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Inducible Expression of Chimeric EWS/ETS Proteins Confers Ewing's Family Tumor-Like Phenotypes to Human Mesenchymal Progenitor Cells[†]

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Ewing's family tumor (EFT) is a rare pediatric tumor of unclear origin that occurs in bone and soft tissue. Specific chromosomal translocations found in EFT cause EWS to fuse to a subset of ets transcription factor genes (ETS), generating chimeric EWS/ETS proteins. These proteins are believed to play a crucial role in the onset and progression of EFT. However, the mechanisms responsible for the EWS/ETS-mediated onset remain unclear. Here we report the establishment of a tetracycline-controlled EWS/ETS-inducible system in human bone marrow-derived mesenchymal progenitor cells (MPCs). Ectopic expression of both EWS/FLI1 and EWS/ERG proteins resulted in a dramatic change of morphology, i.e., from a mesenchymal spindle shape to a small round-to-polygonal cell, one of the characteristics of EFT. EWS/ETS also induced immunophenotypic changes in MPCs, including the disappearance of the mesenchyme-positive markers CD10 and CD13 and the up-regulation of the EFT-positive markers CD54, CD99, CD117, and CD271. Furthermore, a prominent shift from the gene expression profile of MPCs to that of EFT was observed in the presence of EWS/ETS. Together with the observation that EWS/ETS enhances the ability of cells to invade Matrigel, these results suggest that EWS/ETS proteins contribute to alterations of cellular features and confer an EFT-like phenotype to human MPCs.

Ewing's family tumor (EFT) is a rare childhood cancer arising mainly in bone and soft tissue. Since EFT has a poor prognosis, it is important to elucidate the underlying pathogenic mechanisms for establishing a more effective therapeutic strategy. EFT is characterized by the presence of chimeric genes composed of EWS and ets transcription factor genes (ETS) formed by specific chromosomal translocations, i.e., EWS/FLI1, t(11;22)(q24;q12); EWS/ERG, t(21;22)(q12;q12); EWS/ETV1, t(7;22)(p22;q12); EWS/E1AF, t(17;22)(q12;q12); and EWS/FEV, t(2;22)(q33;q12) (26). The products of these chimeric genes behave as aberrant transcriptional regulators and are believed to play a crucial role in the onset and progression of EFT (3, 36). Indeed, recent studies have revealed that the induction of EWS/FLI1 proteins can trigger transformation in certain cell types, including NIH 3T3 cells (36), C2C12 myoblasts (12), and murine primary bone marrow-derived mesenchymal progenitor cells (MPCs) (6, 45, 52). However, studies have also indicated that overexpression of EWS/FLI1 provokes apoptosis and growth arrest in mouse normal

embryonic fibroblasts and primary human fibroblasts (10, 31), hence hampering understanding of the precise role of EWS/ETS proteins in the development of EFT. The function of EWS/ETS proteins would be greatly influenced by cell type, and thus the cells that can originate EFTs might be more susceptible to the tumorigenic effects of EWS/ETS.

Although the cell origin of EFT is still unknown, the expression of neuronal markers in spite of the occurrence in bone and soft tissues has kept open the debate as to a potential mesenchymal or neuroectodermal origin. As described above, ectopic expression of EWS/FLI1 results in dramatic changes in morphology and the formation of EFT-like tumors in murine primary bone marrow-derived MPCs but not in murine embryonic stem cells (6, 45, 52), supporting the notion that MPCs are a plausible cell origin of EFT (45). However, others argue that MPCs cannot be considered progenitors of EFT without further evidence of similarity between human EFT and MPC-EWS/FLI1-induced tumors in mice (29, 46).

The development of experimental systems using murine species is useful for elucidating the mechanisms behind the pathogenesis of EFT. However, several differences between human and murine systems cannot be ignored; these differences include the expression patterns of surface antigens in MPCs, for instance (7, 44, 51, 53). Moreover, human cells are difficult to transform *in vitro*, and the transformed cells of mice seem to produce a more aggressive tumor than those of hu-

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TABLE 1. Cell lines used in this study and fusion transcript types

Cell line	Diagnosis	Fusion transcript type	Reference
EES-1	EFT	EWS/FLI1 type I	20
SCCH196	EFT	EWS/FLI1 type I	21
RD-ES	EFT	EWS/FLI1 type II	5
SK-ES1	EFT	EWS/FLI1 type II	5
NCR-EW2	EFT	EWS/FLI1 type II	19
NCR-EW3	EFT	EWS/EIAF	19
W-ES	EFT	EWS/ERG	13
NB69	NB		15
NB9	NB		15
GOTO	NB		47
NRS-1	RMS	PAX3/FKHR	40

mans (1). The findings suggest the existence of undefined cell-autonomous mechanisms that render human cells resistant to malignant transformation. Therefore, the use of human cell models is ideal for clarifying how EFT develops. Models of the onset of EFT have been generated using primary fibroblasts (31) and rhabdomyosarcoma cells (23). However, these cell types are not appropriate for studying the origins of EFT, and a model that precisely recapitulates EWS/ETS-mediated EFT formation is required.

UET-13 cells are obtained by prolonging the life span of human bone marrow stromal cells by use of the retroviral transgenes hTERT and E7 (38, 50), retain the ability to differentiate into not only mesodermal derivatives but also neuronal progenitor-like cells, and are considered a good model for studying the cellular events in human MPCs. Therefore, we have examined the biological effect of EWS/ETS in human MPCs by use of UET-13 cells by exploiting tetracycline-inducible systems for expressing EWS/ETS (EWS/FLI1 and EWS/ERG). Here we report that overexpression of EWS/ETS mediates an EFT-like phenotype, including morphology, immunophenotype, and gene expression profile, with enhancement of the Matrigel invasion ability of UET-13 cells.

MATERIALS AND METHODS

Cell cultures and establishment of UET-13TR-EWS/ETS cell lines. UET-13 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% Tet system approved fetal bovine serum (T-FBS) (Takara) at 37°C under a humidified 5% CO₂ atmosphere. EFT cell lines (EES-1 [20], SCCH196 [21], RD-ES and SK-ES1 [5], NCR-EW2 and NCR-EW3 [19], and W-ES [13]) and neuroblastoma (NB) cell lines (NB69 and NB9 [15] and GOTO [47]) were cultured in RPMI 1640 with 10% FBS. A rhabdomyosarcoma cell line, NRS-1 (40), was cultured in Eagle's minimal essential medium with 10% FBS. The cell lines used in this study are listed in Table 1.

UET-13 cells were seeded at a density of 5×10^4 cells per well in 24-well tissue culture plates 1 day prior to transfection. For introducing the tetracycline-inducible system, UET-13 cells were transfected with pcDNA6-TR (Invitrogen) by use of Lipofectamine 2000 (Invitrogen). After 72 h, the medium was replaced with fresh medium containing 200 µg/ml of blasticidin S (Invitrogen). Individual resistant clones were selected for a month and designated UET-13TR cells. UET-13TR cells were further transfected with pcDNA4-EWS/ETSs constructed as described below, and individual resistant clones were selected in DMEM containing 10% T-FBS and 200 to 300 µg/ml of Zeocin (Invitrogen). The Zeocin-resistant clones were expanded and tested for the induction of EWS/ETS expression upon the addition of tetracycline by use of reverse transcription-PCR (RT-PCR) as described below.

Plasmid construction. A gateway cassette (bases 1 to 1705) was amplified from pBLOCK-IT3-DEST (Invitrogen) by PCR, and the PCR product was inserted into the EcoRV site of pcDNA4-TO (Invitrogen) (termed pcDNA4-DEST). Since the type II EWS/FLI1 is a stronger transactivator than the type I product

(32), we used the type II variant in the present study. EWS/ERG was isolated from W-ES, an EFT cell line, joining EWS exon 7 and ERG exon 9. Full-length EWS/FLI1 type II and EWS/ERG cDNAs were amplified from cDNAs prepared from NCR-EW2 and W-ES cells, respectively, by PCR as described below and cloned into the XmnI-EcoRV sites of pENTR11 (Invitrogen). The resulting pENTR11-EWS/ETSs were recombined with pcDNA4-DEST by use of LR recombination reaction as instructed by the manufacturer (Invitrogen) to construct the tetracycline-inducible EWS/ETS expression vector pcDNA4-EWS/ETSs.

Western blot analysis. UET-13 transfectants were cultivated with or without 3 µg/ml of tetracycline for 72 h. Western blot analysis was performed as previously described (37). Briefly, the cell lysates were prepared and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% skimmed milk in phosphate-buffered saline (PBS) containing 0.01% Tween 20 (Sigma) and incubated with primary antibodies. As the primary antibodies, anti-Flt-1, anti-Erg-1/2/3 (Santa Cruz Biotechnology), and anti-actin (Sigma) were used. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibodies (DakoCytomation) were used as secondary antibodies. Blots were detected by chemiluminescence using an ECL Plus Western blotting detection system (GE Healthcare Bio-Science Corp.) and exposed to X-ray film (Kodak) for 5 to 30 min.

MTT assay and detection of apoptosis. Growth curves of UET-13 transfectants were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (18). The apoptosis was detected using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Biovision) according to the manufacturer's instructions and analyzed by flow cytometry (Cytomics FC500; Beckman Coulter).

Immunofluorescence analysis. After 1 week of culture in the absence or presence of tetracycline, UET-13 cells and the transfectants were harvested with 0.25% trypsin plus EDTA (IBL). The cells (2×10^6) were incubated with mouse monoclonal antibodies for 20 min. In the case of fluorescence-labeled antibodies, the cells were washed with PBS and then analyzed. In the case of primary unconjugated mouse antibodies, the cells were washed and then incubated with FITC-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) for 20 min. Cell fluorescence was detected using a Cytomics FC500 instrument as described previously (27).

Antibodies against the following human antigens were used: CD10, CD13, CD14, CD29, CD34, CD40, CD44, CD45, CD49e, CD54, CD56, CD61, CD90, CD105, CD117, and CD166 from Beckman Coulter; CD73 from BD Biosciences-Pharmingen; CD55 from Abcam; CD59 from Cedarlane Laboratories; and CD133 and CD271 from Miltenyi Biotec GmbH.

Immunocytochemistry. Cells were grown on collagen type I-coated cover glasses (Iwaki). After 72 h with or without tetracycline, cells were fixed for 30 min in 4% paraformaldehyde and permeabilized in PBS containing 0.2% Triton X-100 (Sigma) for 30 min. Subsequently, they were washed with PBS and blocked in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (Sigma) for 30 min before being incubated with a monoclonal anti-CD99 antibody, i.e., 12E7 (1:100) (DakoCytomation) or O13 (1:200) (Thermo), and polyclonal anti-Flt-1 antibody (1:100) (Santa Cruz) for 1 h. Bound antibodies were visualized with appropriate secondary antibodies, i.e., Alexa Fluor 488 goat anti-mouse IgG (heavy plus light chains) highly cross-adsorbed and Alexa Fluor 546 goat anti-rabbit IgG (heavy plus light chains) highly cross-adsorbed (Invitrogen) for 1 h at 1:300. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) (Sigma). For the visualization of whole cells, cells were treated with CellTracker Blue (Invitrogen) for 30 min and then fixed. Fluorescence was observed and analyzed using a confocal laser scanning microscope and image software (either FV500 from Olympus or LSM10 from Carl Zeiss). Precise measurements of cell size, nuclear size, and the nucleus-to-cytoplasm (N/C) ratio were performed using Image J (16).

