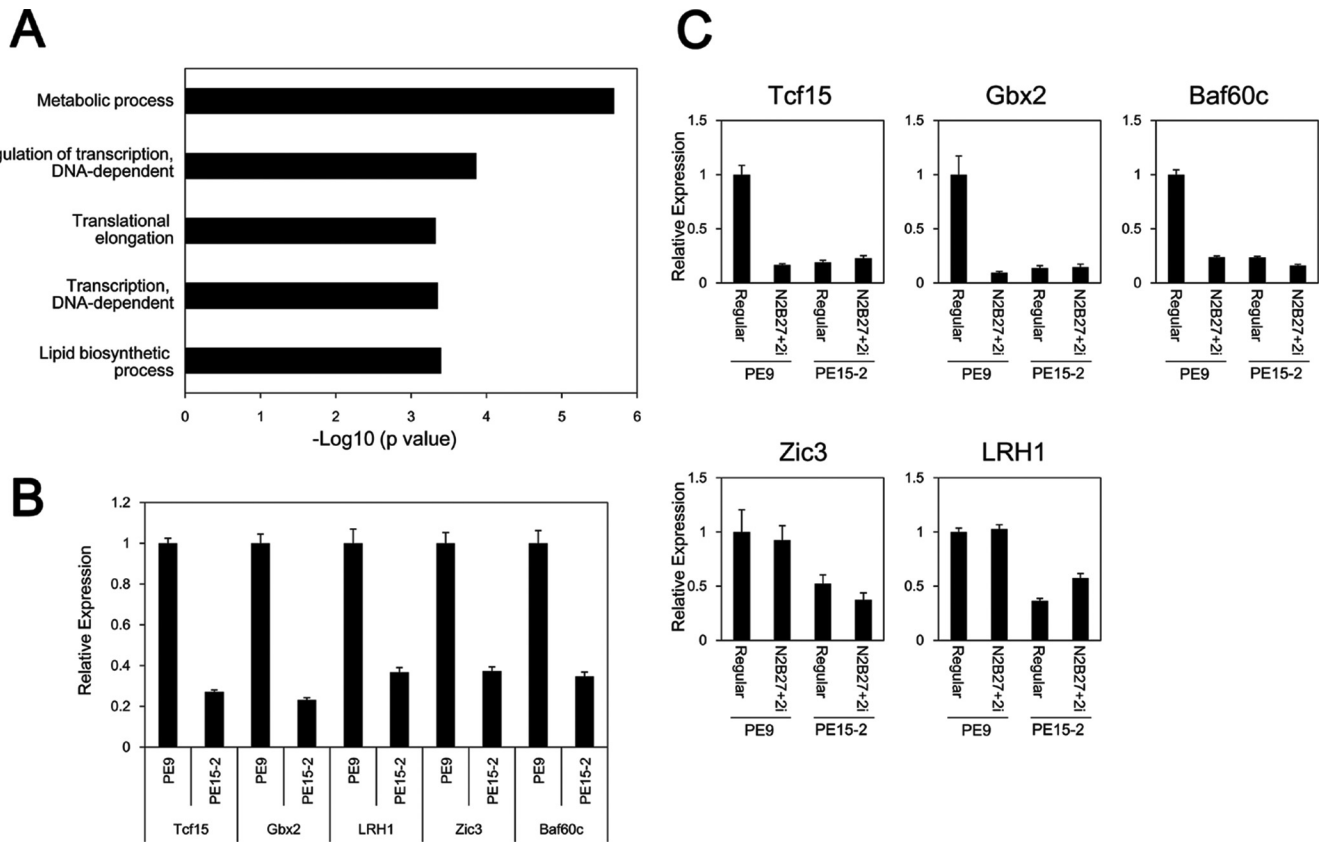

FIGURE 6. **Several transcription factors are down-regulated in *Etv4/5* dKO ES cells.** *A*, GO term analysis of down-regulated candidates in *Etv4/5* dKO ES cells. Genes involved in metabolic processes; regulation of transcription, DNA-dependent; translation elongation; transcription, DNA-dependent; and lipid biosynthetic are enriched significantly. GO categories are ranked according to their *p* value. *B*, expression of several transcription-related genes in PE9 and PE15-2 ES cells. Expression of transcription-related genes, including *Tcf15*, *Gbx2*, *Lrh1*, *Zic3*, and *Baf60c*, is down-regulated in PE15-2 ES cells, as shown by microarray analysis. Expression of the indicated genes in PE9 and PE15-2 ES cells was examined by quantitative RT-PCR. *C*, expression of several transcription-related genes in PE9 and PE15-2 ES cells in the 2i condition. PE9 and PE15-2 ES cells were cultured in the 2i condition, and expression of the indicated genes was examined by quantitative RT-PCR. All samples were analyzed in triplicate, and the data were normalized to *Gapdh* expression. *Error bars*, S.D.

