Human Pluripotent Stem Cell-Derived Tumor Model Uncovers the Embryonic Stem Cell Signature as a Key Driver in Atypical Teratoid/Rhabdoid Tumor

Title

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Graphical Abstract

Highlights
- SMARCB1-deficient human iPSCs give rise to AT/RT-like tumors
- ESC-like signature induces rhabdoid histology and causes a poor prognosis
- AT/RT exhibits ESC-like transcriptional signature and DNA methylation landscape
- ESC-like signature could be a therapeutic target for AT/RT

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In Brief
Terada et al. present SMARCB1-deficient human pluripotent stem cell-derived atypical teratoid/rhabdoid tumor (AT/RT) models and show that ESC-like signature is a critical driver of malignant phenotypes of AT/RT. Genetic ablation targeting the maintenance of pluripotency inhibits AT/RT cell growth, suggesting that the ESC-like signature could be a promising therapeutic target for AT/RT.
Human Pluripotent Stem Cell-Derived Tumor Model Uncovers the Embryonic Stem Cell Signature as a Key Driver in Atypical Teratoid/Rhabdoid Tumor

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SUMMARY

Atypical teratoid/rhabdoid tumor (AT/RT), which harbors SMARCB1 mutation and exhibits a characteristic histology of rhabdoid cells, has a poor prognosis because of the lack of effective treatments. Here, we establish human SMARCB1-deficient pluripotent stem cells (hPSCs). SMARCB1-deficient hPSC-derived neural progenitor-like cells (NPLCs) efficiently give rise to brain tumors when transplanted into the mouse brain. Notably, activation of an embryonic stem cell (ESC)-like signature confers a rhabdoid histology in SMARCB1-deficient NPLC-derived tumors and causes a poor prognosis. Consistently, we find the activation of the ESC-like gene expression signature and an ESC-like DNA methylation landscape in clinical specimens of AT/RT. Finally, we identify candidate genes that maintain the activation of the ESC-like signature and the growth of AT/RT cells. Collectively, SMARCB1-deficient hPSCs offer the human models for AT/RT, which uncover the role of the activated ESC-like signature in the poor prognosis and unique histology of AT/RT.

INTRODUCTION

Atypical teratoid/rhabdoid tumor (AT/RT) is an extraordinarily lethal malignant CNS tumor that occurs mainly in early childhood. The representative morphological feature of AT/RT is a population of cells with classic rhabdoid features: eccentrically located nuclei containing vesicular chromatin, prominent eosinophilic nucleoli, abundant cytoplasm with an obvious eosinophilic globular cytoplasmic inclusion, and well-defined cell borders (Louis et al., 2016). Loss of SMARCB1 (also known as INI1, SNF5, or BAF47) expression at the protein level is observed in almost all AT/RTs, and current consensus holds that immunohistochemical staining for SMARCB1 is a sensitive and specific test for the diagnosis of AT/RT (Louis et al., 2016). Several mouse models of Smarcb1 ablation have been developed to model AT/RT and rhabdoid tumor (Ng et al., 2015; Han et al., 2016). Genetic ablation of Smarcb1 at different developmental stages revealed that intracranial tumors with the rhabdoid histology develop only when Smarcb1 is deleted at embryonic day 6–10 (E6–E10), whereas ablation after birth results in lymphoma development, indicating that a restricted early developmental window is required for the initiation of intracranial rhabdoid tumors (Han et al., 2016). Furthermore, compound deletion of Smarcb1 and p53 at GFAP-expressing neuronal progenitor cells resulted in the development of AT/RT with rhabdoid histological features. Moreover, the expression profile of mouse and human intracranial rhabdoid tumors revealed the highest correlation with neural progenitors and stem cells (Han et al., 2016). Taken together, these findings suggest that AT/RT may arise from neural stem or progenitor cells.

Patients with AT/RT show a rapid clinical deterioration and extremely worse outcome than those with other CNS tumors, despite aggressive surgical and adjuvant radiochemotherapy. Retrospective and epidemiological studies have demonstrated a mean overall survival ranging from 6 to 18 months (Hilden et al., 2004; von Hoff et al., 2011; Chen et al., 2006). Although intensive multimodality regimens have improved the survival rates for AT/RT patients, AT/RT is still refractory to most treatments (Frühwald et al., 2016). Several preclinical studies attempting to identify molecular targets of AT/RT have taken place in recent years. Particularly, the inhibition of enhancer of zeste homolog 2 (EZH2) has been reported to suppress rhabdoid tumor cell growth, and a clinical trial with a specific inhibitor for EZH2 has been in progress against SMARCB1-defective tumors (Knutson et al., 2013; Alimova et al., 2013; Wilson et al., 2010). Besides expectation of these preclinical studies, the development of effective therapeutic approaches has been desired for this deadly cancer.
Figure 1. Generation of an Atypical Teratoid/Rhabdoid Tumor Model using hiPSCs Lacking SMARCB1 and TP53

(A) A schematic illustration of the establishment of hiPSCs SMARCB1/TP53-/- and NPLCs SMARCB1/TP53-/-.

(B) Representative morphology of hiPSCs, hiPSCs TP53+/+ and hiPSCs SMARCB1+/+. Scale bars, 200 μm.

(C) The lack of TP53 and SMARCB1 proteins in hiPSCs SMARCB1/TP53-/- was confirmed by western blot analysis.

(D) Immunofluorescent staining for NESTIN in hiPSCs SMARCB1/TP53-/- and NPLCs SMARCB1/TP53-/-. NESTIN-expressing cells emerged after neural induction.

(E) A schematic illustration of the xenograft transplantation of hiPSCs or NPLCs into the brain of immunocompromised mice.

(F) MRI and representative histological images of a NPLC SMARCB1/TP53-/- derived tumor (NPLC-tumor). NPLCs SMARCB1/TP53-/- give rise to brain tumors, which consist of densely packed undifferentiated small blue round cells with rosette formation. Scale bars, 500 μm (left) and 20 μm (right).

(legend continued on next page)
Previous studies have shown that poorly differentiated tumors in humans often exhibit activation of an embryonic stem cell (ESC)-like gene expression signature, which is correlated with a worse prognosis in adults (Ben-Porath et al., 2008; Wong et al., 2008). Consistently, SALL4, one of the key factors in the maintenance of pluripotency, is re-expressed in a subset of hepatocellular carcinoma cells, especially in patients who have an unfavorable prognosis, suggesting that the acquisition of ESC-like features plays a role in cancer progression (Yong et al., 2013). Takahashi and Yamanaka (2006) succeeded to confer ESC properties to somatic cells upon the transient expression of four reprogramming factors. Recently, an in vivo reprogramming strategy, which enables the fate conversion of differentiated somatic cells to pluripotent stem cells (PSCs) in vivo, has been developed (Taguchi and Yamada, 2017). Notably, a premature termination of the in vivo reprogramming in mice causes the development of pediatric cancer-like tumors with activation of the ESC-like signature (Ohnishi et al., 2014). In addition, the transient expression of reprogramming factors in Kras mutant mice causes the development of alfa-fetoprotein (AFP)-producing cancers, which simultaneously express pluripotency-associated genes and exhibit activation of the ESC-like signature (Shibata et al., 2018). Collectively, activation of the ESC-like signature is involved in the development and progression of particular types of cancer.

Given that PSCs can give rise to various cell types while preserving genetic information, human PSCs (hPSCs) have provided an attractive platform for disease modeling in a genetically defined background in human cells. Indeed, previous studies tried to model human cancers by using hPSCs (Kim et al., 2013; Stricker et al., 2013; Sancho-Martinez et al., 2016), and hESCs with genetic mutation at histone H3.3 succeeded to model diffuse intrinsic pontine glioma in the proper cellular context after neural differentiation (Funato et al., 2014). Here, we established an AT/RT model using human induced pluripotent stem cells (iPSCs) lacking SMARCB1. The human iPSC-derived AT/RT model uncovered an unappreciated feature of AT/RT, which could be a therapeutic target.

RESULTS

Generation of an Atypical Teratoid/Rhabdoid Tumor Model Using Human iPSCs Lacking SMARCB1 and TP53

A mouse study demonstrated that AT/RT-like tumors develop when mutations for both Smarcb1 and p53 are introduced into neural progenitor cells. In the present study, to establish a human AT/RT model, we introduced genetic mutations at SMARCB1 and TP53 into 201B7 human iPSCs (hiPSCs) (hiPSCs SMARCB1; TP53) using the CRISPR/Cas9 system. We first established hiPSCs deficient of TP53 alone (hiPSCs TP53) and then introduced an additional mutation at SMARCB1 to generate hiPSCs (SMARCB1; TP53) (Figures 1A–1C). Subsequently, we induced neural differentiation in hiPSCs (SMARCB1; TP53), hiPSCs TP53, and control 201B7 hiPSCs (Yan et al., 2013). iPSCs with all genotypes efficiently changed their morphology into neural progenitor-like cells (NPLCs) (Figure 1D). Consistently, NANOG and OCT4 expression were reduced in NPLCs at day 14 of the neural induction (Figure S1A). In contrast, an increased expression of neural progenitor cell (NPC)-related genes, such as NESTIN, NCAM, and PAX6, was observed in these NPLCs (Figures S1A–S1C). However, the expression levels of NPC-related genes were lower in NPLCs with SMARCB1 deficiency when compared with wild-type control NPLCs (Figure S1A). Further induction of neuronal differentiation in NPLCs confirmed that neuronal differentiation is impaired in SMARCB1-deficient cells in vitro (Figure S1B). We also examined the effect of SMARCB1 deficiency on the cell growth of hiPSCs and NPLCs in vitro. The TP53 ablation promoted cell proliferation in both hiPSCs and NPLCs, whereas the SMARCB1 ablation inhibited the cell growth of hiPSCs (Figure S1D). Although SMARCB1 deficiency inhibited neuronal differentiation, it did not increase cell proliferation in NPLCs in vitro (Figure S1D).

To test the effect of SMARCB1 deficiency on the tumor-forming ability of NPLCs in vivo, we performed xenograft transplantation of NPLCs (SMARCB1; TP53; control 201B7 NPLCs and NPLCs into mouse brain (Figure 1E). The orthotopic transplantation of NPLCs (SMARCB1; TP53; control 201B7 NPLCs and NPLCs) caused the development of aggressive tumors (n = 26/26) (Figures 1F and S1E). In contrast, control 201B7 NPLCs and NPLCs (TP53) gave rise to only microscopic tumors (n = 2/2, 4/5, respectively) (Figures S1F and S1G), and no macroscopic tumor was developed after the transplantation (n = 0/4, 0/6, respectively) (Figure S1E). Together, SMARCB1 deficiency markedly promoted tumor formation from NPLCs after orthotopic transplantation in vivo.

The histological analysis demonstrated that NPLC-derived tumors (hereafter NPLC-derived tumors) were medulloblastoma-like or embryonal tumor with multilayered rosettes (ETMR)-like tumors, which mainly consisted of densely packed undifferentiated small, blue, round...
Figure 2. hiPSC-Derived Tumors Exhibit Activation of the ESC-like Gene Expression Signature, which Drives Rhabdoid Tumors In Vivo

(A) Clustering analysis using microarray data revealed that both a hESC-like module genes (left) (Wong et al., 2008) and ESC Core module genes (right) (Kim et al., 2010) are similarly expressed in an hiPSC-tumor, a NPLC-tumor, an AT/RT cell line, and hESC lines, whereas medulloblastoma lines and a glioblastoma cell line are clustered with NSCs. The microarray data in Figure 1K were used. Data for NSCs were obtained from GSE18296 and GSE27667.

