Oral Diseases (2008) 14, 144–149. doi:10.1111/j.1601-0825.2007.01360.x

http://www.blackwellmunksgaard.com

ORIGINAL ARTICLE

ESE-1 inhibits the invasion of oral squamous cell carcinoma in conjunction with MMP-9 suppression

S Iwai[1], S Amekawa[1], K Yomogida[2], T Sumi[1], M Nakazawa[1], Y Yura[1], Y Nishimune[2], M Nozaki[2]

[1]*Department of Oral and Maxillofacial Surgery II, Faculty of Dentistry;* [2]*Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*

OBJECTIVES: Matrix metalloproteinases (MMPs) regulated by ets transcription factors facilitate carcinoma cell invasion. An ets family member, ESE-1, is expressed specifically in epithelial tissues, but its association with MMPs is obscure. In this study, we investigated whether ESE-1 regulates invasion of oral squamous cell carcinoma (SCC) via transcriptional activity of MMP-9.

METHODS: HSC-3 and KB were used as human oral SCC lines. The expression of ESE-1 and MMP-9 was detected by *in situ* hybridization and immunohistochemistry. Invasion assay, gelatin zymography and Northern blotting were used to detect the invasion activity, the gelatinolytic activity and the expression of MMP-9 in the ESE-1 transfectants. Luciferase assays and mutation analysis were used for the transcriptional analysis of MMP-9 promoter region by ESE-1.

RESULTS: ESE-1 was expressed in the intermediate layer but not in the invasive area, in which MMP-9 was expressed, in the oral SCC tissues. ESE-1 suppressed invasion activity and 92 kDa gelatinolytic activity in HSC-3 as a result of transfection. ESE-1 regulates MMP-9 expression in a negative manner and the ets binding site on the MMP-9 promoter contributed to suppression by ESE-1.

CONCLUSIONS: These findings indicate that ESE-1 negatively regulates the invasion of oral SCC via transcriptional suppression of MMP-9.

Oral Diseases (2008) **14**, 144–149

Keywords: ESE-1; transcription factor; invasion; MMP-9; oral squamous cell carcinoma

Introduction

Matrix metalloproteinases (MMPs), which are a highly regulated superfamily of enzymes that degrade almost all extracellular matrix and basement membrane components play a key role as enzymes in cancer progression (Liotta *et al*, 1980). Two of these, a 92-kDa type IV collagenase (MMP-9) and 72-kDa type IV collagenase (MMP-2) could degrade type IV collagen, and may contribute to tumor cell invasion by facilitating the degradation of type IV collagen-containing basement membrane (Hurwitz *et al*, 1993; Bernhard *et al*, 1994). The regulation of MMP-9 protein levels has been ascribed to transcriptional activation of the gene, reduced mRNA turnover and altered translational efficiency. The 5′-flanking sequence contains binding sites for AP-1, NFκB, SP-1 and ets transcription factors within the first 670 bp, and these have been implicated in the regulation of MMP-9 gene expression by means of a variety of extracellular signals (Gum *et al*, 1996).

The ets family comprises more than 30 transcription factors that share a highly conserved DNA binding ets domain (Donaldson *et al*, 1994). ESE-1 is the prototype member of a subclass of ets transcription factors (Oettgen *et al*, 1997), which play a role during keratinocyte differentiation in normal epithelia by activating some keratinocyte differentiation-relating genes, including EndoA, SPRR2A, SPRR1A and SPRR3 (Oettgen *et al*, 1997; Stark *et al*, 1998; Fisher *et al*, 1999).

ESE-1 is also expressed in some carcinoma cell lines such as HeLa cervical carcinoma cells, T84 colon carcinoma cells and A431 vulvar carcinoma cells (Oettgen *et al*, 1997), but its function in cancer cells remains unclear. In this study we investigated the expression of ESE-1 and MMP-9 in human oral squamous cell carcinoma (SCC), and showed that ESE-1 reduced the invasive activity of the oral SCC cell line via suppression of MMP-9 expression.

