

The *EWS–Oct-4* fusion gene encodes a transforming gene

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The t(6;22)(p21;q12) translocation associated with human bone and soft-tissue tumours results in a chimaeric molecule fusing the NTD (N-terminal domain) of the *EWS* (Ewing's sarcoma) gene to the CTD (C-terminal domain) of the *Oct-4* (octamer-4) embryonic gene. Since the N-terminal domains of *EWS* and *Oct-4* are structurally different, in the present study we have assessed the functional consequences of the *EWS–Oct-4* fusion. We find that this chimaeric gene encodes a nuclear protein which binds DNA with the same sequence specificity as the parental *Oct-4* protein. Comparison of the transactivation properties of *EWS–Oct-4* and *Oct-4* indicates that the former has higher transactivation activity for a known target reporter gene containing *Oct-4* binding. Deletion analysis of the functional domains of *EWS–Oct-4* indicates that the *EWS* (NTD), the POU domain and the CTD of

EWS–Oct-4 are necessary for full transactivation potential. *EWS–Oct-4* induced the expression of *fgf-4* (fibroblast growth factor 4) and *nanog*, which are potent mitogens as well as *Oct-4* downstream target genes whose promoters contain potential *Oct-4*-binding sites. Finally, ectopic expression of *EWS–Oct-4* in *Oct-4*-null ZHBTc4 ES (embryonic stem) cells resulted in increased tumorigenic growth potential in nude mice. These results suggest that the oncogenic effect of the t(6;22) translocation is due to the *EWS–Oct-4* chimaeric protein and that fusion of the *EWS* NTD to the *Oct-4* DNA-binding domain produces a transforming chimaeric product.

Key words: bone and soft-tissue tumour, chromosomal translocation, *EWS–Oct-4* fusion protein, transactivation, transformation.

INTRODUCTION

Cancers arise as a result of a series of mutations, the most obvious of which are chromosomal abnormalities. Chromosomal translocations cause cancer by activating existing genes or creating new fusion proteins [1]. Recently, some bone and soft-tissue tumours have been shown to harbour a translocation t(6;22)(p21;q12) involving the *EWS* (Ewing's sarcoma) gene at 22q12 and the *Oct-4* (octamer-4) gene at 6p21 [2]. Striking features of this tumour are the diffuse proliferation pattern of the undifferentiated tumour cells and the positive immunoreactivity for vimentin, S-100 and neuron-specific enolase. An approx. 1.8-kb chimaeric transcript was detected by Northern blotting with *EWS* and *Oct-4* probes, and an *EWS–Oct-4* fusion transcript, but not the reciprocal *Oct-4–EWS* fusion, was detected in tumours by RT (reverse transcriptase)-PCR [2].

The *EWS* gene is involved in several tumour-related translocations, generating fusions with genes postulated to function as transcription factors [3]. In each case, the translocation produces chimaeric molecules containing the NTD (N-terminal domain) of *EWS* fused to the DNA-binding domain of the partner. The *EWS* gene encodes a 656-amino-acid protein that contains three arginine- and glycine-rich tracts and an 85-amino-acid RNA recognition motif at its C-terminus. The NTD (amino acids 1–285) of the *EWS* gene is composed almost exclusively (~90%) of tyrosine, glycine, alanine, serine, threonine and proline residues organized in a repeated and degenerate polypeptide motif having the consensus, NSYGQQS. This domain has weak homology to the C-terminal region of eukaryotic RNA polymerase II [4].

Oct-4, also referred to as *Oct-3*, is a member of the POU family of transcription factors, which is expressed in pluripotent ES (embryonic stem) cells and germ cells [5–10]. Members of the

POU transcription factor family share the conserved POU DNA-binding domain, originally identified in the transcription factors Pit-1, Oct-1, Oct-2 and Unc-86 [11]. *Oct-4* activates transcription via octamer motifs located proximal or distal to transcriptional start sites. Its binding sites have been found in various genes including *fgf-4* (fibroblast growth factor 4), *pdgfar* (platelet-derived growth factor α receptor), *osteopontin* and *nanog* [12–16]. In addition, genes, such as *IFN- τ* (tau interferon) and those encoding the α and β subunits of hCG (chorionic gonadotropin), which are expressed in the trophoblast but not in embryos prior to blastocyst formation, may be targets for silencing by *Oct-4* [17–19]. This suggests that *Oct-4* functions as a master switch during differentiation by regulating gene expression in pluripotent cells or cells that can develop pluripotent potential [20,21].

It has previously been reported that *Oct-4* is a key player in the genesis of human TGCTs (testicular germ cell tumours) [22,23]. Human TGCTs are the most common malignancy in adolescent and young adult Caucasian males and are the cause of one in seven deaths in this group [24,25]. The *Oct-4* transcript is consistently detected in a specific set of human TGCTs of adolescents and young adults: the seminomas and embryonal carcinomas [26]. In addition, the precursor lesions of human TGCT, known as CIS (testicular carcinoma *in situ*), also express *Oct-4* [26]. 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 it may be implicated in tumorigenesis by up-regulating downstream target genes [22,27–29]. Consistent with these findings, *Oct-4* expression in a heterologous cell system, transformed non-tumorigenic cells and endowed tumorigenicity in nude mice. Activation of *Oct-4* in adult mice using a doxycycline-dependent expression system resulted in dysplastic growth of epithelial tissues that are dependent on continuous *Oct-4* expression [30].

Abbreviations used: CTD, C-terminal domain; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility-shift assay; ES, embryonic stem; *EWS*, Ewing's sarcoma; *fgf-4*, fibroblast growth factor-4; GST, glutathione S-transferase; HEK-293T, human embryonic kidney-293 cell expressing the large T-antigen of simian virus 40; HRP, horseradish peroxidase; NTD, N-terminal domain; *Oct-4*, octamer-4; RT, reverse transcriptase; TGCT, testicular germ cell tumour.

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In comparison with Oct-4, little is known about the function of the *EWS-Oct-4* gene product. As a first step in investigating how EWS-Oct-4 protein contributes to tumorigenesis, in the present study we analysed its transcriptional activation behaviour and oncogenic properties. We found that it is a nuclear protein which binds DNA with a sequence specificity indistinguishable from that of the parental Oct-4 protein. However it has a greatly increased transcriptional activation potential that is dependent on several functional domains. We also demonstrated that EWS-Oct-4 is a dominantly acting oncogene, as measured by activation of oncogenic Oct-4 downstream target genes and tumour formation in nude mice. These results indicate that EWS-Oct-4 may play a critical role in the formation of bone and soft-tissue tumours by activating the transcription of Oct-4 target genes.