(B) Immunohistochemical analysis of SALL4, LIN28A, and LIN28B in hiPSC-tumors and NPLC-tumors. Scale bars, 50 μm.

(C) Quantification of the SALL4-positive cells in hiPSC-tumors and NPLC-tumors. Note that hiPSC-tumors contain SALL4-positive cells more frequently than NPLC-tumors. Data are represented as the mean with 95% confidence interval. **p < 0.01 (unpaired t test with Welch’s correction).

(D) A qRT-PCR analysis for SALL4 and LIN28 expression in hiPSC- and NPLC-tumors. Data are presented as the mean of technical triplicates. The mean expression level of hiPSC-tumors was set to 1. *p < 0.05 (unpaired t test with Welch’s correction).

(E) GSEA showing that the ESC-like module is enriched in hiPSC-tumors compared to NPLC-tumors.
cells with rosettes (Figure 1F). However, the majority of NPLC-derived tumors did not contain rhabdoid cells (Figures 1G and 1H), a characteristic histology of AT/RT. Although NPCs have been suggested to be a cell-of-origin for AT/RT, other studies proposed that pluripotent fetal cells, which are more immature than NPCs, could be an origin based on the morphology in an electron microscope analysis and the unique gene expression in AT/RTs (Bouffard et al., 2004; Deisch et al., 2011). Therefore, we next transplanted hiPSCs \textit{SMARCB1-/-; TP53-/-} without neural induction (Figure 1E). The transplantation of hiPSCs \textit{SMARCB1-/-; TP53-/-} resulted in aggressive tumor formation (n = 18/18) (Figures 1I and S1E), which was similarly observed after the transplantation of NPLCs \textit{SMARCB1-/-; TP53-/-}. In contrast, the transplantation of control 201B7 hiPSCs and hiPSCs \textit{TP53-/-} caused only microscopic tumor formation (n = 2/5 and 1/8, respectively), and no macroscopic tumor formation was observed after the transplantation (Figures S1E and S1F). Notably, the histological analysis demonstrated that hiPSC \textit{SMARCB1-/-; TP53-/-} derived tumors (hereafter hiPSC-derived tumors) frequently contained a large number of rhabdoid cells with vesicular chromatin, prominent nucleoli, and eosinophilic globular cytoplasmic inclusions, which are representative histological features of AT/RT (Figures 1G and 1I). The tumor area with rhabdoid histology was significantly larger in hiPSC-derived tumors than NPLC-derived tumors (Figure 1H). Immunohistochemical analyses revealed that hiPSC-derived tumors exhibited shared features with AT/RT, including high proliferative activity, lack of SMARCB1 expression, and positive staining for vimentin, glial fibrillary acidic protein (GFAP), synaptophysin, CD99, S-100, EMA, and smooth muscle actin (SMA) (Figure 1J). Consistent with the histological observations, clustering analysis of global gene expression revealed that a hiPSC-derived tumor exhibited a similar expression pattern with an AT/RT cell line, whereas medulloblastoma and glioblastoma cell lines fell into a different cluster (Figure 1K). To exclude the possibility of a PSC clone-specific phenotype, we also established an \textit{SMARCB1}-deficient PSC line by using a different iPSC line, 1383D6 (Figure S1H). The orthotopic transplantation of both 1383D6 iPSCs \textit{SMARCB1-/-} and 1383D6 NPLCs \textit{SMARCB1-/-} caused aggressive tumors (5/7 and 5/5, respectively) (Figure S1I), which phenocopied the tumors from 201B7 iPSCs and NPLCs lacking \textit{SMARCB1} and \textit{TP53}. Notably, four out of five 1383D6 iPSC \textit{SMARCB1-/-} derived tumors contained rhabdoid cells, although the area of the rhabdoid cells was smaller than in 201B7 hiPSC \textit{SMARCB1-/-; TP53-/-} derived tumors (Figures S1I and S1J). In sharp contrast, no rhabdoid cells were detected in 1383D6 NPLC \textit{SMARCB1-/-; TP53-/-} derived tumors (Figures S1I and S1J). Collectively, we succeeded to model human AT/RT by inoculation of hiPSCs lacking \textit{SMARCB1} into mouse brain.

**hiPSC-Derived Tumors Exhibit an ESC-like Gene Expression Signature**

Our results demonstrating that iPSC-derived tumors exhibited a robust rhabdoid phenotype raised the possibility that a pluripotency-related program is associated with the characteristic rhabdoid histology. Therefore, we next investigated the expression of ESC-related modules in hiPSC- and NPLC-derived tumors together with various brain tumor cell lines. Notably, both hiPSC- and NPLC-derived tumors together with an AT/RT cell line exhibited the ESC-like activation patterns of ESC-related modules, including the ESC Core module, which is composed of genes co-occupied by multiple factors in the core pluripotency network (Figure 2A) (Kim et al., 2010; Wong et al., 2008). We next analyzed the expression of SALL4, LIN28A, and LIN28B, which are associated with the maintenance and acquisition of pluripotency, in hiPSC- and NPLC-derived tumors. Immunohistochemical analysis revealed that both hiPSC- and NPLC-derived tumors expressed SALL4, LIN28A, and LIN28B, but the expression was particularly pronounced in hiPSC-derived tumors (Figures 2B and 2C). We also confirmed the elevated expression of SALL4 and LIN28B in hiPSC-derived tumors by qRT-PCR (Figure 2D). Consistently, gene set enrichment analysis (GSEA) showed an enrichment of the ESC-like module (Wong et al., 2009) in hiPSC-derived tumors compared to NPLC-derived tumors (Figure 2E) (Subramanian et al., 2005). Together, we confirmed the ESC-like signature is activated in hiPSC-derived tumors.

**Induction of the ESC-like Signature Leads to Rhabdoid Phenotypes in NPLC-Derived Tumors**

To further investigate the role of the pluripotency-related signature in the histogenesis of rhabdoid cells, we next tried to induce the ESC-like signature during tumor development from NPLCs \textit{SMARCB1-/-; TP53-/-} by the forced expression of four reprogramming factors, namely, \textit{OCT4}, \textit{SOX2}, \textit{KLF4}, and \textit{c-MYC (OSKM-NPLCs)} (Figure S2A). It was reported that the removal of polysialylated-neural cell adhesion molecule (PSA-NCAM)-negative cells could prevent mesodermal tumor formation in hiPSC-derived NPC transplantation, indicating that PSA-NCAM antibody is useful for excluding undifferentiated, tumor-forming PSCs after neural induction (Lee et al., 2015). To eliminate the tumorigenic undifferentiated PSCs after neural induction into NPLCs \textit{SMARCB1-/-; TP53-/-}, we sorted neural-differentiated cells with the PSA-NCAM antibody using...
magnetic-activated cell sorting (MACS). We confirmed the high efficiency of MACS of PSA-NCAM-positive cells by a flow cytometric analysis (Figure S2B), OCT4, SOX2, KLF4, and c-MYC were retrovirally transduced into NPLCs or PSA-NCAM-positive NPLCs (Figure S2C), and the OSKM-NPLCs were inoculated into the mouse brain to obtain NPLC-derived tumors (Figures 2F and S2A). All mice transplanted with OSKM-NPLCs developed aggressive brain tumors (n = 9). Although OSKM-NPLCs did not exhibit a prominent upregulation of pluripotency-related genes in vitro (Figure S2D), an enrichment of the ESC-like module when compared to control NPLC-derived tumors (Figure 2G), suggesting that OSKM induction conferred the ESC-like gene expression signature in NPLC-derived tumors (Wong et al., 2008). Of particular note, OSKM-NPLC-derived tumors exhibited an apparent rhabdoid histology in most areas (Figure 2H). Consistently, the rhabdoid area in OSKM-NPLC-derived tumors was significantly larger than that in control NPLC-derived tumors (Figure 2I). Collectively, we concluded that activation of the ESC-like signature is responsible for the characteristic rhabdoid histology.

Orthotopic Transplantation of hiPSCsSMARCB1−/−;TP53−/− Transplantation
Previous studies demonstrated that activation of the ESC-like signature in tumors is associated with a worse prognosis of adult cancer patients (Ben-Porath et al., 2008). Therefore, we next investigated the survival period of mice after the orthotopic transplantation of hiPSCsSMARCB1−/−;TP53−/−and NPLCsSMARCB1−/−;TP53−/−. Consistent with a positive correlation between activation of the ESC-like signature and the poor prognosis, the overall survival of hiPSCSMARCB1−/−;TP53−/−-transplanted mice that developed tumors with rhabdoid histology was substantially shorter than that of NPLCSMARCB1−/−;TP53−/−-transplanted mice (Figure 2J). Similarly, mice inoculated with OSKM-NPLCs, which also developed tumors with rhabdoid histology, exhibited poor survival when compared to mice inoculated with control NPLCs (Figures S2E and S2F). Together, these results indicate that activation of the ESC-like signature is associated with a worse prognosis of SMARCB1-deficient tumors.

c-MYC Induces Activation of the ESC-like Signature in NPLC-Derived Tumors and Drives Rhabdoid Tumor Development In Vivo
Our results indicate that activation of the ESC-like signature is associated with the unique rhabdoid histology and poor prognosis of AT/RT. However, how AT/RT cells acquire the ESC-like signature remains unclear. A previous study demonstrated that c-Myc activates the ESC-like signature in adult epithelial cells and cancer cells and increases the fraction of tumor-initiating cells (Wong et al., 2008). Considering that c-MYC is overexpressed in a subset of AT/RTs (Johann et al., 2016), we next investigated the effect of c-MYC induction on the activation of the ESC-like signature and the rhabdoid phenotype in NPLC-derived tumors. c-Myc was retrovirally transduced in NPLCSMARCB1−/−;TP53−/− or PSA-NCAM-positive NPLCSMARCB1−/−;TP53−/−(MYC-NPLCs), and then MYC-NPLCs were inoculated into mouse brain (Figures 3A, S3A, and S3B). Notably, MYC-NPLCs gave rise to aggressive tumors containing a large number of rhabdoid cells (Figures 3B, 3C, S3C, and S3D). Moreover, these tumors exhibited an activation of ESC-like module genes (Figure 3D) and increased expression of SALL4 and LIN28 (Figure 3E) compared to control tumors.

To investigate the effect of c-MYC overexpression on the disease outcome, we next investigated the survival period of mice transplanted with MYC-NPLCs. The overall survival of these mice was significantly shorter than that of controls (Figures 3F and S3E). Collectively, these results demonstrate that c-MYC overexpression alone is sufficient for activation of the ESC-like signature and rhabdoid histology in NPLC-derived tumors, which was linked to a worse prognosis.

