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***POU5F1*, encoding a key regulator of stem cell pluripotency, is fused to *EWSR1* in hidradenoma of the skin and mucoepidermoid carcinoma of the salivary glands**

Emely Möller^{1*}, Göran Stenman², Nils Mandahl¹, Hans Hamberg³, Lena Mölne², Joost J. van den Oord⁴, Otte Brosjö⁵, Fredrik Mertens¹, Ioannis Panagopoulos¹

¹Department of Clinical Genetics, Lund University Hospital, SE-221 85 Lund, Sweden

²The Lundberg Laboratory for Cancer Research, Department of Pathology, Göteborg University, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden

³Department of Pathology, Uppsala University Hospital, SE-751 85 Uppsala, Sweden

⁴Department of Pathology, Laboratory of Morphology and Molecular Pathology, University Hospital Leuven, Katholieke Universiteit Leuven, Minderbroedersstraat 12, B-3000 Leuven, Belgium

⁵Department of Orthopedics, Karolinska Hospital, SE-171 76 Stockholm, Sweden

***Correspondence to:** Emely Möller, Department of Clinical Genetics, Lund University Hospital, SE-221 85 Lund, Sweden.

Tel: +46-46-173398, Fax: +46-46-131061

E-mail: emely.moller@med.lu.se

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Abstract

The *EWSR1* gene is known to play a crucial role in the development of a number of different bone and soft tissue tumours, notably Ewing sarcoma. *POU5F1* is expressed during early development to maintain the totipotent status of embryonic stem and germ cells. In the present study, we report the fusion of *EWSR1* and *POU5F1* in two types of epithelial tumours: hidradenoma of the skin and mucoepidermoid carcinoma of the salivary glands. This finding not only broadens considerably the spectrum of neoplasms associated with *EWSR1* fusion genes but also strengthens the evidence for shared pathogenetic mechanisms in the development of adnexal and salivary gland tumours. Reminiscent of the previously reported fusion genes involving *EWSR1*, the identified transcript is predicted to encode a chimaeric protein consisting of the *EWSR1* amino-terminal domain and the *POU5F1* carboxy-terminal domain. We assessed the transcriptional activation potential of the chimaera compared to the wildtype proteins, as well as activation of transcription through the oct/sox composite element known to bind *POU5F1*. Among other *POU5F1* target genes, this element is present in the promoter of *NANOG* and in the distal enhancer of *POU5F1* itself. Our results show that although the chimaera is capable of significant transcriptional activation, it may in fact convey a negative regulatory effect on target genes.

Keywords: *EWSR1*, *POU5F1*, OCT-3/4, fusion gene, translocation, hidradenoma, mucoepidermoid carcinoma, dual luciferase assay, transcriptional activation

Introduction

The *EWSR1* gene at 22q12 was originally identified in the Ewing sarcoma family of tumours as part of the fusion gene *EWSR1-FLI1* created by the translocation t(11;22)(q24;q12) [1]. Subsequently, *EWSR1* has been found to be fused to a number of different genes encoding transcription factors in bone and soft tissue tumours and leukaemias [2]. In each case, the amino (NH₂)-terminal domain of *EWSR1* and the carboxy (COOH)-terminal DNA-binding domain of the partner gene form an aberrant transcription factor, the expression of which is driven by the *EWSR1* promoter [3]. The NH₂-terminal domain of *EWSR1*, containing the consensus serine-tyrosine-glutamine-glycine (SYGQQS) repeat, is believed to function as a potent transcriptional activator in the fusion products. The *EWSR1* chimaeric proteins are likely to contribute to tumorigenesis through specific deregulation of target genes depending on both the *EWSR1* transactivation domain and the DNA-binding specificity of the fusion partner [4-6].

A fusion between *EWSR1* and *POU5F1* has previously been reported in a single case of undifferentiated bone tumour of the pelvis with the translocation t(6;22)(p21;q12) [7]. *POU5F1* encodes a transcription factor which binds the octamer motif (ATGCAAAT) present in the promoter or enhancer regions of target genes [8, 9]. Murine *Pou5f1* is expressed in the embryonic stem cells (ESCs) and germ cells of the pregastrulation embryo to maintain an undifferentiated phenotype. Downregulation of *Pou5f1* during development allows cells to differentiate into somatic lineages while the germ cell lineage with maintained *Pou5f1* expression retains totipotency [10]. The level and duration of *Pou5f1* expression has been found to be tightly regulated; a critical amount of *Pou5f1* sustains the stem-cell phenotype while up- or down-regulation of expression induces divergent developmental programmes

[11]. The expression of human POU5F1 resembles the pattern in mouse, suggesting that POU5F1 plays a similar role in maintaining totipotency in human as it does in mice [12-14].

Here we report the finding of a fusion of *EWSR1* and *POU5F1* in hidradenoma of the skin and mucoepidermoid carcinoma (MEC) of the salivary glands, two epithelial tumour types previously shown to be molecularly related through the presence of a *CRTC1-MAML2* fusion gene in approximately half of the cases [15, 16]. We also assessed the transcriptional activation potential of the EWSR1-POU5F1 chimaera compared to the wildtype (wt) proteins and activation of transcription through DNA-binding sites.