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. PfuTurbo polymerase was purchased from Stratagene and [γ - 32 P]ATP (3000 Ci/mmol) was obtained from PerkinElmer. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, bacterial transformations and SDS/PAGE of proteins were carried out using standard methods as previously described [31]. Subclones generated from PCR products were sequenced by the chain termination method with double-stranded DNA templates to ensure the absence of mutations.

Constructs

To construct pcDNA3-EWS-Oct-4, EWS (NTD) was amplified from pSG5/FLAG-EWS by PCR using primers 5'-EWS-1HindIII (5'-GATCAAGCTTATGGCGTCCACGGATTAC-3'; a HindIII site is underlined) and 3'-SpacerEWS (5'-pATAGTGAAACCCACCTGGTAGGAGGGTAGGA-3'). Oct-4 [CTD (C-terminal domain)] was amplified from pcDNA3/hOct-4 by PCR using primers 5'-POU5F1 (5'-pTGAGGCTGGAGAAGGAG-3') and 3'-hOct-4-437 (5'-CTAGGAATTCTCAGTTTGAATGCATGGG-3'; an EcoRI site is underlined). The two PCR products [EWS (NTD) and Oct-4 (CTD)] were ligated, digested with HindIII and EcoRI, and cloned into the same sites of pBluescript II KS vector (Stratagene) to generate pKSII-EWS-Oct-4. To construct pcDNA3-EWS-Oct-4, the HindIII-EcoRI fragment of pKSII-EWS-Oct-4 was subcloned into the same sites of a pcDNA3 vector (Invitrogen).

To generate GST-EWS-Oct-4 (where GST is glutathione S-transferase), pKSII-EWS-Oct-4 was digested with ClaI, repaired with the Klenow fragment, and re-digested with NotI. pGEX(4T-1) vector was also digested with BamHI, repaired with the Klenow fragment, and then re-digested with NotI. The excised EWS-Oct-4 insert was directly ligated with the NotI and blunted BamHI sites of the pGEX(4T-1) vector. The plasmid encoding GST-hOct-4 has been described previously [32].

pcDNA3/FLAG-EWS-Oct-4 deletion mutants were generated using the following steps. The synthetic FLAG insert, which was generated by annealing the oligonucleotides 5'-HindIII-FLAG-HindIII (5'-pAGCTTATGGATTACAAGGATGACGACGATAAGA-3') and 3'-HindIII-FLAG-HindIII (5'-pAGCTTCTATCGTCGTCATCCTTGTAATCCATA-3'), was cloned into the HindIII site of pKSII/EWS-Oct-4 to generate pKSII/FLAG-EWS-Oct-4 (2xHindIIIplusMet). To generate pKSII/FLAG-EWS-Oct-4, deletions of the second HindIII site and the

first methionine residue at the N-terminus of EWS-Oct-4 were introduced by site-directed mutagenesis using the mutagenesis primer set 5'-Flag-9 (5'-CAAGGATGACGACGATAAGGCGTCCACGGATTACAGTAC-3') and 3'-EWS-23 (5'-GTACTGTAATCCGTGGACGCCTTATCGTCGTCATCCTTG-3') and the QuikChange kitTM (Stratagene). For pcDNA3/FLAG-EWS-Oct-4, plasmid pKSII/FLAG-EWS-Oct-4 was digested with HindIII and EcoRI and cloned into the same sites of pcDNA3. For pcDNA3/FLAG-EWS-Oct-4 (Δ EWS), a deletion of amino acids 1-193 was introduced by site-directed mutagenesis using the mutagenic primer set 5'-EWS-Oct-4 Δ EWS (5'-AAGGATGACGACGATAAGAGGTGGGGTTCCTACTATTG-3') and 3'-EWS-Oct-4 Δ EWS (5'-CAATAGTGAACCCACCTCTTATCGTCGTCATCCTTG-3') and the QuikChange kitTM to generate pKSII/FLAG-EWS-Oct-4 (Δ EWS). Then, the FLAG-EWS-Oct-4 (Δ EWS) fragment of pKSII/FLAG-EWS-Oct-4 (Δ EWS) was digested with HindIII and EcoRI and cloned into the same sites of pcDNA3. For pcDNA3/FLAG-EWS-Oct-4 (Δ Ext), a deletion of a further six amino acids (amino acids 194-199) was introduced by site-directed mutagenesis using the mutagenic primer set 5'-EWS-Oct-4 Δ Ext (5'-CCATCCTACCTCCTACCAAGCTGGAGAAGGAGAAG-3') and 3'-EWS-Oct-4 Δ Ext (5'-CTTCTCCTTCTCCAGCTTGGTAGGAGGGTAGGATGG-3') and the QuikChange kitTM to generate pKSII/FLAG-EWS-Oct-4 (Δ Ext). Then, the FLAG-EWS-Oct-4 (Δ Ext) fragment of the pKSII/FLAG-EWS-Oct-4 (Δ Ext) construct was digested with HindIII and EcoRI and cloned into the same sites of pcDNA3. For pcDNA3/FLAG-EWS-Oct-4 (Δ NTD), a deletion of Oct-4 (NTD) (amino acids 200-210) was introduced by site-directed mutagenesis using the mutagenic primer set 5'-DeltaOct-4NTD (5'-AGGTGGGGTTCCTACTATTGGAGGAGTCCCAGGACATC-3') and 3'-DeltaOct-4NTD (5'-GATGTCTGGACTCCTCCAATAGTGAACCCACCTG-3') to generate pKSII/FLAG-EWS-Oct-4 (Δ NTD). Then, the FLAG-EWS-Oct-4 (Δ NTD) fragment of the pKSII/FLAG-EWS-Oct-4 (Δ NTD) construct was digested with HindIII and EcoRI and cloned into the same sites of pcDNA3. For pcDNA3/FLAG-EWS-Oct-4 (Δ POU), a deletion of Oct-4 (POU) (amino acids 211-366) was introduced by site-directed mutagenesis using the mutagenic primer set 5'-E/OdPOU-613 (5'-AAGCTGGAGCAAAACCCGAGCGACTATGCACAACGAG-3') and 3'-E/OdPOU-1116 (5'-TCGTTGTGCATAGTCGCTCGGGTTTTGCTCCAGCTTC-3') to generate pKSII/FLAG-EWS-Oct-4 (Δ POU). Then, the FLAG-EWS-Oct-4 (Δ POU) fragment of the pKSII/FLAG-EWS-Oct-4 (POU) construct was digested with HindIII and EcoRI and cloned into the same sites of pcDNA3. For pcDNA3/FLAG-EWS-Oct-4 (Δ CTD), the FLAG-EWS-Oct-4 (Δ CTD) was amplified from pKSII/FLAG-EWS-Oct-4 by PCR using primers 5'-FLAG(-6)BamHI (5'-GATCGGATCCGCCACCATGGATTACAAG-3'; a BamHI site is underlined) and 3'-hOct4-867EcoRI (5'-GATCGAATTTCGCTTGATCGCTTGCCCTT-3'; an EcoRI site is underlined), digested with BamHI and EcoRI, and cloned into the same sites of pcDNA3. For pcDNA3/FLAG-EWS-Oct-4 (V351P), a missense mutation of POU (V351P) was introduced by site-directed mutagenesis using the mutagenic primer set 5'-hOct4 V274P (5'-GAAGGATGTGCCCCGAGTGTGG-3') and 3'-hOct4 V274P (5'-CCACACTCGGGGCACATCCTTC-3') to generate pKSII/FLAG-EWS-Oct-4 (V351P). Then, the FLAG-EWS-Oct-4 (V351P) fragment of the pKSII/FLAG-EWS-Oct-4 (V351P) construct was digested with HindIII and EcoRI and cloned into the same sites of pcDNA3.

