STEM CELLS®
Original Article

Stimulation of Oct-4 Activity by Ewing's Sarcoma Protein

JUNGWOON LEE,[a] BYUNG KIRL RHEE,[a] GAB-YONG BAE,[b] YONG-MAHN HAN,[b] JUNGHO KIM[a]

[a]Laboratory of Molecular and Cellular Biology, Department of Life Science, Sogang University, Seoul, Korea; [b]Laboratory of Development and Differentiation, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea

Key Words. Oct-4 • Ewing's sarcoma protein • Proto-oncogene • Transcriptional coactivator Protein–protein interaction • Bacterial two-hybrid screening • Embryonic stem cells

ABSTRACT

The *Oct-4* gene encodes a transcription factor that is expressed in embryonic stem (ES) cells and germ cells. Oct-4 is known to function as a transcriptional activator of genes involved in maintaining an undifferentiated totipotent state and possibly in preventing expression of genes activated during differentiation. In addition, it is a putative proto-oncogene and a critical player in the genesis of human testicular germ cell tumors. Although much effort has gone toward characterizing Oct-4, there is still little known about the molecular mechanisms and the proteins that regulate Oct-4 function. To identify cofactors that control Oct-4 function in vivo, we used a recently developed bacterial two-hybrid screening system and isolated a novel ES cell–derived cDNA encoding Ewing's sarcoma protein (EWS). EWS is a proto-oncogene and putative RNA-binding protein involved in human cancers. By using glutathione-S-transferase (GST) pull-down assays, we were able to confirm the interaction between Oct-4 and EWS in vitro, and moreover, coimmunoprecipitation and colocalization studies have shown that these proteins also associate in vivo. We have mapped the EWS-interacting region to the POU domain of Oct-4. In addition, three independent sites on EWS are involved in binding to Oct-4. In this study, we report that Oct-4 and EWS are coexpressed in the pluripotent mouse and human ES cells. Consistent with its ability to bind to and colocalize with Oct-4, ectopic expression of EWS enhances the transactivation ability of Oct-4. Moreover, a chimeric protein generated by fusion of EWS (1-295) to the GAL4 DNA-binding domain significantly increases promoter activity of a reporter containing GAL4 DNA-binding sites, suggesting the presence of a strong activation domain within EWS. Taken together, our results suggest that Oct-4–mediated transactivation is stimulated by EWS. STEM CELLS *2005;23:738–751*

INTRODUCTION

Oct-4, also referred to as Oct-3, is a member of the POU family of transcription factors, which is expressed in pluripotent embryonic stem (ES) and germ cells [1–6]. Members of the POU transcription factor family share a conserved DNA-binding domain, namely the POU domain, that was originally identified in the transcription factors Pit-1, Oct-1, Oct-2, and Unc-86 [7]. Oct-4 activates transcription via octamer motifs located proximally or distally from transcriptional start sites [8]. Oct-4–binding sites have been found in various genes, including *fgf 4* (fibroblast growth factor 4), *pdgfαr* (platelet-derived growth factor α receptor), and osteopontin [9–11]. In addition, genes, such as tau interferon (*IFN-τ*) and the α and β subunits of chorionic gonadotropin (*hCG*), expressed in the trophectoderm but not in embryos before blastocyst formation may be targets for silencing by Oct-4 [12–14]. This suggests that Oct-4 functions as a master switch during differentiation by regulating cells that have pluripotent potential or can develop such potential [15, 16].

Oct-4 mRNA is normally found in the totipotent and pluripotent stem cells of pregastrulation embryo, including oocytes, early cleavage-stage embryos, and the inner cell mass (ICM) of the blastocyst [1, 3, 17, 18]. In addition, the expression of this gene is

Correspondence: Jungho Kim, Ph.D., Laboratory of Molecular and Cellular Biology, Department of Life Science, Sogang University, Seoul 121-742, Korea. Telephone: 82-2-705-8461; Fax: 82-2-716-2092; e-mail: jkim@sogang.ac.kr Received December 30, 2004; accepted for publication February 14, 2005. 1066-5099/2005/$12.00/0 doi: 10.1634/stemcells.2004-0375

downregulated during differentiation, suggesting that Oct-4 plays a pivotal role in the mammalian development [19]. Furthermore, knocking out the *Oct-4* gene in mice causes early lethality due to lack of ICM formation [20], indicating critical function for self-renewal of ES cells [21]. During human development, expression of Oct-4 is found at least until the blastocyst stage, during which it is involved in gene expression regulation [22].

It was recently reported that Oct-4 is a key player in the genesis of human testicular germ cell tumors (TGCTs) [23, 24]. Human TGCTs are the most common malignancy in adolescent and young adult white males and are the cause of one in seven deaths in this group [25, 26]. The Oct-4 transcript can be consistently detected in a specific set of human TGCTs of adolescents and young adults: the seminomas and embryonal carcinomas [27]. In addition, the precursor lesions of human TGCT, known as CIS, also express Oct-4 [27]. The expression of Oct-4 has also been reported in human primary breast carcinomas, human breast cancer cell lines, and other types of carcinoma cell lines, suggesting that its expression may be implicated in tumorigenesis via upregulating its downstream target genes [28–30]. Consistent with these findings, Oct-4 expression in a heterologous cell system transforms nontumorigenic cells and endows tumorigenicity in the nude mouse, suggesting the possibility that aberrant expression of Oct-4 may contribute to the neoplastic process in cells [23].

The N- and C-termini of Oct-4 function as transactivation domains. Interestingly, although the N-terminus is active in various cultured cell types, the activity of the C-terminal domain depends on the cell type [31]. The POU domain of Oct-4 is a conserved DNA-binding domain that binds as a monomer to the octamer sequence motif, 5'-ATGCAAAT-3' [32]. This *cis*-acting element is important in determining the activity of many promoters and enhancers, including those of housekeeping and of cell type–specific genes [7]. In ES cells, the octamer sequence motif is active irrespective of the distance from its site of transcriptional initiation [33, 34]. However, in differentiated cells, Oct-4 can transactivate only from an octamer motif at proximal positions [1, 31, 35]. To be active from distal sites, Oct-4 requires stem cell–specific bridging factors that link an Oct-4 molecule bound to a remote DNA region to the transcription initiation site [35]. To date, the only identified putative Oct-4 cofactors are the viral oncoproteins E1A and E7 that seem to mimic yet-to-be-defined, stem cell–specific coactivators [35, 36].

EWS was originally identified through its fusion with the *Fli-1* gene, a member of the ETS transcription factor family, in Ewing's sarcoma harboring a t(11;22) chromosomal translocation [37]. Subsequent studies indicated that other ETS transcription factor genes are also fused with the *EWS* gene and produce chimeric proteins in Ewing's sarcoma. In addition, the *EWS* gene has been shown to form fusion proteins in other human cancers, including with *ATF-1* in malignant melanoma of soft parts, *WT1* in desmoplastic small round cell tumors, and orphan family nuclear receptor *TEC* in myxoid chondrosarcomas [38–40]. EWS has high homology to TLS, $hTAF_{II}68$, and *Drosophila* protein SARFH [38–40]. Thus, these proteins are collectively called the TET family member [41].

EWS contains a transcriptional activation domain in its N-terminal domain (NTD) and an RNA recognition motif and RGG repeats, both of which are found in RNA-binding proteins, in its C-terminal domain [38–40]. Interestingly, EWS was found to associate with a subpopulation of the transcription factor IID (TFIID) complex in cells [41, 42]. Furthermore, SARFH, a *Drosophila* homologue of EWS, has been reported to colocalize with RNA polymerase II at active chromatin [43]. Recently, EWS was found associated with the transcriptional coactivator cyclic AMP-responsive element–binding (CREB) protein (CBP) and the hypophosphorylated RNA polymerase II, both of which are enriched in transcription preinitiation complexes [44, 45]. These interactions indicate that EWS may be involved in gene transcription, in turn suggesting that EWS may function as a coactivator of CBP-dependent transcription factors [45].

To identify cofactors that physically interact and potentially cooperate with Oct-4 in allowing cells to remain in the cycle of totipotency, we conducted a bacterial two-hybrid screen of an ES cell cDNA library using Oct-4 as bait. We found EWS to be a binding partner of Oct-4. We confirmed the interaction between EWS and Oct-4 in vitro using bacterially expressed fusion proteins and in vivo through immunoprecipitation/Western blot analyses. We also demonstrate that Oct-4 and EWS are coexpressed in the ES and carcinoma cells. In transient transfection assays, EWS activated Oct-4–dependent transactivation. These data indicate that transcriptional activity of Oct-4 is modulated by EWS.

Materials and Methods

Materials and General Methods

Restriction endonucleases, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). [α-^{32}P]dCTP (3,000 Ci/mmol) and [^{35}S]-methionine (1,000 Ci/mmol) were obtained from PerkinElmer (Wellesley, MA) and GE Healthcare (Piscataway, NJ), respectively. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, bacterial transformations, and SDS-polyacrylamide gel electrophoresis of proteins were carried out using standard methods [46].

Constructs

To generate pBT–Oct-4, full-length Oct-4 cDNA was amplified from pCEP4/Oct-4 (obtained from Dr. A. Okuda, Saitama Medical School, Saitama, Japan) by polymerase chain reaction

(PCR) using primer 5'-Oct4-1 (5'-GATCGGATCCATGGCTG GACACCTGGCT-3') and primer 3'-Oct4-352 (5'-GATCCTC GAGCTATCAGTTTGAATGCATGGG-3'), digested with *Bam*HI and *Xho*I, and cloned into the same sites of pBT bait vector (Stratagene, La Jolla, CA, http://www.stratagene.com) in frame with the bacteriophage λ*cI* gene. To construct pTRC·HisA-Oct-4, the *Bam*HI-*Xho*I fragment of pBT–Oct-4 was subcloned into the same sites of pTRC·HisA vector (Invitrogen, Carlsbad, CA, http://www.invitrogen.com).