Materials and methods

Construction of recombinant plasmids and probes

Human ESE-1 open reading frame (ORF) was amplified by RT-PCR using total RNA of PC-3 cells. A 1.2 kb cDNA fragment of human ESE-1 (1-1128) was amplified with PCR primers (5′-AGTGAATTCGCCACCA

Correspondence: Masami Nozaki, Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan. Tel:/fax: +81 6 6879 8339, E-mail: mnozaki@biken.osaka-u.ac.jp
Received 20 May 2006; revised 11 October 2006; accepted 28 November 2006

TGGCTGCAACCTGTGAGATTAGC-3′ and 5′-GTCAGATCTCCCTCAGTTCCGACTCTGGA-3′) digested with *Eco*RI and *Bgl*II, and inserted into the *Eco*RI/*Bgl*II site of the pSG5 eucaryote expression vector (ESE-1/pSG5). MMP9 wt-Luc was constructed by ligating a 723-bp *Xba*I fragment (−670 to +53) of MMP9-CAT to the pGL3-luciferase reporter gene (Promega, Madison, WI, USA). MMP9mut-Luc was mutated by GGAA into MMP9wt-Luc containing the ets binding site (EBS) (−543 to −533) to GATA by PCR.

In situ *hybridization (ISH)*
A 1.2-kb ESE-1 cDNA fragment digested with *Eco*RI and *Bgl*II from ESE-1/pSG5 plasmids was inserted into the *Eco*RI/*Bam*HI site of Bluescript(II)-SK(−) (Stratagene, Cedar Creek, TX, USA) (ESE-1/pBS). ESE-1/pBS was linearized with *Eco*RI (antisense) or *Xba*I (sense) digestion. ISH was performed with the digoxigenin-labeled Riboprobe system (Roche Diagnostics GmbH, Mannheim, Germany) as described previously (Hirota *et al*, 1992). After hybridization, the bound probe was detected with anti-digoxigenin-Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim).

Cell culture and transfection
Established cell lines originated from human oral SCC cell lines HSC-3 (Momose *et al*, 1986) and KB (Eagle, 1955) and a human melanoma cell line RPMI7951 (Nakahara *et al*, 1997) were used for the present study. The cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (FCS). KB and HSC-3 cell lines were transfected with ESE-1/pSG5 using the lipofection method (Lipofectamine Plus® reagent; Invitrogen, Tokyo, Japan). Following incubation with lipofectamine for 15 min, cells were incubated for 48 h, harvested and used for assay. The efficiency of the transient transfection was 30–50% evaluated by the rate of green fluorescent protein (GFP)-positive cells transfected with the pCAG-enhanced green fluorescent protein (EGFP) construct.

Invasion assay
Cellular *in vitro* invasive properties were analyzed using the BioCoat matrigel invasion chambers (pore size 8 μm; Becton Dickinson, Franklin Lakes, NJ, USA). The procedure was essentially as that described by Albini *et al* (1987). Cells that had penetrated through the lower surface of the filter were counted. Each assay was done in triplicate. Statistical analysis was performed with Student's *t*-test.

Zymography
Zymography was performed as described elsewhere (Johansson and Smedsrod, 1986). SDS-PAGE was performed with 0.1% gelatin and 12.5% polyacrylamide in the gels. After electrophoresis, the gel was washed and stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA). Gelatinase activity was visualized as areas of clear bands in the blue gels.