The construct pCAG-IP/EGFP (enhanced green fluorescent protein) has been described previously [32]. To construct pCAG-IP/EWS-Oct-4-EGFP, EWS-Oct-4 was amplified from pcDNA3/FLAG-EWS-Oct-4 by PCR using primers

5'-BamHIFlagE-O (5'-GATCGGATCCATGGATTACAAGGATGAC-3'; a BamHI site is underlined) and 3'-hOct4CTDGFP (5'-GATCGGATCCGCTCCGTTTGAATGCATGGG-3'; a BamHI site is underlined). The PCR product was digested with BamHI and cloned into the same sites of the pEGFP(N1) vector (Clontech) to generate pEGFP(N1)-EWS–Oct-4. To construct pCAG-IP/EWS–Oct-4, the SalI and NotI fragment of pEGFP(N1)-EWS–Oct-4 was subcloned into the XhoI and NotI sites of the pCAG-IP vector.

Expression and purification of GST fusion proteins

GST–EWS–Oct-4 and GST–hOct-4 proteins were expressed in *Escherichia coli* as described previously [32]. After binding to glutathione-Sepharose and washing, the proteins were eluted with reduced glutathione (Sigma). Protein concentrations were determined using the Bradford method (Bio-Rad). The purity and size of the eluted proteins were evaluated by Coomassie Blue staining of SDS/PAGE.

EMSAs (electrophoretic mobility-shift assays)

Probes for EMSAs were prepared from synthetic oligonucleotides whose sequences have been described previously [32]. The probe was prepared by end-labelling annealed complementary oligonucleotides with [γ -³²P]ATP using T4 polynucleotide kinase. DNA-binding reactions were performed with the recombinant GST-fusion EWS–Oct-4 and hOct-4 proteins for 30 min at 4°C in binding buffer [20 mM Hepes (pH 7.4), 40 mM KCl, 1 mM MgCl₂, 0.1 mM EDTA, 0.1% Nonidet P40 and 0.2 μ g of poly (dI-dC)-(dI-dC)]. Following binding, the reaction mixtures were run on 4% polyacrylamide gels (37:1 acrylamide/bisacrylamide) in 0.5 \times TBE (44.5 mM Tris/HCl, 44.5 mM boric acid and 1 mM EDTA, pH 8.0) at 150 V for 2–3 h at 4°C. The gels were dried and exposed to Kodak X-Omat film at –70°C with an intensifying screen.

Subcellular localization

Immunocytochemical analyses were performed as previously described [33]. Briefly, COS-7 cells were plated on glass coverslips and transfected with the respective DNA plasmids using VivaMagic Reagent (Vivagen) or FuGene™ (Roche Diagnostic). After 48 h, the cells were washed in PBS and fixed for 10 min at –20°C in a mixture of acetone and methanol (1:1, v/v). To detect EWS–Oct-4 or hOct-4, we used an anti-Oct-4 antibody (C-20; Santa Cruz Biotechnology) and a HRP (horseradish peroxidase)-conjugated secondary antibody (Santa Cruz Biotechnology), and fluorescence was detected with a fluorescence microscope (Olympus, IX51) equipped with a CoolSNAP digital camera (Olympus).

Reporter gene assays

Cells were transiently transfected with plasmids by electroporation using the Gene Pulser II RF module system (Bio-Rad). Luciferase assays were performed with the Dual-Luciferase assay system (Promega). *Renilla* luciferase activities were used to normalize transfection efficiencies.

Establishment of ZHBTc4 ES cell line expressing EWS–Oct-4

To generate stably expressing ES cell lines, pCAG-IP/EGFP and pCAG-IP/EWS–Oct-4–EGFP were linearized with PvuI and 20 μ g of each was transfected into ZHBTc4 ES cells (1×10^7)

using the Gene Pulser II RF module system (Bio-Rad). At 48 h post-electroporation, puromycin (Sigma) was added to a final concentration of 1 μ g/ml to select clones carrying stably integrated plasmid DNA. After selection of transfected ZHBTc4 cells, monoclonal cell lines were isolated by picking individual puromycin-resistant colonies.

RT-PCR

Total RNA was prepared from ZHBTc4 ES cells expressing EWS–Oct-4 or vector alone using an RNeasy mini kit (Qiagen) with on-column DNase treatment, and mRNA was purified with an Oligodex-dT mRNA mini kit (Qiagen) followed by cDNA synthesis using a Superscript First-strand Synthesis System for RT-PCR (Invitrogen). RT-PCR reactions for *fgf-4* and *nanog* genes were performed with gene-specific primer sets as described previously [32].