Activation of the ESC-like Gene Expression Signature in Human AT/RT Specimens
Above, we showed that activation of the ESC-like signature is related to the emergence of rhabdoid cells, a unique histological feature of AT/RT, and the worse prognosis of NPLCSMARCB1−/−;TP53−/−-transplanted mice. We next examined the gene expression profile of clinical samples of human AT/RTs, medulloblastomas, and glioblastomas by using previously published datasets. Clustering analysis revealed that ESC-like module genes (Wong et al., 2008) are similarly activated in AT/RT samples and ESCs and iPSCs, whereas medulloblastomas and glioblastomas fall into a different cluster (Figure 3A). Similar results were obtained in the clustering analysis using the ESC Core module (Figure 3A) (Kim et al., 2010). We also examined activation of the ESC-like signature in ETMRs, which often show LIN28A immunoreactivity. Notably, some ETMRs also exhibited ESC-like activation of the ESC Core module genes (Figure S4A). Consistent with the ESC-like gene expression signature, the expression of SALL4, LIN28A, and LIN28B were elevated in AT/RTs compared to medulloblastomas (Figure 3B). SALL4 was highly expressed in AT/RTs compared with medulloblastomas, but the expression level of LIN28B was higher in medulloblastomas (Figure 3B). The increased expression of SALL4, LIN28A, and LIN28B were also confirmed in AT/RT clinical specimens by immunohistochemistry (Figure S4A) (Deisch et al., 2011) (Weingart et al., 2015). Taken together, we confirmed that human AT/RTs harbor the ESC-like signature.

ESC-like DNA Methylation Landscape in Human AT/RT Specimens
Given that human AT/RTs exhibit the ESC-like gene expression signature, we next examined DNA methylation patterns between AT/RTs and PSCs. We performed a comprehensive genome-wide methylation analysis of CpG islands (CGIs) in AT/RTs together with other brain tumors by using previously published datasets. In this analysis, we first extracted CpG sites within CGIs or around transcription start sites (TSSs; ±1,500 bp), which are differentially methylated between PSCs and adult brain tissues (DNAm difference [Dif], >0.6), and then examined DNA methylation levels in AT/RTs, medulloblastomas, ETMRs, glioblastomas, fetal brain tissues, and neural stem cells (NSCs). We found that CGI methylation levels at brain-unmethylated sites (PSC-methylated sites) are often increased in AT/RTs but...
not so much in other tumors or NSCs (Figures 4C and S5). Although ETMRs exhibited the ESC-like gene expression signature, PSC-methylated sites remain unmethylated in ETMRs (Figure S5). In contrast, CGI methylation levels at adult brain-specifically methylated sites tended to be decreased in AT/RTs but not in other tumors, including ETMRs compared to adult
Figure 4. Human AT/RT Specimens Exhibit Activation of the ESC-like Gene Expression Signature and ESC-like DNA Methylation Landscape

(A) Clustering analysis using microarray data revealed that both hESC-like module genes (left) and ESC Core module genes (right) are similarly expressed in AT/RT samples and ESCs and iPSCs. Note that medulloblastoma (MB) and glioblastoma (GBM) samples are clustered separately from ESCs and iPSCs. Microarray data of hPSCs, AT/RTs, medulloblastomas, and glioblastomas were obtained from GSE22392 (hESC/iPSC), GSE70678 (AT/RT), GSE37418 (MB), and GSE53733 (GBM).

(B) Expressions of SALL4 and LIN28 in AT/RTs, medulloblastomas, and glioblastomas compared to ESCs and iPSCs. Data are represented as the median with interquartile range. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (Kruskal-Wallis test and Dunn’s multiple comparisons test). The same microarray data as Figure 4A were used.

(C) The DNA methylation landscape in AT/RTs analyzed using Infinium450K data. In this analysis, we first extracted differentially methylated CpG sites within CpG islands (CGIs) between hPSCs and adult brains. hPSC-specific methylated CpG sites and adult brain-specific methylated CpG sites within CGIs were analyzed.
brain tissues or NSCs (Figures 4C and S5). Similar patterns of DNA methylation alterations were observed at CpG sites around TSSs (Figure S5). Taken together, we concluded that AT/RTs harbor an ESC-like DNA methylation landscape.

**Pediatric Cancers Exhibit Activation of the ESC-like Gene Expression Signature**

Previous studies demonstrated that pluripotency-related genes, such as LIN28B, are frequently upregulated in other pediatric cancers that occur in early childhood, which raised the possibility that activation of the ESC-like signature is a shared feature in pediatric cancers. Therefore, we next examined the expression of the ESC-like module genes in three other pediatric cancers, namely, neuroblastoma (NB), Wilms’ tumor (WT), and hepatoblastoma (HB), by comparing them with their corresponding adult cancer, namely, adenocortical carcinoma (ACC), renal cell carcinoma (RCC), and hepatocellular carcinoma (HCC), respectively. Notably, a principal-component analysis (PCA) revealed that all three pediatric cancer types exhibited similar expression profiles with PSCs and activation of the ESC-like module genes compared to the corresponding adult cancers (Figures 5A and S6). Consistently, the pediatric cancers showed increased expression of SALL4 and LIN28B compared to their counterparts (Figure S5B). Together, our data indicate that activation of the ESC-like signature is a common characteristic of pediatric cancers.

**Genetic Screening with CRISPR/Cas9 to Develop a Strategy for AT/RT Therapy**

Our findings about activation of the ESC-like signature suggest the signature could make a promising therapeutic target for AT/RT. Therefore, we performed genetic screening to identify genes that play a role in the malignant features of AT/RT (Figure 6A). Accordingly, we picked out 110 target genes that are associated with the maintenance of ESC identity based on previous reports (Tables S1 and S2). A lentiviral CRISPR/Cas9 system was employed to disrupt the candidate genes in a cancer cell line, which was established from hiPSC-derived tumor (Figure 6A; Table S1). The high efficiency of non-homologous end joining by this lentiviral CRISPR/Cas9 system was confirmed 7 days after lentiviral transduction in the pediatric cancer cell line SK-N-BE(2) (Figure S7A). The genetic screening revealed decreased cancer cell growth after the transduction of CRISPR/Cas9 together with single guide RNA (sgRNA) for dozens of genes compared to non-targeting control (NTC) sgRNA (Figure 6B; Table S3). Notably, among the candidate genes was Enhancer of zeste homolog 2 (EZH2), which has been reported as a target of AT/RT treatment (Knudson et al., 2013; Alimova et al., 2013; Wilson et al., 2010), indicating that our screening successfully identified potential therapeutic targets. Indeed, we confirmed that GSK126, a specific inhibitor for EZH2, efficiently suppressed growth of the hiPSC-derived cancer cell line. Moreover, GSK126 efficiently inhibited the ESC-like signature in the hiPSC-derived cancer cell line (Figure S7B).

Given that other pediatric cancers similarly exhibit activation of the ESC-like signature, we also performed the same genetic screening in two neuroblastoma cell lines, namely, SK-N-AS and SK-N-BE(2). We found that RAD21 knock out efficiently reduced cell proliferation in the hiPSC-derived cancer cell line and neuroblastoma cell lines (Figures 6B and 6C). The inhibitory effect of cell growth was confirmed in another guide RNA targeting RAD21 (Figure S7C). Notably, RAD21 as well as EZH2 was highly expressed in AT/RTs compared to glioblastomas (Figure 6D). Moreover, the increased expression of RAD21 and EZH2 was similarly observed in other pediatric cancers, including neuroblastomas, Wilms’ tumors, and hepatoblastomas, when compared to the corresponding adult cancers (Figure 6E). Therefore, we focused on RAD21 and EZH2 and conducted further experiments.

The inhibitory effect on tumor cell growth in the hiPSC-derived cancer cell line by the knockout of RAD21 and EZH2 was validated in a large-scale culture (Figure 6F). Xenograft experiments revealed that the lentiviral CRISPR/Cas9-mediated knock out of RAD21 or EZH2 extended the overall survival of mice after the inoculation of hiPSC-derived cancer cells (Figures 6G and 6H). Therefore, we concluded that RAD21 and EZH2 are potential therapeutic targets for AT/RTs.

RAD21 is a key central component within the multi-protein cohesin complex. A previous study demonstrated that histone deacetylase 8 (HDAC8) functions as a deacetylase of SMC3, another component of the cohesin complex, and plays a role in recycling cohesin during cell division (Deardorff et al., 2012). Moreover, it was shown that PCI34051, a HDAC8-specific inhibitor, reduces the localized cohesin, indicating that the inhibitor impairs cohesin function. Considering that RAD21 knock out inhibited cancer cell growth in the AT/RT model, we next investigated the effect of PCI34051 on cancer cell growth in this model. PCI34051 treatment resulted in a modest reduction in the cell growth of hiPSC-derived cancer cells (Figures 6I and S7D). The growth inhibitory effect was not obvious in the presence of siRAD21, which supports the notion that PCI34051 inhibits cell growth by impairing cohesin function (Figure S7D). Most notably, simultaneous treatment with PCI34051 and GSK126 markedly inhibited the cell proliferation (Figure 6I). Mechanistically, suppression of the ESC-like gene expression signature by GSK126 was more pronounced by the combination treatment of PCI34051 and GSK126 (Figures 6J and S7E), although PCI34051 alone did not significantly suppress the ESC-like signature (Figure S7F). Consistently, a gene ontology enrichment analysis revealed that the combination treatment induced genes associated with nervous system development (Figure 6K), suggesting that the combined inhibition of HDAC8 and EZH2 induced neuronal differentiation. We also confirmed that the

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*Infinium450K data of hPSCs, normal brains, fetal brains, NSCs, AT/RTs, and medulloblastomas were obtained from GSE60821 (hPSC), GSE92462 (hPSC, normal brain, fetal brain, and NSC), GSE36278 (normal brain and fetal brain), GSE70460 (AT/RT), and GSE75153 (MB). See also Figures S1 and S5.*
combination treatment remarkably suppressed proliferation in other rhabdoid and AT/RT cell lines (Figure 6L), as well as neuroblastoma cell lines (Figure 6M). In sharp contrast, the suppressive effect was not prominent in two of three glioblastoma cell lines (Figure S7G), which is consistent with the fact that most glioblastomas do not exhibit activation of the ESC-like signature.

**DISCUSSION**

In the present study, we established hPSCs deficient for SMARCB1 to model AT/RT. Although previous studies suggested that NPCs are a cell-of-origin for AT/RT, the majority of tumors from SMARCB1-deficient NPLCs lacked typical rhabdoid cells. On the other hand, despite having the same genetic abnormality, the transplantation of SMARCB1-deficient hPSCs caused tumors containing a large number of rhabdoid cells, indicating that PSC-related embryonic cell properties are associated with the histogenesis of rhabdoid cells, which was further supported by the fact that forced activation of the ESC-like signature confers the rhabdoid histology in SMARCB1-deficient NPLC-derived tumors. Consistent with that notion, we found activation of the ESC-like signature in clinical specimens of AT/RTs but not in medulloblastomas or glioblastomas. Of particular note, SMARCB1-deficient hPSC-transplanted mice showed poor survival compared to SMARCB1-deficient NPLC-transplanted mice, indicating a positive correlation between activation of the ESC-like signature and poor prognosis. Collectively, we developed a human AT/RT model using hPSCs and identified activation of the ESC-like signature as an important determinant of the unique histology and poor prognosis of AT/RT.