Material and methods

Patients

The index case (Case 1) was a 24-year-old, previously healthy, pregnant woman who had noticed a slowly growing tumour in her left shoulder region. Following an initial, superficial excision, the tumour recurred and the patient was referred for surgery at a tertiary care center where a wide local excision was performed. The tumour measured 6 x 4 x 3.5 cm and had a mostly solid, yellowish appearance. The tumour appeared epithelial with solid sheets of poroid and cleared, well delimited cells showing moderately pleomorphic nuclei and focally, nests of tumour cells were seen to bud off into the surrounding connective tissue. Approximately half of the tumour revealed degenerative changes with cyst formation, stromal hyalinisation and calcification. Multinucleate giant cells were frequent in these parts but were absent from the solid poroid and clear cell areas. The multinucleate giant cells were concluded to most likely represent syncytial aggregates of epithelial tumor cells, demonstrating a distinct EMA-positivity around the perimeter and focally a weak cytokeratin-positivity but no

reactivity with antibodies directed towards the histiocytic marker CD 68 (clones KP-1 and PGM-1).

Small amounts of glycogen were demonstrated in the clear cells. No ductal structures were observed (Figure 1A-D). There were less than 5 mitoses per 50 high-power microscopic fields. Paraffin section immunohistochemistry was performed using an automated immunostainer (Ventana, Tucson, AZ). The tumour cells were positive for EMA and vimentin, and focally positive for pankeratin, CK5, progesterone receptor and S-100. Mononuclear as well as giant cells were negative for CD68, FXIIIa, estrogen receptor, HCG and inhibin. GCDFP-15 and CEA stains appeared negative. Based on these findings the tumour was diagnosed as a hidradenoma. Since the tumour margins appeared partly infiltrative, possible malignant potential could not be excluded (atypical hidradenoma). Immunohistochemistry was also performed using the antibody C-20 against the POU5F1 COOH-terminal domain (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Fourteen months after surgery, the patient remains disease-free.

We also had access to RNA or cDNA from frozen tumour tissue from two hidradenomas (Cases 2 and 3) and three MECs (Cases 4-6) that had previously been shown to be negative for the *CRTC1-MAML2* fusion gene [16, 17]. Case 2 was a poroid hidradenoma with few (< 10 %) clear cells and Case 3 was a solid hidradenoma with 10-15 % clear cells [17]. Cases 4-6 correspond to cases 18, 29 and 25, respectively, in [15], and were classified as poorly differentiated MECs. In addition, paraffin sections from 10 *CRTC1-MAML2*-negative hidradenomas [16] were available for interphase FISH analysis (Cases 7-16) using a probe specific for the *EWSR1* gene. These cases contained no or very few clear cells (< 10 %). The cases are summarised in Table 1. All samples were obtained after informed consent and the studies were approved by the local ethical committees.

Chromosome and FISH analyses

Cells from Case 1 were short-term cultured and metaphase preparations were made for G-banding and FISH as previously described [18]. The breakpoint on chromosome 22 was investigated using an *EWSR1* (22q12) break apart probe (Vysis, Downers Grove, IL). To characterise the breakpoint in 6p21 a series of bacterial artificial chromosome (BAC) clones, obtained from the BACPAC Resources Center at the Children's Hospital Oakland Research Institute in Oakland, CA (<http://bacpac.chori.org>), was used. In addition, to identify unequivocally chromosomes 6 and 22, whole chromosome painting (wcp) probes (Vysis) were used.

The same *EWSR1* break apart probe was also used to study interphase nuclei in paraffin sections from Cases 7-16 and Case 4. Interphase FISH was performed as previously described [16].

RT-PCR analysis

RNA was extracted from frozen samples from Cases 1-3 using the TRIzol reagent (Invitrogen, Carlsbad, CA). 2.5 µg RNA was reverse transcribed as previously described [19]. cDNA from a previous study of MECs was available from Cases 4-6 [15]. PCR to detect a putative *EWSR1-POU5F1* chimaeric transcript was performed in 50 µl of 1x PCR buffer, 0.2 mM of each dNTP, 1.25 mM MgCl₂, 0.5 µM of each of the forward and reverse primers, 1 unit of Platinum *Taq* DNA polymerase (Invitrogen), and 1 µl cDNA. The primers EWS-306F forward and POU5F1-1137R reverse were used in the first round of PCR and the primers EWS-366F forward and POU5F1-1001R reverse in the second round (Supplementary Table S1). The PCR was run on a PCT-200 DNA engine (Bio-rad, Hercules, CA) with the cycling profile of initial denaturation for 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C. PCR products were purified from agarose gels and directly

sequenced using the Big Dye terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster city, CA), on an ABI-3100 Avant genetic analyser (Applied Biosystems). Sequences were analysed through searching for homologous regions (BLASTN) in the genome database (GenBank).