Tumorigenic assays in nude mice

Tumorigenic assays were performed in nude mice as previously described [34]. Briefly, 5-week-old athymic nude mice (CD1 nu/nu; Charles River) were pretreated with the tetracycline analogue doxycycline (10 μ g/ml) in their drinking water for 2 weeks before injection. Then, 2×10^7 cells from each clone in 100 μ l of PBS were injected subcutaneously into nude mice and the mice continued to be exposed to doxycycline. Those that developed tumours were killed after 26 days. All procedures were carried out in accordance with the animal experimentation guidelines of Sogang University, Seoul, South Korea.

RESULTS

EWS–Oct-4, like Oct-4, binds to an Oct-4 consensus sequence

Characterization of the EWS–Oct-4 translocation fusion product in bone and soft-tissue tumours revealed a chimaeric gene resulting from in-frame fusion of *EWS* exons 1–6 to *oct-4* exons 1–4 [2]. The breakpoint in *EWS* is in intron 6 and the breakpoint in *oct-4* is in exon 1. The chimaeric gene encodes a protein in which the first 122 amino acids present in the NTD of Oct-4 are replaced by the N-terminal 193 amino acids of EWS via an additional 6 amino acid sequence encoded by the normal intron 6 of *EWS* (Figure 1). There is an atypical consensus sequence for a splicing acceptor in *EWS* intron 6 that could add a cryptic splice acceptor signal to *oct-4* exon 1.

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' [35]. Although there is considerable structural difference between EWS–Oct-4 and Oct-4, the POU DNA-binding domain remains intact. To determine whether EWS–Oct-4 binds the natural target genes of Oct-4, EMSAs were performed. An oligonucleotide containing the consensus Oct-4 DNA-binding sequence [35] was synthesized and used as a target in the binding reactions. GST fusions of EWS–Oct-4 and hOct-4 were expressed in *E. coli*, purified and coupled to glutathione-Sepharose beads. To quantify the amount of each protein used, the affinity-purified GST, GST–EWS–Oct-4 and GST–hOct-4 proteins were fractionated by SDS/PAGE and visualized by Coomassie Blue staining or transferred to a PVDF membrane and immunoblotted with an anti-Oct-4 antibody (results not shown). EMSAs were then performed with the concentration of the Oct-4 probe kept constant and the amount of input protein varied. Protein–DNA complexes were formed with both EWS–Oct-4 and hOct-4 proteins (Figure 2A, lanes 5–7 and 8–10), whereas GST alone

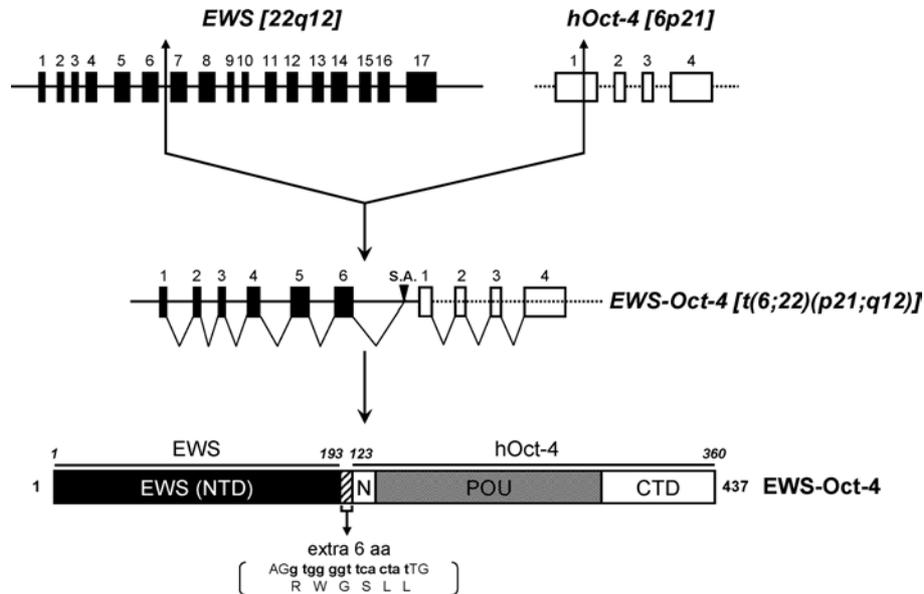


Figure 1 Schematic representation of the $t(6;22)(p21;q12)$ translocation

The exon/intron structures of the *EWS* and *oct-4* genes are shown. The amino acid position is indicated above the schematic diagram representing the fusion protein. The first 193 amino acids (residues 1–193) of *EWS* are fused to the truncated coding sequence (residues 123–360) of *Oct-4* via an additional 6 amino acid sequence encoded by the normal intron 6 of *EWS* in the fusion transcript, which lacks the first 122 amino acids (residues 1–122) present in *Oct-4*. Functionally important domains of the *EWS-Oct-4* chimaera are indicated. N, truncated N-terminal domain of *Oct-4*; S.A., the cryptic splice acceptor signal in *EWS* intron 6.