The fact that rhabdoid histology is prominent in PSC-derived tumors compared to NPLC-derived tumors suggests that an earlier embryonic program than the NPC program is involved in the unique histology. However, it remains unclear how AT/RT cells acquire the ESC-like signature in the postnatal brain. Notably, we found that ESC-methylated Cpg sites are unmethylated in NSCs and fetal brains, suggesting that the ESC-like methylation patterns observed in AT/RTs do not simply reflect the DNA methylation patterns in NSCs or fetal cells. These findings may support the assumption that the ESC-like signature is acquired during AT/RT development. It is also interesting to note that genetic ablation of TP53 seems to enhance the emergence of the rhabdoid histology, although clinical AT/RTs hardly harbor the TP53 mutation. Given that the loss of TP53 greatly promotes somatic cell reprogramming into iPSCs (Hong et al., 2009), it is possible that TP53 deficiency contributed to the acquisition of the ESC-like signature by accelerating the reprogramming
process in this particular model. In contrast, we found that TP53 deficiency alone did not promote the tumor formation in vivo, suggesting that the TP53 deficiency enhances tumor formation in conjunction with SMARCB1 deficiency.

Notably, we showed that c-MYC overexpression induces activation of the ESC-like signature in NPLC-derived tumors and drives tumor development with the rhabdoid phenotype. A previous study demonstrated that c-MYC activates the embryonic transcriptional program and causes stem cell-like phenotypes (Wong et al., 2008). Moreover, a recent study demonstrated that c-MYC-driven dedifferentiation supports the onset of a stem cell-like state and tumorigenesis in mammary epithelial cells (Poli et al., 2018). Together, we propose that c-MYC induces dedifferentiation and activates the ESC-like signature during tumor development from SMARCB1-deficient NPLCs. The fact that c-MYC is frequently amplified in AT/RT in patients at higher age (Johann et al., 2016) may further support the notion that c-MYC induces dedifferentiation during AT/RT development.

Taking advantage of our human AT/RT model, we showed that activation of the ESC-like signature is correlated with the poor prognosis, which raised the possibility that the ESC-like signature is a promising therapeutic target for AT/RT. Accordingly, we performed a CRISPR/Cas9 knock out screening targeting the maintenance of ESC identity and identified genes that potentially maintain the growth of tumor cells. Notably, the identified genes included EZH2, which was previously reported as a potential therapeutic target in AT/RT (Choi et al., 2016; Weingart et al., 2015). Importantly, we found that an EZH2 inhibitor efficiently inhibited the ESC-like signature in AT/RT cells, which is consistent with a previous study that demonstrated Ezh2 maintains the stem cell-associated signature in Smarcb1-deficient mouse embryonic fibroblasts (Wilson et al., 2010).

We also found that knock out of RAD21, which encodes a component within the cohesin complex, significantly suppresses the growth of AT/RT cells, suggesting that the function of cohesin too could be a target for AT/RT treatment. Consistently, an HDAC8-specific inhibitor, which indirectly reduces localized cohesin, together with the EZH2 inhibitor synergistically inhibited activation of the ESC-like signature and markedly suppressed the proliferation of AT/RT cells but had minimal effect on glioblastoma cells. Although a mechanistic basis for the potent inhibition of the ESC-like signature by the combined inhibition of EZH2 and HDAC8 remains to be solved, we propose that inhibition of the ESC-like signature is an effective strategy for AT/RT treatment.

It is interesting that activation of the ESC-like signature was similarly detectable in other pediatric cancers, such as neuroblastomas, Wilms’ tumors, and hepatoblastomas. Notably, the same knock out screening in neuroblastoma cell lines revealed that knock out of RAD21 often suppressed the growth of neuroblastoma cells. Furthermore, the combined treatment with EZH2 and HDAC8 inhibitors synergistically reduced the proliferation of neuroblastoma cells. Together with previous findings that partial reprogramming in vivo induces activation of the ESC-like signature and causes the development of cancers that resemble pediatric cancers (Ohnishi et al., 2014), it is possible that activation of the ESC-like signature may be a general driver of pediatric cancers.
cancer development and, therefore, could be a therapeutic target for pediatric cancers. Indeed, previous studies showed that EZH2 inhibitors are often effective for pediatric cancers (Chen et al., 2019) and that an HDAC8 inhibitor suppresses cell proliferation and induces differentiation in neuroblastoma cells (Oehme et al., 2009). Collectively, we propose that the combined inhibition of EZH2 and HDAC8 could be a promising strategy to treat pediatric cancers by targeting the ESC-like signature.

In summary, we established a human AT/RT model using SMARCB1-deficient hPSCs. Taking advantage of the AT/RT model, we unveiled that activation of the ESC-like signature plays a central role in the unique rhabdoid histology and poor prognosis of AT/RT. Finally, we showed that this signature could be a promising therapeutic target for AT/RT as well as other pediatric cancers.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found with this article online at https://doi.org/10.1016/j.celrep.2019.02.009.

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AUTHOR CONTRIBUTIONS

Y.T., N.J., and Yasuhiro Yamada proposed the research project, designed the experiments, performed the experiments, and wrote the manuscript. M.S., Yosuke Yamada, and T.U. performed the experiments. T.Y., M.K., and S.O. analyzed the microarray and RNA-sequencing data. S.M., Y.A., M.N., Y.M., K.M., and Y.M. provided materials and technical instructions.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-SALL4          | Abnova | Cat# clone 6E3; RRID: AB_566160 |
| Anti-LIN28A         | Cell Signaling Technology | Cat# 8706; RRID: AB_10896850 |
| Anti-LIN28B         | Cell Signaling Technology | Cat# 5422; RRID: AB_10697489 |
| Anti-Ki67 (SP6)     | Abcam  | Cat# ab16667; RRID: AB_302459 |
| Anti-IN1/SNF5       | Sigma-Aldrich | Cat# SAB4200202; RRID: AB_10697389 |
| Anti-Vimentin (V9)  | SantaCruz | Cat# sc-6260; RRID: AB_628437 |
| Anti-GFAP           | DAKO   | Cat# IR524 |
| Anti-CD99           | DAKO   | Cat# clone 12E7; RRID: AB_2076419 |
| Anti-S100           | DAKO   | Cat# GA504 |
| Anti-EMA            | Novocastra | Cat# NCL-L-EMA; RRID: AB_563531 |
| Anti-SMA            | Nichirei | Cat# clone1A4 |
| Anti-Synaptophysin  | Nichirei | Cat# clone27G12 |
| Anti-NESTIN         | Millipore | Cat# MAB5326; RRID: AB_2251134 |
| Anti-TP53           | Santa Cruz | Cat# sc-126; RRID: AB_628082 |
| Anti-β-actin        | Santa Cruz | Cat# sc-47778; RRID: AB_626632 |
| ECL anti-mouse IgG, HRP-linked whole antibody from sheep | GE Healthcare | Cat# NA931; RRID: AB_772210 |
| ESC anti-rabbit IgG and HRP-linked whole antibody from donkey | GE Healthcare | Cat# NA934; RRID: AB_772206 |
| Anti-PSA-NCAM antibody conjugated with microbeads | Miltenyi Biotec | Cat# 130-92-981 |
| Anti-PSA-NCAM       | Millipore | Cat# MAB5324; RRID: AB_95211 |
| StemFit AK03N       | Ajinomoto | Cat# AK03N |
| StemFit AK02N       | Ajinomoto | Cat# AK02N |
| Laminin-511         | Wako   | Cat# 892012 |
| PSC Neural Induction Medium | Life Technologies | Cat# A1647801 |
| Y27632              | Wako   | Cat# 253-00513 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| GSK126              | Funakoshi | Cat# A-1275 |
| PCI34051            | Selleck | Cat# S2012 |
| siGENOME SMARTpool siRNA, RAD21 | Dharmacon | Cat# M-006332-01-0005 |
| siGENOME SMARTpool siRNA, Non-Targeting | Dharmacon | Cat# D-001206-13-05 |
| Critical Commercial Assays |        |            |
| AlamarBlue cell viability reagent | Bio-Rad | Cat# BOF012B |
| Cell Counting Kit-8 | Dojindo | Cat# 341-07761 |
| Human Gene 1.0 ST Array | Affymetrix | Cat# 901086 |
| Truseq Stranded mRNA LT sample prep kit | Illumina | Cat# RS-122-2101, RS-122-2102 |
| Deposited Data      |        |            |
| Microarray data     | This paper | GSE118653 |
| RNA-seq data        | This paper | GSE118654 |
| Affymetrix Human Gene 1.0ST Array datasets | Gene Expression Omnibus | GEO: GSE26313, GSE45265, GSE36947, GSE18296 and GSE27667 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Affymetrix Human Genome U133 Plus 2.0 Array datasets** | Gene Expression Omnibus | GEO: GSE70678, GSE37418, GSE53733, GSE16476, GSE11151, GSE53224, GSE75271, GSE10927, GSE66272, GSE62232, GSE73038 and GSE22392 |
| **Illumina HumanMethylation450 BeadChip datasets** | Gene Expression Omnibus | GEO: GSE60821, GSE92462, GSE70460, GSE75153, GSE6278 and GSE73801 |

**Experimental Models: Cell Lines**

| Human: 201B7 hiPSC | Laboratory of Masato Nakagawa | hPSC Cell Line: 201B7 |
|---------------------|-------------------------------|-----------------------|
| Human: 1383D6 hiPSC | Laboratory of Masato Nakagawa | hPSC Cell Line: 1383D6 |
| Human: KUP-ATRT-1 | Kyoto University | N/A |
| Human: A204 | ATCC | HTB-82 |
| Human: G401 | ATCC | CRL-1441 |
| Human: G402 | ATCC | CRL-1440 |
| Human: SK-N-AS | ATCC | CRL-2137 |
| Human: SK-N-BE(2) | ATCC | CRL-2271 |
| Human:T98G | Cell Resource Center for Biomedical Research, Institute of Development Aging and Cancer, Tohoku University | TKG0471 |
| Human:A172 | Cell Resource Center for Biomedical Research, Institute of Development Aging and Cancer, Tohoku University | TKG0183 |
| Human:U87-MG | ATCC | HTB-14 |

**Experimental Models: Organisms/Strains**

| Mouse: NOD/ShiJic-scidJcl | CLEA Japan | N/A |

**Oligonucleotides**

| Oligos for sgRNA, see Table S1 | This study and Brunello Library | Human CRISPR Knockout Pooled Library (Brunello) |
|---------------------------------|---------------------------------|-----------------------------------------------|
| Primers for quantitative PCR, see Table S4 | This study | N/A |
| Oligos for TP53 sgRNA: CGCTATCTGAGCAGCGCTCA | This study | N/A |
| Oligos for SMARCB1 sgRNA: TGAGAACGCATCTCAGCCCG | This study | N/A |

**Recombinant DNA**

| lentiCRISPR v2 | Sanjana et al., 2014 | Addgene: #52961 |
|---------------|----------------------|------------------|
| pX330-U6-Chimeric BB-CBh-hSpCas9 | Cong et al., 2013 | Addgene: #42230 |
| pMYs-IRES-GFP | Cell Biolads | Cat# RTV-021 |
| pMYs-c-MYC-IRES-GFP | This study | N/A |
| pMx-GFP | Cell Biolads | Cat# VPK-302 |
| pMxs-hOCT4 | Takahashi et al., 2007 | Addgene: #17217 |
| pMxs-hSOX2 | Takahashi et al., 2007 | Addgene: #17218 |
| pMxs-hKLF4 | Takahashi et al., 2007 | Addgene: #17219 |
| pMxs-hMYC | Takahashi et al., 2007 | Addgene: #17220 |
| pCMV-VSV-G | Cell Biolads | Cat# VPK-302 |
| pMD2.G | Addgene | Cat# 12259 |
| psPAX2 | Addgene | Cat# 12260 |

**Software and Algorithms**

| GSEA software (version 3.0) | Subramanian et al., 2005 | http://software.broadinstitute.org/gsea/index.jsp |
|-------------------------------|-------------------------|--------------------------------------------------|
| DAVID bioinformatics database website | Huang et al., 2009 | https://david.ncifcrf.gov/home.jsp |

(Continued on next page)
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

All animal experiments were approved by the Animal Experiment Committee at CiRA and IMSUT, and the care of the animals was in accordance with institutional guidelines. All mice used for the present study were on NOD/ShiJic-scidJcl (NOD SCID) mice of both sexes (8-10 weeks). NOD SCID mice were purchased from CLEA Japan, Inc. Mice were kept in the animal facility with 12 hours of light and dark cycle with food and water ad libitum.

