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Review Article

Mourning Dr. Alfred G. Knudson: the two-hit hypothesis, tumor suppressor genes, and the tuberous sclerosis complex

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On July 10, 2016, Alfred G. Knudson, Jr., MD, PhD, a leader in cancer research, died at the age of 93 years. We deeply mourn his loss. Knudson's two-hit hypothesis, published in 1971, has been fundamental for understanding tumor suppressor genes and familial tumor-predisposing syndromes. To understand the molecular mechanism of two-hit-initiated tumorigenesis, Knudson used an animal model of a dominantly inherited tumor, the Eker rat. From the molecular identification of *Tsc2* germline mutations, the Eker rat became a model for tuberous sclerosis complex (TSC), a familial tumor-predisposing syndrome. Animal models, including the fly, have greatly contributed to TSC research. Because the product of the *TSC2*/*Tsc2* gene (tuberin) together with hamartin, the product of another TSC gene (*TSC1*/*Tsc1*), suppresses mammalian/mechanistic target of rapamycin complex 1 (mTORC1), rapalogs have been used as therapeutic drugs for TSC. Although significant activity of these drugs has been reported, there are still problems such as recurrence of residual tumors and adverse effects. Recent studies indicate that there are mTORC1-independent signaling pathways downstream of hamartin/tuberin, which may represent new therapeutic targets. The establishment of cellular models, such as pluripotent stem cells with *TSC2*/*Tsc2* gene mutations, will facilitate the understanding of new aspects of TSC pathogenesis and the development of novel treatment options. In this review, we look back at the history of Knudson and animal models of TSC and introduce recent progress in TSC research.

On July 10, 2016, Alfred G. Knudson, Jr, died at the age of 93 years. He was a man with great insight into cancer genetics and a leader in the cancer research field. He inspired many younger scientists to seek understanding of the mechanisms of tumorigeneis, especially the cell-of-origin of cancer, and to strive to prevent this disease. He was a personal mentor of this review's author (O.H.) as well as a valued adviser to cancer geneticists in Japan. We deeply mourn his loss and dedicate this review to him, starting with the story of his research history on cancer genetics and animal models (Fig. 1).[1]

The Two-hit Hypothesis, a "Driver" in the Development of the Field of CANCER GENETICS

The dominant nature of hereditary cancer had been recognized as a trait long before the basic techniques of molecular biology were established. Although there were reports describing the loss of specific regions of certain chromosomes in particular types of cancer, no information on the driver mutations or specific genes related to carcinogenesis was available in the early 1970s. At that time, Knudson developed the two-hit hypothesis by statistical methods, without any experimental approaches.[2] He simply compared the time at which both eyes would be affected by retinoblastoma if one or two hits were required and predicted the relative chance of this occurring (Fig. 1b).[2] Knudson's hypothesis clearly postulated the recessive nature of tumor-initiating gene mutations and the mode of inheritance in familial cancer. This led to the concept of the existence of tumor suppressor genes and loss-of-heterozygosity (LOH) as relevant to carcinogenesis. Notably, Knudson already mentioned the possibility of "delayed mutation" that may correspond to germline-mosaic mutations.[2–4] Including this possibility, his insights facilitated the development of the field of cancer genetics. The first molecular cloning of the tumor suppressor gene *RB1*, the predisposing gene for retinoblastoma, was achieved in 1986.[5]

Preserving an Animal Model of Inherited Tumor: A "Tale" of the Eker Rat

In 1954, a Norwegian pathologist, R. Eker found a rat strain that developed bilateral, multiple and dominantly inheritable renal tumors.[6,7] Later, this new rat strain was named the Eker rat.[8] From those days up until the late 1980s, to the best of