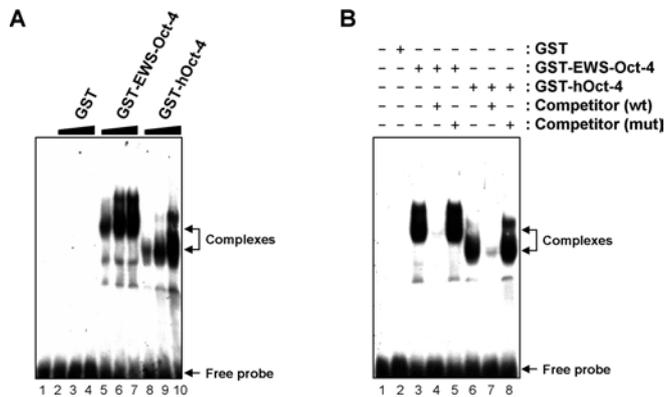


Figure 2 Specific DNA binding by the *EWS-Oct-4* chimaera

(A) EMSAs of the DNA-binding properties of *EWS-Oct-4* and *hOct-4*. EMSAs were performed with either no recombinant protein (lane 1), recombinant GST (lane 2, 10 ng; lane 3, 20 ng; lane 4, 30 ng), GST-*EWS-Oct-4* (lane 5, 10 ng; lane 6, 20 ng; lane 7, 30 ng) or GST-*hOct-4* (lane 8, 10 ng; lane 9, 20 ng; lane 10, 30 ng) and radiolabelled probe, as described in the Materials and methods section. The recombinant proteins used in each EMSA are indicated above the gel. Protein–DNA complexes were resolved on non-denaturing 4% polyacrylamide (37:1, acrylamide/bisacrylamide) gels run at 4°C in 0.5 × TBE (44.5 mM Tris/HCl, 44.5 mM boric acid and 1 mM EDTA, pH 8.0). The positions of free probe and protein–DNA complexes are indicated. (B) Sequence-specific DNA binding by *EWS-Oct-4*. Competition experiments were performed with 100-fold excess of wild-type *Oct-4* oligonucleotide (lanes 4 and 7) or a mutated *Oct-4* oligonucleotide (lanes 5 and 8). The positions of free probe and protein–DNA complexes are indicated by arrows.

hardly bound at all (Figure 2A, lanes 2–4). Binding was specific, as these were displaced by a 100-fold excess of cold oligonucleotide containing the *Oct-4*-binding site but not by a 100-fold excess of cold mutant oligonucleotide containing a mutant *Oct-4* binding sequence not recognized by *Oct-4* (Figure 2B). These results indicate that the DNA-binding specificity of *EWS-Oct-4* resembles that previously defined for *hOct-4*.

EWS-Oct-4 is a nuclear protein

Since the NTD of *hOct-4* is replaced with the NTD of *EWS*, we determined the subcellular localization of the *EWS-Oct-4* by indirect immunofluorescence. We and several other groups have shown that *hOct-4* localizes to the nucleus and that the *Oct-4* POU domain harbours a nuclear localization signal [32,33,36]. Therefore *EWS-Oct-4* should also be a nuclear protein.

To begin to determine the subcellular analysis of *EWS-Oct-4*, COS-7 cells were transfected with either an empty pcDNA3 expression vector (results not shown), pcDNA3-*hOct-4* (Figures 3a and 3b) or pcDNA3-*EWS-Oct-4* (Figures 3c and 3d), and processed for immunofluorescence. Consistent with previous reports [32], *hOct-4* protein was localized to the nucleus (Figures 3a and 3b). Interestingly, indirect immunohistochemistry revealed that the chimaeric *EWS-Oct-4* protein was also localized to the nucleus (Figures 3c and 3d). Together, these data indicate that both *hOct-4* and *EWS-Oct-4* are nuclear proteins.

EWS-Oct-4 activates transcription from reporter genes containing *Oct-4*-binding sites

The NTD of *EWS* appears to contribute to transcriptional activation by *EWS*-fusion proteins by providing a strong transactivation domain [3]. To assess the transcriptional effects of the NTD of *EWS* in *EWS-Oct-4*, we compared transcription by *EWS-Oct-4* and *hOct-4* by co-transfection of the respective expression vectors with a reporter plasmid containing ten copies of *Oct-4*-binding sites and a TATA box upstream of the luciferase gene [33]. Also included was a control plasmid consisting of a cytomegalovirus-driven *Renilla* luciferase gene (Figure 4A). As shown in Figure 4(B), co-transfected *EWS-Oct-4* caused a 735-fold increase in reporter expression in HEK-293T cells [human embryonic kidney-293 cells expressing the large T-antigen of SV40 (simian virus 40)] (Figure 4B, bars 5–7) compared with an approximately 50-fold increase by *hOct-4* (Figure 4B, bars

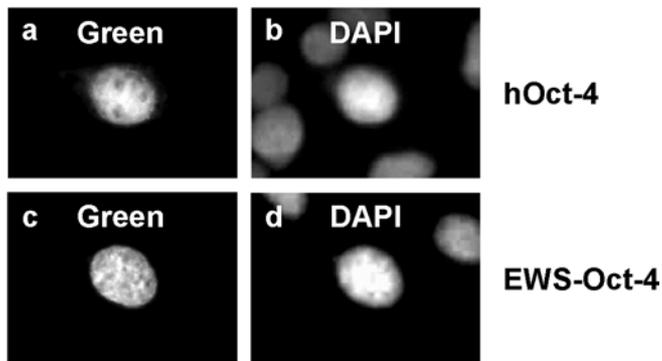


Figure 3 Subcellular localization of EWS–Oct-4

COS-7 cells grown on coverslips were transfected with mammalian expression vectors encoding hOct-4 (a and b) or EWS–Oct-4 (c and d). Cells expressing either hOct-4 or EWS–Oct-4 were subjected to immunofluorescence microscopy with an anti-Oct-4 antibody (C-20; Santa Cruz Biotechnology) and DNA was visualized with DAPI (4',6-diamidino-2-phenylindole; b and d).

2–4). Clearly EWS–Oct-4 is a much more potent transcriptional activator than hOct-4. Western blotting of cell extracts of the transfected cells demonstrated that increasing amounts of hOct-4 and EWS–Oct-4 proteins were synthesized in response to increasing amounts of the corresponding plasmids (Figure 4B, bottom panel). In addition, Western blot analysis showed that the difference in transactivation potential between Oct-4 and EWS–Oct-4 was not due to differences in the amounts of these proteins. Similar results were obtained in COS-7 cells (results not shown).

Three functional domains are important for transactivation by EWS–Oct-4

To define the critical regions within EWS–Oct-4 required for transactivation, we performed transient transfection experiments with FLAG-tagged deletions. The structures of the EWS–Oct-4 deletion mutants are shown schematically in Figure 4(C). The results in the right-hand panel show that deletions of the EWS (NTD) [named as EWS–Oct-4 (Δ EWS)] or the POU domain [EWS–Oct-4 (Δ POU)] abolished transactivation activity, whereas removal of the C-terminal domain of EWS–Oct-4 [EWS–Oct-4 (Δ CTD)] reduced it. Plasmid EWS–Oct-4 (V351P) (harbouring a missense mutation in the POU DNA-binding domain converting Val³⁵¹ into Pro³⁵¹) also failed to activate reporter gene expression. On the other hand deletions of the six extra amino acids (RWGSL) or a part of the Oct-4 (NTD) failed to reduce reporter gene expression (Figure 4C). We interpret these results to indicate that the EWS (NTD), Oct-4 (CTD) and Oct-4 POU domains of EWS–Oct-4 are important for its full transactivation potential. Similar results were obtained in COS-7 cells (results not shown). The expression levels of all mutant proteins were examined by Western blotting (Figure 4D, top panel). For reasons not completely understood, but which may be related to the nature of the NSYGQQS repeat and/or the presence of 23 prolines within the EWS domain of EWS–Oct-4, the estimated molecular mass of the EWS–Oct-4 protein produced in transfected cells was ~58 kDa, slightly larger than the predicted molecular mass of ~48 kDa (Figure 4D, top panel). The same phenomenon has been noted with the EWS–ATF1 and EWS–WT1 fusions, which also have apparent molecular masses greater than predicted [37,38]. EGFP expression served as an internal control for monitoring transfection efficiency (Figure 4D, bottom panel).

