Tumorigenesis and Neoplastic Progression

Function of *EWS-POU5F1* in Sarcomagenesis and Tumor Cell Maintenance

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POU5F1 is a transcription factor essential for the self-renewal activity and pluripotency of embryonic stem cells and germ cells. We have previously reported that *POU5F1* is fused to *EWSR1* in a case of undifferentiated sarcoma with chromosomal translocation t(6;22)(p21;q12). In addition, the *EWS-POU5F1* chimeras have been recently identified in human neoplasms of the skin and salivary glands. To clarify the roles of the *EWS-POU5F1* chimera in tumorigenesis and tumor cell maintenance, we used small-interfering RNA-mediated gene silencing. Knockdown of *EWS-POU5F1* in the t(6;22) sarcoma-derived GBS6 cell line resulted in a significant decrease of cell proliferation because of G1 cell cycle arrest associated with p27^{Kip1} up-regulation. Moreover, senescence-like morphological changes accompanied by actin polymerization were observed. In contrast, *EWS-POU5F1* down-regulation markedly increased the cell migration and invasion as well as activation of metalloproteinase 2 and metalloproteinase 14. The results indicate that the proliferative activity of cancer cells and cell motility are discrete processes in multistep carcinogenesis. These findings reveal the functional role of the sarcoma-related chimeric protein as well as POU5F1 in the development and progression of human neoplasms. (Am J Pathol 2010, 176:1973–1982; DOI: 10.2353/ajpath.2010.090486)

POU5F1/OCT4 is an essential transcription factor for the formation and/or maintenance of the inner cell mass of the mammalian blastocyst, the origin of pluripotent em-

bryonic stem (ES) cells.^{1–3} Suppression of *POU5F1* expression converts ES cells to trophoblasts, whereas overexpression of *POU5F1* leads to differentiation toward endoderm and mesoderm.^{3,4} The self-renewal activity and pluripotency of ES cells are suppressed by knockdown of *POU5F1*.⁵ These data suggest that POU5F1 orchestrates target gene expression in a tightly regulated manner during development and cellular differentiation. Also, POU5F1 induces reprogramming of somatic cells into iPS cells in combination with Sox2, c-Myc, and Klf4.⁶ Moreover, two factors, either POU5F1 and Klf4 or POU5F1 and c-Myc, are apparently sufficient to generate iPS cells.⁷

In carcinogenesis, up-regulated expression of *POU5F1* is significantly correlated to certain lineages of human malignancies including germ cell tumors and breast and bladder cancer.^{8–11} Reactivation of POU5F1 in somatic cells may induce dedifferentiation and may disrupt homeostasis, resulting in malignant transformation. Direct involvement of POU5F1 has been detected in a case of undifferentiated bone sarcoma with t(6;22)(p21;q12) translocation in which *POU5F1* is fused to *EWSR1*.¹² The chimeric *EWS-POU5F1* protein is composed of a transactivation domain of EWS and the entire DNA-binding domain of POU5F1. Ectopic overexpression of the POU5F1 component is achieved by the strong promoter activity of *EWSR1*.¹² Similar gene fusions between *EWSR1* and *POU5F1* have been identified in hidradenoma of the skin and mucoepidermoid carcinoma of the salivary glands.¹³ These results underscore the important role of dysregulated *POU5F1* expression in human cancer and the important contributions of *EWS-POU5F1* to the development and maintenance of cancer cells.

In this study, we knocked down *EWS-POU5F1* by using *POU5F1*-specific small-interfering RNAs (siRNAs) in

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the GBS6 cell line established from the t(6;22) undifferentiated sarcoma.¹² Cellular growth was significantly suppressed by *EWS-POU5F1* depletion and was accompanied by up-regulation of p27^{Kip1} expression, and senescence-like morphological alterations were observed. On the other hand, cell motility and invasive capacity were dramatically increased, and promotion of actin polymerization and activation of metalloproteinase (MMP)14 and MMP2 were observed. These results suggest that *EWS-POU5F1* promotes proliferation of cancer cells but is dispensable for or even inhibits cell motility and invasiveness. This study provides important insights into *EWS-POU5F1* function in carcinogenesis and tumor cell maintenance.