RNA isolation and Northern blot analysis
Total RNA was prepared from cultured cells using the TRIsol reagent (Invitrogen). RNA (20 μg) was resolved on a 1% agarose gel containing 6.7% formaldehyde, transferred to a Zeta-probe blotting membrane (Bio-Rad Laboratories, Tokyo, Japan), hybridized to [α−^{32}P]dCTP-labeled ESE-1 and MMP-9 cDNA probes, exposed and analyzed with a Bio-image Analyzer (BAS2000; Fuji Photo Film Co. Ltd). Human ESE-1 cDNA probes (1.2 kb) were generated from restriction fragments with *Eco*RI/*Bgl*II of ESE-1/pSG5 vectors. Human MMP-9 cDNA probes (0.4 kb) were generated by RT-PCR with a 5′primer (5′-CTGAATTCATTGACGACGCCTTGC-3′) and a 3′primer (5′-ACGGATCCGTGTCGTAGTTGGCCGT-3′) from HSC-3 RNA.

Luciferase assay
MMP9wt-Luc or MMP9mut-Luc was co-transected with various doses of the ESE-1/pSG5 expression vector into the cells. Luciferase activity was measured with the Dual-Luciferase Reporter assay system (Promega). Renilla luciferase activity was used for standardization of transfection efficiency. Statistical analysis was performed with Student's *t*-test.

Immunohistochemistry
Specimens were surgically removed from 10 patients with oral SCC. They were collected at the Oral and Maxillofacial Surgery II, Osaka University Hospital. In 10 cases, eight were moderately differentiated SCCs and two were well-differentiated SCCs, six originated from the lower gingiva and four originated from the tongue. A standard immunohistochemical technique was used for the detection of MMP-9 proteins with anti-human MMP-9 mouse monoclonal antibody (clone 56-2A4; Fuji Yakuhin Co., Ltd, Saitama, Japan). Tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned to 8 μm thickness. Sections were incubated with the primary antibodies 56-2A4 diluted at 1:250 in phosphate-buffered saline, washed in the same buffer, and incubated with biotinylated secondary anti mouse IgG followed by the avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA). The reactions were developed with diaminobenzidene (DAB) and counterstained with methylgreen.

Results

Expression of ESE-1 and MMP-9 in oral SCC tissues
We first investigated the expression of ESE-1 and MMP-9 in oral SCCs ($n = 10$) by using ISH and immunostaining, respectively. In all cases, ESE-1 mRNA was detected in the intermediate layer of carcinoma cells (Figure 1c). Conversely, MMP-9 was detected in the invasive front, but not in the intermediate layer (Figure 1b). These findings show that the expression of ESE-1 and MMP-9 were mutually exclusive in carcinomas. This suggests that downregulation of transcription factor ESE-1 is related to MMP-9 expression as well as to invasiveness.

