EWS-Oct-4B, an alternative EWS-Oct-4 fusion gene, is a potent oncogene linked to human epithelial tumours

S Kim1, B Lim1 and J Kim*,1
1Laboratory of Molecular and Cellular Biology, Department of Life Science, Sogang University, Seoul 121-742, Korea

BACKGROUND: Characterisation of EWS-Oct-4 translocation fusion product in bone and soft-tissue tumours revealed a chimeric gene resulting from an in-frame fusion between EWS (Ewing’s sarcoma gene) exons 1–6 and Oct-4 exons 1–4. Recently, an alternative form of the fusion protein between the EWS and Oct-4 genes, named EWS-Oct-4B, was reported in two types of epithelial tumours, a hidradenoma of the skin and a mucoepidermoid carcinoma of the salivary glands. As the N-terminal and POU domains of the EWS-Oct-4 and EWS-Oct-4B proteins are not structurally identical, we decided to investigate the functional consequences of the EWS-Oct-4B fusion.

METHODS: In this report, we have characterised the EWS-Oct-4B fusion protein. To investigate how the EWS-Oct-4B protein contributes to tumourigenesis in human cancers, we analysed its DNA-binding activity, subcellular localisation, transcriptional activation behaviour, and oncogenic properties.

RESULTS: We found that this new chimeric gene encodes a nuclear protein that binds DNA with the same sequence specificity as the parental Oct-4 protein or the fusion EWS-Oct-4 protein. We show that the nuclear localisation signal of EWS-Oct-4B is dependent on the POU DNA-binding domain, and we identified a cluster of basic amino acids, RKRKR273, in the POU domain that specifically mediates the nuclear localisation of EWS-Oct-4B. Comparison of the properties of EWS-Oct-4B and EWS-Oct-4 indicated that EWS-Oct-4B is a less-potent transcriptional activator of a reporter construct carrying the Oct-4-binding sites. Deletion analysis of the functional domains of EWS-Oct-4B revealed that the EWS N-terminal domain (NTD)B, POU, and C-terminal domain (CTD) are necessary for its full transactivation potential. Despite its reduced activity as a transcriptional activator, EWS-Oct-4B regulated the expression of fgf-4 (fibroblast growth factor-4) and nanog, which are potent mitogens, as well as of Oct-4 downstream target genes, the promoters of which contain potential Oct-4-binding sites. Finally, ectopic expression of EWS-Oct-4B in Oct-4-null ZF-hBTc4 ES cells resulted in increased tumourigenic growth potential in nude mice.

CONCLUSION: These results suggest that the oncogenic effect of the t(6;22) translocation is due to the EWS-Oct-4B chimeric protein, and that alternative fusion of the EWS amino terminal domain to the Oct-4 DNA-binding domain produces another transforming chimeric protein in human epithelial tumours.

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Cancer is an abnormal type of tissue growth in which cells proliferate in an uncontrolled, relatively autonomous manner, leading to a continual increase in the number of dividing cells. Chromosomal translocations cause cancer by activating existing genes or creating new fusion proteins (Rabbits, 1999). Some bone and soft-tissue tumours were shown to harbour a translocation t(6;22)(p21;q12) involving Ewing’s sarcoma (EWS) gene at 6p21 and the Oct-4 gene at 6p21 (Yamaguchi et al, 2005). Striking features of these tumours are the diffuse proliferation pattern of the undifferentiated tumour cells and the positive immunoreactivity for vimentin, S-100, and neuron-specific enolase. A chimeric transcript of ~1.8 kb was detected by northern blotting using EWS and Oct-4 probes, and an EWS-Oct-4 fusion transcript, but not the reciprocal Oct-4-EWS fusion transcript, was detected in tumours by RT-PCR (Yamaguchi et al, 2005). Recently, a new fusion between EWS and Oct-4 was reported in hidradenoma of the skin and mucoepidermoid carcinoma of the salivary glands (Moller et al, 2008). Reverse transcription-PCR and DNA sequence analyses revealed that a part of exon 6 of EWS is fused in-frame to exon 2 of Oct-4 in these tumours, which indicated that an alternative form of EWS-Oct-4 exists owing to a variation in the locations of the EWS and Oct-4 genomic break points. To distinguish it from the previous EWS-Oct-4 fusion detected in human bone and soft-tissue tumours (Yamaguchi et al, 2005), we called it EWS-Oct-4B.

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 N-terminal domain (NTD) (amino acid 1–285) of EWS is composed almost exclusively (~90%) of tyrosine, glycine, alanine, serine, threonine, and proline residues organised in a repeated and degenerate
polypeptide motif with the consensus, NSYGQQS. This domain has weak homology to the C-terminal region of eukaryotic RNA polymerase II (Delattre et al., 1992). The EWS gene is involved in several tumour-related translocations, which generate fusions with genes for putative transcription factors (Kim and Pelletier, 1999). In each case, the translocation produces chimeric molecules containing the NTD of EWS fused to the DNA-binding domain of the partner protein. The C-terminal fusion partners are cellular transcription factors that contribute a sequence-specific DNA-binding domain, which determines the tumour phenotype (Lee, 2007). The highly tissue-restricted expression of the fusion partners contrasts with that of the native EWS gene, which is expressed ubiquitously at high levels. The EWS promoter drives the expression of EWS fusion proteins directly in human cancers owing to the genomic structure of the EWS chimeras (Lin et al., 1999).

Oct-4, also referred to as Oct-3, is a transcriptional regulator of genes involved in maintaining the undifferentiated pluripotent state, and may prevent the expression of genes activated during differentiation (Brehm et al., 1997). It functions as a master switch during differentiation by regulating gene expression in pluripotent cells, or in cells that can develop pluripotent potential (Ovitt and Scholer, 1998; Pesce and Scholer, 2001). In addition, Oct-4 is a key factor in the genesis of human testicular germ cell tumours (TGCTs) (Gidekel et al., 2003; Loojenga et al., 2003). 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 (Olive, 1999; Hellerstedt and Pienta, 2003). The Oct-4 transcript is consistently detected in a specific set of human TGCTs found in adolescents and young adults, namely, seminomas and embryonal carcinomas (Palumbo et al., 2002). In addition, the precursor lesions of human TGCT, known as carcinoma in situ, also express Oct-4 (Palumbo et al., 2002). 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 involvement in tumourigenesis may be related to the upregulation of its downstream target genes (Jin et al., 1999; Monk and Holding, 2001; Gidekel et al., 2003; Wang et al., 2003). Consistent with these findings, Oct-4 expression in a heterologous cell system transformed non tumourigenic cells and produced tumours in nude mice. Activation of Oct-4 in adult mice using a dosenicacycle-dependent expression system resulted in dysplastic growth of epithelial tissues that are dependent on continuous Oct-4 expression (Hochedlinger et al., 2005). In addition, Oct-4 is involved in the reprogramming of mouse and human somatic cells to the pluripotent state (Takahashi and Yamanaka, 2006; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007, 2008; Asse et al., 2008; Dimos et al., 2008; Hockemeyer et al., 2008; Lowery et al., 2008; Maherali et al., 2008; Nakagawa et al., 2008; Park et al., 2008a,b), suggesting that induced pluripotent stem cells, derived from somatic cells of patients, represent a powerful tool for use in replacement therapies (Soldner et al., 2009).

