The Possible Role of EWS-Fli1 in Evasion of Senescence in Ewing Family Tumors

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Abstract
The chromosomal translocation t(11;22) yields the EWS-Fli1 fusion gene and is associated with oncogenesis of Ewing family tumors (EFT). In this study, using the RNA interference method, we show that EWS-Fli1–targeting small interfering RNAs (siRNA) depleted EWS-Fli1 protein and caused growth inhibition in EFT cells with the accumulation of p27 protein and the down-regulation of Skp2 protein in dose-dependent, time-dependent, and sequence-specific manners. Depletion of EWS-Fli1 subacutely elicited a senescence-like phenotype, but not apoptosis, in EFT cells. Furthermore, not only the knockdown of p27, but also the forced expression of Skp2, reduced the expression levels of p27 protein and partially rescued senescence-like phenotype caused by EWS-Fli1–targeting siRNAs. The accumulation of p27 protein in EWS-Fli1–depleted cells inhibited cdk2 kinase activity and was related to the stability of p27 protein, which resulted from a decrease in Skp2 protein. Immunohistochemical analysis of p27 and Skp2 proteins in EFT samples revealed that there was an inverse relationship between the expression profiles of p27 and Skp2 proteins. These findings indicate an important role of EWS-Fli1 in the prevention of senescence, leading to the unlimited growth and oncogenesis of EFT cells through a decrease in the stability of p27 protein due to increased action of Skp2-mediated 26S proteasome degradation.

Introduction
Ewing family tumors (EFT) are rare tumors and account for ~2% of childhood cancers (1). A specific translocation between chromosomes 11 and 22 is found in over 85% of EFTs (2), which results in the generation of the EWS-Fli1 chimeric gene (3). The chimeric gene product, EWS-Fli1 protein, functions as an aberrant transcription factor. A number of studies have suggested that EWS-Fli1 can act as an oncogene (4, 5). The possible roles of EWS-Fli1 in the cell cycle, differentiation, and apoptosis have already been reported (6–14). It is widely appreciated that the program of apoptosis is retained in many different types of leukemias and solid tumors and that apoptosis contributes to the tumor response to anticancer agents. It has been widely accepted that a disturbance of the apoptotic pathway is implicated in the acquisition of a malignant phenotype. On the other hand, it would seem that sufficient attention has not been paid to the relationship between senescence and neoplastic transformation, especially in fusion gene–related tumors.

Previously, we have reported that the use of antisense oligodeoxyribonucleotides to suppress EWS-Fli1 expression significantly reduced the growth of tumor cells both in vitro and in vivo and that EWS-Fli1 modulates the cell cycle regulatory genes, such as cyclin D1, cyclin E, p21, and p27, whereas the inhibition of EWS-Fli1 expression resulted in arrest in the G1 phase of the cell cycle (6–9, 14). Notably, a recent study indicated that p27 plays an important role in the inhibition of tumor development and shows the characteristics of a tumor suppressor gene (15). Moreover, p27 has been reported to be related with cellular senescence, especially premature senescence (16–18). Because we have previously reported the prognostic and therapeutic relevance of p27 in EFTs (8), it is very interesting to investigate whether EWS-Fli1 could be intimately related to p27 or senescence.

Herein, we propose a new concept that EWS-Fli1 has an antisenescent function. In this study, we report that small interfering RNAs (siRNA) against the breakpoint of EWS-Fli1 mRNA might be a very efficient agent with which to inhibit the expression of EWS-Fli1 and the growth of EFT cells, and that EWS-Fli1 might have functions that prevent the induction of senescence in cells through the promotion of Skp2-mediated and 26S proteasome–dependent degradation of p27 protein. To improve our understanding of the role and clinical relevance of p27 and Skp2 in EFTs, we immunohistochemically studied their expression in 25 patients. The present study provides a new insight suggesting that the evasion of senescence is one of the important mechanisms of oncogenesis of EFTs by EWS-Fli1.

