

the earlier-stage lymphoma. This result is compatible with our data, showing overexpression of wt D4-GDI to reduce the invasiveness of human leukemic cells. Thus, D4-GDI may assure the progression and invasion of human leukemia through its mutations and/or its downregulation.

Accumulating recent evidence shows that D4-GDI is expressed not only in hematopoietic tissues, but also in non-hematopoietic neoplasms. Results of cDNA microarray analyses revealed that D4-GDI is upregulated in ovarian [50], and downregulated in bladder carcinomas [51]. On the other hand, Theodorescu et al. [52] found that D4-GDI protein expression in bladder tumors is reduced as a function of bladder tumor progression. This result has suggested that D4-GDI is a metastasis suppressor gene in models of bladder cancer. In contrast, the results of Zhang et al. [43] show that increased expression of D4-GDI promotes cell invasiveness in breast cancer cells. These results suggest that the D4-GDI may have certain roles in the progression of different types of cancer. Thus, it is reasonable to propose that D4-GDI may be involved more generally in the invasive phenotype of human cancer.

Acknowledgments

We thank Dr. Martin Alexander Schwartz (The Scripps Research Institute, La Jolla, CA, USA) for generously providing an expression vector of GST-RBD. We thank Ms. Megumi Takamatsu, Mrs. Michiko Takahashi, and Dr. Yan Xiu, for cell culture and animal care, Mr. Hiroshi Suzuki for immunohistochemistry and Mr. Kohji Takeichi for technical photographic support.

This work was supported by Grants-in Aid for Pediatric Research (9C-5, 12C-1) from the Ministry of Health and Welfare, a research grant from the Ministry of Education in Japan (05404022, 07670258, 07770163, 1770124, 11670193, 10307004), National Grant-in-Aid for the Establishment of a High-Tech Research Center in a Private University, Sankyo Foundation of Life Science, Tsumura Foundation for Medical Research, Kawano Foundation for Children Cancer Research, and Keio Gijyuku Academic Development Funds and a special grant-in-aid for innovative and collaborative research projects at Keio University.

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ORIGINAL ARTICLE: CLINICAL

Outcome of childhood B-cell non-Hodgkin lymphoma and B-cell acute lymphoblastic leukemia treated with the Tokyo Children's Cancer Study Group NHL B9604 protocol

AKIRA KIKUCHI¹, TETSUYA MORI², JUN-ICHIRO FUJIMOTO³, MASAOKI KUMAGAI⁴, SHOSUKE SUNAMI⁵, YURI OKIMOTO⁶, & MASAHIRO TSUCHIDA⁷

¹Division of Hematology/Oncology, Saitama Children's Medical Center, Saitama-shi Saitama 339-8551, Japan,

²Department of Pediatric Oncology, National Center for Child Health and Development, Tokyo, Japan, ³National Research Institute for Child Health and Development, Tokyo, Japan, ⁴Department of Hematology, National Center for Child Health and Development, Tokyo, Japan, ⁵Department of Pediatrics, Japanese Red Cross Narita Hospital, Chiba, Japan, ⁶Department of Hematology-Oncology, Chiba Children's Hospital, Chiba, Japan, and ⁷Department of Pediatrics, Ibaraki Children's Hospital, Ibaraki, Japan

(Received 10 September 2007; revised 30 November 2007; accepted 3 December 2007)

Abstract

From June 1996 to January 2001, 91 patients with B-cell non-Hodgkin lymphoma or B-cell acute lymphoblastic leukemia up to 18 years of age were enrolled in Tokyo Children's Cancer Study Group (TCCSG) NHL B9604 protocol study. Five-day intensive chemotherapy courses including high-dose methotrexate and high-dose cyclophosphamide were used for localized disease (Groups A and B). High-dose cytarabine was added for advanced disease (Groups C and D). Fifteen patients experienced an adverse event. There were three induction failures, eight relapses (three local, four bone marrow (BM), one BM + local), two toxic deaths and two second malignant neoplasm. Event-free survival at 6 years in Group D and in all patients was $82.4\% \pm 9.2\%$ and $81.9\% \pm 4.4\%$, respectively. The TCCSG NHL B9604 protocol achieved an excellent treatment outcome especially in patients with the most advanced disease (Group D: high BM blast cell burden and/or central nervous system involvement).

Keywords: B-NHL, B-ALL, intensified chemotherapy, TCCSG NHL B9604

Introduction

The malignant cells of B-cell (surface immunoglobulin-positive [Ig+]) non-Hodgkin lymphoma (B-NHL) and B-cell acute lymphoblastic leukemia (B-ALL), which are classified as Burkitt lymphoma/leukemia in WHO classification [1], share morphologic, immunophenotypic, cytogenetic and clinical features and are considered to represent a continuum of the same disease. Diffuse large B-cell lymphoma is a distinct disease entity from Burkitt lymphoma/leukemia. However, the treatment for patients with large cell lymphoma is the same as that for patients with Burkitt histology. In early trials, children with advanced B-NHL and B-ALL had a worse outcome

characterized by early recurrences despite a high initial complete remission rate [2,3]. After the introduction of short, intensive therapy courses primarily based on cyclophosphamide (CY), methotrexate (MTX), and intrathecal (i.t.) therapy, the prognosis of these mature B-cell neoplasms has improved significantly [4–11].

Most previous clinical experiences about childhood B-NHL and B-ALL were reported from European and North American study groups, however, there are few data on Japanese or Asian patients. We report here the treatment and results of 91 Japanese children with B-NHL or B-ALL registered to the Tokyo Children's Cancer Study Group (TCCSG) NHL B9604. The aim of this study

Correspondence: Akira Kikuchi MD, Division of Hematology/Oncology, Saitama Children's Medical Center, 2100 Magome Iwatsuki-ku, Saitama-shi Saitama 339-8551, Japan. Tel: +81-48-7581811. Fax: +81-48-7581818. E-mail: a1091069@pref.saitama.lg.jp

was to evaluate the efficacy and safety of the short and intensive chemotherapy regimen designed by TCCSG for childhood B-NHL or B-ALL.