RT-PCR analysis. Total RNA was extracted from cells by use of an RNeasy kit (Qiagen) and reverse transcribed using a first-strand cDNA synthesis kit (GE Healthcare Bio-Science Corp). RT-PCR was performed with a HotStarTaq master mix kit (Qiagen). As an internal control, human GAPDH cDNA was also amplified. The sequences of gene-specific primers for RT-PCR were as follows: for EWS/FLI1 (forward), 5'-ATGGCGTCCACGGATTACAGTACCT-3'; for EWS/FLI1 (reverse), 5'-GGGTCTCTTTGACACTCAATCG-3'; for EWS/ERG (forward), 5'-ATGGCTCCACGGATTACAGTACCT-3'; for EWS/ERG (reverse), 5'-TTAGTAGTAGTAAAGTCCAGATGAGAA-3'; for GAPDH (forward), 5'-CCACCCTGGCAATTCATGGCA-3'; and for GAPDH (reverse), 5'-TCTAGACGGCAGGTGACGT/CCACC-3'. PCR products were electrophoresed with a 1% agarose gel and stained with ethidium bromide.

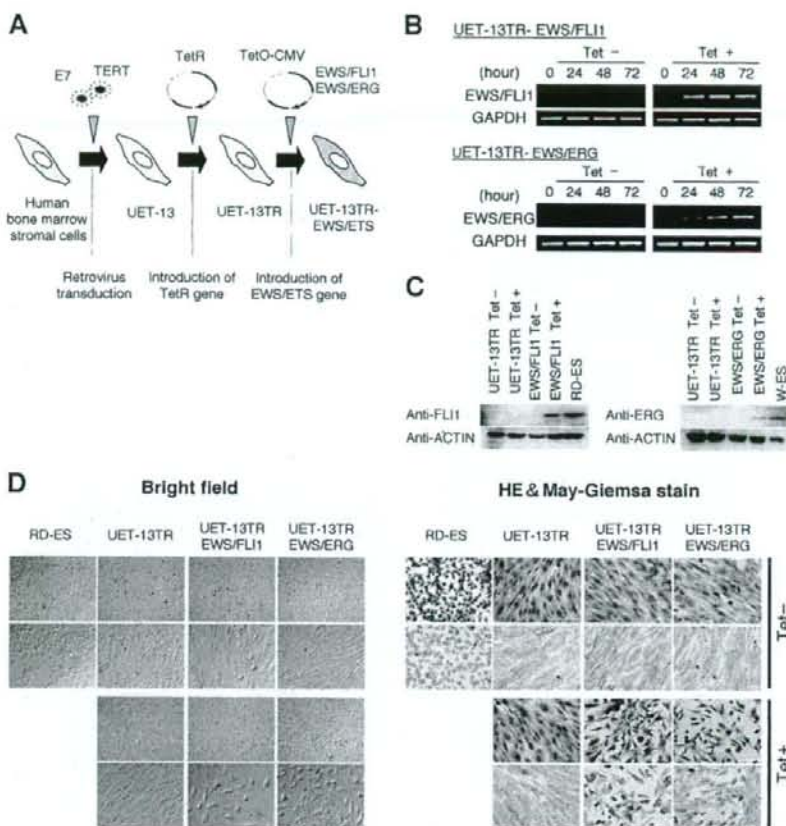


FIG. 1. The effect of EWS/ETS on the morphology of UET-13 cells. (A) The establishment of a tetracycline-inducible EWS/ETS expression system in UET-13 cells. CMV, cytomegalovirus. (B) Analyses for confirming the inducible expression of EWS/ETS genes. EWS/ETS mRNAs were detected in UET-13 transfectants UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG by RT-PCR. These cells were treated with or without 3 μ g/ml of tetracycline (Tet) for the indicated periods. As an internal control, a human GAPDH gene was used. (C) Analyses for confirming the inducible expression of EWS/ETS proteins. The extracts of RD-ES and W-ES cells were also examined as positive controls. Membranes were probed with anti-actin antibody as a loading control. (D) Morphological change after tetracycline treatment of UET-13 transfectants. UET-13 cells and the transfectants were cultured in the absence or presence of tetracycline for 72 h and observed by light microscopy. Magnification, $\times 40$ (top); $\times 200$ (bottom). Cells were also examined using hematoxylin-eosin (HE) (top) and May-Giemsa (bottom) staining (magnification, $\times 200$).

Real-time RT-PCR. Real-time RT-PCR was performed using TaqMan universal PCR master mix and TaqMan gene expression assays and an inventoried assay on an ABI Prism 7900HT sequence detection system (Applied Biosystems) according to the manufacturer's instructions. The human GAPDH gene was used as an internal control for normalization.

DNA microarray analysis. Total RNA isolated from cells was reverse transcribed and labeled using one-cycle target labeling and control reagents as instructed by the manufacturer (Affymetrix). The labeled probes were hybridized to the human genome U133 Plus 2.0 array (Affymetrix). The arrays were performed in a single experiment and analyzed using GeneChip operating software, version 1.2 (Affymetrix). Background subtraction, normalization, and principal component analysis (PCA) were performed by GeneSpring GX 7.3 software (Agilent Technologies). Signal intensities were prenormalized based on the median of all measurements on that chip. To account for the difference in detection efficiencies between the spots, prenormalized signal intensities on each gene were normalized to the median of prenormalized measurements for that gene. The data were filtered using the following steps. (i) Genes that were scored as absent in all samples were eliminated. (ii) Genes for which the signal intensities were lower than 100 were eliminated. (iii) Performing cluster analysis using

filtering genes, we selected the genes that exhibited increased expression or decreased expression in tetracycline-treated cells. Accession numbers for the microarray data are given below.

Invasion assay. The invasion assay was performed using Matrigel (BD Bioscience) according to the previous description (34) with some modification. Polycarbonate filter inserts containing 8- μ m pores (BD Falcon) were coated with 50 μ l of a 6:1 mixture of culture medium and Matrigel and placed into 24-well culture plates containing DMEM supplemented with 10% T-FBS as chemoattractants. Cells (2.5×10^4) treated with or without tetracycline for 72 h were suspended in DMEM containing 0.01% T-FBS and plated on top of each filter insert. After 20 h in culture in the presence or absence of tetracycline, noninvading cells were removed from upper surface of the filter with a cotton swab. The invading cells on the lower surface of the filter were fixed with formalin, stained with hematoxylin-eosin, and counted in five fields per membrane with light microscopy. As a control, cells were also cultured on uncoated filter inserts. The invasion efficiency was presented as the ratio of the number of invading cells on Matrigel-coated inserts to that on uncoated inserts. Experiments were performed in triplicate, and the means with standard deviations of the values are shown in the graphs in Fig. 8.

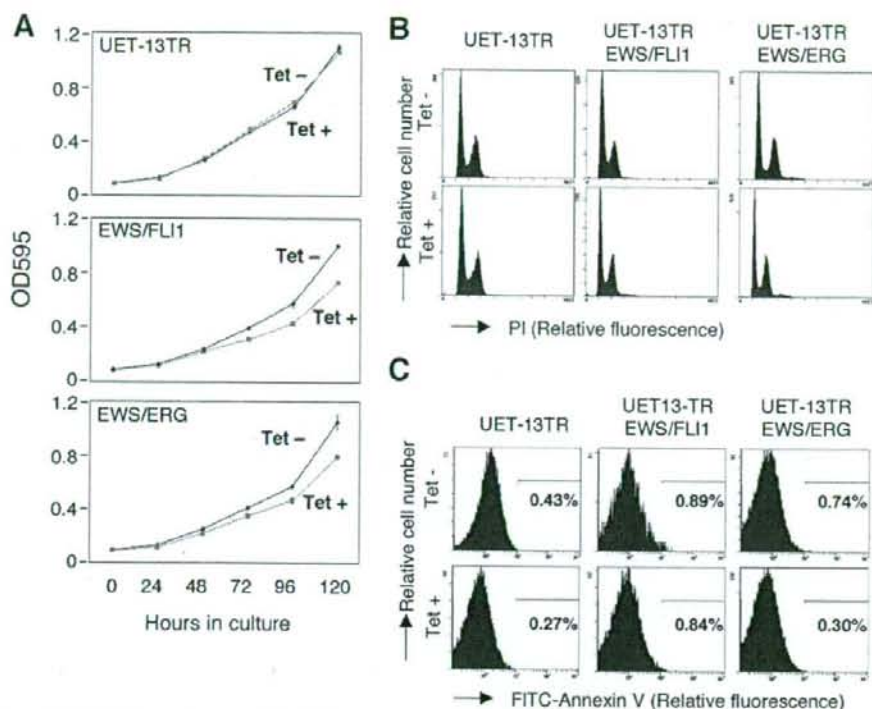


FIG. 2. Effects of EWS/ETS on cell growth in UET-13 cells. (A) Growth curve for UET-13 transfectants. Cells were seeded at 10^3 /well and cultured as described for Fig. 1. The increase in cell number was analyzed by MTT assay. Values are means with the standard errors (SE) from three independent experiments. Diamond symbols indicate UET-13 transfectants in the absence of tetracycline (Tet); box symbols indicate UET-13 transfectants in the presence of tetracycline. (B) Cells were cultured as described for panel A in the absence or presence of tetracycline for 3 days and then stained with PI, and DNA contents were analyzed by flow cytometry (x axis, relative intensity of fluorescence; y axis, relative cell number). (C) Cells treated as described for panel B were stained with FITC-annexin V and analyzed.

Microarray data accession numbers. Microarray data have been deposited in the Gene Expression Omnibus database GEO (www.ncbi.nlm.nih.gov/geo) (accession numbers GSE8665 and GSE8596).

RESULTS

EWS/ETS expression results in morphological changes in UET-13 cells. To investigate how the expression of EWS/ETS affects human MPCs, we used UET-13 cells as a model of human MPCs and expressed EWS/FLI1 (UET-13TR-EWS/FLI1) and EWS/ERG (UET-13TR-EWS/ERG) in a tetracycline-inducible manner (Fig. 1A). As shown in Fig. 1B and C, we confirmed that the tetracycline treatment could induce EWS/ETS expression by RT-PCR analysis and Western blotting. The inducibility upon the addition of doxycycline was comparable to that upon the addition of tetracycline.

Using these cell systems, first we examined the effect of EWS/ETS expression on morphology in UET-13 transfectants. When tetracycline was added to the culture, the morphologies of both UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were dramatically changed (Fig. 1D). Tetracycline-treated UET-13TR-EWS/ETS cells consisted of a mixture of small round-to-polygonal cells and short spindle cells. The cell morphology resembled that of EFT cell lines. To assess the repro-

ducibility of this phenotypic change, other UET-13TR-EWS/ETS clones were examined, and similar morphological changes were observed. Since tetracycline treatment did not affect the morphology of UET-13TR cells (Fig. 1D), it was suggested that the morphological alteration in UET-13 cells from a mesenchymal cell shape to small round cells, one of the characteristics of EFT, can be attributed to EWS/ETS expression.

EWS/ETS expression inhibits cell growth in UET-13 cells.

Next, the effect of EWS/ETS expression on the growth of UET-13 cells was analyzed. As shown in Fig. 2A, an MTT assay revealed that the addition of tetracycline had no effect on the growth of UET-13TR cells but slightly inhibited that of UET-13TR-EWS/ETS cells. We also assessed the cell growth of UET-13 transfectants after tetracycline addition by cell counting and obtained results well in accord with those from the MTT assay (data not shown). To determine the mechanism of this inhibition, DNA content and the binding of annexin V to UET-13 transfectants were examined. No significant increase in either sub-G₁-phase cells (Fig. 2B) or annexin V binding cells (Fig. 2C) was detected, suggesting that EWS/ETS-mediated growth inhibition in UET-13 cells was not due to the activation of an apoptotic pathway. Moreover, no significant decrease in S-G₂-phase cells was observed (Fig. 2B).