Construction of reporter gene plasmids

PCR amplifications and sequence verifications were performed as previously described [20]. The full length *EWSR1* wt (Image ID: 3449145, Invitrogen), *POU5F1* wt 1 (Image ID: 40125986, RZPD, Berlin, Germany) and *POU5F1* wt 2 (Image ID: 4717277, RZPD) cDNA clones were sequenced and used as templates in subsequent PCR amplifications using the primers EWS-44FSalI, EWS-1997REagI, POU5F1wt1-51FSalI, POU5F1wt2-84FSalI and POU5F1wt-864REagI, respectively, to obtain the open reading frames (Table S1). The full length *EWSR1-POU5F1* transcript was attained using the cDNA fragment from the index case (amplified with RT-PCR) together with parts of the *EWSR1* wt and *POU5F1* wt 1 clones; a description of this procedure is available as supplementary material. For the transcription activation assay, the cDNA fragments coding for the (1) full length *EWSR1* wt, (2) full length *POU5F1* wt 1, (3) full length *POU5F1* wt 2, and (4) chimaeric *EWSR1-POU5F1* proteins were inserted in-frame between the *SalI* and *BstZI* restriction sites of the pBIND vector (Promega, Madison, WI) (Figure 2A).

To study transcriptional activation through DNA-binding sites, the GAL4 DNA-binding domains were removed from the pBIND-based constructs by *NheI* and *SalI* digestion, filled in with T4 DNA polymerase and self-ligated. The *NANOG* proximal promoter and the *POU5F1* distal enhancer fragments, both containing the oct-sox composite element [21, 22] were amplified from a healthy individual using the primers NANOGprom1F-KpnI, NANOGprom2R-BglII, POU5F1prom5F-KpnI and POU5F1prom6R-SphI, respectively

(Table S1). The *NANOG* promoter was cloned between the *KpnI* and *BglII* sites of the pGL4.10 [*luc2*] vector (Promega) and the *POU5F1* enhancer between the *KpnI* and *SphI* sites of the pGL4.13 [*luc2*/SV40] vector (Promega) (Figure 2B).

Transfection experiments

HeLa (human cervix carcinoma, DSMZ no. ACC 57) cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10 % fetal bovine serum (FBS), 4 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. The PolyFect transfection reagent (Qiagen, Valencia, CA) was used for transfection according to the manufacturer's instructions. Cells were seeded at a density of 2×10^4 cells/well in 96-well plates and 24 h later transfected with the appropriate plasmid combinations using 1 µl of PolyFect transfection reagent/well. Control plasmids were transfected together with the test constructs in each 96-well plate. The luciferase activity was quantified 48 h after transfection. For the transcription activation assay, 50 ng of pBIND plasmid DNA, empty or with insert, was transfected together with 200 ng of the pG5*luc* vector (Promega). To analyse transcription activation through DNA-binding sites, cells were transfected with 50 ng of pBIND-based construct lacking the GAL4 DNA-binding domain (empty or with insert) together with 200 ng of the pGL4.10-*NANOG* promoter, the pGL4.13-*POU5F1* enhancer or the control pGL4.13-SV40 promoter plasmids.

The cell lysis and luciferase measurements were performed as previously described [20]. Each construct was measured in at least eight replicas. The results are presented as medians together with the 25th and 75th percentiles. The Mann-Whitney two-tailed test was used for the statistical analysis using the statistiXL software version 1.6

(<http://www.statistixl.com>).

Results

Mapping of the t(6;22) translocation breakpoints

Analysis of G-banded metaphase spreads from Case 1 revealed the following karyotype: 46,XX,t(6;22)(p21;q12)[7]/46,XX[3] (Figure 3A). FISH analysis of the two breakpoints showed that the break in 22q12 had occurred within the region covered by the break apart probe flanking the *EWSRI* locus (Figure 3B), indicating that this gene was rearranged through the translocation. The breakpoint in 6p21 was investigated with a series of BAC probes until a split signal hybridising to both derivative chromosomes was obtained with BAC probe CTD-2534O14 (data not shown), identifying *POU5F1* as a good candidate for further molecular genetic analysis.

Interphase FISH with the *EWSRI* break apart probe on paraffin sections from 10 additional hidradenomas (Cases 7-16) and one MEC (Case 4) detected split signals, indicative of disruption of the *EWSRI* gene, in two of the hidradenomas (Case 8 is shown in Figure 3C) and in Case 4 (data not shown). The two positive hidradenomas had been noted for after 1 and 10 years, respectively. The frequency of nuclei showing split signals varied between 10 and 20 %.

Expression of the *EWSRI-POU5F1* chimaera

The RT-PCR analysis of the tumour material using primers EWS-306F and POU5F1-1137R in the first round and EWS-366F and POU5F1-1001R in the second round amplified a DNA fragment of approximately 800 bp in all three (Cases 1-3) investigated hidradenomas and in one (Case 4) of the three MECs (Figure 4A). Sequence analysis revealed that the detected fragment corresponds to an *EWSRI-POU5F1* chimaeric transcript in which a part of

exon 6 of *EWSR1* is fused in-frame to exon 2 of *POU5F1* (Figure 4B). The same fusion transcript was seen in all four positive cases. The cases with available RNA (Cases 1-3) were individually analysed with RT-PCR to eliminate the risk of contamination.

