Research article

EWS/ATF1 expression induces sarcomas from neural crest–derived cells in mice

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Clear cell sarcoma (CCS) is an aggressive soft tissue malignant tumor characterized by a unique t(12;22) translocation that leads to the expression of a chimeric *EWS/ATF1* fusion gene. However, little is known about the mechanisms underlying the involvement of *EWS/ATF1* in CCS development. In addition, the cellular origins of CCS have not been determined. Here, we generated *EWS/ATF1*-inducible mice and examined the effects of *EWS/ATF1* expression in adult somatic cells. We found that forced expression of *EWS/ATF1* resulted in the development of *EWS/ATF1*-dependent sarcomas in mice. The histology of *EWS/ATF1*-induced sarcomas resembled that of CCS, and *EWS/ATF1*-induced tumor cells expressed CCS markers, including S100, SOX10, and MITF. Lineage-tracing experiments indicated that neural crest–derived cells were subject to *EWS/ATF1*-driven transformation. EWS/ATF1 directly induced Fos in an ERK-independent manner. Treatment of human and *EWS/ATF1*-induced CCS tumor cells with FOS-targeted siRNA attenuated proliferation. These findings demonstrated that FOS mediates the growth of *EWS/ATF1*-associated sarcomas and suggest that FOS is a potential therapeutic target in human CCS.

Introduction

Clear cell sarcoma (CCS) is an aggressive malignancy of adolescents and young adults that was first described by Enzinger (1). It typically arises in the deep soft tissues of the lower extremities closed to tendon, fascia, and aponeurosis (2). Chemotherapy and radiotherapy are not of any benefit (3–5), and a high rate of local and distant recurrence results in poor survival rates (3, 6, 7). CCSs harbor the potential for melanocytic differentiation and melanin synthesis (8). Gene expression profiles support the classification of CCS as a distinct genomic subtype of melanomas (9). These melanocytic features often make the distinction from malignant melanoma (MM) difficult. However, in contrast to MM, CCS is characterized by a chromosomal translocation, t(12;22)(q13;q12), that leads to the fusion of activating transcription factor 1 (*ATF1*) gene localized to 12q13 to Ewing's sarcoma oncogene (*EWS*) gene at 22q12 in up to 90% of cases, resulting in expression of the *EWS/ATF1* fusion gene (10–12). Given that CCS and MM have such similar characteristics, it has been proposed that CCSs may arise from a neural crest progenitor. However, the exact origin of CCS still remains to be determined.

The biological role of the EWS/ATF1 fusion protein is still unclear. EWS contains a transcriptional activation domain in the N-terminal region (13–15) and several conserved RNA binding motifs in the C-terminal region (16). Binding of the N-terminal region of EWS to the RNA polymerase II subunit hsRPB7 has been proposed to be important for transactivation of the target genes (17). In contrast, ATF1 is a member of the CREB transcription factor family, whose activity is regulated through phosphorylation of its kinase inducible domain (KID) by protein kinase A (18). ATF1 mediates the activation of cAMP-responsive genes through binding to a conserved cAMP-responsive element (CRE) as a dimmer (19, 20). However, the N-terminal activation domain of EWS replaces the KID in the EWS/ATF1 fusion protein, rendering it unable to support a typical inductive signal (21). Therefore, EWS/ATF1 can act as constitutive transcriptional activator in a cAMP-independent fashion with normal CRE DNA binding activity (14, 22, 23).

Previous studies have revealed some target genes of EWS/ATF1, but their true function in tumorigenesis is still not well understood (24). Expression of *MITF* is constitutively activated by *EWS/ATF1* in CCS in vitro (25). Consistent with this finding, several studies have identified the expression of MITF protein or mRNA in CCS (26–28). MITF is a master regulator of melanocyte development and plays a role in melanoma development (29, 30). Importantly, activation of MITF by EWS/ATF1 is required for CCS proliferation as well as for melanocytic differentiation of CCS in vitro (25).

Although previous studies have demonstrated that EWS/ATF1 is associated with oncogenic potential in CCS, the effect of in vivo expression of *EWS/ATF1* on sarcoma formation is still not known. In the present study, we established *EWS/ATF1* transgenic mice using a doxycycline-dependent expression system in order to investigate the role of *EWS/ATF1* on CCS development in vivo. Our results showed that forced expression of *EWS/ATF1* induced CCS-like sarcoma in the transgenic mice. This mouse model was used to identify the origin of *EWS/ATF1*-induced sarcomas as well as the direct target of EWS/ATF1 in these sarcomas.

Results

Inducible expression of EWS/ATF1 in mice. We first generated doxycycline-inducible *EWS/ATF1* ES cells, in which the human *EWS/ATF1* type 2 fusion gene (26, 31) can be induced under the control of

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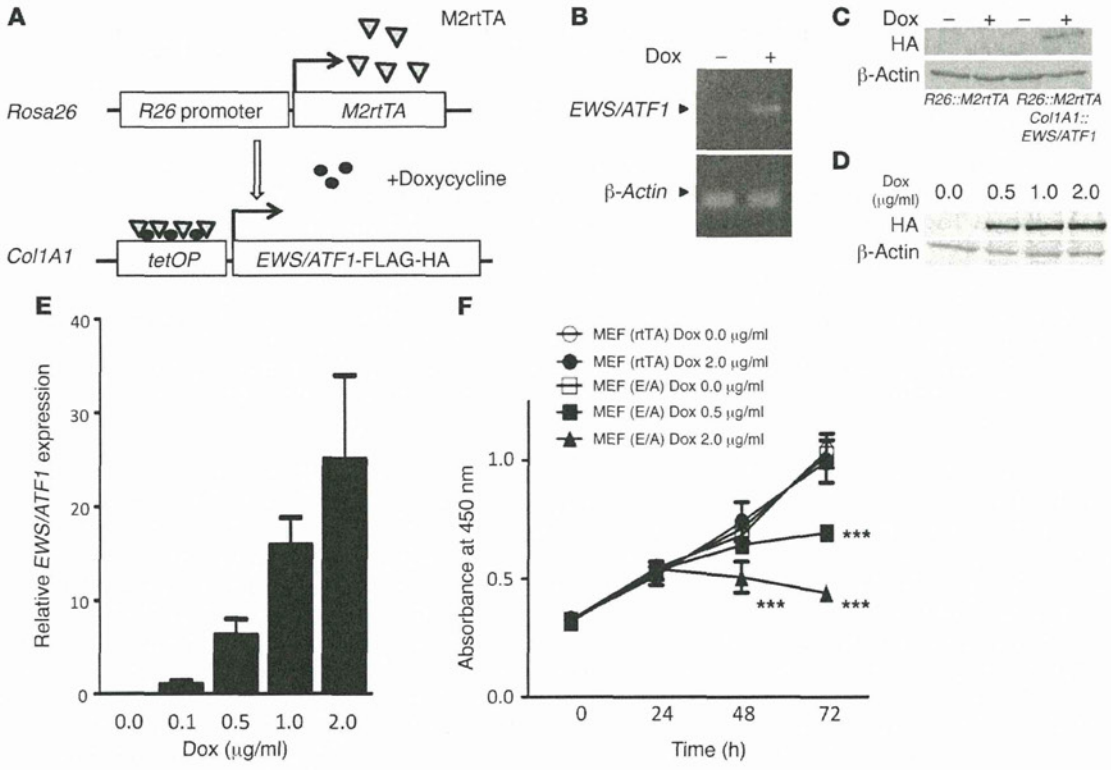

Figure 1

Inducible expression of *EWS/ATF1*. (**A**) Schematic of the doxycycline-inducible *EWS/ATF1* alleles. (**B**) *EWS/ATF1* expression in ES cells, detected by RT-PCR, after exposure to doxycycline for 12 hours. (**C**) EWS/ATF1 expression in ES cells, detected by Western blot, after exposure to doxycycline for 24 hours. (**D**) Dose-dependent induction of EWS/ATF1 protein in *EWS/ATF1*-inducible ES cells by doxycycline. ES cells were exposed to doxycycline concentrations up to 2 μg/ml for 24 hours. Western blot analysis was performed using an anti-HA antibody. (**E**) Dose-dependent doxycycline induction of *EWS/ATF1* mRNA in *EWS/ATF1*-inducible MEFs. MEFs were exposed to different concentrations of doxycycline for 24 hours. Transcript levels were normalized to β-*actin*. Data are mean ± SD ($n = 3$). (**F**) *EWS/ATF1* expression suppressed MEF growth. Cell viability was determined by WST-8 assay. Data are mean ± SD ($n = 4$). Control MEFs (rtTA) and *EWS/ATF1*-inducible MEFs (E/A) were derived from heterozygous *Rosa26::M2rtTA* and *Col1A1::tetO-EWS/ATF1* mice, respectively. ***$P < 0.001$ vs. MEF (rtTA) Dox 0.0 μg/ml, MEF (rtTA) Dox 2.0 μg/ml, and MEF (E/A) Dox 0.0 μg/ml.

a tetracycline-responsive regulatory element (Figure 1A). Upon treatment of these ES cells with doxycycline, expression of the *EWS/ATF1* fusion transcript was detected by RT-PCR (Figure 1B). We also confirmed the expression of EWS/ATF1 protein upon doxycycline treatment (Figure 1C), which was regulated in a dose-dependent manner (up to 2 μg/ml; Figure 1D).

Heterozygous *Rosa26::M2rtTA* mice with heterozygous *tetO-EWS/ATF1* allele were used to induce the *EWS/ATF1* fusion gene. Cultured murine embryonic fibroblasts (MEFs) derived from *EWS/ATF1*-inducible mice were first exposed to doxycycline to test the effect of *EWS/ATF1* expression on somatic cells. *EWS/ATF1* expression at the mRNA level was confirmed 24 hours after exposure (Figure 1E). Unexpectedly, the cell proliferation rate of MEFs decreased after *EWS/ATF1* induction in a doxycycline dose–dependent manner (Figure 1F).

EWS/ATF1 induces sarcoma formation in mice. To investigate the effect of *EWS/ATF1* expression in vivo, we treated *EWS/ATF1*-inducible mice at 6 weeks of age with doxycycline in the drinking water (50 μg/ml). The *EWS/ATF1*-inducible mice given doxycycline started to develop multiple macroscopic soft tissue tumors after 4 weeks. After doxycycline treatment, EWS/ATF1 protein was detected in a variety of tissues, including the intestine, liver, epidermis, and deep soft tissue (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI63572DS1). Doxycycline treatment for 3 months resulted in tumor formation in the deep soft tissues of all mice ($n = 39$), whereas control mice without doxycycline treatment developed no detectable tumors. *EWS/ATF1*-induced tumors typically arose in the trunks, heads, limbs, and whisker pads (Figure 2A). Macroscopically, tumors consisted of circumscribed and lobulated gray-white mass (Figure 2A). In most cases, the tumors were attached to fascia or aponeuroses (Figure 2, A and B), which indicates that the tumors specifically arose from the deep soft tissues. Importantly, 36 of 39 mice (92%) developed tumors in the trunk, which suggests that cells located in the trunk are particularly permissive for tumorigenesis by *EWS/ATF1* expression. Despite expression of EWS/ATF1 protein, no tumor formation was observed in other tissues, such as the epidermis and intestine, even in mice given doxycycline for 3 months.

Microscopic examination of these tumors revealed striking similarities to human CCSs. The tumors showed a rather uniform pattern of compact nests or fascicles of rounded or fusiform cells, which were divided by a framework of fibrocollagenous tissue (Figure 2B).

