Aberrant differentiation of Tsc2-deficient teratomas associated with activation of the mTORC1-TFE3 pathway

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Abstract. The model animal of renal cell carcinoma (RCC), the Eker rat, has a germline mutation in the tuberous sclerosis 2 (Tsc2) gene. Heterozygous mutants develop RCCs by second hit in the wild-type Tsc2 allele, whereas homozygous mutants are embryonic lethal. In the present study, a new cell differentiation model was developed to study the mechanism of Tsc2 mutation-associated pathogenesis by generating Tsc2-deficient embryonic stem cells (ESCs) from Eker rats. Tsc2+/−, Tsc2−/− and Tsc2−/− ESCs were all capable of generating three germ layers: mesoderm, ectoderm, and endoderm. Interestingly, epithelial tumor-like abnormal ductal structures were reproducibly observed in Tsc2−/− teratomas from different ESC lines. Immunohistochemical analysis revealed that mammalian target of rapamycin complex 1 (mTORC1) signaling was activated in abnormal ducts of Tsc2−/− teratomas, on the basis of positive staining for p-S6 and p-4EBP1. In these abnormal ducts, expression levels of epithelial markers (i.e., megalin and cubilin) and the cytoplasmic localization of E-cadherin and β-catenin were similar to those in Eker rat RCCs. Moreover, a transcription factor regulated by mTORC1, named TFE3, was located in the nuclei of abnormal ducts and Eker rat RCCs. As a negative regulator of ESC differentiation, TFE3 may result in tissue-specific differentiation defects related to tumorigenesis in Eker rats and Tsc2−/− teratomas. The present study suggests that ESCs derived from Eker rats constitute a novel experimental tool with which to analyze differentiation defects and cell-type specific pathogenesis associated with Tsc2 deficiency.

Introduction

The Eker rat is a useful animal model with which to study renal cell carcinoma (RCC) (1). Spontaneous tumors develop in the kidney due to a germline mutation in the tuberous sclerosis 2 (Tsc2) gene (2,3). Homozygous mutants are embryonic lethal during midgestation (equivalent to mouse E9.5-E13.5) (4). In contrast, heterozygous mutants develop bilateral multicentric renal cell carcinomas within one year after birth (5). The development of multistage renal carcinogenesis can be monitored at the histological level (6). TSC2 (encoding tuberin) is a tumor-suppressor gene identified as a causative gene of TSC as well as TSC1 (encoding hamartin) (7-9). These products form a complex that inhibits the mammalian target of rapamycin complex 1 (mTORC1), a serine/threonine protein kinase essential for the regulation of cell growth and proliferation (10). Mammalian cells express two functionally distinct mTOR complexes: mTORC1 and mTORC2. mTORC1 contains mTOR, Raptor and LST8 as primary subunits and is inhibited by rapamycin (11). In TSC-associated tumors, the loss of TSC1 or TSC2 induces mTORC1-dependent phosphorylation of p70S6 kinase, ribosomal protein S6 and 4EBP1 (12). On the other hand, mTORC2 contains Rictor, LST8 and Sin1 as primary subunits. This complex functions as a rapamycin-insensitive regulator of the cytoskeleton and cell survival through Akt phosphorylation (13).

Few previous studies have explored the cancer-related cellular mechanisms triggered by the loss of tumor-suppressor genes using embryonic stem cells (ESCs) such as Apc-mutated ESCs (14). To establish a new research model for the analysis of renal carcinogenesis, we developed ESCs from Eker rat blastocysts (15). We successfully established Tsc2+/−, Tsc2−/− and Tsc2−/− Eker rat ESCs with pluripotency. Furthermore, in the teratoma formation assay, which is an important process for pluripotent stem cells (PSCs) to prove differentiation ability, epithelial tumor-like abnormal ductal structures resembling Eker rat RCCs were identified in Tsc2−/− teratomas. Immunohistochemical analysis revealed the activation of mTORC1 signaling in Tsc2−/− teratomas, particularly in abnormal ductal structures, which was suppressed by rapamycin treatment. We suggested that the appearance of these abnormal structures indicates a pathogenic mechanism.
related to renal tumorigenesis in Eker rats. These teratomas constitute a new research tool for the analysis of tissue-specific tumorigenesis by suppression of mTORC1 signaling.

Materials and methods

Animal studies. All animal experiments were performed according to protocols approved by the Animal Care Committee of Juntendo University of Medicine (Approval no. 250105). All surgeries were performed under isoflurane anesthesia and measures were taken to minimize animal suffering.

