

図3 現行の歯周組織再生療法

現在、GTR法とエムドゲイン®による歯周組織再生療法が臨床応用されており、ともに臨床の場で一定の成果をあげている。これらとともに、歯根膜中に存在する内在性「歯周組織幹細胞」の潜在能力を引き起こすことにより、歯周組織再生を誘導しようとするものである。

なった(図3)。これは、歯周組織欠損部を生体親和性のGTR膜で覆うことにより、歯肉上皮由来および歯肉結合組織由来の細胞が同上欠損部へ侵入するのを防ぎ、歯根膜由来細胞を同上欠損部へ到達させることにより、歯周組織再生を誘導しようとする治療法である。その後1990年代に入り、「エナメルマトリクスタンパク(EMD):エムドゲイン®」が臨床応用されるようになる(図3)。この

タンパクは歯の発生期にヘルトヴィッヒ上皮鞘(Hertwig's epithelial sheath)から分泌されるタンパクでセメント質の形成を促す作用を有しているといわれている。6ヵ月齢ブタの下顎骨歯胚から生成されたEMDが現在臨床応用されており、EMDを歯周外科時に歯周組織欠損部へ投与することによりセメント質形成が、ひいては歯周組織再生が誘導されると考えられている。

II 歯周組織再生療法の将来展望

1 サイトカイン療法による歯周組織再生誘導

先に述べた現行の歯周組織再生療法は、臨床の場で一定の成果をあげているものの、適応症・予知性などにつき、改善されるべき点があることが指摘されている。そこで、I-1で述べた歯周組織再生の過程をサイトカインの局所投与により活性化し、歯周組織再生を積極的に促進しようとする新

たな治療法の確立が試みられている。表1にはこれまでに動物実験などでその有効性が報告されているサイトカインを示している。このうち、PDGF-BBと β -TCPを組み合わせたものが歯周組織再生誘導用のdeviceとして米国FDAの承認を受け、現在米国にて臨床応用が開始されている²⁾。

筆者らの研究室では、強力な血管新生作用と間葉系細胞の増殖誘導能を有する塩基性線維芽細胞増殖因子[basic fibroblast growth factor:

表1 サイトカインによる歯周組織再生誘導

①PDGF-BB (platelet-derived growth factor)	+	IGF-1 (insulin-like growth factor-1)
②BMP-2 (bone morphogenetic protein-2)		
③TGF- β (transforming growth factor- β)		
④OP-1 (BMP-7) (osteogenic protein-1)		
⑤BDNF (brain-derived neurotrophic factor)		
⑥VEGF (vascular endothelial growth factor)		
⑦PDGF-BB (platelet-derived growth factor)	+	β -TCP (GEM21S®) (b-tricalcium phosphate)
⑧FGF-2 (bFGF) (basic fibroblast growth factor)		

bFGF (FGF-2)に着目し、FGF-2を歯周外科時に歯槽骨欠損部に局所投与することにより、歯周病により失われた歯周組織の再生を人為的に誘導・促進する、新しい歯周組織再生療法の開発に取り

組んできた。

a. 動物実験における塩基性線維芽細胞増殖因子 FGF-2の歯周組織再生誘導効果の検討

筆者らは、FGF-2が実際に歯周組織再生を促進するか否かを以下の動物実験により検証した^{3,4)}。ビーグル犬およびカナクイザルの下顎臼歯部複根歯に歯周病モデル(2級根分岐部病変)を作製し、架橋ゼラチンを基剤としたFGF-2を実験側の歯周組織欠損部に填入し、対照側には同基剤のみを填入した。そして、FGF-2投与後それぞれ6週および8週経過した後に、FGF-2投与部位に歯周組織の再生が誘導されているか否かを検討した。その結果、FGF-2投与側では、肉眼的にも明らかな骨の新生が認められた(図4)^{3,4)}。そして、組織学的にも新生歯槽骨、新生歯根膜、新生セメント質が観察され、統計学的にも有意な歯周組織再生が誘導、促進されているのが明らかにされた(表2)。これ以外の歯周組織欠損モデルに対しても同様の

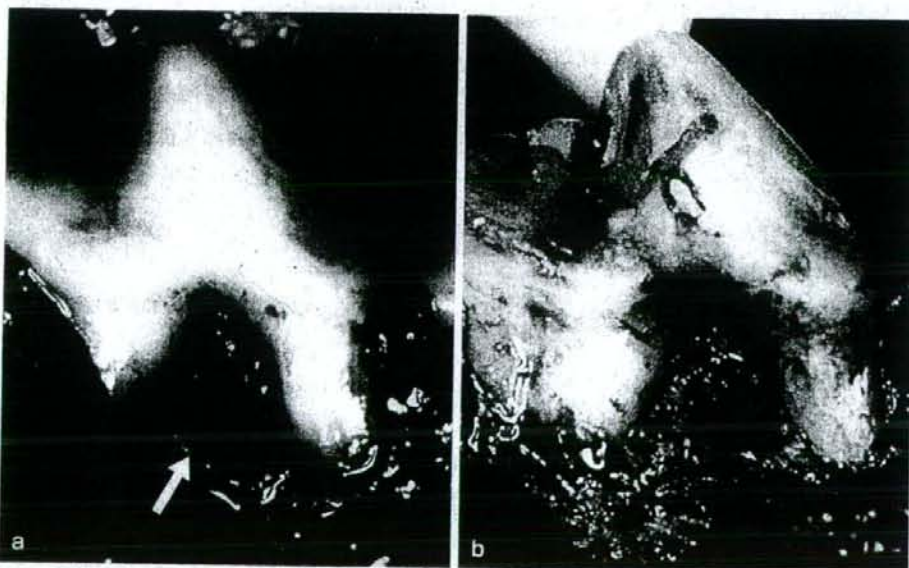


図4 FGF-2投与によりビーグル犬の根分岐部病変に誘導された歯周組織再生

0.1%FGF-2投与前a, 投与6週後bの2級根分岐部病変における歯槽骨の新生(骨充填)の様子を示す。

(村上伸也, 高山真一: bFGFの現状と将来展望. 歯界展望, 医歯薬出版, 536, 2002.)