Materials and Methods

Cell Culture

The GBS6 cell line was established from a pelvic bone undifferentiated sarcoma with t(6;22)(p21;q12).¹² The cells were maintained at 37°C under 5% CO₂ in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10 mmol/L of HEPES buffer, pH7.4. NIH3T3, HeLa, and HCT116 cells were grown at 37°C under 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

RNA Interference and DNA Transfection

RNA interference and DNA transfection experiments were performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). GBS6 cells were seeded on 12-well plates 24 hours before transfection at a density of 1×10^5 or 2.5×10^5 cells per well for siRNAs or plasmid DNAs, respectively. GBS6 cells were then transfected with 60 pmol or 1.6 μ g of siRNAs or plasmids, respectively. The following siRNAs were purchased from Qiagen (Hilden, Germany): siRNA-POU5F1-1 (SI00690389) and siRNA-POU5F1-2 (SI026617) and control (non-sil). A FLAG-tagged p27 expression plasmid was a kind gift from Dr. Kei-ichi Nakayama.

Senescence-Associated β -galactosidase Assay

Senescence-associated β -galactosidase was detected histochemically by using a Senescence Detection Kit (Biovision, Mountain View, CA) 4 days after transfection of siRNAs.

Western Blotting

Whole cell lysates were size-fractionated by SDS-polyacrylamide gel electrophoresis and were transferred onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline (pH 7.5) containing 0.2% Tween 20 and 5% nonfat dry milk. Primary antibodies used were as follows: goat anti-Oct3/4 (1:500 dilution; C-20, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-lamin

A/C (1:500 dilution; Santa Cruz Biotechnology), rabbit anti-p27 (1:200 dilution; Santa Cruz Biotechnology), mouse anti-p53 (1:200 dilution; DO-1, Santa Cruz Biotechnology), mouse anti-p21 (1:100 dilution; BD Biosciences, San Diego, CA), mouse anti-Rb (1:500 dilution; IF8, Santa Cruz Biotechnology), rabbit anti-Phospho-Rb (Ser807/811; 1:500 dilution; Cell Signaling Technology, Beverly, MA), mouse anti-cyclin D1 (1:500 dilution; A-12, Santa Cruz Biotechnology), rabbit anti-CDK2 (1:500 dilution; M2, Santa Cruz Biotechnology), rabbit anti-CDK4 (1:500 dilution; H-22, Santa Cruz Biotechnology), rabbit anti-CDK6 (1:500 dilution; C-21, Santa Cruz Biotechnology), mouse anti-MMP14 (1:200 dilution; Daiichi Fine Chemical, Tokyo, Japan), and mouse anti-RhoA (1:200 dilution; Upstate Biotechnology, Temecula, CA). The signals were detected by using appropriate secondary antibodies and an enhanced chemiluminescence kit (GE Health care, Piscataway, NJ).

Flow Cytometric Analysis

Single cell suspensions were permeabilized with 0.1% triton X-100 in PBS, and 50 mg/ml of propidium iodide and 1 mg/ml of RNase A were added. The cell suspensions were then analyzed by using a FACS-calibur flow cytometer (Beckton Dickinson, Franklin Lakes, NJ) and Modifit software (Beckton Dickinson).

Cell Invasion and Migration Assays

A quantitative invasion assay was performed by using a BD BioCoat Matrigel invasion chamber with 8- μ m pore size membranes (BD Biosciences) according to the manufacturer's instruction. Briefly, cells incubated with siRNAs or plasmid DNAs for 24 hours were trypsinized and resuspended at a density of 1×10^5 cells per 1 ml of RPMI without serum. Cells (5×10^5) were then loaded onto inserts of the upper chambers. RPMI with 10% fetal bovine serum was added to the lower chambers. After 24 hours of incubation, cells on the upper surface membranes were removed gently with a cotton swab. Cells on the lower surface were stained with Wright-Giemsa solutions and air-dried. Cell migration was also evaluated by using the same chambers without Matrigel by assessing the cell numbers within the lower chamber. The invading or migrating cells were counted, and images were obtained by using an Olympus BX41 microscope with a 20 $\times$ objective (Olympus, Tokyo, Japan). For the wound healing assay, GBS6 cells were cultured for 48 hours after transfection of siRNAs to reach 90% confluence in 12-well plates. A linear scratch, 100 μ m in width, was produced by using a plastic tip. Cells were incubated in growth medium for the indicated period. Images were photographed by using an Olympus IX70 phase contrast microscope. The distance of cell migration from the scratch line was measured in micrometers on the photographs.

Gelatin Zymography

Conditioned media from GBS6 cell cultures were harvested 48 hours after siRNA transfection, loaded on 10% gelatin gels (Invitrogen), and electrophoresed. The gels were stained with 0.25% Coomassie Blue and were destained in 5% acetic acid/10% methanol to visualize bands corresponding to the gelatinolytic activity.