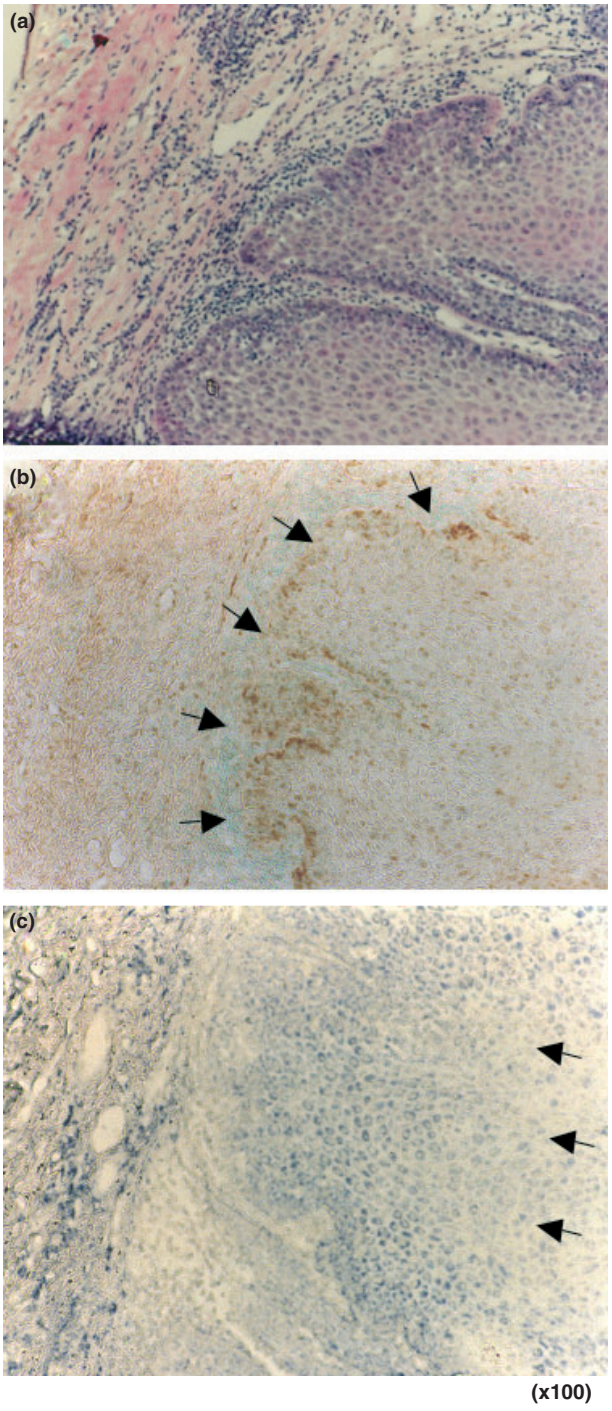

Figure 1 Expression of ESE-1 and MMP-9 in oral SCC tissues. Two independent samples of oral SCC tissues (hematoxylin and eosin staining). (**a**) Oral SCC have invaded stromal tissues. MMP-9 proteins were immunohistochemically detected in the invasive front by using the monoclonal antibody against MMP-9 (**b**). ESE-1 mRNA was detected in the intermediate layer and not in the invasive front (arrows) by *in situ* hybridization with an antisense probe for ESE-1 (**c**) (original magnification: ×100)

Invasion activity of ESE-1 mRNA transfectants

To examine the role of ESE-1 on invasion, we performed matrigel invasion assays using transfection of the ESE-1 expression vector into the HSC-3 cell line,

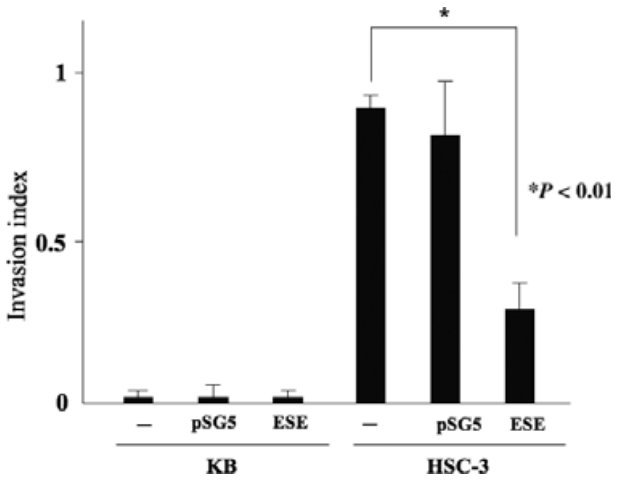

Figure 2 Invasion reduced by ESE-1 transfection. Invasion indices for the matrigel invasion assay of parental cells (KB and HSC-3) and of mock transfectants pSG5 and ESE-1/pSG5 transfectants (ESE) are shown. Cells were cultured on the matrigel-coated membrane of a culture insert and incubated at 37 °C in 5% CO_2 for 48 h. Cells that penetrated through the matrigel membrane were counted. The experiment was repeated three times. The invasion index is indicated on the left. Statistical analysis was performed with Student's *t*-test ($P < 0.01$)

which is highly invasive, and the KB cell line, which is highly noninvasive (Figure 2). The number of HSC-3 cells penetrating the matrigel membrane was significantly reduced by the overexpression of ESE-1 ($P < 0.01$). On the other hand, the number of penetrating KB cells was low and not changed by ESE-1/pSG5 transfection (Figure 2). Expression of ESE-1 mRNA was low in each of the parental cell lines and extremely high in transfectants. No morphological change was detected in these transfectants (data not shown). These results demonstrated that ESE-1 expression suppressed the invasiveness of HSC-3 cells in matrigel.