Cell culture. Rat ESCs were cultured on mitomycin C-treated mouse embryonic fibroblasts in N2B27-2i medium (1:1 ratio of 2i medium:N2B27 medium; Axon Medchem, Groningen, The Netherlands) containing 1,000 U/ml rat leukemia inhibitory factor (LIF; ESGRO Millipore, Bedford, MA, USA), 3 µM GSK3β inhibitor CHIR99021 (Axon), and 1 µM MEK inhibitor PD0325901 (Axon).

Teratoma formation. A total of 1.5x10^6 Tsc2-/+ or Tsc2-/- Eker ESCs suspended in Matrigel were subcutaneously injected into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. After 5 weeks, 1.5 mg/kg body weight of rapamycin (Sigma-Aldrich, St. Louis, MO, USA) was intraperitoneally injected every other day, for a total of three injections. Mice were sacrificed the day after the last injection.

Immunohistochemistry. Immunohistochemistry was carried out using a standard method using formalin-fixed, paraffin-embedded tissues. For chromogenic analysis, EnVision+ System HRP-labeled polymer for rabbit or mouse Abs (dako) were primarily used as secondary antibodies. Alternatively, biotinylated anti-sheep/goat immunoglobulin from donkey (1:100 dilution) and streptavidin-biotinylated horseradish peroxidase complex (1:100) (GE Healthcare Life Sciences, USA) were used for the staining of goat polyclonal antibodies. For immunofluorescence, the following secondary Abs were used: Alexa Fluor 568-conjugated donkey anti-goat Ab (Molecular Probes, Eugene, OR, USA); Alexa Fluor 488-conjugated donkey anti-mouse Ab (Jackson ImmunoResearch Laboratories, West Grove, PA, USA); Alexa Fluor 488-conjugated donkey anti-rabbit Ab (Molecular Probes); Alexa Fluor 488 or 568-conjugated goat anti-mouse or anti-rabbit Abs (Molecular Probes). Nuclei were stained with 6-diamidino-2-phenylindole (DAPI). Fluorescent images were captured and analyzed using an Axioplan 2 microscope (Carl Zeiss, Germany).

Following primary mouse monoclonal antibody (mAb), rabbit polyclonal (pAb), rabbit monoclonal (rAb) and goat polyclonal (gAb) antibodies were used at indicated dilution rates: anti-p-S6K (Thr389) rAb (108d; 1:1,000); anti-p-4EBP1 (Thr37/46) rAb (C20; 1:100) (both from Cell Signaling Technology, Beverly, MA, USA); anti-LRP2 (megalin) mAb (CD7D5; 1:300; Novus Biologicals, Littleton, CO, USA); anti-cubilin gAb (A-20; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Ki67 pAb (1:350; Novus Biologicals); anti-TFE3 pAb (1:500; Atlas Antibodies AB, Stockholm, Sweden); anti-TFEB pAb (1:100; Bioss USA Antibodies, Woburn, MA, USA); anti-β-catenin pAb (1:1,000; Merck Millipore, Darmstadt, Germany); anti-E-cadherin mAb (1:50; BD Biosciences, San Jose, CA, USA).

Cell counting was performed using at least 4 fields (magnification, x100) of each sample. Ki67 positive-cells were compared between ductal and abnormal ductal cells of Tsc2-/+ and Tsc2-/- teratomas, respectively. For E-cadherin and β-catenin, a cell clearly and continuously stained at the perimeter was defined as positive. Statistical analyses were performed with SAS software version R8.1 (SAS Institute Japan, Ltd., Tokyo, Japan). Values of p<0.05 were considered significant.

Western blotting. For western blotting, teratomas were dissected, snap frozen and stored until use. Protein samples were obtained by lysing teratomas in standard sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol) for SDS polyacrylamide gel electrophoresis. The following primary antibodies were used at the indicated dilutions: anti-TSC2 pAb (C20; 1:500; Santa Cruz Biotechnology); anti-p-S6K (Thr389) rmAb (108D; 1:1,000; Cell Signaling Technology); anti-S6K pAb (C-18; 1:500; Santa Cruz Biotechnology); anti-p-Akt (Ser473) rmAb (D9E; 1:1,000); anti-Akt pAb (1:1,000); and β-actin pAb (1:1,000) (all from Cell Signaling Technology).

Results

Tsc2-/- ESCs forms epithelial tumor-like abnormal ductal structures in teratomas. In the previous study, we found that Tsc2-+/- or Tsc2-/- Eker ESCs could differentiate to all three germ layers and epithelial tumor-like abnormal cells forming ductal structures in Tsc2-/- teratomas by renal capsule injection in nude mice (15). To confirm this phenomenon in more mature teratomas, we constructed teratomas by subcutaneous-inject-protocol in NOD/SCID mice. H&E-stained sections revealed that Tsc2-/- ESCs had the potential to differentiate into all three germ layers in this system (Fig. 1A).