Total RNA Extraction, Conventional RT-PCR, and Real-Time Quantitative RT-PCR

Total RNA extraction, reverse transcription, and RNA quantification were performed according to methods described previously.¹⁴ Conventional RT-PCR was performed by using a Gene Amp 9700 thermal cycler (Applied Biosystems, Foster City, CA). We conducted 40 cycles of three-step PCR (95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute) for miR302-367 locus and 25 cycles of three-step PCR (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) for actin. The specific forward and reverse primers for optimal amplification were designed as follows: miR302-367 locus, 5'-GGGCTCCCTTCAACTTTAAC-3' and 5'-ATTCTGTCATTGGCTTAACAATCCATCACC-3'; β -actin, 5'-AGGCATCCTCACCTGAAGTACCC-3' and 5'-GCCAGGTCCAGACGCAGG-3'. Real-time RT-PCR was performed by using a 7500 Fast Real-Time PCR System (Applied Biosystems) using the following parameters: 40 cycles of three-step PCR (95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds). The specific forward and reverse primers to produce approximately 60-bp amplicons for optimal amplification in real-time PCR were designed as follows: MMP2, 5'-CCGCAGTGACGGAAGATGT-3' and 5'-GCCCCACTTGCGGTCAT-3'; MMP14, 5'-CGAGAGGAAGGATGGCAAATT-3' and 5'-AGGGA-CGCCTCATCAAACAC-3'; β -actin, 5'-TGGATCAGCAAG-CAGGAGTATG-3' and 5'-GCATTTGCGGTGGACGAT-3'; and miR302-367 locus, 5'-TTTGAGTGTGGTGGTTCC-TACCT-3' and 5'-AGCCAAGAACTGCACACAGTGT-3'.

Actin Staining

GBS6 cells were seeded onto four-chamber culture slides (BD Biosciences) at a density of 3000 cells per well 24 hours after transfection and were incubated for an additional 24 hours in growth medium. Cells were then fixed and stained with phalloidin-rhodamine (Invitrogen). Images were photographed by using a Leica DM6000B laser scanning microscope with a 40 $\times$ objective (Leica Microsystems, Cambridge, UK).

RhoA Activity Assay

To confirm RhoA activation, the amount of RhoA-GTP bound to the Rhotekin Rho-binding domain (RBD) was determined by using the Rho Activation Assay Kit (Upstate Biotechnology). Forty-eight hours after transfection of siRNAs, whole cell lysates were incubated with Rhotekin RBD-agarose for 45 minutes at 4°C. After washing, agarose beads were resuspended in Laemmli sample

buffer, boiled for 5 minutes, and subjected to immunoblotting with an anti-RhoA antibody.

Microarray

The oligonucleotide array Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA), composed of 38,500 human genes and expressed sequence tags, was hybridized with cRNA probes generated from GBS6 cells 4 days after siRNA transfection and was scanned according to the method previously described.¹⁴ The data were deposited in a public database (<http://www.ncbi.nlm.nih.gov/geo>, accession number: GSE12320, last accessed November 5, 2009). Clustering analysis was performed by using dChip software (<http://biosun1.harvard.edu/complab/dchip/> last accessed October 14, 2009). The gene network was analyzed by Webgestalt (<http://bioinfo.vanderbilt.edu/webgestalt/> last accessed October 31, 2009).

Statistical Analysis

Results were evaluated statistically by using Student's *t*-test. A value of *P* < 0.05 was considered significant.

Results

EWS-POU5F1 Knockdown Induces p27^{Kip1} Up-Regulation and G1 Arrest

The GBS6 cell line was established from a t(6;22) undifferentiated sarcoma that expressed the chimeric *EWS-POU5F1* but not wild-type *POU5F1*.¹² To investigate the biological role of *EWS-POU5F1*, we knocked down *EWS-POU5F1* in GBS6 cells by RNA interference. Effective knockdown of *EWS-POU5F1* on 2 days after transfection was confirmed for two independent *POU5F1*-specific siRNAs (Figure 1A, 88.3% of reduction by siRNA-1 and 85.9% by siRNA-2). The effects of the two siRNAs were similar to each other in every experiment, and the results using siRNA-*POU5F1*-1 are exhibited subsequently as a representative.

Suppression of *EWS-POU5F1* in GBS6 cells was significantly inhibited proliferation, the cell numbers being 58% or 54% of those treated with a control siRNA on day 2 or day 4, respectively (Figure 1B). A terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay did not show an apparent increase of apoptotic cells during treatment with RNA interference (data not shown). This result suggests that the suppression of cell growth might be because of inhibition of the cell cycle. Flow cytometric analysis demonstrated that knockdown of *EWS-POU5F1* significantly decreased the S-phase population and increased the G1 fraction compared with the control (Figure 1C), indicating that cell growth of GBS6 was suppressed because of G1 arrest.