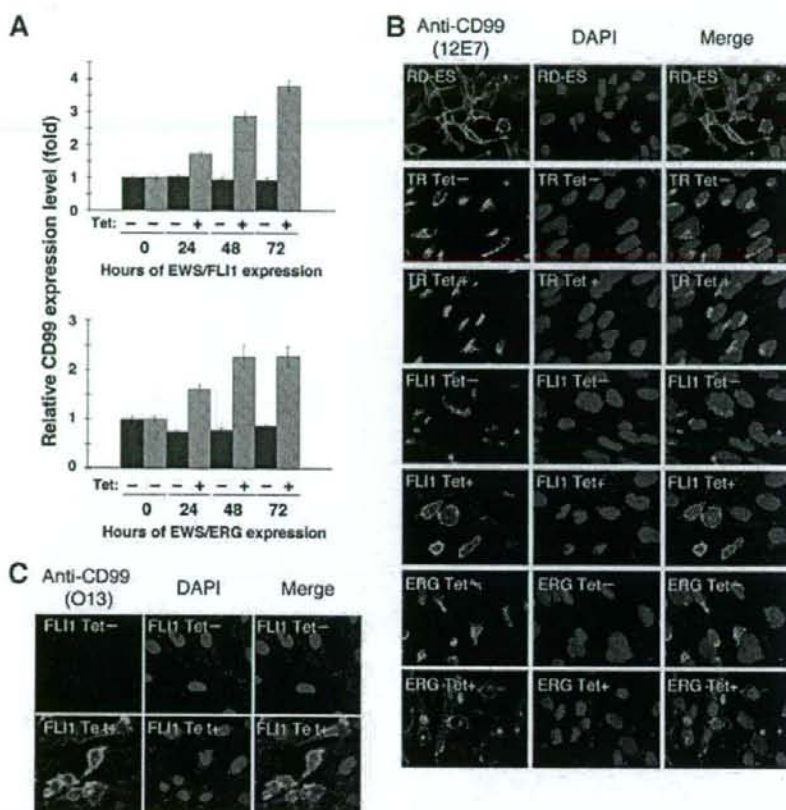


FIG. 3. Effects of tetracycline-mediated EWS/ETS expression on the expression and distribution of CD99 in UET-13 cells. (A) Relative CD99 levels in UET-13 transfectants in the absence or presence of tetracycline (Tet). UET-13 transfectants were treated with or without 3 μ g/ml of tetracycline for the indicated periods. Real-time RT-PCR was performed to investigate the expression pattern of CD99. Signal intensities of CD99 were normalized using those of a control housekeeping gene (human GAPDH gene). Data are relative values with standard deviations from triplicate wells and are normalized to the mRNA level at 0 h, which is arbitrarily set to 1 in the graphical presentation. (B and C) Immunocytochemical staining of CD99 in UET-13 transfectants. Cells were cultured on coverslips in the absence or presence of tetracycline for 72 h and then stained with anti-CD99 antibody 12E7 (B) or O13 (C) as described in Materials and Methods. RD-ES cells were also examined as a positive control. For the staining of nuclei, DAPI was used.

Effect of EWS/ETS on CD99 expression in UET-13 cells. The p30/32MIC-2 gene product, CD99, is a cell surface glycoprotein expressed in EFT with a strong membranous staining pattern and thus constitutes a useful marker for EFT (2, 30). Knowing the dramatic change of morphology in UET-13 cells, we next investigated the mRNA level of CD99 in tetracycline-treated and untreated UET-13 transfectants by quantitative real-time RT-PCR. CD99 levels were clearly elevated by tetracycline treatment in both UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells in a time-dependent manner (Fig. 3A).

We also examined the protein expression of CD99 by immunostaining using 12E7 antibody, which is most widely used as an anti-CD99 antibody. An EFT cell line, RD-ES, showed strong membranous staining of CD99 (Fig. 3B), while neither UET-13TR cells nor UET-13 cells had such a staining. Of note is the fact that although 12E7 reactivity was observed only in the cytoplasm in perinuclear regions in both UET-13TR (Fig.

3B) and UET-13 (data not shown) cells, this antibody is well known to cross-react with a cytoplasmic protein not yet characterized. Since another anti-CD99 antibody, O13, did not react with either UET-13TR (Fig. 3C) or UET-13 (data not shown) cells, we concluded that the perinuclear staining of 12E7 mentioned above was a cross-reaction with unrelated proteins.

In the absence of tetracycline, both UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were also negative with anti-CD99 antibodies (a pattern designated CD99⁻), similar to UET-13 cells. Surprisingly, however, tetracycline induced a membranous staining pattern (designated CD99⁺) in UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells, and some CD99⁺ cells had irregularly contoured nuclei (Fig. 3B). The same results were observed with another anti-CD99 antibody, O13 (Fig. 3C), indicating that the membranous staining observed for UET-13 transfectants with the anti-CD99 antibodies

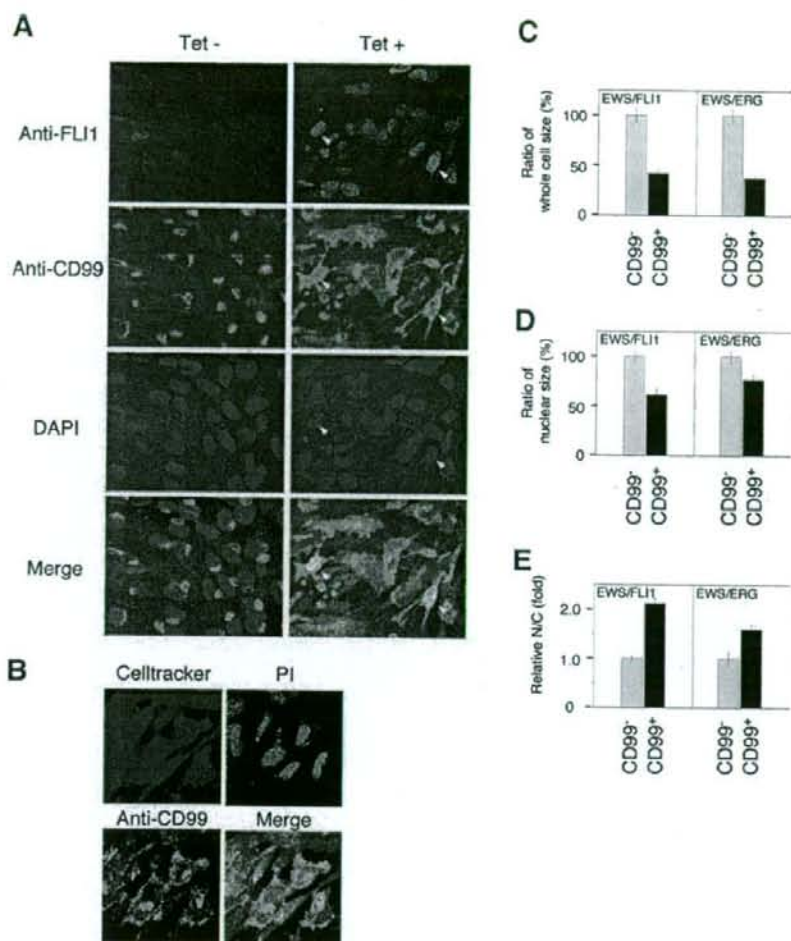


FIG. 4. EWS/ETS expression, alteration of CD99 distribution, and cell morphological changes in UET-13 cells. (A) Immunofluorescence studies using anti-Flil1 (red), anti-CD99 (green), and DAPI (blue). UET-13TR-EWS/FLI1 cells were cultured on coverslips in the absence or presence of tetracycline (Tet) for 72 h and then stained as described in Materials and Methods. White arrowheads indicate CD99⁺ cells that have a strong staining pattern with anti-Flil1 antibodies and also have remarkable CD99 expression and morphological features. (B) Immunofluorescence analysis by triple staining with whole cells (Celltracker; blue), CD99 (anti-CD99; green), and nuclei (PI; red). UET-13TR-EWS/FLI1 cells were cultured as described for panel A and then stained as described in Materials and Methods. (C to E) Measurements of whole-cell size (C), nuclear size (D), and N/C ratio (E) in tetracycline-treated UET-13 transfectants. UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were cultured on coverslips in the presence of tetracycline for 72 h and then stained as described in Materials and Methods. These samples were analyzed by the image analysis software Image J ($n = 50$). (C and D) Data are relative values with the SE and are normalized to the size of CD99⁻ cells, which is arbitrarily set to 100. (E) Data are relative values with the SE and are normalized to the size of CD99⁻ cells, which is arbitrarily set to 1.

was really CD99 derived. Despite the fact that cells were single colony derived, there was a heterogeneous response to tetracycline treatment in UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells, but most of the CD99⁺ cells had a small round morphology, one of the characteristics of EFT. To assess the correlation between EWS/FLI1 expression and the change of the CD99 expression pattern, we performed immunofluorescence studies using anti-Flil1 and anti-CD99 antibodies. As shown in Fig. 4A, tetracycline treatment induced a marked

enhancement of nuclear staining with anti-Flil1 antibodies in a large number of UET-13TR-EWS/FLI1 cells, indicating the induction of EWS/FLI1 proteins. Furthermore, we observed that the cells with a strong signal for Flil1 tended to reveal a membranous staining pattern with anti-CD99 antibodies and a small round morphology (Fig. 4A). To further verify the correlation between CD99 expression pattern and cell morphology, we estimated the size of cells by triple staining using Celltracker Blue, PI, and anti-CD99 antibody (Fig. 4B). As

TABLE 2. Immunophenotypic characterization of UET-13 transfectants and EFT cells

MPC status ^a	CD marker	Result for ^b :								EFT status ^c	SK-ES1	
		UET-13		UET-13TR		UET-13TR-EWS/FLI1		UET-13TR-EWS/ERG				RD-ES
		Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺			
M+	CD29	+	+	+	+	+	+	+	+	+	+	
M+	CD59	+	+	+	+	+	+	+	+	+	+	
M+	CD90	+	+	+	+	+	+	+	+	+	+	
M+	CD105	+	+	+	+	+	+	+	+	+	+	
M+	CD166	+	+	+	+	+	+	+	+	+	+	
M+	CD44	+	+	+	+	+	+	+	+	-	-	
M+	CD73	+	+	+	+	+	+	+	+	-	-	
M+	CD10	+	+	+	+	+	+	+	+	-	-	
M+	CD13	+	+	+	+	Down	Down	Down	Down	-	-	
M+	CD49e	+	+	+	+	Down	Down	Down	Down	+	-	
M+	CD61	+	+	+	+	Down	Down	Down	Down	-	-	
M+	CD55	+	+	+	+	Down	Down	Down	Down	+	-	
M+	CD54	-	-	-	-	Up	Up	Up	Up	+	+	
M(-)	CD117	-	-	-	-	Up	Up	Up	Up	+	+	
M+/-	CD271	-	-	-	-	Up	Up	Up	Up	+	+	
	CD40	-	-	-	-	-	-	-	-	+	+	
	CD56	-	-	-	-	-	-	-	-	+	+	
M(-)	CD133	-	-	-	-	-	-	-	-	+	+	
M(-)	CD14	-	-	-	-	-	-	-	-	-	-	
M(-)	CD34	-	-	-	-	-	-	-	-	-	-	
M(-)	CD45	-	-	-	-	-	-	-	-	-	-	

^a M(-), negative for MPCs; M+/-, positive for BM-derived MPCs but negative after in vitro culture; M+, positive for MPCs.