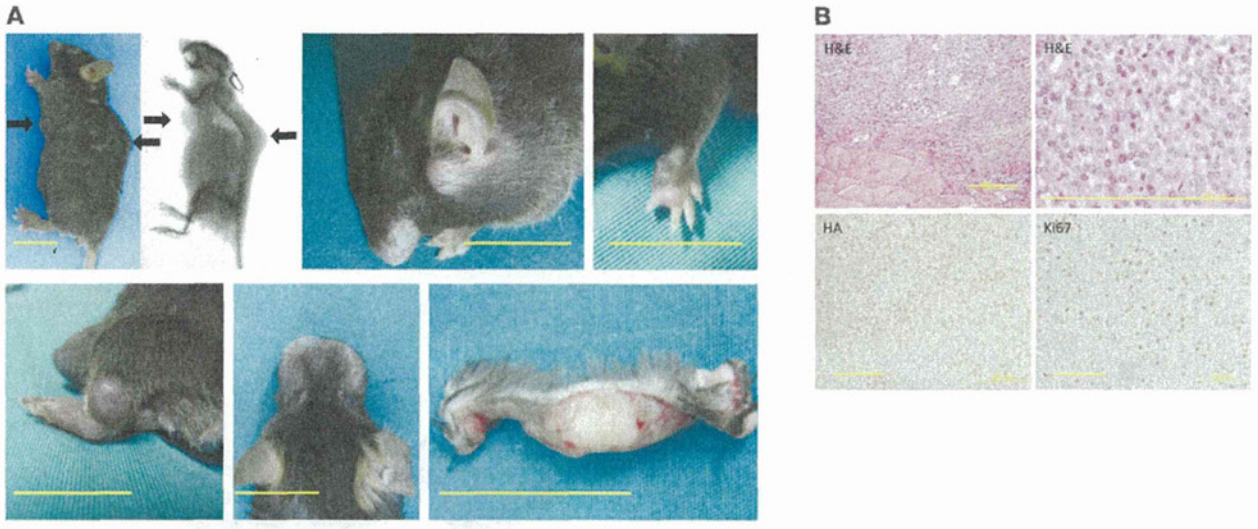

Figure 2

EWS/ATF1-induced tumors resemble human CCS. *EWS/ATF1* transgenic mice were administered 50 μg/ml doxycycline in their drinking water for 3 months. (**A**) *EWS/ATF1* expression caused tumor formation (arrows) in various locations: trunk, head, limbs, and whisker pads. X-ray examination revealed multiple tumors in deep soft tissue. The cut surface of a large tumor on the ventral trunk of an *EWS/ATF1*-inducible mouse revealed a lobulated gray-white mass in the deep soft tissue. Scale bars: 20 mm. (**B**) Histological analysis of *EWS/ATF1*-induced tumors. Tumors were composed of round or fusiform cells with prominent basophilic nuclei and clear cytoplasm, which were surrounded by fibrous fascicles. HA immunostaining confirmed EWS/ATF1 expression in the tumor cells. Frequent Ki67-positive cells were present throughout the lesions. Scale bars: 200 μm (H&E, left); 50 μm (H&E, right); 100 μm (HA and Ki67).

The individual tumor cells had a homogeneous appearance. They had round to ovoid vesicular nuclei with prominent basophilic nucleoli and clear or pale-staining cytoplasm (Figure 2B). The majority of the tumor cells expressed EWS/ATF1 fusion protein in nuclei (Figure 2B and Supplemental Figure 2A). Ki67-positive proliferating cells were observed in about 30%–40% of tumor cells (Figure 2B), indicative of active proliferative activity. The survival curves of *EWS/ATF1*-induced mice were analyzed to evaluate the overall effect of *EWS/ATF1* expression on life span. The transgenic mice treated with doxycycline became moribund within 3–10 months, suggestive of multiple tumor formation in the deep soft tissue, whereas mice without doxycycline treatment survived much longer, and no tumor formation was observed. The median survival time of *EWS/ATF1*-inducible mice treated with doxycycline was 20 weeks (Supplemental Figure 2B).

Previous studies demonstrated that human CCSs express markers for neural crest lineage as well as melanocytic differentiation (8, 9). Therefore, to examine the similarity of mouse *EWS/ATF1*-induced tumors with human CCSs, we performed immunohistochemical analysis for CCS-expressing markers; *EWS/ATF1*-induced tumor cells showed the expression such markers, including S100, Sox10, and Mitf (Figure 3A).

Neural crest–lineage cells are permissive to EWS/ATF1-driven sarcoma development. The cell of origin for CCS remains to be determined. Based on the potential of CCSs for melanocytic differentiation and melanin synthesis, previous studies proposed that CCS may arise from a neural crest progenitor. To determine whether *EWS/ATF1*-induced sarcomas actually arise from neural crest–derived cells, we performed a lineage-tracing experiment in which neural crest–derived cells were tagged by reporter in vivo (32). To label neural crest–derived cells in vivo, we first used transgenic mice containing *Wnt1-Cre* and floxed *LacZ* reporter alleles. We further introduced doxycycline-inducible *EWS/ATF1* alleles into the reporter mice to generate compound transgenic mice (Figure 3B). We confirmed that *EWS/ATF1*-induced tumor cells did not express *Wnt1* (Supplemental Figure 3A). Transgenic mice were treated with doxycycline in the drinking water to induce subcutaneous tumors and the developed tumors were then analyzed for the expression of the reporter gene. Importantly, all 14 *EWS/ATF1*-induced tumors were ubiquitously positive for X-gal staining (Figure 3C and Supplemental Figure 3D), which suggests that neural crest–lineage cells are a cell of origin for *EWS/ATF1*-associated sarcomas. We further performed another lineage-tracing experiment using transgenic mice containing *P0-Cre* and floxed *EYFP* reporter alleles (Figure 3D), which have been also widely used to label neural crest–derived cells. Again, we found that all 6 *EWS/ATF1*-induced tumors were positive for EYFP (Figure 3E and Supplemental Figure 4, C and D).

Establishment of tumor cell lines. Tumor samples were obtained from primary tumors of *EWS/ATF1*-induced mice to establish cell lines from *EWS/ATF1*-induced tumors. We established 2 tumor cell lines, G1297 and G1169, from 2 independent mice. These cells grew in the form of an adherent monolayer in the presence of doxycycline (0.2 μg/ml). We cultured the cells up to the fourth passage in medium containing 0.2 μg/ml doxycycline in order to avoid contamination by fibroblasts. We examined the effect of different concentrations of doxycycline on the growth and morphology of the established cell lines. We confirmed that the expression of *EWS/ATF1* transcript and protein increased in response to doxycycline in a dose-dependent manner in both established cell lines (Supplemental Figure 5, A–C). The growth and morphology of the tumor cells varied in a doxycycline dose–dependent manner: small, round tumor cells grew rapidly at concentrations above 0.1 μg/ml,

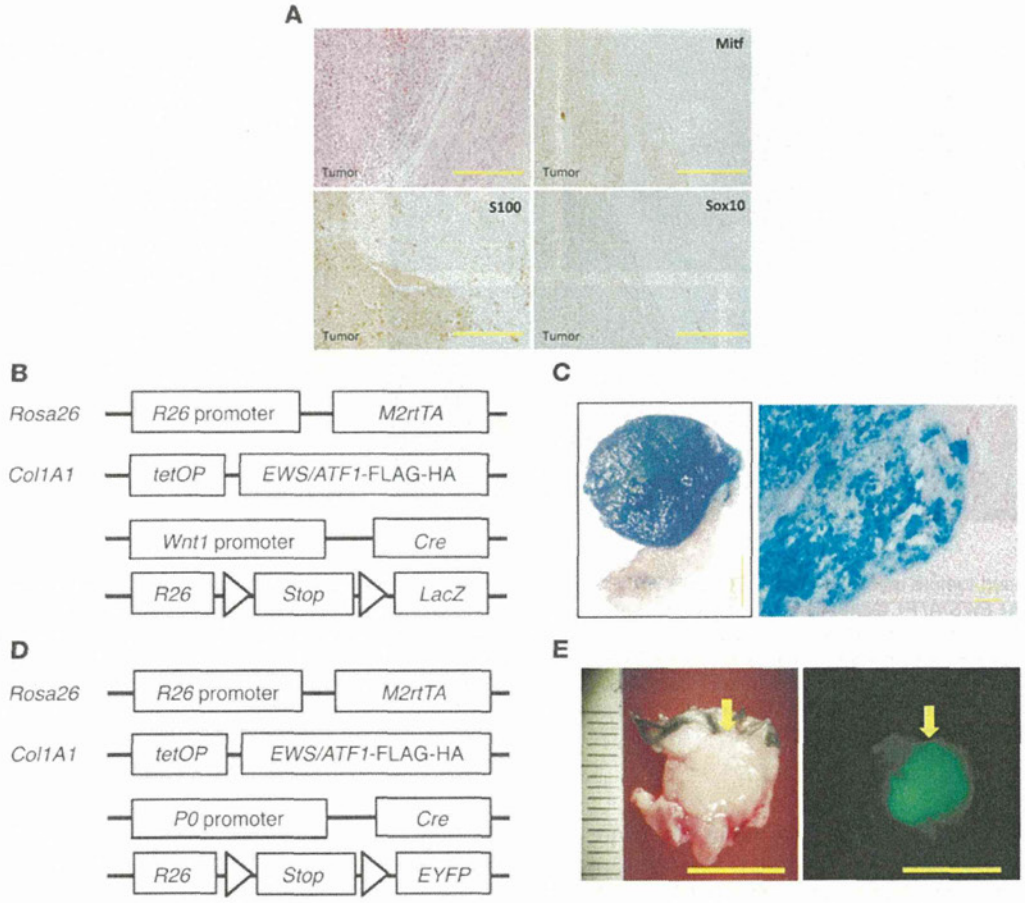

Figure 3

EWS/ATF1-induced tumors arise from neural crest–lineage cells. (**A**) Immunohistochemical analysis for CCS markers. Nuclear staining for S100, Sox10, and Mitf was observed in tumor cells. Sections were counterstained with hematoxylin. Scale bars: 100 μm. (**B**) Schematic representation of reporter alleles for the lineage-tracing experiment using *Wnt1-Cre* allele. Doxycycline-inducible *EWS/ATF1* alleles were introduced into reporter mice containing the *Wnt1-Cre* and floxed *LacZ* reporter alleles. (**C**) X-gal staining for *EWS/ATF1*-induced tumors with *Wnt1-Cre* and floxed *LacZ* reporter alleles. Positive staining for X-gal indicated that the tumor arose from a neural crest–lineage cell. Histological analysis revealed that neoplastic cells were stained with X-gal. Counterstaining was performed with fast red. Scale bars: 2 mm (left); 50 μm (right). (**D**) Schematic representation of reporter alleles for the lineage-tracing experiment using *P0-Cre* allele. Doxycycline-inducible *EWS/ATF1* alleles were introduced into reporter mice containing the *P0-Cre* and floxed *EYFP* reporter alleles. (**E**) Representative image of a tumor (arrow) in the trunk of an *EWS/ATF1*-induced mouse with *P0-Cre* and floxed *EYFP* reporter alleles. Fluorescent signals for EYFP expression were detected in the. Scale bars: 10 mm.

whereas dendritic fibroblast–like spindle cells were observed below 0.05 μg/ml (Figure 4A). Notably, doxycycline withdrawal caused rapid morphological changes, into a fibroblast-like shape, and these tumor cells did not proliferate up to the next passage (Figure 4A). Consistent with these findings, cell viability assay revealed that the number of cells was increased by doxycycline treatment in a dose-dependent manner (Figure 4B). We next examined the effect of *EWS/ATF1* expression on tumorigenesis ability in the subcutaneous tissue of immunocompromised mice. The established cell line G1297 was cultured in medium containing 0.2 μg/ml doxycycline, and 5.0×10^6 cells were transplanted into the subcutaneous tissue of nude mice. It is important to note that all mice treated with 50 μg/ml doxycycline in the drinking water developed tumors within 3 weeks, whereas no tumor formation was observed in mice without doxycycline treatment (Figure 4C). Histological analysis revealed that the subcutaneous tumors in nude mice consisted of neoplastic cells that resembled the primary tumor cells in *EWS/ATF1*-induced transgenic mice (Figure 4D). Positive immunoreactivity for HA-Tag was observed in all tumor cells (Figure 4D).