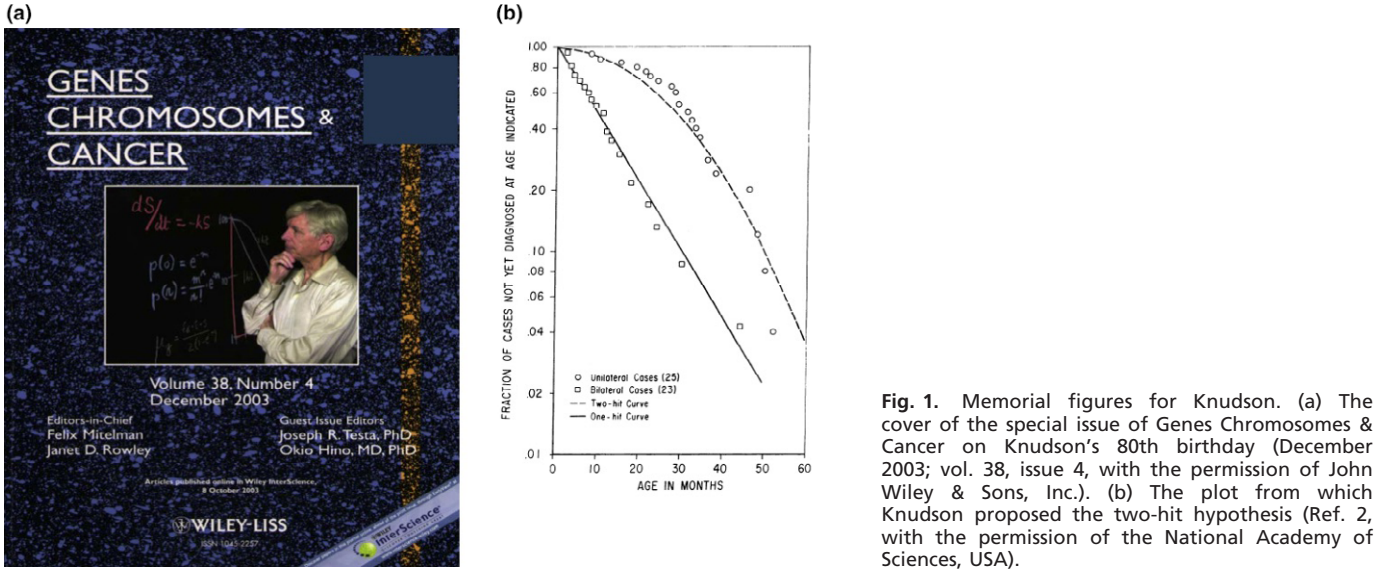

Fig. 1. Memorial figures for Knudson. (a) The cover of the special issue of Genes Chromosomes & Cancer on Knudson's 80th birthday (December 2003; vol. 38, issue 4, with the permission of John Wiley & Sons, Inc.). (b) The plot from which Knudson proposed the two-hit hypothesis (Ref. 2, with the permission of the National Academy of Sciences, USA).

our knowledge, no other laboratory animal model of inherited tumor with a high penetration was reported. Knudson saw that experimental animal models of hereditary tumors would be useful tools for cancer science. Thus, he sought reports of animal models of hereditary cancers and found information about the Eker rat. He brought these animals to the USA and maintained the mutation on a Long Evans background. By the mid-1980s to early 1990s, significant progress in research on polymorphic DNA markers had been achieved not only for human but also for rodent genomes. Using syntenic homologies between humans and rats, a positional cloning approach was taken and, finally, a germline insertion was identified in the homolog of the tuberous sclerosis complex (TSC) 2 gene (*Tsc2*). This was the tumor predisposing mutation of the Eker rat.[9–12]

There is a number of similarities between human TSC and the Eker rat, such as the dominant inheritance and LOH of *TSC2*/*Tsc2* region in tumorous lesions. However, unlike the epithelial renal tumors of the Eker rat, tumorous lesions in TSC patients are mainly hamartomas, such as renal angiomyolipoma (AML), lung lymphangioleiomyomatosis (LAM), and subependymal giant cell astrocytoma (SEGA), etc.[13] Our studies on the somatic, intragenic *Tsc2* mutations (second hits) in tumors and suppression of phenotypes by transgenic expression of wild-type *Tsc2* confirmed that the *Tsc2* is a bona fide predisposing gene in the Eker rat.[14,15]

TSC: A Multi-Organ Disorder with a Defect in the Tumor Suppressor Complex

Tuberous sclerosis complex is caused by a mutation either in *TSC1* or *TSC2* and is characterized by tumorous lesions and neuropsychiatric disorders. Symptoms in patients with either TSC gene mutation are similar, although those with the *TSC2* mutation may have more severe neurological phenotypes.[13] Both genes are tumor suppressor genes and their products, hamartin (TSC1) and tuberin (TSC2), form a functional complex. In addition to the Eker rat, mice with either TSC gene homolog knocked out have now been established and the development of renal and other tumors was reported.[16–19] Using those animal models, embryonic lethality of homozygous mutants of TSC genes was shown, and various cell lines deficient for TSC genes were established. In addition, several tissue-specific conditional gene-targeting systems have been established.[20] Although the detailed explanation of each system is beyond the scope of this review, suffice it to say that the functions of TSC genes in various tissues and recapitulation of TSC-related pathology, especially for brain lesions, have been documented using such conditional knockout systems.[20]