To investigate how the recently identified EWS-Oct-4B protein contributes to tumourigenesis in two types of human epithelial tumours, namely, hidradenoma of the skin and mucoepidermoid carcinoma of the parotid glands, we analysed this transcriptional activation behaviour and oncogenic properties. We found that it is a nuclear protein that binds DNA with a sequence specificity indistinguishable from that of the parental Oct-4 or the chimeric EWS-Oct-4 proteins. The nuclear localisation signal (NLS) of EWS-Oct-4B was dependent on the POU DNA-binding domain, and we identified a cluster of basic amino acids, 269RKRKR273, in the POU domain that specifically mediates the nuclear localisation of EWS-Oct-4B. When we compared transactivatioc of reporter constructs carrying Oct-4-binding sites by the different EWS-Oct-4 proteins, EWS-Oct-4B was a less-potent transcriptional activator than EWS-Oct-4. Results from deletion analysis also suggested that several functional domains of EWS-Oct-4B are necessary for it to achieve its full activation potential. However, although EWS-Oct-4B is a less-potent transcriptional activator than EWS-Oct-4, it functions as a dominantly acting oncogene, as measured by the activation of oncogenic Oct-4 downstream target genes and tumour formation in nude mice. These data indicate that EWS-Oct-4B may have a critical role in the formation of human epithelial tumours by activating the transcription of Oct-4 target genes.

MATERIALS AND METHODS

Molecular cloning

To generate pcDNA3/Flag-EWS-Oct-4B, we used the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), the mutagenic primer set 5'-EOB (5'-AACTCAGTTATCCTCCGATCCAGGACAATGAAACGCT-3') and 3'-EOB (5'-AGGTGGCAGTCTCCCTGGAGCTGGGATAAATGTGTA-3'), and pKSII/Flag-EWS-Oct-4 (Lee et al., 2007) as a template to construct pKSII/Flag-EWS-Oct-4B. Then, pKSII/Flag-EWS-Oct-4B was digested with EcoRI and HindIII and cloned into the corresponding sites of the pcDNA3 vector (Invitrogen Molecular Probes, Carlsbad, USA).

The GST-EGFP-EWS-Oct-4B deletion mutants were generated as follows: (A) pGST-EGFP, details on the construction of pGST-EGFP have been previously reported (Kim et al., 2008); (B) pGST-EGFP/EWS (NDD), an EWS (NDD) fragment was amplified from pcDNA3/EWS-Oct-4 by PCR using primers 5'-GST-EGFP-EWS(N) (5'-GATCCGGATCCAGGCTCCAACGGATACGAC-3') and BamHI site underlined (and 3'-GST-EGFP-EWS(N) (5'-GATCCGGATCCAGCTGGGATAAATGTGTA-3'); (C) pGST-EGFP/POUB, a POUB fragment was amplified from pcDNA3/EWS-Oct-4B by PCR using primers 5'-GST-EGFP-POUB (5'-GATCCGGATCCAGGCTCCAACGGATACGAC-3') and 3'-GST-EGFP-POUB (5'-GATCCGGATCCAGTCCCTGGGATAAATGTGTA-3'); (D) pGST-EGFP-POUB (pGST-EGFP-POUB) was amplified from pKSII/EWS-Oct-4B (pGST-EGFP-POUB) and 3'-GST-EGFP-POUB (pGST-EGFP-PouB) was digested with BamHI and cloned into the corresponding site of the pGST-EGFP vector to generate pGST-EGFP/PouB (pGST-EGFP-POUB); (E) pGST-EGFP-PouB (pGST-EGFP-PouB) was digested with BamHI and cloned into the corresponding site of the pGST-EGFP vector to generate pGST-EGFP/PouB (pGST-EGFP-POUB); (F) pGST-EGFP-PouB (pGST-EGFP-POUB) was digested with BamHI and cloned into the corresponding site of the pGST-EGFP vector to generate pGST-EGFP/PouB (pGST-EGFP-POUB).

To construct pGAL4-EGFP, details on the construction of pGAL4-EGFP have been previously reported (Kim et al., 2008); (G) pGAL4-EGFP/EWS (NDD), an EWS (NDD) fragment was amplified from pcDNA3/EWS-Oct-4B by PCR using primers 5'-GAL4-EGFP/EWS-NTD (5'-GATCCGGATCCAGGCTCCAACGGATACGAC-3') and 3'-GAL4-EGFP/EWS-NTD (5'-GATCCGGATCCAGTCCCTGGGATAAATGTGTA-3'); (H) pGAL4-EGFP/EWS-POUB, a POUB fragment was amplified from pcDNA3/EWS-Oct-4B by PCR using primers 5'-GAL4-EGFP/EWS-POUB (5'-GATCCGGATCCAGGCTCCAACGGATACGAC-3') and 3'-GAL4-EGFP/EWS-POUB (5'-GATCCGGATCCAGTCCCTGGGATAAATGTGTA-3'); (I) pGAL4-EGFP/EWS-NTD (pGAL4-EGFP/EWS-NTD) was digested with BamHI and cloned into the corresponding site of the pGAL4 vector to generate pGAL4/EWS-NTD (pGAL4/EWS-NTD); (J) pGAL4-EGFP/EWS-POUB (pGAL4-EGFP/EWS-POUB) was digested with BamHI and cloned into the corresponding site of the pGAL4 vector to generate pGAL4/EWS-POUB (pGAL4/EWS-POUB); (K) pGAL4-EGFP/EWS-POUB (pGAL4-EGFP/EWS-POUB) was digested with BamHI and cloned into the corresponding site of the pGAL4 vector to generate pGAL4/EWS-POUB (pGAL4/EWS-POUB).