We next examined the expression of a series of cell cycle regulators. Increased expression of p27 was observed in GBS6 cells during *EWS-POU5F1* knockdown (Figure 1D, left). An RT-PCR experiment showed no significant decrease of *p27* mRNA during *EWS-POU5F1*

Figure 1. Knockdown of *EWS-POU5F1* inhibits proliferation of GBS6 cells accompanied by G1 cell cycle arrest and up-regulation of p27. **A:** RNA interference. GBS6 cells were transfected with control or *POU5F1* siRNAs and harvested 2 days after transfection; lysates were subjected to Western blotting by using anti-*POU5F1* antibody. Lamin A/C was used as a control. **Lane 1**, wild-type; **lane 2**, negative control siRNA (non-sil); **lane 3**, *POU5F1* siRNA-1; **lane 4**, *POU5F1* siRNA-2. **B:** Proliferation assay of GBS6 cells treated with non-sil or *POU5F1* siRNA. Mean of relative cell numbers $\pm$ SE of three independent experiments are presented (* $P < 0.005$). **C:** Flow cytometric analysis of GBS6 cells treated with siRNAs. Average percentages of G1, G2, and S phases in three experiments are indicated. * $P < 0.005$. **D:** Western blotting of GBS6 cells transfected with control (–) or *POU5F1* (+) siRNAs by using antibodies specific for the indicated protein. Histone H1 was used as a loading control (**left**); proliferation assay (**right**) of GBS6 cells in which p27 was introduced. Mean of relative cell numbers $\pm$ SE of three independent experiments are presented (*, ** $P < 0.005$). **E:** Western blotting of GBS6 cells and NIH3T3 cells transfected with an empty or *EWS-POU5F1* vectors by using anti-*POU5F1* or anti-p27. **Lane 1**, GBS6 cells; **lane 2**, NIH3T3 treated with an empty vector; and **lane 3**, NIH3T3 treated with an *EWS-POU5F1* expression vector. Ponceau staining and Histone H1 were used as a loading control.

suppression (data not shown), suggesting that the change might be indirect and p27 was not transcriptionally regulated by *EWS-POU5F1*. During knockdown, phosphorylation of Rb protein on Ser807/811 was significantly decreased, whereas expression of total Rb protein remained unchanged (Figure 1D, left). Expression of p21 and p53 was not affected (Figure 1D, left). In addition, a comparative genomic hybridization analysis revealed a homozygous loss of *p16^{INK4A}/p14^{ARF}* (Supplemental Figure S1, see <http://ajp.amjpathol.org>). A significant decrease of cyclin D1 expression was also noted (Figure 1D, left). On the other hand, expression of CDK2, CDK4, and CDK6 was unchanged (Figure 1D, left).

Exogenous introduction of p27 into GBS6 cells resulted in 82% and 61% decreased proliferation compared with the transfected controls on days 2 and 4, respectively (Figure 1D, right). Conversely, exogenous expression of *EWS-POU5F1* in NIH3T3 cells markedly depleted p27 (Figure 1E). However, expression of *EWS-POU5F1* did not affect proliferation of NIH3T3 cells, suggesting that the effect might be cell context-dependent. Taken together, these results indicate that *EWS-POU5F1* supports tumor cell growth, at least in part, through down-regulating the p27^{Kip1} activity.

Induction of the Senescence-Like Morphology by *EWS-POU5F1* Knockdown

GBS6 cells possess a short spindle-shaped morphology with a narrow cytoplasm and a small nucleus with rough heterochromatin (Figure 2A, left), reflecting the original phenotype *in vivo*.¹² After introduction of *POU5F1*-specific siRNAs, we observed prompt enlargement of GBS6 cell bodies. Most GBS6 cells demonstrated large and flat cyto-

plasms as well as enlarged nuclei with fine chromatin 4 days after siRNA transfection. This morphology mimicked that observed in cellular senescence (Figure 2A, right). Most of the GBS6 cells enlarged by *EWS-POU5F1* knockdown expressed senescence-associated β -galactosidase (Figure 2B), a well-established biomarker of senescence.¹⁵ However, senescence-associated heterochromatin foci, another biomarker of senescence,¹⁶ were not observed (data not shown). Importantly, the enlarged GBS6 phenotype (and growth arrest) mediated by *POU5F1*-specific siRNAs disappeared 10 days after transfection when *EWS-POU5F1* expression returned (data not shown). Thus, the change was transient and reversible. These data suggest that the phenotypic changes were not because of senescence but rather indicated G1 arrest. Interestingly, overexpression of p27^{Kip1} did not induce morphological changes in GBS6 cells (data not shown), indicating that different molecular pathways downstream of *EWS-POU5F1* are responsible for the senescence-like morphologies.