Cell culture and neural induction

201B7 and 1383D6 hiPSCs (provided by Masato Nakagawa, CiRA) were cultured in StemFit AK03N or AK02N (Ajinomoto) on cell culture plates coated with laminin-511 (Wako) at 37 °C with 5% CO₂. For neural induction, culture medium was switched to GIBCO PSC Neural Induction Medium (Life Technologies) containing Neurobasal medium and GIBCO PSC neural induction supplement. At day 7 of the neural induction, these cells were dissociated and plated in neural expansion medium containing 50% Neurobasal medium, 50% Advanced DMEM/F12 and neural induction supplement. Cells were treated with 10 μM ROCK inhibitor Y27632 (Wako) at the time of plating overnight to prevent cell death. To further induce differentiation, culture medium was switched to Neurobasal medium containing 2% B-27 Supplement (GIBCO) and GlutaMAX Supplement (GIBCO).

KUP-ATRT-1, an AT/RT cell line, was established at Kyoto University. Other tumor cell lines were purchased from ATCC or Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. Tumor cells were cultured with RPMI 1640 (GIBCO) or MEM, GlutaMAXTM supplement (GIBCO) containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂.

Establishment of hiPSC SMARCB1−/−; TP53−/−-derived cancer cell line

A block of hiPSC SMARCB1−/−; TP53−/−-derived tumor was mechanically minced. The minced tissue was digested with StemPro Accutase Cell Dissociation Reagent (GIBCO) at 37 °C for 15 minutes. After washing, the tissue was triturated and passed through a 100 μm cell strainer. Cells were plated onto cell culture plates coated with laminin-511 in AK03N media. Passaging of the cultures was performed approximately once a week.

METHOD DETAILS

TP53 and SMARCB1 knockout using CRISPR/Cas9 system

The TP53 and SMARCB1 genes were knocked-out with P3 primary cell 4D-Nucleofector X Kit (Lonza). 201B7 cells were transfected with modified px330-U6-Chimeric_BB-CDh-hSpCas9 plasmid (Addgene: Plasmid #42230) (Cong et al., 2013) expressing a sgRNA against TP53 (CGCTATCTGAGCAGCGCTCA) or SMARCB1 (TGAGAACGCATCTCAGCCCG) with puromycin-resistance gene. To avoid genomic integration, puromycin selection (1 μl/ml) was performed only for two days. Each single colony was picked-up and expanded. The knockout for TP53 and SMARCB1 was confirmed by sequencing and western blot.

Retroviral transduction

To generate retroviral vectors, Plat-GP cells on 150 mm dishes were cultured with DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin (P/S) (Nacalai tesque) and 10% FBS (GIBCO) until 70%–80% confluency and were transfected with 12.375 μg retroviral
vector plasmid (pMx-GFP, pMxs-hOCT4, pMxs-hSOX2, pMxs-hKLF4, pMxs-hc-MYC and pMYs-IREs-GFP or pMYs-hc-MYC-IREs-GFP) (Addgene, Cell Biolabs, Inc.) (Takahashi et al., 2007) independently in combination with 5.625 μg pCMV-VSV-G (Cell Biolabs, Inc.) using Lipofectamin 2000 (Life technologies). 24 hours after the transfection, the culture media was refreshed, and the supernatant was collected over 3 consecutive days. The filtered supernatant was concentrated by PEG-it (System Biosciences), re-suspended, aliquoted and stored at -80 °C. 1 x 10^6 of NPLCs^SMARCB1^−/−; TP53−/−; SMARCD1−/− or PSA-NCAM-positive NPLCs^TP53−/−; SMARCD1−/− were incubated with the concentrated virus-containing supernatant overnight. Five days after the infection, cells were harvested and transplanted into the mouse brain. At the same time, RNA was extracted from the infected cells.

**Xenograft tumor model**

1 μl of 4 x 10^5 cells were injected into the left striatum of NOD SCID mice at the coordinates of 1.5 mm lateral from the bregma and 2 mm deep from the dura using a 10 μl Hamilton syringe with a flow rate of 1 μl per minute. MRI studies were performed on a 1.5-Tesla MRI scanner (MRmini SA1508; DS Pharma Biomedical). Transplanted mice were traced until they died or showed some neurological signs (observation period: 4-31 weeks).

**Histological analysis and immunostaining**

Mice were transcardially perfused with 1 x PBS followed by 4% paraformaldehyde. Following dissection, brain tissues were transferred to PBS and subsequently embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin (H&E), and serial sections were used for the immunohistochemical analysis. The primary antibodies, which were incubated at 4 °C overnight in blocking buffer, were as follows: anti-SALL4 (Abovna: #clone 6E3), anti-LIN28A (Cell Signaling Technology: #8706), anti-LIN28B (Cell Signaling Technology: #5422), anti-Ki67 (SP6) (Abcam: #ab16667), anti-INI1/SNF5 (Sigma-Aldrich: #91735), anti-Vimentin (V9) (SantaCruz: #sc-6260), anti-GFAP (DAKO: #IR524), anti-CD99 (DAKO: #clone 1E7), anti-S100 (DAKO: #GA504), anti-EMA (Novocastro: #NCL-L-EMA), anti-SMA (Nichirei: #clone1A4) and anti-Synaptophysin (Nichirei: #clone27G12). The sections were incubated with the appropriate species of HRP-conjugated secondary antibodies (Nichirei, Histofine) at room temperature for 30 minutes, and immunostaining development was performed using DAB (Nichirei). The stained slides were counterstained with Meyer hematoxylin. The histopathological sections were reviewed by two different pathologists (Yo Y and Ya Y) who have been certified by the Japanese Society of Pathology.

**Immunofluorescent staining**

The samples were fixed with 4% paraformaldehyde for five minutes and soaked in PBS. They were then incubated with anti-NESTIN (Millipore: #MAB5326) at 4 °C overnight in blocking buffer and were processed with × 500 DAPI (Invitrogen) and × 150 fluorescence-labeled secondary antibodies diluted with 0.5% BSA in PBS for 90 minutes at room temperature. After washing in PBS for 5 minutes twice, the samples were mounted and evaluated with a confocal laser-scanning microscope (Zeiss LSM700 or 710). Western blot analysis

Cell lysates were prepared in RIPA buffer with protease inhibitors. Protein samples were carried out according to standard methods. The primary antibodies used were anti-INI1/SNF5 (Sigma-Aldrich: #91735), anti-TP53 (Santa Cruz Biotechnology: #sc-126) and anti-β-actin (Santa Cruz, sc-47778). The secondary antibodies used were ECL anti-mouse IgG, HRP-linked whole antibody from sheep (NA931, GE Healthcare), ECL anti-rabbit IgG and HRP-linked whole antibody from donkey (NA934, GE Healthcare). ImageQuant LAS4000 (GE Healthcare) was used for band detection.

**Magnetic-based cell sorting (MACS) and flow cytometric analysis**

Expanded NPLCs^SMARCB1^-/-; TP53^-/- were exposed to 10 μM of Y27632 (Wako) for more than one hour to prevent cell death prior to the MACS procedure. After dissociation, the cells were briefly blocked in 0.5% BSA-PBS solution and then incubated with anti-PSA-NCAM antibody conjugated with microbeads (Miltenyi Biotec: #130-92-981) for 15 minutes at 4 °C. After extensive washing, the cell suspension was loaded on a separation column (LS column) that was attached to a magnetic stand. Positively-labeled cells that remained in the column were eluted to a tube with culture medium for further manipulation and analysis. After magnetic cell separation, we further used flow cytometry to examine the purity of PSA-NCAM-positive cells. MACS-sorted cells were cultured for 24 hours and stained with anti-PSA-NCAM antibody (Millipore: #MAB5324) for 15 minutes at 4 °C followed by CF488-conjugated secondary antibody for 10 minutes at 4 °C. The positive fraction was evaluated by flow cytometry (Aria II, BD) using non-stained cells as a control.

**Genetic screening with lentiviral CRISPR/Cas9 system**

We utilized the lentCRISPR v2 (Addgene #52961) (Sanjana et al., 2014) for one-by-one gene disruption. The candidate genes were selected based on the previous literatures and databases (Table S1). The lentiviral vector has both a sgRNA scaffold and a Cas9 to induce insertions and deletions (indels) at the genomic locus of the cells into which they are transduced.

The lentiviral cloning and production were performed as described previously (Sanjana et al., 2014). Briefly, the complementary oligonucleotides for sgRNAs (Table S1) were annealed by heating to 95 °C for 3 minutes and subsequent cooling to 60 °C for 3 minutes on ice. The lentCRISPR v2 plasmid was digested with BsmBI (New England Biolabs) at 55 °C overnight and purified by FastGene...
Gel/PCR Extraction Kit (Genetics). The annealed oligonucleotides were ligated into the digested lentCRISPR v2 plasmid by Ligation high Ver.2 (TOYOBO) at 16°C for 30 minutes. The reactants were transformed into Stbl3 chemically competent E. coli (Thermo Fisher Scientific). The grown colonies were analyzed by Sanger sequencing with ABI 3500xL (Applied Biosystems) to confirm correctly recombined clones. The lentiviral plasmids were extracted by GenElute Plasmid Miniprep Kit (Sigma).

To generate lentiviral vectors, HEK293T cells on 60 mm dishes were cultured with DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin (P/S) (Nacalai tesque), 10% FBS (GIBCO) until 70%–80% confluency and transfected with 2.4 μg lentiCRISPR v2 plasmid, 0.8 μg pMD2.G (Addgene #12259) and 1.6 μg psPAX2 (Addgene #12260) using PEI max (Polysciences). 24 hours after transfection, culture media were refreshed, and the supernatant was collected over 3 consecutive days. The filtered supernatant was concentrated by PEG-it (System Biosciences), re-suspended, aliquoted and stored at –80°C. No lentiviral functional titration was performed. Instead, we measured base-line cell viability at days 3-5 during the screening process to adjust the transduction efficiency bias as explained below. The screening was performed with 96-well plate format in duplicate. Each cell line was plated to be 70%–80% confluent at the day of lentiviral transduction (day 0). We avoided using outer lanes, where the value could be variable because of the media evaporation. At day 0, virus-containing supernatant was added to each well, and 24 hours later, puromycin was added to eliminate non-transfected cells. At days 3-5, the baseline cell viability was assessed using alamarBlue (Bio-Rad). Fluorescence was detected with 2104 EnVision Multi Detection Microplate Reader (Perkin Elmer). The average fluorescence intensity value in blank wells was subtracted to determine the fluorescence intensity value of each well. Media were replaced every 2-3 days. Whenells transduced with non-targeting control (NTC) sgRNA reached 70%–80% confluency, cell viability was measured again by alamarBlue to assess the effect of each knockout on cell growth. Cell growth rates were calculated as follows: the average of duplicate fluorescence intensity values was divided by the average at baseline. Cell growth rates were compared with NTC sgRNA-transduced cells.