To isolate human Oct-4 (hOct-4), total RNA was prepared from human ES cells using RNeasy mini kit (Qiagen, Valencia, CA, http://www.qiagen.com) with on-column DNAse treatment. Then, the messenger RNAs were purified using an Oligodex-dT mRNA mini kit (Qiagen). cDNA was synthesized using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) as instructed by the manufacturer. For pcDNA3–hOct-4, the human Oct-4 gene was amplified with the human Oct-4 primers 5'-GATCGGATCCATGGCGGGACACCTGGCT-3' and 5'-GATCGAATTCTCAGTTTGAATGCATGGG-3', digested with *Bam*HI and *Eco*RI, and cloned into the same sites of pcDNA3.

GST-fusion Oct-4 plasmids GST–Oct-4, GST–Oct-4 (NTD), GST–Oct-4 (POU), and GST–Oct-4 (CTD) were generated through the following steps. For GST–Oct-4, plasmid pBT–Oct-4 was digested with *Bam*HI and *Xho*I and cloned into the same sites of pGEX (4T-1). For GST–Oct-4 (NTD), the NTD of Oct-4 was amplified from pCEP4/Oct-4 by PCR using primers 5'-Oct4-1 and 3'-Oct4-131 (5'-GATCCTCGAGCTAGTCCT GGGACTCCTCGGG-3'), digested with *Bam*HI and *Xho*I, and cloned into the same sites of pGEX (4T-1). For GST-Oct-4 (POU), the POU domain of Oct-4 was amplified from pCEP4/Oct-4 by PCR using primers 5'-Oct4-122 (5'-GATCGGATCCGTG GAACCAACTCCCGAG-3') and 3'-Oct4-287 (5'-GATCCTC GAGCTATTGGGAATACTCAATACT-3'), digested with *Bam*HI and *Xho*I, and cloned into the same sites of pGEX (4T-1). For GST-Oct-4 (CTD), the CTD of Oct-4 was amplified from pCEP4/Oct-4 by PCR using primers 5'-Oct4-278 (5'-GATCG GATCCGGCAAAAGATCAAGTATT-3') and 3'-Oct4-352 (5'-GATCCTCGAGCTATCAGTTTGAATGCATGGG-3'), digested with *Bam*HI and *Xho*I, and cloned into the same sites of pGEX (4T-1).

GST-fusion EWS plasmids were generated by the following steps. For GST-EWS, plasmid pSG5-Flag-EWS (kindly provided by Dr. L. Yang, University of Washington School of Medicine, Seattle) was digested with *Hin*dIII, blunted with Klenow fragment, and redigested with *Eco*RI. The excised fragment was ligated into the *Eco*RI and *Sma*I sites of pGEX (4T-1). For GST-EWS (NTD), the NTD of EWS was amplified from pSG5-Flag-EWS by the PCR using primers 5'-EWS-1 (5'-GATC GAATTCATGGCGTCCACGGATTAC-3') and 3'-EWS-295 (5'-GATCCTCGAGCTAACTCATGCTCCGGTTCTC-3'), digested with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX (4T-1). For GST-EWS (GRPI), the glycine, arginine, proline-rich motif I of EWS was amplified from pSG5-Flag-EWS by the PCR using primers 5'-EWS-290 (5'-GATCGAATTCGAGAACCG GAGCATGAGT-3') and 3'-EWS-350 (5'-GATCCTCGAGC TATGGGCCTAGATCAAGATC-3'), digested with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX (4T-1). For GST-EWS (RRM), the RNA recognition motif of EWS was amplified from pSG5-Flag-EWS by the PCR using primers 5'-EWS-352 (5'-GATCGAATTCGTAGATCCAGATGAAGAC-3') and 3'-EWS-463 (5'-GATCCTCGAGCTAGCCCTCACGGGGTGGCAG-3'), digested with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX (4T-1). For GST-EWS (GRP II and III), plasmid pSG5-Flag-EWS was digested with *Mse*I and *Hin*dIII, repaired with the Klenow fragment, and then ligated into the *Sma*I site of pGEX (4T-1). The descriptions of plasmids encoding GST-EWS (1-35), GST-EWS (70-163), and GST-EWS (192-265) have been previously described [47].

For expression in mammalian cells, the *Bam*HI and *Xho*I fragment containing Oct-4 was isolated from pGEX(4T-1)–Oct-4 and subcloned into the same sites of pcDNA3 (Invitrogen) to generate pcDNA3/Oct-4.

To generate DsRed–Oct-4, Oct-4 was amplified with pcDNA3–Oct-4 by PCR using primers 5'-Oct4-1 and 3'-Oct4-351Age (5'-GATCACCGGTGCTCCGTTTGAATGCAT GGG-3'), digested with *Bam*HI and *Age*I, and cloned into the same sites of pDsRed1-N1 (Clontech, Palo Alto, CA, http://www.clontech.com). For EGFP-EWS, the 5' portion of EWS was amplified from pSG5-Flag-EWS by PCR using primers 5'-EWS-1Hind (5'-GATCAAGCTTATGGCGTCCACGGAT TAC-3') and 3'-EWS-250 (5'-AGCTTGGCTGTAGGATCC-3') and digested with *Hin*dIII and *Bam*HI. The 3' portion of EWS was amplified from pSG5-Flag-EWS by PCR using primers 5'-EWS-241 (5'-CCACCCCAAACTGGATCC-3') and 3'-EWS-656Age (5'-GGCGACCGGTGGGTAGGGCCGATCTCTGCG-3') and digested with *Bam*HI and *Age*I. These excised products were directly ligated with the *Hin*dIII- and *Age*I-digested pEGFP (N1) vector (Clontech) to generate pEGFP-EWS.

To generate GAL4-EWS (1-295), pGEX (4T-1)-EWS (NTD) was digested with *Xho*I, repaired with Klenow fragment, and redigested with *Eco*RI. The excised fragment was subcloned into the *Eco*RI and *Sma*I sites of pM vector (Clontech). To construct GAL4-EWS (290-656), the CTD of EWS was amplified by PCR using primer 5'EWS 290 and 3'EWS-656Age, digested with *Age*I, and repaired with Klenow fragment. The excised fragment was redigested with *Eco*RI and subcloned into *Eco*RI and *Sma*I sites of pM vector.

The reporter plasmid, pOct-4(10x)TATA luc, comprises 10 copies of the i-opn element that binds to Oct-4 [48], which was generated by annealing and multimerizing the

oligonucleotides 5'-AAGTTAAAATCACATTTGAAATG CAAATGGAAAAGCAAGTTAAAATCACATTTG-3' and 3'-TTCAATTTTAGTGTAAACTTTACGTTTACCTTTTC GTTCAATTTTAGTGTAAAC-5' and precloned into TA vector (Invitrogen). Ten copies of this element were then digested with *Xho*I and *Hin*dIII and recloned into the same sites of the pGL3 Basic vector (Promega, Madison, WI, http://www.promega.com). To construct pOct-4(10x)TATA luc, the TATA box was generated by annealing the synthetic oligonucleotides 5'TATA (5'-phospho-GATCTGAGGGTATATAATGGAA-3') and 3'TATA (5'-phospho-AGCTTTCCATTATATACCCTCA-3') and subcloned into the *Bam*HI and *Hin*dIII sites of the pOct-4(10x) luc, downstream from the Oct-4–binding sites. The human *Rex-1* promoter, which contains an Oct-4 binding site (5'-ATTTGCAT-3') [49], was cloned into the *Xho*I-*Hin*dIII sites of the promoterless pGL3 Basic vector (Promega) by PCR amplification from genomic DNA. The amplification primers used for this purpose were 5'Rex (5'-GATCCTCGAGTCGGGATTCAGAAGAGGC-3') and 3'Rex (5'-GATCAAGCTTGGCGGCTAGGAGTTCAGC-3').

Cell Culture, Transfection, and Reporter Assays

Mouse and human ES cells were grown as previously described [50, 51]. P19, COS-7, HEK293T, or NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplied with 10% heat-inactivated fetal calf serum (Gibco-BRL, Gaithersburg, MD, http://www.gibcobrl.com), penicillin, and streptomycin. The cells were transiently transfected with plasmids by electroporation using the Gene Pulser II RF module system (Bio-Rad, Hercules, CA, http://www.bio-rad.com) or by lipofection using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany, http://www.roche-applied-science.com) as instructed by the manufacturer. Luciferase assays were performed with the dual-Luciferase Assay System, in accordance with the supplier's protocol (Promega). Renilla luciferase activities were used to normalize the transfection efficiency.

Bacterial Two-Hybrid Screening

To generate mouse ES cell–derived cDNA library, total RNA was extracted from mouse ES cells and cDNA library was constructed using StrataScript reverse transcriptase as instructed by the manufacturer (Stratagene). Also, following the instruction of the manufacturer, the BacterioMatch Two-Hybrid system reporter strain (Stratagene) was transformed sequentially with pBT-Oct-4 and plasmid pTRG-cDNA, which contains the amino-terminal domain of RNA polymerase α subunit and a cDNA library from mouse ES cells subcloned into the multiple cloning site (Stratagene). Positive clones were screened for their potential to grow on selective medium and for the expression of a reporter enzyme, β-galactosidase. After secondary screening assays eliminated false positives, plasmids containing potential Oct-4–binding partners were isolated and sequenced.

Northern Blot Analysis

For Northern blotting, total cellular RNAs were prepared using Trizol reagent (Invitogen), and aliquots (10 μg/lane) were separated on 1.5% agarose-formaldehyde gels. RNA was transferred to Hybond nylon membranes (Amersham) and crosslinked to the membrane in a GS Gene Linker UV Chamber (Bio-Rad). The *Pst*I and *Xho*I (0.65 kb), *Hin*dIII and *Eco*RI (0.98 kb), or *Eco*RI and *Bam*HI (0.73 kb)-digested DNA fragments from mouse Oct-4, human Oct-4, or EWS cDNAs, respectively, were gel purified and ^{32}P-labeled using a Prime-It II Random Primer Labeling kit (Stratagene). Hybridizations, using the radiolabeled probe, were carried out overnight in ExpressHyb Solution (Clontech) at 68ºC. The blots were washed twice at 68ºC with 2 × standard saline citrate/0.1% SDS, and radiolabeled bands were visualized by autoradiography.