Continuous expression of EWS/ATF1 is required for tumor growth maintenance. To further examine whether continuous expression of *EWS/ATF1* is necessary for the growth of *EWS/ATF1*-induced tumors, we withdrew doxycycline in tumor-bearing EWS/ATF1 transgenic mice that had been given doxycycline for 3 months. Importantly, doxycycline withdrawal resulted in a rapid reduction of tumor mass in 4 independent mice (7 tumors total). The regressed tumors contained fibrous tissue, but no viable neoplastic cells were observed 3 months after doxycycline withdrawal (Figure 4E), which suggests that *EWS/ATF1*-induced tumor growth depends on continuous *EWS/ATF1* expression. We next examined the histological changes shortly after doxycycline withdrawal in order to investigate the mechanisms of tumor regression.

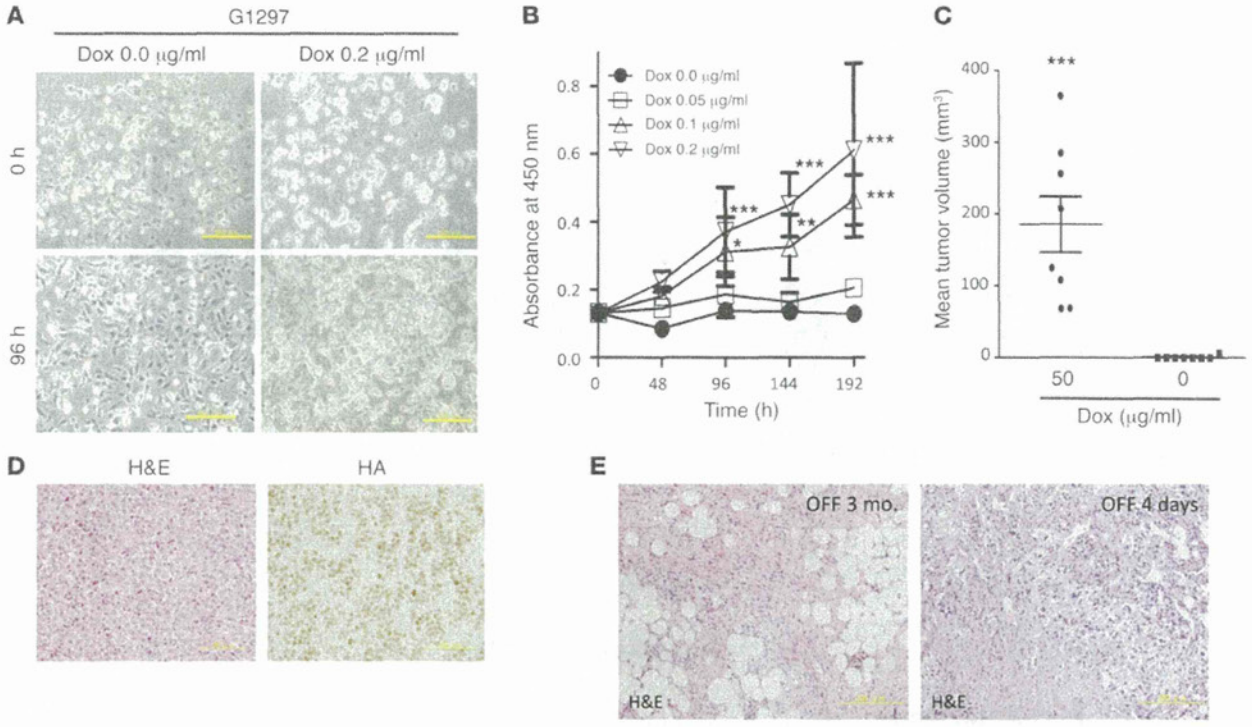

Figure 4

Establishment and analysis of tumor cell lines. G1297 and G1169 cell lines were established from 2 independent *EWS/ATF1*-induced tumors. (**A**) Morphology of the G1297 line after treatment without or with doxycycline (0 and 0.2 μg/ml, respectively). At concentrations above 0.1 μg/ml, small, round tumor cells grew rapidly, while dendritic fibroblast-like spindle cells were observed; tumor cell growth almost stopped at concentrations less than 0.05 μg/ml. Scale bars: 50 μm. (**B**) Effect of different levels of *EWS/ATF1* on tumor cell growth. G1297 cells were cultured in different concentrations of doxycycline (0, 0.05, 0.1, and 0.2 μg/ml), and cell viability was determined by WST-8 assay. Data are mean ± SD ($n = 8$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Dox 0.0 μg/ml. (**C**) Subcutaneous transplantation of 5.0×10^6 G1297 cells in immunocompromised mice resulted in tumor formation in mice treated with 50 μg/ml doxycycline ($n = 8$). Mean tumor volumes ± SEM are shown. $^{***}P < 0.005$. (**D**) Representative histology and HA immunostaining of tumors in nude mice. The tumor resembled the original sarcoma with EWS/ATF1 expression. Scale bars: 100 μm. (**E**) Doxycycline withdrawal led to rapid tumor regression. At 3 months after doxycycline withdrawal, no viable tumor cells were observed, and tumors were replaced by fibrous tissue. Widespread cell death was observed 4 days after doxycycline withdrawal. Scale bars: 200 μm.

We found widespread cell death within the tumor mass, accompanied by massive infiltration of inflammatory cells, at 4 days after doxycycline withdrawal (Figure 4E), which indicates that neoplastic cells cannot survive in vivo without *EWS/ATF1* expression. Taken together, these results clearly indicate that *EWS/ATF1* plays a pivotal role in the proliferation and maintenance of *EWS/ATF1*-induced tumor cells in vivo.

Fos is a direct target of EWS/ATF1. To determine the downstream targets regulated by EWS/ATF1, we next performed gene expression analysis using G1297 cells. First, we confirmed that withdrawal of doxycycline for 96 hours resulted in no detectable expression of *EWS/ATF1* RNA or protein in cultured tumor cells. Next, the tumor cells were exposed again to doxycycline at a concentration of 0.05 or 0.2 μg/ml, and microarray analysis was performed at 3 and 48 hours after doxycycline exposure. Induction of *EWS/ATF1* resulted in altered expression of a number of genes associated with cell growth, such as growth factor genes (*Areg* and *Ereg*), cell cycle regulators (*Cenpa*, *Ccna2*, *Ccnb2*, *Cdkn1b*, *Plk1*, and *Aurka*), and a proto-oncogene (*Fos*) at either time point (Supplemental Figure 6A). Although a previous study demonstrated that *MITF-M* is a direct target of EWS/ATF1 in human CCS cell lines (25), we failed to detect its expression in our *EWS/ATF1*-induced tumor cell lines and primary tumor samples (Supplemental Figure 7, A and B). Among the transcripts upregulated by *EWS/ATF1*, we focused on the proto-oncogene *Fos*, because this was one of the most highly upregulated genes by *EWS/ATF1* after doxycycline exposure in the microarray analysis (Supplemental Figure 6A). Quantitative real-time RT-PCR (qRT-PCR) confirmed upregulation of both *Fos* and *EWS/ATF1* transgenes in 2 independent tumor cell lines as early as 3 hours after doxycycline exposure (Figure 5A and Supplemental Figure 7C). We also found that the *EWS/ATF1*-induced tumor specimens expressed higher levels of both *Fos* and *EWS/ATF1* transgenes (Figure 5B). Expression of *Fos* is induced by numerous stimuli, which are transmitted through the RAS/Raf/MAP kinase or cAMP-dependent protein kinase pathway (33). In order to investigate the mechanism of *Fos* induction by *EWS/ATF1*, we next examined whether the RAS/Raf/MAP kinase pathway is involved in *EWS/ATF1*-mediated *Fos* activation. In contrast to the rapid and transient induction of Fos in MEFs after serum stimulation (Supplemental Figure 7D), expression of Fos in the *EWS/ATF1*-expressing tumor cell line was detected even under serum-free conditions, and it gradually increased after serum stimulation (Supplemental Figure 7E). Interestingly, whereas serum-stimulated MEFs revealed immediate phosphorylation of ERK1 and ERK2

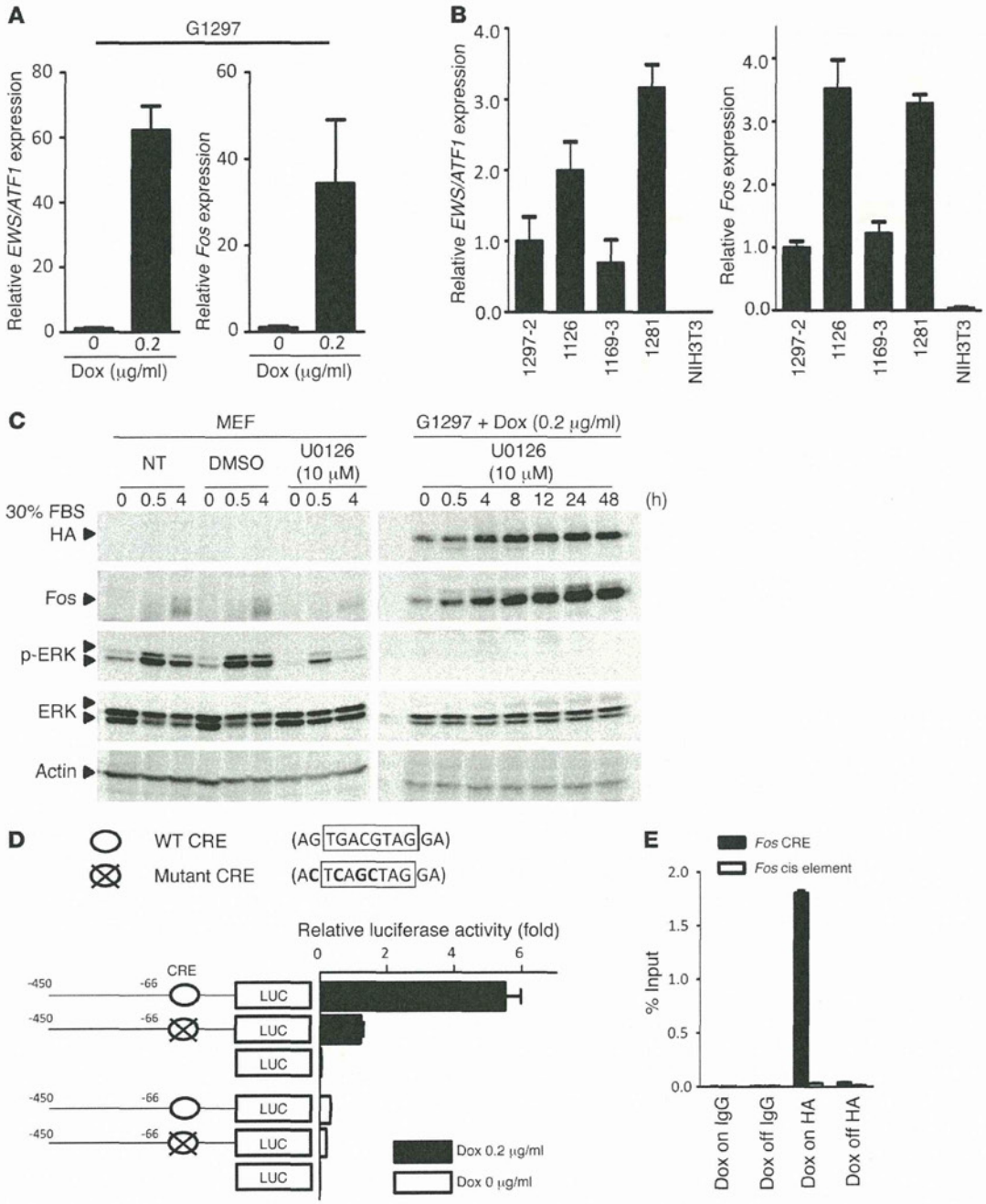

Figure 5

Fos is a direct target of EWS/ATF1. (**A**) Real-time RT-PCR analysis of G1297 cells revealed significant upregulation of both *EWS/ATF1* and *Fos* 3 hours after doxycycline exposure. (**B**) Relative expression of *EWS/ATF1* and *Fos* in 4 *EWS/ATF1*-induced tumors from 4 independent mice. NIH3T3 cells served as a control. Transcript levels were normalized to β-*actin*. Data are mean ± SD ($n = 3$). (**C**) Fos induction by *EWS/ATF1* was independent of the ERK pathway. Serum-starved MEFs and G1297 cells were stimulated with 30% FBS for the indicated times. Cells were also treated with 10 μM of the MEK inhibitor U0126. Whereas ERK1/2 inhibition by U0126 decreased Fos in MEFs, U0126 failed to suppress *Fos* expression in G1297 cells. NT, not treated. (**D**) *Mouse Fos promoter–luciferase reporter constructs and pRL-SV40 vector* (as an internal control) were cotransfected in G1297 cells treated with or without 0.2 μg/ml doxycycline. Luciferase activity of each construct was normalized to internal control activity. Data are mean ± SD ($n = 3$). (**E**) ChIP-PCR analysis was performed for the *Fos* promoter region containing CRE or the negative control cis element using HA-tag antibody or IgG as nonimmune immunoprecipitation, respectively. EWS/ATF1 was enriched at the CRE element of the *Fos* promoter in G1297 cells after treatment with 0.2 μg/ml doxycycline. Data (mean ± SD) were quantified by qRT-PCR and expressed as percent of input DNA.

