Establishment of *Tsc2*-deficient rat embryonic stem cells

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Abstract. Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by *TSC1* or *TSC2* mutations. TSC causes the development of tumors in various organs such as the brain, skin, kidney, lung, and heart. The protein complex TSC1/2 has been reported to have an inhibitory function on mammalian target of rapamycin complex 1 (mTORC1). Treatment with mammalian target of rapamycin (mTOR) inhibitors has demonstrated tumor-reducing effects in patients with TSC but is also associated with various adverse effects. In recent years, experiments involving *in vivo* differentiation of pluripotent stem cells have been reported as useful in elucidating mechanisms of pathogenesis and discovering new therapeutic targets for several diseases. To reveal the molecular basis of the pathogenesis caused by the *Tsc2* mutation, we derived embryonic stem cells (ESCs) from Eker rats, which have the *Tsc2* mutation and develop brain lesions and renal tumors. Although several studies have reported the necessity of *Tsc1* and *Tsc2* regulation to maintain ESCs and hematopoietic stem cells, we successfully established not only $Tsc2^{+/+}$ and $Tsc2^{+/-}$ ESCs but also $Tsc2^{-/-}$ ESCs. We confirmed that these cells express pluripotency markers and retain the ability to differentiate into all three germ layers. Comprehensive gene expression analysis of $Tsc2^{+/+}$ and $Tsc2^{+/-}$ ESCs revealed similar profiles, whereas the profile of $Tsc2^{-/-}$ ESCs was distinct from these two. *In vitro* differentiation experiments using these ESCs combined with *in vivo* experiments may reveal the mechanism of the tissue-specific pathogenesis caused by the *Tsc2* mutation and identify specific new therapeutic targets.

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Introduction

Tuberous sclerosis complex (TSC) is a genetic disorder characterized by multisystem involvement and wide phenotypic variability. This condition results in the development of non-cancerous tumors in various organs and most frequently affects the brain, skin, kidney, lung, heart, and retina. TSC manifestations in the central nervous system include cortical tubers, subependymal nodules, subependymal giant cell astrocytomas, and scattered abnormal cells throughout the brain (1). A majority of patients with TSC reveal neurological and/or psychiatric symptoms, including epilepsy, intellectual disability, autism spectrum disorder (ASD), attention deficit, depression, and anxiety disorder, which range from mild to severe and may impair their ability to live an independent life.

Mutation of either *TSC1* or *TSC2* causes TSC (2,3). Protein products of *TSC1* (hamartin) and *TSC2* (tuberin) form a complex that inhibits the Ras homologue enriched in the brain (Rheb), a small G protein that activates mammalian target of rapamycin complex 1 (mTORC1). Defects of *TSC1* or *TSC2* cause excessive mTORC1 activation, which in turn provokes abnormal regulation of important cellular processes such as cellular growth and proliferation (4,5). The Knudson's 'two-hit' model (6) has been the working molecular model for tumor development in TSC for several years. In fact, loss of heterozygosity (LOH) of *TSC1* or *TSC2* has been demonstrated in renal angiomyolipomas (7-9) and in subependymal giant cell astrocytomas (10). However, evidence for LOH in TSC cortical tubers is limited (11). On the other hand, haploinsufficiency of these genes is also speculated to be involved in TSC pathogenesis. To reveal *Tsc* mutation-related mechanisms of the pathogenesis, rodents harboring a defect of the *Tsc1* or *Tsc2* gene have been extensively investigated (12-15). For instance, $Tsc1^{+/-}$ and $Tsc2^{+/-}$ mouse models exhibit learning and memory deficits (16,17). Eker rats are heterozygous for a mutation of *Tsc2* and develop hereditary kidney cancer by the age of 1 year (18-20). Although kidney cancer is rare in human patients with TSC, it is the only cancer known to occur at an increased incidence in TSC. The embryonic lethality of $Tsc2^{-/-}$ Eker rat embryos is characterized by disrupted neuroepithelial growth (21). Although cortical tubers are rare (22), 63% of Eker rats develop brain lesions comprising a mixture of large and elongated cells in both subependymal and subcortical regions (23,24). In contrast, among *Tsc1* and *Tsc2* knock-out

mouse models, only conditional ablation in the brain can induce such lesions (25). Consequently, with regard to brain lesions, the Eker rat model is more similar to the human patients compared with other mouse models.

We observed that the tumorigenicity of *Tsc2*$^{-/-}$ cells derived from mice was effectively inhibited by rapamycin treatment (26). Other groups reported a similar effect when Eker rats or knock-out mice were treated with rapamycin, although some residual tumors were detected (27,28). These findings have provided the rationale for therapy with rapalogues to treat TSC lesions such as lymphangioleiomyomatosis, SEGAs, and angiomyolipomas, directed at the abnormal activation of mTORC1 (29-31). Although decreased tumor volume has been documented, complete cure was not achieved in most cases. In addition, there are several problems associated with long-term use of rapalogues, including various undesirable side-effects. Consequently additional therapeutic molecular targets are required. The pathogenesis of TSC is assumed to be related to abnormal differentiation as a result of *TSC1/2* deficiency. For instance, abnormal giant cells that appear in brain lesions of patients with TSC express both neuronal and glial lineage markers (32). In recent years, a number of articles have revealed differentiation- and cell type-specific abnormalities using *in vitro* differentiation protocols to investigate differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). To evaluate roles of *Tsc2* from the viewpoint of differentiation and tissue-specific pathogenesis as well as to compare and combine *in vivo* and *in vitro* models, we established ESCs from Eker rats.