Recombinant Six Histidine–Tagged Oct-4 Protein Purification

The recombinant pTRC·HisA-Oct-4 plasmid was transformed into *Escherichia coli*, the expression of $(His)_6$-Oct-4 was induced by isopropyl-β-D-thiogalactopyranoside, and the recombinant protein was purified by chromatography on Ni-nitrilotriacetic acid (NTA)–agarose in accordance with the supplier's protocol (Qiagen). In brief, the cell pellet from a culture was resuspended in lysis/wash buffer (50 mM Tris·HCl [pH 8.0], 300 mM NaCl, 10 mM imidazole) and clarified by centrifugation (16,000 rpm for 15 minutes) at 4ºC, and the supernatant was incubated with NTA-agarose resin for 1 hour at 4ºC on a slowly rotating wheel. The Oct-4–bound NTA-agarose resin was then packed into a Micro Bio-Spin Chromatography Column (Bio-Rad) and washed extensively with lysis/wash buffer. The protein was eluted with elution buffer (50 mM Tris·HCl [pH 8.0], 300 mM NaCl, 20 mM imidazole). The elution profile of Oct-4 protein was monitored by Western blotting using monoclonal Xpress antibody (Invitrogen).

In Vitro Transcription and Translation

In vitro transcriptions and translations were performed in the presence of [^{35}S]-methionine in rabbit reticulocyte lysates using the TNT T7–coupled reticulocyte system, as specifically instructed by the manufacturer (Promega).

GST Pull-Down Assays

GST pull-down assays were performed as described previously [52] using GST-fusion protein and [^{35}S]-methionine–labeled EWS or recombinant $(His)_6$-tagged Oct-4. Bound proteins were detected using autoradiography or immunoblotting as previously described [47].

Immunoprecipitation and Western Blot Analysis

For Oct-4/EWS immunoprecipitation, plasmid pSG5/Flag-EWS was cotransfected into COS-7 cells with either pcDNA3 alone or pcDNA3/Oct-4. After 48 hours, cell pellets were disrupted in ice-

cold NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 × complete protease inhibitor cocktail solution [Roche Diagnostics]) on ice for 30 minutes. Supernatants were then collected by centrifugation at 16,000*g* for 15 minutes at 4ºC and incubated for 1 hour with monoclonal antibody directed against Oct-4 (C-10, Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com). Complex precipitated with protein G-Sepharose (Pierce, Rockford, IL, http://www.piercenet.com) was washed four times with ice-cold NP-40 lysis buffer. The immunoprecipitated proteins were eluted in SDS loading buffer (62.5 mM Tris·HCl [pH 6.9], 10% glycerol, 2% SDS, 5% β-mercaptoethanol), separated on 8% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride (PVDF) membrane. EWS was detected by immunoblotting using M2 anti-Flag monoclonal antibody (Sigma, St. Louis, http://www.sigmaaldrich.com), whereas Oct-4 was detected with the same antibody as described above. Detection was performed by chemiluminescence using Western Lightening (PerkinElmer Life Sciences, Boston, http://www.perkinelmer.com). Coimmunoprecipitation experiments of Oct-4 and EWS in P19 cells were performed as described in earlier reports [53, 54].

Subcellular Localization Experiment

COS-7 cells were plated on glass coverslips and transfected with the respective DNA plasmids using FuGENE 6 following the manufacturer's instructions. After 48 hours of transfection, the cells were washed in phosphate-buffered saline (PBS) and fixed for 10 minutes at –20ºC in a mixture of acetone and methanol (1:1, vol/vol). The coverslips were mounted with 50% glycerol/PBS. Green or red fluorescence was detected under a fluorescence microscope (IX51, Olympus, Tokyo, http://www.oylmpus.com) equipped with a CoolSNAP digital camera (Olympus).

Results

Identification of EWS as an Oct-4–Interacting Partner

Bacterial two-hybrid screening [55] was performed to identify protein molecules interacting with Oct-4. Because Oct-4 is exclusively found in totipotent embryonic cells and mouse germ cells [51, 56], mouse ES cells were chosen to generate a cDNA expression library. A pBT–Oct-4 fusion was constructed and used as bait for bacterial two-hybrid screen. Among approximately one million recombinant clones that were screened, we identified five interesting clones as the Oct-4–binding partners. In addition, through nucleotide sequence determination and comparison with GenBank and SwissProt databases, we found that one clone contained the cDNA sequence of EWS, which is a putative RNA-binding protein and proto-oncogene [37, 39]. Interestingly, it has been reported recently that EWS associates with a transcriptional coactivator, CBP, and the hypophosphorylated form of RNA polymerase II, suggesting that EWS may be a transcriptional coactivator [44, 45].

As depicted in Figure 1A, the kanamycin-resistant reporter strain for the bacterial two-hybrid system was transformed with a series of plasmids containing Oct-4, EWS, or appropriate controls. All bacterial reporter cells harboring both pBT fusions (chloramphenicol-resistant) and pTRG fusions (tetracycline-resistant) were able to grow on Lurina-Bertani (LB) medium containing kanamycin (+Kan), chloramphenicol (+Chl), and tetracycline (+Tet), indicating that both plasmids are present (Fig. 1B). However, apart from the LGF2-Gal11 interaction, which serves as a positive control, we found that only the expression of both pBT–Oct-4 and pTRG-EWS in bacterial reporter cell line allowed growth on +Kan/+Chl/+Tet LB plates containing 0.47 or 0.59 mM carbenicillin, indicating a physical interaction between Oct-4 and EWS (Figs. 1C, 1D).

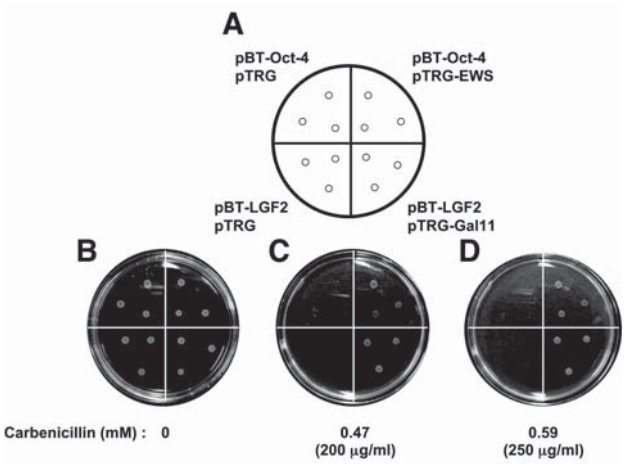

Figure 1. Interaction between Oct-4 and Ewing's sarcoma protein (EWS) in the bacterial two-hybrid system. **(A):** Schematic diagram of bacterial reporter cells transformed with the indicated plasmid set. Bacterial reporter cells were cotransformed with the indicated plasmids as shown and plated onto the corresponding sectors. The bait vector, pBT, encodes the full-length bacteriophage cI protein under the control of the *lac*UV5 promoter. The pBT-Oct-4 encodes a fusion of bacteriophage λcI and Oct-4. The target plasmid, pTRG, contains the amino-terminal domain of RNA polymerase α-subunit. pTRG-EWS indicates the pTRG cDNA clone obtained from screening, which encodes a fusion protein of RNA polymerase α-subunit and EWS. **(B–D):** Growth of bacterial reporter cells harboring the plasmids shown in **(A)**. Cells were visualized after 16 hours of incubation at 37ºC on +Kan/+Chl/+Tet selective medium **(B)** lacking carbenicillin and **(C)** containing 0.47 mM or **(D)** 0.59 mM carbenicillin, respectively. Bacterial reporter cells cotransformed with the indicated plasmids were plated onto +Kan/+Chl/+Tet plates to verify expression of both bait (chloramphenicol-resistant, Chr^r) and prey (tetracycline-resistant, Tet^r) plasmids in reporter cells (kanamycin-resistant, Kan^r) or onto +Kan/+Chl/+Tet/+Carb plates for examining the interaction between bait and prey proteins. Bacterial reporter cells were transformed with indicated plasmids, and individual $Kan^rChl^rTet^r$ transformants were spotted on +Kan/+Chl/+Tet selective culture plates containing 0, 0.47, or 0.59 mM carbenicillin, respectively. pBT-LGF2 and pTRG-Gal11 plasmids were used as a positive control.

Coexpression of Oct-4 and EWS in Mouse and Human ES Cells and Mouse Embryonal Carcinoma Cells

It has been reported that the *Oct-4* gene is expressed in ES cells and germ cells of the mouse embryo [1-6]. Interestingly, cells that differentiate into somatic tissues lose Oct-4 expression. However, it remains unknown as to whether EWS is expressed in ES or embryonal carcinoma (EC) cells, although it was reported to be ubiquitously expressed in several mouse tissues investigated [57]. Thus, to determine the expression pattern of these two mRNAs, Oct-4 and EWS transcripts were investigated by Northern blot analysis of total RNA from six different cell lines, including human and mouse ES cells. As reported previously [34, 58, 59], Oct-4 expression was detected in mouse and human ES cells and mouse EC cells but not in COS-7, HEK293T, and NIH3T3 cell lines (Fig. 2, two upper panels).

The expression profile of EWS in these same cell lines was assessed by Northern blotting (Fig. 2, third panel). The *EWS* gene is expressed in all cell lines investigated, including ES and EC cells. These results clearly show that Oct-4 and EWS are expressed in ES and EC cells. As a control for RNA loading, ethidium bromide staining of 28S rRNA indicated relatively equal amounts of total RNA present from the different cell lines analyzed (Fig. 2, lower gel).

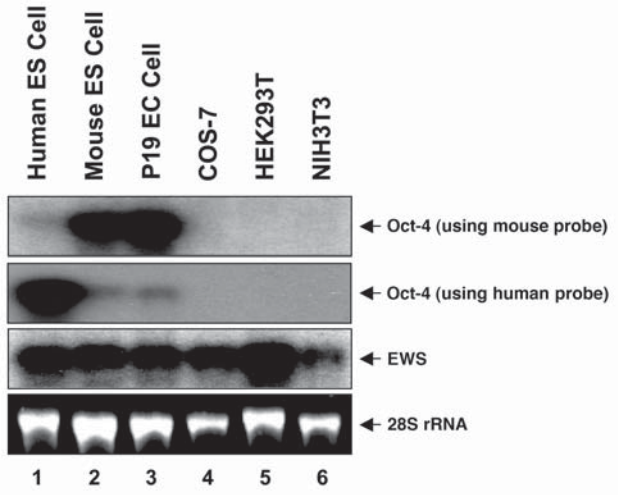

Figure 2. Northern blot analysis of Oct-4 and EWS mRNA in mouse and human cells. Human ES (lane 1), mouse ES (lane 2), P19 mouse EC (lane 3), COS-7 (lane 4), HEK293T (lane 5), or NIH3T3 (lane 6) cells were harvested to be used for preparing total RNA. Total RNA was fractionated on a 6% formaldehyde–1.5% agarose gel, transferred to a nylon membrane, and probed with mouse Oct-4 (upper panel), human Oct-4 (second panel), or EWS (third panel) cDNAs, respectively, as described in Materials and Methods. The EtBr staining of the agarose gel used for the Northern blotting is shown to demonstrate that equal amounts of total RNA were loaded in each lane (lower panel). Arrows indicate the position of migration of the respective RNAs. Abbreviations: EC, embryonal carcinoma; ES, embryonic stem; EtBr, ethidium bromide; EWS, Ewing's sarcoma protein.