Gelatin zymography analysis of MMP-9 and MMP-2, and expression of MMP-9 mRNA

To investigate whether ESE-1 transfectants reduce the capacity to degrade type IV collagen, which is a main component of matrigel, MMP release from ESE-1 transfected HSC-3 into conditioned media was assayed by gelatin zymography. Parental HSC-3 cells released 92 kDa type IV collagenase (Pro-MMP-9) at a high and 72 kDa type IV collagenase (Pro-MMP-2) at a low level. ESE-1 transfection reduced the gelatinolytic activity of Pro-MMP-9 as well as Pro-MMP-2 (Figure 3). No gelatinolysis was detected in KB with or without transfection of ESE-1 (Figure 3). RPMI7951 released 92 kDa type IV collagenase (Pro-MMP-9) and 72 kDa type IV collagenase (Pro-MMP-2) were used as positive controls. Northern blot analysis was used to investigate whether a decline in the transcriptional level of the MMP-9 gene contributes to a decrease in the enzyme activity of MMP-9 in HSC-3 cells. The expression level of MMP-9 mRNA in HSC-3 cells overexpressing ESE-1

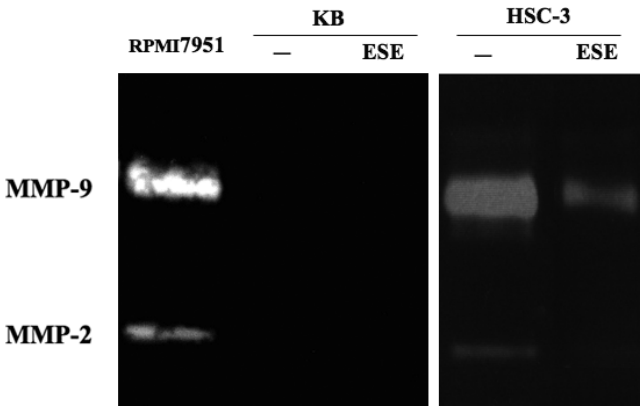

Figure 3 Gelatin zymography analysis of MMP-9 and MMP-2 in parental cells (KB and HSC-3) and ESE-1 transfectants (ESE). Equal amount (20 μg) of samples was electrophoresed on SDS-PAGE gels containing gelatin (0.1%) as a substrate for direct enzyme activity. RPMI7951 cells were used for positive control. Pro-MMP-9 and Pro-MMP-2 were detected at 92 and 72 kDa

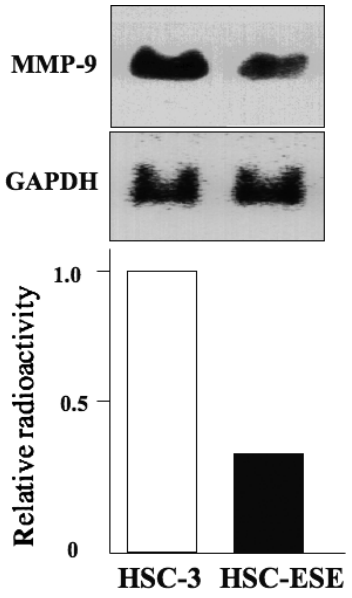

Figure 4 Expression of MMP-9 mRNA in parental HSC-3 cells and ESE-1 transfectants (HSC-ESE). Upper panels show the results of Northern blotting, and the lower panel relative radiogating, which was obtained by dividing the intensity of MMP-9 mRNA in each sample by the intensity of GAPDH mRNA for the internal control

decreased 30% compared with that of parental HSC-3 cells (Figure 4).