Materials and Methods
Construction of siRNA duplexes. We purchased 21 nucleotides of siRNAs corresponding to the sequence of the breakpoint of EWS-Fli1 type I (siBPEFI; 5'-GGCGACGAGACCCUUCAUUAGCUU-3'), type II (siBPEFII; 5'-GCCCGACGAGACCCUUCAUUAGCUU-3'), 21 nucleotides of siRNA corresponding to the sequence within the 5' upstream half of EWS-Fli1 mRNA (siEWS1; 5'-GGCGACGAGACCCUUCAUUAGCUU-3'), 21 nucleotides of siRNA corresponding to the sequence within the 5' upstream half of EWS-Fli1 mRNA (siEWS1; 5'-GGCGACGAGACCCUUCAUUAGCUU-3'), siRNA for p27 (sip27; 5'-GGACGCAUGUGCGAGAAUAUU-3'), the scrambled siRNA (siScr) was used as a control from Dharmacon (LaFayette, CO). Transfection of siRNA was carried out with OligofectAMINE (Invitrogen, Rockville, MD) according to the protocol of the manufacturer. Cotransfection of expression vectors and siRNAs was carried out with LipofectAMINE 2000 (Invitrogen) as described by the manufacturer for EFT cells or HEK 290 cells. To ensure that the target molecules were efficiently depleted, cells for apoptosis-related assays and senescence-associated β-galactosidase staining assay were transfected twice with the corresponding siRNA, with the second transfection 4 days after the first transfection.

Cell lines and cell culture. The EFT cell lines SK-N-MC, WE-68, PNKT-1, and RD-ES were cultured as described previously (8, 19). SK-N-MC, PNKT-1, and WE-68 have EWS-Fli1 type I fusion, whereas RD-ES has the type II
fusion. Human embryonic kidney HEK-293 cells and a breast carcinoma cell line MCF-7 obtained from the American Type Culture Collection (Rockville, MD) were cultured at 37°C, 5% CO2 in DMEM (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Invitrogen). The subtypes of EWS-Fli1 in the EFT cells were determined by reverse transcription-PCR (RT-PCR) and sequencing. The number of cells was counted using a COULTER Hematology Analyzer (Beckman Coulter, Fullerton, CA).

**Microscopy.** For immunofluorescence microscopy, the cell cultured on the chamber slides were fixed with 4% formaldehyde in PBS for 10 minutes at room temperature and permeabilized with 0.1% Triton X-100 (Sigma, St. Louis, MO) in PBS, and then filamentous actin was stained for 1 hour with 10 μL of rhodamine-phalloidin (Invitrogen) in 200 μL PBS (0.3 μmol/L final concentration), and washed extensively with PBS as described previously (20). The cells were examined using confocal laser scanning microscopy (Olympus, Tokyo, Japan).

**Western blot analysis, immunoprecipitation, and cdk2 kinase activity assay.** Western blot analysis, cell extract preparation, immunoprecipitation, and cdk2 kinase activity assay using histone-H1 as a substrate were carried out exactly as described previously (6–9). Nuclear extracts were prepared as described previously (9). The antibodies used were as follows: mouse monoclonal anti-human p27 (Kip1; Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-human p27, phospho-specific (Thr187; Calbiochem, San Diego, CA), rabbit polyclonal anti-human Fli1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antipoly(A) ribose) polymerase (Roche, Mannheim, Germany), rabbit polyclonal anti-human cdk2 (Santa Cruz Biotechnology), rabbit polyclonal anti-human Skp2 (Santa Cruz Biotechnology), mouse monoclonal antiactin (Chemicon, Temecula, CA), and horseradish peroxidase–conjugated antimouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology).

**Real-time quantitative RT-PCR.** Real-time quantitative RT-PCR (TaqMan PCR) was done as described previously (8). The primers used for the detection of EWS-Fli1 transcripts were the forward primer 5'-GGCA-GCGGCTCTCCAGTAC-3' and the reverse primer 5'-CATGGGTCTCTTCTCT-TGAGT-3'. The sequence of the TaqMan probe used to quantify the RT-PCR products of EWS-Fli1 was 5'-(Fam)CCACCCAAAACCTGGATCTCA-CCGG(TAMRA)-3'. The standard DNA templates containing EWS-Fli1 type I and type II were generated by PCR and subcloned into the pCR 2.1 TOPO (Invitrogen). The mixtures of the primers and the probe for glycolaldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were purchased from PE Applied Biosystems (Foster City, CA). The relative amount of EWS-Fli1 was standardized against the amount of GAPDH mRNA (8).

**Senescence-associated β-galactosidase staining.** Cells were seeded onto the slide chamber in appropriate media, transfected with siRNAs (100 nmol/L). Senescence-associated β-galactosidase staining was done as described previously (21). Senescence was scored by determining the percentage of the population that exhibited a senescence-associated β-galactosidase activity (21).