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The pcDNA3.Flag-EWS-Oct-4B deletion mutants were generated as follows: (A) pcDNA3.Flag-EWS-Oct-4B (ΔEWS), a Flag-EWS-Oct-4B (ΔEWS) fragment was amplified from pKSII.Flag-EWS-Oct-4B by PCR using primers 5'-FlagOct-4BΔPOU (5'-GATGTAACGCTATGACACCAAGATGGCATTTACCTGCAAAGCTCTC-3'; HindIII site underlined) and 3'-EOcIR (5'-GATGCG AATTCTCATAGATGATTAC-3; EcoRI site underlined), digested with HindIII and EcoRI, and cloned into the corresponding sites of the pcDNA3 vector to generate pcDNA3.Flag-EWS-Oct-4B (ΔEWS); (B) pcDNA3.Flag-EWS-Oct-4B (ACTD), a Flag-EWS-Oct-4B (ACTD) fragment was amplified from pKSII.Flag-EWS-Oct-4B by PCR using primers 5'-BamHIFlagE-O (5'-GATGCGATCCATGAGAATGCTAGTGAAGTGCC-3; BamHI site underlined) and 3'-BoD5A-86/BamRI (5'-GATGCGATCCATGAGAATGCTAGTGAAGTGCC-3; EcoRI site underlined), digested with BamHI and EcoRI, and cloned into the corresponding sites of the pcDNA3 vector to generate pcDNA3.Flag-EWS-Oct-4B (ACTD); (C) pcDNA3.Flag-EWS-Oct-4B (V313P), pcDNA3.Flag-EWS-Oct-4B (V313P) (V351P) (Kim et al, 2009) was digested with EcoRI to isolate an EcoNI-V313P-EcoNI fragment. Then, pcDNA3.Flag-EWS-Oct-4B was digested with EcoRI to generate pcDNA3.Flag-EWS-Oct-4B (ΔEWS), and pcDNA3.Flag-EWS-Oct-4B (ΔEcoNI) was ligated with the EcoNI-V313P-EcoNI fragment to generate pcDNA3.Flag-EWS-Oct-4B (V313P).

Construct pCAG-IP/EGFP has been described previously (Lee et al, 2006). To create pCAG-IP/EGFP-Oct-4B/EGFP, EWS-Oct-4B was amplified from pcDNA3.Flag-EWS-Oct-4B by PCR using primers 5'-BamHIFlagE-O (5'-GATGCGATCCATGAGAATGCTAGTGAAGTGCC-3; BamHI site underlined) and 3'-BoD5A/BamRI (5'-GATGCGATCCATGAGAATGCTAGTGAAGTGCC-3; BamHI site underlined). The PCR product was digested with BamHI and cloned into the same sites of the pEGFP (N1) vector (Clontech Laboratories) to generate pEGFP(N1)-EWS-Oct-4B. To construct pCAG-IP/EGFP-Oct-4B-EGFP, the blunt HindIII (repaired with the Klenow fragment) and NotI fragments of pEGFP(N1)-EWS-Oct-4B were subcloned into the blunted XhoI (repaired with the Klenow fragment) and NotI sites of the pCAG-IP vector.

Electrophoretic mobility shift assays

Probes (5'-GGGACATCTCAATTACAAACTGAGGGGTC-3' and 5'-GAGCGCTCATTGTTGTATGCGAATGCGGTC-3'; underlines indicate the Oct-4 recognition site) for electrophoretic mobility shift assays (EMSA) were prepared from synthetic oligonucleotides for which the sequences have been described previously (Nishimoto et al, 1999). The probe was prepared by end-labelling annealed complementary oligonucleotides with [γ-32P] ATP using T4 polynucleotide kinase. The DNA-binding reactions were performed using purified glutathione-S-transferase (GST), GST-EWS-Oct-4, or GST-EWS-Oct-4B proteins for 30 min at 4°C in binding buffer containing 10 mM Tris-HCl (pH 8.0), 40 mM KCl, 6% glycerol, 1 mM DTT, 0.05% NP-40, and 10 ng/ml−1 of poly (dI dC) (dI dC). After binding, the reaction mixtures were run on 4% polyacrylamide gels (acylamide/bisacrylamide ratio. 37:1) in 0.5× TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA) buffer at 150 V for 2–3 h at 4°C. The gels were dried and exposed to Kodak X-omat film (Kodak, Rochester, NY, USA). 4°C using an intensifying screen.

Transfection, subcellular localisation, and reporter gene assays

Subcellular localisation experiments were performed as previously described (Kim et al, 2008). Briefly, to examine the localisation of the EWS-Oct-4B-EGFP or GST-EGFP fusion proteins of the EWS-Oct-4B truncation mutants, cells were transfected with pEGFP, pEWS-Oct-4B-EGFP, pGST-EGFP, pGST-EGFP/EWS (NTD)B, pGST-EGFP/POU8, pGST-EGFP/POU1B (LILIL), or pGST-EGFP/CTD using the VivaMagic reagent (Vivagen, Seoul, Korea).

Figure 1 Structural comparison of EWS-Oct-4 alternative forms. Two different forms of the EWS-Oct-4 protein (EWS-Oct-4 and EWS-Oct-4B) and the human Oct-4 isoforms (Oct-4A and Oct-4B) are represented schematically. Amino acid (aa) positions are indicated above and below the schematic representing the proteins. In the case of EWS-Oct-4, the first 193 aa (residues 1–193) of EWS are fused to the truncated coding sequence (residues 123–360) of Oct-4A through an additional six aa sequence. This six aa sequence is encoded by the normal intron 6 of EWS, representing the proteins. In the case of EWS-Oct-4, the first 193 aa (residues 1–193) of EWS are fused to the truncated coding sequence (residues 1–193) of Oct-4A through an additional six aa sequence. This six aa sequence is encoded by the normal intron 6 of EWS, representing the proteins. In the case of EWS-Oct-4, the first 193 aa (residues 1–193) of EWS are fused to the truncated coding sequence (residues 1–193) of Oct-4A through an additional six aa sequence. This six aa sequence is encoded by the normal intron 6 of EWS, representing the proteins.
washed in PBS, and then fixed for 10 min at –20°C in a mixture of acetone and methanol (1:1, v/v). Coverslips were mounted with 50% glycerol/PBS, and the green fluorescence of EGFP was detected using a fluorescence microscope (IX71, Olympus, Tokyo, Japan) equipped with DP71 digital camera (Olympus). For reporter gene assays, cells were transiently transfected with plasmids by electroporation using the VivaMagic reagent (Vivagen). Luciferase assays were performed with the Dual-luciferase Assay System (Promega, Madison, WI, USA). Renilla luciferase activities were used to normalise transfection efficiencies.