Drastic modification of the cytoskeleton was also observed in siRNA-treated enlarged GBS6 cells. Phalloidin staining revealed prominent networks of F-actin throughout the cytoplasm of siRNA-treated cells (Figure 2C, right). Control GBS6 cells showed only a small amount of actin fibers in the cytoplasmic rim (Figure 2C, left). A close link between actin polymerization and a small G protein Rho has been reported.¹⁷ Indeed, a GTP-bound activated form of RhoA protein was apparently increased on *EWS-POU5F1* knockdown (Figure 2D). These data indicate that *EWS-POU5F1* affected the RhoA signaling pathway and morphology of tumor cells by modulating the actin fiber network. Finally, transfection of *POU5F1*-specific siRNA into HeLa cells that do not express *POU5F1* did not affect cell morphology (Figure 2E), indi-

Figure 2. Morphological changes and actin polymerization in siRNA-treated GBS6 cells. **A:** Photomicrographs of GBS6 cells transfected with control siRNA or *POU5F1*-specific siRNA. Papanicolaou staining, $\times 400$ original magnification. Scale bars = 100 μm . **B:** Senescence-associated β -galactosidase (SA- β -Gal) assay 4 days after transfection of siRNAs. Original magnification, $\times 400$. The columns indicate mean % SA- β -Gal-positive cells in three independent experiments ($*P < 0.005$). **C:** Enhanced actin polymerization in *EWS-POU5F1* silent GBS6 cells. F-actin was visualized by phalloidin-rhodamine staining. Original magnification, $\times 400$. Scale bars = 100 μm . **D:** Increased RhoA-GTP in *EWS-POU5F1* silenced GBS6 cells (+) compared with control (-). **E:** *POU5F1* knockdown does not affect morphology of HeLa cells. Papanicolaou staining, $\times 200$ original magnification. Scale bars = 100 μm .

cating that the above findings are not because of non-specific effects of *POU5F1* siRNAs.

Knockdown of *EWS-POU5F1* Promotes Cell Migration and Invasion

Uncontrolled proliferation and metastatic activities are important biological characteristics of cancer.¹⁸ Indeed, in the t(6;22) sarcoma case, the patient died of multiple pulmonary metastases.¹² Therefore, it is intriguing to clarify whether *EWS-POU5F1* promotes cell migration and invasiveness. Migration and invasion of GBS6 cells treated with siRNA for *EWS-POU5F1* were assessed in a Matrigel invasion assay. *EWS-POU5F1* knockdown resulted in marked increases in migration and invasion activities compared with the control GBS6 cells (Figure 3A and Table 1). The original GBS6 cells rarely migrated *in vitro*; however, the number of cells migrating in the absence of *EWS-POU5F1* increased more than 50-fold. The increase in cell motility after *EWS-POU5F1* knockdown was also confirmed by a wound healing assay, showing that GBS6 cells with *EWS-POU5F1*

knockdown migrated 2.5-fold faster than the control cells (Figure 3B).

We next asked whether the enhanced invasiveness of GBS6 cells in Matrigel was solely because of increased cell motility or whether invasiveness itself was also accelerated. Because cell invasion activity is closely associated with increased metalloproteinase activity,^{19,20} MMP2 and MMP9 activities were assessed by gelatin zymography. The zymogram exhibited a significant increase of the gelatinolytic activity of MMP2, whereas the MMP9 activity was not altered (Figure 3C, top). MMP14/MT1-MMP, a membrane-type MMP, activates pro-MMP2 in collaboration with a tissue inhibitor of metalloproteinase 2.^{19,21} An immunoblot analysis demonstrated increased expression of the MMP14 protein (Figure 3C, bottom), consistent with promotion of MMP2 activity. Thus, *EWS-POU5F1* knockdown increased cell motility and also enhanced invasiveness through accelerated degradation of matrix by MMPs.