In 2008, authentic rat ESCs were established for the first time (33,34), lagging behind the establishment of mouse (35,36) and human ESCs (37). Using methods described by Buehr *et al* (33), we generated ESCs from blastocysts of Eker rats to establish an *in vivo* experimental system to explore the role of *Tsc2* in TSC pathogenesis. Although several reports have indicated the necessity of *Tsc1* and *Tsc2* regulation to maintain ESCs (38) and somatic stem cells (39) or to establish iPS cells (40), we were able to establish not only *Tsc2*$^{+/+}$ and *Tsc2*$^{+/-}$ ESCs but also *Tsc2*$^{-/-}$ ESCs. To our knowledge, this is the first report describing the generation of *Tsc2*-deficient ESCs.

Materials and methods

Ethics statement. All animal experiments were conducted in strict accordance with the institutional guidelines of Juntendo University for animal experiments. The protocol was approved by the Animal Experimentation Committee of Juntendo University (Tokyo, Japan) (approval no. 250105). All surgical procedures were performed under isoflurane anesthesia, and all efforts were made to minimize animal suffering.

Animals. Genetic homogeneity of Eker rats was maintained in our laboratory by brother-sister mating. Wistar rats, Brown Norway rats, C57BL/6J mice, and nude mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). All animals were housed under specific pathogen-free conditions.

Mouse embryonic fibroblasts (MEFs). MEFs were derived from embryonic day 14.5 C57BL6/J mouse embryos. MEFs were cultured in Knockout DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and penicillin streptomycin (all from Gibco Life technologies, Carlsbad, CA, USA) on gelatin-coated dishes. MEFs were treated with mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for use as feeder cells.

Culture of ESCs. We generated ESCs from Eker rats according to the method reported by Buehr *et al* (33). After double heterozygous mating of Eker rats, E4.5 blastocysts were gently flushed out from uteri using the N2B27 medium (StemCells, Inc., Newark, CA, USA). After removal of zonae pellucidae with acid Tyrode's solution, whole blastocysts were plated and cultured on mitomycin C-treated MEFs in N2B27 medium supplemented with 3 μM of CHIR99021, 1 μM of PD0325901 (both from Axon Medchem BV, Groningen, The Netherlands), 1,000 U/ml rat leukemia inhibitory factor (LIF) (ESGRO®; Millipore, Bedford, MA, USA) [two inhibitors (2i) + LIF condition]. After 5-7 days, blastocyst outgrowths were cut into pieces and replated in the same 2i + LIF medium. Thereafter, emerging ESC colonies were dissociated using Accutase (Innovative Cell Technologies, Inc., San Diego, CA, USA) and passaged every 2-4 days.

Alkaline phosphatase staining. Alkaline phosphatase staining was performed with an alkaline phosphatase kit (85L3R; Sigma-Aldrich) according to the manufacturer's instructions.

Chromosomal analysis. A standard chromosome preparation method using colchicine treatment was employed. Chromosome preparations were analyzed after Giemsa staining. At least 30 metaphase chromosome sets were analyzed for each line.

Genotyping polymerase chain reaction (PCR). Genotyping of ESCs was conducted using PCR on ESC DNA. To discriminate *Tsc2* mutant or wild-type alleles, the following primers were used: 5MFJ (5'-ACC ATC AGG ATG CTG CTG AA-3'), 3MFJ2 (5'-CTA TGG CCA CAT GTG ACC AA-3'), and TSR27 (5'-GCG CCA GAT TCA CCT CAT TA-3') (41). PCR was used to identify the gender of ESCs by amplification of the rat Y chromosome-specific *Sry* gene using the primer pair Sry-F (5'-CAT CGA AGG GTT AAA GTG CCA-3') and Sry-R (5'-ATA GTG TGT AGG TTG TTG TCC-3') (33).

Reverse transcription (RT)-PCR. Total RNA was obtained using a NucleoSpin® RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Complementary DNA was synthesized using a SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and an oligo-dT primer, according to the manufacturer's instructions. PCR was performed in a thermal cycler (Hybaid MBS 0.2G Thermal Cycler; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The following primer pairs were used: Oct4-F (5'-GGG ATG GCA TAC TGT GGA C-3'), Oct4-R (5'-CTT CCT CCA CCC ACT TCT C-3'), Sox2-F (5'-GGC GGC AAC CAG AAG AAC AG-3'), Sox2-R (5'-GTT GCT CCA GCC GTT CAT GTG-3'), rat Nanog-F (5'-GCC CTG AGA AGA AAG AAG AG-3'), rat Nanog-R (5'-CGT ACT GCC CCA TAC TGG AA-3') (33), rat nestin-F (5'-AGC CAT TGT GGT CTA CTG A-3'), rat nestin-R (5'-TGC AAC TCT GCC TTA TCC-3'), Sox17-F

(5'-AGG AGA GGT GGT GGC GAG TAG-3'), and Sox17-R (5'-GTT GGG ATG GTC CTG CAT GTG-3') (34).

Western blotting. Cells were harvested and lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol). Proteins were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was blocked with 1% skimmed milk in Tris-buffered saline containing 0.05% Tween-20 and probed with appropriate antibodies using the EnVision System (DakoCytomation, Glostrup, Denmark). Antibody signals were developed using ECL reagents and Hyperfilm ECL film (both from GE Healthcare, Little Chalfont, UK), which were then scanned using CEPROS SV (Fujifilm, Tokyo, Japan). The following primary antibodies were used: anti-Tsc2 antibody (C20; 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-Tsc1 primary antibody (c-Tsc1, 1:500), anti-phospho-S6 ribosomal protein (Ser235/236) rabbit polyclonal antibody (1:1,000, no. 2211), anti-S6 ribosomal protein rabbit monoclonal antibody (1:1,000, no. 2217) (both from Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-β-actin mouse monoclonal antibody (1:1,000; Sigma-Aldrich).