POU5F1 immunohistochemistry on paraffin sections from Case 1 demonstrated strong nuclear and a comparatively weaker cytoplasmic staining of the tumour cells (Figure 1E and F).

Transcriptional activation potentials of EWSR1-POU5F1, EWSR1 and POU5F1

The transfected EWSR1-POU5F1 chimaera fused to GAL4 activated the reporter expression (Figure 2C), indicating that the chimaera contains an activation domain. The chimaera and EWSR1 wt activated transcription more than POU5F1 wt 1 and 2, respectively ($P < 0.001$), and activation by POU5F1 wt 1 was stronger than by POU5F1 wt 2 ($P < 0.001$). We found no difference in activity between EWSR1 wt and EWSR1-POU5F1.

In the experiment with the *NANOG* promoter, both EWSR1 wt and POU5F1 wt 1 activated the luciferase expression stronger than EWSR1-POU5F1 and POU5F1 wt 2, respectively ($P < 0.001$), and EWSR1-POU5F1 activated the expression more than POU5F1 wt 2 ($P < 0.001$) (Figure 2D). In the experiment with the *POU5F1* distal enhancer, POU5F1 wt 1 activated transcription most efficiently ($P < 0.001$), followed by EWSR1 wt, EWSR1-POU5F1 and POU5F1 wt 2 (Figure 2E). In all experiments, POU5F1 wt 1 activated the luciferase expression more than POU5F1 wt 2 ($P < 0.001$).

Discussion

We have identified the fusion of *EWSR1* and *POU5F1* and *EWSR1* rearrangements in five hidradenomas of the skin and in one salivary gland MEC. Although clinically distinct, these two tumour types have earlier been shown to display significant biologic and genetic similarities; according to previous, admittedly still limited, molecular studies, close to half of hidradenomas and MECs display the same *CRTC1-MAML2* fusion gene [15, 16, 23]. According to the results presented here, it seems as if a large proportion of the cases that are negative for this fusion gene instead shares an *EWSR1-POU5F1* chimera. Furthermore, there are histopathologic similarities between the two entities. Hidradenoma, which is a benign adnexal tumour primarily affecting adults, is composed of different cell types, the relative frequencies of which may vary from lesion to lesion [24]. When clear cells, containing abundant glycogen, predominate the tumour is often referred to as clear cell hidradenoma. In a previous study on 20 cases of hidradenoma, the *CRTC1-MAML2* fusion was particularly frequent in this subtype of hidradenoma; all 10 fusion-positive cases contained clear cells, whereas they were absent or very few in 10 fusion-negative cases [16]. It has been suggested that clear cell variants of hidradenoma are of apocrine derivation and that variants predominated by poroid and cuticular cells (poroid hidradenoma) are of eccrine derivation. Possibly, hidradenomas may transform into hidradenocarcinomas [25]. Previous studies have also suggested that nuclear pleomorphism and the occurrence of multinucleate giant cells, as seen in Case 1, may indicate a potential for local recurrence or malignant change in these tumours [26].

Like hidradenoma, MEC, which is the most common malignant salivary gland tumour, most frequently occurs in middle-aged adults and consists of three cell types that may be present in varying proportions: epidermoid, mucus-forming and intermediate cells [27]. Tumours in which epidermoid cells predominate have been associated with high-grade lesions. Also in MECs, a strong relationship between morphologic and clinical features and

fusion gene status has emerged; tumours with the *CRTC1-MAML2* chimaera are, on average, smaller, occur in younger individuals, are more common in low-grade lesions, and are associated with better outcome [15, 28].

Apart from the index case (Case 1), which was included because of its karyotypic features, the hidradenomas and MECs investigated in the present report were selected on the basis of being negative for the *CRTC1-MAML2* fusion [15-17]. Thus, the finding of an *EWSR1-POU5F1* chimaera in substantial subsets of these tumours indicates that the creation of these two fusion genes constitutes two important, alternative molecular pathways in hidradenoma and MEC tumourigenesis. All three hidradenomas from which RNA was available were positive for the fusion, and at least 2 of the 10 cases that could be studied by interphase FISH had *EWSR1* rearrangements. We can, of course, not exclude that these cases harbour variant *EWSR1* fusion genes with other 3'-partners than *POU5F1*. However, the interphase FISH analysis may have underestimated the true incidence of *EWSR1* rearrangements, as the quality of some of the available slides was suboptimal. Thus, our results indicate that at least 20% of hidradenomas carry a fusion gene in which *EWSR1* constitutes the 5'-part; the frequency of the *EWSR1-POU5F1* fusion in MECs is more difficult to predict since only three cases, one of which was positive, were studied. Furthermore, our results strongly indicate that the *EWSR1-POU5F1* fusion is associated with the less well differentiated variants of both hidradenoma and MEC.