Western blot analysis

Western blot analysis was performed using anti-Oct-4 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-EGFP (Invitrogen Molecular Probes), anti-GST (B-14, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAL4 (RR5C1, Santa Cruz Biotechnology), anti-Flag (M2, Sigma, St Louis, MO, USA), and anti-GAPDH (V-18, Santa Cruz Biotechnology) antibodies, and reactive bands were detected by chemiluminescence using Western Lightening (PerkinElmer Life Sciences, Boston, MA, USA).

Establishment of stable cell lines and tumourigenic assays in nude mice

Details on the construction of ZHBTc4 ES cell line expressing EGFP have been previously reported (Lee et al., 2006). To generate stably expressing ES cell lines, pCAG-IP/EWS-Oct-4B-EGFP was linearised with PvuI and 10 μg of each was transfected into ZHBTc4 ES cells (4 × 10⁶) using the MicroPorator (Digital Bio, Seoul, Korea). At 48 h after electroporation, puromycin (Sigma) was added to a final concentration of 1 μg ml⁻¹ 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. Tumourigenic assays were performed in nude mice as previously described (Kim et al., 1998a). In brief, 5-week-old athymic nude mice (CD1 nu/nu; Charles River, Wilmington, MA, USA) were pre-treated with tetracycline analogue doxycycline (10 μg ml⁻¹) in their drinking water for 2 weeks before injection. Then, 0.6 × 10⁷ cells from each clone in 100 μl PBS were injected subcutaneously into nude mice and the mice continued to be exposed to doxycycline. Those mice that developed tumours were killed after 28 days.

RESULTS

Structural features of EWS-Oct-4B

The EWS-Oct-4 chimeric gene was originally isolated from bone and soft-tissue tumours (Yamaguchi et al., 2005). This chimeric 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 through an additional six amino acid sequence encoded by the normal intron 6 of EWS (Figure 1). Recently, a different type of EWS-Oct-4 chimeric transcript, EWS-Oct-4B, was isolated in a hidradenoma of the skin and a mucoepidermoid
cancer of the salivary gland (Moller et al., 2008). Sequence analysis of the EWS-Oct-4B gene revealed that a part of EWS exon 6 was fused in-frame to exon 2 of Oct-4, but unlike the EWS-Oct-4 fusion found in bone and soft-tissue tumours, neither the additional six amino acid sequence nor the NTD of Oct-4A was retained in the EWS-Oct-4B fusion (Figure 1). In addition, the POU domain of EWS-Oct-4B (POU\textsuperscript{b}, total 154 amino acids) lacks two amino acids of the N-terminal sequence of the EWS-Oct-4 POU domain (POU\textsuperscript{a}, total 156 amino acids). As a result of this particular chromosome translocation, the structure of the C-terminal portion of EWS-Oct-4B is more similar to the human Oct-4B isoform than to Oct-4A.

**EWS-Oct-4B is a nuclear protein**

We have shown previously that EWS-Oct-4 localises to the nucleus (Lee et al., 2007). As the structure of EWS-Oct-4B is more similar to human Oct-4B rather than to Oct-4A (Figure 1), and as Oct-4B is mainly localised to the cytoplasm (Lee et al., 2006), we decided to investigate the subcellular localisation of EWS-Oct-4B. As a first step, we transfected 293T cells with the pEGFP-N1 vector or with pEWS-Oct-4B-EGFP and carefully monitored the localisation of green fluorescence. In the transiently transfected cells, EGFP-tagged EWS-Oct-4B was clearly localised to the nucleus (Figure 2A), whereas EGFP alone was found in both the nucleus and cytoplasm (Figure 2Aa). These data indicate that EWS-Oct-4B is a nuclear protein, unlike human Oct-4B, which is mainly localised to the cytoplasm.

To map the region of EWS-Oct-4B responsible for its nuclear localisation, we generated a set of EWS-Oct-4B deletion mutants (Figure 2B). Because polypeptides with molecular masses of 40–50 kDa can passively diffuse into the nucleus (Breeuwer and Goldfarb, 1990), we fused the isolated functional domains of EWS-Oct-4B to GST and EGFP. The 293T cells were transfected with expression vectors for the GST-EGFP fusion proteins of the EWS-Oct-4B truncation mutants, and the localisation of EGFP was analysed by fluorescence microscopy. GST-EGFP/EWS (NTD)\textsuperscript{b} (Figure 2Cb) and CTD (Figure 2Ce) localised to the cytoplasm, whereas GST-EGFP/POU (Figure 2Cc) clearly localised to the nucleus. GST-EGFP was used as a control and localised to the cytoplasm of 293T cells (Figure 2Ca).

To define NLS further in the POU\textsuperscript{b} domain of EWS-Oct-4B, we generated a POU\textsuperscript{b} mutant in which several highly conserved basic amino acids, 269RRKR273, were replaced with the 269LILIL\textsuperscript{273} sequence using site-directed mutagenesis. This region of basic amino acids was chosen as a putative NLS on the basis of earlier evidence that states that positively charged sequences are good candidates for nuclear targeting signals (Dingwall and Laskey, 1991). Consistent with our prediction, the substitution of 269RRKR273 with the 269LILIL\textsuperscript{273} sequence resulted in the cytoplasmic accumulation of GST-EGFP/POU\textsuperscript{a} (Figure 2Cd). This result suggested that this cluster of basic amino acids in the POU\textsuperscript{b} domain does indeed function as an NLS of EWS-Oct-4B.

**EWS-Oct-4B is a less-potent transcriptional activator than EWS-Oct-4**

To assess the transactivation potential of EWS-Oct-4B, we cotransfected 293T cells with the pEGFP-N1 vector or with pEWS-Oct-4B-EGFP and carefully monitored the localisation of green fluorescence. In the transiently transfected cells, EGFP-tagged EWS-Oct-4B was clearly localised to the nucleus (Figure 2B). Because polypeptides with molecular masses of 40–50 kDa can passively diffuse into the nucleus (Breeuwer and Goldfarb, 1990), we fused the isolated functional domains of EWS-Oct-4B to GST and EGFP. The 293T cells were transfected with expression vectors for the GST-EGFP fusion proteins of the EWS-Oct-4B truncation mutants, and the localisation of EGFP was analysed by fluorescence microscopy. GST-EGFP/EWS (NTD)\textsuperscript{b} (Figure 2Cb) and CTD (Figure 2Ce) localised to the cytoplasm, whereas GST-EGFP/POU (Figure 2Cc) clearly localised to the nucleus. GST-EGFP was used as a control and localised to the cytoplasm of 293T cells (Figure 2Ca).