Good news from the fly and bad news after treatment. The importance of the GAP (GTPase activating protein)-related domain of tuberin in tumor suppression *in vivo* was identified using a transgenic Eker rat system.[21] A functional link between the hamartin/tuberin complex and the mammalian/mechanistic target of rapamycin (mTOR) kinase and the target of GAP-related domain, Rheb, was revealed through studies of *Drosophila*.[22] The Rheb-mTORC1 pathway activates mRNA translation by stimulating p70S6K-rpS6- and eIF4E-mediated signals. Hamartin/tuberin negatively regulates mTORC1 as does GAP for Rheb (Fig. 2). Tumors in animal models and TSC patients exhibit mTORC1-related pathway activation.[23] Indeed, the growth of TSC gene-deficient tumors can be suppressed *in vivo* by rapamycin, concomitant with the downregulation of mTORC1.[24,25] Rapamycin and its homologs (rapalogs) have been clinically used for the treatment of SEGA, AML and LAM, and substantial efficacy has been reported in many cases.[26] However, the effects of rapalogs are not cytotoxic but rather cytostatic.[25,26] The complete elimination of tumor cells is impossible and the volume of tumor increases after therapy is stopped.[26] Also, there are considerable adverse effects with rapalogs. Thus, other treatment options with different drug targets are needed now.

New downstream pathways of mTORC1. Many research groups have tried to identify novel pathways regulated by mTORC1 by means of comprehensive gene expression analysis and proteomics. Over several years recently, the *de novo* pyrimidine synthesis pathway has been characterized as such a candidate.[27,28] The key enzyme of this pathway, CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase), is phosphorylated and activated by S6K in an mTORC1-dependent manner. Thus, hyper-activation of mTORC1 induces pyrimidine synthesis to promote cell growth and cell cycle progression. The mTORC1-independent binding of CAD to GTP-bound Rheb was also reported, suggesting that

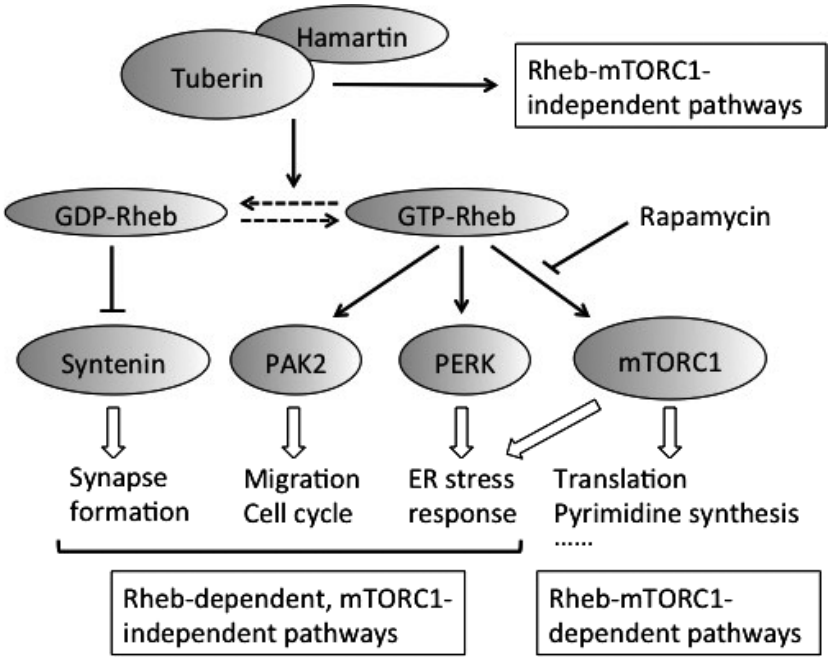

Fig. 2. mTORC1-dependent- and -independent pathways downstream of hamartin/tuberin. There are two categories of mTORC1-independent pathways according to their dependence on Rheb. Only representative pathways are shown.