(文献3)より)

表2 ビーグル犬およびカニクイザルに作製した実験的2級根分岐部病変に対するFGF-2の歯周組織再生誘導効果

a	ビーグル犬 (%)	対照側 (n=6)	FGF-2側 (n=6)
	新生骨形成率	35.4±8.9	83.6±14.3*
	新生骨梁形成率	16.6±6.2	44.1±9.5*
	新生セメント質形成率	37.2±15.1	97.0±7.5*
*: p<0.01 (文献3)より改変)			
b	カニクイザル (%)	対照側 (n=6)	FGF-2側 (n=6)
	新生骨形成率	54.3±8.0	71.3±13.5*
	新生骨梁形成率	31.6±3.5	48.7±8.9**
	新生セメント質形成率	38.8±8.6	72.2±14.4**

*: p<0.05 **: p<0.01

FGF-2投与時の欠損量を100%とし、6週後aおよび8週後bに新生が確認された骨・骨梁・セメント質量をそれぞれ百分率で表している。対照側には基剤のみを投与した。

(文献4)より改変)

処置を行い、そのすべてにおいて、歯肉上皮の下方増殖・骨性癒着・歯根吸収などの異常な治療形態を生じない、統計学的に有意な歯周組織再生が誘導されてくることを確認している。

b. 臨床試験におけるFGF-2の歯周組織再生誘導効果

2001年よりFGF-2の歯周組織再生誘導効果ならびに安全性の検討を目的として、多施設参加の第II相臨床試験(プラセボを含む用量反応同時対照による二重盲検試験)が行われた。その結果、ヒトの2壁性および3壁性歯槽骨欠損に対し、0.3% FGF-2含有ヒドロキシプロピルセルロース(HPC)製剤の局所投与がX線写真上で統計学的に有意な歯槽骨新生を誘導し得ることが確認されている。また、同試験期間中には安全性上問題になるような事例は認められなかったと報告されている。FGF-2のヒトに対する安全性および歯周組織再生誘導に対する有効性についてのさらなる検討のために、2005年8月より後期第II相の臨床試験(プラセボを含む二重盲検・並行群間比較用量反応試験)が展開されている。

2 細胞移植による歯周組織再生療法の開発

a. 細胞移植による歯周組織再生療法の必要性

先に述べたGTR膜、EMDおよびサイトカインを用いた歯周組織再生療法は、すべて内在性の歯根膜組織由来幹細胞のもつ自己修復力を活性化することにより歯周組織再生を図るアプローチと捉えることができる。一方、生体内の幹細胞数は加齢とともに減少することが知られている。したがって、歯周組織再生に関しても、高齢者の方や、重度歯周病の場合には内在性の歯根膜細胞の活用だけでは十分な再生量が期待できないことが危惧される。そのような場合には、ほかの組織より採取した間葉系幹細胞を歯周組織欠損部へ移植することにより、歯周組織再生を促すことが必要になるものと考えられる。

b. 移植細胞の選択

—脂肪組織由来幹細胞の可能性—

従来、再生医療のための幹細胞源としては、骨髄より採取したものが広く使用されている。しかしながら、骨髄からの幹細胞の採取は、身体への侵襲が大きく、また採取できる細胞数も限定される。そこで、筆者らの研究室では、採取に際して患者への負担がより少なく、安全性も高いと考えられる脂肪組織中に存在する未分化間葉系幹細胞に着目した。すでに*in vitro*において、脂肪組織由来未分化間葉系幹細胞(ADSC)が、脂肪、骨、軟骨、筋肉など中胚葉性の細胞へ分化することが報告されており、ADSCがmultipotentな細胞であることが明らかにされている⁵⁾。筆者らの研究室でも、ヒト脂肪組織より単離したADSCが骨芽細胞lineageへの分化能を有していることを*in vitro*にて確認している。そこで、ビーグル犬を用いた歯周病モデルを用いて、ADSC移植による歯周組