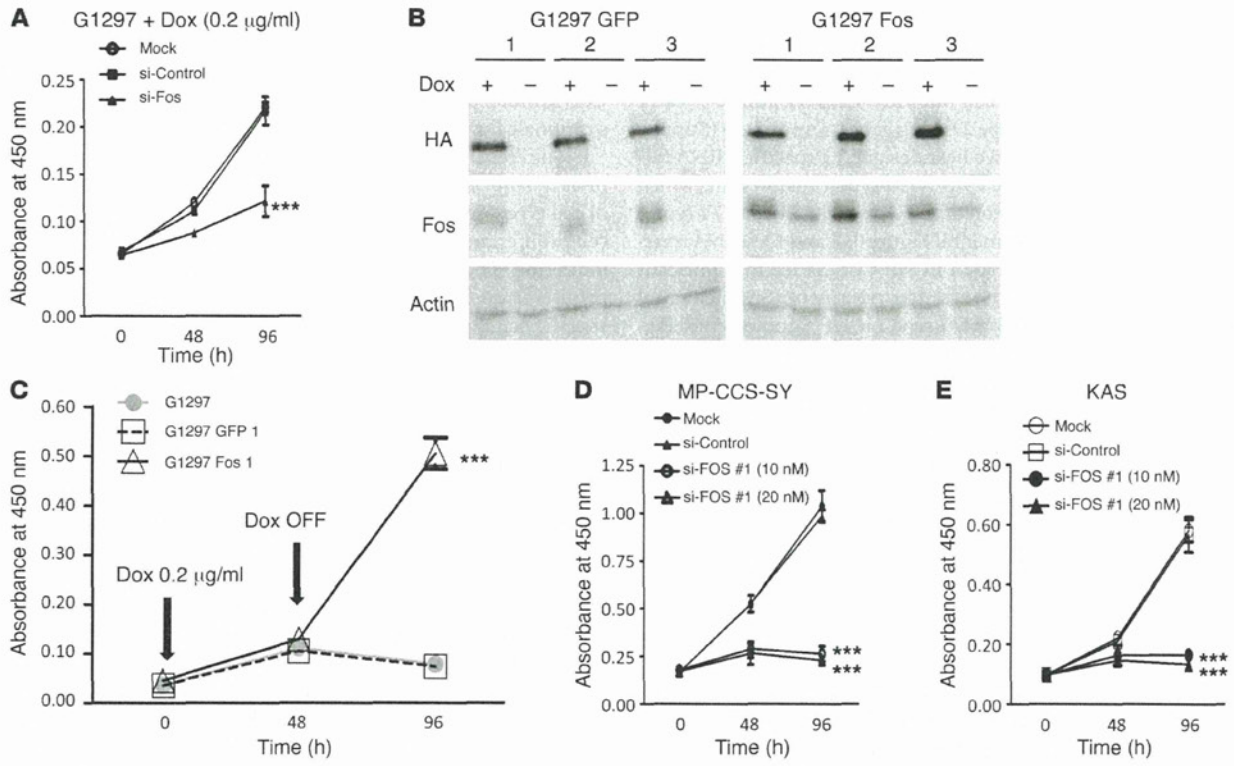

Figure 6

Fos plays a key role in *EWS/ATF1*-induced cell proliferation. (**A**) Effect of *Fos* knockdown on proliferation of *EWS/ATF1*-induced cells. G1297 cells were treated with siRNA targeting *Fos* (si-Fos; 10 nM), a control siRNA (si-Control; 10 nM), or lipofectamine alone (Mock). 48 and 96 hours later, cell viability was determined by WST-8 assay. Results are mean ± SD ($n = 4$). ***$P < 0.001$ vs. si-Control and Mock. (**B**) *EWS/ATF1*-induced tumor cell lines overexpressing *Fos* or *EGFP* (G1297 Fos and G1297 GFP, respectively). pCAG-Fos-IZ vector or pCAG-EGFP-IZ vector were stably transfected in G1297 cells. Western blot analysis revealed that G1297 Fos cells stably expressed Fos protein even in the absence of doxycycline. (**C**) Cell proliferation assay for G1297, G1297 GFP, and G1297 Fos cells before and after doxycycline withdrawal. Doxycycline treatment (0.2 μg/ml) was withdrawn for 48 hours. Cell viability was determined by WST-8 assay. ***$P < 0.001$ vs. G1297 and G1297 GFP. (**D** and **E**) Effect of *FOS* knockdown on growth of human CCS cell lines. MP-CCS-SY and KAS cells were treated with siRNA#1 targeting *FOS* (si-FOS #1; 10 nM and 20 nM), control siRNA (si-Control; 20 nM), or lipofectamine alone (Mock). 48 and 96 hours later, cell viability was determined by WST-8 assay. Data are mean ± SD ($n = 4$). ***$P < 0.001$ vs. si-Control and Mock.

(Supplemental Figure 7D), phosphorylation of ERK1/2 was not observed in the *EWS/ATF1*-induced tumor cell line, even after serum stimulation (Supplemental Figure 7E), which suggests that continuous upregulation of Fos in *EWS/ATF1*-induced tumor cells is independent of the RAS/Raf/ERK signaling pathway. We treated *EWS/ATF1*-induced tumor cells with the MEK inhibitor U0126 to block activation of ERK1/2 in order to further confirm the ERK-independent activation of Fos. Although inhibition of ERK1/2 resulted in a substantial decrease of Fos in MEFs, U0126 failed to suppress Fos expression in *EWS/ATF1*-induced tumor cells (Figure 5C). These data indicate that constitutive overexpression of Fos in *EWS/ATF1*-induced tumor cells was mediated by an ERK-independent mechanism.

Previous studies demonstrated an interaction of ATF1 at a CRE in the *Fos* promoter (34, 35), which suggests that EWS/ATF1 may induce *Fos* expression through interaction with the CRE. Conversely, in the present study, regulatory motif analysis of the upregulated genes by EWS/ATF1 demonstrated enrichment of CRE near the transcription start site (from −1,000 bp to +200 bp; Supplemental Figure 6B). To evaluate the functional importance of this element in EWS/ATF1-mediated activation of *Fos*, we constructed a reporter plasmid containing the mouse *Fos* promoter with wild-type and mutated CRE and examined transcriptional activity by luciferase assay (Figure 5D). We confirmed that induction of EWS/ATF1 resulted in remarkably increased *Fos* promoter activity with wild-type CRE in G1297 cells. Importantly, luciferase activity of the mutated promoter significantly decreased compared with that of the wild-type promoter. We further examined whether EWS/ATF1 directly binds to the CRE of the *Fos* promoter. ChIP-PCR analysis revealed that doxycycline-induced EWS/ATF1 was enriched at the CRE of the *Fos* promoter, but not at the negative control cis element (Figure 5E). Our results indicated that the CRE is crucial for EWS/ATF1-mediated transcriptional activity of *Fos* in *EWS/ATF1*-induced tumor cells.

Expression of FOS in human CCS. To investigate whether overexpression of *FOS* is linked to human CCS, we analyzed *FOS* expression in the human CCS cell lines MP-CCS-SY and KAS and in the control lung fibroblast cell line WI38 by qRT-PCR. *FOS* was found to be highly expressed in both human CCS cell lines compared with WI38 (Supplemental Figure 8A). We also found that surgically resected clinical CCS specimens also expressed higher levels of *FOS* than did WI38 (Supplemental Figure 8B), which indicates that human CCS expresses higher levels of *FOS*.

We examined the effect of *EWS/ATF1* knockdown on *FOS* expression in human CCS cell lines to further investigate the association between *EWS/ATF1* expression and increased *FOS* expression in human CCS. Human CCS cell lines MP-CCS-SY and KAS carry the *EWS/ATF1* type 1 and type 2 fusion genes, respectively (Supplemental Figure 9, A and B). We next designed a specific siRNA targeting the breakpoint of the *EWS/ATF1* type 1 fusion gene, which had no effect on the expression of *ATF1* or of the *EWS/ATF1* type 2 fusion gene in KAS (Supplemental Figure 9, E and F). siRNA treatment targeting *EWS/ATF1* type 1 in MP-CCS-SY led to significant downregulation of *FOS* 48 hours after treatment (Supplemental Figure 9G) as well as of *EWS/ATF1* type 1 itself (Supplemental Figure 9, C and D), which indicates that *FOS* is a direct target of EWS/ATF1 in human CCS. In contrast to *FOS*, we observed a modest reduction of *MITF-M* expression after *EWS/ATF1* knockdown in MP-CCS-SY cells (Supplemental Figure 9H).

FOS could be a promising therapeutic target for human CCS. To examine whether *Fos* overexpression facilitates proliferation of tumor cells expressing *EWS/ATF1*, we knocked down *Fos* in *EWS/ATF1*-induced tumor cells using siRNA. The G1297 cell line was treated with siRNA for *Fos* in the presence of doxycycline. siRNA treatment (10 nM) decreased the expression of *Fos* at the mRNA level by 75% at 24 hours after transfection, although it had no effect on expression of the *EWS/ATF1* transgene compared with the control siRNA (Supplemental Figure 10A). In addition, we confirmed that Fos protein levels were also decreased 48 hours after transfection (Supplemental Figure 10B). A WST-8 assay was performed in *EWS/ATF1*-induced tumor cells transfected with the nonfunctional control siRNA or with functional *Fos* siRNA to examine the effect of *Fos* knockdown on the cellular kinetics. The siRNA targeting *Fos* efficiently inhibited cell proliferation of *EWS/ATF1*-induced tumor cells, even in the presence of doxycycline (Figure 6A). In order to further confirm the importance of *Fos* expression for *EWS/ATF1*-induced tumor cell growth, we established *EWS/ATF1*-induced tumor cell lines in which *Fos* is overexpressed (Figure 6B). We found that *Fos*-overexpressed *EWS/ATF1*-inducible cells retained the ability to proliferate for at least 48 hours after doxycycline withdrawal, whereas control cells in which *GFP* is overexpressed stopped their proliferation soon after withdrawal (Figure 6C). We also examined the effect of *FOS* knockdown on cell growth of human CCS cell lines using siRNA targeting *FOS*. Consistent with the results in *EWS/ATF1*-induced tumor cells, siRNA treatment strongly suppressed the growth of CCS cell lines (Figure 6, D and E, and Supplemental Figure 11, A–F). Taken together, these data suggest that *FOS*, a direct target of EWS/ATF1, mediates the oncogenic growth of *EWS/ATF1*-related sarcomas and could be a potent therapeutic target for human CCS.

Discussion

The current study revealed that forced expression of the *EWS/ATF1* fusion gene induced sarcoma formation in *EWS/ATF1* transgenic mice. The histology of the tumors in *EWS/ATF1* transgenic mice showed a striking similarity to that of human CCS. In addition, immunohistochemistry demonstrated that *EWS/ATF1*-induced tumor cells express neural crest–associated markers, such as S100, Mitf, and Sox10, which are also expressed in human CCS. Given that the *EWS/ATF1* fusion gene is detected in CCS, our *EWS/ATF1* transgenic mouse is the first mouse model for investigating CCS pathogenesis. Our present results demonstrated that continuous expression of *EWS/ATF1* was required for growth and tumor formation of *EWS/ATF1*-induced tumor cells. These results indicate that *EWS/ATF1* plays a pivotal role in both development and maintenance of *EWS/ATF1*-associated sarcomas, implying that CCS exhibits oncogene addiction (36) to *EWS/ATF1*, and provide a rationale for targeting EWS/ATF1 itself to treat CCS.