Transcriptional control of MMP-9 promoter by ESE-1

The MMP-9 gene promoter contains several regulation motifs including the EBS (Figure 5a). To examine the possibility that ESE-1 suppresses MMP-9 through this EBS, we performed co-transfection experiments using ESE-1 expression vector and Luciferase reporter constructs which are driven by wild type or mutated EBS MMP-9 promoters (Figure 5a). Luciferase activity of the wild-type construct was significantly reduced by

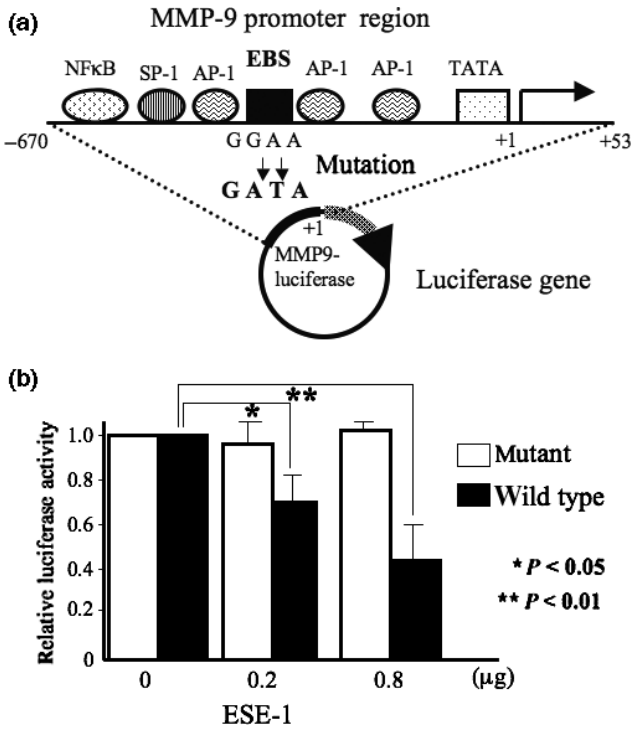

Figure 5 MMP-9 promoter activity is reduced by ESE-1 through EBS. (**a**) Construction of MMP9wt-Luc and MMP9mut-Luc. For studying the effects of ESE-1 on MMP-9 gene promoter activity, the 5′-upstream region (−670 to +53) of the human MMP-9 gene was ligated to the pGL3 luciferase reporter plasmid. Several transcription binding sites including EBS, AP-1, SP-1 and NFkB were present in this region. To confirm the role of EBS, nucleotide substitutions were introduced into the sequence (GGAA to GATA) in the MMP-9 promoter. (**b**) Suppression of MMP-9 promoter activity by ESE-1. 0.1 μg of the reporter plasmid or mutant plasmid was co-transfected with 0–0.8 μg of ESE-1 expression plasmid, and luciferase activity expressed in the cells 48 h after transfection was measured. Transcription from the promoter was significantly downregulated in ESE-1 in a dose-dependent manner (black bar). The mutant did not respond to ESE-1 stimulation (white bar). The standard deviations of the various experiments are indicated by error bars. Statistical analysis was performed with Student's *t*-test (*$P < 0.05$, **$P < 0.01$)

ESE-1 transfection in a dose-dependent manner. The mutant reporter construct, on the other hand, did not respond to ESE-1 (Figure 5b).