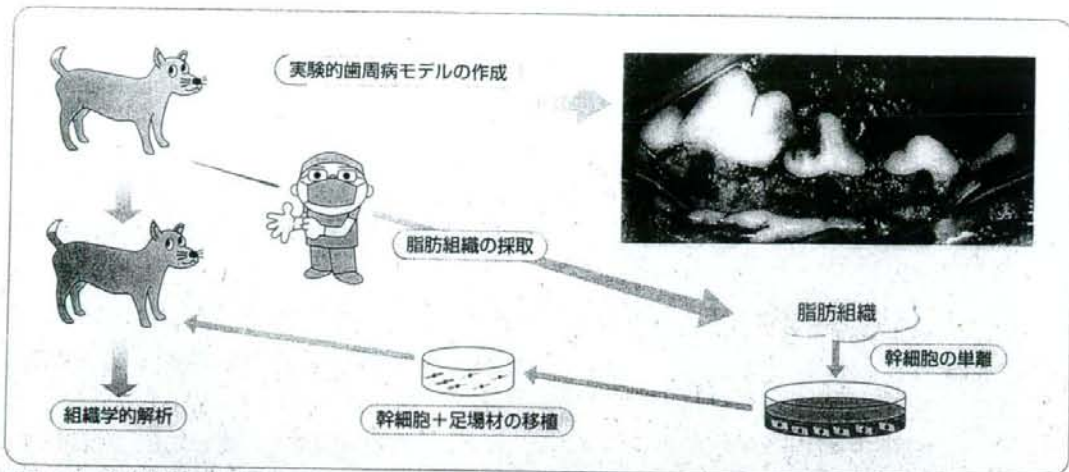


図5 ビーグル犬を用いたADSC移入による歯周組織再生誘導実験

ビーグル犬の下顎臼歯頬側分岐部に人工的2級分岐部病変を作製し、同部に同一ビーグル犬の腹部大網脂肪組織より分離したADSCを、足場材としてのフィブリンゲルとともに移植する。

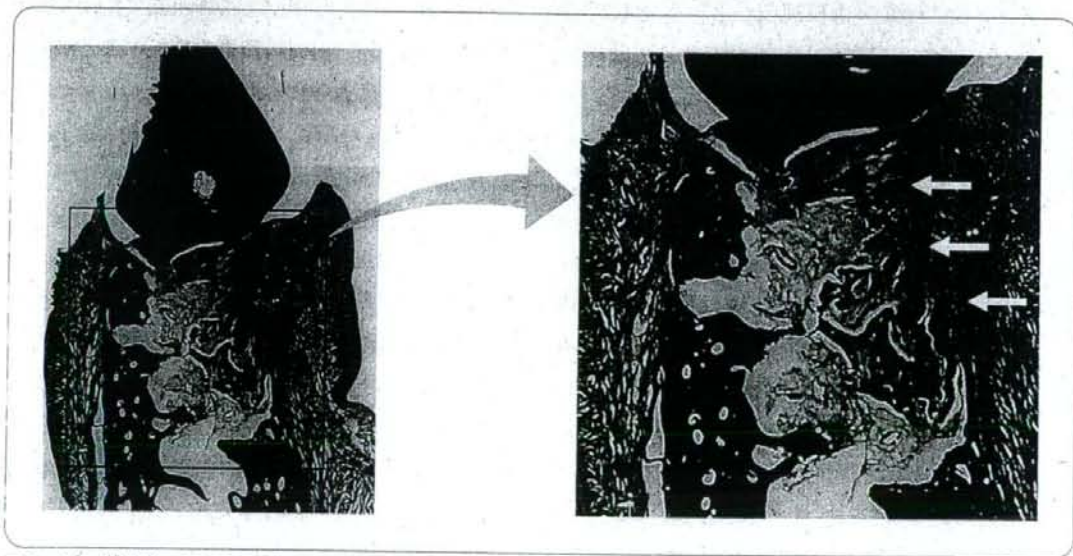


図6 ビーグル犬へのADSC移入による歯周組織再生

作成した左右両側の人工的骨欠損のうち一側を被験部位として、ADSC+フィブリンゲルを移植した。一方、対照部位にはフィブリンのみを移植した。移植後6週目に屠殺し、組織切片を作成して組織学的に歯周組織再生効果を評価した。ADSC移植後6週目の組織学的解析を示す。矢印部に著明な骨の新生を伴う歯周組織再生が認められた。

織再生誘導効果について検討を行った(図5)。ビーグル犬の第四前臼歯分岐部に人工的歯周組織欠損(2級分岐部病変)を作成し、腹部より採取した脂肪より単離したADSCをフィブリンゲルとともに同欠損部へ移植した結果を図6に示してい

る。組織学的解析を行った結果から、移植部には著明な新生骨が確認されている。このように、生体内に豊富に存在し採取も簡便かつ安全に行える脂肪組織は、歯周組織再生療法において、有用な幹細胞源となり得ることが強く示唆されている。