^b +, most cells positive; -, negative; Up, up-regulated by tetracycline treatment; Down, down-regulated by tetracycline treatment. Boldface indicates the antigens the immunophenotypes of which were changed in favor of EFT. Tet⁻, tetracycline negative; Tet⁺, tetracycline positive.

^c E+, positive for EFTs.

presented in Fig. 4C and D, the results clearly showed that the majority of CD99⁺ cells were significantly smaller in both whole-cell size and nuclear size than the CD99⁻ cells. Moreover, CD99⁺ cells also had a substantially increased N/C ratio (Fig. 4E). These results indicated that EWS/ETS expression promoted CD99 expression in UET-13 cells, and CD99 expression status is correlated with the degree of morphological change.

EWS/ETS expression altered the immunophenotype of UET-13 cells. Human MPCs reveal a characteristic expression of several surface antigens and can be identified on the basis of the reactivity with a set of monoclonal antibodies against CD antigens (25, 42). On the other hand, some CD antigens are characteristically expressed on EFT cells (17, 28, 33). Using the combinations of these antibodies listed in Table 2, which are useful for the immunodetection of either MPCs or EFT cells, we further examined whether EWS/ETS expression affects the immunophenotype of UET-13 cells and compared its effect with that on the immunophenotype of EFT cell lines (Table 2 and Fig. 5). As shown in Table 2, UET-13 cells express most of the human primary MPCs markers. Some of the antigens expressed in MPCs, namely, CD29, CD59, CD90, CD105, and CD166, were also found to be expressed in EFT cell lines, but others, namely, CD10, CD13, CD44, CD61, and CD73, were not. In contrast, antigens recognized to be present in EFT cells, including CD40, CD56, and CD133, were absent from UET-13 cells. Interestingly, when the effect of tetracycline-mediated EWS/ETS expression on the immunophenotype of UET-13 cells was tested, levels of some of the antigens present in UET-13 cells, such as CD10, CD13, and CD61, were found to be decreased (Fig. 5). In contrast, some of the markers found

in EFT cells, i.e., CD54, CD117, and CD271, became positive in UET-13TR-EWS/ETS cells after tetracycline treatment. Because UET-13TR cells did not show such immunophenotypic change upon treatment with tetracycline, these results indicated that, at least in part, the immunophenotype of UET-13 cells was changed in favor of EFT in the presence of EWS/ETS.

EWS/ETS in UET-13 cells modulates EFT-like gene expression. To further examine the molecular mechanism of EWS/ETS-dependent cellular modulation in human mesenchymal progenitor background, we performed DNA microarray-based expression profiling using the Affymetrix human genome U133 Plus 2.0 array. As a first step to this approach, we validated our experimental systems by analyzing the sequential changes of known EWS/ETS target genes, i.e., inhibitor of differentiation 2 (ID2) (14, 39), NK2 transcription factor related, locus 2 (NKX2.2) (9, 48), and insulin-like growth factor binding protein 3 (IGFBP3) (41). Consistent with previous reports, levels of ID2 and NKX2.2 increased with the expression of EWS/ETS in a time-dependent manner, whereas the expression level of IGFBP3 decreased (Fig. 6A). Employing the same procedure, we also examined whether the change of surface antigen expression was regulated at the transcriptional level and determined the mRNA expression levels of some surface antigens in UET-13 transfectants with or without tetracycline treatment. In accordance with the results of immunocytometric and immunohistological experiments, the mRNA expression levels of CD10, CD13, CD49e, and CD61 were decreased, while those of CD54, CD99, CD117, and CD271 were markedly increased in tetracycline-treated UET-13TR-EWS/ETS cells (Fig. 6B and C), indicating that the expression of these antigens is

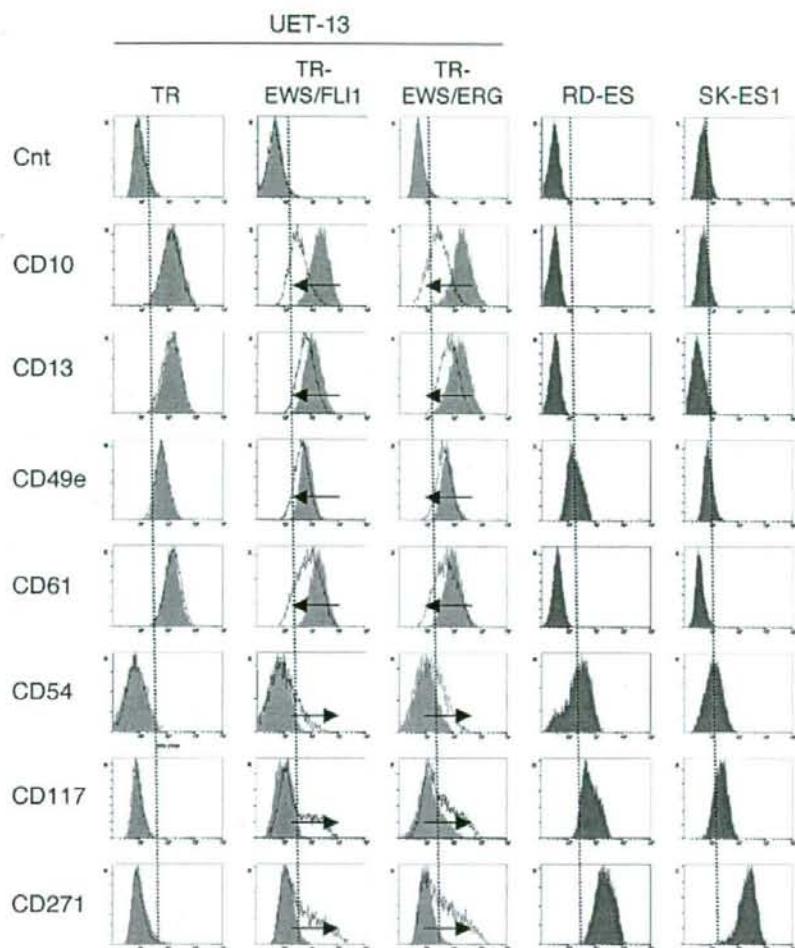


FIG. 5. Immunophenotypic change on induction of EWS/ETS expression in UET-13 cells. UET-13 transfectants were cultured with or without $3 \mu\text{g/ml}$ of tetracycline for 1 week and flow cytometric analyses were performed by using a set of antibodies as indicated. The histograms of UET-13 transfectants with (empty) and without (gray) tetracycline treatment were overlaid. Dotted lines indicate fluorescence intensities in negative control panels (Cnt). Arrows indicate the immunophenotypic change caused by tetracycline. The immunophenotypes of the EFT cell lines RD-ES and SK-ES1 were also examined.

controlled at the transcriptional level in the presence of EWS/ETS.

We next investigated the candidate genes whose expression is regulated by EWS/ETS in human MPCs. First, we selected the genes with up-regulated or down-regulated expression by EWS/ETS induction using gene cluster analysis (Fig. 7A; UET-13TR-EWS/FLI1 up, 4,294 probes; down, 4,103 probes; UET-13TR-EWS/ERG up, 3,358 probes; down, 3,705 probes). To reduce the number of the candidate genes, we selected up-regulated genes that are expressed in tetracycline-treated cells at least 1.5-fold higher than in untreated cells (UET-13TR-EWS/FLI1, 1,137 probes; UET-13TR-EWS/ERG, 835 probes). Similarly, the down-regulated genes that are expressed in tetracycline-treated cells at least 0.75-fold lower than in untreated cells (UET-

13TR-EWS/FLI1, 1,803 probes; UET-13TR-EWS/ERG, 773 probes). By selecting common probes in both cells, we finally identified a group of candidate genes significantly controlled by EWS/ETS induction in the human mesenchymal progenitor background. Since microarray analysis was performed as a global screening in a single experiment, it is likely that there is a fair bit of noise in the derived gene profiles due to the lack of replicate data. This may account in part for the limited overlap between the profiles induced by EWS-FLI1 and EWS-ERG, whereas we still identified 349 probes of common up-regulated genes and 293 probes of common down-regulated genes (see the supplemental material). In addition to the EFT-specific genes mentioned above, these contained those previously described as EFT-specific genes, such as those for OB-cadherin/cadherin-11 (31), Janus

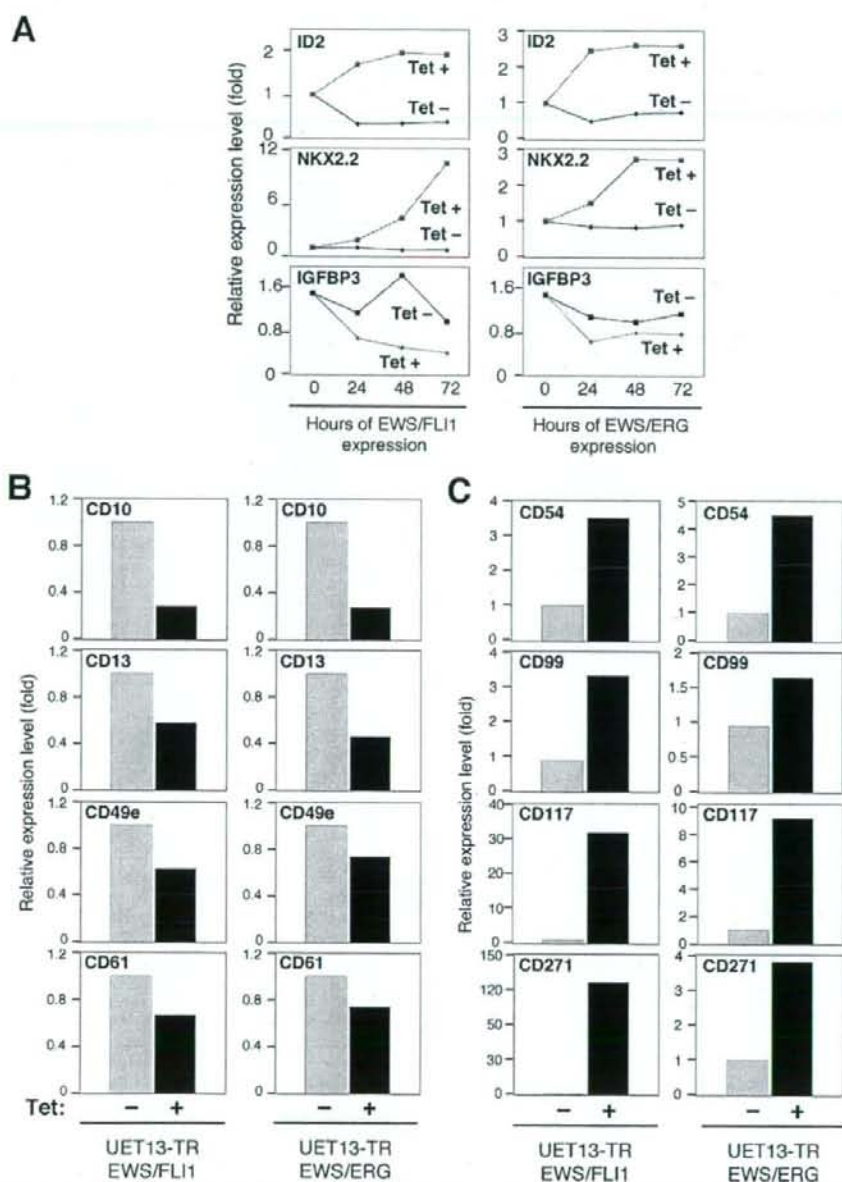


FIG. 6. The change of expression profile on induction of EWS/ETS in UET-13 cells. UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were cultured in the absence or presence of tetracycline (Tet) for the indicated periods and analyzed using the Affymetrix human genome U133 Plus 2.0 array as described in Materials and Methods. (A) The sequential changes of ID2, NKX2.2, and IGFBP3 mRNA levels in UET-13 transfectants upon treatment with or without tetracycline. Diamond symbols indicate UET-13 transfectants in the absence of tetracycline; box symbols indicate UET-13 transfectants in the presence of tetracycline. (B and C) Microarray studies for the determination of expression profiles of surface antigens in UET-13 transfectants. UET-13 transfectants were treated with or without 3 μ g/ml of tetracycline for 72 h. mRNA levels were determined with the Affymetrix human genome U133 Plus 2.0 array.