It is interesting to note that sarcoma formation was observed only in deep soft tissue, although *EWS/ATF1* was induced in a variety of cell types in this experimental system (37, 38). In addition, the cell proliferation rate of MEFs in vitro was reduced by *EWS/ATF1* induction. These results clearly demonstrated that the abnormal proliferation by the forced expression of *EWS/ATF1* requires a specific cell type of origin, accompanied by a specific microenvironment. Consistent with these findings, recent studies of other sarcoma-related genes revealed that introduction of *SYT/SSX*, a synovial sarcoma-related gene, into *Myf5*-positive immature myoblasts specifically resulted in sarcoma formation, whereas its expression in more differentiated cells induced myopathy without tumor induction (39). In addition, introduction of *EWS/FLI1*, a fusion gene detectable in Ewing sarcomas, results in transformation specifically in bone marrow–derived mesenchymal progenitor cells in vitro (40). Taken together, these findings are suggestive of cell type–specific carcinogenesis by expression of sarcoma-related fusion oncogenes.

Our lineage-tracing experiments in vivo suggested that *EWS/ATF1*-associated tumor cells are derived from neural crest–derived cells. This result is consistent with several lines of evidence that CCS often shows melanocytic differentiation and resembles MM. However, our present results do not exclude the possibility that *EWS/ATF1*-induced tumors can arise from non–neural crest–derived cells. In addition, the exact cell type of origin of *EWS/ATF1*-induced tumors remains unclear, since neural crest–lineage progenitors can differentiate into many different cell types, such as neuronal cells, melanocytes, and Schwann cells. Recently, Schwann cell precursors along the peripheral nerve have been shown to be a cellular source of large numbers of melanocytes in the skin during development in mice and chicks (41). Moreover, Schwann cells also retain the potential to differentiate into melanocytes, resulting from a loss of nerve contact (41). Given the finding that *EWS/ATF1*-induced tumor cells expressed markers for melanocytic differentiation, it is possible that the neural crest–derived Schwann cells could be the origin of *EWS/ATF1*-associated sarcomas.

We found that *Fos* was one of the direct targets of EWS/ATF1 in *EWS/ATF1*-induced tumor cells. *Fos* is an immediate early gene that can be activated by a variety of mitogens and growth factors. The present study showed that *Fos* induction by forced expression of *EWS/ATF1* was independent of the ERK signaling pathway. In contrast, we found that *Fos* upregulation was mediated by a CRE of the *Fos* promoter, accompanied by direct interaction of EWS/ATF1 with the CRE on the *Fos* promoter. The direct interaction of EWS/ATF1 at CRE may induce continuous transcriptional activation of *Fos* in *EWS/ATF1*-induced tumor cells. Previous studies have demonstrated a higher expression level of *FOS* to be involved in tumor growth in several cancers (42–44), and overexpression of *Fos* results in osteosarcoma formation in transgenic mice (45, 46). Here we showed that *FOS* was also upregulated in CCS by the *EWS/ATF1* fusion transcript and that the increased *FOS* promoted the growth of *EWS/ATF1*-related sarcomas. Accordingly, blocking the FOS pathway might be a promising therapeutic strategy for treating CCS (Supplemental Figure 12).

Methods

Molecular cloning and gene targeting in ES cells. Human *EWS/ATF1*-FLAG-HA was amplified by RT-PCR from the human CCS cell line KAS using primers ACATGGCGTCCACGGATTACAG and CCTAGGCGTAGTCGGGCACGTCGTAGGGGTATCCTCCAGCGGCCGACTTGTCATCGTCGTCCTTGTAGTCTCCTCCAACACTTTTATTGGAATAAAGAT and cloned into pcr2.1-TOPO. Sequence-verified *EWS/ATF1*-FLAG-HA cDNA was subcloned into a unique *EcoRI* site of pBS31 prime (37, 38). KH2 ES cells (obtained from Open Biosystems) were used to insert a single copy of *EWS/ATF1*-FLAG-HA by Flipase (PLP) recombination into the *Col1A1* locus under the control of a minimal CMV tetracycline-inducible promoter using a previously described method (37), and ES cells were selected for hygromycin resistance.

Mouse generation. For blastocyst injections, fertilized zygotes were isolated from the oviducts of day-0.5 pregnant B6D2F1 females and allowed to develop to the blastocyst stage in culture. 7–12 ES cells were injected per blastocyst. The injected blastocysts were transferred into day-2.5 pseudopregnant recipient females.

Doxycycline treatment. 6-week-old mice were administered 50 μg/ml doxycycline (Sigma-Aldrich) in their drinking water supplemented with 2 mg/ml sucrose. For cultured cells, doxycycline was used at a concentration of 0.05–0.2 μg/ml.

RNA preparation and RT-PCR. Total RNA was isolated using a RNeasy mini kit (Qiagen). Total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems). qRT-PCR analysis using the fluorescent SYBR green method (Bio-Rad) was performed in accordance with the manufacturer's instructions. The data generated from each reaction were subjected to gene expression analysis using an iCycler iQ Real-Time PCR Detection System (Bio-Rad). See Supplemental Table 1 for specific primer pairs used for amplification. Microarray analysis was performed with SurePrint G3 Mouse GE 8X60K microarray (Agilent Technologies) and Mouse Gene 1.0 ST Array (Affymetrix) according to the manufacturer's instructions. All analyses were performed by Genespring GX (version 12; Agilent Technologies).

Western blot analysis. Western blot analyses were carried out as described previously (47, 48). The following antibodies were used: anti-HA (rabbit IgG, 1:1,000 dilution; Cell Signaling), anti-Fos (rabbit IgG, 1:1,000 dilution; Cell Signaling), anti-ERK1/2 (rabbit IgG, 1:1,000 dilution; Cell Signaling), anti–phospho-ERK1/2 (rabbit IgG, 1:1,000 dilution; Cell Signaling), anti-ATF1 (rabbit IgG, 1:5,000 dilution; EPITOMICS), and anti–β-actin (mouse IgG, 1:5,000 dilution; Calbiochem).

Cell proliferation assay. Cell growth was determined by WST-8 assay using a Cell Counting Kit-8 (Dojindo Laboratories). Absorbance at 450 nm is indicative of the die amount of formazan, which is directly proportional to the number of living cells.

Histological analysis. Normal and tumor tissue samples were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. 4-μm sections were stained with H&E, and serial sections were used for immunohistochemical analyses. Immunostaining was performed using an avidin-biotin immunoperoxidase assay. The primary antibodies used were anti–HA-Tag (1:600 dilution; Cell Signaling), anti-Ki67 (1:250 dilution; Dako), anti-S100 (1:800 dilution; Dako), anti-SOX10 (1:200 dilution; R&D Systems), anti-MITF (1:500 dilution; Exalpha), and anti-GFP (1:1,000 dilution; Abcam).

X-gal staining. Briefly, tumor tissue samples were embedded in OCT compound and frozen. 8-μm cryostat sections were immediately fixed in 0.2% glutalaldehyde for 10 minutes. The sections were stained overnight in an X-gal staining solution, then counterstained with fast red for 3 minutes.

Tumorigenicity studies. 4-week-old male BALB/c athymic mice were obtained from Japan SLC. A total of 5.0×10^6 G1297 cells in 0.1 ml serum-free DMEM was inoculated subcutaneously through a 26-gauge needle into the posterior flank of each mouse. 3 weeks after inoculation, the tumor diameters were measured with digital calipers, and tumor volume was calculated as $(w^2 \times l)/2$ and expressed in mm^3.

siRNA transfection. We performed transient knockdown assays with a siRNA targeting *Fos* (Santa Cruz), *FOS* (Santa Cruz and Dharmacon) or the breakpoint of *EWS/ATF1* type 1 (sense, GCGGUGGAAUGGGAAAAAUTT; antisense, AUUUUUCCCAUUCCACCGCTT; KOKEN) using Lipofectamine RNAiMAX (Invitrogen). We used nontargeting siRNA (Cosmo Bio Co.) as a control.

Cell lines. MP-CCS-SY and KAS are CCS cell lines carrying *EWS/ATF1* type 1 and type 2, respectively. MP-CCS-SY was established as described previously (49), and KAS was provided by T. Nakamura (Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan; ref. 24). HOS (osteosarcoma), U2OS (osteosarcoma), NIH3T3 (embryonic fibroblast), and WI38 (lung fibroblast) cells were purchased from the American Type Culture Collection. B16-F1 (mouse melanoma) and A375 (human MM) were purchased from the European Collection of Cell Cultures.

Mice. Wnt1-Cre mice were provided by S. Iseki (Tokyo Medical and Dental University, Tokyo, Japan; ref. 50), *P0-Cre* mice were provided by K. Yamamura (Kumamoto University, Kumamoto, Japan; ref. 51), and floxed *LacZ* mice were provided by M. Okabe (Osaka University, Suita, Japan; ref. 52). Floxed *EYFP* mice (53) were obtained from Jackson Laboratory.

Construction of the reporter plasmid. To obtain the *Fos* reporter plasmid (pGL3A-1486), the genomic DNA fragment containing –450 to +0 of the 5′-flanking sequence was amplified by PCR with the primer set 5′-TCTATCGATAGGTACGAATGTTCGCTCGCCTTCTC-3′ and 5′-ACGCGTAAGAGCTCGGGAGTAGTAGGCGCCTCAGC-3′ and subcloned into the *KpnI* site of the pGL3 vector (Promega). The *Fos* reporter plasmid with mutant CRE element was generated by PCR-targeted mutagenesis with the primer set 5′-CCAGTTCCGCCCACTCAGCTAGGAAGTCCATCC-3′ and 5′-GGATGGACTTCCTAGCTGAGTGGGCGGAACTGG-3′.

Luciferase assay. Reporter genes were transfected into the G1297 *EWS/ATF1*-induced tumor cell line together with phRL-SV40 (Promega) using lipofectamine LTX (Invitrogen), and luciferase activity was measured with a luminometer (VERITAS; Promega). Firefly luciferase activities, derived from each reporter construct, were normalized to Renilla luciferase activities from phRL-SV40.

Stable transfection. To obtain *Fos* expression plasmid (pCAG-Fos-IZ vector), *Fos* cDNA was amplified by RT-PCR from the G1297 cell line with the primer set 5′-ACATGATGTTCTCGGGTTTCAA-3′ and 5′-ACTCACAGGGCCAGCAGCGTGG-3′ and subcloned into the *ECoR* site of the pCAG-*EGFP*-IZ vector (provided by H. Niwa, RIKEN, Kobe, Japan). pCAG-*EGFP*-IZ vector or pCAG-*Fos*-IZ vector was transfected into the G1297 cell line using Lipofectamine LTX Reagent (Invitrogen) and selected for Zeocine resistance (600 μg/ml).

Patients and tumor tissue collection. Anonymized tumor specimens were obtained by surgical resection or biopsy at Gifu University Hospital or Kyoto University Hospital in accordance with an approved protocol from the Institutional Review Board. Total RNA was isolated using a RNeasy mini kit (Qiagen).

ChIP analysis. A total of 5.0×10^6 *EWS/ATF1*-inducible tumor cells was fixed in 1% formaldehyde for 10 minutes, followed by treatment with 1 ml glycine buffer for 5 minutes. Cells were pelleted, washed, and then resuspended in lysis buffer for 30 minutes. After centrifugation, the pellet was resuspended in NP40 buffer with protease inhibitors (Sigma-Aldrich). Sonication was performed using a XL-2000 (MISONIX), after which the supernatant was used as the input sample for immunoprecipitation experiments. Antibodies used were rabbit HA (Cell Signaling) and rabbit normal IgG (Abcam). Protein G–coated magnetic beads were used to purify specific antibody/DNA complexes. After washes, immunoprecipitated DNA was decrosslinked by elu-

tion buffer at 65°C for 12 hours. To remove protein and RNA, samples were incubated with RNaseA for 1 hour at 37°C and proteinase K treatment for 1 hour at 37°C. Samples were purified by PCR purification kit (Qiagen). The amount of DNA immunoprecipitated with HA-tagged protein was quantified by real-time PCR with primers flanking the *Fos* promoter, including the CRE element (forward, 5′-TCCTACACGCGGAAGGTCTAGG-3′; reverse, 5′-TAGAAGCGCTGTGAATGGATGG-3′). Primers 5′-GCACTAATTAGTCGCGGTGGTGG-3′ (forward) and 5′-CAGGGTCTTAGTGGGATCAAGG-3′ (reverse) were used as a negative control.