Hidradenoma is thought to be closely related to another adnexal tumour, poroma. No cytogenetic data exist for this tumour, but one karyotype from its malignant counterpart, porocarcinoma, has been published, and also this case contained a translocation affecting the long arm of chromosome 22, suggesting that *EWSR1* may be involved in more subtypes of appendageal tumours [29]. Unfortunately, there was no material for further analyses from that case.

In all the *EWSR1-POU5F1* fusion-positive cases, the breakpoint in *EWSR1* was in exon 6, fusing a part of this exon in-frame to exon 2 of *POU5F1*. In the previously reported case of undifferentiated bone tumour with *EWSR1-POU5F1* fusion, the breakpoint in *EWSR1* was in intron 6 and the breakpoint in *POU5F1* was in exon 1 [7]. Nevertheless, in both events, the NH₂-terminal domain of *EWSR1* is fused to the part of *POU5F1* which contains the DNA-binding domains. The *EWSR1-POU5F1* chimaera is thus predicted to resemble other *EWSR1* fusion proteins. Chimaeric *EWSR1* transcripts where the breakpoints split the exons of one, or both, of the involved genes, have been described before [30, 31] and may be explained by the usage of intra-exonic cryptic splice sites during pre-mRNA processing.

EWSR1 fusion proteins have previously been suggested to be potent transcriptional activators [3] and we therefore evaluated the transcriptional activation potential of the *EWSR1-POU5F1* chimaera compared to the wt proteins using a GAL4 DNA-binding domain fusion model. Our results show that the *EWSR1-POU5F1* chimaera activates transcription stronger than the *POU5F1* wt variants, and as strongly as wt *EWSR1*. Similar to other *EWSR1* fusions, it seems reasonable to assume that the transactivation property is derived from the *EWSR1* NH₂-terminal domain and that it may be involved in deregulating genes targeted by the chimaera.

We cloned the *NANOG* proximal promoter and the *POU5F1* distal enhancer fragments, both containing the conserved pou5f1/sox composite element known to bind *POU5F1* [21, 22], in order to estimate the activation of reporter gene expression through this site. In both experiments, *EWSR1* wt and *POU5F1* wt 1 activated expression significantly stronger than the *EWSR1-POU5F1* chimaera and *POU5F1* wt 2. Based on these findings, it seems likely that the *EWSR1-POU5F1* chimaera would act through the *POU5F1* enhancer or *NANOG* promoter to down-regulate expression (as compared to *POU5F1* wt 1) *in vivo*, resembling the *EWSR1-ATF1* chimaera which has been found to activate some promoters

with ATF1-binding sites while repressing others [32]. In a recent study by Lee *et al.*, the transcriptional activation potential of the EWSR1-POU5F1 chimaera, through the pou5f1-binding site, was compared to that of wt POU5F1, and the chimaera was found to activate transcription more strongly than wt POU5F1 [33]. However, in that study a reporter plasmid carrying 10 copies of the pou5f1-binding site was used whereas we utilised the *NANOG* proximal promoter and the *POU5F1* distal enhancer, resembling the *in vivo* scenario. Moreover, we included EWSR1 wt, yielding the interesting result that EWSR1 wt seems to activate transcription more efficiently than the chimaera.

EWSR1 lacks obvious DNA-binding motifs [1] and may exert its transcriptional regulatory effects on the *POU5F1* enhancer and *NANOG* promoter through interaction with DNA-binding proteins. In fact, POU5F1 has been identified as an EWSR1-interacting partner [34]. In all our experiments, especially when using the *NANOG* promoter and *POU5F1* enhancer constructs, POU5F1 wt 1 activated transcription more efficiently than POU5F1 wt 2 which is in agreement with previous findings [35]. The two *POU5F1* wt variants differ in their 5'-portions, suggesting that the NH₂-terminal domain not retained in POU5F1 wt 2 or in the chimaera may be important for transcriptional activation.

POU5F1 reactivation has been found to be implicated in human tumours; aberrant *POU5F1* expression was detected in germ cell tumours [36, 37] and recently in bladder cancer [38]. Furthermore, ectopic *Pou5f1* expression in adult mice resulted in dysplastic growth of epithelial tissues [39]. However, analyses of *POU5F1* expression in human adult tissues and cancers have been complicated by the presence of several *POU5F1* pseudogenes which may cause experimental bias and therefore need to be treated with caution [40]. Bearing in mind the findings of the present study, it is also of interest to note that murine epidermal basal keratinocytes transiently transfected with *Pou5f1* can differentiate into neuronal cells, suggesting that aberrant expression of *Pou5f1* is sufficient for reverting

differentiated somatic cells into more developmentally potent cells [41]. Also, the factors Pou5f1, Sox2, c-Myc and Klf4 can induce the reprogramming of murine fibroblasts into ES-like cells with activated endogenous *Pou5f1* and *Nanog* expression [42, 43]. These four factors, or alternatively Pou5f1, sox2, Nanog and Lin28, were subsequently found to induce the analogous reprogramming of human somatic cells [44, 45]. Translocation could be a mechanism of *POU5F1* reactivation, causing inappropriate expression of the POU5F1 DNA-binding domains. It is tempting to postulate that this reactivation may cause cells to acquire an undifferentiated phenotype as a mechanism of tumourigenesis. While POU5F1 has been frequently associated with the maintenance of an undifferentiated phenotype, the *CTRC1-MAML2* fusion protein was initially associated with activation of Notch signalling and subsequently found to induce genes regulated by the cAMP/CREB pathway [23, 46, 47]. Possibly, alternative pathways in hidradenoma and MEC might contribute to the distinct behaviour of *CTRC1-MAML2* fusion-positive and -negative tumours.