Real-time quantitative RT-PCR showed that expression of MMP2 and MMP14 was also increased at the RNA level (Figure 3D), suggesting that *EWS-POU5F1* may also

Figure 3. Knockdown of *EWS-POU5F1* promotes cell migration and invasion. **A:** Matrigel invasion assay. Giemsa-Wright staining, $\times 200$ original magnification. Scale bars = 100 μm . **B:** Wound healing assay. **Left panel** shows representative results and **right panel** exhibits mean distance of migration calculated at 10 selected points (* $P < 0.005$). **C:** Knockdown of *EWS-POU5F1* activates MMP2 and MMP14. **Top panel** shows gelatin zymography and **bottom panel** shows immunoblotting using an anti-MMP14 antibody. **D:** Quantitation of MMP2 and MMP14 mRNAs by real-time quantitative RT-PCR in three independent assays (* $P < 0.005$; ** $P < 0.01$).

regulate MMP expression directly or indirectly. The Matrigel invasion assay was also performed by using HeLa cells after introduction of the *EWS-POU5F1* expression vector. Cellular invasiveness was again suppressed (Figure 4A and Table 1; $P < 0.01$), though cell migration was decreased only moderately. In addition, depletion of MMP14 protein was demonstrated by introduction of *EWS-POU5F1* into both HeLa and HCT116 colon carcinoma cells (Figure 4B). Overexpression of *EWS-POU5F1* did not affect the expression level of p27, MMP2, or MMP9 in HeLa or HCT116 cells (data not shown). These results suggest that *EWS-POU5F1* suppresses cellular motility and invasion in the broad cellular context. In contrast, overexpression of *p27^{Kip1}* did not affect either cell migration or invasion (Table 1), clearly indicating that cell motility/invasiveness is modulated in a p27-independent manner in GBS6 cells and that simple growth suppression is not sufficient to enhance the invasive activity of tumor cells.

Modulation of the Gene Expression Profile by *EWS-POU5F1* Suppression

To investigate important downstream molecules regulated by *EWS-POU5F1*, alteration of global gene expression profiles by *EWS-POU5F1* knockdown was examined. We compared RNAs derived from *POU5F1*-specific siRNA-treated and control GBS6 cells (4 days after siRNA treatment) by using 54,676 probe sets of Affymetrix GeneChip Human Genome U133 Plus 2.0. We identified 98 probe sets (80 genes), the expression of which was increased more than 1.5-fold, and 55 probe sets (45 genes), the expression of which was decreased more than 1.5-fold (Figure 5A and Supplemental Table S1 at <http://ajp.amjpathol.org>). The genes whose expression was modified significantly were then classified according to gene ontology categories (Figure 5B). Interestingly,

Table 1. Invasiveness and Migration of GBS6 and HeLa Cells

Cells and treatment	<i>EWS-POU5F1</i> knockdown in GBS6		p27 expression in GBS6		<i>EWS-POU5F1</i> expression in HeLa	
	–	+	–	+	–	+
No. invasion	0.3 $\pm$ 0.4	101 $\pm$ 19*	0	0	1108 $\pm$ 85	357 $\pm$ 64*
No. migration	9.3 $\pm$ 3.2	>500*	11.1 $\pm$ 1.1	3.0 $\pm$ 0.7	3628 $\pm$ 401	1725 $\pm$ 229

Mean values $\pm$ SE of cell numbers of invasion and migration per 5×10^5 cells are exhibited.
 * $P < 0.01$ versus control (–).

Figure 4. Inhibition of cell migration and invasion by *EWS-POU5F1* in HeLa cells. **A:** Matrigel invasion assay. Giemsa-Wright staining, $\times 200$ original magnification. Scale bars = 100 μ m. **B:** Immunoblotting of HeLa cells transfected with control (–) or *EWS-POU5F1* (+) expression vectors using anti-MMP14 (top), anti-FLAG (middle), and anti- β -tubulin (bottom).

23.8% of up-regulated genes were involved in cell motility, invasion, or cytoskeleton, consistent with the remarkable alteration of the phenotypes in GBS6 cells. In addition, 13.3% of down-regulated and 13.8% of up-regulated genes belong to differentiation and development categories, indicating importance of the *POU5F1* function in pluripotency. However, *EWS-POU5F1* knockdown did not induce GBS6 cells to differentiate toward any specific lineage.

Representative differentially expressed genes of interest belonging to motility, adhesion/invasiveness, morphology/cytoskeleton, mesodermal differentiation, and growth suppression categories are shown in Figure 5C. In motility and adhesion/invasiveness categories, up-regulation of *MMP2* was again observed, though *MT1-MMP* was up-regulated only marginally. We also noted up-regulation of *CAV1*, the mutation of which is associated with mammary carcinoma invasiveness.²² In addition, another up-regulated gene, *F2R*, has been reported as overexpressed in human cancers with high metastatic potency.²³ Down-regulation of *ELMO1* is intriguing because it is required for promoting phagocytosis and cell shape changes.²⁴

A number of genes involved in the differentiation process were up-regulated by *EWS-POU5F1* knockdown.