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**Figure 3** Comparison of transactivation potential by EWS-Oct-4B. Cells (293T) were cotransfected with expression vectors encoding the indicated amounts of EWS-Oct-4 (grey bars) or EWS-Oct-4B (black bars), the pOct-4(10 x ) TATA luc reporter plasmid, and the Renilla luciferase (top panel). Reporter activity was normalised 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 thrice independently and the mean values are plotted with their standard errors (± s.e., vertical bars). Extracts for luciferase assays were resolved using 12% SDS–PAGE, transferred to a PVDF membrane, and immunoblotted with anti-Oct-4 (C-20) (middle panel) or anti-EGFP (bottom panel) antibodies, as indicated.

**EWS-Oct-4B similar to EWS-Oct-4 binds to an Oct-4 consensus sequence**

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' (Botquin et al., 1998). Although there is considerable structural similarity between EWS-Oct-4B and EWS-Oct-4 (Figure 1), POU\textsuperscript{b}, the DNA-binding domain of EWS-Oct-4B, lacks two amino acids of the N-terminal sequence found in the EWS-Oct-4 POU domain (POU\textsuperscript{a}), and the structure of the EWS-Oct-4B C-terminal portion (POU plus CTD) is more similar to that of the Oct-4B isoform than to that of Oct-4A. Interestingly, the Oct-4B isoform does not bind to a probe carrying the Oct-4 consensus-binding sequence, and two separate regions of its NTD are responsible for inhibiting DNA binding (Lee et al., 2006). To determine whether EWS-Oct-4B binds the target sequence of Oct-4, we performed EMSAs. An oligonucleotide containing the consensus Oct-4 DNA-binding sequence (Botquin et al., 1998)
was synthesised and used as target in the binding reactions. Glutathione-S-transferase fusions of EWS-Oct-4B and EWS-Oct-4 were expressed in Escherichia coli, purified, and coupled to glutathione–sepharose beads. To quantify the amount of each protein used, affinity-purified GST, GST-EWS-Oct-4B, and GST-EWS-Oct-4 proteins were fractionated by SDS–PAGE, transferred to a PVDF membrane, and immunoblotted with an anti-GST antibody (B-14, Santa Cruz Biotechnology) (Figure 4A). Electrophoretic mobility shift assays were then performed by keeping the concentration of the Oct-4 probe constant and varying the amount of input protein. Protein–DNA complexes were formed with both EWS-Oct-4B and EWS-Oct-4 proteins (Figure 4B lanes 4–6 and 7–9), whereas GST alone hardly bound at all (Figure 4A lanes 1–3). Binding was specific, as these complexes 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 that is not recognised by Oct-4 (data not shown). These results indicate that, although EWS-Oct-4B shares its C-terminal portion with the human Oct-4B isoform, the DNA-binding specificity of EWS-Oct-4B resembles that previously defined for Oct-4A.

EWS (NTD)B is a less-potent transcriptional activation domain than EWS (NTD)

Although protein–DNA complexes were formed with both EWS-Oct-4B and EWS-Oct-4 proteins (Figure 4) and both are localised to the nucleus (Figure 2), EWS-Oct-4B was a less-potent transcriptional activator than EWS-Oct-4 (Figure 3), suggesting that this difference could be due to the differences in their NTD s. To verify this hypothesis, we created fusion proteins in which the GAL4 DNA-binding domain was fused to the EWS (NTD) of EWS-Oct-4 or to the EWS (NTD)B of EWS-Oct-4B (Figure 5A). The pG5 luc reporter contains five GAL4 DNA-binding sites upstream of the TATA box and was used as a reporter in these experiments.

Figure 4 DNA-binding property of the EWS-Oct-4B chimera. (A) Immunoblot analysis of EWS-Oct-4B and EWS-Oct-4 to quantify GST fusion proteins. The GST-EWS-Oct-4 fusion proteins used in EMSAs were fractionated on 10% SDS–PAGE and visualised by western blotting with an anti-GST antibody (B-14, Santa Cruz Biotechnology). (B) EMSAs of the DNA-binding properties of EWS-Oct-4B and EWS-Oct-4. The EMSAs were performed using recombinant GST (lane 1, 0.15 μg; lane 2, 0.45 μg; lane 3, 1.35 μg), GST-EWS-Oct-4 (lane 4, 0.15 μg; lane 5, 0.45 μg; lane 6, 1.35 μg), or GST-EWS-Oct-4B (lane 7, 0.15 μg; lane 8, 0.45 μg; lane 9, 1.35 μg), and radiolabeled probe, as described in Materials and Methods. The recombinant proteins used in each EMSA are indicated above the gel. Protein–DNA complexes were resolved on non-denaturing 4% polyacrylamide (acylamide:bisacrylamide ratio, 37 : 1) gels run at 4°C in 0.5 × TBE (44.5 mM Tris–HCl, 44.5 mM boric acid, 1 mM EDTA). The positions of free probe and protein–DNA complexes are indicated.

Figure 5 Transactivation potential of EWS (NTD)B. (A) Schematic representation of the GAL4-fusion expression plasmids used in this study. The expression vectors driving the production of GAL4-EWS (NTD) or GAL4-EWS (NTD)B are shown. GAL4, GAL4 DNA-binding domain; EWS (NTD), EWS N-terminal domain of EWS-Oct-4; EWS (NTD)B, EWS N-terminal domain of EWS-Oct-4B. (B) Comparison of transactivation potential of EWS (NTD) and GAL4-EWS (NTD)B into 293T cells (top panel). 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). Each transfection was performed independently at least thrice and the mean values are plotted with their standard errors (i.e., vertical bars). Extracts for luciferase assays were resolved using 12% SDS–PAGE, transferred to a PVDF membrane, and immunoblotted with anti-GAL-4 (RK5C1) (middle panel) or anti-EGFP (bottom panel) antibodies as indicated.
An expression vector pGAL4 (pM, Clontech Laboratories), which drives the synthesis of only the GAL4 DNA-binding domain, had no significant effect on the level of luciferase produced from pG5 luc when transfected into 293T cells (Figure 5B, lane 1). Interestingly, pGAL4-EWS (NTD) strongly activated luciferase production from pG5 luc by 25-fold (Figure 5B, lane 3), indicating that the EWS (NTD) of the EWS-Oct-4 protein has an intrinsic transcriptional activation property. On the other hand, the EWS (NTD) of EWS-Oct-4 is capable of activating transcription, but is a less potent transactivator than the EWS (NTD) of EWS-Oct-4.