Embryoid body (EB) formation. ESCs were plated into low-adhesion 96-well dishes (MS-9096; Sumitomo Bakelite Co., Ltd., Tokyo, Japan). After 10 days of suspension culture, EBs were plated onto Matrigel-coated dishes in GMEM/10% fetal bovine serum medium (both from Gibco Life Technologies).

Immunocytochemistry. Cells were fixed and permeabilized with 4% paraformaldehyde and 0.25% Triton X-100 (both from Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS for 30 min at 4˚C and then washed (3x5 min) with PBS/0.1% bovine serum albumin (BSA) (Iwai Kagaku Co., Tokyo, Japan). Cells were incubated with a primary antibody in PBS with 1% BSA for 1 h at room temperature. Thereafter, cells were washed and incubated with fluorophore-conjugated secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) for 1 h at room temperature. Immunofluorescent images were captured using a Leica TCS SP5 v2.0 system (Leica, Heidelberg, Germany). The following primary antibodies were used: anti-Oct3/4 mouse monoclonal antibody (1:50, C-10; Santa Cruz Biotechnology, Inc.), anti-Sox2 rabbit polyclonal antibody (1:100, poly6309; BioLegend, San Diego, CA, USA), anti-β-III tubulin mouse monoclonal antibody (1:500, Tuj-1; Covance Laboratories, Princeton, NJ, USA), anti-myosin heavy chain mouse monoclonal antibody (1:50, MF20; R&D Systems, Minneapolis, MN, USA), and anti-Gata4 mouse monoclonal antibody (1:50; Santa Cruz Biotechnology, Inc.). Alexa Fluor (488 or 568)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Invitrogen Life Technologies) were used at 1:1,000 dilutions.

Teratoma formation. Approximately 5x10^5 cells were injected under kidney capsules of nude mice. Tumors were dissected after 4-5 weeks and fixed in 10% buffered formalin. Tumor tissues were embedded in paraffin wax, sectioned, and examined after hematoxylin and eosin staining.

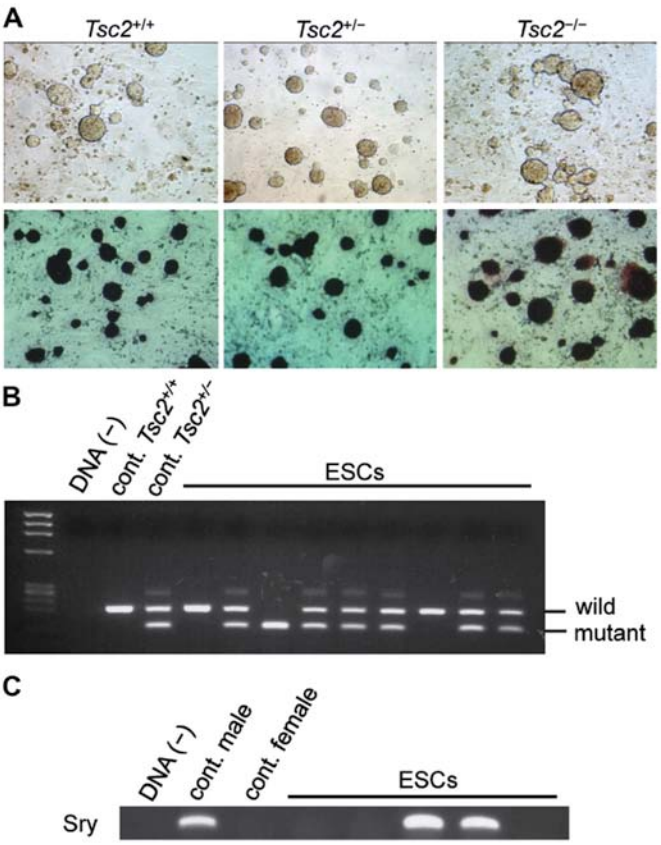

Figure 1. Establishment of *Tsc2*-deficient cell lines from blastocysts of Eker rats. (A) Colonies of established cell lines. *Tsc2*$^{+/+}$, *Tsc2*$^{+/-}$, and *Tsc2*$^{-/-}$ represent wild-type, *Tsc2* heterozygous mutant, and *Tsc2* homozygous mutant, respectively. Morphology of colonies established from blastocysts of Eker rats cultured on mouse embryonic fibroblasts (MEFs) in two inhibitors (2i) with leukemia inhibitory factor (LIF) (upper panels). Alkaline phosphatase staining of colonies (lower panels). Representative colonies are presented. (B) Polymerase chain reaction (PCR) genotyping for the *Tsc2* gene. Upper and lower bands represent wild-type and mutant *Tsc2* alleles, respectively. DNA(-), negative control; cont. *Tsc2*$^{+/+}$, wild-type rat; cont. *Tsc2*$^{+/-}$, *Tsc2* heterozygous mutant rat. Results of representative lines [embryonic stem cells (ESCs)] are presented. (C) PCR of the *Sry* gene for gender determination of established cell lines. DNA(-), negative control; cont. male, male control; and cont. female, female control. Results of representative lines (ESCs) are presented.

Blastocyst injection and generation of chimeric rats. Because collection of many blastocysts from Brown Norway rats is inefficient, we attempted the chimera formation assay using Brown Norway as well as Wistar rats. Rat blastocysts at E4.5 days were collected on the day of injection and cultured for 2-3 h to ensure cavitation. ESCs were disaggregated using Accutase, and 10-12 cells were injected into blastocyst cavities. Injected embryos were transferred into uteri of pseudopregnant rats.

Dead embryos were collected from uteri by cesarean section. For *Tsc2* genotyping PCR, genomic DNA was obtained from several parts of each embryo or pup.