CAD is regulated at multiple levels in TSC gene-deficient tumors.[29]

Many other important phenomena downstream of mTORC1, such as autophagy and ER stress, have now been characterized.[30–36] Pathways involved in those phenomena are already being exploited to develop new therapeutic targets and combination treatments with inhibitors of such pathways and rapalogs have been tested at the basic research level. A detailed understanding of the different pathways downstream of mTORC1 is important for developing therapeutic options encompassing independent modes of inhibition of each pathway. This will facilitate the establishment of personalized medication avoiding the adverse effects of rapalogs.

mTORC1-independent downstream pathways of the hamartin/tuberin complex. If rapamycin-insensitive, i.e. mTORC1-independent, signaling pathways are regulated by hamartin/tuberin complexes, they could be good candidates for new molecular targeting therapeutic drugs. Recently, an increasing number of hamartin/tuberin-regulated but rapamycin-insensitive pathways have been identified. These can be classified into two categories according to their dependence on Rheb (Fig. 2).

By kinome analysis, Alves *et al.* found that PAK2 activity is increased in *Tsc2*-deficient MEFs in a Rheb-dependent, but mTORC1-independent manner.[37] They also found increases in PAK2 phosphorylation in brain lesions from TSC patients. Interestingly, Yamagata *et al.* reported that GDP-bound Rheb is not merely an inactive form but selectively binds to and degrades syntenin in a proteasome-dependent manner.[38,39] In neuronal dendrites of *Tsc2* heterozygous mice, the reduction of tuberin renders Rheb into a more GTP-bound state and causes accumulation of syntenin, which, in turn, disrupts spine structures.[38,39] Although the functional relationship between hamartin/tuberin and syntenin has not been elucidated in other tissues and tumorous lesions, syntenin-mediated cytoskeletal regulation might be a candidate drug target for TSC. Paradoxically, considering the stimulatory function of mTORC1 on translation, Tyagi *et al.* reported inhibition of global translation in Rheb-overexpressing HEK293 cells as well as in *Tsc2*-deficient MEFs.[40] Rheb enhances phosphorylation of eIFα, thereby inhibiting translation, and directly binds to and stimulates the kinase activity of PERK *in vitro*.[40] Thus, Rheb may directly regulate the balance between two major translation-controlling pathways dissociated from mTORC1.

There are many reports describing the mTORC1-independent dysregulation of gene expression.[41–44] However, only some of these indicated that the dysregulation was Rheb-independent. Thus, a more detailed understanding of Rheb-independent pathways downstream of hamartin/tuberin would be expected to increase options for developing novel therapeutic approaches to TSC.

Towards next generation treatment for TSC. Because mTORC1 plays a pivotal role in the integration of multiple cellular reactions, its chronic activation seems to influence many downstream pathways, facilitating vulnerabilities and adaptive reprogramming of metabolism. During such reprogramming, cell type-specific addiction to particular metabolites or reactions frequently occurs.[34,45] Using high-throughput drug screening and/or recently-developed genome editing techniques, researchers have explored pathways related to such addictive factors in TSC gene-deficient cells. To identify growth-suppressive gene mutations (downregulations) synergistic with TSC gene-deficiency, Housden *et al.*[46] established a system consisting of CRISPR-Cas9-mediated targeting and RNAi screens in *Drosophila* cells. Confirming their data using *Tsc2*-deficient MEFs and TSC AML cells, they identified three candidates, *RNGTT*, *CDK11* and *CCNT1*, as synthetic interacting genes.[46] Notably, *RNGTT* and *CDK11* are implicated in mRNA synthesis and the formation of cap structures, suggesting that increased cap-dependent translation caused by mTORC1 activation additionally requires the higher rate of mRNA synthesis in TSC gene-deficient cells. As Houdson *et al.* focused on knocking out kinases and phosphatases in their study, it should be possible to identify other synthetic lethal pathways with targets other than global knockouts.

Employing a screening approach using many inhibitors of metabolism and signaling pathways, Blenis and colleagues found that the simultaneous inhibition of HSP90 and glutaminase, i.e. induction of ER stress and reduction of glutathione

level, induces apoptosis in TSC gene-deficient cells.[47] The effect of this combined inhibition is minimal on TSC gene-proficient cells, suggesting its usefulness for the treatment of TSC.