**Multiple domains are important for EWS-Oct-4B function**

To define the critical regions within EWS-Oct-4B required for transactivation, we performed transient transfection experiments using flag-tagged EWS-Oct-4B deletions. The structures of the EWS-Oct-4B deletion mutants are shown schematically in Figure 6A. The data in the right panel show that deletion of the EWS (NTD)B (named as EWS-Oct-4B (V313P)) reduced activity. We interpret these results to indicate that EWS (NTD) and CTD, as well as the DNA-binding activity of EWS-Oct-4B, are important for its full transactivation potential. The expression levels of all mutant proteins were examined by western blotting (Figure 6B, top panel). EGFP expression served as an internal control for monitoring transfection efficiency (Figure 6B, bottom panel).

**Regulation of endogenous Oct-4 downstream target genes by EWS-Oct-4B**

To determine whether ectopic expression of EWS-Oct-4B could modulate the expression of endogenous Oct-4 downstream target genes such as fibroblast growth factor-4 (fgf-4) (Ambrosetti et al., 1997; Wang et al., 2003) and nanog (Kuroda et al., 2005; Rodda et al., 2005), we stably transfected Oct-4-null ZHBTc4 ES cells with pCAG-IP/EGFP and pCAG-IP/EWS-Oct-4B constructs (Figure 7A). We used the ZHBTc4 ES cell line because EWS-Oct-4B-positive human epithelial tumour cell lines or their equivalents are not available. In addition, it was convenient to introduce expression of the EWS-Oct-4B gene ectopically, because both the endogenous alleles of Oct-4 have been inactivated by gene targeting in the ZHBTc4 ES cell line, which also harbours the tetracycline-repressible mouse Oct-4 transgene (Niwa et al., 2000).

The amount of EWS-Oct-4B protein was determined by western blotting. The stable cell line carrying EWS-Oct-4B produced the EWS-Oct-4B-EGFP protein as an EGFP fusion form (Figure 7B, lane 2). No EWS-Oct-4B-EGFP protein was detected in the control cell line.

**Figure 6** Functional regions of EWS-Oct-4B. (A) Transcriptional properties of EWS-Oct-4B deletion mutants. Shown on the left are schematic representations of EWS-Oct-4B deletion constructs. A reporter plasmid, pOct-4(10 C2) TATA luc, was cotransfected into 293T cells with various flag-tagged EWS-Oct-4B mutants. Relative transcriptional activation values are shown on the right as mean increases ± s.e. relative to a value of 100% for transfection of EWS-Oct-4B. The results are the means of three independent experiments performed in duplicate. (B) Immunoblot analysis showing expression of the EWS-Oct-4B deletion mutants in transiently transfected cells. Total cell lysates were fractionated by 12% SDS – PAGE and visualised by western blotting with anti-Flag (M2, Sigma, top panel) or anti-EGFP (Invitrogen Molecular Probes, bottom panel) antibodies. The positions of pre-stained molecular weight markers (New England Biolabs, Hitchin, UK) are indicated to the left (kDa). Lane 1, empty vector; lane 2, EWS-Oct-4B; lane 3, EWS-Oct-4B (ΔEWS); lane 4, EWS-Oct-4B (ΔCTD); and lane 5, EWS-Oct-4B (V313P).
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The stable cell lines from which the total RNAs used in the northern blot analysis were derived are shown above the panel.

**Methods.** Ethidium bromide (EtBr) staining of the agarose gel used for northern blotting shows that equal amounts of total RNA were loaded in each lane et al, 2007). These same results were obtained with two other independent clones of ZHBTc4 cells expressing EWS-Oct-4B (Figure 7C, lane 1). Thus, EWS-Oct-4B is capable of activating Oct-4 downstream target genes in vivo.

ZHBTc4 ES cells harbouring EWS-Oct-4B have tumourigenic growth potential in nude mice

Finally, we examined the ability of the EWS-Oct-4B chimeric protein to induce tumours in nude mice. Nude mice were exposed to the tetracycline analogue, doxycycline, in their drinking water for 2 weeks before injection of EWS-Oct-4B cells, and the exposure was continued thereafter. As shown in Figure 8, all six mice injected with ZHBTc4 cells expressing EWS-Oct-4B developed large tumours within a relatively short latent period, showing that EWS-Oct-4B functions as an oncogene, at least as efficiently as EWS-Oct-4 (Lee et al, 2007) or Oct-4 (Gidekel et al, 2003). The same results were obtained with two other independent clones of ZHBTc4 cells expressing EWS-Oct-4B (data not shown). These results show that, similar to EWS-Oct-4 (Lee et al, 2007), overexpression of the EWS-Oct-4B chimeric protein is sufficient to induce tumourigenesis in nude mice.

**DISCUSSION**

Chromosome translocation is an important mechanism for creating oncogenes, and is the one that occurs frequently in human neoplasms (Rabbits and Stocks, 2003). In this report, we have characterised the EWS-Oct-4B fusion protein, an alternative form of fusion between the EWS and Oct-4 genes. This product is produced by chromosome translocation and is found in two types of human epithelial tumours (Møller et al, 2008). These tumours contain a characteristic t(6;22)(p21;q12) translocation, which results in the fusion of the amino terminal domain of EWS with the carboxy-terminal domain of human Oct-4. Here, we show that the alternative fusion protein, EWS-Oct-4B, is a nuclear protein that binds DNA with a sequence specificity indistinguishable from that of the parental Oct-4 or the original EWS-Oct-4 fusion product. In addition, this fusion gene is another powerful transforming gene, although it encodes a less-potent transcriptional activator than EWS-Oct-4.

The tumourigenic potential of the chimeric EWS-Oct-4B gene product is consistent with the idea that it has a crucial role in the formation of hidradenomas or mucoepidermoid carcinomas in humans. Although the EWS-Oct-4B form is revealed as a less-potent transcrip-tional activator than EWS-Oct-4 (Figure 3), the efficient and rapid growth of ZHBTc4 ES cells expressing EWS-Oct-4B protein in nude mice indicates that EWS-Oct-4B is also a potent oncogene (Figure 8). In human cancer, genes involved in controlling cell proliferation and survival are altered in ways that change either the behaviour or the amounts of proteins they produce. The chromosome rearrangement between EWS and Oct-4 genes suggests the reactivation of silencing Oct-4 gene expression as a form of EWS-Oct-4B fusion. Accumulating evidence shows that anomalous expression of Oct-4 is involved in several human cancers. For example, the introduction of Oct-4 into Swiss 3T3 cells causes their tumourigenic transformation and produces tumours.