TABLE 1

Down-regulated genes (GO:0006355; regulation of transcription, DNA-dependent) in *Etv4/5* dKO ES cells

Genes whose expression difference is log2 (ratio (PE15-2/PE9)) < -2.0 are listed.

Symbol	Description	Relative -fold changes, log2
Zfp386	Zinc finger protein 386 (Kruppel-like)	−4.02
Tcf15	Transcription factor 15	−3.64
Trps1	Trichorhinophalangeal syndrome I (human)	−3.34
Lrh1/Nr5a2	Nuclear receptor subfamily 5, group A, member 2	−3.31
Gbx2	Gastrulation brain homeobox 2	−3.29
Bcl11a	B-cell CLL/lymphoma 11A (zinc finger protein)	−2.98
Nkx6-3	NK6 transcription factor related, locus 3 (*Drosophila*)	−2.94
Baf60c/Smarcd3	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 3	−2.58
Dbp	D site albumin promoter binding protein	−2.51
Rhox10		−2.43
Tcea3	Transcription elongation factor A (SII), 3	−2.42
Suhw3	Suppressor of hairy wing homolog 3 (*Drosophila*)	−2.22
Nkx6-2	NK6 transcription factor related, locus 2 (*Drosophila*)	−2.21
Zic3	Zinc finger protein of the cerebellum 3	−2.19
Zfp459	Zinc finger protein 459	−2.11
A930001N09Rik	RIKEN cDNA A930001N09 gene	−2.04
Spic	Spi-C transcription factor (Spi-1/PU.1-related)	−2.03
Supt16h	Suppressor of Ty 16 homolog (*Saccharomyces cerevisiae*)	−2.01

FIGURE 5. ***Etv4/5* dKO ES cells have impaired abilities to induce ectoderm marker gene expression.** *A*, morphological signatures of EBs derived from PE9 (WT) and PE15-2 (*Etv4/5* dKO) ES cells. PE9 and PE15-2 ES cells were cultured in suspension without LIF for 3–9 days. Morphological signatures were observed. *Scale bar*, 100 μm. *B*, expression of differentiation marker genes in PE9 and PE15-2 EBs. Expression of the indicated differentiation marker genes in the conditions of *A* in PE9 and PE15-2 EBs was examined by quantitative RT-PCR. Expression of *Etv4* and *Etv5* mRNA was only shown in PE9 ES cells. All samples were analyzed in triplicate, and the data were normalized to *Gapdh* expression. *Error bars*, S.D.