Beyond the Two-Hit Hypothesis: Pathogenesis Initiated by One-Hit On a Tumor Suppressor Gene Revisited

There have been many reports concerning haploinsufficiencies of tumor suppressor genes.[48,49] There may be cases where cells with a heterozygous mutation of a tumor suppressor gene do initiate tumorigenesis, without a second hit, in a cell-autonomous manner.[50] Moreover, it is likely that the heterozygosity of tumor suppressor genes in the cells of the tumor microenvironment promotes tumor development through interactions with tumor cells in a non-cell-autonomous manner, as reported using the mouse homolog of the neurofibromatosis type 1 (NF1) gene (*Nf1*).[51,52] The consequences of haploinsufficiency may be tissue-specific. Apart from tumorigenesis, various abnormal conditions, such as via effects on cell differentiation and in systemic metabolism, have been documented as consequences of the haploinsufficiency of tumor suppressor genes.[53–56]

tuberous sclerosis complex patients often suffer from neuropsychiatric symptoms, such as autism and epilepsy, in addition to their tumorous lesions.[13] Animal models of TSC also exhibit defects in cognitive ability and/or social interaction, without any macroscopic pathology in the brain.[57–61] These phenotypes seem to be caused, at least in part, by haploinsufficiency of TSC genes. The administration of rapamycin partly corrects such neuropsychiatric symptoms, suggesting that activated mTORC1 is involved in such haploinsufficiency-induced pathogenesis (Fig. 3). Whether there is a second hit in TSC-associated small lesions in the brain is controversial.[62–64] In the animal models of TSC, tumor-related pathology without a second hit to any predisposing gene has been reported.[65,66] Indeed, no aberrant activation of mTORC1 has been detected in those second hit-negative lesions.[65,66] Thus, some mTORC1-independet downstream pathways of hamartin/tuberin may contribute to the effects of haploinsufficiency of TSC genes. Sporadic- or TSC-associated LAM lesions and TSC-associated angiofibromas are mixed populations of differentiated cells.[67,68] Some of these cells harbor two-hit mutations and interact with heterozygous mutant- or wild-type cells (in the sporadic cases).[67,68] As is true for *Nf1*, heterozygous mutations in cells of the microenvironment may promote pathogenesis initiated by a second hit. In addition, like *PTEN*, TSC genes may show tissue-specific haploinsufficiency affecting metabolism, as revealed by the Eker rat.[69] To determine the molecular basis of haploinsufficiency, Knudson and colleagues compared the gene expression profile of non-tumorous tissue between TSC patients and those with other inherited syndromes, and control subjects.[70,71] Through the understanding of detailed molecular mechanism of haploinsufficiency, new targets for therapeutic intervention in inherited tumor syndromes including TSC may be found. In terms of the endpoint of treatment, it is ideal that haploinsufficiency-induced-, as well as two hit-initiated-tumors can be completely eliminated by drug administration for a particular term. In contrast, to control haploinsufficiency-induced non-tumorous symptoms, such as neuropsychiatric and metabolic ones, continuous treatment of patients during all of their life might be necessary. Thus, the strategies and endpoint to develop treatment might be different between ones for tumorous and non-tumorous symptoms.

Exploring Cell Type-Specific Tumorigenesis Using Pluripotent Stem Cells

The tissue-specific nature of different inherited tumors reflects the cell type-specific effects of mutations in oncogenes and tumor suppressor genes. Tumorigenesis is tightly linked to abnormalities in the differentiation process of stem or precursor cells. To explore the cell type-specific and cell fate-related mechanism of tumorigenesis, *in vitro* differentiation models using pluripotent stem cells (PSCs) are useful. Once such a model has been established, it can be used for high-throughput screening of drug candidates. Recently, an *in vitro* model of osteoblast differentiation using induced PSCs (iPSCs) from a Li-Fraumeni syndrome family member was reported.[72] Unexpectedly, p53-mutant osteoblasts induced from patient-derived iPSCs exhibited defective differentiation and increased tumorigenicity, associated with aberrant regulation of *H19*. These results suggest that p53 deficiency induces osteosarcomas by