In summary, our results show that an *EWSR1-POU5F1* fusion gene is present in hidradenoma as well as in MEC, providing further evidence for a genetic link between these two tumour types. Furthermore, it appears that this fusion gene is more common in less well differentiated variants of the tumours, suggesting that it may be of prognostic significance; this aspect, however, requires analysis of larger tumour series. It also remains to be investigated whether the two fusion genes – *CTRC1-MAML2* and *EWSR1-POU5F1* – affect the same or different cellular pathways.

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Figure legends

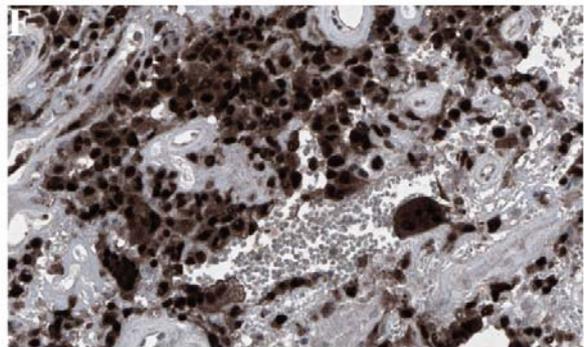
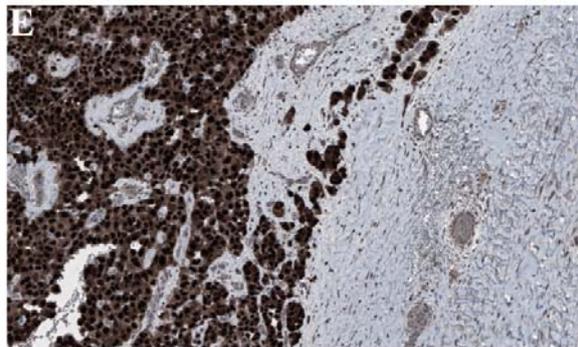
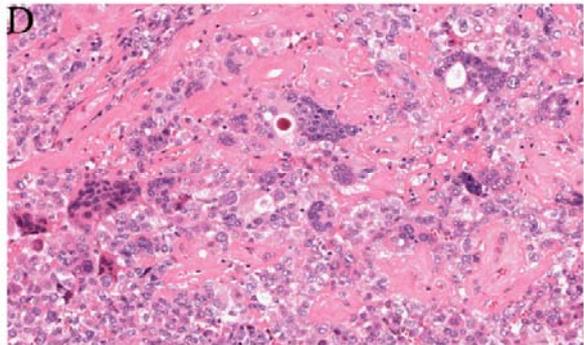
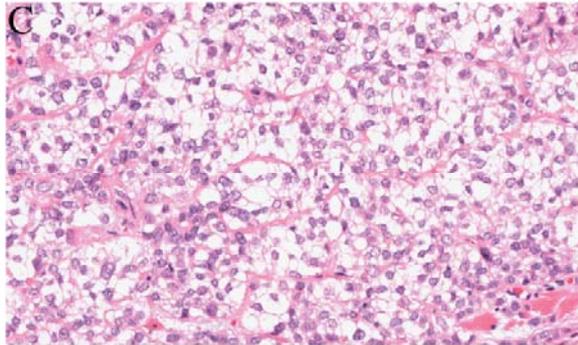
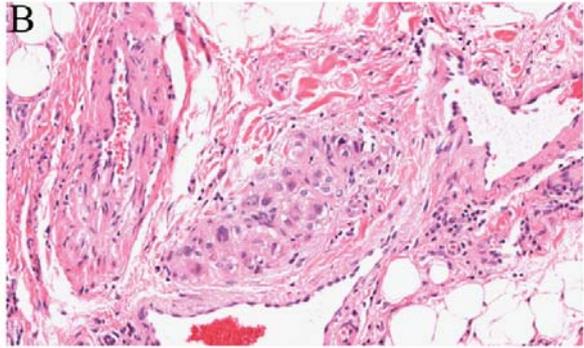
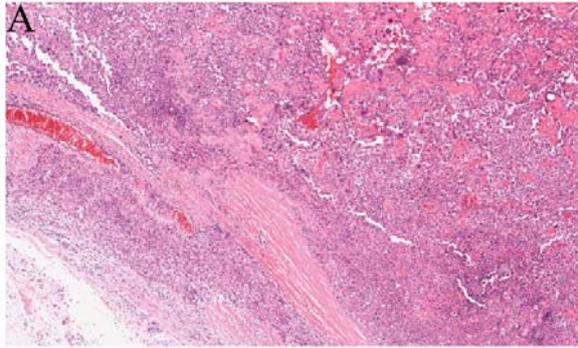
Figure 1. Morphologic appearance (Hematoxylin & Eosin) of hidradenoma with t(6;12)(p21;q12) and *EWSR1-POU5F1* gene fusion. (A) Section with capsular invasion and hyalinised central parts; (B) A small aggregate of tumour cells outside the tumour capsule; (C) Clear cell component; (D) Multinucleate giant cells in the central part of the tumour. Immunohistochemical stain for POU5F1; (E) A strong nuclear and a comparatively weaker cytoplasmic staining in a peripheral area of the tumour, note unstained epithelium of preexisting hair follicles (lower right); (F) In central parts of the tumour, multinucleated giant cells appear equally strongly stained as the mononuclear epithelial cells.