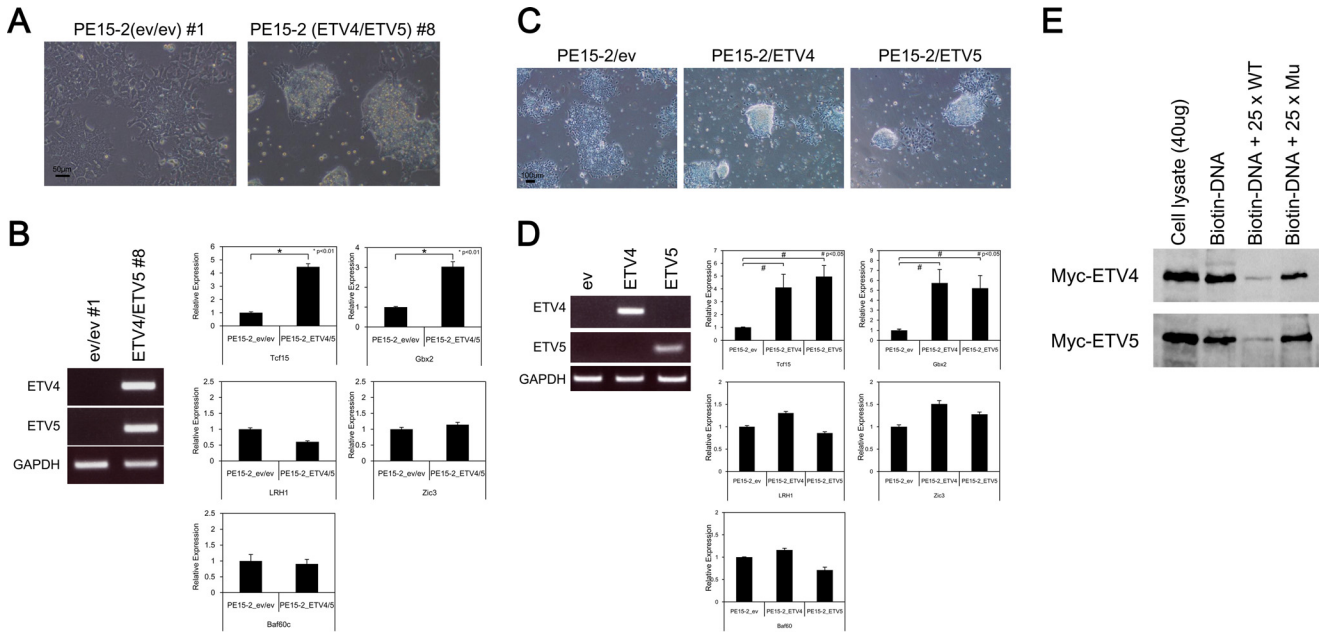

FIGURE 7. **Both ETV4 and ETV5 are able to rescue the epithelial like morphology and expression of *Tcf15* and *Gbx2* in dKO ES cells.** *A*, overexpression of both ETV4 and ETV5 in PE15-2 (*Etv4/5* dKO) ES cells. ETV4/5-expressing PE15-2 ES cells and control cells were established as described under "Experimental Procedures." Morphological signatures were observed. *Scale bar*, 50 μm. *B*, induction of *Tcf15* and *Gbx2* expression in ETV4/5-expressing PE15-2 ES cells. Expression of *Tcf15* and *Gbx2* mRNA, as well as *Lrh1*, *Zic3*, and *Baf60c* mRNA, in the PE15-2 ES cell lines was examined by quantitative RT-PCR. All samples were analyzed in triplicate, and the data were normalized to *Gapdh* expression. Expression of *Etv4* and *Etv5* mRNA was examined by regular RT-PCR. *C*, overexpression of either ETV4 or ETV5 in PE15-2 (*Etv4/5* dKO) ES cells. ETV4- or ETV5-expressing PE15-2 ES cells and control cells were established as described under "Experimental Procedures." Morphological signatures were observed. *Scale bar*, 100 μm. *D*, induction of *Tcf15* and *Gbx2* expression in ETV4- and ETV5-expressing PE15-2 ES cells. Expression of *Tcf15* and *Gbx2* mRNA as well as *Lrh1*, *Zic3*, and *Baf60c* mRNA in the PE15-2 ES cell lines was examined by quantitative RT-PCR. All samples were analyzed in triplicate, and the data were normalized to *Gapdh* expression. Expression of *Etv4* and *Etv5* mRNA was examined by regular RT-PCR. *E*, ETV4 and ETV5 directly bind to the putative binding sequences of the *Gbx2* gene. Biotin-labeled oligonucleotide containing the putative binding site was incubated with cell extracts from Myc-ETV4- or Myc-ETV5-transfected HEK293 cells either with or without 25-fold non-labeled WT or mutated (*Mu*) oligonucleotide. The result shown is representative of three separate experiments. *Error bars*, S.D.

phenotype remain to be clarified. Of note, the expression of several stem cell-related genes (*Tcf15*, *Lrh1/Nr5a2*, *Gbx2*, *Baf60c/Smarcd3*, and *Zic3*) was repressed, whereas the expression of differentiation-associated genes (*Olig1*, *Olig2*, *Gata2*, and *Hand1*) was enhanced in the cells from microarray analysis (data not shown). The self-renewal capacity and pluripotency of ES cells is thought to be controlled by gene regulatory networks, of which OCT3/4 and NANOG serve as master regulators. ETV5 is one of a large number of additional transcription factors in the network (33). Our investigation revealed that ETV4/5 are dispensable for self-renewal ability. However, induction of ectodermal gene expression was impaired in *Etv4/5* dKO ES cells. Therefore, we conclude that *Etv4/5* dKO ES cells are not completely differentiated and instead remain between an undifferentiated and a differentiated state or prodifferentiated state. Alternatively, because ES cells are thought to be a heterogeneous population, the *Etv4/5* dKO ES cell line may represent a mixed population of undifferentiated and differentiated cells.