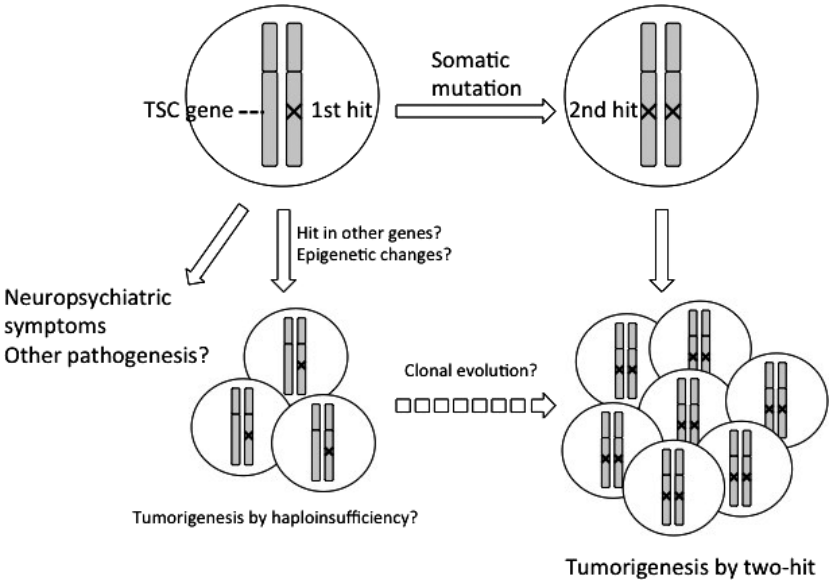

Fig. 3. Haploinsufficiency- and two-hit-initiated pathologies in tuberous sclerosis complex (TSC). Germline mutations (1st hit) cause neuropsychiatric symptoms during infancy without a 2nd hit. Other lesions, such as cortical tubers in brain may be caused by haploinsufficiceny of TSC genes. The majority of tumorous lesions develop after two hits of TSC genes. In animal models, tumorigenesis without a 2nd hit has been reported. Although not depicted in this figure, there are interactions between two-hit-initiated tumorous cells and surrounding non-tumorous cells to form lesions.

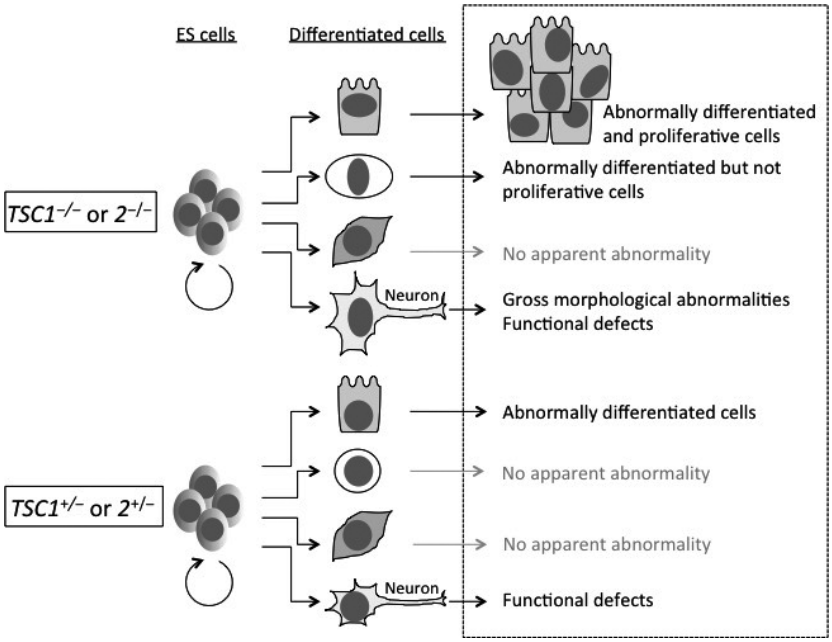

Fig. 4. A model of differentiation abnormalities in tuberous sclerosis complex (TSC) using pluripotent stem cells (PSCs). There are tissue-specific mechanisms in the pathogenesis of TSC. Modeling of differentiation abnormalities related to TSC (in the box delineated by dotted lines) will be useful to explore the mechanism of pathogenesis and to establish high-throughput screening systems. Upon differentiation, compared with wild-type cells (not depicted in this figure) homozygous PSCs mutant for TSC genes (*TSC1*$^{-/-}$ or *2*$^{-/-}$) may show differentiation abnormalities with or without aberrant proliferation in some specific lineages. In the case of neurons, heterozygous cells (*TSC1*$^{+/-}$ or *2*$^{+/-}$) may cause functional defects related to neuropsychiatric symptoms of TSC. For neuronal differentiation of human *TSC2*-mutant ES cells, refer to Costa, V, *et al.*[80]