Oct-4 Interacts with EWS In Vitro and In Vivo

To further define the specificity of the Oct-4/EWS interaction identified in the bacterial two-hybrid screen, we performed in vitro GST pull-down assays (Fig. 3A). Bacterially expressed GST or GST–Oct-4 fusion proteins immobilized on glutathione-Sepharose beads were mixed with in vitro–produced [^{35}S]-methionine–labeled EWS protein. After extensive washing, the bound proteins were analyzed by 8% SDS-PAGE and autoradiography. As shown in Figure 3A, approximately 10% of input EWS protein was specifically retained on the Oct-4–conjugated Sepharose beads. Because EWS did not bind to GST alone (Fig. 3A, lane 2), the interaction was considered to be specific.

The interaction between Oct-4 and EWS was also investigated using cell lysates and recombinant proteins to confirm whether the recombinant proteins generated in bacteria or synthesized by in vitro transcription/translation could still interact with the endogenous partner. Failure to reproduce this interaction in this system could indicate that the recombinant proteins are nonfunctional or require post-translational modification of interaction. Cell extract was prepared from P19 EC cell line and incubated with either GST-fusion Oct-4 (Fig. 3B, top panel) or EWS (bottom panel). After extensive washing of the beads, the bound proteins were eluted with sample buffer, separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane, and probed with anti-sera to EWS. GST–Oct-4 beads, but not control GST beads, efficiently retained endogenous EWS (Fig. 3B, top panel). In the reciprocal experiment, the GST-EWS fusion protein was also able to interact with endogenous Oct-4 (Fig. 3B, bottom panel). These results indicate that the proteins made in bacterial cells, synthesized by in vitro transcription/translation, and from cell lysates seem to be equivalent with respect to Oct-4–EWS interaction.

To determine whether the interaction between Oct-4 and EWS occurs in vivo, we performed immunoprecipitation experiments after transient transfection of COS-7 cells with expression vectors that expressed both proteins. Plasmid pcDNA3/Oct-4 or pcDNA3 was cotransfected with pSG5/Flag-EWS into COS-7 cells. Cells were lysed for 48 hours after the transfection and Oct-4 protein–immunoprecipitated with an α-Oct-4 antibody (C-10, Santa Cruz Biotechnology). Immunoblotting was performed on eluents using an anti-Flag antibody (M2, Sigma) to detect the presence of EWS. EWS was found to specifically coprecipitate with Oct-4 (Fig. 3C, top panel). Probing for EWS (middle panel) or Oct-4 (lower panel) indicated the presence of the two proteins in the extracts from the transfected cells. To further examine whether endogenous Oct-4 and EWS associate in mammalian cells in vivo, immunoprecipitation experiments were also performed with cell extract from P19. Antibody against Oct-4 (C-10, Santa Cruz Biotechnology), and not control serum, coprecipitated EWS (Fig. 3D, top panel), whereas anti-EWS (C-19, Santa Cruz Biotechnology) coprecipitated Oct-4 (Fig. 3D, lower panel). These results suggest that Oct-4 and EWS can associate in vivo.

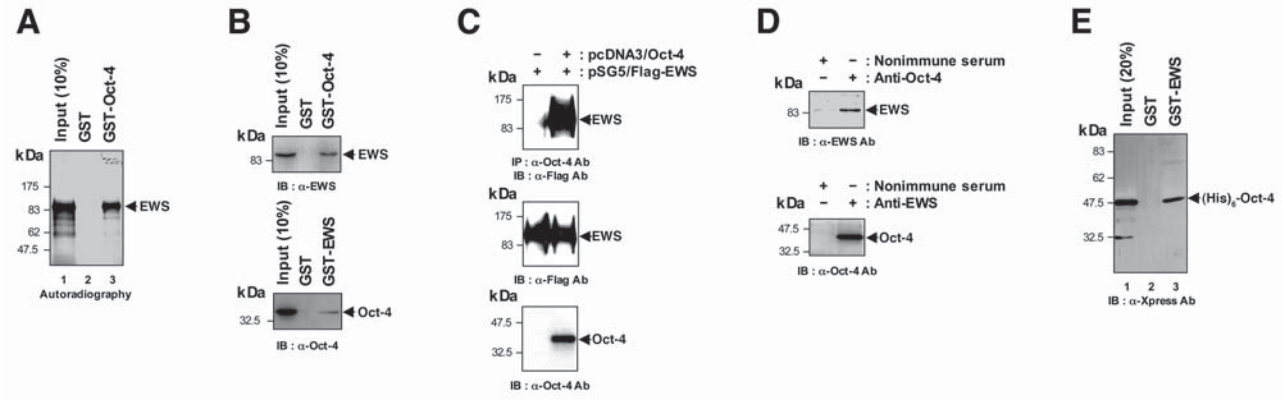

Figure 3. Interaction between Oct-4 and Ewing's sarcoma protein (EWS) in vitro and in vivo. **(A):** Association of Oct-4 with EWS in vitro. [^{35}S]-methionine–labeled EWS was incubated with GST alone or GST fusion protein containing full-length Oct-4. An aliquot of input (10%) and the pellet from GST alone or GST–Oct-4 pull-downs were analyzed on an 8% SDS-PAGE, and the bound EWS protein was visualized by fluorography. The presence of GST or GST-Oct-4 is indicated at the top of each lane. The positions of migration of the molecular weight markers are indicated to the left, and EWS is indicated by an arrow to the right. **(B):** Interactions of GST-Oct-4 or GST-EWS with in vivo expressed EWS or Oct-4 proteins. P19 cell lysate was incubated with either GST-Oct-4 (top panel) or GST-EWS (bottom panel). An aliquot of input (10%) and the pellets from either GST-Oct-4 or GST-EWS pull-downs were resolved on SDS-PAGE, and the bound proteins were detected by Western blot. The presence of GST-fusion proteins is indicated at the top of each lane. The positions of migration of the molecular weight markers are indicated to the left, and EWS or Oct-4 is indicated by arrows to the right. **(C):** Association of Oct-4 with EWS in vivo. Forty-eight hours after cotransfection of COS-7 cell lines with 10 μg of pSG5/Flag-EWS and either 10 μg of pcDNA3 or pcDNA3/Oct-4, cell extracts were prepared as described in Materials and Methods and immunoprecipitated with an α-Oct-4 antibody (C-10; Santa Cruz Biotechnology). After fractionation on 8% SDS-PAGE, the immunoprecipitates were analyzed to verify the presence of EWS protein by Western blotting using an α-Flag antibody (M2; Sigma). Lysates were also analyzed for EWS (middle) and Oct-4 (bottom) proteins by Western blotting. The positions of the molecular weight markers are indicated to the left, and the position of migration of EWS and Oct-4 are indicated by arrows to the right. **(D):** Coimmunoprecipitation of Oct-4 and EWS in P19 cell. P19 embryonic carcinoma cell lysates were immunoprecipitated with α-Oct-4 antibody (top panel) or α-EWS antibodies (bottom panel), resolved by SDS-PAGE, and probed with anti-EWS antibodies (C-19; Santa Cruz Biotechnology) or anti–Oct-4 antibody (C-10; Santa Cruz Biotechnology), respectively. The positions of the molecular weight markers are indicated to the left, and the position of migration of EWS and Oct-4 are indicated by arrows to the right. **(E):** The interaction between Oct-4 and EWS is direct. Bacterially produced $(His)_6$-tagged Oct-4 protein was incubated with bacterially produced GST alone or GST-EWS at 4°C for 1 hour. After extensive washing, bound Oct-4 protein was assessed by 15% SDS-PAGE and Western blot analysis with an α-Xpress antibody (Clontech). The positions of the migration of the molecular weight markers are indicated to the left, and six histidine–tagged Oct-4 is indicated by an arrow to the right. Abbreviations: Ab, antibody; IB, immunoblot; IP, immunoprecipitation.

However, neither the in vivo interaction in bacterial (Fig. 1) or mammalian cells (Figs. 3C, 3D) nor the GST pull-down assay using in vitro–translated EWS protein (Fig. 3A) rules out the possibility that the observed interaction may occur through an intermediate bridging partner. Therefore, to address this, Oct-4 was expressed as a six histidine–containing fusion protein in *E. coli* and purified by Ni^{+2}-NTA agarose resin. A GST pull-down assay was then performed using recombinant $(His)_6$-Oct-4 with GST or GST-EWS fusion proteins, which had also been produced in *E. coli*. After extensive washing, the amount of Oct-4 retained was determined by SDS-PAGE and Western blotting with an α-Xpress antibody (Clontech). As shown in Figure 3E, bacterially produced GST-EWS protein interacts with recombinant Oct-4, suggesting that the Oct-4-EWS interaction does not require an adaptor protein.

The POU Domain of Oct-4 Is Involved in EWS Interaction

To define the minimal region within Oct-4 required for binding to EWS, in vitro binding experiments were performed with truncation forms of Oct-4 functional domains. The structure of the Oct-4 deletion mutants used in the study is shown schematically in Figure 4A. First, we performed GST pull-down assays using in vitro translated [^{35}S]-methionine–labeled EWS protein, together with deletion mutant Oct-4 fusion proteins. As shown in Figure 4B, EWS bound to GST-Oct-4 (POU) but did not interact with GST-Oct-4 (NTD) or GST-Oct-4 (CTD). The GST-Oct-4 (POU) showed similar binding affinity with full-length Oct-4 protein, indicating that the POU domain of Oct-4 contains the domains responsible for EWS interaction (J.L. and J.K., unpublished data). The same amounts of GST fusion proteins were used in these assays, as confirmed by fractionation on 15% SDS-PAGE (J.L. and J.K., data not shown).