To confirm the CRISPR/Cas9-mediated cleavage efficiency in this screening, we randomly selected 20 genes among the candidate genes and assessed the indel frequency at days 3 and 7 in the SK-N-BE(2) cell line. The indel frequency was calculated by TIDE software (https://tide.nki.nl)(Brinkman et al., 2014). The TIDE software parameters used in this study were as follows: left boundary, 100 bp; right boundary, –10 bp; decomposition window, 115-685 bp; indel size range, 20 bp.

The titration of puromycin was pre-determined for each cell line to efficiently eliminate non-transduced cells at days 3-5. The concentrations of puromycin used in this study were 1.0, 1.0 and 1.5 μg/ml for hiPSC SMARC81/−/−; TP53/−/−-derived cancer cell line, SK-N-BE(2) and SK-N-AS, respectively.

**Cell growth inhibition assays and cell proliferation assays**

*In vitro* drug sensitivity was determined using alamarBlue cell viability reagent (Bio-Rad) according to the manufacturer’s protocol. Cells were plated into 96-well culture plates at a density of 2 × 10³ cells/well. Cells were treated with 10 μM of DMSO, GSK126, PCI34051 or a combination of GSK126 and PCI34051 at day 0 and incubated for 7 days. The experiment was performed in triplicate, and each sample was measured three times. Fluorescence intensity was detected with 2104 EnVision Multi Detection Microplate Reader. The average fluorescence intensity value of blank wells was subtracted to determine the fluorescence intensity of each well at each time point. The average fluorescence intensity of DMSO-treated cells at day 7 was set to 1. Cell Counting Kit-8 (Dojindo) was also used to examine *in vitro* cell proliferation. Absorbance was measured using iMark Microplate Absorbance Reader (Bio-Rad).

**siRNA transfection**

siRNA transfection was performed using Lipofectamine RNAi Max (Invitrogen). We performed knockdown assays with a siRNA targeting RAD21 (Dharmacon). Nontargeting siRNA (Dharmacon) was used as a control. Culture medium was exchanged every 2 days, and cell proliferation was determined using Cell Counting Kit-8 (Dojindo).

**RNA Preparation, qRT-PCR and microarray analysis**

Total RNA was isolated using the RNeasy Plus Mini kit (QIAGEN). The qRT-PCR analysis was performed using GoTaq qPCR Master Mix (Promega). The specific primer pairs used for amplification are shown in Table S4. The transcript levels were normalized to the GAPDH level. The microarray analysis was performed using Human Gene 1.0 ST Array (Affymetrix) in accordance with the manufacturer’s instructions, GeneSpring GX software program (version 12; Agilent Technology), GSEA software (version 3.0) and the DAVID bioinformatics database website (https://david.ncifcrf.gov/home.jsp). The gene sets of the hESC-like module (Wong et al., 2006) and the Core Human module (Kim et al., 2010) were used for the clustering analysis. The gene set of “WONG_EMBRYONIC_STEM_CELL_CORE” in MSigDB (version 6.0) was used for the GSEA analysis.

**Library preparation for RNA sequencing**

200 ng of total RNA was prepared for the library construction. High-quality RNA (RNA Integrity Number value ≥ 7) assessed by Bioanalyzer was used for the library preparation. RNA-seq libraries were generated using the Truseq Stranded mRNA LT sample prep kit (Illumina). PolyA-containing mRNA was purified by poly-T oligo-attached magnetic beads, and the RNA was fragmentated and primed for cDNA synthesis. Cleaved RNA fragments were reverse transcribed into first strand cDNA using transcriptase and random primers. Second strand cDNA was synthesized by the incorporation of dUTP, and ds cDNA was separated using AMPure XP beads (BECKMAN COULTER). A single ‘A’ nucleotide was added to the 3’ ends of the blunt fragments, and then adapters with
index were ligated to the ends of the ds cDNA. ds cDNA fragments were amplified by PCR with PCR primer Cocktail. The number of PCR cycles was minimized (15 cycles) to avoid skewing the representation of the libraries. RNA-seq libraries were sequenced on NextSeq 500 (75 bp or 86 bp single, Illumina).

**RNA-seq data analyses**

The sequenced reads were mapped to the human reference genome (hg38) using Tophat2 (version 2.1.1) with the GENCODE (version 27) annotation gtf file and the aligner Bowtie2-2.3.4 (Langmead and Salzberg, 2012) after trimming adaptor sequences and low-quality bases by cutadapt-1.16 (Martin, 2011). The uniquely mapped reads were used for further analyses. Using cufflinks-2.2.1 (Trapnell et al., 2010) with the human GENCODE (version 27, protein coding) annotation gtf file, reads per kilobase of exon per million mapped reads (RPKM) were calculated as the expression levels of each gene. RPKM values were used for the GSEA method.

**DNA methylation analyses for infinium methylation array data**

Infinium450K data were obtained from GSE60821 (hESC), GSE92462 (hESC, NSCs, normal brain and fetal brain), GSE36278 (normal brain, fetal brain and GBM), GSE75153 (MB), GSE73801 (ETMR) and GSE70460 (AT/RT). Previously described human CGIs (Illingworth et al., 2010) were used for the methylation analysis. The UCSC LiftOver tools (http://genome.ucsc.edu) (Rosenbloom et al., 2015) were used to convert the coordinates of hg18 assembly into those of hg19 assembly. After the conversion of the CGI regions, overlap regions were merged into a single region. The UCSC refGene table was used to determine the TSS sites. The median signal value of the probes within each CGI and TSS ± 1,500 bp was calculated as the methylation signal of the region. Brain-methylated regions and brain-unmethylated regions were defined as the CGI and TSS ± 1,500 bp that represent higher (> 0.6) and lower (< 0.6) brain-methylation (median methylation signals in 7 brain samples), respectively, compared to PSC-methylation (median methylation signals in 6 PSC samples). The all probe methylation signals within the indicated regions were used in violin plots.

**Quantification and Statistical Analysis**

To quantify the dominance of rhabdoid cells in tumors, an H&E stained section was randomly photographed at 10 × magnification. Three or 4 pictures for each sample were processed with ImageJ software (NIH) to evaluate the positive area of rhabdoid cells. The positive area was determined by the area of rhabdoid cells divided by the area of the tumor in the histological image. To assess SALL4-positive cells, each section was randomly photographed at 200 × magnification. Positive nuclei in tumor cells were counted using five images. The number of positive nucleus was divided by the number of total nuclei of tumor cells in each image. These results were evaluated with Graphpad Prism 6 software.

All values and graphs are expressed as the mean with 95% confidence interval or the median with interquartile range, and statistical analyses were performed using unpaired t test with Welch’s correction or Mann-Whitney U test for continuous variables and Fisher’s exact test for categorical variables. One-way ANOVA or Kruskal-Wallis test was performed to compare multiple groups. Dunnett’s or Dunn’s multiple comparisons test was used for multiple comparisons. Kaplan-Meier survival curves were compared using the log-rank test. Statistical parameters including statistical significance and n values are described in the figures and figure legends. A value of p < 0.05 was considered significant. All analyses were conducted using Graphpad Prism 6 software.

**DATA AND SOFTWARE AVAILABILITY**

All data analyzed by microarray and RNA-seq have been deposited in the Gene Expression Omnibus (GEO) under accession numbers GSE118653 and GSE118654.
Supplemental Information

Human Pluripotent Stem Cell-Derived Tumor Model
Uncovers the Embryonic Stem Cell Signature as a
Key Driver in Atypical Teratoid/Rhabdoid Tumor

Yukinori Terada, Norihide Jo, Yoshiki Arakawa, Megumi Sakakura, Yosuke Yamada, Tomoyo Ukai, Mio Kabata, Kanae Mitsunaga, Yohei Mineharu, Sho Ohta, Masato Nakagawa, Susumu Miyamoto, Takuya Yamamoto, and Yasuhiro Yamada
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Figure S1: Generation of an atypical teratoid/rhabdoid tumor model using hiPSCs lacking SMARCB1, related to Figure 1.
Figure S1: Generation of an atypical teratoid/rhabdoid tumor model using hiPSCs lacking SMARCB1, related to Figure 1

(A) A qRT-PCR for pluripotency genes and neural differentiation genes. Data are presented as the mean ± SD of biological triplicates. The mean expression level of hiPSCs 201B7 or NPLCs 201B7 was set to 1.
(B) A qRT-PCR for neuronal differentiation genes after the neuronal differentiation of NPLCs for 7 days. Data are presented as the mean ± SD of biological triplicates. The mean expression level of NPLCs (differentiation 7 days) was set to 1.
(C) Gene ontology enrichment analysis of NPLCs SMARCB1−/−, TP53−/− compared to undifferentiated hiPSCs SMARCB1−/−, TP53−/−. Analysis was performed using DAVID (Huang da et al., 2009). Genes showing ≥ 2-fold changes in NPLCs SMARCB1−/−, TP53−/− compared to hiPSCs TP53−/−, SMARCB1−/− were used in the analysis.
(D) Cell proliferation assays of hiPSCs and NPLCs. **p<0.01, ***p<0.001 (One-way ANOVA and Dunnett's multiple comparisons test).
(E) Macroscopic tumor incidence after xenograft transplantation of hiPSCs and NPLCs into the brain of immunocompromised mice.
(F) Microscopic tumor incidence after xenograft transplantation of hiPSCs and NPLCs into the brain of immunocompromised mice.
(G) Representative histological images of microscopic tumors. The iPSC 201B7-derived tumor exhibits a teratoma-like histology, which contains ciliated epithelial cells (left), while the NPLCs TP53−/− tumor shows a scar-like histology (right). Scale bars, 500 µm (top) and 50 µm (bottom).
(H) Western blot analysis of 1383D6 iPSCs SMARCB1−/− for SMARCB1 and β-actin shows the lack of SMARCB1 in 1383D6 iPSCs SMARCB1−/−.
(I) Representative histological images of a 1383D6 iPSC SMARCB1−/−-derived tumor and 1383D6 NPLC SMARCB1−/−-derived tumor. Note that the 1383D6 iPSC SMARCB1−/−-derived tumor contains rhabdoid cells. Scale bars, 500 µm (top) and 20 µm (bottom).
(J) Quantification of the rhabdoid area within 1383D6 iPSC SMARCB1−/−-derived tumors and 1383D6 NPLC SMARCB1−/−-derived tumors. Data are represented as the median with interquartile range. *p<0.05 (Mann-Whitney U test).
Figure S2: Induction of the ESC-like signature leads to rhabdoid phenotype in tumors, related to Figure 2
Figure S2: Induction of the ESC-like signature leads to rhabdoid phenotype in tumors, related to Figure 2

(A) A schematic illustration of a xenograft transplantation study of NPLCs $^{\text{SMARCB1}^{-/-}; \text{TP53}^{-/-}}$ transduced with GFP (GFP-NPLCs) or four reprogramming factors ($\text{OCT4}$, $\text{SOX2}$, $\text{KLF4}$ and $\text{c-MYC}$) (OSKM-NPLCs).