**Vectors.** The expression vectors containing human wild-type p27 cDNA and p27 mutant cDNA, in which methionine at amino acid position 187 and isoleucine at the position 188 are substituted for threonine and proline, respectively (p27mt), were purchased from InvivoGen (San Diego, CA). An expression vector containing human Skp2 cDNA (22), a gift from Dr. Masaki Mori (Departments of Molecular and Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan), was digested with EcoRI/XhoI and the excised cDNA was ligated to the Kpn1/EcoRI site of the Xpress-tagged expression system pCNA51/His, containing an aminoterminal Xpress-epitope (Invitrogen). Expression vectors for EWS-Fli1 were gifts from Dr. C.T. Denny (Gwynne Hazen Cherry Memorial Laboratories, University of California at Los Angeles, Los Angeles, CA).

**Transfection of human cells.** WE-68 cells were transfected after transfection of siScr or siBPEFI were treated with 10 μg/mL cycloheximide for various times. Cell lysates were immunoblotted for EWS-Fli1, p27, and actin protein. Densitometry was done by means of the NIH Image version 1.61 to quantify relative amounts of protein detected on Western blot analysis.

**Statistics.** With regard to statistics about senescence-associated β-galactosidase–associated experiments in vitro and relationship between Skp2 protein and p27 protein expression in EFTs (Table 1), P value calculations were done with Mann-Whitney test and Fisher's exact test, respectively (StatView). Differences were considered significant when P < 0.05.

### Results

**Reduction of EWS-Fli1 protein and mRNA expression by siRNA molecules in dose-dependent, time-dependent, and sequence-specific manners.** To knock down the expression of EWS-Fli1 type I (siBPEFI) or type II (siBPEFI). We also designed siEWS1, which is a 21-nucleotide siRNA corresponding to the sequence within the 5' upstream half of EWS-Fli1 mRNA (i.e., 21-nucleotide siRNAs corresponding to EWS mRNA) because we had previously found that antisense oligodeoxynucleotides directed against the sequence within the 5' upstream half of EWS-Fli1 mRNA was more effective for inhibiting EFT cell growth than that corresponding to the sequence of the breakpoint of EWS-Fli1 type I mRNA and that such EWS-targeting antisense oligodeoxynucleotides did not affect the growth of cells without the EWS-Fli1 chimeric gene (14).

To examine whether siRNAs would abolish the expression of EWS-Fli1, the effects of siRNAs on the protein and mRNA levels of EWS-Fli1 were examined by Western blot analysis and real-time quantitative RT-PCR (TaqMan PCR assay), respectively. Treatment with siBPEFI decreased the EWS-Fli1 protein expression in EFT cells with the type I fusion in both a time- and dose-dependent manner (Fig. 1A, left and middle, respectively). Treatment with siBPEFI decreased the EWS-Fli1 expression in WE-68 harboring the type II fusion, whereas siEWS1 decreased EWS-Fli1 protein expression much more effectively than siBPEFI did (Fig. 1A, right).

The protein levels of EWS-Fli1 started to decrease within 12 hours after the transfection of siBPEFI and had been suppressed over the entire 8 days (Supplementary Fig. S1B and C). Consistent with our previous experiments using antisense oligodeoxynucleotides (7, 8), the suppression of EWS-Fli1 expression using siRNAs in WE-68, SK-N-MC, PNKT-1, and RD-ES resulted in the accumulation of p27 protein, a cyclin-dependent kinase inhibitor, in both dose- and time-dependent manners (Fig. 1A; see also Fig. 4D). The expression level of p27 protein started to increase at least at 16 hours after the transfection of siBPEFI and continued to be high over the entire 8 days (Supplementary Fig. S1B and C). For EWS-Fli1 chimeric transcripts in EFT cells, real-time quantitative RT-PCR revealed that the level of EWS-Fli1 mRNA decreased to <20% of that of the control and had been suppressed over the entire 8 days (Fig. 1B; Supplementary Fig. S1D). Consistent with the data from Western

| Table 1. Relationship between Skp2 protein and p27 protein expression in EFTs |
|---------------------------------|------------------|
| **p27 LI** | **Skp2 LI** |
| **Negative** | **Positive** |
| Negative | 4 | 10 |
| Positive | 9 | 2 |