EWS Contains at Least Three Independent Oct-4–Interacting Motifs

The *EWS* gene contains an open reading frame of 1,968 bp and encodes a protein of 656 amino acids [37, 39]. On the basis of homology features of the primary amino acid sequence, the EWS coding region can be divided into three different domains, namely N-terminal domain (NTD), RNA recognition motif (RRM), and glycine,

arginine, and proline-rich (GRP-rich) motifs I, II, and III (Fig. 5A). To delineate the amino acids in EWS responsible for the interaction with Oct-4, we fused EWS (NTD) (amino acids 1 through 295), EWS (1-35) (amino acids 1 through 35), EWS (70-163) (amino acids 70 through 163), EWS (192-265) (amino acids 192 through 265), EWS (GRP I) (amino acids 290 through 350), EWS (RRM) (amino acids 352 through 463), or EWS (GRP II and III) (amino acids 441 through 656) to GST. These truncation mutants were then individually expressed as GST fusion proteins in *E. coli* and coupled to glutathione-Sepharose beads. After incubation with recombinant His_6-tagged Oct-4 protein and after extensive washing, we found that four GST fusions containing the NTD (70-163), GRP I, and GRP II and III domains specifically retained Oct-4 protein (Fig. 5B), whereas Oct-4 did not interact with GST alone (Fig. 5B, lane 2), GST-EWS (1-35) (lane 4), or GST-EWS (RRM) (lane 8). This result suggests that EWS has at least three sites (amino acids 70 through 163, GRP I, and GRP II and III) that can bind to Oct-4 independently.

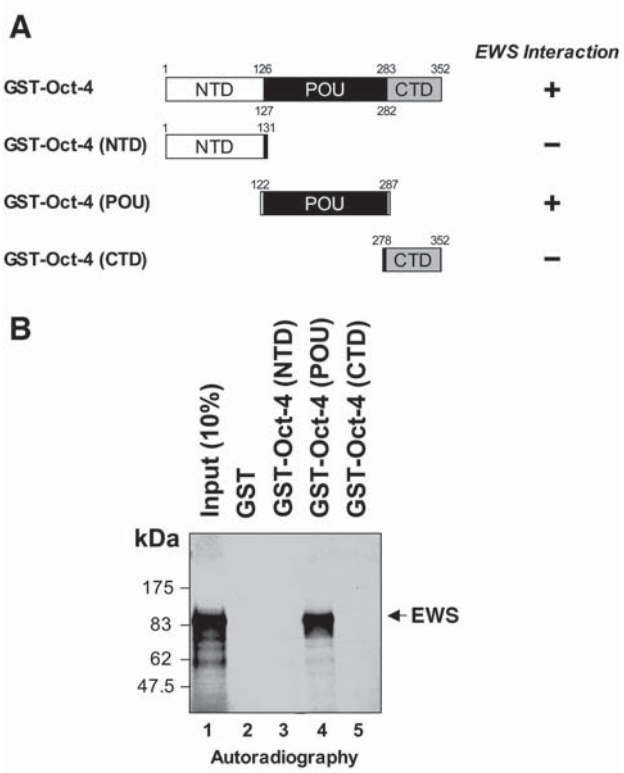

Figure 4. Mapping Ewing's sarcoma protein (EWS)–interacting motif on Oct-4. **(A):** Schematic diagram of the Oct-4 cDNA fragments fused in-frame to the GST gene in pGEX vector. Numbers refer to the amino acid residues, and the + symbol to the right indicates the ability to bind to EWS. **(B):** Strong binding affinity of Oct-4 POU domain to EWS. Proteins from *Escherichia coli* that expressed recombinant pGEX vectors encoding the various GST-Oct-4 fragments were incubated with [^{35}S]-methionine–labeled in vitro translated EWS protein. After GST pull-down assays, the bound proteins were eluted with SDS loading buffer and analyzed by 8% SDS-PAGE. The positions of molecular weight markers are indicated on the left, and the position of EWS is indicated on the right.

Colocalization of Oct-4 and EWS in the Nucleus

The existence of an Oct-4–EWS complex was further confirmed through the intracellular localization of Oct-4 and EWS in cotransfected COS-7 cells (Fig. 6A). For this purpose, we constructed plasmids expressing fluorescent DsRed or GFP fusion proteins of Oct-4 or EWS, respectively. A pDsRed1–Oct-4 construct that expresses DsRed–Oct-4 protein was transiently transfected into COS-7 cells, and subcellular localization of the protein was detected by fluorescent microscopy for red fluorescence. In transiently transfected cells, the DsRed-tagged Oct-4 protein was clearly localized to the nucleus (Figs. 6Aa, 6Ab). In addition, we transfected enhanced green fluorescent protein (EGFP) fusion EWS construct into the same cell lines to determine its intracellular localization using green fluorescence. As shown in

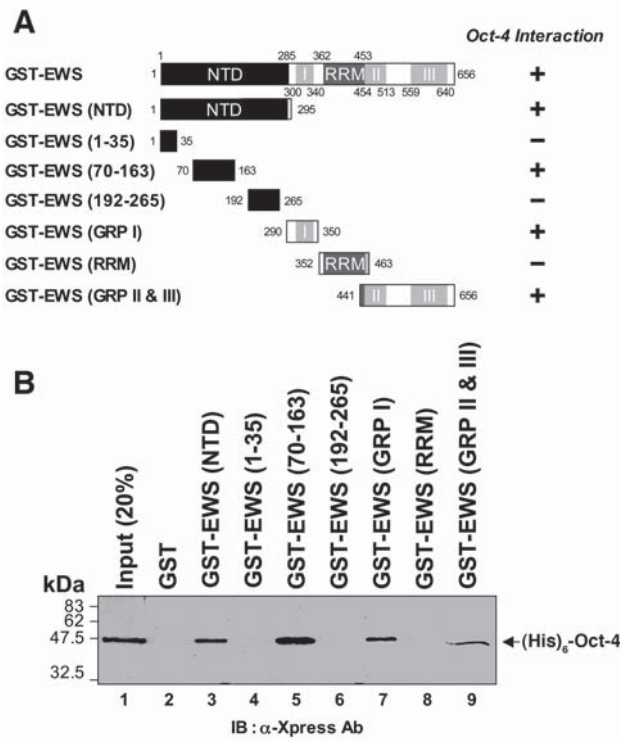

Figure 5. Involvement of three independent domains of Ewing's sarcoma protein (EWS) in the interaction of Oct-4. **(A):** Schematic diagram of the EWS cDNA fragments fused in-frame to the GST gene in pGEX vector. Numbers refer to the amino acid residues, and the + symbols to the right indicate the ability to bind to Oct-4. **(B):** Binding of Oct-4 to the EWS (70-163), GRP I, and GRP II and III domains. Recombinant six histidine–tagged Oct-4 protein was incubated with 2 μg of GST (lane 2), GST-EWS (NTD) (lane 3), GST-EWS (1-35) (lane 4), GST-EWS (70-163) (lane 5), GST-EWS (192-265) (lane 6), GST-EWS (GRP I) (lane 7), GST-EWS (RRM) (lane 8), or GST-EWS (GRP II and III) (lane 9) proteins bound to glutathione-Sepharose beads. An aliquot of the input (20%, lane 1) and the pellets (lanes 2 through 9) from GST pull-down assays were analyzed by 15% SDS-PAGE, and the bound Oct-4 proteins were detected by Western blot using α-Xpress antibody (Clontech). The position of migration of Oct-4 is indicated by an arrow to the right. Abbreviations: Ab, antibody; IB, immunoblot.

Figures 6Ac and 6Ad, COS-7 cells transiently transfected with EGFP-tagged EWS also contained EWS protein to the nucleus. To further examine a possible colocalization of Oct-4 and EWS proteins, we analyzed the simultaneous expression of these constructs in cotransfected cells. Both DsRed-tagged Oct-4 and GFP-tagged EWS proteins were distributed in a more or less fine punctate pattern in the nucleus (Figs. 6Ae–6Ah). Although they displayed a very similar pattern, Oct-4 seemed to be more evenly distributed. The overlay image indicated that Oct-4 and EWS in the nucleoplasm partially overlapped (Fig. 6Ag).

To further validate these results and to eliminate any possibility that the overexpression of the proteins in the COS-7 cells may yield confounding results relative to their subcellular localizations, the colocalization of the endogenously expressed Oct-4 and EWS proteins was also determined by indirect immunofluorescence studies using α-Oct-4 (C-10, Santa Cruz Biotechnology) or α-EWS (C-19, Santa Cruz Biotechnology) antibodies. As shown in Figure 6B, both proteins are exclusively localized to the nucleus in P19 cells, suggesting that the nuclear localization of Oct-4 and EWS proteins was not affected by the nature of transient transfection or by the cell line studied. Therefore, we could conclude that Oct-4 and EWS proteins colocalize or are in close proximity in cells.