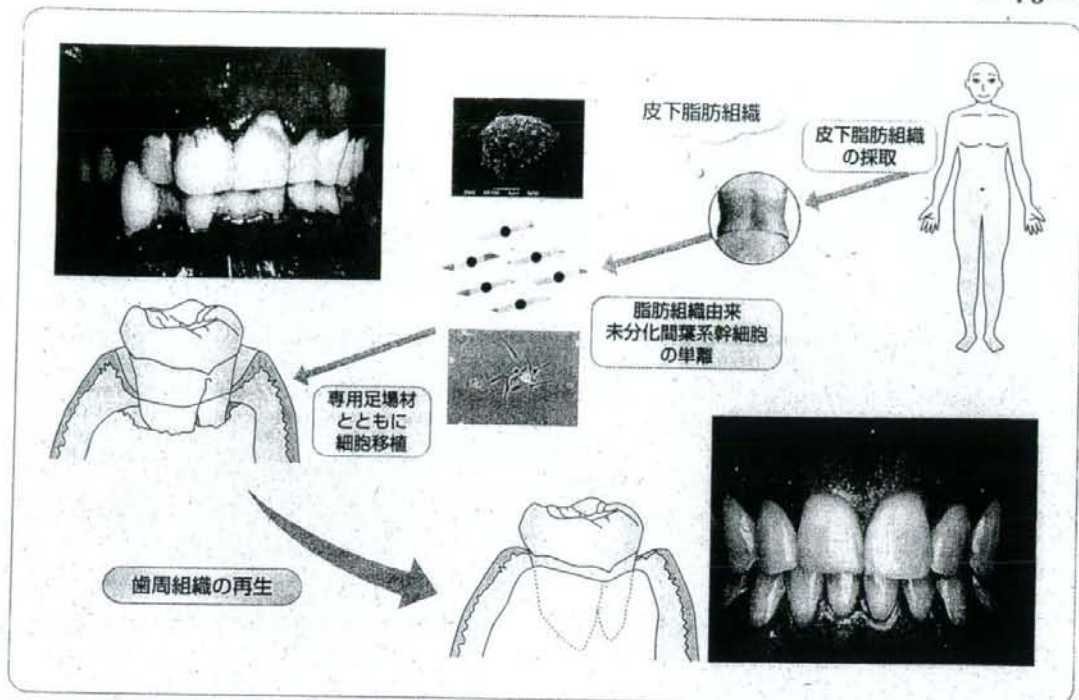


図7 ADSC移入による次世代型歯周組織再生療法の開発

患者から皮下脂肪を採取し、そこからADSCを分離した後、適切な足場材とともに同細胞を歯周組織欠損部に移入することにより、同部の歯周組織再生を促そうとするものである。

c. 至適足場材の選定

上述したような中等度歯周病の症例においては、レシビエント由来フィブリンを足場材として使用することにより細胞を移植することが可能であるが、より重度の歯周組織破壊を伴う症例においては、組織工学的な工夫を加味することが必要とされる。すなわち、組織再生を期待する場(スペース)を保持するスペースメイキング能力を有し、かつ、適度の賦形性とさらには骨伝導性を有するような適切な歯周組織再生誘導用足場材の開発が期待される。しかしながら、現時点では、ヒドロキシアパタイト、乳酸・グリコール酸共重合体など足場材候補となる既存の製品が数種類存在するが、重度歯周病再生治療用にカスタマイズされた効果的な足場材の報告はきわめて少なく、至適足場材の開発および選定は今後の重要な課題と

して残されている。

d. 幹細胞移植を用いた歯周組織再生療法の今後の展望

歯周組織は、歯槽骨・セメント質・歯根膜・歯肉から構成される複雑な組織であり、各組織を歯周組織破壊の度合いに応じて自由自在に再生させることが最終目標である。今後、安全性が担保されており、かつ幹細胞の遊走・増殖、さらには骨芽細胞やセメント芽細胞への分化を支える至適足場材を選定することにより、細胞移入による歯周組織再生療法の適応症例はおおいに拡大するものと思われる(図7)。そして近未来には、サイトカイン・幹細胞・足場材の3要素を最適条件で融合させた、真のオーダーメイド歯周組織再生療法の確立が強く望まれる。



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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/E1AF	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-Fli-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 (2×10^5) 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-Fli-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 LSM510 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'-ATGGCGTCCACGGATTACAGTACTC-3'; for EWS/FLI1 (reverse), 5'-GGGTCTCTTTGACACTCAATCG-3'; for EWS/ERG (forward), 5'-ATGGCGTCCACGGATTACAGTACTC-3'; for EWS/ERG (reverse), 5'-TTAGTAGTAAGTGGCCAGATGAGAA-3'; for GAPDH (forward), 5'-CCACCATGGCAATCCATGGCA-3'; and for GAPDH (reverse), 5'-TCTAGACGGCAGGTCCAGTCC-3'. PCR products were electrophoresed with a 1% agarose gel and stained with ethidium bromide.