Tsc2-/- teratomas also contained epithelial tumor-like abnormal cells forming ductal structures (Fig. 1B). They comprised large cells with clear, finely granular and occasionally vacuolated cytoplasm. They ruptured the basement membrane and invaded the surrounding parenchyma. These characteristics are reminiscent of Eker rat renal carcinoma (16).

RCCs of Eker rats originate from renal proximal tubules (17). To explore the cell types of abnormal ductal structures in Tsc2-/- teratomas, tissue sections were stained for megalin and cubilin. Expressed in epithelial cells of renal proximal tubules, these receptors mediate the endocytosis of numerous ligands. Although megalin expression is widespread among various epithelial cell types, cubilin expression is restricted to cells in renal proximal tubules, small intestine and yolk sac (18). In tissue sections, non-tumorous renal proximal tubules and Eker rat RCCs stained positive for megalin and cubilin (Fig. 2A). Interestingly, abnormal ductal structures of Tsc2-/- teratomas were also positive for megalin and cubilin, suggesting epithelial characteristics (Fig. 2B).

Moreover, tissue sections were stained for Ki67 to check the mitotic activity. Ki67-positive cells were frequently detected in abnormal ductal structures as observed in Eker.
rat RCCs (Fig. 2C). Compared with ductal parts in Tsc2<sup>+/+</sup> teratomas, abnormal ductal structures in Tsc2<sup>-/-</sup> teratomas showed a significant increase in the number of Ki67-positive cells (Fig. 2D). These observations suggest that cells forming abnormal ductal structures of Tsc2<sup>-/-</sup> teratomas possess an epithelial phenotype. Moreover, these findings suggest that the appearance of abnormal ductal structures reflects abnormal cellular differentiation caused by Tsc2 deficiency.

**Activation of mTORC1 signaling in abnormal ductal structures of Tsc2<sup>-/-</sup> teratomas.** The activation state of the mTORC1 pathway in the Tsc2<sup>+/+</sup>, Tsc2<sup>+</sup><sup>-/-</sup> and Tsc2<sup>-/-</sup> teratomas was investigated by immunostaining of downstream protein targets: phosphorylated S6 (p-S6) and phosphorylated 4EBP1 (p-4EBP1).

Positive staining of p-S6 and p-4EBP1 was broadly observed throughout Tsc2<sup>-/-</sup> teratomas and was considerably more intense in abnormal ductal structures as in Eker rat RCCs (Fig. 2B). These results indicated that mTORC1 signaling is activated in abnormal ducts of Tsc2<sup>-/-</sup> teratomas. The importance of the mTORC1 pathway in the formation of abnormal ductal structures was investigated by treatment with the mTOR inhibitor rapamycin after solid teratomas were formed. All ESCs maintained differentiation of three germ layers but the development of abnormal ductal structures in Tsc2<sup>-/-</sup> teratomas was suppressed under rapamycin-treated conditions (Fig. 3A).

Upregulation of S6 phosphorylation in Tsc2<sup>-/-</sup> teratomas and its suppression by rapamycin were confirmed by western blot analysis (Fig. 3B). Interestingly, Akt (S473) phosphorylation was suppressed in Tsc2<sup>-/-</sup> teratomas but was significantly reactivated by rapamycin, suggesting that the negative feedback on Akt was conferred by an activated mTORC1 signal. These observations support a relationship between mTORC1 hyperactivation and the development of abnormal ductal structures in Tsc2<sup>-/-</sup> teratomas.

**E-cadherin and β-catenin localization are dysregulated in Tsc2<sup>-/-</sup> teratomas.** Recent evidence suggests that the localization of E-cadherin is regulated by tuberin via an Akt/mTORC1-dependent signaling pathway and that Tsc2<sup>-/-</sup> epithelial cells display a loss of plasma membrane E-cadherin leading to decreased cell-cell adhesion (19). Regulation of β-catenin by hamartin/tuberin complex was also reported (20, 21). Therefore, we examined the expression of both E-cadherin and β-catenin by dual staining with cubilin.
In Eker rat RCCs, both E-cadherin and β-catenin primarily localized to the cytoplasm and were weakly detectable in the plasma membrane (Figs. 4A and 5A). In Tsc2⁻⁻ teratomas, abnormal ductal structures exhibited both E-cadherin and β-catenin staining at the plasma membrane, but the intensity was weaker and more heterogeneous compared with