Patients and methods

Children and adolescents up to 18 years of age newly diagnosed with B-NHL or B-ALL were eligible for this study. From June 1996 to January 2001, 91 patients with B-NHL or B-ALL were enrolled in this study. Diagnosis was made by pathologists at a regional hospital and registration was made based on the regional diagnosis. A central review was performed retrospectively by the reference laboratory according to the WHO classification [1]. The St. Jude system was used for disease staging [12]. Patients were stratified into four groups according to stage, resection status, bone marrow (BM) blast cell burden and central nervous system (CNS) involvement as shown in Figure 1.

Five-day intensive chemotherapy courses including high-dose MTX (HD-MTX) and high-dose CY (HD-CY) were used for Groups A and B. High-dose cytarabine (HD-AraC) was added for Groups C and D. Patients in Groups B, C and D received a 5-day cytoreductive prephase before the first course was administered (Table I). Courses were administered at 3–4-week intervals. The number of treatment courses was three in Group A, six in B, six in C and seven in D, respectively. Treatment duration ranged from 10 to 27 weeks (Figure 1).

Events were defined as induction failure, relapse, death in induction, death in remission and second malignant neoplasm. Survival curves were calculated by the Kaplan–Meier method. Toxicities were graded according to National Cancer Institute Common Toxicity Criteria version 2. All treatments were performed with informed consent from the patients' guardians.

Risk Group	Definition	Therapy Courses
A	Stage I, Stage II completely resected	A1 A2 A1
B	Stage I, Stage II not resected	PP B1 B2 B1 B2 B1 B2
C	Stage III, Stage IV and CNS(-) and BM < 70%	PP C1 C2 C3 C1 C2 C3
D	Stage IV CNS(+) or BM > 70%	PP D1 D1 D2 D3 D1 D2 D3

Figure 1. Treatment strategy. Patients were stratified into four risk groups; A, B, C and D. The composition of therapy courses is shown in Table I.

Results

The clinical characteristics of the 91 patients are shown in Table II. The percentage in Group A patients in our series was quite small (3.3%) compared with that of equal disease status patients in other studies (7–17%) [6,8,11]. A retrospective central review was performed in 64.8% (59/91) of the enrolled patients and the concordance rate was 88.1% (52/59). Fifteen patients experienced an adverse event. There were three induction failures, eight relapses (three local, four BM, one BM + local), two toxic deaths and two second malignant neoplasm. Nine patients died of induction failure in three, relapse in four and toxicity in two patients [intracranial hemorrhage (ICH) and sepsis]. Two patients developed a second malignant neoplasm. Grade 4 non-hematological toxicity was noted in 4 cases. Event-free survival (EFS) and overall survival (OS) were calculated as follows: EFS and OS in total were $81.9\% \pm 4.4\%$ and $90.1\% \pm 3.1\%$, respectively. EFS was $66.7\% \pm 27.2\%$ in Group A ($n=3$), $95.8\% \pm 4.1\%$ in Group B ($n=25$), $77.6\% \pm 6.3\%$ in Group C ($n=46$) and $82.4\% \pm 9.2\%$ in Group D ($n=17$) (Figure 2). OS was in 100% in Groups A and B ($n=28$), $86.8\% \pm 5.0\%$ in Group C and $82.4\% \pm 9.2\%$ in Group D.

Discussion

In this article, we reported the outcome of 91 childhood B-NHL and B-ALL patients treated with TCCSG NHL B9604 protocol. OS and EFS at 6 years in total were $90.1\% \pm 3.1\%$ and $81.9\% \pm 4.4\%$, respectively. OS of our study was comparable with SFOP study [8], BFM study [6] and UKCCSG study [7] and better than CCG study [9] or Venezuela study [11] whereas the observation period of our study was longer than other studies. Although the EFS of our study was worse than the SFOP study [8] and BFM study [6], it was comparable with the UKCCSG study [7] and better than the CCG study [9], POG study [4] and Venezuela study [11]. Especially in patients with the most advanced disease (Group D: high BM blast cell burden and/or CNS involvement), EFS was $82.4\% \pm 9.2\%$. This was comparable with the SFOP study [8] and BFM study [6]. The overall good prognosis of this study is thought to be mainly due to the relatively larger dose of chemotherapeutic drugs and intensified regimens resulting in a good outcome.

There were 15 event cases in this study, as shown in Table III. Among these cases, the diagnosis of two relapsed patients (UPN 132,146), whose diagnosis was DLBCL by the regional hospital, was corrected to B-LBL by retrospective central review. These

Table I. Therapeutic regimen of TCCSG NHL B9604 protocol.