affecting the program of osteogenic differentiation. In another report, Yamada and colleagues established iPSCs from *EWS-FLI1*-driven mouse osteosarcomas and demonstrated their impaired differentiation to osteogenic cells irrespective of *EWS-FLI1* expression.[73] These results suggest that the genetic and/or epigenetic alterations generated in osteosarcomas affect osteogenic differentiation. Moreover, osteosarcomas were induced from such osteosarcoma-derived iPSCs when *EWS-FLI1* is expressed, suggesting cooperation between driver gene alterations.[73,74] There have also been attempts to establish embryonic stem cells (ESCs) from families with inherited tumor-predisposing syndromes. *NF1*-heterozygous ESCs derived from an affected family member show hyperpigmentation when differentiated into the melanocyte lineage.[75] This is a typical model of haplosufficiency-induced pathology.

In the case of TSC, studies with PSCs are ongoing. Ito *et al.* established *Tsc2*$^{-/-}$ ESCs from Eker rat embryos and found that they have the potential to differentiate into three germ layers, despite mTORC1 being hyper-activated.[76] This result is in contrast to the case of the adenomatous polyposis coli gene (*Apc*) in which mouse *Apc*$^{-/-}$ ESCs fail to differentiate effectively in the teratoma formation assay.[77] Thus, each tumor suppressor gene mutation might give rise to different effects at different levels of pluripotent and cellular differentiation states. Interestingly, among *Tsc2*$^{-/-}$ teratoma tissues, tumor-like aberrant structures are seen, which are suppressed by administration of rapamycin.[78] These results may be relevant to renal tumorigenesis in the Eker rat. In another paper, the importance of *Tsc2* in the regulation of pluripotency in mouse ESC was also reported.[79] Very recently, a human ESC model of TSC was established by targeting *TSC2* gene with a zinc-finger nuclease technique.[80] As in the case of rat ESCs, mTORC1 activity is increased in human *TSC2*$^{-/-}$ ESCs relative to wild-type and *TSC2*$^{+/-}$ ESCs. Upon neuronal differentiation experiments, *TSC2*$^{-/-}$ ESCs exhibit abnormalities in proliferation and morphology from the early stage as well as in synaptic function at the later stage. *TSC2*$^{+/-}$ ESCs also exhibit abnormalities in synaptic function, but not in proliferation and morphology. These phenotypes are reversed by rapamycin, clearly indicating the dosage-dependent, differentiation stage-specific effects of mTORC1 (Fig. 4).

The Ultimate Goal: Prevention of Pathogenesis in Familial Tumor-Predisposing Syndromes

In this review, we introduced and discussed familial tumor predisposing syndromes, taking TSC as the main example. As mentioned earlier, the tumor phenotype of Eker rat is different from that of TSC patients. To advance research, not only for TSC, but also for other diseases, we must consider the phenotypic difference between human patients and animal models. Although the basic mechanism, such related to the function of tumor suppressor gene product, might be conserved, the "final output" of phenotype is sometimes different. Needless to say, animal models are important to understand the conserved mechanisms in which molecular targets of drugs are involved. However, unraveling the mechanism causing difference, i.e. human-specific tissue specificity, might be the key to finding highly selective molecular targets for therapy.

The goal of cancer research is to prevent deaths from cancer. The suppression of recurrence and metastasis is the most important objective. For many hereditary tumor-predisposing syndromes, however, it is also feasible to consider preventing the development of pathology in the first place. For the purpose of chemoprevention and treatment, the primary research target is the target pathway of the drug in question. To minimize adverse effects, it is optimal to find a target pathway that is specific to the cell type from which the tumors initiate. Such pathways need to be elucidated through the understanding of cell type-specific or differentiation-related mechanisms of tumorigenesis. In addition, the differences in drug efficacy and adverse effects among patients must be considered. These may be determined by genetic background as already revealed for adverse effects on treatment with irinotecan.[81] Large-scale pharmacogenomic studies and the development of patient-specific cell or tissue models may facilitate the establishment of methods for personalized prevention.

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The authors have no conflict of interest to disclose.

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