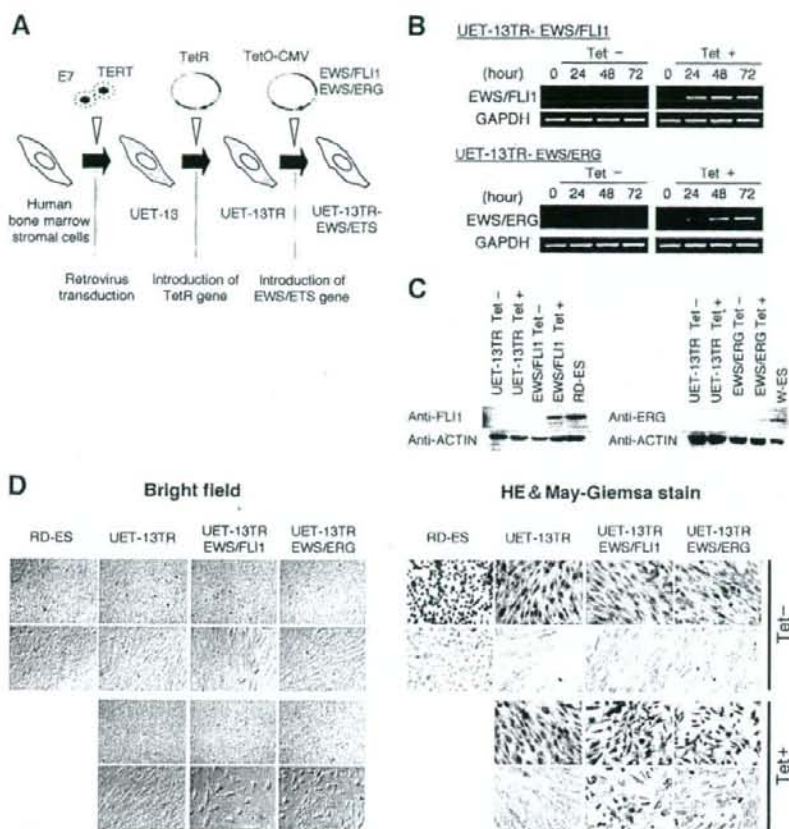


FIG. 1. The effect of EWS/ETS on the morphology of UET-13 cells. (A) The establishment of a tetracycline-inducible EWS/ETS expression system in UET-13 cells. CMV, cytomegalovirus. (B) Analyses for confirming the inducible expression of EWS/ETS genes. EWS/ETS mRNAs were detected in UET-13 transfectants 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 cells were treated as described for panel B and subjected to Western blotting for the detection of EWS/ETS proteins. The extracts of RD-ES and W-ES cells were also examined as positive controls. Membranes were reprobbed 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, non-invading 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.

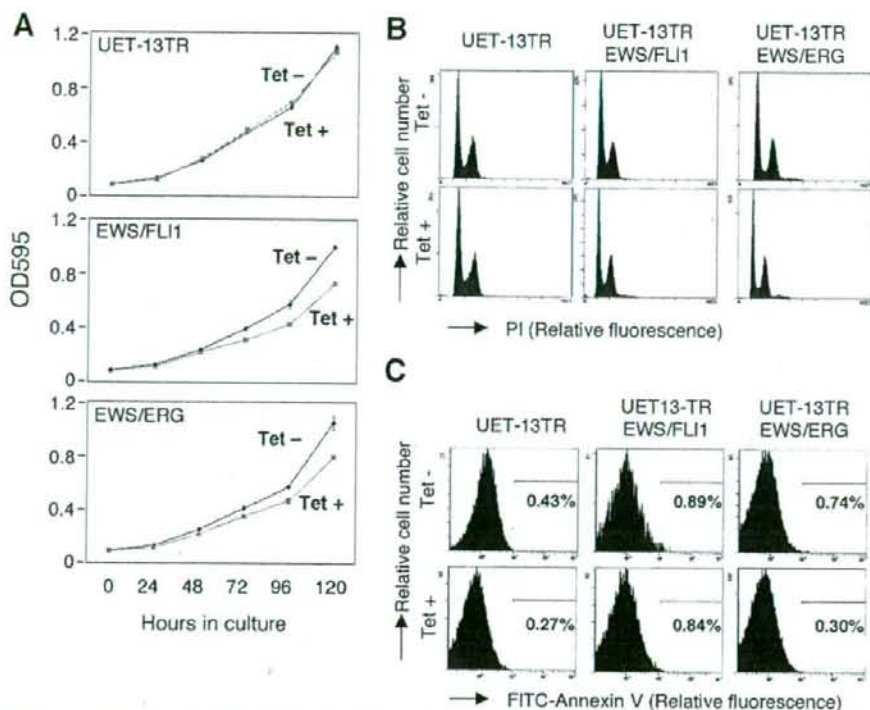


FIG. 2. Effects of EWS/ETS on cell growth in UET-13 cells. (A) Growth curve for UET-13 transfectants. Cells were seeded at 10^3 /well and cultured as described for Fig. 1. The increase in cell number was analyzed by MTT assay. Values are means with the standard errors (SE) from three independent experiments. 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).

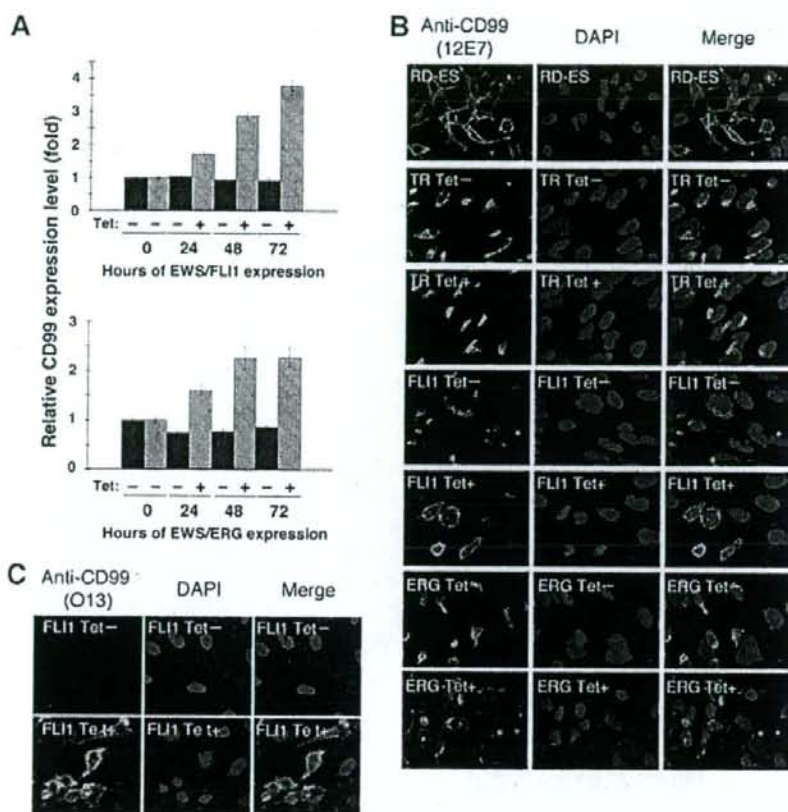


FIG. 3. Effects of tetracycline-mediated EWS/ETS expression on the expression and distribution of CD99 in UET-13 cells. (A) Relative CD99 levels in UET-13 transfectants in the absence or presence of tetracycline (Tet). UET-13 transfectants were treated with or without 3 μ g/ml of tetracycline for the 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 (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