Gene expression microarray analysis. The Rat Affymetrix GeneChip Gene 1.0 ST Array (Affymetrix, Inc., Santa Clara, CA, USA) was used for microarray analysis. Amplification and labeling of probes and hybridization were performed according to the manufacturer's instructions. Hierarchical clustering analysis was performed using GeneSpring software version 12.1 (Agilent Technologies, Inc., Santa Clara, CA, USA).

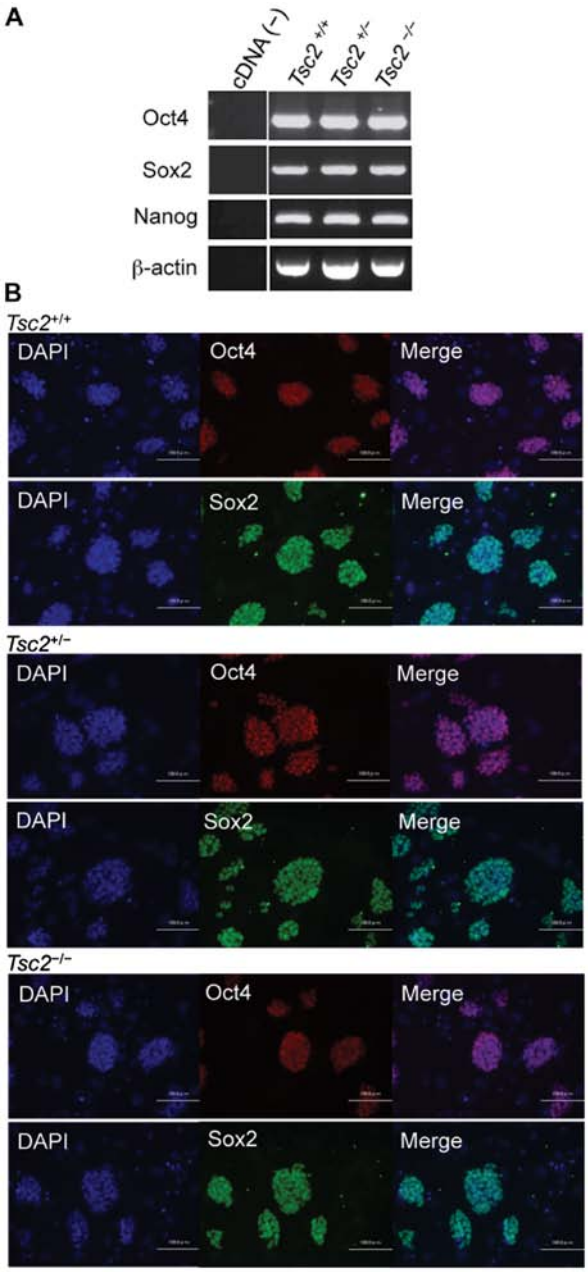

Figure 2. Expression of pluripotency markers in *Tsc2*$^{-/-}$ embryonic stem cells (ESCs). (A) Reverse transcription (RT)-PCR analysis of *Oct4*, *Sox2*, *Nanog*, and *β-actin* expression in established cell lines. cDNA(-); negative control. Representative results are presented. (B) Immunofluorescent staining of pluripotency markers in established cell lines: *Tsc2*$^{+/+}$ (top panels), *Tsc2*$^{+/-}$ (middle panels), and *Tsc2*$^{-/-}$ (bottom panels). Oct4 (red) and Sox2 (green) are used as pluripotency markers. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Right panels are merged images of left and center panels. Scale bars, 100 μm.

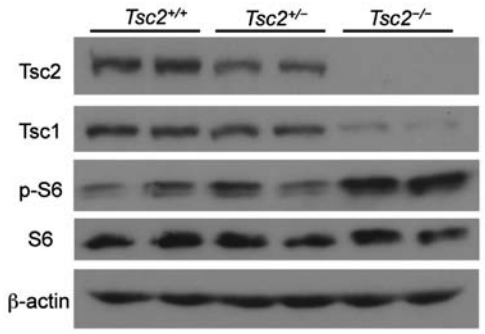

Figure 3. Activated mammalian target of rapamycin complex 1 (mTORC1) pathway in *Tsc2*$^{-/-}$ embryonic stem cells (ESCs). Analysis of mTORC1 pathway activation by western blotting in established cell lines. Tsc1, Tsc2, S6 ribosomal protein (S6), and phosphorylated S6 ribosomal protein (p-S6) were analyzed. β-actin was used as the control.

Results

Establishment of Tsc2-deficient stem cells from Eker rat embryos. After mating of double heterozygous Eker rats, a total of 34 blastocysts were collected. Zonae pellucidae were removed, and most blastocysts were successfully cultured on feeder cells, revealing outgrowths from embryonic fibroblasts (MEFs) in N2B27 medium supplemented with 2i + LIF (33). After several passages, a total of 26 cell lines were established. We routinely passaged these cells every 2-4 days by dissociating them into single cells and replating onto new feeder cells. They grew as dome-shaped or spherical colonies and were maintained for >25 passages without losing their morphology (Fig. 1A). A majority of colonies expressed alkaline phosphatase, an indicator of stem cell character (Fig. 1A). Thereafter, we checked *Tsc2* genotypes of established cell lines by PCR. Surprisingly, we identified that not only *Tsc2*$^{+/+}$ and *Tsc2*$^{+/-}$ cell lines but also *Tsc2*$^{-/-}$ cell lines had been established (Fig. 1B). Considering that previous reports had indicated that *Tsc2* is necessary for the maintenance of stem cell characteristics, this result was unexpected. Both male and female cell lines were established for each genotype (Fig. 1C). Chromosome analysis revealed that most cell lines had normal ploidy (n=42, data not shown). RT-PCR analysis revealed that *Tsc2*$^{-/-}$ cells expressed the pluripotency markers *Oct4*, *Sox2*, and *Nanog* (Fig. 2A). Oct4 and Sox2 expressions were confirmed by immunofluorescence microscopy (Fig. 2B). These results indicate that *Tsc2*-deficient stem cells could be established from Eker rat embryos. We performed further experiments using two independent cell lines of each genotype.