Regulation of endogenous Oct-4 downstream target genes by EWS–Oct-4

To see whether ectopic expression of EWS–Oct-4 could modulate expression of endogenous Oct-4 downstream target genes such as *fgf-4* [14,27] and *nanog* [12,16], we stably transfected Oct-4-null ZHBTc4 ES cells with the pCAG-IP/EGFP and pCAG-IP/EWS–Oct-4 constructs (Figure 5A). We used the ZHBTc4 ES cell line because, (i) EWS–Oct-4-positive bone and soft-tissue tumour cell lines or their equivalents are not available, (ii) Oct-4 is expressed in ES cells, and (iii) the expression of the Oct-4 transgene in ZHBTc4 ES cells can be completely turned off by tetracycline. Both endogenous alleles of Oct-4 have been inactivated by gene targeting in this ZHBTc4 ES cell line, which also harbours the tetracycline-repressible mouse Oct-4 transgene [39].

The amount of EWS–Oct-4 protein in three different clones (EWS–Oct-4–EGFP #1–3) were determined by Western blotting. The three clonally derived cell lines produced similar levels of EWS–Oct-4 protein (Figure 5B, lanes 2–4) and no EWS–Oct-4 protein was detected in the control cell line (Figure 5B, lane 1). As shown in Figure 5(C), expression of *fgf-4* and *nanog* was detected in tetracycline-treated ZHBTc4 ES cells expressing EWS–Oct-4 chimaeric protein (Figure 5C, lanes 2–4). However, these genes were not detectable in ZHBTc4 ES cells expressing EGFP vector alone (Figure 5C, lane 1). Thus EWS–Oct-4 is capable of activating Oct-4 downstream target genes *in vivo*.

ZHBTc4 ES cells harbouring EWS–Oct-4 have tumorigenic growth potential in nude mice

Finally, we examined the ability of the EWS–Oct-4 chimaera to induce tumours in nude mice. The nude mice were exposed to the tetracycline analogue, doxycycline, in their drinking water for 2 weeks before injection of EWS–Oct-4 cells and the exposure was continued thereafter. As shown in Figure 6, all six mice receiving ZHBTc4 cells expressing EWS–Oct-4 developed large tumours within a relatively short latent period, showing that EWS–Oct-4 functions as an oncogene at least as efficiently as Oct-4 [22]. The same results were obtained with two other independent clones of ZHBTc4 cells expressing EWS–Oct-4 (results not shown). These results demonstrate that overexpression of EWS–Oct-4 chimaeric protein is sufficient to induce tumorigenesis in nude mice.

DISCUSSION

We have characterized the EWS–Oct-4 fusion protein produced by chromosome translocation in human bone and soft-tissue tumours. These tumours contain a characteristic t(6;22)(p21;q12) translocation resulting in fusion of the NTD of EWS and the CTD of human Oct-4, a gene of the POU family of transcription factors [2]. Chromosome translocations occur frequently in human neoplasms and constitute an important mechanism for proto-oncogene activation [40]. In the present study we show that EWS–Oct-4 is a nuclear protein which binds DNA with a sequence specificity indistinguishable from that of the parental Oct-4 protein. In addition, this fusion gene encodes a more potent transcriptional activator than Oct-4 and is a powerful transforming gene.

The EWS NTD of EWS–Oct-4 appeared to contribute to the transcriptional activation function of EWS–Oct-4 by providing a novel activation domain (Figure 4). This EWS NTD has several structural features. It contains a large number of tyrosine, glutamine, alanine, serine, threonine, glycine and proline residues,