Accession number. The profiling data cited in Supplemental Figure 6 are available at GEO (accession no. GSE41123).

Statistics. Statistical analyses were carried out using GraphPad Prism (version 5.01; GraphPad Software). Data were analyzed using ANOVA, and *P* values less than 0.05 were considered statistically significant.

Study approval. Animal experiments were approved by the Gifu University Animal Experiment Committee, and the care of the animals was in accordance with institutional guidelines. All clinical samples were approved for analysis by the Ethics Committee at Kyoto University Graduate School and Faculty of Medicine (Kyoto, Japan). Written informed consent was obtained from all patients with cancers analyzed in this study.

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Cellular reprogramming and cancer development

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Cancer develops through the accumulation of genetic and epigenetic abnormalities. The role of genetic alterations in cancer development has been demonstrated by reverse genetic approaches. However, evidence indicating the functional significance of epigenetic abnormalities remains limited due to the lack of means to actively modify coordinated epigenetic regulations in the genome. Application of the reprogramming technology may help researchers to overcome this limitation and shed new light on cancer research. Reprogramming is accompanied by dynamic changes of epigenetic modifications and is therefore considered to be a useful tool to induce global epigenetic changes in cancer genomes. We herein discuss the similarities between reprogramming processes and carcinogenesis and propose the potential use of reprogramming technology to help understanding of the significance of epigenetic regulations in cancer cells. We, also discuss the application of induced pluripotent stem cell technology to cancer modeling based on the similar characteristics between pluripotent stem cells and cancer cells.

Epigenetic Modifications in Carcinogenesis

Cancer development is caused by a series of genetic mutations in cancer related genes, including oncogenes, tumor suppressor genes and genes related to genome stability. However, recent studies suggest that epigenetic modifications also play critical roles in tumorigenesis.[1] "Epigenetics" is defined as meiotically and mitotically inherited regulations of gene expression that are not accompanied by alteration of the DNA sequence. Two major epigenetic regulations observed in mammals are DNA methylation and post-translational modification of histone proteins. Accumulating evidence highlights the significance of epigenetic dysregulation in tumorigenesis and in the maintenance of cancer cells.[2,3]

Abnormal patterns of genomic methylation in cancer cells are characterized by global losses of genomic methylation and increased methylation at specific loci, predominantly CpG islands that are often localized near transcription start sites. DNA methylation is a type of covalent modification in which a methyl group is added to a cytosine in the genome. In mammalian cells, DNA methyltransferases (DNMTs) are involved in the establishment and maintenance of DNA methylation. DNMT1 has high catalytic activity and shows a preference for hemimethylated DNA. It plays a role in maintaining the genomic methylation levels during DNA replication. In contrast, DNMT3A and DNMT3B carry out *de novo* methylation at non-methylated CpG dinucleotides. Although DNMT3L does not exhibit enzymatic activity, it participates in DNA methylation by regulating other methyltransferase activities. Human tumors often display an aberrant expression of DNMTs. Higher levels of DNMT1 and DNMT3A/3B are frequently observed in a wide variety of cancers. Previous studies have suggested that such altered expressions of DNMTs could partly explain the abnormal patterns of genomic methylation observed in cancer cells.[4]

The functional significance of both global hypomethylation and site-specific hypermethylation in cancer has been suggested in previous studies.[2] Global genomic hypomethylations are frequently observed in both benign and malignant tumors.[5,6] The clinical outcomes observed in several malignancies indicate that genomic hypomethylation is correlated with poor prognoses in cancer patients, suggesting that loss of DNA methylation can be a marker for tumor prognosis. It is noteworthy that global DNA hypomethylation is associated with abnormal chromosomal structures, as observed in patients with ICF (Immunodeficiency, Centromeric instability and Facial abnormalities) syndrome[7,8] who harbor mutations of *DNMT3B*. In addition, embryonic stem (ES) cells lacking *Dnmt1* with decreased genomic methylation levels demonstrate increased mutation rates, implying the importance of maintaining the genomic methylation level for preserving genomic integrity.[9,10] Indeed, DNA hypomethylated mice that harbor hypomorphic alleles of the *Dnmt1* consistently succumb to thymomas, indicating that global DNA hypomethylation promotes carcinogenesis possibly through the induction of chromosomal abnormalities.[11] This notion has been further supported by the results of experiments using mice with heterozygous mutations in the tumor suppressor gene *Nf1* in conjunction with *Dnmt1* hypomorphic alleles. In these mice, global DNA hypomethylation promotes the loss

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of heterozygosity in the mutated gene, leading to tumor formation.

Conversely, site-specific hypermethylation is associated with the silencing of tumor suppressor genes.[4] For instance, treatment of cancer cells with 5-aza-2-deoxycytidine (5-Azadc), which blocks DNMTs activity, causes increased expression of tumor suppressor genes and results in growth arrest, differentiation and/or apoptosis both *in vitro* and *in vivo*.[12] In addition, *Apc* mutant mice heterozygous for *Dnmt1* develop significantly decreased numbers of intestinal tumors.[13–15] Although the opposing effects of the genomic methylation on tumor development seem to be contradictory, these effects were demonstrated in one experiment utilizing *Apc* mutant mice with global DNA hypomethylation. In this study, the forced reduction of genomic methylation resulted in the promotion of early lesions with losses of *Apc*; however, the overall development of large lesions was inhibited.[16,17]

Imprinted genes display a characteristic parent-of-origin-specific DNA methylation pattern that results in the expression of only a single allele. DNA methylation that maintains the monoallelic expression of imprinted genes is established during gametogenesis and is important for fetal growth regulation and perinatal development.[18,19] Loss of imprinting (LOI) (either biallelic expression or complete silencing of imprinted genes) has been implicated in the progression of several tumors.[20,21] For instance, the aberrant biallelic expression of the insulin-like growth factor-2 (*IGF2*) gene is thought to promote tumorigenesis through inhibition of apoptosis or expansion of undifferentiated cells.[22] Indeed, LOI is a significant risk factor for human colorectal carcinogenesis. In addition, LOI at *PEG3*, *P57* and *IGF2R* has been implicated in the development of oligodendrogliomas, breast cancer and hepatocellular carcinomas, respectively.[23–25] Although there is reported evidence that LOI at the *IGF2* locus promotes tumorigenesis,[26] it remains unclear if the observed LOI is merely a consequence of altered epigenetic regulation in already transformed cells. Furthermore, the question whether LOI at other various imprinted genes also plays a causal role in cancer development is yet to be answered. Importantly, chimeric mice derived from imprinting-free ES cells develop multiple tumors,[27] suggesting a causal link between LOI and cancer development in mice.

Histone is a structural unit of nucleosomes that is important for packing of genomic DNA. Accumulating evidence suggests that post-translational modification of histones (e.g., acetylation, methylation, ubiquitylation, phosphorylation, sumoylation and ribosylation) controls the activity of transcription of surrounding DNA. Epigenetic silencing is associated with at least two distinct histone modifications: polycomb-based histone H3 lysine 27 trimethylation (H3K27me3) and H3K9 dimethylation (H3K9me2).[28] Polycomb group (PcG) proteins are components of polycomb repressor complex 1 (PRC1) and PRC2. Enhancer of zeste 2 (EZH2), a member of PRC2, exhibits histone methyltransferase activity with substrate specificity to H3K27.[29] H3K27me3 serves as a signal for specific binding of the chromodomain of the other PRC, PRC1, that includes BMI-1, RING1, HPC and HPH.[30] The binding of PRC1 to the methylated histone hampers the recruitment of transcriptional activation factors such as SWI/SNF to surrounding genomic regions, resulting in the prevention of RNA polymerase II from transcription initiation.[31,32] Conversely, H3K9me2 primarily plays a role in transcriptional control. G9a is a mammalian H3K9 methyltransferase that can repress gene transcription activity by inducing local H3K9me2 and H3K9me3 at target promoters.[33,34]

Recent studies suggest that histone methyltransferases play roles in the promotion and progression of human cancers. For example, dysregulation of PcG proteins has been observed in a variety of cancers. EZH2 is upregulated in several types of cancer, including metastatic prostate cancer, lymphomas and aggressive breast cancer.[35] An increased expression of EZH2 is correlated with a poor prognosis in patients with prostate and breast cancer. In addition, frequent somatic mutations in *EZH2* have been reported in hematopoietic malignancies, consistent with the hypothesis that histone modifications are functionally involved in cancer development.[36] BMI-1, a member of PRC1, is frequently overexpressed in human medulloblastoma cell lines and primary tumors.[37] In addition to the findings that altered expressions of PcG proteins are closely related to tumorigenesis, the expression of G9a is also upregulated in cancers such as hepatocellular carcinomas.[38] It has been shown that G9a is associated with epigenetic silencing of tumor suppressor genes and with the maintenance of malignant phenotypes, providing additional evidence that the aberrant regulation of histone modifications contributes to cancer development.

As described above, emerging evidence indicates that cancer cells harbor altered epigenetic modifications correlated with altered expressions of cancer-related genes. It is important to note that DNA methylation, histone modification, chromatin remodeling and transcriptional regulation are closely interconnected in mammals.[39,40] Several methyl-CpG-binding proteins (e.g., MeCP2 and MBD2) interact with histone deacetylase and recruit corepressor proteins. This evidence implies a mechanism linking DNA methylation and histone modification. Therefore, a comprehensive analysis of epigenetic modifications, including global analyses of DNA methylation, histone modifications and miRNA expression, in conjunction with a gene expression analysis is necessary to understand cancer epigenetics. Reverse genetic approaches have enabled us to elucidate the functional roles of specific "genetic" alterations in cancer cells. However, to elucidate the functional roles of "epigenetic" alterations, there have not yet been available experimental methods to efficiently control the coordinated epigenetic modifications. Active modification of epigenetic regulation would be a powerful tool to perform functional analyses of epigenetic regulation in cancer cells. In this context, it is noteworthy that technologies for cellular reprogramming enable us to induce dynamic changes in epigenetic modifications. Application of the reprogramming technology to cancer cells to actively alter their epigenetic status might eventually enhance our understanding of cancer epigenomes.

Epigenetic Regulations in Cellular Reprogramming

Global changes in epigenetic modifications can be observed during mammalian embryogenesis and are directly linked to the regulation of pluripotency and cellular differentiation. Although the developmental process in mammals is considered to be unidirectional, previous studies have demonstrated that differentiated cells can be converted into undifferentiated stem cells by artificial manipulation. For example, nuclear transplantation (NT) can reprogram a terminally differentiated cell into a pluripotent stem cell that can give rise to all types of cells in the body.[41–43] Recent groundbreaking studies have demonstrated that the overexpression of four transcription factors, *Oct4*, *Sox2*, *Klf4* and *Myc*, reprograms differentiated cells into "induced pluripotent stem cells" (iPSCs).[44,45] These studies have extended our understanding of the mechanisms underlying pluripotency acquisition, maintenance and differentiation.