kinase 1 (JAK1) (49), keratin 18, and six-transmembrane epithelial antigen of the prostate (STEAP) (22). The expression pattern of these genes (642 probes) in UET-13 transfectants in the absence or presence of tetracycline is shown in the gene cluster in

Fig. 7B. The expression of these genes was indeed changed significantly after EWS/ETS expression in both cells. They included genes associated with signal transduction (such as those for epidermal growth factor receptor, FAS [CD95], and fibroblast

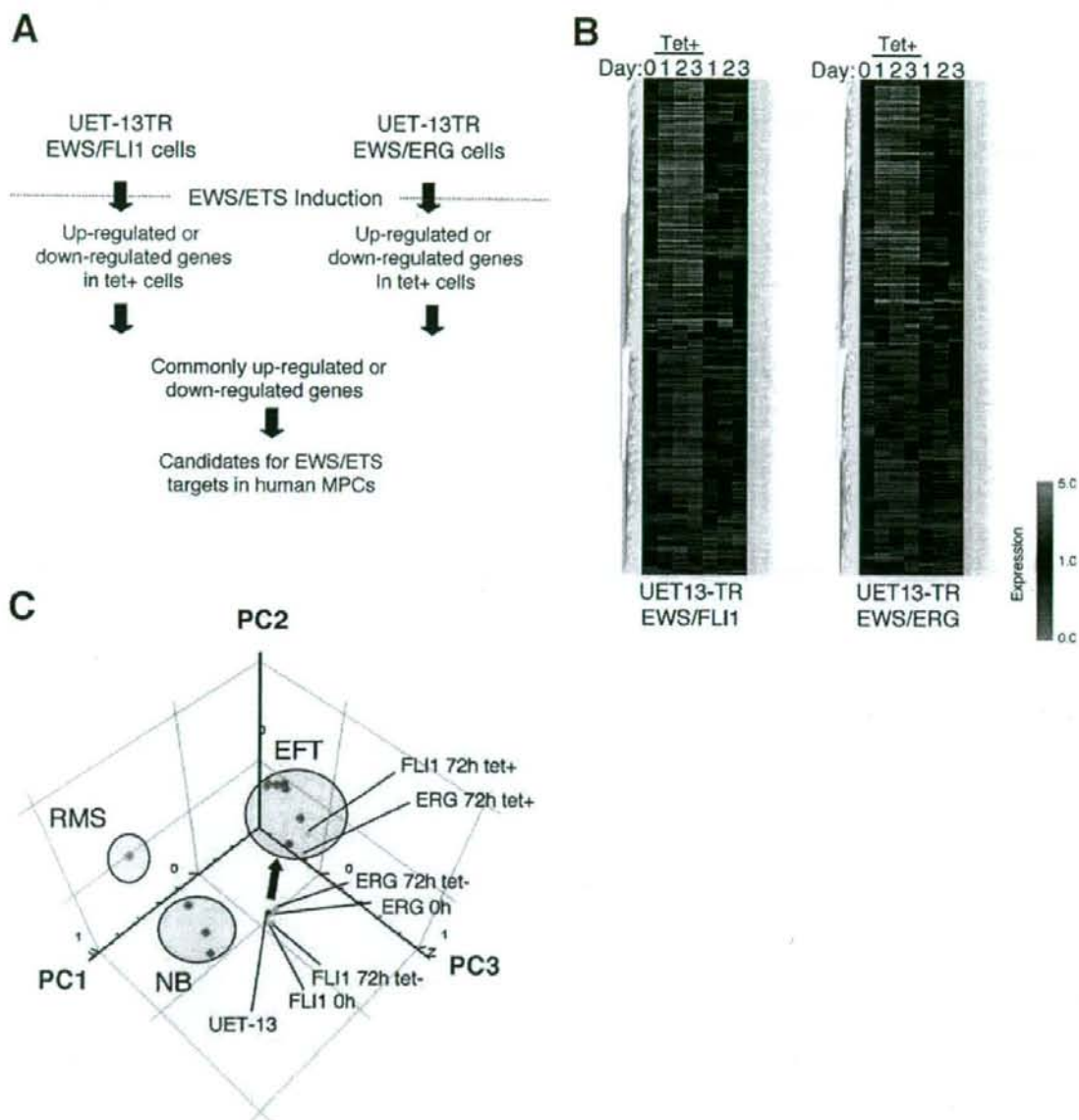


FIG. 7. Identification of candidates for the target of EWS/ETS in human MPCs by use of a microarray. UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were cultured as described for Fig. 6 and analyzed using the Affymetrix human genome U133 Plus 2.0 array as described in Materials and Methods. (A) Scheme for the analysis of microarray data. (B) Gene cluster analysis of UET-13 transfectants in the absence or presence of tetracycline by use of 642 candidate genes for targets of EWS/ETS in human MPCs. (C) Visualization of sequential change by the gene expression profile in UET-13 transfectants following tetracycline-mediated EWS/ETS expression based on a PCA of 642 candidate genes. Deep blue plots indicate UET-13 cells. Light blue plots indicate UET-13 transfectants in the absence of tetracycline for 72 h. Yellow plots indicate UET-13 transfectants in the presence of tetracycline for 72 h. The pink circle indicates EFT cell lines expressing EWS/FLI1 (purple plots), EWS/ERG (red plot), and EWS/E1AF (light green plot). The light blue circle with blue plots indicates NB cell lines. The yellow circle with an orange plot indicates a rhabdomyosarcoma (RMS) cell line. Cutoff induction and repression levels are 1.5-fold and 0.75-fold, respectively. Tet, tetracycline.

growth factor receptor 1) and development (such as jagged-1 and frizzled-4, -7, and -8). Interestingly, in addition to the surface antigens presented in Fig. 6B and C, the expression profiling of EWS/ETS-expressing UET-13 cells displayed the modulation of several genes associated with cell adhesion, cytoskeletal structure, and membrane trafficking, such as those for collagen-11 and -21, ephrin receptor-A2, -B2, and -B3, ephrin-B1, claudin-1, integrin- α 11, - α M, and - β 2, CD66 (carcinoembryonic antigen-related cell adhesion molecule-1), and CD102 (intercellular cell adhesion molecule-2). They also included genes of chemokines CCL-2 and -3. These data raise the possibility that EWS/ETS can contribute to the membrane condition in human MPCs via the regulation of these cell surface molecules and chemokines.

Using these genes, we performed a PCA to visualize the shift in the gene expression pattern among the 642 probes. As shown in Fig. 7C, the plots of UET-13 transfectants treated with tetracycline became closer to those of EFT cells than to those of UET-13 transfectants without tetracycline treatment. These results indicated that the expression pattern of these genes was altered from that of UET-13 cells to that of EFT cells in an EWS/ETS-dependent manner. Since the gene expression profile of UET-13 cells is similar to those of other cell types of mesenchymal origin (data not shown), our results highlighted that the phenotypic alteration from mesenchyme to EFT-like cells in UET-13 cells induced by tetracycline treatment was accompanied by a change in the global gene expression profile.

EWS/ETS expression enhances the Matrigel invasion of UET-13 cells. To assess the role of EWS/ETS in malignant transformation in human MPCs, UET-13 transfectants were examined by invasion assay. As shown in Fig. 8A, tetracycline treatment did not affect the Matrigel invasion ability of UET-13TR cells. When examined similarly, however, tetracycline treatment resulted in an apparently increased invasion ($P < 0.05$) for both UET-13TR-EWS/FLI1 (Fig. 8B) and UET-13TR-EWS/ERG (Fig. 8C) cells. The results indicated that EWS/ETS expression can induce Matrigel invasion properties in human MPCs.

DISCUSSION

In the present study, using UET-13 cells as a model of human MPCs, we demonstrated that ectopic expression of EWS/ETS promoted the acquisition of an EFT-like phenotype, including cellular morphology, immunophenotype, and gene expression profile. Moreover, EWS/ETS expression enhances the ability of UET-13 cells to invade Matrigel. This assay is thought to mimic the early steps of tumor invasion *in vivo* (34), and the ability to penetrate the Matrigel has been positively correlated with invasion potential in several studies. Therefore, we concluded that EWS/ETS expression could mediate a part of the feature of tumor transformation in human MPCs. Thus, our culture system would provide a good model for testing the effects of EWS/ETS in human MPCs.

Several lines of evidence have indicated the transforming ability of EWS/FLI1, whereas that of EWS/ERG is not yet to be clarified. Therefore, it is noteworthy that our data demonstrated that EWS/ERG could promote an EFT-like phenotype in UET-13 cells similarly to EWS/FLI1. Thus, EWS/ERG also has the ability to induce an EFT-like phenotype in the human

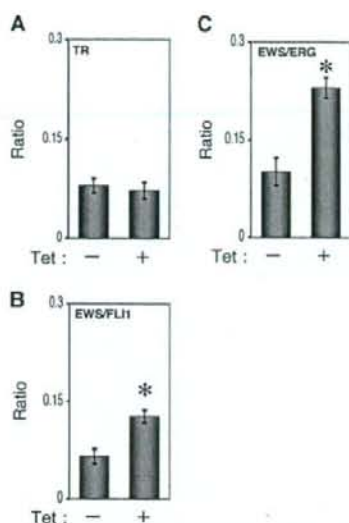


FIG. 8. Effects of EWS/ETS expression on the Matrigel invasion ability of UET-13 cells. UET-13TR (A), UET-13TR-EWS/FLI1 (B), and UET-13TR-EWS/ERG (C) cells were cultured in the absence or presence of tetracycline (Tet) for 72 h and then plated (2.5×10^4) on Matrigel-coated or uncoated filter inserts. After 20 h of culture, invading cells were stained with hematoxylin-eosin and counted in five fields per membrane as described in Materials and Methods. *, $P < 0.05$.

system. The major steps in the development of EFT should be commonly regulated by distinct chimeric EWS/ETS proteins. Indeed, several genes are common transcriptional targets of different chimeric EWS/ETS proteins in the murine system (11, 24, 35). Our data also showed that the 642 probes are coregulated in both EWS/FLI1-expressing cells and EWS/ERG-expressing cells. Further comparative studies of both the EWS/FLI1- and the EWS/ERG-mediated onset of EFT could allow us to understand the common functions of EWS/FLI1 and EWS/ERG in EFT. In addition, our systems are also useful for precisely distinguishing between the functions of these chimeric molecules in the development of EFT.