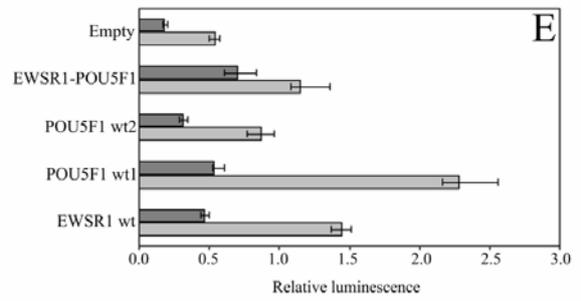
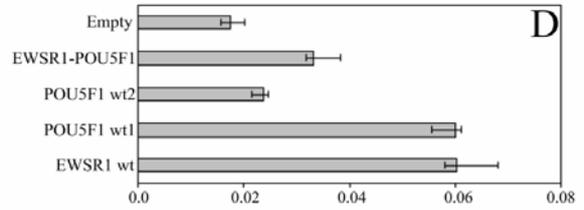
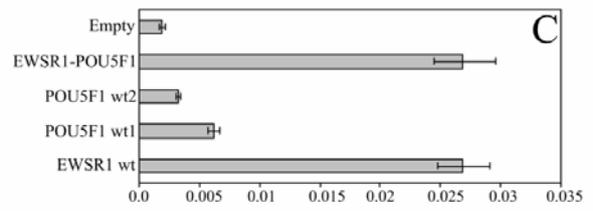
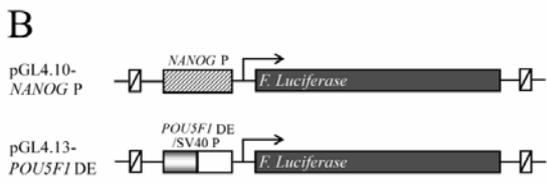
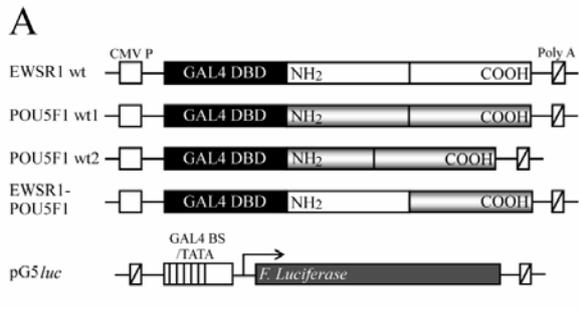
Figure 2. The transcriptional activation potential of the chimaera compared to the wt proteins and transcription activation through DNA-binding sites. (A) The pBIND-based constructs expressing *EWSR1* wt (656 aa), *POU5F1* wt 1 (360 aa), *POU5F1* wt 2 (265 aa) or *EWSR1-POU5F1* (390 aa) fused to the GAL4 DNA-binding domain (GAL4 DBD). The predicted *EWSR1-POU5F1* chimaera contains 174 aa of the *EWSR1* NH₂-terminal transactivation domain and 225 aa of the *POU5F1* COOH-terminal DNA-binding domains. The constructs were cotransfected with vector pG5*luc* that contain GAL4 binding sites (GAL4 BS). (B) The pBIND-based constructs, with GAL4 removed, were cotransfected with luciferase reporter constructs containing either the *NANOG* proximal promoter (*NANOG* P) or the *POU5F1* distal enhancer (*POU5F1* DE) together with the SV40 promoter (SV40 P). (C) *EWSR1* wt and the *EWSR1-POU5F1* chimaera are equally strong as transcriptional activators and 4.5 to 9 times stronger than the *POU5F1* wt 1 and 2, respectively ($P < 0.001$). Empty vector pBIND contains the GAL4 DNA-binding domain. (D) Through the *NANOG* promoter, *EWSR1* wt and *POU5F1* wt 1 activated the luciferase expression equally efficiently and 1.8 to 2.5 times stronger than *EWSR1-POU5F1* and *POU5F1* wt 2, respectively ($P < 0.001$). (E) Through the