Activation of the mTORC1 pathway in Tsc2$^{-/-}$ *ESCs*. To evaluate the mTORC1 activation status, we analyzed ESCs by western blotting. Tsc2 protein was detected in *Tsc2*$^{+/+}$ and *Tsc2*$^{+/-}$ cells but not in *Tsc2*$^{-/-}$ cells, thereby confirming results of the genotype analysis. Tsc1 protein levels were decreased in *Tsc2*$^{-/-}$ ESCs, thereby reflecting the reciprocal stabilization between Tsc1 and Tsc2 proteins (42). As expected, an increase in S6 phosphorylation was detected in *Tsc2*$^{-/-}$ cells compared with that in *Tsc2*$^{+/+}$ and *Tsc2*$^{+/-}$ cells, which indicates abnormal activation of the mTORC1 pathway in *Tsc2*$^{-/-}$ ESCs (Fig. 3). These results indicate that despite abnormal activation of the mTORC1 pathway, *Tsc2*$^{-/-}$ ESCs can be established.

In vitro differentiation of Tsc2$^{-/-}$ *ESCs into three germ layers*. Using the EB formation assay, we evaluated the differentiation potential of the established cell lines. We assessed the expression of differentiation markers by RT-PCR. Expression of markers for ectoderm (*Nestin*), endoderm (*Sox17*), and mesoderm (*Flk1*) were all observed in EBs (Fig. 4A). We plated EBs onto Matrigel-coated dishes and assessed their differentiation status by immunofluorescent staining for β-III tubulin (neuroectoderm), myosin (mesoderm), and Gata4

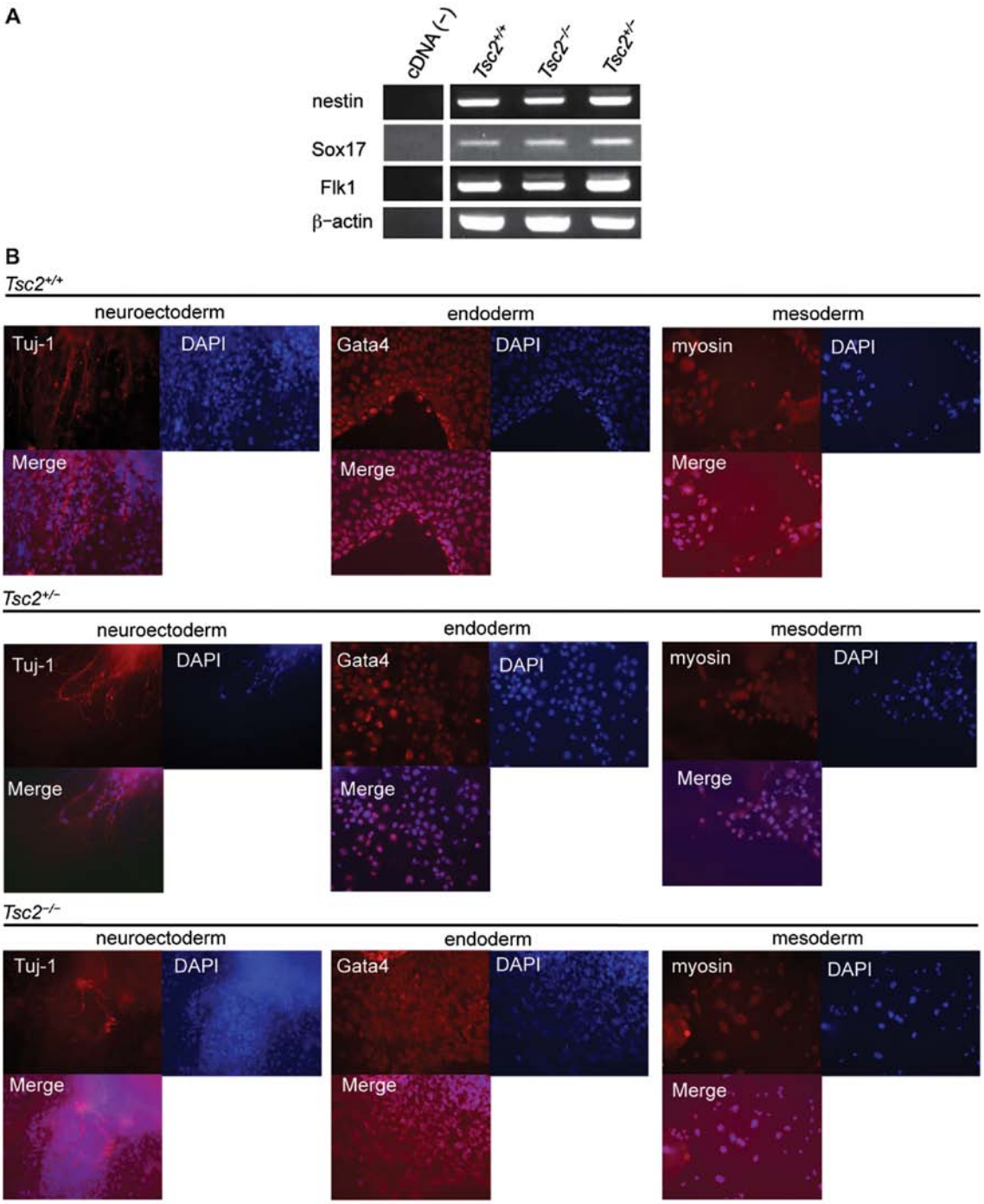

Figure 4. *In vitro* differentiation of *Tsc2*$^{-/-}$ embryonic stem cells (ESCs). (A) Reverse transcription (RT)-PCR analysis of differentiation marker expression in embryoid bodies (EBs) formed from established cell lines. Expression of *Nestin* (ectoderm), *Sox17* (endoderm), and *Flk1* (mesoderm) were analyzed with *β-actin* as control. cDNA(-); negative control. (B) Immunofluorescent staining of Tuj-1/β-III tubulin (neuroectoderm, red), Gata4 (endoderm, red), and myosin (mesoderm, red). Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI) (blue).