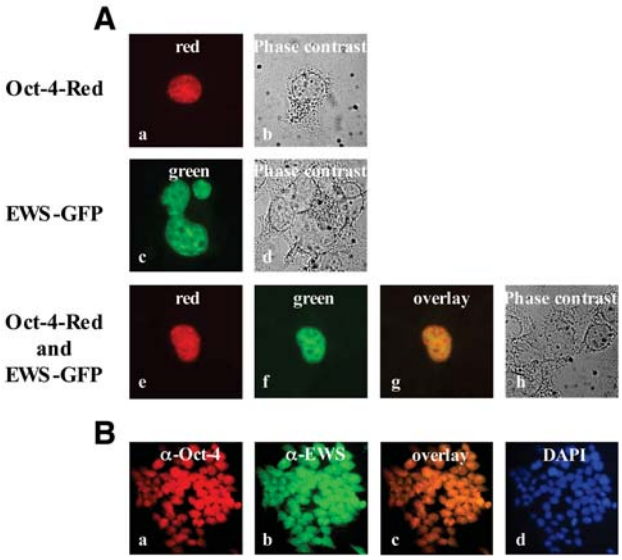

Figure 6. Subcellular localization of Oct-4 and Ewing's sarcoma protein (EWS) in cells. **(A):** Colocalization of Oct-4 and EWS in the transiently transfected COS-7 cells. COS-7 cells grown on coverslips were transfected with mammalian expression vectors encoding DsRed-tagged Oct-4 and/or GFP-tagged EWS proteins. Subcellular distribution of single (a or c) or cotransfected (e–g) COS-7 cells was analyzed by fluorescent microscopy to detect red or green fluorescence. Merge image (g) is shown for colocalization. Expression constructs used (abbreviated on left side of panels) were pDsRed1–Oct-4 and pEGFP-EWS. **(B):** Subcellular distribution of endogenous Oct-4 and EWS in P19 cell. The P19 cells were fixed with an acetone/methanol mixture and incubated with primary antibodies for Oct-4 (C-10; Santa Cruz Biotechnology) or EWS (C-19; Santa Cruz Biotechnology). Subcellular distribution of Oct-4 (a) or EWS (b) was examined using a florescence microscope. Merge image (c) is shown for colocalization. Cell nuclei were stained with DAPI (d).

EWS Activates Oct-4–Mediated Transactivation

Given the suggested physical association and colocalization between Oct-4 and EWS in vitro and in vivo, we investigated the potential functional consequence of the interaction between Oct-4 and EWS. For these assays, we constructed an Oct-4 reporter plasmid, pOct-4(10x)TATA luc, containing 10 copies of Oct-4–binding sites and a TATA box cloned upstream of the luciferase gene (Fig. 7A). The effect of EWS was examined on gene expression from this reporter plasmid by introducing pcDNA3–Oct-4 with or without pSG5-EWS in 293T cells. As shown in Figure 7A, Oct-4 activated gene expression from the pOct-4(10x)TATA luc reporter by 18-fold (lane 3). However, cotransfection with the EWS expression construct led to a 53-fold increase in reporter expression (~300% augmentation by the effect of EWS) (lane 4), with no significant effect on the basal transcription level (lane 2). Similarly, EWS augmented Oct-4–mediated gene expression from human *Rex-1* promoter containing one binding site for Oct-4 (Fig. 7B). These results strongly indicate that EWS potentiates Oct-4–mediated transactivation.

To further confirm that these effects of EWS were not the result of increased Oct-4 protein levels, cell extracts prepared from 293T cells transfected with Oct-4 expression plasmid in the presence or absence of EWS expression were examined by Western blotting (Fig. 7C). The EGFP expression plasmid served as an internal control for monitoring transfection efficiency (bottom panel). Fractionated cell extracts probed by anti–Oct-4 antibody demonstrated no increase in exogenously expressed Oct-4 protein (top panel).

To dissect how EWS activates Oct-4–mediated transcription, we created fusion proteins in which the GAL4 DNA-binding domain was fused to NTD (aa 1-295) or CTD (aa 290-656) domains of EWS, respectively (Fig. 7D). The pG5 luc reporter contains five GAL4 DNA-binding sites upstream of the TATA box and was used as a reporter in these experiments. An expression vector driving the synthesis of only the GAL4 DNA-binding domain, pcDNA3/GAL4, had no effect on the level of luciferase produced from pG5 luc when transfected into 293T cells (Fig. 7D, lane 1). Interestingly, pcDNA3/GAL4-EWS (1-295) strongly activated luciferase production from pG5 luc by 150-fold (lane 2), indicating that the NTD of EWS protein has intrinsic transcription activation property. On the other hand, EWS (290-656) (lane 3) was not a potent transactivator when fused to the DBD of GAL4. Furthermore, pcDNA3/EWS (1-295), which lacks a GAL4 DNA-binding domain, did not activate luciferase expression from pG5 luc (J.L. and J.K., data not shown). This suggests the need for the

NTD of EWS to bind to the reporter construct to achieve activation of transcription. These results demonstrate that the NTD of EWS is capable of activating transcription. In sum, these results suggest that EWS specifically activates Oct-4–mediated transcriptional activation through its physical interaction with Oct-4.

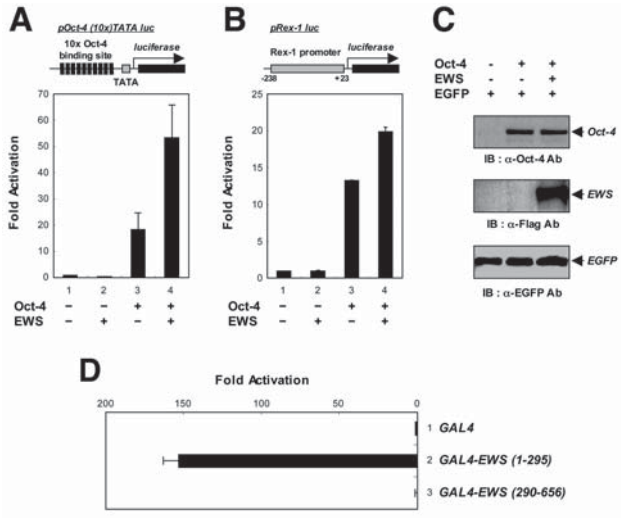

Figure 7. Functional influence of transactivation activity of Oct-4 by Ewing's sarcoma protein (EWS). **(A):** Stimulation of Oct-4–mediated transactivation by EWS. Schematic representation of reporter plasmid, pOct-4(10x)TATA luc, for Oct-4 is shown on top. The 10 copies of Oct-4 recognition sites are indicated by 10 solid bars, the TATA box is presented by a shaded box, and the luciferase gene is indicated by a solid box. Oct-4 0.25 μg (bars 3 and 4) or empty vector plasmid (bars 1 and 2) were cotransfected with 0.75 μg of empty vector (bars 1 and 3) or EWS expression plasmid (bars 2 and 4) into HEK293T cells. After 48 hours, the cells were harvested and luciferase assays were performed. The average of the two independent experiments is presented, and the error bars are shown. **(B):** Potentiation of the Oct-4–dependent *Rex-1* promoter activity by EWS. Schematic representation of human Rex-1 promoter reporter plasmid, pRex-1 luc, for Oct-4 is shown on top. This reporter plasmid contains genomic DNA sequence for human *Rex-1* from nucleotides –238 to +23. The human Rex-1 promoter region is indicated by a shaded box, whereas the luciferase gene is indicated by a solid box. Oct-4 4 μg (bars 3 and 4) or empty vector plasmid (bars 1 and 2) were cotransfected with 12 μg of empty vector (bars 1 and 3) or EWS expression plasmid (bars 2 and 4) into HEK293T cells. After 48 hours, the cells were harvested and luciferase assays were performed. The average of the two independent experiments is presented, and the error bars are shown. **(C):** Western blot analysis of Oct-4 levels in transfected cell extracts to confirm that an equal amount of the exogenous Oct-4 is expressed irrespective of EWS overexpression. HEK293T cells were transfected with expression plasmids for Oct-4 in combination with EWS. At 48 hours after transfection, the cells were harvested and extracted. A portion of each cell extract was separated by SDS-PAGE and immunoblotted with anti–Oct-4 (top panel), anti-EWS (middle panel), or anti-EGFP (bottom panel) antibodies as indicated. The pEGFP-N1 vector was included as a control to determine transfection efficiency. **(D):** Transactivation potential of EWS (1-295). The reporter plasmid, 5x Gal4 TATA luc, was cotransfected with the indicated EWS deletion mutants into HEK293T cells. Luciferase activity was expressed as fold activation relative to the basal level observed with the reporter plasmid and the GAL4 DNA-binding domain alone (lane 1). The average of two independent experiments is presented. Abbreviations: Ab, antibody; IB, immunoblot.

Discussion

In this study, we report that the transactivation properties of Oct-4 can be modulated by the EWS. The relation between embryonic genes and human cancer has drawn great interest, because there is interconnection among the processes of cell growth, differentiation, and tumorigenesis. It has been demonstrated that several genes responsible for developmental alterations have been incontrovertibly linked to human malignancy [60]. Furthermore, preimplantation of embryonic cells has been found to show similarity in phenotype to cancer cells. Interestingly, both types of cell undergo deprogramming to a proliferative stem cell state and become potentially immortal and invasive [60–62]. Although Oct-4 is normally expressed in germ cells and is required for maintaining their pluripotency, it can also promote tumorigenesis when expressed inappropriately in these same cells [23]. Recent studies suggest that Oct-4 plays a part in human cancer development, including testicular germ cell tumor and breast carcinoma [23, 24, 28–30]. At the cell level, introduction of Oct-4 into Swiss 3T3 cells has been found to cause their tumorigenic transformation and endows tumor formation in the nude mouse [23], indicating the possibility that aberrant activity of Oct-4 may contribute to the neoplastic process in vivo.

Oct-4 encodes a POU transcription factor that is expressed by all pluripotent cells during embryogenesis and is also abundantly expressed by ES, embryonic germ, and EC cell lines [17, 56, 59, 63, 64]. Differentiation of totipotent cells to somatic lineages occurs at the blastocyst stage and during gastrulation, coinciding with Oct-4 downregulation. The unique *Oct-4* expression pattern in the mouse embryo led to the hypothesis on the totipotent cycle [65]. Consistent with this speculation, *Oct-4*–null embryos die at the time of implantation due to a failure to form the ICM [20]. These results demonstrate that Oct-4 is required for preventing somatic differentiation of the ICM and is important for maintaining the undifferentiated state during embryonic development. Despite the fact that Oct-4 function is critical for controlling totipotency of ES cells and promoting tumorigenesis in human tissues, there is still little known about the detailed molecular mechanisms and the proteins that regulate Oct-4 function.