Derivation of iPSCs from somatic differentiated cells is accomplished by erasing the epigenetic modifications associated with the maintenance of cellular identity and inducing epigenetic modifications similar to those found in early embryos while leaving genetic sequences unaltered. The earliest event in iPSC derivation has been suggested to be genome-wide changes in the histone modification H3K4me2 at more than a thousand loci, including large subsets of pluripotency-related or developmentally regulated gene promoters and enhancers. In contrast, the patterns of repressive H3K27me3 modification remain largely unchanged except for focused depletion occurring specifically at positions where H3K4 methylation is gained. Notably, these chromatin regulation events precede transcriptional changes within corresponding loci.[46] In addition, "partially" (or "unsuccessfully") reprogrammed cell lines display incomplete repression of lineage-specifying transcription factors. These observations suggest that changes in epigenetic modifications play critical roles in the early stages of cellular reprogramming and that incomplete epigenetic repression of key genes may be a bottleneck in the transition to pluripotent states.[47] Several studies have demonstrated that treatment with DNMT inhibitors and histone modifiers such as VPA can improve the overall efficiency of the reprogramming process.[48,49] This evidence emphasizes the importance of global changes in epigenetic modifications for successful reprogramming.

Similarities Between Cancer Cells and Pluripotent Stem Cells

The process of iPSC derivation shares similar characteristics with cancer development. During the reprogramming, somatic differentiated cells acquire unlimited proliferation properties and self-renewing activities.[50] This is also one of the most important events in carcinogenesis. It has also been suggested that iPSCs and cancer cells share similar characteristics of cell metabolism.[51] Metabolites involved in transmethylation, cellular respiration and energy production functionally affect cellular reprogramming.[52] Changes in metabolic status which occur during somatic cell reprogramming are largely similar to those observed in cancer development.[53] This is in agreement with the results of previous studies demonstrating that similar pathways are associated with both oncogenesis and the induction of pluripotency.[54] Such similarities between pluripotent stem cells and cancer cells suggest that tumorigenesis and reprogramming processes may be partly promoted by overlapping mechanisms. Indeed, previous studies have suggested that reprogramming factors are involved in the development of various types of cancer.

Oct3/4 (also known as *Pou5f1*), a transcription factor that has been recognized as a fundamental factor, maintains pluripotency in ES cells and primordial germ cells.[55,56] Although Oct3/4's role during preimplantation development is to maintain embryonic cells in a pluripotent undifferentiated state, previous studies have suggested that Oct3/4 expression may possibly play a role in cancer development. OCT3/4 has been proposed to be a useful marker for germ cell tumors (GCTs) such as seminomas and embryonal carcinomas.[57] OCT3/4 is functionally involved in the self-renewal of GCT cells possibly through the maintenance of undifferentiated states. Importantly, the forced induction of *Oct3/4* in adult somatic cells results in dysplastic growth in epithelial tissues through the inhibition of cellular differentiation in a manner similar to that in embryonic cells.[58] These findings suggest that Oct3/4 expression affects epigenetic regulations and contributes to the maintenance of undifferentiated proliferating states in somatic cells. The notion may provide a possible link between transcription factor-mediated reprogramming and carcinogenesis.

A wide variety of cancers express increased levels of *MYC*. *MYC* oncogene is frequently translocated in multiple myeloma and is one of the most highly amplified oncogenes in many different human cancers. It should be noted that Myc is an important transcriptional regulator in ES cells and it significantly promotes the process of iPSC derivation. Myc targets in ES cells are predominantly involved in cellular metabolism, the cell cycle and protein synthesis pathways, whereas the targets of core reprogramming factors such as Oct3/4, Nanog and Sox2 are frequently related to developmental processes. Interestingly, Myc preferentially binds to promoters of actively transcribed genes with the histone H3 lysine 4 trimethylation (H3K4me3) signature and enhance their transcriptions in both pluripotent stem cells and cancer cells.[59,60] List of the genes activated under these promoters might explain the background mechanism of the unlimited proliferation of both pluripotent stem cells and cancer cells.

As described above, transcription factor-mediated reprogramming of somatic cells might be also involved in cancer development. Considering that epigenetic regulations are important during reprogramming, it is possible that epigenetic regulations modulated by the reprogramming factors also play a role in carcinogenesis. Therefore, uncovering the process of cellular reprogramming might eventually provide a better understanding of cancer epigenomes that are responsible for the unlimited growth properties of cancer cells.

Risk of Tumor Development in the Clinical Application of iPSCs to Cell Transplantation Therapy

Pluripotent stem cells are expected to be a promising source of cells for cell transplantation therapies. iPSCs have offered a solution to the ethical objections of ESC usage and to possible immune rejection against ESC-derived cells after transplantation into unmatched individuals. Therefore, iPSCs provide a novel and efficient approach for patient-specific regenerative therapy. One of the risks of using iPSCs in cell transplantation therapy is cancer development from iPSC-derived cells. As mentioned above, pluripotent stem cells share a number of cellular and molecular properties with cancer cells. Both divide rapidly with unlimited proliferative activity,[61] both lack contact inhibition[62] and both exhibit high telomerase activity.[63] Similarities between iPSCs and cancer cells have also been observed with respect to overall gene expression patterns[64–66] and epigenetic status.[67] Indeed, one of the important characteristics of pluripotent stem cells is the ability to form teratomas in immunocompromised mice.

For the safe application of iPSCs in regenerative medicine, a number of possible mechanisms in which iPSC-derived cells become cancerous should be considered to avoid such cancer development. First, it is possible that contamination with undifferentiated pluripotent stem cells may result in teratoma formation when transplanted in patients. Given that an iPSC line may consist of a heterogeneous rather than a homogeneous cell population, it is also possible that contamination with unsuccessfully reprogrammed cells or mutated iPSCs might lead to cancer development. Second, since the derivation process of iPSCs requires a number of cell divisions, genetic mutations may be accumulated during the establishment of iPSCs *in vitro*, which may be a potential risk for cancer development. In addition, the reprogramming process is likely to participate in the activation of stress response pathways in the cells, which might also cause the accumulation of genetic mutations, regardless of the reprogramming method used.[68] Third, previous studies have demonstrated that epigenetic modifications play a role in genomic integrity. Therefore, dynamic changes in epigenetic modifications that occur during iPSC generation might induce genomic instability, leading to genetic alterations in cancer-related genes. Finally, based on the proposed concept of "epigenetic field defect" which is involved in the increased risk for cancer development,[69] it should be also considered that altered epigenetic modifications in iPSCs-derived cells might create cancer-susceptible populations after the integration of transplanted iPSC-derived cells.

Recent studies have described unique genetic and epigenetic properties of iPSCs distinct from those of ESCs, which might increase the risk of cancer development in iPSC-mediated transplantation therapy. These studies have examined copy number variations (CNVs)[70] and point mutations of protein-coding regions[71] across the genome by utilizing next generation sequencers. Additionally, genome-wide DNA methylation patterns of ESCs and iPSCs have been analyzed at the single-base level using whole genome bisulfite sequencing.[72] These studies, along with other investigations, have proposed that reprogramming and the subsequent expansion of iPSCs *in vitro* may result in the accumulation of various genetic and epigenetic abnormalities at the chromosomal, subchromosomal and single-base levels.[73] Such chromosomal abnormalities appear soon after the establishment of iPSCs,[74] whereas chromosomal abnormalities are not generally observed in ESCs. The frequency of mutations in iPSCs has been estimated to be ten times higher than that in fibroblasts.[71] In addition, there are greater number of subsequent CNVs in iPSCs, which are not found in the cells of origin or in human genomes of comparable background, than in ESCs.[70,74] Similarly, epigenetic analyses of iPSCs feature retained epigenetic marks of the cells of origin. Another potential difference between ESCs and iPSCs is the status of genomic imprinting. Recent studies have found variety in the expression of imprinted genes among different lines of both murine and human iPSCs.[75,76] Considering the critical role of genomic imprinting during the developmental process, aberrant silencing or the activation of imprinted genes during reprogramming might affect the differentiation capacity of pluripotent stem cells. Indeed, aberrant imprinting at a single imprinted gene cluster in murine iPSCs results in an impaired developmental potential, thus suggesting that epigenetic variations at imprinted loci can affect the biological behaviors of iPSCs. It is important to note that aberrant imprinting is evident in some types of human cancers and it is also associated with cancer development.[77,78] Therefore, an aberrant expression of imprinted genes in iPSCs might also affect the tumorigenicity of iPSC-derived cells after cell transplantation therapy.

In contrast, a particularly sensitive analysis for genetic sequencing have subsequently demonstrated that a subset of point mutations found in iPSC lines already exist in a small minority of fibroblasts used for reprogramming, suggesting that the reprogramming process itself may not induce genetic instability.[70] In addition, large number of CNVs acquired during early passages of iPSCs disappear after subsequent growth *in vitro*, which implies that iPSCs with altered CNVs are negatively selected during the maintenance of iPSCs *in vitro*.[70] With regard to alterations in DNA methylation patterns, it is also noteworthy that other studies examining a large number of pluripotent stem cells have concluded that it is difficult to distinguish iPSCs from ESCs by DNA methylation patterns. Furthermore, previous studies suggested that the different induction and culture conditions in each laboratory can cause the variable expression patterns.

Taken together, it is still controversial whether the featured genetic and/or epigenetic alterations are inevitable risks of iPSCs for the future clinical therapies (Figure 1). As ESCs also show considerable variation in terms of both genetic and epigenetic contexts, the proposed alterations in iPSCs reported in previous studies may simply represent heterogeneity among different pluripotent stem cell lines. In addition, it has been demonstrated that iPSC lines, as well as ESC lines, show huge variations in differentiation propensity, which implies the presence of variation in tumorigenic risk

as well. Therefore, further studies using a large number of different iPSC/ESC lines are required before we conclude the stability of iPSCs in terms of genome/epigenome integrity. The development of proper strategies to determine and select better iPSCs will lead to the safe application of iPSCs in cell transplantation therapy.

Attempts to Generate Safe iPSCs for Clinical Application

As we described in the previous section, the potential obstacles to the efforts to develop secure iPSCs for clinical application are attributable to the lack of knowledge regarding the mechanisms of tumor development from iPSC-derived cells. In addition, it has not been determined if iPSC-derived cells are actually associated with an increased risk of cancer development. Yet several attempts have already been made to decrease the risk of cancer development from iPSC-derived cells. Based on the assumption that *c-Myc* plays a role in iPSC-associated cancer development, previous studies have been conducted to search for alternatives to replace *c-Myc* in the induction of iPSCs. *L-Myc* and *Glis1* were found to exhibit stronger and more specific activities in promoting iPSC generation in the absence of *c-Myc*.[79,80] L-Myc has shorter amino acid sequences in the N-terminal region compared with the other two Myc members. Glis1 is a Gli-like transcription factor that is enriched in unfertilized oocytes and embryos at one cell stage. Notably, although both factors markedly enhance the generation of iPSCs from both murine and human fibroblasts, no increases in the number of tumor formation events is observed in iPSC-derived chimeric mice, suggesting that these factors might have advantages in the establishment of secure iPSCs for clinical application.

iPSCs were initially derived by the transduction of reprogramming factors in fibroblasts with integrating viruses carrying transcription factors.[45] It should be noted that at least one of these reprogramming factors are frequently expressed in various types of cancer. It has also been suggested that reactivation of reprogramming factors predisposes iPSCs to genomic instability.[81] Because an ectopic expression of either *Oct3/4* or *Klf4* can induce dysplasia *in vivo*,[58,82] reactivation of integrated transgene might cause cancer development from iPSC-derived cells. Accordingly, various methods have been developed to eliminate such safety concerns by means of elimination of genome integrations. For example, transduction of purified recombinant reprogramming proteins,[83,84] synthetic RNA introduction,[85] infection with nonintegrating Sendai virus,[86] *piggyBac* transposon[87,88] and gene introduction using episomal vectors[89] have been used to generate iPSCs free from genomic integration of exogenous genes. However, the reprogramming efficiency of these approaches is often substantially lower than that of the methods using genome-integrating approaches, and the quality of resultant iPSCs remains to be scrutinized. Further studies will be required to determine the safe reprogramming method that is applicable to the future cell-based regenerative medicine.