As mentioned above, the immunophenotypic analysis also revealed that the expression profiles of surface antigens in UET-13 cells were changed in favor of EFT cells in the presence of EWS/ETS (Fig. 4). Notably, the expression of CD54 (intercellular cell adhesion molecule-1 [ICAM1]), CD117 (c-kit), and CD271 (low-affinity nerve growth factor receptor [LNGFR]) increased in EWS/ETS-expressing UET-13 cells. These markers are positive in EFT cell lines (17, 28, 33), and in addition, CD117 is detected in about 40% of patient samples (17) and is negative in human primary MPCs (4, 43). Thus, it is reasonable to consider that a phenotypic marker of EFT was induced in UET-13 cells by EWS/ETS expression. On the other hand, CD54 and CD271 are positive in human primary MPCs (8, 25, 42), whereas these markers are negative in UET-13 cells. However, a previous report showed the disappearance of some positive markers, including CD271, from primary human MPCs during the process of *ex vivo* expansion

(25), and it has been speculated that the expression of these molecules in MPCs is induced *in vivo* via interaction with the bone marrow microenvironment and that the necessary stimuli are absent from *ex vivo* culture conditions. Therefore, the immunophenotype of UET-13 cells rather might be related to that of *ex vivo*-expanded primary human MPCs. In addition, it may be possible that EWS/ETS expression led to the reexpression of these disappeared markers in UET-13 cells without the necessary stimuli. In this case, the maintenance of CD271 expression outside of the bone marrow microenvironment might be a characteristic of EFT. Thus, our results proved that both EWS/FLI1 and EWS/ERG can be major causes of the expression of these markers and that human MPCs that precisely recapitulate the expression are strong candidates for the cell origins of EFT cells. The findings also imply that these antigens are suitable targets for diagnostic tools and new therapeutic agents. In fact, imatinib mesylate, which demonstrates anticancer activity against malignant cells expressing BCR-ABL as well as CD117 and platelet-derived growth factor receptor, inhibits proliferation and increases sensitivity to vincristine and doxorubicin in EFT cells (17).

Notably, our results also indicate that UET-13 cells, which have the MPC phenotype, possess the potential to acquire an EFT-like phenotype upon the expression of EWS/ETS. Unlike what is seen for human primary fibroblasts (31), ectopic EWS/ETS expression induces an EFT-like morphological change in human MPCs, suggesting that the cell type affects susceptibility to the events following EWS/ETS expression. In murine MPCs, retrovirally transduced EWS/FLI1 has been reported to induce the expression of CD99, a most useful marker for EFT, though the results are controversial (6, 45). However, our direct evidence obtained with UET-13 cells clearly demonstrated that CD99 expression is induced by EWS/ETS proteins in human MPCs. Moreover, we showed that the expression of CD99 might correlate with EWS/ETS-mediated morphological change, whereas the functional role of CD99 and the correlation between CD99 expression status and EWS/ETS-mediated morphological change in the development of EFT remain unclear.

Consistent with the morphological and immunophenotypic changes, the expression pattern of a set of genes in EWS/ETS-expressing UET-13 cells shifted to that in EFT cells (Fig. 7C). Although EWS/ETS expression enhanced the ability of UET-13 cells to invade Matrigel, it did not promote migratory ability and surface-independent growth, as assessed by migration assay and soft agar colony formation assay (data not shown). We also failed to develop EFT-like tumors by injecting EWS/ETS-inducing UET-13 cells into irradiated nude mice treated with tetracycline (data not shown). These results imply that EWS/ETS expression is not sufficient to induce the full transformation in UET-13 cells, and other genetic abnormalities not regulated by EWS/ETS could still be required for the full transformation of human MPCs into EFT cells. An identification of these genes will greatly improve our understanding of the additional genetic lesions that occur after EWS/ETS expression. The genes expressed in EFT cell lines but not in EWS/ETS-expressing UET-13 cells would be candidates for such genes.

In summary, we reported the development of an inducible EWS/ETS expression system in UET-13 cells as a model for

the development of EFT in MPCs. In our system, the chimeric genes alone are sufficient to confer EFT-like phenotypes, EFT-specific gene expression pattern, and partial but not full features of malignant transformation. Further analysis using our system should elucidate the pathogenic mechanism by which EFTs develop from MPCs, especially the initiating events mediated by EWS/ETS expression. Our system should also aid in the identification of novel targets of the EWS/ETS-mediated pathway as potential anticancer targets.

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ERRATUM

Inducible Expression of Chimeric EWS/ETS Proteins Confers Ewing's Family Tumor-Like Phenotypes to Human Mesenchymal Progenitor Cells

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Volume 28, no. 7, p. 2125–2137, 2008. Page 2131: The boxheads for Table 2 should appear as shown below.

MPC status ^a	CD marker	Result for ^b :								EFT status ^c	
		UET-13	UET-13R		UET-13TR-EWS/FLI		UET-13TR-EWS/ERG		RD-ES		SK-ES1
			Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺			

LETTERS

IGFBP-4 is an inhibitor of canonical Wnt signalling required for cardiogenesis

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Insulin-like growth-factor-binding proteins (IGFBPs) bind to and modulate the actions of insulin-like growth factors (IGFs)¹. Although some of the actions of IGFBPs have been reported to be independent of IGFs, the precise mechanisms of IGF-independent actions of IGFBPs are largely unknown^{1,2}. Here we report a previously unknown function for IGFBP-4 as a cardiogenic growth factor. IGFBP-4 enhanced cardiomyocyte differentiation *in vitro*, and knockdown of *Igfbp4* attenuated cardiomyogenesis both *in vitro* and *in vivo*. The cardiogenic effect of IGFBP-4 was independent of its IGF-binding activity but was mediated by the inhibitory effect on canonical Wnt signalling. IGFBP-4 physically interacted with a Wnt receptor, Frizzled 8 (Frz8), and a Wnt co-receptor, low-density lipoprotein receptor-related protein 6 (LRP6), and inhibited the binding of Wnt3A to Frz8 and LRP6. Although IGF-independent, the cardiogenic effect of IGFBP-4 was attenuated by IGFs through IGFBP-4 sequestration. IGFBP-4 is therefore an inhibitor of the canonical Wnt signalling required for cardiogenesis and provides a molecular link between IGF signalling and Wnt signalling.

The heart is the first organ to form during embryogenesis, and abnormalities in this process result in congenital heart diseases, the most common cause of birth defects in humans³. Molecules that mediate cardiogenesis are of particular interest because of their potential use for cardiac regeneration^{4,5}. Previous studies have shown that soluble growth factors such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Wnts and Wnt inhibitors mediate the tissue interactions that are crucial for cardiomyocyte specification^{3,4}. We proposed that there might be additional soluble factors that modulate cardiac development and/or cardiomyocyte differentiation.

P19CL6 cells differentiate into cardiomyocytes with high efficiency in the presence of 1% dimethylsulphoxide (DMSO)⁶. We cultured P19CL6 cells with culture media conditioned by various cell types in the absence of DMSO, and screened the cardiogenic activity of the conditioned media. The extent of cardiomyocyte differentiation was assessed by the immunostaining with MF20 monoclonal antibody that recognizes sarcomeric myosin heavy chain (MHC). Among the several cell types tested, culture media conditioned by a murine stromal cell line OP9 induced cardiomyocyte differentiation of P19CL6 cells without DMSO treatment (Fig. 1a, left and middle panels). Increased MF20-positive area was accompanied by the induction of cardiac marker genes such as *αMHC*, *Nkx2.5* and *GATA-4*, and by the increased protein levels of cardiac troponin T (cTnT) (Fig. 1a,

right panel). In contrast, culture media conditioned by COS7 cells, mouse embryonic fibroblasts, NIH3T3 cells, HeLa cells, END2 cells (visceral endoderm-like cells), neonatal rat cardiomyocytes and neonatal rat cardiac fibroblasts did not induce cardiomyocyte differentiation of P19CL6 cells in the absence of DMSO (Fig. 1a and data not shown). From these observations, we postulated that OP9 cells secrete one or more cardiogenic growth factors.

To identify an OP9-derived cardiogenic factor, complementary DNA clones isolated by a signal sequence trap method from an OP9 cell cDNA library⁷ were tested for their cardiogenic activities by transient transfection. When available, recombinant proteins were also used to confirm the results. Among candidate factors tested, IGFBP-4 induced cardiomyocyte differentiation of P19CL6 cells, as demonstrated by the increase in MF20-positive area and the induction of cardiac markers (Fig. 1b). We also cultured P19CL6 cells with OP9-conditioned media pretreated with an anti-IGFBP-4 neutralizing antibody. The application of an anti-IGFBP-4 neutralizing antibody attenuated the efficiency of cardiomyocyte differentiation induced by OP9-conditioned media (Fig. 1c). These findings strongly suggest that IGFBP-4 is a cardiogenic factor secreted from OP9 cells.

Because IGFBPs have been characterized as molecules that bind to and modulate the actions of IGFs, we tested whether IGFBP-4 promotes cardiogenesis by either enhancing or inhibiting the actions of IGFs. We first treated P19CL6 cells with a combination of anti-IGF-I and IGF-II-neutralizing antibodies or a neutralizing antibody against type-I IGF receptor. If IGFBP-4 induces cardiomyocyte differentiation by inhibiting IGF signalling, treatment with these antibodies should induce cardiomyocyte differentiation and/or enhance the cardiogenic effects of IGFBP-4. In contrast, if IGFBP-4 promotes cardiogenesis by enhancing IGF signalling, treatment with these antibodies should attenuate IGFBP-4-mediated cardiogenesis. However, treatment with these antibodies did not affect the efficiency of IGFBP-4-induced cardiomyocyte differentiation (Fig. 1d and data not shown). Treatment of P19CL6 cells with IGF-I and IGF-II also did not induce cardiomyocyte differentiation (data not shown). Furthermore, treatment with an IGFBP-4 mutant (IGFBP-4-H74P; His 74 replaced by Pro)⁸ that is unable to bind IGFs induced cardiomyocyte differentiation of P19CL6 cells even more efficiently than wild-type IGFBP-4 (Fig. 1e). This is presumably due to the sequestration of wild-type IGFBP-4 but not mutant IGFBP-4-H74P by endogenous IGFs. In agreement with this idea, exogenous IGFs attenuated wild-type IGFBP-4-induced but not IGFBP-4-H74P-induced cardiogenesis (Fig. 1f). Taken together, these observations indicate

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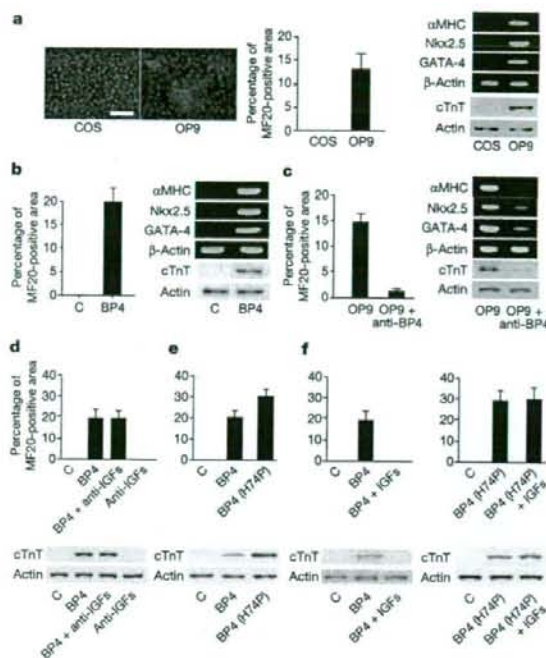


Figure 1 | IGFBP-4 promotes cardiomyocyte differentiation in an IGF-independent manner. **a**, Culture media conditioned by OP9 cells but not by COS7 cells induced cardiomyocyte differentiation of P19CL6 cells as assessed by MF20-positive area, cardiac marker-gene expression and cTnT protein expression. Scale bar, 100 μ m. Error bars show s.d. **b**, Treatment with IGFBP-4 (1μ g ml⁻¹) induced cardiomyocyte differentiation of P19CL6 cells in the absence of DMSO. Error bars show s.d. **c**, Treatment with a neutralizing antibody against IGFBP-4 (anti-BP4; 40μ g ml⁻¹) attenuated cardiomyocyte differentiation of P19CL6 cells induced by OP9-conditioned media. Error bars show s.d. **d**, Treatment with neutralizing antibodies against IGF-I and IGF-II (anti-IGFs; 5μ g ml⁻¹ each) had no effect on IGFBP-4-induced cardiomyocyte differentiation of P19CL6 cells. Error bars show s.d. **e**, Mutant IGFBP-4 (BP4(H74P)) that is incapable of binding to IGFs retained cardiomyogenic activity. Error bars show s.d. **f**, IGFs (100 ng ml^{-1} each) attenuated wild-type IGFBP-4-induced but not mutant IGFBP-4-H74P-induced cardiomyocyte differentiation of P19CL6 cells. Error bars show s.d.

that IGFBP-4 induces cardiomyocyte differentiation in an IGF-independent fashion.