(endoderm) (Fig. 4B). Not only *Tsc2*$^{+/+}$ and *Tsc2*$^{+/-}$ cells but also *Tsc2*$^{-/-}$ cells demonstrated the potential to differentiate into all three germ layers. In addition, we observed spontaneously beating areas in EBs of all *Tsc2* genotypes (data not shown). These results suggest that most differentiation processes of ESCs were not blocked by *Tsc2* deficiency.

Differentiation of Tsc2$^{-/-}$ *ESCs into multiple lineages in teratomas*. When *Tsc2*$^{-/-}$ ESCs were transplanted under the kidney capsule of nude mice, they differentiated into tissues derived from all three germ layers, including gut-like epithelium (endoderm), cartilage and adipocytes (mesoderm), stratified squamous epithelium, and neuroepithelium (ectoderm) (Fig. 5). These results indicate that *Tsc2*$^{-/-}$ ESCs are multipotent, although detailed characterization of each of the differentiated tissues remains to be elucidated. Interestingly, we observed that abnormal ductal structures appeared in *Tsc2*$^{-/-}$ teratomas (Kawano H, *et al*, unpublished data). Further characterization of these abnormal structures is described in another report (Kawano H, *et al*, unpublished data).

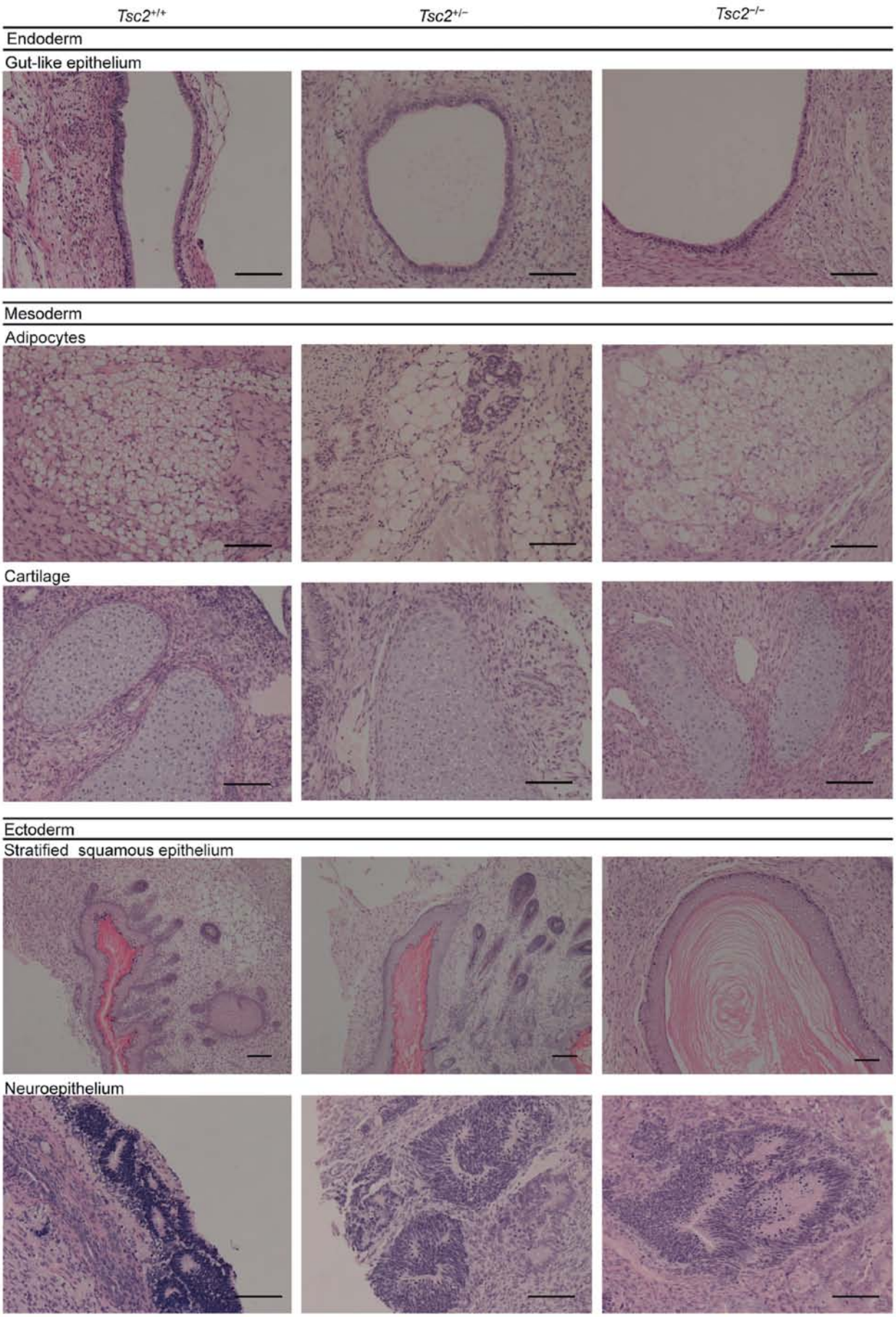

Figure 5. Differentiation of *Tsc2*$^{-/-}$ embryonic stem cells (ESCs) in teratomas. Tissue samples of teratomas derived from established ESCs were stained with hematoxylin and eosin. From top to bottom: gut-like epithelium (endoderm), adipocytes (mesoderm), cartilage (mesoderm), stratified squamous epithelium (ectoderm), and neuroepithelium (ectoderm). Scale bars, 100 μm.