Group	Day	Treatment	Day	Treatment	Day	Treatment
Group A						
Block A1:						
PDN 60 mg/m ² P.O.	Days 1-5		Block C2:	Dex 10 mg/m ² P.O.	Days 1-5	
MTX 3 g/m ² 6 h div with CFR	Days 1			VCR 1.5 mg/m ²	Days 1	
CY 250 mg/m ² 1 h div	Days 2-5			AraC 150 mg/m ² × 6 l h div q12 h	Days 1-3	
VP16 100 mg/m ² 2 h div	Days 2-5			CY 1 g/m ² 2 h div	Days 2-4	
MH i.t.	Days 1			Epi 60 mg/m ² 1 h div	Days 5	
Block A2:						
PDN 60 mg/m ² P.O.	Day 1-5			MHC i.t.	Day 1	
VCR 1.5 mg/m ²	Day 1			MH i.t.	Day 8 (only in the first course)	
AraC 150 mg/m ² × 6 l h div q12 h	Day 1-3		Block C3:			
CY 1 g/m ² 2 h div	Day 2,3			Dex 10 mg/m ² P.O.	Day 1-5	
MH i.t.	Day 1			VDS 3 mg/m ²	Day 1	
Group B						
Block P(Prephase):						
PDN 60 mg/m ² P.O.	Day 1-5			VP16 100 mg/m ² 2 h div	Day 2-5	
CY 200 mg/m ² 1 h div	Day 1, 2			AraC 2 g/m ² 3 h div q12 h	Day 1-3	
MH i.t.	Day 1			Epi 60 mg/m ² 1 h div	Day 5	
Block B1:						
PDN 60 mg/m ² P.O.	Day 1-5		Group D:			
MTX 3 g/m ² 6 h div with CFR	Day 1		Block P: the same as in Group C			
CY 250 mg/m ² 1 h div	Day 2-5		Block D1:			
VP16 100 mg/m ² 2 h div	Day 2-5			Dex 10 mg/m ² P.O.	Day 1-5	
Epi 50 mg/m ² 1 h div	Day 5			MTX 3 g/m ² 24 h div with CFR	Day 1	
MH i.t.	Day 1			CY 300 mg/m ² × 6 l h div q12 h	Day 2-4	
Block B2:						
PDN 60 mg/m ² P.O.	Day 1-5			(only day 2,3 in the first course)		
VCR 1.5 mg/m ²	Day 1			Epi 80 mg/m ² 1 h div	Day 5	
AraC 150 mg/m ² × 6 l h div q12 h	Day 1-3			MHC i.t.	Day 5 (in the first course)	
CY 1 g/m ² 2 h div	Day 2,3			MH i.t.	Day 1 (in the second course)	
Epi 50 mg/m ² 1 h div	Day 5			MHC i.t.	Day 8 (in the second course)	
MH i.t.	Day 1			MH i.t.	Day 1,8 (in the third course)	
Group C						
Block P:						
PDN 60 mg/m ² P.O.	Day 1-7		Block D2:			
VP16 100 mg/m ² 1 h div	Day 5-7			Dex 10 mg/m ² P.O.	Day 1-5	
MHC i.t.	Day 1,6			VCR 1.5 mg/m ²	Day 1	
Block C1:						
Dex 10 mg/m ² P.O.	Day 1-5			AraC 150 mg/m ² × 6 l h div q12 h	Day 1-3	
MTX 3 g/m ² 24 h div with CFR	Day 1			CY 1 g/m ² 2 h div	Day 2-4	
CY 250 mg/m ² × 6 l h div q12 h	Day 2-4			Epi 80 mg/m ² 1 h div	Day 5	
VP16 100 mg/m ² 2 h div	Day 2-5			MHC i.t.	Day 1 (in the first course)	
(only day 2,3 in the first course)				MH i.t.	Day 8 (in the first course)	
Epi 60 mg/m ² 1 h div	Day 5			MH i.t.	Day 1,8 (in the second course)	
MHC i.t.	Day 5 (in the first course)		Block D3:			
MH i.t.	Day 1 (in the second course)			Dex 10 mg/m ² P.O.	Day 1-5	
				VDS 3 mg/m ²	Day 1	
				VP16 100 mg/m ² 2 h div	Day 2-5	
				AraC 2 g/m ² 3 h div q12 h	Day 1-3	
				Epi 80 mg/m ² 1 h div	Day 5	
				MH i.t.	Day 1,8	

Cranial irradiation for patients with CNS involvement at onset

24 Gy for older than 2 years old

18 Gy for 1 year old

0 Gy for less than 1 year old

Cumulative drug dosage in each group

Group	A	B	C	D
CY (g/m ²)	4	9.4	9	11.4
VP16 (mg/m ²)	800	1200	1700	2100
MTX (g/m ²)	6	9	6	9
CA (g/m ²)	0.9	2.7	25.8	28.8
Epi (mg/m ²)	0	300	360	560

PDN, prednisolone; CFR, citrovorum factor rescue; VP16, etoposide; MH, MTX and hydrocortisone; VCR, vincristine; AraC, cytarabine; q12h, every 12 h; Epi, epirubicine; MHC, MH and AraC; Dex, dexamethazone; VDS, vindesine.

Table II. Clinical characteristics of 91 patients treated with TCCSG NHL B9604 protocol.

Registration period	April 1996–January 2001
Observation period	0–103 months (median 60 months)
Sex (Male/Female)	64/27
Age range	0.9–16.8 years (median 9.4 years)
Pathological diagnosis	Burkitt, 45 Large cell, 26 B-ALL, 9 B-NHL not further specified, 6 Others, 5
Primary site	Head and neck: 32; chest: 3 Abdomen: 36; bone: 8 B-ALL: 9; others: 3
Disease stage	I: 5; II: 23; III: 25; VI: 38
Risk group	A: 3; B: 25; C: 46; D: 17

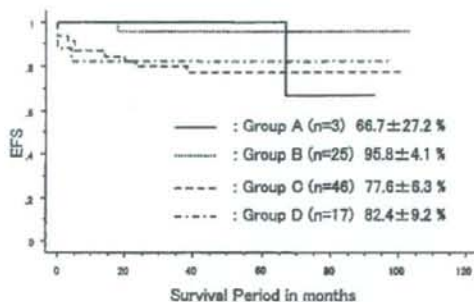


Figure 2. EFS according to the risk groups. EFS was 66.7% ± 27.2% in Group A (n=3), 95.8% ± 4.1% in Group B (n=25), 77.6% ± 6.3% in Group C (n=46) and 82.4% ± 9.2% in Group D (n=17).

patients were rescued with further chemotherapy for B-LBL. These relapses were considered due to inadequate treatment duration rather than drug resistance. In Group A, one patient (UPN 100) whose primary site was the tonsils developed diffuse large B-cell lymphoma at the cecum 5 years after initial diagnosis. Although we could not determine whether this lesion was relapse or secondary neoplasm, the EFS of Group A patients became low because of this rare event. Such a late relapse as in this case was also reported in the SFOP study [8]. Aside from these three cases, the 12 event cases consisted of three induction failures, five relapses, two toxic deaths and two second malignant neoplasm. All three induction failures and four BM involved relapsed patients died of disease progression in spite of further chemotherapy or stem cell transplantation. These cases may have required a quite different treatment concept for the first-line therapy based on stratification with tumor biology profiling. One patient (UPN 44) showed massive ICH at initial presentation and died 2 days after

Table III. Event cases in TCCSG NHL B9604 protocol.