To explore further the mechanisms by which IGFBP-4 induces cardiomyogenesis, we tested the hypothesis that IGFBP-4 might modulate the signals activated by other secreted factors implicated in cardiogenesis. It has been shown that canonical Wnt signalling is crucial in cardiomyocyte differentiation^{3,4}. In P19CL6 cells, Wnt3A treatment activated β -catenin-dependent transcription of the TOPFLASH reporter gene, and this activation was attenuated by IGFBP-4 (Fig. 2a). Wnt/ β -catenin signalling is transduced by the cell-surface receptor complex consisting of Frizzled and low-density-lipoprotein receptor (LDLR)-related protein 5/6 (LRP5/6)⁹ and IGFBP-4 attenuated TOPFLASH activity enhanced by the expression of LRP6 or Frizzled 8 (Frz8) (Fig. 2a). As a control, IGFBP-4 did not alter BMP-mediated activation of a BMP-responsive reporter BRE-luc (Supplementary Fig. 1b). These findings suggest that IGFBP-4 is a specific inhibitor of the canonical Wnt pathway. To examine this possibility *in vivo*, we performed axis duplication assays in *Xenopus* embryos. Injection of *Xwnt8* or *Lrp6* mRNA caused secondary axis formation, and injection of *Xenopus* IGFBP-4 (*XIGFBP-4*) mRNA alone had minimal effects on axis

formation. However, *Xwnt8*-induced or LRP6-induced secondary axis formation was efficiently blocked by coexpression of *XIGFBP-4* (Fig. 2b, c), indicating that IGFBP-4 inhibits canonical Wnt signalling *in vivo*. To explore the mechanisms of Wnt inhibition by IGFBP-4, *Xenopus* animal cap assays and TOPFLASH reporter gene assays were performed. In animal cap assays, IGFBP-4 inhibited LRP6-induced but not β -catenin-induced Wnt-target gene expression (Supplementary Fig. 1c). Similarly, IGFBP-4 attenuated Wnt3A-induced or LRP6-induced TOPFLASH activity but did not alter Dishevelled-1 (Dvl-1)-induced, LiCl-induced or β -catenin-induced TOPFLASH activity (Supplementary Fig. 1d, e). These findings suggest that IGFBP-4 inhibits canonical Wnt signalling at the level of cell-surface receptors. To examine whether IGFBP-4 antagonizes Wnt signalling via direct physical interaction with LRP5/6 or Frizzled, we produced conditioned media containing the Myc-tagged extracellular portion of LRP6 (LRP6N-Myc), the Myc-tagged cysteine-rich domain (CRD) of Frz8 (Frz8CRD-Myc), and V5-tagged IGFBP-4 (IGFBP-4-V5). Immunoprecipitation (IP)/western blot experiments revealed that IGFBP-4 interacted with LRP6N (Fig. 2d) and Frz8CRD (Fig. 2e). A liquid-phase binding assay with ¹²⁵I-labelled IGFBP-4 and conditioned media containing LRP6N-Myc or Frz8CRD-Myc demonstrated that the interaction between IGFBP-4 and LRP6N or Frz8CRD was specific and saturable (Fig. 2f, g). A Scatchard plot analysis revealed two binding sites with different binding affinities for LRP6N (Fig. 2f, inset) and a single binding site for Frz8CRD (Fig. 2g, inset). A similar binding assay with ¹²⁵I-labelled Wnt3A demonstrated that IGFBP-4 inhibited Wnt3A binding to LRP6N (Fig. 2h) and Frz8CRD (Fig. 2i), and a Lineweaver-Burk plot revealed that IGFBP-4 was a competitive inhibitor of the binding of Wnt3A to Frz8CRD (Supplementary Fig. 2a). IP/western blot analyses with various deletion mutants of LRP6 and IGFBP-4 revealed that IGFBP-4 interacted with multiple domains of LRP6 and that the carboxy-terminal thyroglobulin domain of IGFBP-4 was required for IGFBP-4 binding to LRP6 or Frz8CRD (Supplementary Fig. 2b-f). It has been shown that inhibition of canonical Wnt signalling promotes cardiomyocyte differentiation in embryonic stem (ES) cells and in chick, *Xenopus* and zebrafish embryos^{10,11}. These results therefore collectively suggest that IGFBP-4 promotes cardiogenesis by antagonizing the Wnt/ β -catenin pathway through direct interactions with Frizzled and LRP5/6.

Next we investigated the role of endogenous IGFBP-4 in P19CL6 cell differentiation into cardiomyocytes. Reverse transcriptase-mediated polymerase chain reaction (RT-PCR) analysis revealed that the expression of *Igfbp4* was upregulated during DMSO-induced P19CL6 cell differentiation (Fig. 3a). Expression of *Igfbp3* and *Igfbp5* was also upregulated in the early and the late phases of differentiation, respectively. Expression of *Igfbp2* was not altered, and that of *Igfbp1* or *Igfbp6* was not detected. When IGFBP-4 was knocked down by two different small interfering RNA (siRNA) constructs, DMSO-induced cardiomyocyte differentiation was inhibited in both cases (Fig. 3b). In contrast, knockdown of *Igfbp3* or *Igfbp5* did not inhibit DMSO-induced cardiomyocyte differentiation (Fig. 3b, right panel). Treatment with an anti-IGFBP-4 neutralizing antibody also blocked DMSO-induced cardiomyocyte differentiation (Fig. 3c). Secretion of endogenous IGFBP-4 is therefore required for the differentiation of P19CL6 cells into cardiomyocytes. Immunostaining for IGFBP-4 revealed that cardiac myocytes were surrounded by the IGFBP-4-positive cells, suggesting that a paracrine effect of IGFBP-4 on cardiomyocyte differentiation is predominant (Fig. 3d). Essentially the same results were obtained in ES cells (Supplementary Fig. 3d-g). To investigate whether IGFBP-4 promotes the differentiation of P19CL6 cells into cardiomyocytes by the inhibition of the canonical Wnt pathway, we expressed dominant-negative LRP6 (LRP6N) in P19CL6 cells. Expression of LRP6N enhanced cardiomyocyte differentiation of P19CL6 cells and reversed the inhibitory effect of *Igfbp4*

knockdown on cardiomyogenesis (Fig. 3e). These observations suggest that endogenous IGFBP-4 is required for cardiomyocyte differentiation of P19CL6 cells and ES cells, and that the cardiogenic effect of IGFBP-4 is mediated by its inhibitory effect on Wnt/ β -catenin signalling.

The role of endogenous IGFBP-4 in cardiac development *in vivo* was also examined with *Xenopus* embryos. Whole-mount *in situ* hybridization analysis revealed that strong expression of *XIGFBP-4* was detected at stage 38 in the anterior part of the liver adjacent to the heart (Fig. 4a). Knockdown of *XIGFBP-4* by two different morpholino (MO) constructs resulted in cardiac defects, with more than 70% of the embryos having a small heart or no heart (Fig. 4b). The specificity of MO was confirmed by the observation that simultaneous injection of MO-resistant *XIGFBP-4* cDNA rescued the MO-induced cardiac defects (Fig. 4b, Supplementary Fig. 4c). Coexpression of IGF-binding-defective *XIGFBP-4* mutant (*XIGFBP-4*-H74P) or

dominant-negative LRP6 (LRP6N) also rescued the cardiac defects induced by *XIGFBP-4* knockdown (Fig. 4b), whereas overexpression of *Xwnt8* in the heart-forming region resulted in cardiac defects similar to those induced by *XIGFBP-4* knockdown (Supplementary Fig. 4d–f), supporting the notion that the cardiogenic effect of IGFBP-4 is independent of IGFs but is mediated by inhibition of the Wnt/ β -catenin pathway. The temporal profile of cardiac defects induced by *XIGFBP-4* knockdown was also examined by *in situ* hybridization with *cardiac troponin I* (*cTnI*) (Fig. 4c). At stage 34, morphology of the heart was comparable between control embryos and MO-injected embryos. However, at stage 38, when *XIGFBP-4* starts to be expressed in the anterior part of the liver, the expression of *cTnI* was markedly attenuated in MO-injected embryos; expression of *cTnI* was diminished and no heart-like structure was observed at stage 42. Thus, the heart is initially formed but its subsequent growth is perturbed in the absence of *XIGFBP-4*, suggesting that IGFBP-4

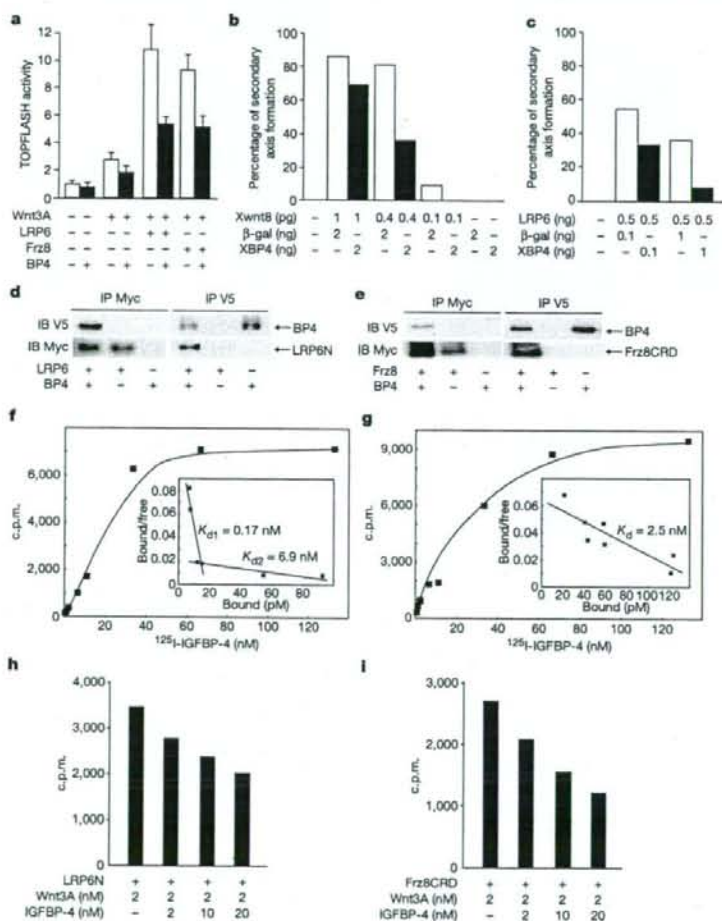


Figure 2 | IGFBP-4 inhibits Wnt/ β -catenin signalling through direct interactions with Wnt receptors. **a**, IGFBP-4 attenuated β -catenin-dependent transcription in P19CL6 cells. P19CL6 cells were transfected with TOPFLASH reporter gene and expression vectors for LRP6 or Frz8, and then treated with Wnt3A or Wnt3A plus IGFBP-4; luciferase activities were then measured. Error bars show s.d. **b**, *XIGFBP-4* (XBP4) inhibited *Xwnt8*-induced secondary-axis formation in *Xenopus* embryos ($n = 20$ for each group). **c**, IGFBP-4 inhibited LRP6-induced secondary-axis formation in *Xenopus* embryos ($n = 30$ for each group). **d**, **e**, IGFBP-4 interacted directly

with LRP6N (**d**) and Frz8CRD (**e**). IB, immunoblotting; IP, immunoprecipitation. **f**, A binding assay between 125 I-labelled IGFBP-4 and LRP6N. The inset is a Scatchard plot showing two binding sites with different binding affinities. **g**, A binding assay between 125 I-labelled IGFBP-4 and Frz8CRD. The inset is a Scatchard plot showing a single binding site. **h**, **i**, IGFBP-4 inhibited Wnt3A binding to LRP6N (**h**) or Frz8CRD (**i**). 125 I-labelled Wnt3A binding to LRP6N or Frz8CRD was assessed in the presence of increasing amounts of IGFBP-4.

promotes cardiogenesis by maintaining the proliferation and/or survival of embryonic cardiomyocytes.