Figure 2. Activation of mTORC1 signaling in abnormal ductal structures of teratomas. Tissue sections of Eker rat RCC (A) and abnormal ductal structures in Tsc2⁻⁻ teratomas (B) were stained with H&E, anti-p-S6, anti-p-4EBP1, anti-megalin, or anti-cubilin antibodies. (C) RCC of Eker rat and abnormal ductal structures in Tsc2⁻⁻ teratomas were stained with Ki67. Scale bars, 100 µm. (D) Ki67-positive cells were counted in the ductal parts of Tsc2⁺⁺ and the abnormal ductal parts of Tsc2⁻⁻ teratomas. *p<0.05, **p<0.01.
that in the normal ducts (Figs. 4B and 5B). Furthermore, rapamycin treatment increased the intensity and homogeneity of plasma membrane E-cadherin and β-catenin in Tsc2⁻/⁻ teratomas, although its effect on β-catenin was relatively weak (Figs. 4B and 5B). Taken together, these observations suggest that the membrane localization of E-cadherin and β-catenin was dysregulated by Tsc2 deficiency in an mTORC1-dependent manner.

Enhanced nuclear localization of TFE3 in Tsc2⁻/⁻ teratomas. A recent study provided evidence regarding the regulation of transcription factor EB (TFEB) and transcription factor E3 (TFE3) by mTORC1 (22,23). Deregulated expression and/or gene rearrangement of these members of the MITF/TFE transcription factor family have been implicated in the development of RCC (24,25). In fact, TFEB is essential for the expression of genes involved in autophagy and lysosome biogenesis and is negatively regulated by mTORC1 (26). On the other hand, TFE3 was recently identified as a key player in mouse ESCs to maintain their self-renewal state and prevent differentiation (22). Interestingly, the knockdown of Tsc2 increased the nuclear TFE3 concentration through the mTORC1 pathway. Therefore, the localization of TFEB and TFE3 was documented in Eker rat kidneys and teratomas by immunohistochemistry analysis.

In normal kidneys, Tsc2⁺/+ and Tsc2⁻/- teratomas, TFE3 was found in the cytoplasm of epithelial cells (Fig. 6). On the other hand, nuclear localization was prominent in Eker rat RCCs, whereas surrounding normal components maintained a cytoplasmic localization (Fig. 6). Likewise, in Tsc2⁻/⁻ teratomas, abnormal ductal structures and the stroma clearly revealed high nuclear accumulation of TFE3. Moreover, rapamycin completely abolished TFE3 accumulation in the
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These results suggest that TFE3 activation by mTORC1 signaling is involved in the development of abnormal ductal structures in Tsc2−/− teratomas.

Discussion

Various aspects of tissue specificity and differentiation provide important insights into the mechanisms of tumorigenesis. Differentiation experiments using pluripotent stem cells (PSCs) constitute valid methods to explore the mechanism of tissue-specific tumorigenesis. In general, tumorigenesis is initiated by the loss of tumor-suppressor gene function according to Knudson's two-hit model (27). Since PSCs experience two hits on the tumor-suppressor gene, they constitute ideal tools to investigate the relationship between differentiation abnormalities and tumor initiation. In humans, it is not easy to establish homozygous mutant PSCs for tumor-suppressor genes. Using reprogramming technology, induced PSCs were generated from a patient heterozygous for BRCA1 mutation (5382insC) (28). These mutant iPSCs exhibited increased protein kinase Cθ, but the differentiation capacity was not different between wild-type and mutant iPSCs.

In rodents, several studies have documented homozygous mutant PSCs for tumor-suppressor genes (14,29). For instance, Kielman et al established mouse ESCs that were homozygously mutated in the Apc gene (14). In these mutant ESCs, β-catenin was upregulated. Apc-mutated teratomas revealed severe differentiation defects in neuroectodermal, dorsal mesodermal and endodermal lineages. These data suggest that constitutive activation of the Apc/β-catenin pathway results in differentiation defects in the possibly underlying tumorigenesis in the colon and other self-renewing tissues. Kawamata and Ochiya established Tp53-mutated rat iPSCs and rat cell lines of Tp53 mutant strains (29). Unexpectedly, female, but

nucleus (Fig. 6). On the other hand, there was no apparent phenotype regarding TFEB localization (data not shown).

Figure 5. Localization of β-catenin in Eker rat RCCs and teratomas. (A) Dual staining for β-catenin (green) and cubilin (red). Nuclei are stained with DAPI (blue). Left panels indicate merged images from the three types of staining, and right panels indicate β-catenin staining alone. (a) RCC. (b) Ductal part of Tsc2−/+ teratomas. (c) Abnormal ductal structure of Tsc2−/− teratomas. (d) Rapamycin-treated Tsc2−/− teratomas. Scale bars, 100 µm. (B) Comparison of the number of β-catenin-positive cells in Tsc2−/+ teratomas without rapamycin and Tsc2−/− teratomas with or without rapamycin. **p<0.001.