**NOTE:** P = 0.0154, Fisher's exact test.

**Abbreviation:** LI, labeling index.
EWS-Fli1 Prevents Cells from Senescence

Figure 1. A, lysates from EFT cells treated with various concentrations of siRNAs for the required time were prepared. Time course (left) and dose-dependent (middle) expressions of EWS-Fli1 and p27 protein in WE-68 after transfection of siRNAs were examined by Western blot analysis. In the dose-dependent experiment, lysates from WE-68 cells treated with various concentrations of siRNAs were prepared 48 hours after transfection. The expression of EWS-Fli1 and p27 protein in RD-ES harboring the EWS-Fli1 type II fusion gene was examined 48 hours after transfection with 100 nmol/L siRNAs (right). Actin protein was used as a loading control throughout the experiments. B, relative amount of EWS-Fli1 mRNA to GAPDH mRNA. RNAs from EFT cells treated with various concentrations of siRNAs for the required time as indicated in the figure were prepared and subjected to real-time quantitative PCR as described in Materials and Methods. Time course expressions of EWS-Fli1 mRNA in WE-68 after transfection with 100 nmol/L of siRNAs (left) were quantified. Dose-dependent expression of EWS-Fli1 mRNA in WE-68 (middle) and the expression of EWS-Fli1 mRNA in RD-ES cells (right) were also quantified 48 hours after transfection of siRNAs. Columns, amount of EWS-Fli1 mRNA extracted from cells treated with siScr (black) and EWS-Fli1–targeting siRNAs (white).

blot analysis, siEWS1 suppressed EWS-Fli1 mRNA expression in RD-ES cells much more effectively than siBPEFII did; however, the difference was not significant. The results for Western blot analysis and RT-PCR in siScr-transfected cells were almost the same as those in untreated cells throughout the experiments. These results indicate that EWS-Fli1–targeting siRNAs suppress EWS-Fli1 expression in dose-dependent, time-dependent, and sequence-specific manners, while also up-regulating p27 expression.

Knockdown of EWS-Fli1 caused inhibition of EFT cell proliferation and elicited senescence-like phenotype. We next investigated the effects of siRNA on the growth of EFT cells because we had previously reported that antisense oligodeoxynucleotides against EWS-Fli1 mRNA inhibited the growth of EFT cells both in vitro and in vivo but not that of non-EFT cells (14). siBPEFII specifically and dose-dependently inhibited the proliferation of WE-68 with EWS-Fli1 type I but not that of RD-ES with the type II fusion (Fig. 2A and B). We obtained similar results using two other EFT cells with the type I fusion, SK-N-MC and PNKT-1 (data not shown). On the other hand, siBPEFII specifically inhibited the proliferation of RD-ES but not the proliferation of WE-68 or any other cells expressing EWS-Fli1 type I (Fig. 2B). siEWS1 was also effective in inhibiting EFT cell growth (Fig. 2B). Although siEWS1 lost the specificity for the fusion types, siEWS1 effectively inhibited the growth of RD-ES and WE-68 cells compared with siBPEFI and siBPEFII, respectively (Fig. 2B). None of the EWS-Fli1–targeting siRNAs affected the growth of MCF-7, a breast carcinoma cell line without the EWS-Fli1 fusion gene (data not shown). siScr used as negative control did not affect the proliferation of the EFT cells (Fig. 2A and B). We concluded that both the siRNA that was directed against the EWS gene and those that were directed against the breakpoint of the fusion gene were able to inhibit the proliferation of EFT cells through the suppression of EWS-Fli1 expression.

It remains an unsettled question as to why the knockdown of EWS-Fli1 expression by siRNAs could result in the inhibition of EFT cell growth. Because there have been some reports suggesting that inhibition of the expression of another oncogenic fusion gene, BCR-ABL, leads to the induction of apoptosis in chronic myeloid leukemia (23), we examined whether the knockdown of EWS-Fli1 could also result in apoptosis in EFT cells. To detect apoptotic cell death in EFT cells, we carried out a DNA fragmentation assay. Western blot analysis for the detection of cleaved PARP protein, and the differential staining of WE-68 cells with Annexin V protein and propidium iodide through dual-color "bivariate" flow cytometry. None of these three methods detected the cell death of EFT cell lines treated with EWS-Fli1–targeting siRNAs (Supplementary Fig. S1A, B, and E). Overall, we concluded that, by itself, the knockdown of EWS-Fli1 expression would not elicit apoptotic cell death in our EFT cells.