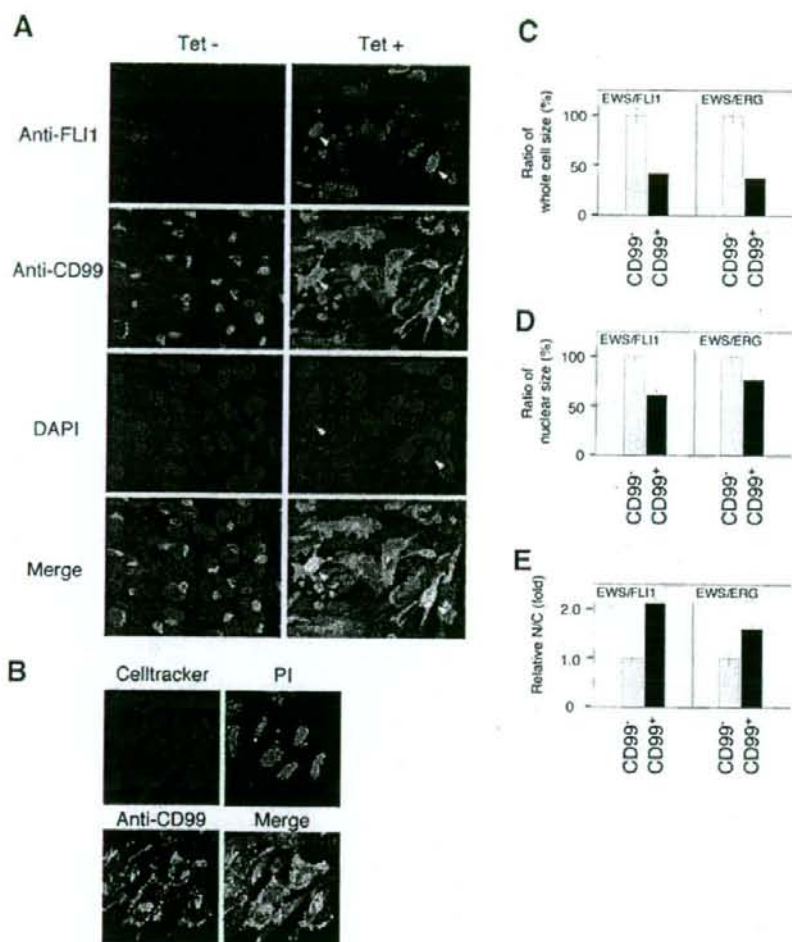


FIG. 4. EWS/ETS expression, alteration of CD99 distribution, and cell morphological changes in UET-13 cells. (A) Immunofluorescence studies using anti-Fli1 (red), anti-CD99 (green), and DAPI (blue). UET-13TR-EWS/FLI1 cells were cultured on coverslips in the absence or presence of tetracycline (Tet) for 72 h and then stained as described in Materials and Methods. White arrowheads indicate CD99⁺ cells that have a strong staining pattern with anti-Fli1 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-Fli1 and anti-CD99 antibodies. As shown in Fig. 4A, tetracycline treatment induced a marked

enhancement of nuclear staining with anti-Fli1 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 Fli1 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 ⁺			
M+	CD29	+	+	+	+	+	+	+	+		
M+	CD59	+	+	+	+	+	+	+	+		
M+	CD90	+	+	+	+	+	+	+	+	E+	
M+	CD105	+	+	+	+	+	+	+	+		
M+	CD166	+	+	+	+	+	+	+	+		
M+	CD44	+	+	+	+	+	+	+	-		
M+	CD73	+	+	+	+	+	+	+	-		
M+	CD10	+	+	+	+	Down	+	Down	-		
M+	CD13	+	+	+	+	Down	+	Down	-		
M+	CD49e	+	+	+	+	Down	+	Down	+		
M+	CD61	+	+	+	+	Down	+	Down	-		
M+	CD55	+	+	+	+	Down	+	+	+		
M+	CD54	-	-	-	-	Up	+	Up	+	E+	
M(-)	CD117	-	-	-	-	Up	-	Up	+	E+	
M+/-	CD271	-	-	-	-	Up	-	Up	+	E+	
	CD40	-	-	-	-	-	-	+	+	E+	
	CD56	-	-	-	-	-	-	+	+	E+	
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