UPN	Age	Sex	Stage	Group	Histology (central review)	Event	Remission period	Outcome	Cause of death	Survival period
11	15	F	III	C	DLBCL (DLBCL)	Secondary malignancy	14	Alive	N.A.	88+
22	5	F	III	C	Burkitt (Burkitt)	Induction failure	0	Dead	Disease progression	9
39	14	M	III	C	Burkitt (Burkitt)	Relapse (BM)	3	Dead	Disease progression	6
44	9	M	IV	D	ALL	ICH	0	Dead	ICH	0
57	11	M	IV	D	Burkitt (Burkitt)	Relapse (BM)	4	Dead	Disease progression	6
58	3	F	IV	C	Burkitt (Burkitt)	Induction failure	0	Dead	Disease progression	6
74	15	M	III	C	Burkitt (Burkitt)	Relapse (BM+local)	6	Dead	Disease progression	7
100	13	F	I	A	DLBCL (DLBCL)	Relapse (cecum)	67	Alive	N.A.	71+
102	10	F	IV	C	ALL	Relapse (BM)	6	Dead	Disease progression	11
103	2	M	III	C	Burkitt (Burkitt)	Septic	0	Dead	Septic	0
132	7	M	II	B	DLBCL (B-LBL)	Relapse (BM)	33	Alive	N.A.	66+
141	14	M	III	C	Medium (N.D.)	Relapse (local)	20	Alive	N.A.	77+
146	7	F	IV	C	DLBCL (B-LBL)	Relapse (local)	23	Alive	N.A.	60+
149	15	M	III	C	MLBCL (MLBCL)	Secondary malignancy	38	Alive	N.A.	62+
154	8	M	IV	D	Burkitt (Burkitt)	Induction failure	0	Dead	Disease progression	10

DLBCL, diffuse large B cell lymphoma; MLBCL, mediastinal large B-cell lymphoma; ICH, intracranial hemorrhage; N.A., not applicable; N.D., not done. Age is shown in years. Remission period and survival period are shown in months. A plus sign indicates that the patient is still alive.

admission. We do not think this was therapy-related toxic death. Toxic death was reported in another patient (UPN 103) who died of sepsis during remission induction therapy. Although Grade 4 non-hematological toxicity was noted in four cases in addition to this patient, this intensified treatment regimen was well tolerated. Two second malignant neoplasm (myelodysplastic syndrome and acute myeloid leukemia) were observed 14 and 38 months after diagnosis. These patients survived with BM transplantation from unrelated donors. As we used a relatively larger dose of chemotherapeutic drugs in this study, we have to cautiously observe the development of a second malignant neoplasm among patients treated with this protocol.

In recent childhood lymphoma studies, international collaborations are essential because large scale studies are needed to prove improved outcome compared with current good one. To participate in these international studies, it is important for us (Japanese or Asian people) to confirm short, intensive chemotherapy for B-NHL and B-ALL is safe and effective regardless of racial differences. We elucidated such short, intensive chemotherapy for B-NHL and B-ALL was safe and effective for Japanese children. Treatment reduction is a main theme of childhood B-NHL therapy. Recently, Patte et al. [13] reported results of FAB/LMB96 trial for intermediate risk B-NHL patients. In this report, they elucidated a four-course treatment is enough for these patients with initial good response. Several studies for B-NHL have attempted therapy reduction to decrease toxicity, however, the dose reduction is associated with an inferior outcome for advanced stage disease so far [14,15].

The TCCSG NHL B9604 protocol achieved an excellent treatment outcome, especially in patients with the most advanced disease (Group D: high BM blast cell burden and/or CNS involvement). Although we have to cautiously observe the development of late adverse effects in treated patients, several study attempts have not succeeded in appropriate therapy reduction without jeopardizing survival and intensified regimen is, at least tentatively, needed for a good prognosis for advanced stage patients.

Acknowledgements

The authors dedicate this article to Dr. Yasunori Toyoda, who died too early, for his dedication to their lymphoma studies. This study was done on behalf of the Tokyo Children's Cancer Study Group (TCCSG). The authors thank Mrs. Kaori Itagaki for preparing and refining the protocol data for non-Hodgkin lymphoma in the Tokyo Children's Cancer Study Group.

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

Yoshitaka Miyagawa,¹ Hajime Okita,^{1*} Hideki Nakajima,¹ Yasuomi Horiuchi,¹ Ban Sato,¹ Tomoko Taguchi,¹ Masashi Toyoda,³ Yohko U. Katagiri,¹ Junichiro Fujimoto,² Jun-ichi Hata,¹ Akihiro Umezawa,³ and Nobutaka Kiyokawa¹

Department of Developmental Biology, National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan¹; National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan²; and Department of Reproductive Biology, National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan³

Received 27 April 2007/Returned for modification 13 July 2007/Accepted 7 January 2008

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-

* Corresponding author. Mailing address: Department of Developmental Biology, National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan. Phone: 81-3-3416-0181. Fax: 81-3-3417-2496. E-mail: okita@nch.go.jp.
† Supplemental material for this article may be found at <http://mcb.asm.org/>.

[∇] Published ahead of print on 22 January 2008.