MGP, *LBH*, *JUN*, *MYOF*, *CTGF*, and *MESDC2* are involved in mesodermal differentiation (Figure 5C and Supplemental Table S1 at <http://ajp.amjpathol.org>). The mesodermal origin of t(6;22) sarcoma was also supported by the fact that a number of genes encoding extracellular matrix proteins were also up-regulated. However, any specific differentiation toward muscle, bone, cartilage, or adipocytes was not supported by gene expression profiling.

Four putative tumor suppressors, *IGFBP7*, *HTRA1*, *TGFBR2*, and *SOCS3*, were up-regulated by *EWS-POU5F1* knockdown.^{25–28} Although it remains unclear whether these genes are the direct targets of *EWS-POU5F1*, modified expression of these genes should be noted in addition to the altered state of p27, cyclin D1, and Rb. In summary, expression profiling provided important information on the molecular networks affected by the oncogenic function of *EWS-POU5F1*.

EWS-POU5F1 Up-Regulates the ES Cell-Specific miR302-367 Cluster

MicroRNAs (miRNAs) are noncoding RNAs consisting of approximately 22 nucleotides, which posttranscriptionally regulate mRNAs. They are important in development and differentiation, and abnormal expression of miRNAs has been reported in various neoplasms.^{29,30} The miR302-367 cluster has been identified recently as ES cell-specific, and the cluster is transcriptionally regulated by Nanog, *POU5F1*, Sox2, and Rex1.^{31,32} RT-PCR analysis revealed remarkable down-regulation of the miR302-367 cluster during knockdown of *EWS-POU5F1* (Figures 6A and 6B), suggesting that chimeric *EWS-POU5F1*, like wild-type *POU5F1*, may regulate miR302-367. The result strongly suggests that *EWS-POU5F1* regulates downstream genes not only by its direct DNA binding but also through modulating the expression of miRNA.

Discussion

In the present study we show that *EWS-POU5F1* enhances cellular proliferation of GBS6 sarcoma cells. Knockdown of *EWS-POU5F1* caused GBS6 cells to arrest in the G1 phase of the cell cycle. We also noted up-regulation of p27^{Kip1}, down-regulation of cyclin D1, and diminished phosphorylation of Rb protein. The tumor suppressor p27^{Kip1} is a CDK2 inhibitor, and it inhibits the cell cycle at the G1/S transition.³³ It is likely that p27^{Kip1} functions downstream from *EWS-POU5F1* in oncogenic transformation. In support of this idea, exogenous introduction of p27^{Kip1} blocked proliferation of GBS6 cells.

During suppression of *EWS-POU5F1*, GBS6 cells showed morphological changes similar to those seen in cellular senescence (eg, spreading of the cytoplasm, marked enlargement of cell size, and expression of senescence-associated β -galactosidase, a hallmark of senescence).¹⁵ However, the lack of senescence-associated heterochromatin foci¹⁶ and the reversible nature of the G1 arrest suggest that the change induced by *EWS-POU5F1* knockdown differs from senescence. Loss of p16^{INK4A} might protect GBS6 cells from senescence,

Figure 5. Gene clustering analysis across the compared populations. **A:** Heat map shows the expression of EWS-POU5F1 regulated genes in GBS6 cells treated with negative control siRNA or *POU5F1*-specific siRNAs. Up-regulated and down-regulated genes are presented in red and green, respectively. **B:** Pie charts show the distribution of the 80 up-regulated and 45 down-regulated genes in GBS6 cells transfected with *POU5F1* siRNAs according to gene ontology (GO) annotations. **C:** Prediction of the major signaling pathways affected by EWS-POU5F1. Lower bound of fold changes in each gene are indicated in parentheses.

and senescence-like morphological changes might be achieved by alteration of the actin fiber network.