Whereas apoptotic cells show distinct morphologic features, including membrane blebbing, chromatin condensation, and cell shrinkage (24), the WE-68 cells treated with siBPEFI did not display such apoptosis-related features. Rhodamine-labeled phalloidin staining revealed that WE-68 and SK-N-MC treated with siBPEFI and RD-ES treated with siEWS1 for 3 days displayed a flattened and enlarged morphology, which could imply a senescence-like phenotype (Fig. 2C; Supplementary Fig. S2A). The siScr-treated cells did not display any morphologic changes. To confirm whether these morphologic changes are associated with senescence-like phenotype, we carried out senescence-associated β-galactosidase staining, a well-established biomarker of senescence (21). The WE-68 cells treated with siBPEFI started to be positively stained for senescence-associated β-galactosidase 3 days after the transfection in a dose- and time-dependent manner, whereas the cells treated with siScr did not show the expression of senescence-associated β-galactosidase (Figs. 2D and 3E; Supplementary Fig. S2B). We obtained similar results using PNKT-1 (Supplementary Fig. S2D). The cells transfected with siBPEFI did not proliferate over the entire 8 days (Supplementary Fig. S2C). Taken together, these subacute, morphologic, and biochemical changes imply that EWS-Fli1–targeting siRNAs caused EFT cells to fall into premature senescence, thereby suggesting that EWS-Fli1 plays an important role in preventing this from happening.

p27-Dependent senescence induced by EWS-Fli1–targeting siRNA in EFT cells. In previous reports, we revealed that the down-regulation of EWS-Fli1 expression resulted in the accumulation of p27 protein in several EFT cells (7, 8). Furthermore, because p27 has been reported to be related to cellular senescence,
Because p27 is a potent inhibitor of cdk2 and has been linked to cell cycle arrest (25), we examined whether an increase in the expression of p27 protein caused by the depletion of EWS-Fli1 expression could suppress cdk2 kinase activity. The protein level of p27 was readily increased in siBPEFI-treated WE-68 cells when compared with PBS-treated or siScr-treated cells, whereas the total intracellular net amount of cdk2 protein did not change with these treatments (Fig. 3A). However, an immunoprecipitation assay using a specific antibody against cdk2 revealed that the cdk2 complex contained p27 protein in siBPEFI-treated WE-68 cells, but not in PBS- or siScr-treated WE-68 cells (Fig. 3B). Furthermore, the subsequent cdk2 kinase activity assay using histone-H1 as the substrate revealed that in comparison with the controls, cdk2 kinase activity was completely abolished 3 days after the transfection of siBPEFI in WE-68 cells (Fig. 3C).

Next, we designed a 21-nucleotide siRNA against human p27 mRNA (sip27) and introduced it into EFT cells. Whereas siBPEFI alone depleted EWS-Fli1 protein and caused accumulation of p27 protein 3 days after transfection in WE-68, the cotransfection of sip27 with siBPEFI prevented the accumulation of p27 protein in a dose-dependent manner (Fig. 3D). As we expected, the cotransfection of sip27 with siBPEFI decreased WE-68 and PNKT-1 cells, which were positively stained for senescence-associated β-galactosidase in a dose-dependent manner 8 days after transfection, whereas sip27 alone did not make these cells senescent (Fig. 3E and F). These findings suggest that EWS-Fli1 protein might be able to evade premature senescence, at least in part, via the suppression of p27 protein expression. Taken together, these data indicate that transition from a proliferative to a quiescent phenotype apparently requires at least the abolition of cdk2 kinase activity by p27 protein.