It has been shown that canonical Wnt signals inhibit cardiogenesis in chick and frog embryos, and that Wnt antagonists such as Dkk1 and Crescent secreted from the anterior endoderm or the organizer region counteract the Wnt-mediated inhibitory signals and induce cardiogenesis in the anterior lateral mesoderm⁴. However, IGFBP-4-mediated Wnt inhibition is required at later stages of development, when the heart is already formed at the ventral portion and starts to grow and remodel to maintain embryonic circulation. It has been shown that Wnt/ β -catenin signalling has time-dependent effects on cardiogenesis in ES cells: canonical Wnt signalling in the early phase of ES-cell differentiation promotes cardiomyogenesis, whereas it inhibits cardiomyocyte differentiation in the late phase^{10–12}. In agreement with this notion, IGFBP-4 promoted cardiomyocyte differentiation of ES cells only when IGFBP-4 was applied in the late phase after embryoid body formation (Supplementary Fig. 3a–c). Similar

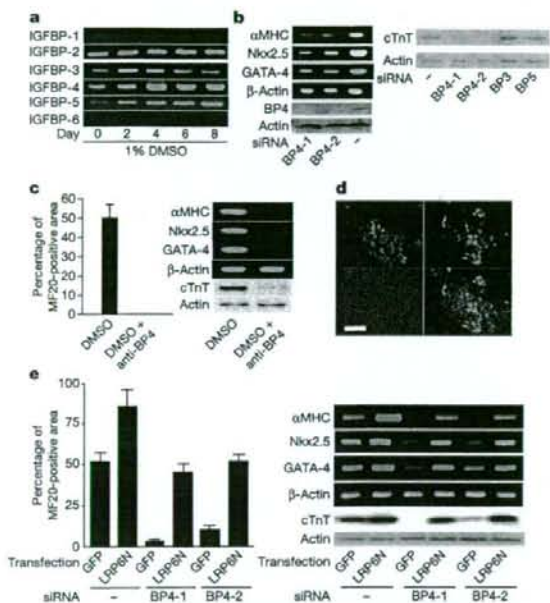


Figure 3 | IGFBP-4 is required for the differentiation of P19CL6 cells into cardiomyocytes. **a**, Expression analysis of IGFBP family members by RT-PCR during DMSO-induced cardiomyocyte differentiation of P19CL6 cells (from day 0 to day 8). **b**, Left: knockdown of *Igfbp4* in P19CL6 cells attenuated cardiac marker expression in response to treatment with DMSO. BP4-1 and BP4-2 represent two different siRNAs for IGFBP-4. Right: knockdown of *Igfbp3* or *Igfbp5* had no effect on cTnT expression in response to DMSO treatment. **c**, Treatment with a neutralizing antibody against IGFBP-4 (anti-BP4; 40 $\mu\text{g ml}^{-1}$) attenuated DMSO-induced cardiomyocyte differentiation of P19CL6 cells. Error bars show s.d. **d**, IGFBP-4 immunostaining during DMSO-induced differentiation of P19CL6 cells stably transfected with αMHC -green fluorescent protein (GFP) reporter gene. Top left, IGFBP-4 staining (red); top right, GFP expression representing differentiated cardiomyocytes; bottom left, nuclear staining with DAPI (4',6-diamidino-2-phenylindole); bottom right, a merged picture. Scale bar, 100 μm . **e**, Attenuated cardiomyocyte differentiation of P19CL6 cells by *Igfbp4* knockdown was rescued by inhibiting Wnt/ β -catenin signalling. Control and *Igfbp4*-knocked-down P19CL6 cells were transfected with an expression vector for GFP or LRP6N (a dominant-negative form of LRP6) and induced to differentiate into cardiomyocytes by treatment with DMSO. LRP6N overexpression rescued the attenuated cardiomyocyte differentiation induced by *Igfbp4* knockdown as assessed by MF20-positive area (left panel), cardiac marker-gene expression and cTnT protein expression (right panel). Error bars show s.d.

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time-dependent effects of Wnt/ β -catenin signalling on cardiogenesis has been shown in zebrafish embryos¹¹. Moreover, several recent reports suggest that Wnt/ β -catenin signalling is a positive regulator of cardiac progenitor-cell proliferation in the secondary heart field¹³. It therefore seems that canonical Wnt signalling has divergent effects on cardiogenesis at multiple stages of development: first, canonical Wnt signalling promotes cardiogenesis at the time of gastrulation or mesoderm specification; second, it inhibits cardiogenesis at the time when cardiac mesoderm is specified in the anterior lateral mesoderm; third, it promotes the expansion of cardiac progenitors in the secondary heart field; and fourth, it inhibits cardiogenesis at later stages when the embryonic heart is growing. It is interesting to note that IGFBP-4 is expressed predominantly in the liver. Mouse IGFBP-4 is

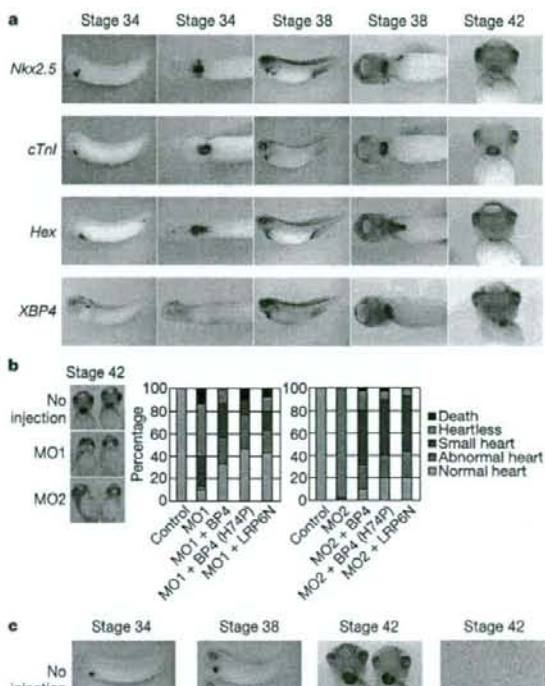


Figure 4 | IGFBP-4 is required for the maturation of the heart in *Xenopus* embryos. **a**, *In situ* hybridization analysis of *Nkx2.5* (an early cardiac marker), *cTnI* (a mature cardiac marker), *Hex* (a liver marker), and *XIGFBP-4* (*XBP4*) mRNA expression at stages 34, 38 and 42. **b**, Knockdown of *XIGFBP-4* by two different morpholinos (MO1 and MO2) resulted in severe cardiac defects as assessed by *cTnI* *in situ* hybridization at stage 42 (left). These cardiac defects were rescued by simultaneous injection of MO-resistant wild-type *XIGFBP-4*, mutant *XIGFBP-4*-H74P (BP4(H74P)) and LRP6N ($n = 30$ for each group). **c**, Temporal profile of cardiac defects induced by *XIGFBP-4* knockdown. Morphology of the heart as assessed by *cTnI* *in situ* hybridization was almost normal at stage 34 but was severely perturbed at stages 38 and 42. The right column shows sections of control and MO-injected embryos. The arrow indicates the heart in control embryos. No heart-like structure was observed in MO-injected embryos.

also strongly expressed in the tissues adjacent to the heart such as pharyngeal arches and liver bud at embryonic day (E)9.5 (Supplementary Fig. 3h). These observations and the results of IGFBP-4 immunostaining in P19CL6 cells and ES cells suggest that IGFBP-4 promotes cardiogenesis in a paracrine fashion. Together with a previous report showing that cardiac mesoderm secretes FGFs and induces liver progenitors in the ventral endoderm¹⁴, these observations suggest that there exist reciprocal paracrine signals between the heart and the liver that coordinately promote the development of each other.

IGFBPs are composed of six members, IGFBP-1 to IGFBP-6. Reporter gene assays and β -catenin stabilization assays revealed that IGFBP-4 was the most potent canonical Wnt inhibitor and that IGFBP-1, IGFBP-2 and IGFBP-6 also showed modest activity in Wnt inhibition, whereas IGFBP-3 and IGFBP-5 had no such activity (Supplementary Fig. 5a–c). In agreement with this, IP/western blot analyses demonstrated that IGFBP-1, IGFBP-2, IGFBP-4 and IGFBP-6 but not IGFBP-3 or IGFBP-5 interacted with LRP6 or Frz8CRD (Supplementary Fig. 5d, e). Thus, the lack of cardiac phenotypes in IGFBP-4-null mice or IGFBP-3/IGFBP-4/IGFBP-5 triple knockout mice¹⁵ may be due to genetic redundancies between IGFBP-4 and other IGFBPs such as IGFBP-1, IGFBP-2 and/or IGFBP-6.

The identification of IGFBP-4 as an inhibitor of Wnt/ β -catenin signalling may also have some implications for cancer biology¹⁶. It was shown that treatment with IGFBP-4 reduces cell proliferation in some cancer cell lines *in vitro*, and that overexpression of IGFBP-4 attenuates the growth of prostate cancer *in vivo*. Decreased serum levels of IGFBP-4 are associated with the risk of breast cancer. Because the activation of Wnt signalling is implicated in several forms of malignant tumours^{17,18}, it is possible that the inhibitory effect of IGFBP-4 on cell proliferation is mediated in part by the inhibition of canonical Wnt signalling.

METHODS SUMMARY

Cell culture. P19CL6 cells and ES cells were cultured and induced to differentiate into cardiomyocytes essentially as described^{6,10}. P19CL6 cells (2,000 cells per 35-mm dish) were treated with various conditioned media for screening of their cardiogenic activities. For siRNA-mediated knockdown, pSIREN-RetroQ vectors (Clontech) ligated with double-stranded oligonucleotides were transfected into P19CL6 cells or ES cells, and puromycin-resistant clones were selected. **IP/western blot analyses and binding assays.** Conditioned media for IP/western blot analyses were produced by using 293 cells. Binding reactions were performed overnight at 4°C. ¹²⁵I-labelling of IGFBP-4 and Wnt3A was performed with IODO-BEADS Iodination Reagent (Pierce). A liquid-phase binding assay was performed essentially as described¹⁹.

Xenopus experiments. Axis duplication assays, animal cap assays, and *in situ* hybridization analyses in *Xenopus* were performed essentially as described²⁰. Electroporation of mRNA was performed at stage 28 essentially as described²¹.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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