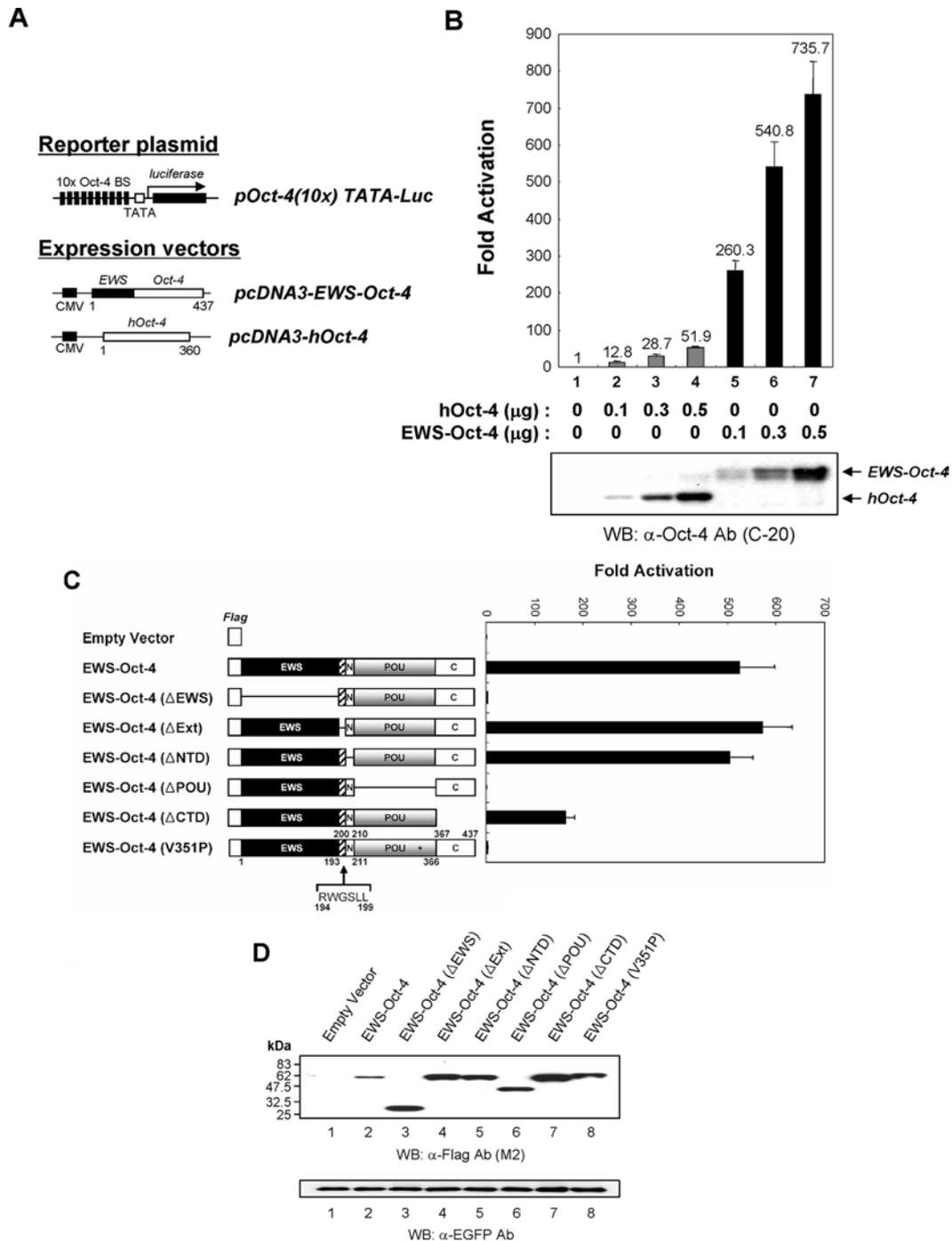


Figure 4 Transactivation potential of EWS-Oct-4

(A) Schematic representation of the reporter and expression plasmids. The pOct-4(10x) TATA-Luc reporter plasmid contains ten copies of the Oct-4 recognition sites upstream of a basal promoter-luciferase construct. The ten copies are indicated by solid bars, the TATA box is represented by an open box, and the luciferase gene by a solid bar. The expression vectors driving the production of EWS-Oct-4 or hOct-4 are also shown. The positions of the first and last amino acids are indicated below each construct. (B) Transcriptional properties of EWS-Oct-4 and hOct-4. HEK-293T cells were co-transfected with expression vectors encoding the indicated amounts of hOct-4 (grey bars) or EWS-Oct-4 (black bars), the pOct-4(10x) TATA luciferase reporter plasmid and the *Renilla* luciferase (top panel). Reporter activity was normalized with *Renilla* luciferase activity to correct for different transfection efficiencies. Fold induction is expressed relative to the empty expression vector. Each transfection was performed at least three times independently and the mean values are plotted \pm S.E.M. (vertical bars). Extracts for luciferase assays were resolved by SDS/PAGE (12% gels), transferred on to PVDF membrane and immunoblotted with an anti-Oct-4 antibody (C-20; bottom panel). (C) Functional regions of EWS-Oct-4. Shown on the left are schematic representations of the deletion constructs of EWS-Oct-4. Numbers refer to amino acid residues. A reporter plasmid, pOct-4(10x) TATA luciferase, was co-transfected into HEK-293T cells with the various FLAG-tagged EWS-Oct-4 mutants. Relative transcriptional activation values are shown on the right as means \pm S.E.M. relative to a value of 1 for transfection of the reporter plasmid alone. Values are means for three independent experiments performed in duplicate. (D) Immunoblot analysis of the EWS-Oct-4 deletion mutant expression in transiently transfected cells. Total cell lysates were fractionated by SDS/PAGE (12% gels) and visualized by Western blotting with anti-FLAG (M2; Sigma-Aldrich) or anti-EGFP (BD Biosciences) antibodies. The positions of prestained molecular mass markers (New England Biolabs) are indicated to the left (kDa). Lane 1, Empty vector; lane 2, EWS-Oct-4; lane 3, EWS-Oct-4 (Δ EWS); lane 4, EWS-Oct-4 (Δ Ext); lane 5, EWS-Oct-4 (Δ NTD); lane 6, EWS-Oct-4 (Δ POU); lane 7, EWS-Oct-4 (Δ CTD); lane 8, EWS-Oct-4 (V351P).

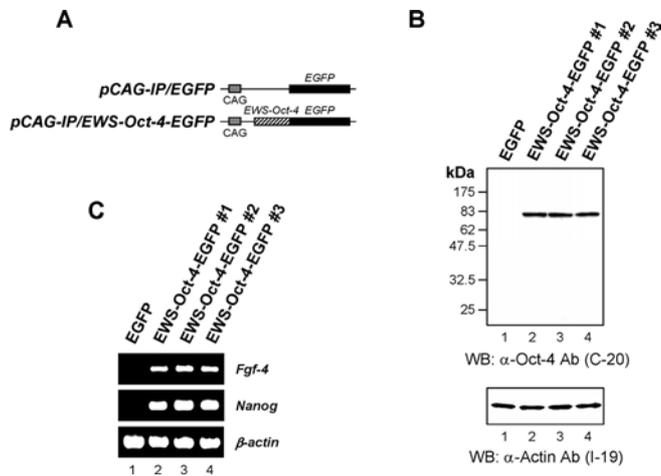


Figure 5 Transcriptional activation of Oct-4 downstream target genes by EWS–Oct-4 *in vivo*

(A) Schematic representation of the expression vectors used. Expression vector pCAG-IP/EWS–Oct-4–EGFP corresponds to EWS–Oct-4 fused to EGFP. The pCAG-IP/EGFP expression vector was used as a control. CAG expression units (CAGs) are indicated by shaded boxes, EWS–Oct-4 is represented by a hatched box, and EGFP is indicated by solid boxes. (B) Immunoblot analysis of EWS–Oct-4 expression in stably transfected ZHBTc4 ES cells. Total cell lysates (35 μ g of protein) were fractionated by SDS/PAGE (12% gels) and visualized by Western blotting with anti-Oct-4 (C-20; Santa Cruz Biotechnology, upper panel) or anti-actin (I-19; Santa Cruz Biotechnology, lower panel) antibodies. (C) Induction of Oct-4 downstream target genes by EWS–Oct-4 *in vivo*. RT-PCR analyses of *fgf-4* and *nanog* mRNAs were performed in ZHBTc4 ES cells expressing vector or EWS–Oct-4 fusion proteins. β -actin was used for normalization. Following amplification, an aliquot of each product was analysed by staining the gel with ethidium bromide. The stable cell lines from which the input RNAs used in the RTs were derived are shown above the panel.