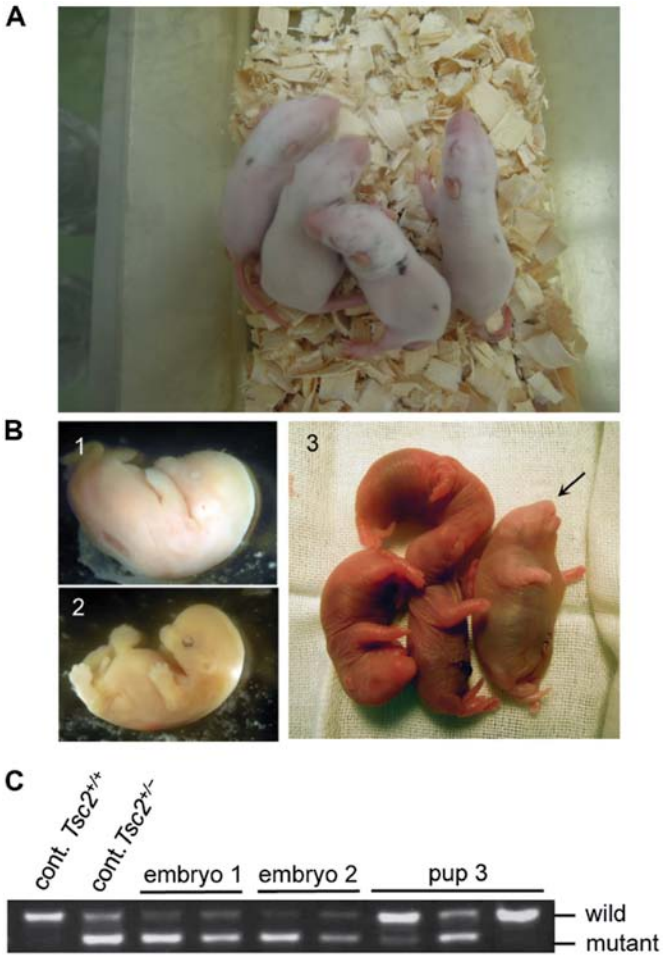

Figure 6. Chimeric rats from established embryonic stem cells (ESCs). (A) Four chimeras (2 males, 2 females) obtained by injection of *Tsc2*$^{+/+}$ ESCs into Wistar blastocysts. Black coat color indicates a contribution of ESCs established from Eker rats. (B) Dead embryos (1 and 2) and a pup (3, arrow) from blastocysts injected with *Tsc2*$^{-/-}$ ESCs. (C) *Tsc2*-genotyping polymerase chain reaction (PCR) of dead embryos and a pup. Genomic DNA was obtained from several parts of each embryo and pup. DNA(-), negative control; cont. *Tsc2*$^{+/+}$, wild-type rat; cont. *Tsc2*$^{+/-}$, *Tsc2* heterozygous mutant rat.

Contribution of Tsc2$^{+/+}$ *ESCs in chimeras*. Next, to determine the ability of established ESCs to form chimeras, we injected *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ ESCs into blastocysts of Wistar rats or Brown Norway rats (Materials and methods). Although ratios were low, four chimeras with black coat color were born from Wistar blastocysts injected with *Tsc2*$^{+/+}$ ESCs, indicating the contribution of ESCs from the Eker rat strain (Fig. 6A). In contrast, we were unable to obtain pups demonstrating chimeric coat color in repeated trials using two independent *Tsc2*$^{-/-}$ ESCs. However, in these trials, we detected dead embryos in the uterus of recipient mother rats at term (Fig. 6B1 and 2). The appearance of dead embryos suggested developmental retardation. In addition, one live pup was delivered by cesarean section but died shortly after birth (Fig. 6B3). This pup revealed various morphological abnormalities such as an enlarged trunk. PCR genotyping of dead embryos and the pup indicated the contribution of *Tsc2*$^{-/-}$ ESCs in their tissues (Fig. 6C). On the basis of the band pattern of two dead embryos, we concluded that they had a greater contribution of *Tsc2*$^{-/-}$ ESCs compared with the live pup. These results suggest that a greater contribution of *Tsc2*$^{-/-}$ ESCs in the chimera results in embryonic lethality.

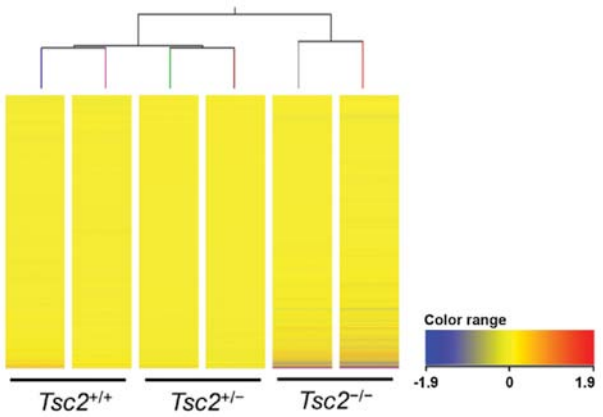

Figure 7. Hierarchical clustering analysis of embryonic stem cells (ESCs). RNAs from ESCs were analyzed using the Affymetrix GeneChip Gene 1.0 ST Array. Data normalization and hierarchical clustering analysis of gene expression profiles were performed using the GeneSpring software.