Application of iPSC Technology to Cancer Research

Reprogramming of cancer cells

Because cellular reprogramming actively modifies the epigenetic state of a cellular genome without affecting its genetic constitution, reprogramming technologies can be used as tools to analyze the impact of epigenetic regulations on cancer cells. Historic experiments conducted in frogs have demonstrated that kidney carcinoma nuclei can be reprogrammed and that the resulting nuclei support the early development of frog embryos to the tadpole stage.[90] It has also been reported that by utilizing nuclear transfer into enucleated oocytes, the nuclei of leukemia, lymphoma and breast cancer cells are able to support normal preimplantation development to the blastocyst stage, although they fail to give rise to ES cells. However, interestingly, under the absence of key oncogene expression, the nuclei from cancer cells show the potential to give rise to ES cells. Namely, murine blastocysts cloned from melanoma cells with doxycycline-dependent *RAS*-inducible system give rise to ES cells in the absence of *RAS* expression with the potential to differentiate into multiple cell types, including melanocytes, lymphocytes and fibroblasts.[91] Chimeric mice produced from these ES cells develop melanomas with higher penetrance, shorter latency and expanded tumor spectrums after retransduction of *RAS* in the melanocyte lineage in comparison to that observed in donor mouse models. Importantly, the chimeric mice produced from melanoma nuclear-transferred ES cells have nontumorigenic melanocytes, even after retransduction of *RAS*. This observation suggests that epigenetic reprogramming can revert melanoma cells to non-neoplastic melanocytes, highlighting the impact of epigenetic regulations in cancer cells.

Reprogramming by means of nuclear transfer requires special equipment and a high skill. In contrast, the discovery of iPSC generation technology provides a simple and reproducible method to reprogram differentiated cells into pluripotent stem cells.[44,45] Taking advantage of the transcription factor-mediated reprogramming method, a number of studies have been conducted to reprogram cancer cells. However, these experiments have revealed that cancer cells are resistant to reprogramming. Recently, several groups have succeeded in reprogramming cancer cells into an iPSC-like state. iPSC-like cells have been generated from chronic myeloid leukemia cells,[92,93] melanoma cells[94] and gastrointestinal cancer cells.[95] Although these studies have demonstrated that particular cancer cell genomes can be reprogrammed into iPSC-like states, reprogramming efficiency seems to be extremely low in cancer cells, suggesting that some properties of cancer cells strongly hamper cellular reprogramming. Considering that cellular reprogramming is a process in which dynamic epigenetic changes are required, the fact that cancer cells are resistant to cellular reprogramming raises the possibility that cancer cells have stable epigenetic regulations that compete with the exogenous reprogramming forces derived from

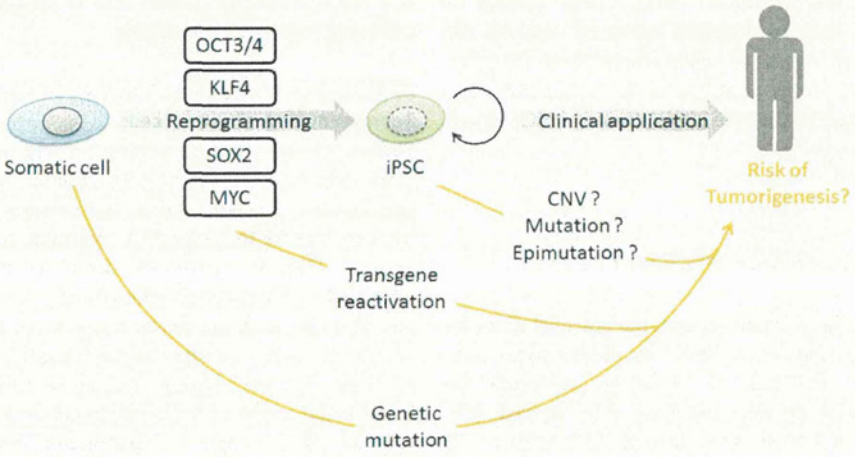

Figure 1. Potential risk of tumor development in the clinical application of iPSCs. Cancer development is a potential risk of using iPSCs in cell transplantation therapy. The genomic and epigenomic instability of iPSCs may cause tumor development from iPSC-derived cells, yet the instability has not been fully elucidated.

induced reprogramming factors. Uncovering the underlying mechanisms that explain the resistance of cancer cells to epigenetic reprogramming may be of great help in advancing understanding of the epigenetic stability of cancer cells, which may eventually contribute to the development of efficient therapeutic strategies targeting epigenetic modifications in cancer cells. In addition, understanding the molecular basis for the reprogramming resistance in cancer cells may further reveal a mechanism for the low efficiency of normal somatic cell reprogramming.

Concept of cell type-specific carcinogenesis

Tumors develop through the accumulation of multiple mutations in oncogenes and tumor suppressor genes. Oncogenic mutations are often observed in a tissue- or cell type-specific manner.[96] Similarly, the cancer mutation spectrum is different between cancers arising from different organs. In hereditary cancer syndromes, patients are predisposed to tumor development in specific tissues despite the presence of mutated cancer-related genes in all somatic cells. This suggests that the effects of cancer-relevant mutations are highly influenced by cell type-specific contexts in different environments. As cellular reprogramming enables us to erase the cellular identity of original cell types without altering the genomic sequences including genetic abnormalities in cancer cells, redifferentiation of cancer cells into cells of other tissue types should be useful method to directly demonstrate the concept of cell type-specific carcinogenesis. Actually, redifferentiation of reprogrammed cancer cells has been demonstrated in leukemia cell model. However, CML-derived iPS cells failed to recapitulate cancer phenotypes, even after the differentiation into hematopoietic cell lineages.[93] Although the concept of cell-type specific carcinogenesis has not yet been proven, application of the iPSC technology to cancer cell reprogramming would be a useful tool for the future achievement of demonstrating this concept (Figure 2a).

Disease modeling using reprogramming technologies

Cancer-derived iPSCs are also expected to provide a novel experimental opportunity to establish disease models. It is interesting to note that iPSCs generated from a blood sample obtained from an imatinib-sensitive CML patient were found to be resistant to imatinib although the CML-derived iPSCs consistently expressed BCR-ABL oncoproteins.[93] Although imatinib treatment is a highly effective therapy for CML,[97] a minority of patients either fail to respond to imatinib. The altered response to imatinib in CML-iPSC suggests that cancer-derived iPSCs could be a novel platform to investigate the effect of the differentiation status on the response of cancer cells to external signals such as therapeutic agents, which could contribute to the development of effective therapeutic strategies. In this context, cancer cell reprogramming may be useful for understanding cancer cell behaviors that are related to distinct differentiation status.

Forced modifications of differentiation states in cancer cells may further lead to increasing understanding of the hierarchical control of differentiation in cancer cells. Such hierarchical control might be associated with the concept of cancer stem cells. Given that epigenetic regulations determine the heterogeneity underlying the concept of cancer stem cells, it might be possible that reprogramming technology could be applicable to modeling cancer stem cells through active modification of both epigenetic regulations and cell differentiation status.

iPSC technology might be also available to examine whether dedifferentiation process occurs during cancer development. Cancer cells frequently express progenitor-related genes that are exclusively expressed in the tissue-specific stem/progenitor cells of the originated tissue.[98] The hypothesis that cancer arises from tissue-specific stem/progenitor cells suggests that the undifferentiated properties of cancer cells may be consequence of the expansion of original undifferentiated cells. Therefore, it remains to be determined whether a dedifferentiation process is

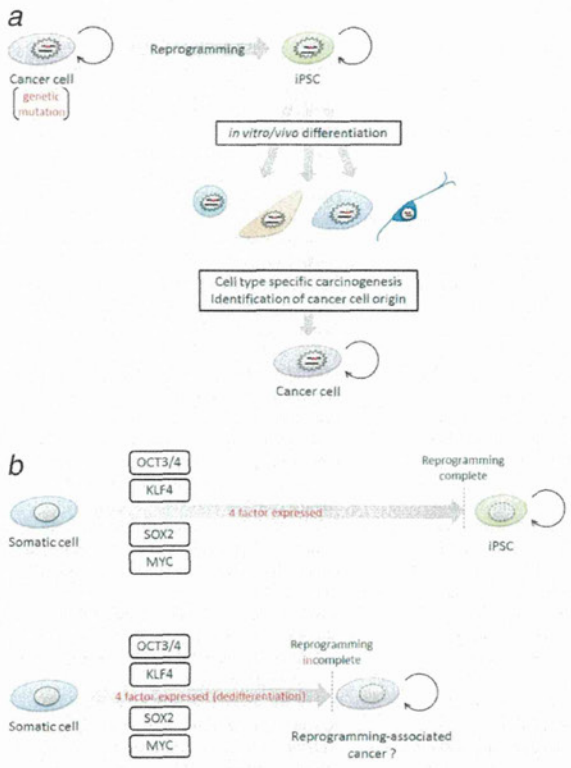

Figure 2. Application of iPSC technology for cancer research. Reprogramming technology is considered to be a useful tool to induce global epigenetic changes and to alter differentiation status of cancer cells. (*a*) Reprogramming of cancer cells and differentiation of the cancer cell-derived iPSCs. Cellular reprogramming actively modifies the epigenetic/differentiation state of cancer cells without affecting its genetic constitution. The reprogrammed cancer cells may provide a novel platform to demonstrate the concept of cell type-specific carcinogenesis and to identify the cancer cell origin. (*b*) Cancer modeling. Expression of Yamanaka 4 factors can reprogram somatic differentiated cells into undifferentiated stem cells with unlimited proliferation properties. Reprogramming technology might be available to model reprogramming-associated cancers, which is accompanied by dedifferentiation process with altered epigenetic regulations.

actually involved in the oncogenic process. Somatic cell reprogramming entails the erasure of gene expression programs characteristic of differentiated somatic cells and the reactivation of embryonic patterns of gene expression characteristic of the pluripotent state.[99,100] Importantly, it has been shown that, not only stem/progenitor cells, but also somatic differentiated cells, can be reprogrammed into pluripotent stem cells, suggesting that differentiated cells can actually acquire self-renewing activities during the process.[101] As an increased expression of pluripotency-related factors is frequently detectable in poorly differentiated cancers,[65] the dedifferentiation process may actively promote the development of certain types of cancers with transcriptional networks similar to those of pluripotent stem cells. Together with the fact that the factors that drive reprogramming are oncogenes or have been linked to cellular transformation, reprogramming technology might be useful to model reprogramming-associated cancers that are accompanied by dedifferentiation (Figure 2b).

Identification of cancer cell origin

In many types of cancers, the cell of origin remains unknown. The identification of the origin of cancer cells enables us to expand our knowledge of cancer development, which facilitates the discovery of more effective chemopreventive approaches. For instance, comparative studies of the cell of origin and the resultant cancer cells may reveal the key events that are directly involved in cancer development. Additionally, recent studies have suggested that the characteristics of the cell of origin often persist in the consequent tumor cells and may play critical roles in the propagation of tumor cells *in vivo*. Therefore, identification of the cell of origin should be beneficial to understand biological properties of cancer cells.

Based on the concept of cell type-specific carcinogenesis, it is expected that the reprogrammed cancer genome can exert their cancerous properties only when cancer genomes are matching with a particular cell type. In this context, reprogrammed cancer cells that harbor genetic mutations sufficient for cancer development but still retain multidifferentiation properties may be available for identifying cell types that give rise to cancer cells. In addition, recent studies have proposed that solid cancers arise from relatively undifferentiated cells such as tissue-specific stem cells. For instance, the conditional knock out of the *Apc* tumor suppressor gene in tissue-specific stem cells specifically results in the frequent development of intestinal tumors,[102] while only a few tumors develop when *Apc* is depleted in progenitor/differentiated cells in crypts. The hypothesis of cancer stem cells also holds that cancer emerges from primitive tissue stem cells, yet it remains to be determined whether the concept can be applied for a wide variety of cancers. Cancer-derived iPSCs may also provide a platform for determining the particular differentiation status permissive for cancer development.

Conclusion

Technologies that induce pluripotent stem cells confer unlimited growth ability on somatic differentiated cells, a hallmark of cancer cells. It is important to note that the reprogramming process does not require changes in genomic sequences, thus indicating that changes in epigenetic modifications play a central role in this process. Application of reprogramming technologies for cancer cells might therefore be useful for uncovering the role of epigenetic regulations in cancer cells. Moreover, cancer development caused by the introduction of reprogramming factors might be applicable to cancer modeling that is predominantly dependent on epigenetic dysregulation with impaired gene expressions and altered differentiation status.

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