(B) A flow cytometric analysis of PSA-NCAM expression in NPLCs $^{\text{SMARCB1}^{-/-}; \text{TP53}^{-/-}}$ after sorting with PSA-NCAM antibody by magnetic activated cell sorting (MACS).

(C) A qRT-PCR for $\text{OCT4}$, $\text{SOX2}$, $\text{KLF4}$ and $\text{c-MYC}$ at 5 days after the transduction of GFP or OSKM in NPLCs $^{\text{SMARCB1}^{-/-}; \text{TP53}^{-/-}}$. Data are presented as the mean of technical triplicates. The mean expression level of OSKM-NPLCs was set to 1.

(D) A qRT-PCR for pluripotent genes and neural differentiation genes at 5 days after the transduction of GFP, c-MYC or OSKM in NPLCs $^{\text{SMARCB1}^{-/-}; \text{TP53}^{-/-}}$ in vitro. Data are presented as the mean of three biological replicates with standard deviation. The mean expression level of GFP-NPLCs was set to 1. *$p<0.05$, **$p<0.01$ (One-way ANOVA and Dunnett's multiple comparisons test).

(E) (F) Survival curve of mice transplanted with GFP-NPLCs or OSKM-NPLCs. Note that mice inoculated with OSKM-NPLCs exhibit poor survival when compared to mice inoculated with control NPLCs. Kaplan-Meier analysis was performed. *$p<0.05$, **$p<0.01$ (Log-rank test).
Figure S3: c-MYC induces activation of the ESC-like signature and drives rhabdoid tumors in vivo, related to Figure 3
Figure S3: c-MYC induces activation of the ESC-like signature and drives rhabdoid tumors in vivo, related to Figure 3

(A) A schematic illustration of a xenograft transplantation study of NPLCs $^{S:\text{MARCB1}^{-/-}; TP53^{-/-}}$ transduced with GFP (GFP-NPLCs) or c-MYC (MYC-NPLCs). Control GFP-NPLCs are the same as Figure S2.

(B) A qRT-PCR for c-MYC after the transduction of GFP or c-MYC in NPLCs $^{S:\text{MARCB1}^{-/-}; TP53^{-/-}}$. Data are presented as the mean of technical triplicates. The mean expression level of MYC-NPLCs was set to 1.

(C) Representative histological images of a MYC-NPLC-derived tumor. Note that a number of rhabdoid cells are observed in the tumor. Scale bars, 500 µm (upper) and 20 µm (lower).

(D) Quantification of the rhabdoid area in GFP-NPLC- and MYC-NPLC-derived tumors. Data are represented as the median with interquartile range. **$p<0.01$ (Mann-Whitney U test).

(E) Survival curves of mice transplanted with GFP-NPLCs or MYC-NPLCs. Note that the overall survival of mice inoculated with MYC-NPLCs is significantly shorter than of control. Kaplan-Meier analysis was performed. ****$p<0.0001$ (Log-rank test).
Figure S4: Human AT/RT specimens exhibit activation of the ESC-like gene expression signature, related to Figure 4
Figure S4: Human AT/RT specimens exhibit activation of the ESC-like gene expression signature, related to Figure 4

(A) Clustering analysis using microarray data revealed that both hESC-like module genes (left) and ESC Core module genes (right) are similarly expressed in AT/RT samples and ESCs/iPSCs. Note that some ETMRs were clustered with AT/RT samples and ESCs/iPSCs. Medulloblastoma (MB) and glioblastoma (GBM) samples are basically clustered separately from ESCs/iPSCs. The microarray data of hPSCs, AT/RTs, ETMRs, medulloblastomas and glioblastomas were obtained from GSE22392 (hESC/hiPSC), GSE73038 (ETMR), GSE70678 (AT/RT), GSE37418 (MB) and GSE53733 (GBM).

(B) Immunohistochemical analysis of SALL4, LIN28A and LIN28B in clinical AT/RT specimens. Scale bars, 50 µm.
Figure S5: DNA methylation landscape in human AT/RT specimens, related to Figure 4
Figure S5: DNA methylation landscape in human AT/RT specimens, related to Figure 4

The DNA methylation landscape in AT/RTs analyzed using Infinium450K data. In this analysis, we first extracted differentially methylated CpG sites within CGIs (left) or around TSSs (right) between hPSCs and adult brains. hPSC-specific methylated CpG sites and adult brain-specific methylated CpG sites were analyzed for AT/RTs, ETMRs, medulloblastomas and glioblastomas as well as fetal brains and NSCs. AT/RTs harbor increased methylation at the PSC-specific methylated CpG sites, while adult brain-specific methylated CpG sites are less methylated in AT/RTs. Each dot indicates the median of DNA methylation at the analyzed CpG sites. Infinium450K data of hPSCs, normal brains, fetal brains, NSCs, AT/RTs, ETMRs, medulloblastomas and glioblastomas were obtained from GSE60821 (hPSC), GSE92462 (hPSC/NSC/normal brain/fetal brain), GSE36278 (normal brain/fetal brain/GBM), GSE73801 (ETMR), GSE75153 (MB) and GSE70460 (AT/RT).
Figure S6: Pediatric cancers exhibit activation of the ESC-like gene expression signature, related to Figure 5
Figure S6: Pediatric cancers exhibit activation of the ESC-like gene expression signature, related to Figure 5

Principal component analysis of the global gene expression profiles in pediatric cancers and adult cancers. Pediatric cancers exhibit ESC-like gene expression patterns when compared to the corresponding adult cancers. AT/RT: atypical teratoid/rhabdoid tumor, NB: neuroblastoma, WT: Wilms tumor, HB: hepatoblastoma, GBM: glioblastoma, ACC: adrenocortical carcinoma, RCC: renal cell carcinoma, HCC: hepatocellular carcinoma. The same microarray data as Figure 5A were used.
Figure S7: Genetic screening with CRISPR/Cas9 to develop a novel therapeutic strategy for AT/RT, related to Figure 6
Figure S7: Genetic screening with CRISPR/Cas9 to develop a novel therapeutic strategy for AT/RT, related to Figure 6

(A) The cleavage efficiency at Day 3 and Day 7 after lentiviral-mediated transduction of CRISPR/Cas9 together with sgRNAs in SK-N-BE(2) in vitro. sgRNAs for 18 randomly selected genes were transduced with CRISPR/Cas9, and the efficiency of non-homologous end joining was measured by TIDE software 3 and 7 days after the lentiviral transduction. Data are represented as the median with interquartile range.

(B) GSEA showing that EZH2 inhibition by GSK126 suppresses the ESC-like module in hiPSC-derived cancer cells.

(C) Growth ratios (Day 14/Day 3) of hiPSC-derived cancer cells, SK-N-AS and SK-N-BE(2) transduced with CRISPR/Cas9 with sgRNA for non-targeting control (NTC) or RAD21. Two independent sgRNAs for RAD21 reduced the cell growth ratio compared to NTC. Data are presented as the mean of three biological replicates with standard deviation. The growth ratio of NTC-transduced cells was set to 1. *p<0.05, **p<0.01 and ****p<0.0001 (One-way ANOVA and Dunnett's multiple comparisons test).

(D) Cell viability of hiPSC-derived cancer cells treated with PCI34051 (10 µM) or the combination of PCI34051 (10 µM) and siRAD21. Note that the growth inhibitory effect of PCI34051 is not obvious in the presence of siRAD21. *p<0.05, ****p<0.0001 (One-way ANOVA and Dunnett's multiple comparisons test).

(E) GSEA revealed that suppression of the ESC-like module by GSK126 is further pronounced by the combination treatment of GSK126 and PCI34051.

(F) Sole inhibition of HDAC8 by PCI34051 does not suppress the ESC-like module in hiPSC-derived cancer cells.

(G) Cell viability of glioblastoma cell lines treated with DMSO, PCI34051 (10 µM), GSK126 (10 µM) or the combination of PCI34051 and GSK126 (10 µM each). *p<0.05, ***p<0.001 (One-way ANOVA and Dunnett's multiple comparisons test).
### Table S1: List of 110 target genes and their oligos for sgRNA, related to Figure 6