The inhibitory role of EWS-POU5F1 in cell migration and invasion was unexpected. It is very likely that multiple

molecular processes were responsible for increased motility and invasiveness of GBS6 cells treated with *POU5F1* siRNAs. It has been reported that RhoA activation induces actin polymerization¹⁷ that is causatively related to cancer cell invasion and migration.³⁴ Paradoxical promotion of tumor invasiveness related to p27^{Kip1}-dependent G1 arrest has been reported in malignant melanoma with Mitf activation in which Mitf promotes melanoma proliferation by down-regulating p27^{Kip1} but suppresses tumor cell invasion by the Dia1-dependent pathway.³⁵ Furthermore, p27^{Kip1} supports cell motility through modulation of the RhoA pathway.³⁶ In GBS6 cells, however, introduction of p27^{Kip1} affected neither cell mobility/invasiveness nor morphological changes. Those results suggest that there might be a p27^{Kip1}-independent pathway in RhoA activation and actin polymerization. MMP2 and MT1-MMP, which were up-regulated by knockdown of *EWS-POU5F1*, are candidate upstream regulators of RhoA because recent studies indicate these MMPs induce RhoA activation in osteosarcoma and vascular endothelial cells.^{37,38} Moreover, our study indicates that increased cell motility was not a simple consequence of growth suppression. Furthermore, the present results raise an important concern for the treatment of cancer in general. That is, when treatment suppresses the expression of oncogenic transcription factors, inhibition of tumor growth might be accompanied by enhanced tumor cell invasion and metastasis.

Carcinogenesis is a multistep process that requires multiple genetic and epigenetic alterations.³⁹ Therefore, the fusion of *EWSR1* and *POU5F1* is not sufficient for complete carcinogenesis, and t(6;22) tumors possess additional mutations such as *p16/p14* loss. Our prelimi-

Figure 6. **A:** RT-PCR analysis of miR302-367 in GBS6 cells transfected with control (-) or POU5F1 (+). β -actin was amplified to confirm quality and quantity of RNA. **B:** Real time quantitative RT-PCR analysis of miR302-367. Average expression was calculated in three independent experiments (* $P < 0.005$).

nary study demonstrated that retrovirus-mediated gene transfer of *EWS-POU5F1* could immortalize but not induce full transformation of murine mesenchymal stem cells (M. Tanaka and T. Nakamura, unpublished observation). Identification of genes cooperative with *EWS-POU5F1* for carcinogenesis is important, and genetic analysis including mutagenesis experiments will provide useful information for understanding the mechanism of *POU5F1*-induced carcinogenesis. In addition, our study suggests that tumor progression toward invasive properties may be caused by genes that do not cooperate with *EWS-POU5F1* but may even counteract *EWS-POU5F1*.

It is intriguing to define important *EWS-POU5F1* target genes in carcinogenesis. Because *POU5F1/Oct3/4* is a transcriptional regulator, it is likely that the fusion to *EWS* modulates the nature of its regulatory activities for downstream target genes. Previous studies suggested that *POU5F1* acquires the enhanced transcriptional activity by addition of the *EWS* N-terminal domain.^{13,40} The target genes for *POU5F1* have been extensively investigated by using ES cells.^{4,41,42} In these studies *POU5F1* is found associated with *SOX2* and/or *Nanog*, both of which are also expressed in GBS6 cells (data not shown). However, the down-regulated genes in *EWS-POU5F1* knockdown GBS6 cells did not always overlap with *POU5F1* target genes in ES cells, probably because of the different cellular context between ES cells and sarcoma cells. Alternatively, the addition of the *EWS* N-terminal domain may alter the binding specificity of *POU5F1* to the target sequences. Nevertheless, it is still possible that there are common target genes for both *EWS-POU5F1* and wild-type *POU5F1*. In a comparison between genes showing altered expression on *EWS-POU5F1* knockdown in the present study and the genes detected in chromatin immunoprecipitation (ChIP)-on-chip or chromatin immunoprecipitation-paired-end ditag (ChIP-PET) studies,^{41,42} *INSIG1*, *EPHA4*, *DHCR7*, *ANKS1B*, *ANO4*, *RDH10*, *PHF19*, *BNIP3*, and *TRIB1* are good candidates for common target genes of *POU5F1* or *EWS-POU5F1* in organogenesis or sarcomagenesis. In addition, the miR302-367 cluster has been identified as a target of ES cell-associated transcription factors, including *POU5F1*.³² Down-regulation of miR302-367 on *EWS-POU5F1* knockdown strongly suggests that *EWS-POU5F1* regulates gene expression by recognition of a target sequence as well as miRNA-mediated mRNA inhibition. In fact, overexpression of miR302 induces cell cycle progression of ES cells.⁴³ Interestingly, miR302 represses protein expression of cyclin D1 in ES cells, the opposite effect for *EWS-POU5F1* in GBS6 cells, suggesting cell context-dependent function of the miR302-467 cluster. Further studies are needed to identify key downstream molecules controlling cell proliferation and/or cell motility and invasiveness.

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