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in nude mice (Gidekel et al., 2003). Furthermore, several groups have reported that Oct-4 is expressed in human tumours, including testicular germ cell tumours and breast carcinomas, and has a part in human cancer development (Jin et al., 1999; Monk and Holding, 2001; Gidekel et al., 2003; Looijenga et al., 2003). In addition, the activation of Oct-4 in somatic tissues of adult mice, using a doxycycline-dependent expression system, results in dysplastic growth in epithelial tissues (Hochdinger et al., 2005), indicating that abnormal expression of Oct-4 may be the cause of tumourigenesis in somatic tissues.

Alternative splicing of human Oct-4 mRNA gives rise to two different protein isoforms designated as Oct-4A and Oct-4B (Takeda et al., 1992). These isoforms are composed of 360 and 265 amino acids, respectively, of which the 225 amino acids at the carboxy-termini are identical. The CTDs (71 amino acids) of human Oct-4A and Oct-4B are identical, but the Oct-4B POU domain (total 154 amino acids) lacks two amino acids of the N-terminal sequence of the Oct-4A POU domain (total 156 amino acids). Outside these regions, Oct-4B has little similarity to Oct-4A. The C-terminal part of EWS-Oct-4B, isolated in a hidradenoma of the skin and a mucoepidermoid carcinoma of the salivary gland (Moller et al., 2008), was found to be more similar to that of Oct-4B than to that of Oct-4A. Similar to Oct-4B, the POU DNA-binding domain of EWS-Oct-4B lacks two amino acids of the N-terminal sequence of the Oct-4A POU domain (total 156 amino acids). Outside these regions, Oct-4B has little similarity to Oct-4A.

EWS-Oct-4A is a nuclear protein (Figure 2) that binds DNA with a sequence specificity indistinguishable from that of Oct-4A (Figure 4) and shows a potent transcriptional activation potential (Figures 3 and 7). Therefore, our data imply that the DNA-binding and transactivation properties of EWS-Oct-4B and Oct-4B differ, although there are structural similarities between these two proteins.

Although the DNA-binding specificity of EWS-Oct-4B resembles that previously defined for EWS-Oct-4 (Figure 4), we show that EWS-Oct-4B is a less-potent transactivator than EWS-Oct-4 (Figure 3). According to our previous report, the EWS NTD of EWS-Oct-4 contributes to its transcriptional activation function by providing a novel activation domain (Lee et al., 2007), which suggests that the difference in transactivation potential between EWS-Oct-4B and EWS-Oct-4 could be due to differences in their EWS NTDs. Consistent with this hypothesis, the EWS (NTD)B of EWS-Oct-4B was capable of activating transcription, but was a less-potent transactivator than the EWS (NTD) of the EWS-Oct-4 fusion protein (Figure 5). The EWS NTD contains a large number of tyrosine, glutamine, alanine, serine, threonine, glycine, and proline residues, some of which are organised in a repeated and degenerate peptide motif with a frequently recurring serine–tyrosine dipeptide (NSYQQS) that shares homology with the CTD of the large subunit of eukaryotic RNA polymerase II (Delattre et al., 1992). The amino acid sequence of the EWS (NTD) does not suggest any particular discrete region that might be important for transcriptional activation (Kim et al., 1998b); thus, it may be possible that the entire EWS (NTD) is necessary for its full transactivation potential.

It remains to be determined whether the structural differences between EWS-Oct-4B and EWS-Oct-4 are clinically significant in human cancer. In the case of Ewing’s sarcoma, there are up to 18 possible types of in-frame EWS-Fli-1 fusion transcripts and most of these isotypes have been observed in vivo (Zucman et al., 1993). Interestingly, regardless of tumour site, stage, or size, the survival rate of Ewing’s sarcoma patients with type 1 EWS-Fli-1 fusion is markedly better than those in whom Ewing’s sarcoma carries other types of EWS-Fli-1 fusions (Zoubek et al., 1996; de Alava et al., 1998; Lin et al., 1999). Although we have shown that EWS-Oct-4B regulates the expression of fgf-4 and nanog, which are known potent mitogens (Figure 7), it remains to be seen whether the activation of fgf-4 or nanog through EWS-Oct-4B is sufficient to produce the neoplastic phenotype found in the two types of epithelial tumours, hidradenoma of the skin and mucoepidermoid carcinoma of salivary glands. The functional characterisations of both EWS-Oct-4 and EWS-Oct-4B isoforms in human cancers should be of potential medical interest.
incomplete mesenchymal-to-embryonic transition (Makino et al., 2009). Because EWS-Oct-4 is a nuclear protein that binds DNA with a sequence specificity indistinguishable from that of the parental Oct-4 protein (Lee et al., 2007), and because ectopic expression of Oct-4 also causes dysplasia by inhibiting progenitor cell differentiation (Hochdelinger et al., 2005), it seems reasonable to speculate that the fusion of EWS NTD to the Oct-4 DNA-binding domain may produce a transforming chimeric product that inhibits cellular differentiation. Despite its reduced activity as a transcriptional activator, EWS-Oct-4B also regulated Oct-4 downstream target genes, which include fggf-4 and nanog. It will be interesting to determine which downstream target gene(s) are critical for tumourigenesis, and whether EWS-Oct-4B collaborates with these gene(s) to generate human epithelial tumours.

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REFERENCES

Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bilic J, Pekarik V, Tiscornia G, Edel M, Boue S, Belmonte JC (2008) Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol 26: 1276 – 1284

Ambrosetti DC, Basilio C, Daley L (1997) 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 17: 6321 – 6329

Botquin V, Hess H, Fuhrmann G, Anastassiadis C, Gross MK, Friend G, Scholer HR (1998) New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2. Genes Dev 12: 2073 – 2090

Breuer M, Goldfarb DS (1990) Facilitated nuclear transport of histone H1 and other small nucleophilic proteins. Cell 60: 999 – 1008

Brehm A, Obko K, Scholer H (1997) The carboxy-terminal transcriptional domain of Oct-4 acquires cell specificity within the POU domain. Mol Cell Biol 17: 154 – 162

de Alava E, Kawai A, Healey JH, Fillman I, Myers PA, Huvos AG, Gerald WL, Jhanwar SC, Argani P, Antonescu CR, Pardon-Mindan FJ, Ginsberg J, Womer R, Lawlor ER, Wunder J, Andrusi I, Sorenson PH, Barr FG, Ladanyi M (1998) EWS-FLI1 fusion transcript structure is an independent determinant of prognosis in Ewing's sarcoma. J Clin Oncol 16: 1248 – 1255