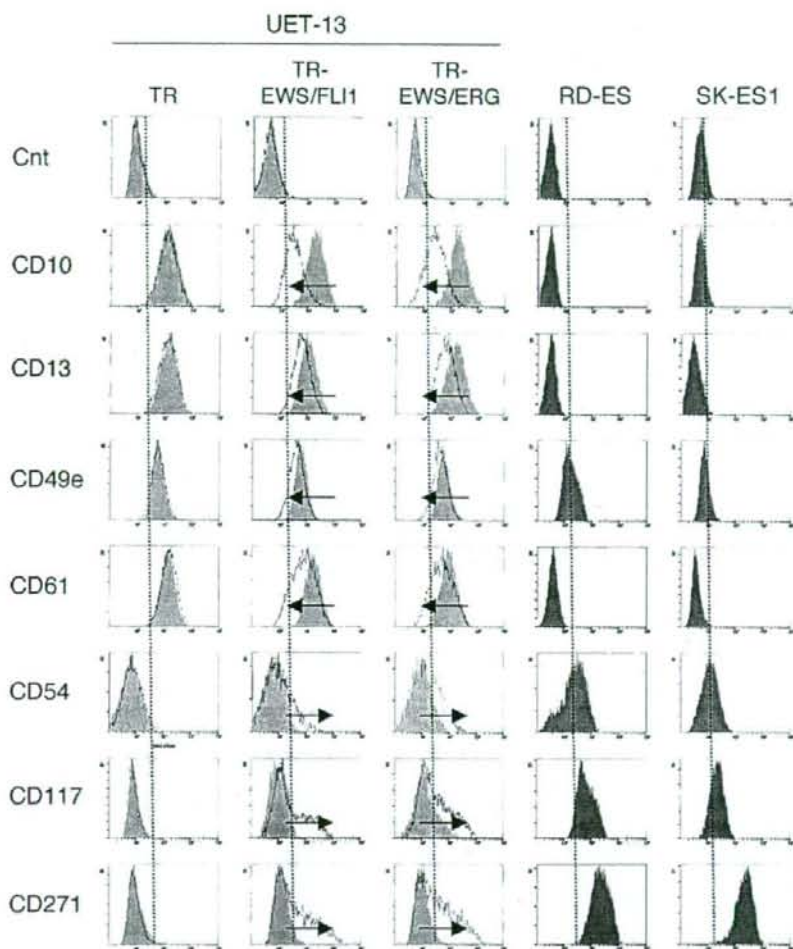


FIG. 5. Immunophenotypic change on induction of EWS/ETS expression in UET-13 cells. UET-13 transfectants were cultured with or without 3 μ M of tetracycline for 1 week and flow cytometric analyses were performed by using a set of antibodies as indicated. The histograms of UET-13 transfectants with (empty) 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

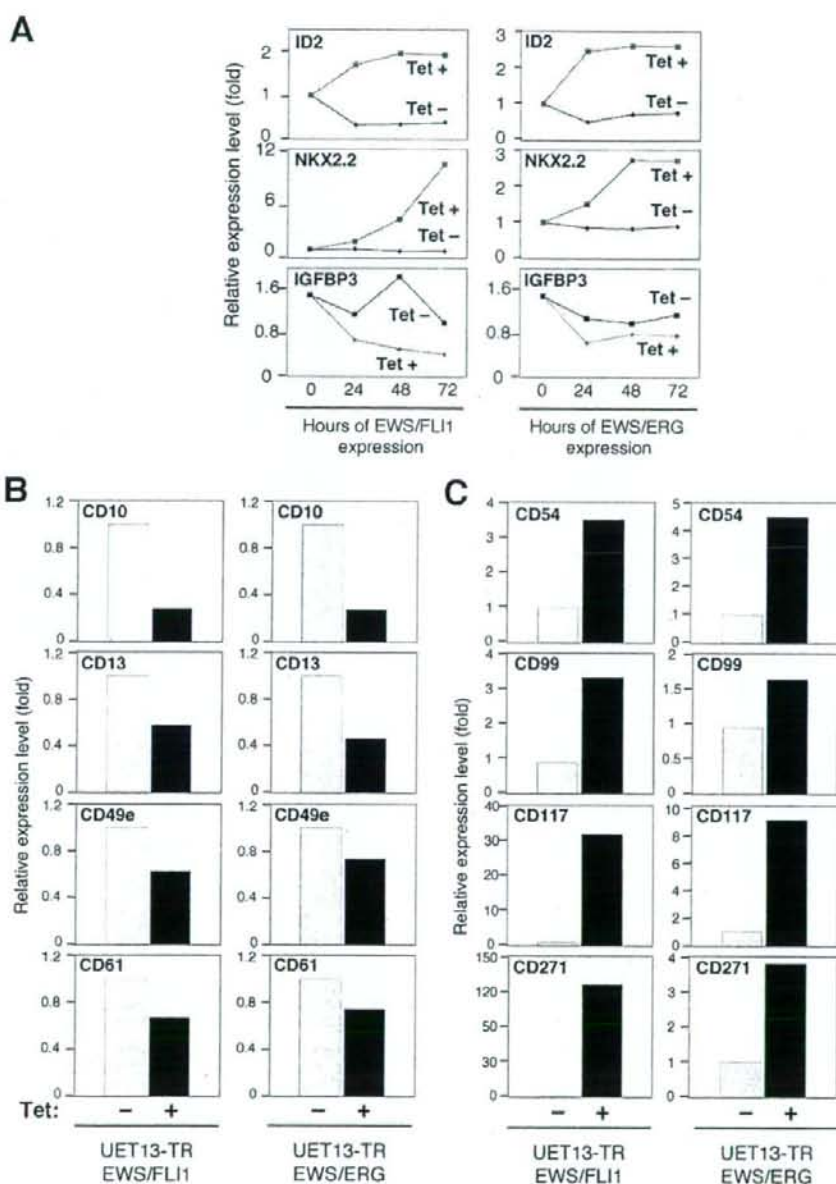


FIG. 6. The change of expression profile on induction of EWS/ETS in UET-13 cells. UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were cultured in the absence or presence of tetracycline (Tet) for the indicated periods and analyzed using the Affymetrix human genome U133 Plus 2.0 array as described in Materials 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