Thus, in search of proteins that can regulate Oct-4 function, we set up a bacterial two-hybrid screening system [55]. Using a mouse ES cell cDNA library, five positive clones were isolated and further characterized by DNA sequencing. A BLAST search indicated that we isolated a cDNA encoding of EWS. EWS is a putative proto-oncogene [37], coactivator [44, 45], and RNA-binding protein [37] originally identified as a fusion protein with Fli-1 in Ewing's sarcoma [37] and later found in several additional malignancies in which its NTD is fused to the DNA-binding

domains of several DNA-binding proteins such as ERG, ETV1, E1A-F, FEV, WT1, ATF1, CHOP, and TEC [39]. The expression of both pBT–Oct-4 and pTRG-EWS in a bacterial two-hybrid system allowed growth on a +Kan/+Chl/+Tet LB plate containing carbenicillin, indicating a physical interaction between Oct-4 and EWS in bacterial cells (Fig. 1). In addition, several lines of evidence indicate that Oct-4 and EWS interact within the cell: *Oct-4* mRNA is coexpressed with *EWS* in self-renewal ES and EC cells that we have examined (Fig. 2); Oct-4 associates with EWS in vitro, indicating that they can interact directly (Figs. 3A, 3B, 3E); and Oct-4 colocalizes with EWS in the nucleus (Fig. 6) and can be immunoprecipitated with EWS in vivo (Figs. 3C, 3D), suggesting that the interaction between Oct-4 and EWS occurs under physiological conditions.

EWS shares an extensive sequence similarity with a subgroup of TLS and $hTAF_{II}68$, which are two human nuclear RNA-binding proto-oncoproteins that are products of genes commonly translocated in human sarcomas [39, 66]. These proteins have been found associated with TFIID complexes and copurified with the human RNA polymerase II, suggesting the involvement of transcriptional activation [66]. TFIID is a multiprotein complex composed of the TATA-binding protein (TBP) and TBP-associated factors ($TAF_{II}s$) and is the factor that nucleates preinitiation complex formation on protein-coding genes [67]. The TAFs in TFIID are essential for activator-dependent transcription.

The EWS NTD shows unique structural features. It contains large numbers of tyrosine, glutamine, alanine, serine, threonine, glycine, and proline residues, some of which are organized in a repeated and degenerated peptide motif with a frequently occurring serine-tyrosine dipeptide (NSYGQQS) [37]. A database search revealed that it shares homology with the CTD of the large subunit of eukaryotic RNA polymerase II [37]. This subunit was efficiently phosphorylated on tyrosine residues by c-Abl tyrosine kinase, and the sites of phosphorylation were found to be within the CTD of the RNA polymerase II [68]. The consequence of this phosphorylation event on RNA polymerase II is to convert transcriptionally paused complexes into elongation-competent molecules [69]. Thus, it will be important to assess the tyrosine phosphorylation status of EWS by c-Abl in ES and EC cells or human testicular germ cell tumors, because it shares homology to the C-terminal region of eukaryotic RNA polymerase II [37] and EWS/WT1, one of EWS-fusion forms, is also a target for c-Abl [52].

We have mapped the interacting domains and demonstrated that Oct-4 binds with the EWS through its POU domain (Fig. 4). Additionally, three independent regions in the EWS, encompassing amino acids 70 through 163 in the NTD, amino acids 290 through 350, and amino acids 441 through 656 in the CTD of EWS, seem important for binding to Oct-4 (Fig. 5). There are several possible models suggested by these data. One is that single EWS binds to three Oct-4 molecules. Another is that three binding sites of EWS have different binding affinities, and there may be a dynamic interaction with Oct-4 in which Oct-4 binds to one site, after which it is transferred to another site during the activation. Lastly, Oct-4–related molecules can bind to some of the other sites through the POU domain. However, additional experiments will be required to distinguish between these possibilities.

Because EWS does not have a DNA-binding motif or significant DNA-binding activity to specific gene promoters, it is suggested that EWS acts as a coactivator of transcription if it is recruited to the vicinity of the promoter. Thus, to confirm this speculation, we investigated the functional consequences of the interaction between Oct-4 and EWS in transient transfection assays. As shown in Figures 7A and 7B, ectopic expression of EWS stimulates Oct-4–dependent transcriptional activation. Association of the EWS to Oct-4, mediated by their binding domains, may stably tether EWS to DNA and increase the activity of intrinsic activation domains within EWS. EWS and/or Oct-4 and Oct-4 and DNA interactions could also lead to reciprocal conformation changes that expose latent activation domains within EWS.

However, unlike intensively studied coactivators, such as p300 and CBP [70–72], the precise transactivation mechanism of EWS as a coactivator has yet to be demonstrated. In light of characterization of other coactivator proteins, there are several possible models for EWS. First, EWS may promote Oct-4–mediated gene expression by bridging between Oct-4 and the basal transcription machinery. It has been recently reported that EWS associates with important factors for transcription, such as a basal transcription factor TFIID [41, 42], a transcriptional coactivator CBP [44, 45], and the hypophosphorylated RNA polymerase II form [44]. Second, EWS may recruit transcription machinery–modifying enzymes necessary for transcription initiation or elongation steps. Protein tyrosine kinase c-Abl efficiently phosphorylates on tyrosine residues of the C-terminal domain of eukaryotic RNA polymerase II [68], and the consequence of this phosphorylation event on RNA polymerase II is to convert transcriptionally paused complexes into elongation-competent molecules [69]. Interestingly, the NTD of EWS contains two putative c-Abl SH3 domain binding sites [52]. Third, EWS may act as an adaptor molecule linking gene transcription and mRNA processing by interacting with splicing factors. It has been reported that transcriptional and post-transcriptional processing are closely coupled events in vivo [73], and, based on the structural features, it is likely that EWS is most likely to become involved in mRNA synthesis and splicing. Consistent with this speculation, EWS also interacts with the several splicing factors SF1 [74], U1C [75], TARS-1/TRSR-2 translocation liposarcoma protein–associated serine-arginine protein [76], and Y-box protein-1 [77].

Transcriptional coactivator proteins operate at the end points of a variety of signal transduction pathways, thereby modulating specific gene expression programs involved in cell growth, differentiation, homeostasis, and viral pathogenesis [78, 79]. We

have reported that EWS/WT1 associates with several signaling proteins, such as SH3 domains of v-Src, c-Abl, and Crk(N) and SH2 domains of v-Src, Grb2, Fyn, and GAP(N+C) [47]. Although it is not clear whether EWS itself interacts with signal-dependent transcription factors, the ability of EWS-fusion protein to interact with several cell-signaling molecules and to modulate its function by signaling proteins (47, 52) suggests that EWS may function as a signal integrator by coordinating complex signal transduction events at the transcriptional level. In fact, CBP and p300 have been proposed to mediate transcription induction via intrinsic and associated histone acetytransferase activities, which may facilitate binding of nuclear factors to their target sites by destabilizing promoter-bound nucleosomes [80]. Thus, it would be valuable to test whether EWS also has these types of activity.

In conclusion, the findings of this study provide additional evidence that Oct-4 function can be modulated by EWS. Oct-4 likely contributes to maintaining cells in an undifferentiated, pluripotent, or totipotent state in two ways: by activating certain key genes [48, 81–83] and by silencing others [12, 14, 84]. This EWS-mediated Oct-4 regulation may be a key mechanism that can stimulate cells of the ICM from expressing products that prevent differentiation toward the trophectoderm. In addition, it would be interesting to determine whether EWS can collaborate with Oct-4 to transform cells in human testicular germ cell tumor.

Acknowledgments

This research was supported by a grant (SC2090) from the Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea. J.K. is also supported by the Advanced Basic Research Laboratory Program (R14-2002-012-01002-0) of the KOSEF. We thank Dr. Jerry Pelletier for helpful comments and a critical reading of the manuscript and Dong Hwa Yang for help with luciferase assays.

References

1 Scholer HR, Dressler GR, Balling R et al. Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. EMBO J 1990;9:2185–2195.

2 Okamoto K, Okazawa H, Okuda A et al. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 1990;60:461–472.

3 Rosner MH, Vigano MA, Ozato K et al. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 1990;345:686–692.

4 Goto T, Adjaye J, Rodeck CH et al. Identification of genes expressed in human primordial germ cells at the time of entry of the female germ line into meiosis. Mol Hum Reprod 1999;5:851–860.

5 Hansis C, Grifo JA, Krey LC. Oct-4 expression in inner cell mass and trophectoderm of human blastocysts. Mol Hum Reprod 2000;6:999–1004.

6 Burdon T, Smith A, Savatier P. Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol 2002;12:432–438.

7 Herr W, Cleary MA. The POU domain: versatility in transcriptional regulation by a flexible two-in-one DNA-binding domain. Genes Dev 1995;9:1679–1693.

8 Lamb KA, Rizzino A. Effects of differentiation on the transcriptional regulation of the FGF-4 gene: critical roles played by a distal enhancer. Mol Reprod Dev 1998;51:218–224.

9 Kraft HJ, Mosselman S, Smits HA et al. Oct-4 regulates alternative platelet-derived growth factor alpha receptor gene promoter in human embryonal carcinoma cells. J Biol Chem 1996;271:12873–12878.

10 Ambrosetti DC, Basilico C, Dailey L. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. Mol Cell Biol 1997;17:6321–6329.

11 Vigano MA, Staudt LM. Transcriptional activation by Oct-3: evidence for a specific role of the POU-specific domain in mediating functional interaction with Oct-1. Nucleic Acids Res 1996;24:2112–2118.

12 Ezashi T, Ghosh D, Roberts RM. Repression of Ets-2-induced transactivation of the tau interferon promoter by Oct-4. Mol Cell Biol 2001;21:7883–7891.

13 Liu L, Leaman D, Villalta M et al. Silencing of the gene for the alpha-subunit of human chorionic gonadotropin by the embryonic transcription factor Oct-3/4. Mol Endocrinol 1997;11:1651–1658.

14 Liu L, Roberts RM. Silencing of the gene for the beta subunit of human chorionic gonadotropin by the embryonic transcription factor Oct-3/4. J Biol Chem 1996;271:16683–16689.

15 Pesce M, Scholer HR. Oct-4: gatekeeper in the beginnings of mammalian development. STEM CELLS 2001;19:271–278.

16 Ovitt CE, Scholer HR. The molecular biology of Oct-4 in the early mouse embryo. Mol Hum Reprod 1998;4:1021–1031.

17 Scholer HR, Ruppert S, Suzuki N et al. New type of POU domain in germ line-specific protein Oct-4. Nature 1990;344:435–439.

18 Yeom YI, Ha HS, Balling R et al. Structure, expression and chromosomal location of the Oct-4 gene. Mech Dev 1991;35:171–179.