**EWS-Fli1 promotes the proteolysis of p27 protein via an Skp2-mediated mechanism.** Our next concern is how EWS-Fli1 regulates p27 expression in EFT cells. In our previous study, we reported that EWS-Fli1 promotes the 26S proteasome-dependent degradation of p27 protein (8). Recently, it was found that p27 protein levels in breast cancer cells can depend on its subcellular localization, which would be regulated through the phosphorylation status of p27 protein (26, 27). Therefore, we examined the
Localization and the phosphorylation status of p27 in EFT cells after the treatment of EWS-Fli1–targeting siRNAs. In the result, Western blot analysis of fractionated cytoplasmic and nuclear lysates of EFT cells transfected with PBS, siScr, or EWS-Fli1–targeting siRNAs for 3 days revealed that depletion of EWS-Fli1 protein resulted in the accumulation of p27 protein at not only the nuclear fraction but also the cytoplasmic fraction (Fig. 4A). Subsequently, Western blot analysis of the phosphorylation of p27 on Thr<sup>187</sup> in WE-68 cells using a commercially available antibody revealed that depletion of EWS-Fli1 protein in WE-68 resulted in a marked accumulation of p27 protein; however, it did not affect the amount of phosphorylated p27 protein (Fig. 3A). Next, we examined whether the phosphorylation of p27 on Thr<sup>187</sup> residue would be required for the EWS-Fli1 effect because it has been reported that mutation of amino acids Thr<sup>187</sup>/Pro<sup>188</sup> to Met<sup>187</sup>/Ile<sup>188</sup> in p27 protein (p27<sub>mt</sub>) is resistant to protein degradation in lung cancer cells (28). HEK293 cells cotransfected with EWS-Fli1 and wild-type p27 expression vectors (p27WT)
of p27 protein in the late G1 phase of the cell cycle (29). It is ubiquitination by F-box protein Skp2 and subsequent degradation of protein synthesis (Fig. 4). To assess whether Skp2 can mediate premature senescence in EFT cells, we attempted to change the expression level of Skp2 protein in EFT cells. To increase Skp2 protein in EFT cells, we introduced an Skp2 expression vector into EFT cells. The forced expression of Skp2 resulted in a decrease in the p27 protein level in EFT cells caused by EWS-Fli1–targeting siRNAs (Fig. 5A). A senescence-associated β-galactosidase assay revealed that the forced expression of Skp2 could partially, but significantly, revert senescence-like phenotype in EFT cells caused by EWS-Fli1–targeting siRNAs (Fig. 5B and C). These data suggest that Skp2 would mediate the prevention of premature senescence in EFT cells through the degradation of p27 protein.

Collectively, we concluded that EWS-Fli1 might promote the proteolysis of p27 protein via the Skp2-mediated mechanism.

**Skp2 mediates p27-related premature senescence in EFT cells.** To assess whether Skp2 can mediate premature senescence in EFT cells, we attempted to change the expression level of Skp2 protein in EFT cells. To increase Skp2 protein in EFT cells, we introduced an Skp2 expression vector into EFT cells. The forced expression of Skp2 resulted in a decrease in the p27 protein level in EFT cells caused by EWS-Fli1–targeting siRNAs (Fig. 5A). A senescence-associated β-galactosidase assay revealed that the forced expression of Skp2 could partially, but significantly, revert senescence-like phenotype in EFT cells caused by EWS-Fli1–targeting siRNAs (Fig. 5B and C). These data suggest that Skp2 would mediate the prevention of premature senescence in EFT cells through the degradation of p27 protein.

**Inverse patterns of Skp2 and p27 protein expression in EFTs.** To examine the clinical importance of Skp2 protein expression, we analyzed the expressions of Skp2 protein and p27 protein using an immunohistochemical staining method in tissue samples obtained from primary EFTs. The relationship between p27 protein expression and Skp2 protein expression was examined in 25 cases of EFTs. The typical immunohistochemistry patterns for p27 and Skp2 in EFTs are shown in Supplementary Fig. S3. Nine of 13 cases in the Skp2-negative group were also in...
the p27-positive group, whereas 10 of 12 cases in the Skp2-positive group were also in the p27-negative group (Table 1). This indicates that Skp2 protein expression is inversely correlated with p27 protein levels in EFT \( (P = 0.0154) \). In EFT tissues, samples positive for Skp2 showed no or very low levels of p27 protein and vice versa, implying that there is an inverse relationship between the expression profiles of Skp2 and p27 proteins.

### Discussion

In this study, we revealed that siRNAs inhibited the expression of the EWS-Fli1 chimeric gene in a sequence-specific manner and the growth of EFT cells in vitro and that the growth of EFT cells mostly depended on the function of EWS-Fli1 because the breakpoint-specific siRNAs only affected those cells with targeted nucleotide sequences and the inhibition ratio of cell growth was parallel with the reduction in EWS-Fli1 expression. We concluded that RNA interference would seem to be a powerful strategy for the gene silencing of EWS-Fli1 in EFTs.

Whereas the possible mechanisms of EWS-Fli1 in tumorigenesis have been reported, few attempts have been made at clarifying the relation between EWS-Fli1 and apoptosis (10, 12, 13). At first, we carried out three different methods to detect apoptotic cell death to elucidate the mechanism of the growth inhibition by EWS-Fli1–targeting siRNAs. However, we found that the mere knockdown of EWS-Fli1 does not result in apoptotic cell death and necrotic cell death. Therefore, we concluded that EWS-Fli1–targeting siRNAs by themselves could not always lead EFT cells to apoptosis.