| Target genes associated with the maintenance of ESC identity | Oligos for sgRNA |
|---------------------------------------------------------------|-----------------|
| ANTC                                                          | CACCCTGAAAGGTGTTCGGCAGGAC |
|                                                                | AAACGCTCTGCCAGACACCTICAG |
| LIN28B                                                        | CACCAGCCATGGCTGGGAAAAATCCCAAG |
|                                                                | AAACATGGAATTACGTCGAGGAC |
| TCF3                                                          | CACCAGCTGCTGGCTGGCTTCGA |
|                                                                | AAACCTGGGAGAGTCGAGGAGTCG |
| MAP4K4                                                        | CACCCTAGGTGTAAGTTATGTA |
|                                                                | AAACCTTTAACATGTCGCCCTGA |
| GLUL                                                          | CACCAGGGAAGGGTGCCCCAGGA |
|                                                                | AAACCTGGATGGGACCCCTGTTG |
| ETV5                                                          | CACCCTAGATATGAAACCGAGTCCA |
|                                                                | AAACCTGGAGGCTTTATATGTCAG |
| RND2                                                          | CACCCTGCGGAAACTGACTACGC |
|                                                                | AAACCGCTAGTGCTGACCACCGCA |
| MYCN                                                          | CACCAGGCAAGCCGATCGTCCT |
|                                                                | AAACGCGGACCCCAGACGTCTCCA |
| JARID2                                                        | CACCCTTATCGCAGCTACCCXG |
|                                                                | AAACCGGGACCACCTGGCTTCGA |
| NIPBL                                                         | CACCAGGTGTACCTACTCCAAC |
|                                                                | AAACGCTTGGCATTGATGGCAAC |
| KLF9                                                          | CACCACGCACAGGCTGGTCCAA |
|                                                                | AAACCTTGGACGGCTATGGACAGC |
| MYBL2                                                         | CACCTCACAGTGTCGAGGAGAG |
|                                                                | AAACCTTTTGTCGACCCCTCTG |
| IFITM1                                                        | CACCAGAGGCCAATATCCGAA |
|                                                                | AAACCTTCTTATGCTGCGTCG |
| APOC1                                                         | CACCAGGTATATGACGATCAGC |
|                                                                | AAACGCTGCTGATGCTGCTG |
| UCK2                                                          | CACCACGACGCTAGGATGATCC |
|                                                                | AAACGTCCTAAATCCGACCTG |
| HLA-DPA1                                                      | CACCAGGGCTACTGCTGGCATCATCG |
|                                                                | AAACCTGATGCGCGAATGGGCCC |
| EYA1                                                          | CACCAGGAAATTTATCACCTGGCT |
|                                                                | AAACGCTTCCTGACTGACAGT |
| HTATIP2                                                       | CACCAGCCTCCTAGGGACCTCAGT |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| PLXCG16                                                       | CACCACAGTGGCTGGCTGGAT |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| HNRNPUL1                                                     | CACCAGGGTCATGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| SLC15A1                                                       | CACCACCTCTGACCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| ENAH                                                          | CACCACAGGCTCGACGATGAGC |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| MYBPH                                                         | CACCAGGGCTACTGCTGGCATCATCG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| IGFBP2                                                        | CACCAGGGCTACTGCTGGCATCATCG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| SOX2                                                          | CACCAGGGCTACTGCTGGCATCATCG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| NANOG                                                         | CACCACGAGCTGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| POU5F1                                                        | CACCAGGGCTACTGCTGGCATCATCG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| EYA2                                                          | CACCAGGGCTACTGCTGGCATCATCG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| SETD8                                                         | CACCACGAGCTGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| CENPE                                                        | CACCACGAGCTGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| CHAFT1A                                                       | CACCACGAGCTGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| INCENP                                                       | CACCACGAGCTGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| MSH6                                                         | CACCACGAGCTGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| FN1                                                           | CACCACGAGCTGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| ANP32A                                                        | CACCACGAGCTGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| NFKBIA                                                       | CACCACGAGCTGCTGAGGAGG |
|                                                                | AAACGCTTAGGGCTTATGGAGGAGG |
| GJA1                                                          | CACCACGAGCTGCTGAGGAGG |
| Gene Name | Sequence |
|-----------|----------|
| CRMP1     | AACTGGACCCAGTGATGGTGCTGAC |
| DST       | CACCGTACCTCACGGTATACAGC |
| CAMK2N1   | CACCAGACAAACATTCTCGGGCC |
| IPITM2    | CACCGGTTACACATCATCCCTATG |
| SIX2      | CACCGGGGAAATTGGCCGACAG |
| PAX8      | CACCGGTCGAGGGACACCTTTC |
| PHOX2B    | CACCGAGGCTTACCCGAGGCTT |
| IGF2      | CACCGGTAGAGGGAAGTCGCTT |
| PAX2      | CACCGGCTCCATACGGCTG |
| LMO1      | AACTGGAGGATGGCTGACAC |
| CRABP2    | CACCGGACCCAGTGATGGTGCTGAC |
| C1QB      | CACCGGTCGAGGGACACCTTTC |
| FRAME     | CACCGTACCTCACGGTATACAGC |
| RGS4      | CACCGGTAGAGGGAAGTCGCTT |
| GPR64     | CACCGGTTATGGACAGGTATGGC |
| RGS5      | CACCGGTCGAGGGACACCTTTC |
| MYC       | CACCGGTCGAGGGACACCTTTC |
| HMGA1     | CACCGGTCGAGGGACACCTTTC |
| HMGA2     | CACCGGTCGAGGGACACCTTTC |
| EZH2_1    | CACCGGTCGAGGGACACCTTTC |
| EZH2_2    | CACCGGTCGAGGGACACCTTTC |
| ARID1A    | CACCGGTCGAGGGACACCTTTC |
| ARID4A    | CACCGGTCGAGGGACACCTTTC |
| CBX3      | CACCGGTCGAGGGACACCTTTC |
| CDK8      | CACCGGTCGAGGGACACCTTTC |
| CHD7      | CACCGGTCGAGGGACACCTTTC |
| EDF1      | CACCGGTCGAGGGACACCTTTC |
| FUBP1     | CACCGGTCGAGGGACACCTTTC |
| H1FX      | CACCGGTCGAGGGACACCTTTC |
| H2AFZ     | CACCGGTCGAGGGACACCTTTC |
| MAX       | CACCGGTCGAGGGACACCTTTC |
| MGA       | CACCGGTCGAGGGACACCTTTC |
| BRF1      | CACCGGTCGAGGGACACCTTTC |
| SETHB1    | CACCGGTCGAGGGACACCTTTC |
| SMAOD2    | CACCGGTCGAGGGACACCTTTC |
| SMAOD3    | CACCGGTCGAGGGACACCTTTC |
| SMARCA4    | CACCGGTCGAGGGACACCTTTC |
| SMARCC1    | CACCGGTCGAGGGACACCTTTC |
| First author    | Reference                                      |
|-----------------|------------------------------------------------|
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| Gene    | Relative growth ratio | ES-NKX | Relative growth ratio | ES-NKX | Relative growth ratio | ES-NKX |
|---------|-----------------------|--------|-----------------------|--------|-----------------------|--------|
| ETV1    | 1.150064716           | 1.057981618 | 1.057425882           | 1.039592187 | 1.027048617           | 0.997706827 |
| CHD1    | 0.128501961           | 0.019827689 | 0.037121991           | 0.024087719 | 0.015416376           | 0.086727669 |
| SETDB1  | 1.620459123           | 1.411135363 | 1.358066892           | 1.106066933 | 1.098326367           | 1.033383322 |
| MSH6    | 0.068890222           | 0.021091304 | 0.038938216           | 0.034706229 | 0.024503032           | 0.066475012 |
| H1FX    | 0.169565387           | 0.102015944 | 0.116679669           | 0.076598735 | 0.015463973           | 0.071611438 |
| SMAD2   | 1.80329171            | 1.10477116  | 0.89024884            | 0.85108255  | 0.68995379            | 0.525616952 |
| ZNF43   | 1.823783525           | 1.623044227 | 1.399116379           | 1.361292041 | 1.335807366           | 1.240871988 |
| IGFBP2  | 1.070148896           | 1.049473870 | 1.020739680           | 1.005750122 | 0.982708611           | 0.964495493 |
| GJA1    | 1.378771137           | 1.346312861 | 1.323205269           | 1.299869180 | 1.276521046           | 1.257709562 |
| RTF1    | 0.080812974           | 0.054012904 | 0.049950114           | 0.045540789 | 0.035540789           | 0.024593584 |
| EIF4A3  | 0.779968272           | 0.644558025 | 0.278088963           | 0.225049999 | 0.034632283           | 0.034632283 |
| TCF3    | 1.089113336           | 0.89124223  | 1.040679454           | 0.934479687 | 0.088771413           | 0.032886971 |
| ETV5    | 0.897191713           | 0.609038620 | 0.560673837           | 0.525805503 | 0.389712904           | 0.389712904 |
| MYBPH   | 1.088508037           | 0.89124223  | 1.040679454           | 0.934479687 | 0.088771413           | 0.032886971 |
| SOX11   | 1.181951071           | 1.089113336 | 0.89124223  | 0.609038620 | 0.560673837           | 0.525805503 |
| HNRNPUL1| 1.054771042           | 0.917999747 | 0.917999747 | 0.917999747 | 0.917999747           | 0.917999747 |
| TCF3    | 0.987252999           | 0.987252999 | 0.987252999 | 0.987252999 | 0.987252999           | 0.987252999 |
| SOX11   | 0.889312282           | 0.917999747 | 0.917999747 | 0.917999747 | 0.917999747           | 0.917999747 |
| H1FX    | 1.070148896           | 1.049473870 | 1.020739680           | 1.005750122 | 0.982708611           | 0.964495493 |
| MTA1    | 1.054771042           | 1.089113336 | 0.89124223  | 0.609038620 | 0.560673837           | 0.525805503 |
| CHD1    | 1.054771042           | 1.089113336 | 0.89124223  | 0.609038620 | 0.560673837           | 0.525805503 |
| TRIM28  | 1.089113336           | 0.89124223  | 1.040679454           | 0.934479687 | 0.088771413           | 0.032886971 |
| SOX2    | 0.987252999           | 0.987252999 | 0.987252999 | 0.987252999 | 0.987252999           | 0.987252999 |
| DHX15   | 1.070148896           | 1.049473870 | 1.020739680           | 1.005750122 | 0.982708611           | 0.964495493 |
| RAD21   | 0.972712158           | 0.972712158 | 0.972712158 | 0.972712158 | 0.972712158           | 0.972712158 |
| EIF4A3  | 0.897191713           | 0.609038620 | 0.560673837           | 0.525805503 | 0.389712904           | 0.389712904 |
| LIN28B  | 1.070148896           | 1.049473870 | 1.020739680           | 1.005750122 | 0.982708611           | 0.964495493 |
| YY1     | 0.987252999           | 0.987252999 | 0.987252999 | 0.987252999 | 0.987252999           | 0.987252999 |

Table S3: Results of genetic screening with lentiviral CRISPR/Cas9 system, related to Figure 6.
Table S4: Primer list, related to STAR Methods

| Genes            | Forward                  | Reverse                  |
|------------------|--------------------------|--------------------------|
| Quantitative PCR |                          |                          |
| GAPDH            | ATGGGGGAAGGTGAAGGTCG     | GGGTCAATGGATGGCAACAATA   |
| NANOG            | TGGATGCTCACAACGGAGA      | GTTGGCTTTTGGAACCTGTTG    |
| OCT4             | CTTGGAGCTGGAGAAGGAGAAGCTG | CAAAGGCAGGGCTTACACATGTTC |
| NESTIN           | CACGCTGGCCGCACTTCAAGATG  | AGGGAGTTGGGCTCAAGGACTC   |
| NCAM1            | GAGGAGAGAGGAGCCAAAA      | CTGGCACTCTGCTGGTTGCTT    |
| Pax6             | CAGCTGGTGTGTGTCTTGTCA    | CTGGGCAACTGTGTTGCTTC     |
| SALL4            | CAACATTTTGAGGCGAGCTT     | GCGGGCTGAAGTTATGGTTC     |
| LIN28            | TGTTGGACCCCCTGGTTAGA     | CATGAGTCAAGGGCACAAC      |
| SOX2             | CAGAAAACCAAGACGGCTCA     | GCGTTCAATGAGTGCTTGGC     |
| c-MYC            | CTGGGTGCTCTCAATGGAGA     | GCTGGCTCTCTTTTCCACAGA    |
| MAP2             | AGAGGATGGCTTTGGAGAGC     | TGAGACACCTGCTCTGGCTG     |
| TUBI             | GCCCAAGGGGTCACTACACG     | GCCAGTGGCAATTTTCCACAC    |
| CDKN2A           | GTGGACCTGGCTGAGGAGA      | CTTTCAGTCGGGGATTGCTG     |
| E2F1             | TCCAGAACCACATCCAGTG      | CTGGGTCACCCCCTCAAG       |
| CCND1            | GAAAGATCGTCGCCACCTG      | GACCTCCCTCTGCGACCTCT     |
| GFAP             | CAGTCAGACGTCTTCAACC      | GCACTGCAAGGCTTCTTCAC     |
| Quantitative PCR for transduction of |                          |                          |
| pMxs-OCT4, SOX2, KLF4 and c-MYC |                          |                          |
| c-MYC            | ATACATCTCTGGCTCCCAAGCAGA | GACATGGGCTGCCCCTGTTATTATTT |
| SOX2             | TTCACATGCTCCAGCCTCACTACG | GACATGGGCTGCCCCTGTTATTATTT |
| KLF4             | CACCTCGCTTACACATGAGA     | GACATGGGCTGCCCCTGTTATTATTT |
| OCT4             | GCCCTCCAGCACTTCAACATGA   | CTTACGCGAAAATACGGGACACA  |
| Direct sequencing |                          |                          |
| SMARCB1          | CTGGCTGACGTGGCTTCCA      | ACGGGACTTCCACGTTA        |
| TP53             | TGTCAGCAGTGGGATTATT      | CGCAAATTTTCTTCCACCTCA    |