some of which are organized in a repeated and degenerate peptide motif with a frequent serine-tyrosine dipeptide (NSYGQQS) and which shares homology with the CTD of the large subunit of eukaryotic RNA polymerase II [4]. The CTD of RNA polymerase II is efficiently phosphorylated on tyrosine residues by c-Abl [41], rendering it able to convert transcriptionally paused complexes into elongation competent molecules [42]. Although EWS–WT1, a representative EWS–fusion oncoprotein, is modified on tyrosine residues by c-Abl, this inhibits its DNA binding [43]. It would be interesting to test whether EWS–Oct-4 is also a substrate for c-Abl and whether its activity is controlled by c-Abl-mediated tyrosine phosphorylation.

The tumorigenic potential of the chimaeric *EWS–Oct-4* gene product is consistent with the idea that it plays a crucial role in the formation of bone and soft-tissue tumours. The efficient and rapid growth of ZHBTc4 ES cells expressing EWS–Oct-4 protein in nude mice indicates that EWS–Oct-4 is a potent oncogene (Figure 6). Although Oct-4 is normally expressed in ES cells and germ cells and is required for maintaining their pluripotency, it can also promote tumorigenesis when expressed inappropriately in cells. For example, introduction of Oct-4 into Swiss 3T3 cells causes their tumorigenic transformation and causes tumours in the nude mouse [22,23,28,29]. In addition, Oct-4 is expressed in human tumours, including TGCT and breast carcinoma, and plays a part in human cancer development [22].

Because the *EWS–Oct-4* gene has been shown to encode a strong transcriptional activator (Figure 4), it is conceivable that it contributes to tumorigenesis by deregulating expression of Oct-4-responsive genes. As shown in Figure 5, ectopically expressed EWS–Oct-4 upregulates expression of *fgf-4* and *nanog*, known Oct-4 target genes. Interestingly, expression of *fgf-4* and *nanog* is involved in the development of human cancers. Transfection

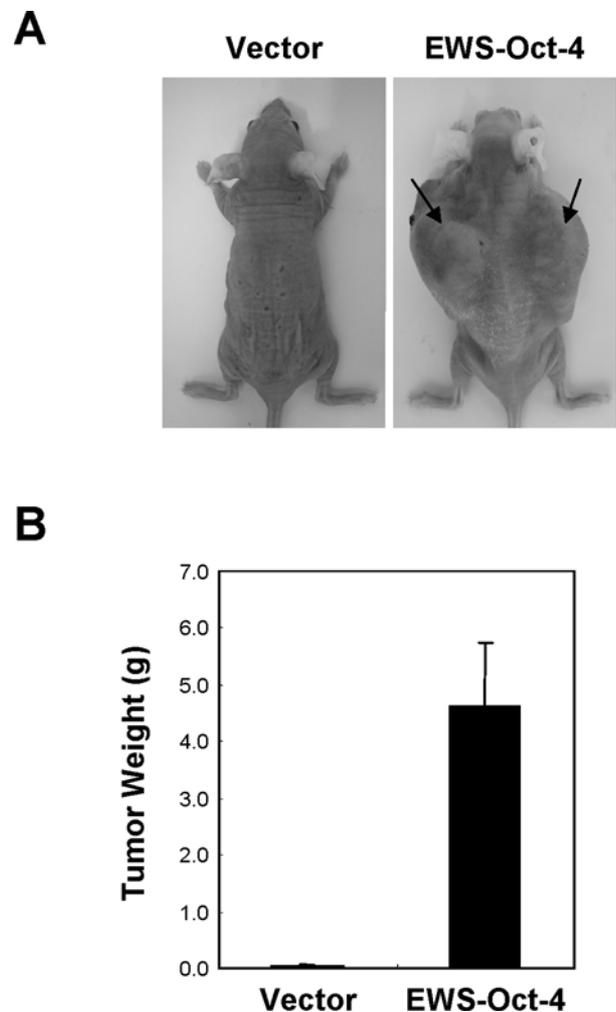


Figure 6 Characterization of EWS–Oct-4-expressing ZHBTc ES cells in nude mice

(A) Effects of EWS–Oct-4 expression on the development of tumours in nude mice. Approx. 2×10^7 Oct-4-null ZHBTc ES cells expressing EWS–Oct-4 or empty vector suspended in 0.1 ml of PBS was injected into 5-week-old Balb/c athymic nude mice. Tumour development was observed and photographs were taken 26 days after injection. Arrows indicate tumours on nude mice. (B) Growth of tumours of Oct-4-null ZHBTc ES cells expressing EWS–Oct-4 in nude mice. Tumour weight in individual animals was measured at 26 days after injection ($n=6$) and is plotted as means \pm S.E.M.

of *fgf-4* into the breast cancer cell line MCF-7 [44] or the non-malignant HBL100 cell line [45] increased their tumorigenicity and metastatic potential. Additionally, introduction of *nanog* into NIH 3T3 cells increased their growth rate and led to the transformed phenotype as demonstrated by focus formation and colony growth in soft agar [46]. Even though definitive identification of the critical target gene(s) is needed, *fgf-4* and *nanog* are potential targets of EWS–Oct-4 in bone and soft-tissue tumour development.

In conclusion, the present study provides evidence that EWS–Oct-4 is an oncogene and that it is necessary for tumorigenesis in bone and soft-tissue tumours. Additional genes may co-operate with EWS–Oct-4 or be required for tumour progression. EWS–Oct-4 probably contributes to oncogenesis by activating key Oct-4 downstream target genes, such as *fgf-4* and *nanog*. Thus it would be interesting to determine which downstream target gene is critical for tumorigenesis and whether EWS–Oct-4 collaborates with this/these gene(s) to generate bone and soft-tissue tumours.

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