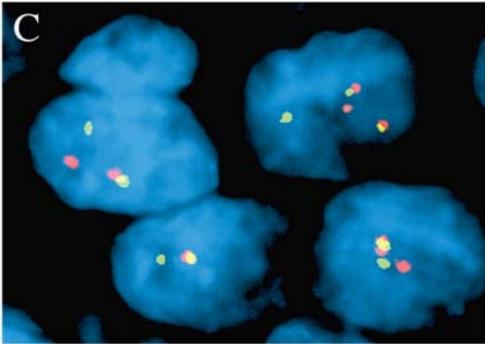
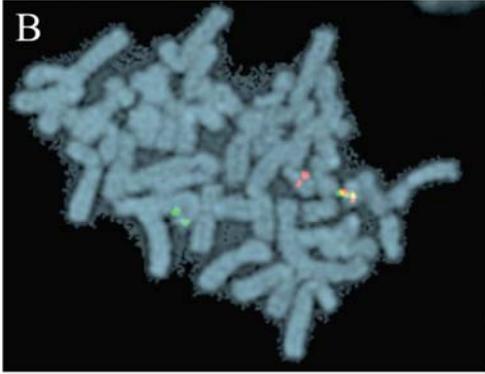
POU5F1 enhancer, *POU5F1* wt 1 activated transcription most efficiently ($P < 0.001$), 2 times stronger than the *EWSR1-POU5F1* chimaera, followed by *EWSR1* wt. Light grey bars represent the transcriptional activity from the *POU5F1* distal enhancer together with SV40 promoter and dark grey bars the SV40 promoter alone.

Figure 3. Chromosome and FISH analyses. (A) Partial karyotype showing the t(6;22)(p21;q12) in Case 1, the breakpoints are indicated with arrows. FISH on (B) metaphase spreads from Case 1 and on (C) interphase nuclei from Case 8 using a break apart probe for the *EWSR1* gene showing intact (red and green signals co-localized) and split (separated red and green signals) probes.

Figure 4. RT-PCR analysis for the *EWSR1-POU5F1* chimaeric transcript. (A) Gel electrophoresis depicting fragments of approximately 800 bp detected in Cases 1-4 using primers EWS-306F and POU5F1-1137R in the first round and EWS-366F and POU5F1-1001R in the second round. Chimaeric transcripts were not detected in Cases 5 and 6. (B) Partial chromatogram showing the *EWSR1-POU5F1* junctions (arrow) and the predicted amino acid sequence of the chimaeric transcripts where a part of *EWSR1* exon 6 is fused in-frame to *POU5F1* exon 2. (C) The quality of the cDNA synthesis was examined by amplification of 300 bp *ABL* cDNA fragments as previously described [19]. M = 1 kbp ladder.







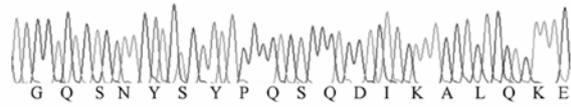
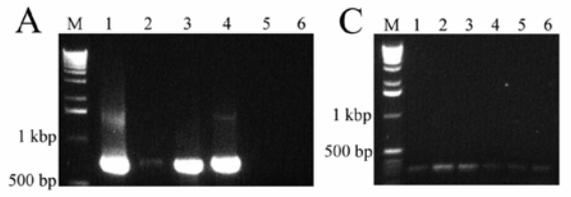


Table 1: Clinical-Pathological data on hidradenoma and MEC cases

Case	Age/ Gender	Tumour site	Tumour size (cm)	Histology	<i>EWSR1-POU5F1</i> fusion transcript	<i>EWSR1</i> FISH ²	Clinical outcome
1	24/F	Shoulder	6	Hidradenoma	+	+	NED 2 years
2	63/F	Axilla	0.5	Poroid Hidradenoma	+	ND	NED 4 years
3	83/F	Head	ND	Solid Hidradenoma	+	ND	No follow-up ³
4 ¹	85/M	Parotid gland	3.5	PD MEC	+	+	DoC 1 year
5 ¹	73/M	Submandibular gland	2.1	PD MEC	-	ND	LR 0.6, 1, and 2 years; lymph node mets 0 and 1.1 years; mets floor of mouth 0.9 years; DoD 5 years
6 ¹	89/F	Oral gland	2	PD MEC	-	ND	DoD 4 months
7	79/F	Thigh	1.7	Hidradenoma	ND	+	No follow-up
8	33/M	Head	ND	Hidradenoma	ND	+	No follow-up
9-16	ND	ND	ND	Hidradenoma	ND	-	No follow-up

¹ Cases published previously in Behboudi *et al* 2006 [15] (denoted case no. 18, 29 and 25, respectively)

² The presence of an *EWSR1* split signal is denoted +, absence of *EWSR1* split -

³ As hidradenoma is a benign tumour, no formal follow-up is required

Abbreviations: NED, no evidence of disease; ND, not determined; PD, poorly differentiated; MEC, mucoepidermoid carcinoma; DoC, dead of other causes; LR, local recurrence; mets, metastases; DoD dead of disease.