Instead, potent EWS-Fli1–targeting siRNAs caused all four EFT cell lines used in this study to become flattened and have enlarged morphology. As we expected that this morphologic change might imply cellular senescence, two of the four EFT cell lines treated with EWS-Fli1–targeting siRNAs were positively stained for senescence-associated \( \beta \)-galactosidase although all of them displayed the flattened and enlarged morphologic change. Because plural EWS-Fli1–targeting siRNAs caused senescence-like phenotype in WE-68 and PNKT-1, we conclude that EWS-Fli1 must definitely be involved in cellular senescence. However, the remaining RD-ES cells and SK-N-MC cells treated with EWS-Fli1–targeting siRNAs were rarely and never stained for senescence-associated \( \beta \)-galactosidase, respectively, although they also displayed the flattened and enlarged morphologic change. Although it has been widely accepted that senescence-associated \( \beta \)-galactosidase staining would be a classic marker of senescent cells (21), senescence-associated \( \beta \)-galactosidase staining might not necessarily be a sensitive strategy for the detection of senescence (30). The mechanism and markers for apoptosis have been genetically and biochemically investigated and established, whereas those for senescence have remained ambiguous. If more sensitive methods for the detection of senescence were to be developed, then they might reveal the senescent status of RD-ES and SK-N-MC cells with the flattened and enlarged morphologic change caused by the depletion of EWS-Fli1 expression. By way of conclusion, we emphasize that EWS-Fli1 protein might bring about the evasion of senescence in EFT cells.

It is very important to reveal how EWS-Fli1 could evade falling into premature senescence. There are reports stating that oncogenic fusion genes, such as EWS-Fli1, prevent fusion gene–related tumors from suffering apoptotic cell death (10, 23); however, a concept whereby such fusion genes might prevent the tumor cells from falling into cellular senescence has rarely been considered (17). It might be important for the products of many fusion genes resulting from chromosomal translocation to have the function of evading senescence, which would then allow oncogenesis to take place. It has been stated that evasion of senescence as well as apoptosis are important events in the production of cancer (31, 32). Recently, it has been reported that the acquisition of ability to escape or recover from chemotherapy-induced cellular senescence is one of major causes for cancer recurrence or progression (33–35). Several genes have been reported to be related to the

![Figure 5. Skp2 mediates p27-related cellular senescence in EFT cells. A. Western blot analysis of lysates from PNKT-1 cells cotransfected with siRNAs and Skp2 expression vector for 3 days. PBS, 100 nmol/L of siScr, or siBPEFI with 4 \( \mu \)g Skp2 expression vector or empty vector (+ or –) were cotransfected into PNKT-1, and the expression levels of the indicated proteins were detected by Western blot analysis. 1/2, 2 \( \mu \)g Skp2 expression vector. Filled arrowhead, exogenous tagged Skp2 protein; open arrowhead, endogenous Skp2 protein. B, representative micrographs of PNKT-1 stained for senescence-associated \( \beta \)-galactosidase to determine senescent cells. Senescence-associated \( \beta \)-galactosidase staining was carried out 8 days after cotransfection into PNKT-1 cells of PBS, 100 nmol/L of siScr, or siBPEFI with Skp2 expression vector or empty vector. Bars, 100 \( \mu \)m. C (columns), the percentages of senescence-associated \( \beta \)-galactosidase staining positive cells shown in (B); bars, SD. The forced expression of Skp2 significantly reverted senescence-associated \( \beta \)-galactosidase staining positive cells in EFT cells caused by siBPEFI. *, \( P = 0.0495 \); Mann-Whitney test.](#)
phosphorylation of p27 on Thr187 residue by cyclin-cdk2 complex. We have also found that antiparallel with the protein by EWS-Fli1 depends on the phosphorylation of p27 on (29, 40, 42). In this report, we found that down-regulation of p27 considered to be very stable and accumulated in EWS-Fli1–depleted EFT cells, a reduction in Skp2 protein might fail to degradation of p27 protein by the 26S proteasome pathway. In EWS-ubiquitination of p27, which is a prerequisite step for the targeted expression of Skp2 or stabilize Skp2 protein. Presumably, EWS-tested, which implies that EWS-Fli1 might up-regulate the accumulation of p27 protein, the expression of F-box protein Skp2, or maintaining cellular senescence. We propose that by actively reducing the level of p27 protein in EFT cells, EWS-Fli1 might be able to alleviate the effect of p27 on its major targets, cyclins/cdk2 complexes. As such, the increased cyclin/cdk2 activities might prompt the progression of the cell cycle and prevent EFT cells from falling into senescence. This led us to arrive at our conclusion that EWS-Fli1 might evade premature senescence through the suppression of p27 expression.