Figure 6. Nuclear localizations of TFE3 in Eker rat RCCs and teratomas. Dual staining for TFE3 (green) and cubilin (red). Nuclei are stained with DAPI (blue). Left panels indicate merged images from the three types of staining, and right panels indicate TFE3 staining alone. (A) Normal renal cortex. (B) RCC. (C) Abnormal ductal structure of Tsc2−/− teratomas. (D) Rapamycin-treated Tsc2−/− teratomas. Scale bars, 100 µm.
not male, homozygous Tp53 mutant rats exhibited neural tube defects. Concurrently, Tp53-null rat ESCs resisted differentiation during the embryoid body (EB) formation assay. Although these studies utilizing PSCs clearly illustrate the importance of tumor-suppressor gene function in differentiation, gross abnormalities observed in embryoid bodies or teratomas failed to capture the detailed tissue specificity.

In the present study, the tissue differentiation of PSCs with a deficiency in tumor-suppressor gene presented a different scenario. In Tsc2−/− teratomas, various tissue types were generated such as neuroepithelial tissue, squamous epithelium, mesenchyme (undefined immature connective tissue), smooth muscle, cartilage, bone, adipose, gastrointestinal epithelium and glandular (unspecified) tissues. Therefore, these findings suggest that Tsc2−/− ESCs possess the ability to differentiate into all three germ layers. Nevertheless, abnormal ductal structures appeared in these differentiated tissues. In Tsc2−/− teratomas, cells of abnormal ducts were positive for cubilin and megalin, suggesting aberrant differentiation of some epithelial components. Although the identity of the cell types remains unknown, some cell type-specific effects of Tsc2 deficiency may emerge during the differentiation of teratomas. We provide evidence that the enhanced activation of mTORC1 pathways contributes to the development of Tsc2−/− abnormal ducts. Since abnormal ducts frequently and reproducibly appeared in Tsc2−/− teratomas from different cell lines, the accumulation of specific mutations may not be required for their development. Some epigenetic mechanisms may support differentiation defects associated with Tsc2 deficiency in teratomas. Such mechanisms may be related to the tissue specificity of tumorigenesis in Eker rats.

E-cadherin plays pivotal roles in epithelial cell behavior, tissue formation and cancer suppression (30). During embryonic development, the expression and function of E-cadherin must be normal for the induction and maintenance of polarized and differentiated epithelia (31). The lethality of E-cadherin knockout mice at an early stage of embryogenesis highlights the significance of E-cadherin in normal development and tissue function (32,33). The epithelial-to-mesenchymal transition and loss of E-cadherin expression are closely related and believed to be involved in tumor initiation as well as metastasis (34). An imbalance in β-catenin signaling often results in disease and deregulated growth related to cancer and metastasis (35). During tumor progression, β-catenin signaling is inappropriately activated by the loss of E-cadherin or mutants in various β-catenin signaling components (21). Eker rat RCC and abnormal ductal structures of Tsc2−/− teratomas revealed decreased plasma membrane localization of E-cadherin and β-catenin, which was partially corrected by rapamycin treatment. These data support findings from previous studies, suggesting that Tsc2 deficiency affects E-cadherin localization through perturbations of transport mechanisms (19). The dysregulation of E-cadherin and β-catenin by mTORC1 hyperactivation may cause a polarity defect during the development of Tsc2−/− abnormal ducts and tumorigenesis in Eker rats.

The transcription factor TFE3 has been implicated in renal carcinogenesis (23). However, details of TFE3 function have not been elucidated. In a recent study, TFE3 activation by mTORC1 was determined to be essential for the maintenance of self-renewal state and the capacity to withstand differentiation (22). When mTORC1 is activated, TFE3 moves to the nucleus to promote the transcription of estrogen-related receptor β genes involved in the maintenance of self-renewal and pluripotency (22). In our analysis, TFE3 was detected in the nucleus of both Eker rat RCCs and Tsc2−/− teratomas, particularly in abnormal ductal structures.

In conclusion, we established a novel experimental system to analyze the differentiation and cell-type specific defects associated with Tsc2 deficiency using ESCs derived from Eker rats. Future studies should elucidate how mTORC1 hyperactivation and other mechanisms contribute to the development of abnormal ductal structures in Tsc2−/− teratomas. Our system will facilitate the understanding of the pathogenesis caused by Tsc2 deficiency in Eker rats as well as in human tumor stem cells.

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