Overexpression of both ETV4 and ETV5, as well as single overexpression of either ETV4 or ETV5, in dKO ES cells led to morphological changes (formation into a compact colony) and reinduction of *Tcf15* and *Gbx2* expression, suggesting a redundant function of ETV4 and ETV5 in ES cells. The proliferation ratio was almost comparable between ETV4/5-expressing dKO ES cells and empty vector-transfected dKO ES cells (data not shown). Because the expression of exogenous *Etv4* and *Etv5* was driven by the CAG promoter, a strict regulation by their native promoters may be required for complete rescue of the phenotype of dKO ES cells.

Our analysis indicates that *Tcf15* and *Gbx2* are target genes of ETV4 and ETV5. TCF15 is a basic helix-loop-helix transcription factor and thought to be a key regulator of mesoderm differentiation (58). Furthermore, somite formation is disrupted in *Tcf15*-deficient mice (59). TCF15 primes pluripotent cells for entry to the somatic lineage, and forced expression of TCF15 in ES cells suppresses endodermal differentiation (60). *Gbx2* is a homeobox gene and downstream target of LIF/STAT3 in ES cells (61). GBX2-overexpressing ES cells are able to maintain the undifferentiated state in the absence of LIF. *Stat3*-deficient ES cells differentiate even in the presence of LIF, although the cells are able to maintain the undifferentiated state by treatment with 2i (46). Importantly, GBX2 suffices to maintain the undifferentiated state in *Stat3*-deficient ES cells without 2i, suggesting that GBX2 is a regulator of ES cell self-renewal. Although *Tcf15* and *Gbx2* are downstream genes of ETV4 and ETV5 and could be involved in determining ES cell properties, these two molecules were insufficient to rescue the phenotypes of *Etv4/5* dKO ES cells. We also overexpressed GBX2 in *Etv4/5* dKO ES cells, but it appeared to have cytotoxic activity, and thus ES cell colonies were not obtained (data not shown). Overexpression of TCF15 in *Etv4/5* dKO ES cells led to compact colony formation, but cell proliferation as well as expression of other repressed genes, including *Zic3* and *Gbx2*, were not recovered (data not shown). Therefore, the compensatory factors for ETV4 and ETV5 remain to be clarified.

ETV4 and ETV5 are overexpressed in various tumors, including those affecting the breast, ovary, endometrium, prostate, and esophagus, and are involved in prognosis (40, 42). Ectopic forced expression of ETV4 in breast cancer cells promotes proliferation of MDA 231 breast cancer cells with induction of cyclin D3 expression (62), and functional inhibition of ETV4 or ETV5 by RNAi in mouse mammary tumor cells reduces cell proliferation with decreased expression of cyclin D1 and D2 (63). Proliferation of esophageal adenocarcinoma cells is also regulated by ETV4 (64). Our present data show reduced proliferation of *Etv4/5* dKO ES cells. Expression levels of D-type and E-type cyclins were comparable between wild-type and *Etv4/5* dKO ES cells from the microarray analysis; however, expression of *p16/p19*, *p15*, and *p57* was significantly enhanced in *Etv4/5* dKO ES cells. Indeed, ectopic expression of either P15 or P16 in human ES cells resulted in an accumulation of cells in the G_1 phase (65). Although the regulation mechanisms of *p16/p19*, *p15*, and *p57* by ETV4 and ETV5 remain to be elucidated, ETV4 and ETV5 can be involved in the proliferation of various types of cells by controlling cell cycle regulators.

In summary, *Etv4/5* dKO ES cells express OCT3/4 and NANOG, which are core factors of the gene regulatory network, allowing their undifferentiated status to be maintained. In contrast, expression of some stem cell-related genes, including *Tcf15*, *Gbx2*, *Zic3*, and *Lrh1*, was dysregulated in *Etv4/5* dKO ES cells, indicating that the network of the cells is partially impaired, resulting in a reduction of proliferation and a lack of induction of ectodermal gene expression. Further studies, including clarification of protein interaction partners and characterization of downstream targets of ETV4/5, will be helpful for understanding the functions of ETV4/5 in mouse ES cells.

Author Contributions—T. A. designed, performed, and analyzed the study and wrote the paper. S. K. and F. C. provided materials and wrote the paper. K. U. and H. K. analyzed data. T. Y. designed and analyzed the study and wrote the paper.

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