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-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 (2×10^5) 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-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 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'-ATGGCGTCCACGGATTACAGTACCT-3'; for EWS/FLI1 (reverse), 5'-GGGTCTCTTTGACACCTCAATCG-3'; for EWS/ERG (forward), 5'-ATGGCGTCCACGGATTACAGTACCT-3'; for EWS/ERG (reverse), 5'-TTAGTAGTAAGTGCCAGATGAGAA-3'; for GAPDH (forward), 5'-CCACCCTATGGCAATTCATGGCA-3'; and for GAPDH (reverse), 5'-TCTAGACGGCAGGTGACGT/CCACC-3'. PCR products were electrophoresed with a 1% agarose gel and stained with ethidium bromide.

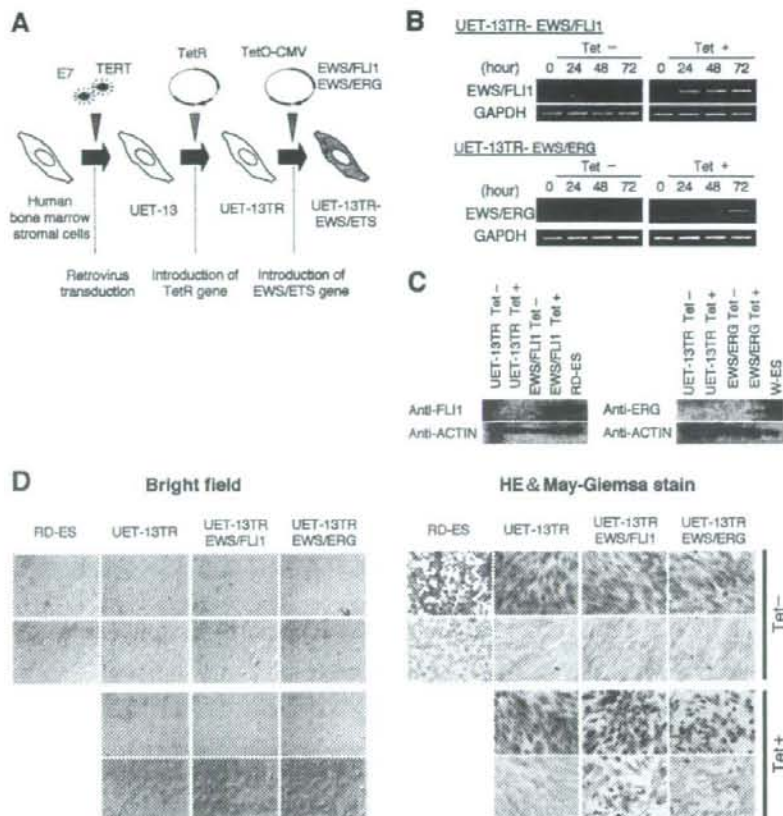


FIG. 1. The effect of EWS/ETS on the morphology of UET-13 cells. (A) The establishment of a tetracycline-inducible EWS/ETS expression system in UET-13 cells. CMV, cytomegalovirus. (B) Analyses for confirming the inducible expression of EWS/ETS genes. EWS/ETS mRNAs were detected in UET-13 transfectants 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 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.

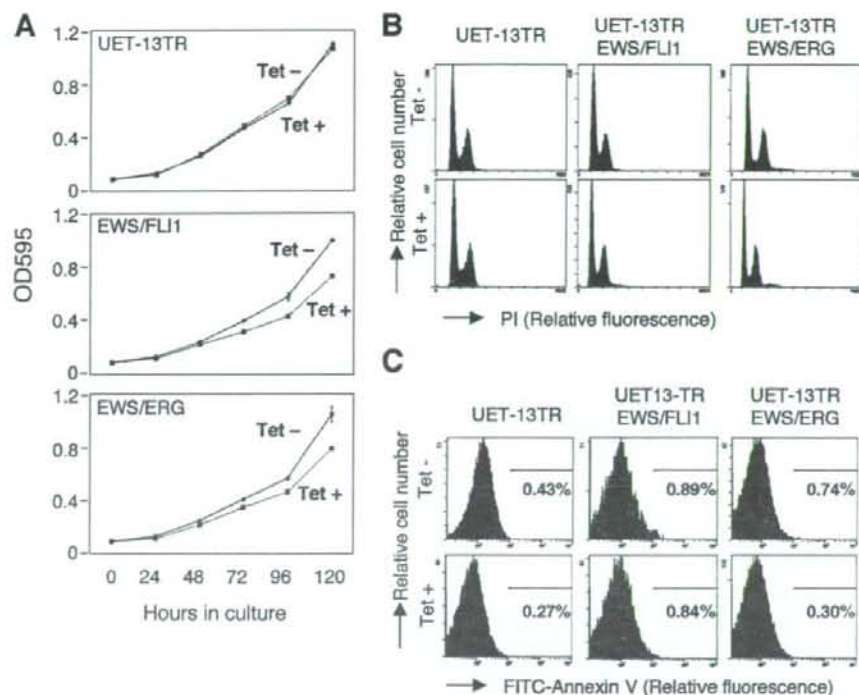


FIG. 2. Effects of EWS/ETS on cell growth in UET-13 cells. (A) Growth curve for UET-13 transfectants. Cells were seeded at 10^3 /well and cultured as described for Fig. 1. The increase in cell number was analyzed by MTT assay. Values are means with the standard errors (SE) from three independent experiments.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