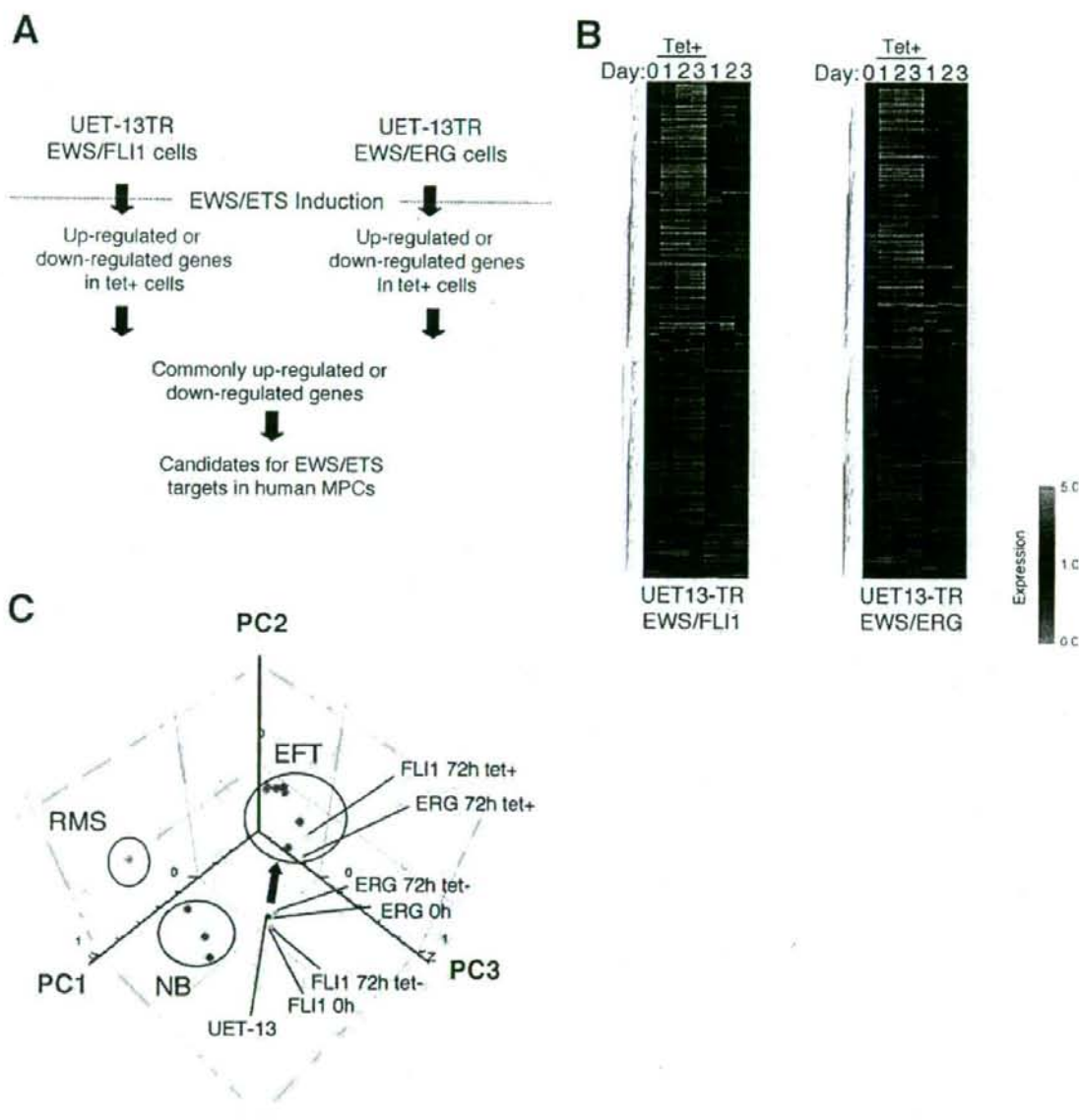


FIG. 7. Identification of candidates for the target of EWS/ETS in human MPCs by use of a microarray. UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were cultured as described for Fig. 6 and analyzed using the Affymetrix human genome U133 Plus 2.0 array as described in Materials and Methods. (A) Scheme 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

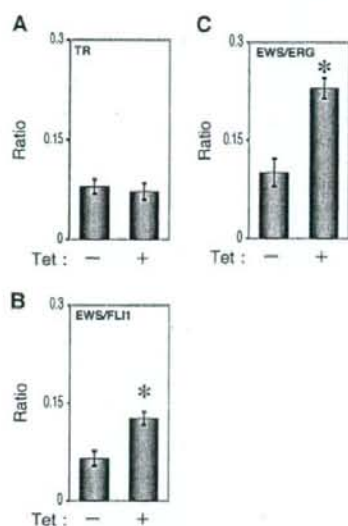


FIG. 8. Effects of EWS/ETS expression on the Matrigel invasion ability of UET-13 cells. UET-13TR (A), UET-13TR-EWS/FLI1 (B), and UET-13TR-EWS/ERG (C) cells were cultured in the absence or presence of tetracycline (Tet) for 72 h and then plated (2.5×10^4) on Matrigel-coated or uncoated filter inserts. 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 unclarified.

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 :						RD-ES	SK-ES1	EFT status ^c
		UET-13R		UET-13TR-EWS/FLI		UET-13TR-EWS/ERG				
		Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺			
	UET-13									