Delattre O, Zucman J, Plougastel B, Desmazes C, Melot T, Peter M, Kovar H, Joubert I, de Jong P, Rouleau G, Aurias A, Thomas G (1992) Gene fusion and other small nucleophilic proteins. Cell 60: 162 – 165

Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Kim J, Lee KA (2007) Ewings family oncoproteins: drunk, disorderly and in search of self-renewal. J Biol Chem 282: 33554 – 33565

Lee J, Kim HK, Rho JY, Kim J (2006) The human OCT-4 isoforms differ in their ability to confer self-renewal. J Biol Chem 281: 33554 – 33565

Lee J, Kim KY, Kang KY, Kim HK, Han YM, Kim J (2007) The EWS-Oct-4 fusion gene encodes a transforming gene. Biochem J 406: 519 – 526

Lee J, Rhee BK, Bae GY, Kam HM, Kim J (2005) Stimulation of Oct-4 activity by Ewing's sarcoma protein. Stem Cells 23: 738 – 751

Lee KA (2007) Ewing's family oncoproteins: drunk, disorderly and in search of partners. Cell Res 17: 286 – 288

Lin PP, Brody RL, Hamelin AC, Bradner JE, Healey JH, Ladanyi M (1999) Differential transcriptional regulation by alternative EWS-FLI1 fusion proteins correlates with clinical heterogeneity in Ewing's sarcoma. Cancer Res 59: 1428 – 1432

Looijenga LH, Stoop H, de Leeuw HP, de Gouveia Brazao CA, Gillis AJ, van Roozendaal KE, van Zoelen EJ, Weber RF, Wolfenbuttel KP, van Dekken H, Honecker F, Bokemeyer C, Perlman EJ, Schneider DT, Kononen J, Sauter G, Gloor SB, Smart EJ, Kononen J, Sauter G, Oosterhuis JW, van den Belt-D regulate Oct-4 downstream target genes, which include fggf-4 and nanog. It will be interesting to determine which downstream target gene(s) are critical for tumourigenesis, and whether EWS-Oct-4B collaborates with these gene(s) to generate human epithelial tumours.
Maherali N, Ahfeldt T, Rigamonti A, Utikal J, Cowan C, Hochdelinger K (2008) A high-efficiency system for the generation and study of human induced pluripotent stem cells. *Cell Stem Cell* 3: 340 – 345

Makino H, Toyoda M, Matsumoto K, Saito H, Nishino K, Fukawatase Y, Machida M, Akutsu H, Uyama T, Miyagawa Y, Okita H, Kiyokawa N, Fujino T, Ishikawa Y, Nakamura T, Umezawa A (2009) Mesenchymal to embryonic incomplete transition of human cells by chimeric OCT4/3 (POUSF1) with physiological co-activator EWS. *Exp Cell Res* 315: 2727–2740

Moller E, Stenman G, Mandahl N, Hamberg H, Molne L, van den Oord JJ, Brosjo O, Mertens F, Panagopoulos I (2008) POUSF1, encoding a key regulator of stem cell pluripotency, is fused to EWSR1 in hirudinoma of the skin and mucoepidermoid carcinoma of the salivary glands. *J Pathol* 215: 78 – 86

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

Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* 26: 101 – 106

Nishimoto M, Fukushima A, Okuda A, Muramatsu M (1999) The gene for PDGF alpha-receptor 1.5 kb transcript, OCT-4, and c-KIT in human testicular germ cell tumours and for our understanding of regulatory mechanisms. *Genes Chromosomes Cancer* 27: 217 – 222

Niwa H, Miyazaki J, Smith AG (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24: 337 – 345

Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent mouse induced pluripotent stem cells. *Nature* 448: 313 – 317

Oliver RT (1999) Germ cell cancer. *Curr Opin Oncol* 11: 236 – 241

Ovitt CE, Scholer HR (1998) The molecular biology of Oct-4 in the early mouse embryo. *Exp Cell Res* 247: 631 – 640

Palumbo C, van Roozendaal K, Gillis A, de Munnik H, Okita K, Mochiduki Y, Takizawa N, Yamanaka S (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* 26: 101 – 106

Pesce M, Scholer HR (2001) Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells* 19: 271 – 278

Rabbitts TH (1999) Perspective: chromosomal translocations can affect genes controlling gene expression and differentiation – why are these functions targeted? *J Pathol* 187: 39 – 42

Rabbitts TH, Stocks MR (2003) Chromosomal translocation products engender new intracellular therapeutic technologies. *Nat Med* 9: 383 – 386

Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, Ng HH, Robson P (2005) Transcriptional regulation of nanog by OCT4 and SOX2. *J Biol Chem* 280: 24731 – 24737

Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R (2009) Parkinsson’s disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* 136: 964 – 977

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131: 861 – 872

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126: 663 – 676

Takeda J, Seino S, Bell GI (1992) Human Oct3 gene family: cDNA sequences, alternative splicing, gene organization, chromosomal location, and expression at low levels in adult tissues. *Nucleic Acids Res* 20: 4613 – 4620

Wang P, Branch DR, Bala M, Schultz GA, Goss PE, Jin T (2003) The POU homeodomain protein OCT3 as a potential transcriptional activator for fibroblast growth factor-4 (FGF-4) in human breast cancer cells. *Biochem J* 375: 199 – 205

Wernig M, Meissner A, Cassady JP, Jaenisch R (2008) c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* 2: 10 – 12

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochdelinger K, Bernstein BE, Jaenisch R (2007) *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448: 318 – 324

Yamaguchi S, Yamazaki Y, Ishikawa Y, Kagawauchi N, Mukai H, Nakamura T (2005) EWSR1 is fused to POUSF1 in a bone tumor with translocation t(6;22)(p21;q12). *Genes Chromosomes Cancer* 43: 217 – 222

Zoubek A, Dockhorn-Dworniczak B, Delattre O, Christiansen H, Niggl F, Gatterer-Menzi I, Smith TL, Jurgens H, Gadner H, Kovar H (1996) Does expression of different EWS chimeric transcripts define clinically distinct risk groups of Ewing tumor patients? *J Clin Oncol* 14: 1245 – 1251

Zucman J, Melot T, Desmaze C, Ghysdael J, Plougastel B, Peter M, Zucker JM, Triche TJ, Sheer D, Turc-Carel C, Ambros P, Combaret V, Lenoir G, Aurias A, Thomas G, Delattre O (1993) Combinatorial generation of variable fusion proteins in the Ewing family of tumours. *EMBO J* 12: 4481 – 4487