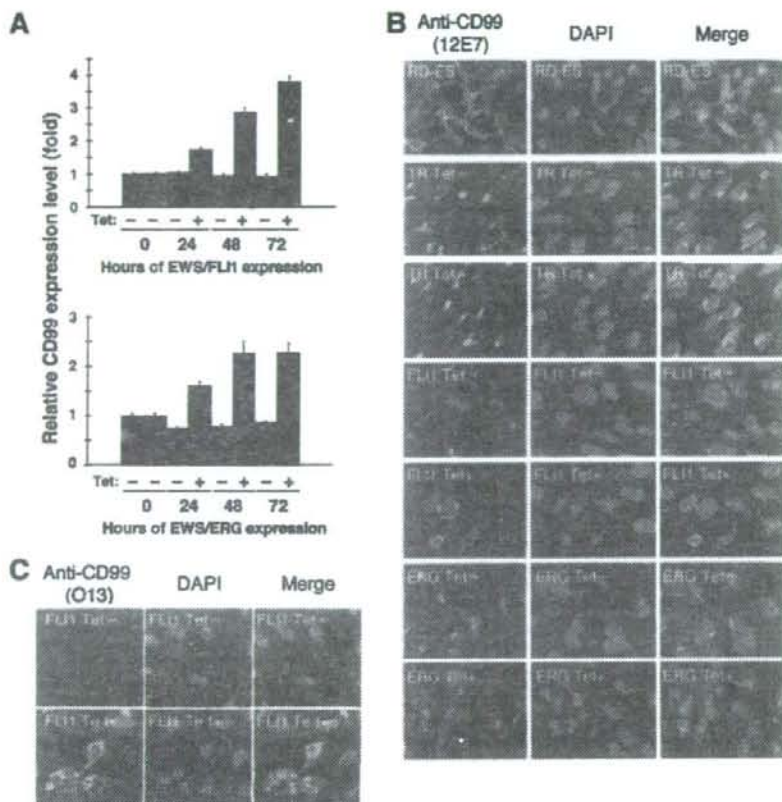


FIG. 3. Effects of tetracycline-mediated EWS/ETS expression on the expression and distribution of CD99 in UET-13 cells. (A) Relative CD99 levels in UET-13 transfectants in the absence or presence of tetracycline (Tet). UET-13 transfectants were treated with or without 3 μ g/ml of tetracycline for the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

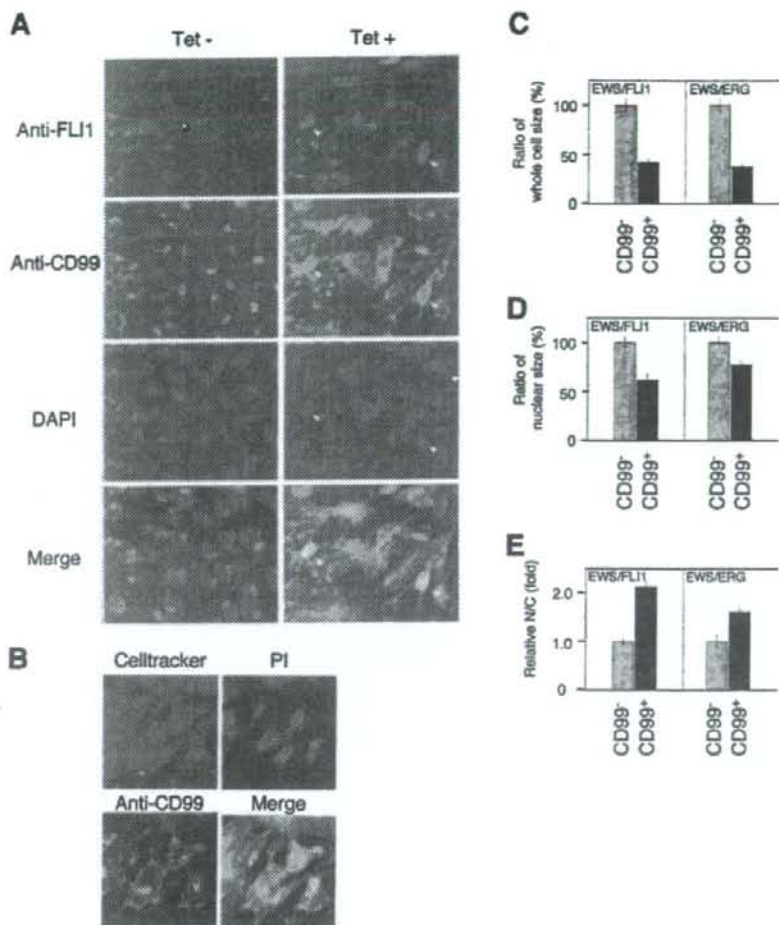


FIG. 4. EWS/ETS expression, alteration of CD99 distribution, and cell morphological changes in UET-13 cells. (A) Immunofluorescence studies using anti-Flil1 (red), anti-CD99 (green), and DAPI (blue). UET-13TR-EWS/FLI1 cells were cultured on coverslips in the absence or presence of tetracycline (Tet) 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 :							RD-ES	EFT status ^c	SK-ES1
		UET-13		UET-13TR		UET-13TR-EWS/FL11		UET-13TR-EWS/ERG			
		Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺	Tet ⁻	Tet ⁺	Tet ⁺			
M+	CD29	+	+	+	+	+	+	+	+	+	
M+	CD59	+	+	+	+	+	+	+	+	+	
M+	CD90	+	+	+	+	+	+	+	+	+	
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	+	+	
M(-)	CD117	-	-	-	-	Up	-	Up	+	+	
M+/-	CD271	-	-	-	-	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