Although germline transmission has not been confirmed yet, the contribution in chimeras suggests that ESCs established in this study possessed characteristics of multipotent stem cells.

Distinct gene expression pattern in Tsc2$^{-/-}$ *ESCs on microarray analysis*. To compare gene expression profiles of established ESCs, we employed microarray analysis (Fig. 7). Similar expression levels of pluripotency-related genes were identified in all these cells. Moreover, hierarchical clustering analysis revealed that gene expression profiles of *Tsc2*$^{+/+}$ and *Tsc2*$^{+/-}$ ESCs resembled each other, but those of *Tsc2*$^{-/-}$ ESCs revealed an apparently distinct pattern. These results suggest that the homozygous *Tsc2* mutation causes extensive gene expression changes in rat ESCs.

Discussion

In this study, we successfully established *Tsc2*$^{-/-}$ ESCs from Eker rats. These cells possessed characteristic features of ESCs, including expression of pluripotency markers, long-term self-renewal, and the capacity to differentiate into derivatives of all three germ layers. Although detailed mechanisms are still not clear, there have been several reports indicating the importance of the Tsc2-mTOR pathway in stem cell maintenance and differentiation (38,39,43). Gan *et al* reported that *Tsc1* is a critical regulator of self-renewal, mobilization, and multilineage development in hematopoietic stem cells and that it executes these phenomena via both mTORC1-dependent and -independent pathways (39). Further, it was reported that the activation of S6K by expression of the constitutively active S6K1 or siRNA-mediated knockdown of *TSC2* and *RICTOR* induced differentiation of human ESCs (38). Recently, Betschinger *et al* reported that siRNA-mediated knockdown of *Tsc2* or *Flcn* inhibits differentiation of ESCs (43). In contrast to results in these reports, we successfully established *Tsc2*$^{-/-}$ ESCs possessing multipotent differentiation capacity despite the presence of the activated mTORC1 pathway. There are several possible reasons why the derivation of *Tsc2*$^{-/-}$ ESCs was possible in this study. Previous studies utilized siRNA- or shRNA-mediated knockdown of *Tsc2* in already established ESCs or conditional knockout of *Tsc1* in

somatic stem cells. Such 'acute' downregulation of *Tsc1*/*Tsc2* may cause some aberrant gene regulation that restrains the maintenance of the multipotent nature and differentiation capacity of stem cells. Microarray analysis revealed a distinct gene expression pattern in $Tsc2^{-/-}$ ESCs compared with their $Tsc2^{+/+}$ and $Tsc2^{+/-}$ counterparts. Our system enables comparison of gene expression profiles between $Tsc2^{-/-}$ ESCs and $Tsc2^{+/-}$ ESCs with *Tsc2* knockdown. Such analysis is of interest to further explore *Tsc2* mutation-related pathogenesis.

We were unable to obtain pups demonstrating chimeric coat color using $Tsc2^{-/-}$ ESCs. Results of dead embryos suggested that higher contribution of $Tsc2^{-/-}$ cells in chimeras induced embryonic lethality. Further, it has been reported that when human *TSC2*-deficient fibroblast-like cells were grafted into mice, differentiated tissues revealed features of TSC skin tumors and that *TSC2*-deficient cells directly or indirectly induce abnormal follicular neogenesis and epidermal proliferation (44). Because $Tsc2^{-/-}$ ESCs may cause abnormal differentiation of hair in chimeras, it may not be appropriate to determine the contribution of ESCs on the basis of hair color of chimeras.

He *et al* reported that reprogramming of somatic cells derived from $Tsc2^{-/-}$ mouse embryos to iPSCs was not possible (40). In this study, we provide evidence that the derivation of $Tsc2^{-/-}$ ESCs from Eker rat embryos is possible. In somatic cells, some epigenetic abnormalities caused by *Tsc2* deficiency may not be corrected even under reprogramming conditions. Conversely, during early embryogenesis, epigenetic abnormalities in $Tsc2^{-/-}$ cells may be tuned to maintain the stemness. With reprogramming experiments using Eker rat-derived embryonic fibroblasts, ESCs established in this study will serve as useful tools to compare effects of *Tsc2* deficiency on epigenetic status in reprogramming and ESC derivation.

In recent years, various patient-derived iPSCs have been used for *in vitro* differentiation experiments to mimic the pathogenesis of human diseases (45,46). Moreover, such cellular models are useful to research novel drug target molecules by high-throughput screening (47). With regard to tumorigenesis, tissue specificity and abnormal differentiation are relevant to its molecular basis. Lineage-specific *in vitro* differentiation of tumor suppressor-deficient ESCs will provide valuable experimental models to explore the mechanism of pathogenesis. However, in humans, establishment of tumor suppressor-deficient (i.e., homozygously inactivated) ESCs or iPSCs has been technically difficult. In rodents, homozygous mutant ESCs for tumor suppressors, including *Rb*, *Tp53*, and *Apc*, have been established (48-50). To date, none of those ESCs have been extensively used for *in vitro* differentiation experiments. For example, *Apc*-deficient ESCs failed to differentiate into multiple lineages in the teratoma formation assay, suggesting that the induction of various cell types was not applicable to these ESCs (50). In contrast, $Tsc2^{-/-}$ ESCs exhibited the potential to differentiate into all germ layers and multiple cell lineages, both *in vitro* and *in vivo*. We already observed development of abnormal ductal structures in $Tsc2^{-/-}$ teratomas, suggesting that cell type-specific effects of *Tsc2* deficiency could be reproduced in differentiation of ESCs (Kawano H, *et al*, unpublished data). Combined with *in vivo* experiments, *in vitro* differentiation models using ESCs established in this study will facilitate understanding of *Tsc2* mutation-related pathogenesis as well as aid in the search for therapeutic target pathways.

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