We have reported that EWS-Fli1 might attenuate the p27 protein level via the activation of the protease-mediated degradation pathway (8); however, the precise mechanism is still unclear. Many researchers, including ourselves, have reported that the concentration of p27 protein in cells is mainly regulated by posttranslational mechanisms (8, 40–43). F-box protein Skp2, the substrate-specific ubiquitin ligase of the SCF complex (Skp1/Cul1/F-box), is responsible for ubiquitin-dependent degradation of p27 protein both in vitro and in vivo. Binding of Skp2 to p27 protein requires phosphorylation of p27 on Thr residue by cyclin-cdk2 complex (29, 40, 42). In this report, we found that down-regulation of p27 protein by EWS-Fli1 depends on the phosphorylation of p27 on Thr residue. We have also found that antiparallel with the accumulation of p27 protein, the expression of F-box protein Skp2, the substrate-specific E3 ubiquitin ligase of the SCF complex, was selectively down-regulated in all the EWS-Fli1–depleted EFT cells tested, which implies that EWS-Fli1 might up-regulate the expression of Skp2 or stabilize Skp2 protein. Presumably, EWS-Fli1 might increase and permit Skp2 protein to accelerate the ubiquitination of p27, which is a prerequisite step for the targeted degradation of p27 protein by the 26S proteasome pathway. In EWS-Fli1–depleted EFT cells, a reduction in Skp2 protein might fail to polyubiquitinate p27 protein. Accordingly, p27 protein can be considered to be very stable and accumulated in EWS-Fli1–depleted EFT cells, which would result in the inhibition of cyclins/cdk2 kinase activity. The precise process of regulation of Skp2 expression by EWS-Fli1 warrants further investigation.

In senescence-related experiments, Skp2 plays an important role in the prevention of premature senescence by EWS-Fli1 in EFT cells. Skp2 reduced the expression of p27 protein and partially prevented EFT cells from premature senescence caused by EWS-Fli1–targeting siRNAs. We suppose that Skp2 could be substituted for EWS-Fli1. Taken together, it seems that EWS-Fli1 promotes the degradation of p27 protein and prevents cellular senescence through the Skp2-mediated and 26S proteasome–dependent mechanism in EFT cells.

It has been reported that an inverse correlation between Skp2 and that p27 was found in several malignant tumors (44, 45). In the present study, we also showed that Skp2 protein levels were inversely correlated with p27 protein levels in EFTs in this report. Although the survival curves drawn according to the Kaplan-Meier method showed that patients with EFTs, which were positive for Skp2, tend to have a worse prognosis than patients whose tumors were negative for Skp2, an analysis of survival using the log-rank test revealed that the difference was not significant (data not shown). Skp2 is irrefutably linked to proteolysis of p27 protein, but it is not the only molecule needed for the regulation of p27 protein (46). Therefore, we concluded that Skp2 might have an important consequence for EFTs patients; however, other mechanisms would be involved in the proteolysis of p27 protein.

In summary, the present study showed that EWS-Fli1 selectively accelerates p27 protein degradation by the Skp2-dependent 26S proteasome pathway and finally prevents EFT cells from falling into premature senescence. The findings of this report are very significant, not only because of the important role of EWS-Fli1 in the evasion of premature senescence but also because of the novel concept that oncogenic fusion genes have an important role in the prevention of senescence, as well as apoptosis. Insight into the mechanism behind senescence may provide a promising lead to the development of therapeutic strategies for the treatment of EFTs.

Acknowledgments

Received 6/7/2006; revised 9/29/2006; accepted 11/10/2006.


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We thank Dr. F. van Valen (Department of Orthopaedics, University Hospital, Munster, Germany) for VII-64 and WE-68, Dr. Yoshikiko Maehara (Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan) for the ABI PRISM 7700 Sequence Detection System, and Hiroko Eguchi and Yoko Yagawa for their technical assistance.

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