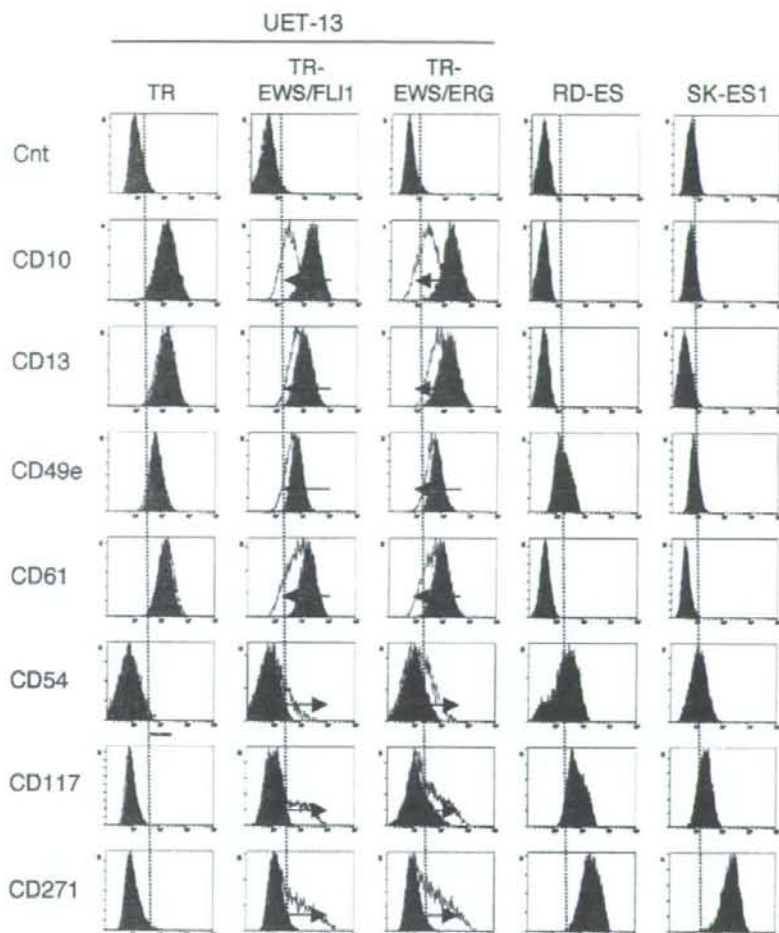


FIG. 5. Immunophenotypic change on induction of EWS/ETS expression in UET-13 cells. UET-13 transfectants were cultured with or without 3 μ g/ml of tetracycline for 1 week and flow cytometric analyses were performed by using a set of antibodies as indicated. The histograms of UET-13 transfectants with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

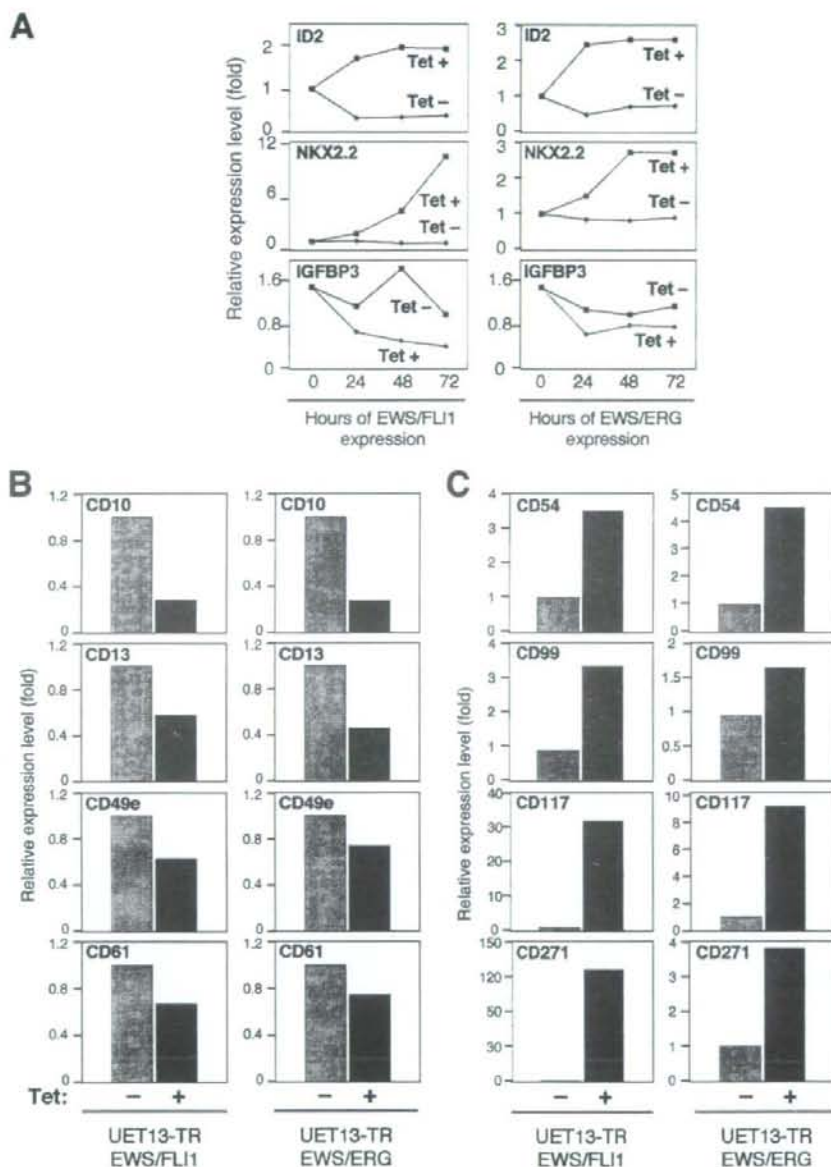


FIG. 6. The change of expression profile on induction of EWS/ETS in UET-13 cells. UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were cultured in the absence or presence of tetracycline (Tet) for the indicated periods and analyzed using the Affymetrix human genome U133 Plus 2.0 array as described in Materials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