19 Pesce M, Wang X, Wolgemuth DJ et al. Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. Mech Dev 1998;71:89–98.

20 Nichols J, Zevnik B, Anastassiadis K et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 1998;95:379–391.

21 Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 2000;24:372–376.

22 Abdel-Rahman B, Fiddler M, Rappolee D et al. Expression of transcription regulating genes in human preimplantation embryos. Hum Reprod 1995;10:2787–2792.

23 Gidekel S, Pizov G, Bergman Y et al. Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell 2003;4:361–370.

24 Looijenga LH, Stoop H, de Leeuw HP et al. POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. Cancer Res 2003;63:2244–2250.

25 Hellerstedt BA, Pienta KJ. Germ cell tumors: review of selected studies from 2002. Curr Opin Oncol 2003;15:234–238.

26 Oliver RT. Germ cell cancer. Curr Opin Oncol 1999;11:236–241.

27 Palumbo C, van Roozendaal K, Gillis AJ et al. Expression of the PDGF alpha-receptor 1.5 kb transcript, OCT-4, and c-KIT in human normal and malignant tissues: implications for the early diagnosis of testicular germ cell tumours and for our understanding of regulatory mechanisms. J Pathol 2002;196:467–477.

28 Monk M, Holding C. Human embryonic genes re-expressed in cancer cells. Oncogene 2001;20:8085–8091.

29 Wang P, Branch DR, Bali M et al. The POU homeodomain protein OCT3 as a potential transcriptional activator for fibroblast growth factor-4 (FGF-4) in human breast cancer cells. Biochem J 2003;375(pt 1):199–205.

30 Jin T, Branch DR, Zhang X et al. Examination of POU homeobox gene expression in human breast cancer cells. Int J Cancer 1999;81:104–112.

31 Brehm A, Ohbo K, Scholer H. The carboxy-terminal transactivation domain of Oct-4 acquires cell specificity through the POU domain. Mol Cell Biol 1997;17:154–162.

32 Scholer HR. Octamania: the POU factors in murine development. Trends Genet 1991;7:323–329.

33 Okamoto K, Okazawa H, Okuda A et al. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 1990;60:461–472.

34 Scholer HR, Balling R, Hatzopoulos AK et al. Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. EMBO J 1989;8:2551–2557.

35 Scholer HR, Ciesiolka T, Gruss P. A nexus between Oct-4 and E1A: implications for gene regulation in embryonic stem cells. Cell 1991;66:291–304.

36 Brehm A, Ohbo K, Zwerschke W et al. Synergism with germ line transcription factor Oct-4: viral oncoproteins share the ability to mimic a stem cell-specific activity. Mol Cell Biol 1999;19:2635–2643.

37 Delattre O, Zucman J, Plougastel B et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 1992;359:162–165.

38 Ladanyi M. The emerging molecular genetics of sarcoma translocations. Diagn Mol Pathol 1995;4:162–173.

39 Kim J, Pelletier J. Molecular genetics of chromosome translocations involving EWS and related family members. Physiol Genomics 1999;1:127–138.

40 Arvand A, Denny CT. Biology of EWS/ETS fusions in Ewing's family tumors. Oncogene 2001;20:5747–5754.

41 Bertolotti A, Melot T, Acker J et al. EWS, but not EWS-FLI-1, is associated with both TFIID and RNA polymerase II: interactions between two members of the TET family, EWS and hTAFII68, and subunits of TFIID and RNA polymerase II complexes. Mol Cell Biol 1998;18:1489–1497.

42 Petermann R, Mossier BM, Aryee DN et al. Oncogenic EWS-Fli1 interacts with hsRPB7, a subunit of human RNA polymerase II. Oncogene 1998;17:603–610.

43 Immanuel D, Zinszner H, Ron D. Association of SARFH (sarcoma-associated RNA-binding fly homolog) with regions of chromatin transcribed by RNA polymerase II. Mol Cell Biol 1995;15:4562–4571.

44 Araya N, Hirota K, Shimamoto Y et al. Cooperative interaction of EWS with CREB-binding protein selectively activates hepatocyte nuclear factor 4-mediated transcription. J Biol Chem 2003;278:5427–5432.

45 Rossow KL, Janknecht R. The Ewing's sarcoma gene product functions as a transcriptional activator. Cancer Res 2001;61:2690–2695.

46 Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual, 3rd Ed. New York: Cold Spring Harbor Laboratory Press, 2001.

47 Kim J, Lee JM, Branton PE et al. Modulation of EWS/WT1 activity by the v-Src protein tyrosine kinase. FEBS Lett 2000;474:121–128.

48 Botquin V, Hess H, Fuhrmann G et al. New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2. Genes Dev 1998;12:2073–2090.

49 Hosler BA, Rogers MB, Kozak CA et al. An octamer motif contributes to the expression of the retinoic acid-regulated zinc finger gene Rex-1 (Zfp-42) in F9 teratocarcinoma cells. Mol Cell Biol 1993;13:2919–2928.

50 Lee MH, Williams BO, Mulligan G et al. Targeted disruption of p107: functional overlap between p107 and Rb. Genes Dev 1996;10:1621–1632.

51 Park JH, Kim SJ, Oh EJ et al. Establishment and maintenance of human embryonic stem cells on STO, a permanently growing cell line. Biol Reprod 2003;69:2007–2014.

52 Kim J, Lee JM, Branton PE et al. Modification of EWS/WT1 functional properties by phosphorylation. Proc Natl Acad Sci U S A 1999;96:14300–14305.

53 Kim J, Lee K, Pelletier J. The desmoplastic small round cell tumor t(11;22) translocation produces EWS/WT1 isoforms with differing oncogenic properties. Oncogene 1998;16:1973–1979.

54 Lee HJ, Kim S, Pelletier J et al. Stimulation of hTAFII68 (NTD)-mediated transactivation by v-Src. FEBS Lett 2004;564:188–198.

55 Dove SL. Studying protein-protein interactions using a bacterial two-hybrid system. Methods Mol Biol 2003;205:251–265.

56 Palmieri SL, Peter W, Hess H et al. Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. Dev Biol 1994;166:259–267.

57 Aman P, Panagopoulos I, Lassen C et al. Expression patterns of the human sarcoma-associated genes FUS and EWS and the genomic structure of FUS. Genomics 1996;37:1–8.

58 Scholer HR, Hatzopoulos AK, Balling R et al. A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. EMBO J 1989;8:2543–2550.

59 Okamoto K, Okazawa H, Okuda A et al. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 1990;60:461–472.

60 Calvo M, Artuch R, Macia E et al. Diagnostic approach to inborn errors of metabolism in an emergency unit. Pediatr Emerg Care 2000;16:405–408.

61 Fujimoto T, Miyayama Y, Fuyuta M. The origin, migration and fine morphology of human primordial germ cells. Anat Rec 1977;188:315–330.

62 Cillo C, Cantile M, Faiella A et al. Homeobox genes in normal and malignant cells. J Cell Physiol 2001;188:161–169.

63 Rosner MH, Vigano MA, Ozato K et al. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 1990;345:686–692.

64 Yeom YI, Fuhrmann G, Ovitt CE et al. Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 1996;122:881–894.

65 Pesce M, Gross MK, Scholer HR. In line with our ancestors: Oct-4 and the mammalian germ. Bioessays 1998;20:722–732.

66 Bertolotti A, Lutz Y, Heard DJ et al. hTAF(II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins TLS/FUS and

EWS is associated with both TFIID and RNA polymerase II. EMBO J 1996;15:5022–5031.

67 Roeder RG. Nuclear RNA polymerases: role of general initiation factors and cofactors in eukaryotic transcription. Methods Enzymol 1996;273:165–171.

68 Baskaran R, Dahmus ME, Wang JY. Tyrosine phosphorylation of mammalian RNA polymerase II carboxyl-terminal domain. Proc Natl Acad Sci U S A 1993;90:11167–11171.

69 O'Brien T, Hardin S, Greenleaf A et al. Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation. Nature 1994;370:75–77.

70 Janknecht R. The versatile functions of the transcriptional coactivators p300 and CBP and their roles in disease. Histol Histopathol 2002;17:657–668.

71 Chan HM, La Thangue NB. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J Cell Sci 2001;114(pt 13):2363–2373.

72 Goodman RH, Smolik S. CBP/p300 in cell growth, transformation, and development. Genes Dev 2000;14:1553–1577.

73 Bentley D. Coupling RNA polymerase II transcription with pre-mRNA processing. Curr Opin Cell Biol 1999;11:347–351.

74 Zhang D, Paley AJ, Childs G. The transcriptional repressor ZFM1 interacts with and modulates the ability of EWS to activate transcription. J Biol Chem 1998;273:18086–18091.

75 Knoop LL, Baker SJ. The splicing factor U1C represses EWS/FLI-mediated transactivation. J Biol Chem 2000;275:24865–24871.

76 Yang L, Chansky HA, Hickstein DD. EWS.Fli-1 fusion protein interacts with hyperphosphorylated RNA polymerase II and interferes with serine-arginine protein-mediated RNA splicing. J Biol Chem 2000;275:37612–37618.

77 Chansky HA, Hu M, Hickstein DD et al. Oncogenic TLS/ERG and EWS/Fli-1 fusion proteins inhibit RNA splicing mediated by YB-1 protein. Cancer Res 2001;61:3586–3590.

78 Janknecht R, Hunter T. Transcription: a growing coactivator network. Nature 1996;383:22–23.

79 Janknecht R, Hunter T. Nuclear fusion of signaling pathways. Science 1999;284:443–444.

80 Vo N, Goodman RH. CREB-binding protein and p300 in transcriptional regulation. J Biol Chem 2001;276:13505–13508.

81 Ovitt CE, Scholer HR. The molecular biology of Oct-4 in the early mouse embryo. Mol Hum Reprod 1998;4:1021–1031.

82 Rosfjord E, Rizzino A. The octamer motif present in the Rex-1 promoter binds Oct-1 and Oct-3 expressed by EC cells and ES cells. Biochem Biophys Res Commun 1994;203:1795–1802.

83 Yuan H, Corbi N, Basilico C et al. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev 1995;9:2635–2645.

84 Liu L, Leaman D, Villalta M et al. Silencing of the gene for the alpha-subunit of human chorionic gonadotropin by the embryonic transcription factor Oct-3/4. Mol Endocrinol 1997;11:1651–1658.