Discussion

In this study, we examined the expression of ESE-1 and MMP-9 not only in the oral SCC cell line, but also in oral SCC tissues. ESE-1 mRNA was detected in the intermediate layer as described previously (Oettgen *et al*, 1997), but not in the basal cell layer of the normal oral epithelium. This indicates that ESE-1 has a biological function in cell differentiation and stratification in the oral epithelium. In the invasive oral SCC, ESE-1 mRNA was expressed in the intermediate layer and not in the invasive front, where MMP-9 was detected. These results show that ESE-1 and MMP-9 are exclusive in their expression in oral SCC. Matrigel invasion was reduced by ESE-1 overexpression, and

gelatin zymography and Northern blotting indicated that ESE-1 suppresses the MMP-9 enzyme activity through reduction in the amount of mRNA. On the other hand, adhesion capacity and motility are thought to be factors influencing matrigel invasion activity in addition to proteolytic enzyme activity (Fenrich *et al*, 1999). However, the morphology of ESE-1 transfectants and parental cells did not differ. The invasion index was standardized with the number of cells passing through the non-coated chamber. Taken together, these findings suggest that the reduction in invasion by ESE-1 is mostly caused by the downregulation of MMP-9 gene expression. Mutation analysis of the MMP-9 promoter showed that ESE-1 suppressed the transcription of the MMP-9 gene through EBS. In addition to direct MMP-9 regulation, however, another mechanism remains possible. The expression of the tissue inhibitor of metalloproteinase-1 (TIMP-1), which is an inhibitor of MMPs, is controlled by several DNA response elements that respond to variations in the level and activity of AP-1 and ets transcriptional regulatory proteins (Logan *et al*, 1996). Accordingly, we could not exclude the possibility that ESE-1 also has a positive effect at least in part on the expression of the TIMP-1 gene, of which TIMP-1 suppresses the enzyme activity of MMP-9. We postulated that the reduction in ESE-1 expression could result in upregulation of the invasive capacity of tumor cells. This hypothesis may be useful for investigating the expression of ESE-1 in oral leukoplakia with several levels of dysplasia, as an indication of the presence of premalignant lesions. ESE-1 is specifically expressed in epithelial cells (Oettgen *et al*, 1997). These findings indicate that ESE-1 may activate many genes and sometimes suppress gene expression in various epithelial cells. In this study ESE-1 suppressed the MMP-9 promoter through EBS binding (Figure 5). ESE-1 also suppressed the keratin 4 gene promoter in an EBS-independent manner in esophageal squamous epithelial cells and in HeLa cells. In the study, the pointed domain in ESE-1 N-terminus is shown to act as a transcriptional suppressor motif (Brembeck *et al*, 2000). Another ets factor, TEL, acts as a transcriptional suppressor. This suppression is mediated by interaction with the mSin3A co-suppressor through the pointed domain (Fenrich *et al*, 1999). TEL also interacts with Fli-1 and inhibits Fli-1-mediated transactivation on the GPIX promoter (Kwiatkowski *et al*, 1998). It is possible that the pointed domain of ESE-1 interacts with a co-suppressor or another ets factor, which mediates MMP-9 promoter suppression. Under these conditions, E1AF, an ets family transcription factor, is expressed in oral SCC cells (Hanzawa *et al*, 2000) and at the invasive front in colorectal cancer (Horiuchi *et al*, 2003). E1AF can upregulate the promoter activities of several MMP genes, including MMP-9. ESE-1 may thus compete with E1AF for EBS and inhibit MMP-9 promoter activity, when ESE-1 is expressed in the HSC-3 cell line or in the invasive front of oral SCC. The potency of the ESE-1 protein may depend on particular promoter and cell targets.

Taken together, these results indicate that ESE-1 suppressed the transcription of the MMP-9 gene through EBS, which in turn inhibited the invasion of oral SCC line HSC-3 in conjugation with MMP-9 suppression.

References

Albini A, Iwamoto Y, Kleinman HK *et al* (1987). A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res* **47:** 3239–3245.

Bernhard EJ, Gruber SB, Muschel RJ (1994). Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. *Proc Nat Acad Sci USA* **91:** 4293–4297.

Brembeck FH, Opitz OG, Libermann TA *et al* (2000). Dual function of the epithelial specific ets transcription factor, ELF3, in modulating differentiation. *Oncogene* **19:** 1941–1949.