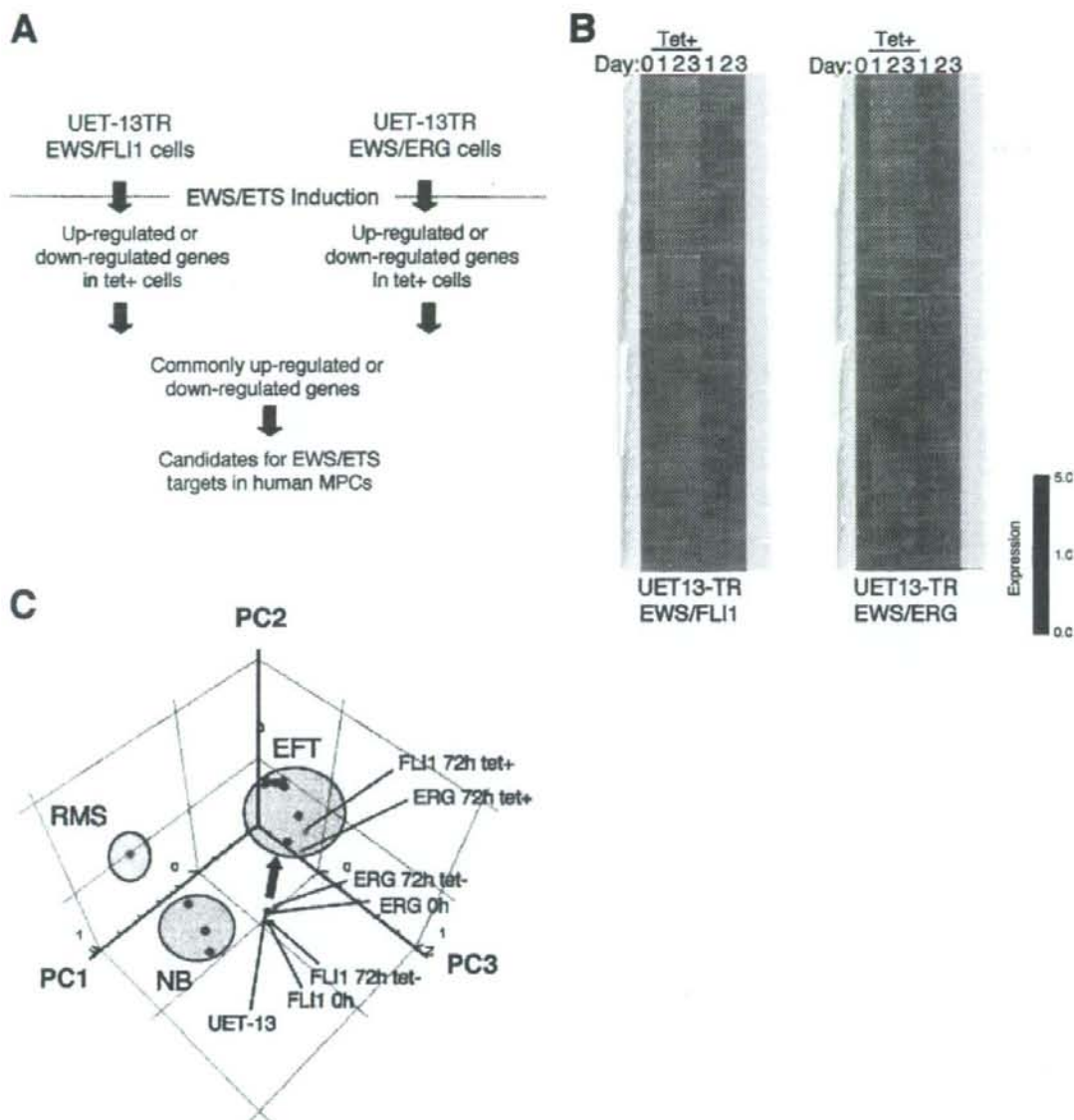


FIG. 7. Identification of candidates for the target of EWS/ETS in human MPCs by use of a microarray. UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were cultured as described for Fig. 6 and analyzed using the Affymetrix human genome U133 Plus 2.0 array as described in Materials and Methods. (A) Scheme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

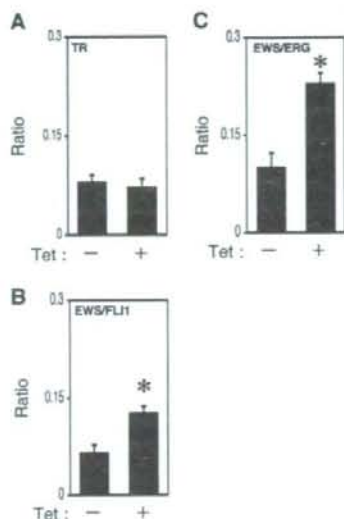


FIG. 8. Effects of EWS/ETS expression on the Matrigel invasion ability of UET-13 cells. UET-13TR (A), UET-13TR-EWS/FLI1 (B), and UET-13TR-EWS/ERG (C) cells were cultured in the absence or presence of tetracycline (Tet) for 72 h and then plated (2.5×10^4) on Matrigel-coated or uncoated filter inserts.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

ACKNOWLEDGMENTS

This work was supported in part by health and labor sciences research grants (the 3rd-Term Comprehensive 10-Year Strategy for Cancer Control [H19-010], Research on Children and Families [H18-005 and H19-003], Research on Human Genome Tailor Made, and Research on Publicly Essential Drugs and Medical Devices [H18-005]) and a grant for child health and development from the Ministry of Health, Labor and Welfare of Japan, JSPS (Kakenhi 18790263). This work was also supported by a CREST, JST grant from the Japan Health Sciences Foundation for Research on Publicly Essential Drugs and Medical Devices and the Budget for Nuclear Research of the Ministry of Education, Culture, Sports, Science and Technology, based on screening and counseling by the Atomic Energy Commission. Y. Miyagawa is an awardee of a research resident fellowship from the Foundation for Promotion of Cancer Research (Japan) for the 3rd-Term Comprehensive 10-Year Strategy for Cancer Control.

We are grateful to T. Motoyama for the NRS-1 cell line. We respectfully thank S. Yamauchi for her secretarial work and M. Itagaki for many helpful discussions and support.

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