Donaldson LW, Petersen JM, Graves BJ *et al* (1994). Secondary structure of the ETS domain places murine Ets-1in the superfamily of winged helix-turn-helix DNA-binding proteins. *Biochemistry* **33:** 13509–13516.

Eagle H (1955). Propagation in fluid medium of a human epidermoid carcinoma, strain KB. *Proc Soc Exp Biol Med* **89:** 362–364.

Fenrich R, Amann JM, Lutterbach B *et al* (1999). TEL and AML-1 contribute repression domains to the t (12;21) fusion protein. *Mol Cell Biol* **19:** 6566–6574.

Fisher DF, Stark MW, Lehtola MM *et al* (1999). Structure and evolution of the human SPRR3 gene: implications for function and regulation. *Genomics* **55:** 88–99.

Gum R, Lengyel E, Juarez J *et al* (1996). Stimulation of 92-kDa gelatinase B promoter activity by ras is mitogen-activated protein kinase kinase 1-dependent and requires multiple transcription factor binding sites including closely spaced PEA3/ets and AP-1 sequences. *J Biol Chem* **271:** 10672–10680.

Hanzawa M, Shindoh M, Higashino F *et al* (2000). Hepatocyte growth factor upregulates E1AF that induces oral squamous cell carcinoma cell invasion by activating matrix metalloprotease genes. *Carcinogenesis* **21:** 1079–1085.

Hirota S, Ito A, Morii E *et al* (1992). Localization of mRNA for c-kit receptor and its ligand in the brain of adult rats: an analysis using in situ hybridization histochemistry. *Brain Res Mol Brain Res* **15:** 47–54.

Horiuchi S, Yamamoto H, Min Y *et al* (2003). Association of ets-related transcriptional factor E1AF expression with tumour progression and overexpression of MMP-1 and matrilysin in human colorectal cancer. *J Pathol* **200:** 568–576.

Hurwitz A, Dushink M, Solomon H *et al* (1993). Cytokine-mediated regulation of rat ovarian function: interleukin-1 stimulates the accumulation of 92-kilodalton gelatinase. *Endocrinology* **132:** 2709–2714.

Johansson S, Smedsrod B (1986). Identification of a plasma gelatinase in preparations of fibronectin. *J Biol Chem* **261:** 4363–4366.

Kwiatkowski BA, Bastian LS, Bauer TR *et al* (1998). The ets family member Tel binds to the Fli-1 oncoprotein and inhibits its transcriptional activity. *J Biol Chem* **273:** 17525–17530.

Liotta LA, Tryggvason K, Gaevisa S *et al* (1980). Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* **284:** 67–68.

Logan SK, Garabedian MJ, Campbell CE *et al* (1996). Synergistic transcriptional activation of the tissue inhibitor of metalloproteinases-1 promoter via functional interaction of AP-1 and Ets-1 transcription factors. *J Biol Chem* **271:** 774–782.

Momose F, Hirata T, Araida N *et al* (1986). The characterization of three cell lines derived from oral squamous cell carcinomas. *J Jap Stomatol Soc* **35:** 485–495.

Nakahara H, Howard L, Thompson EW *et al* (1997). Transmembrane/cytoplasmic domain-mediated membrane type 1-matrix metalloprotease docking to invadopodia is required for cell invasion. *Proc Natl Acad Sci U S A* **94:** 7959–7964.

Oettgen P, Alani RM, Barcinski MA *et al* (1997). Isolation and characterization of a novel epithelium-specific transcription factor, ESE-1, a member of the *ets* family. *Mol Cell Biol* **17:** 4419–4433.

Stark MWJ, Fisher DF, Meijer E *et al* (1998). AP-1 and Ets transcription factors regulate the expression of the human SPRR1A keratinocyte terminal differentiation marker. *J Biol Chem* **273:** 24683–24692.