

## **E1AF, an ets-oncogene family transcription factor.**

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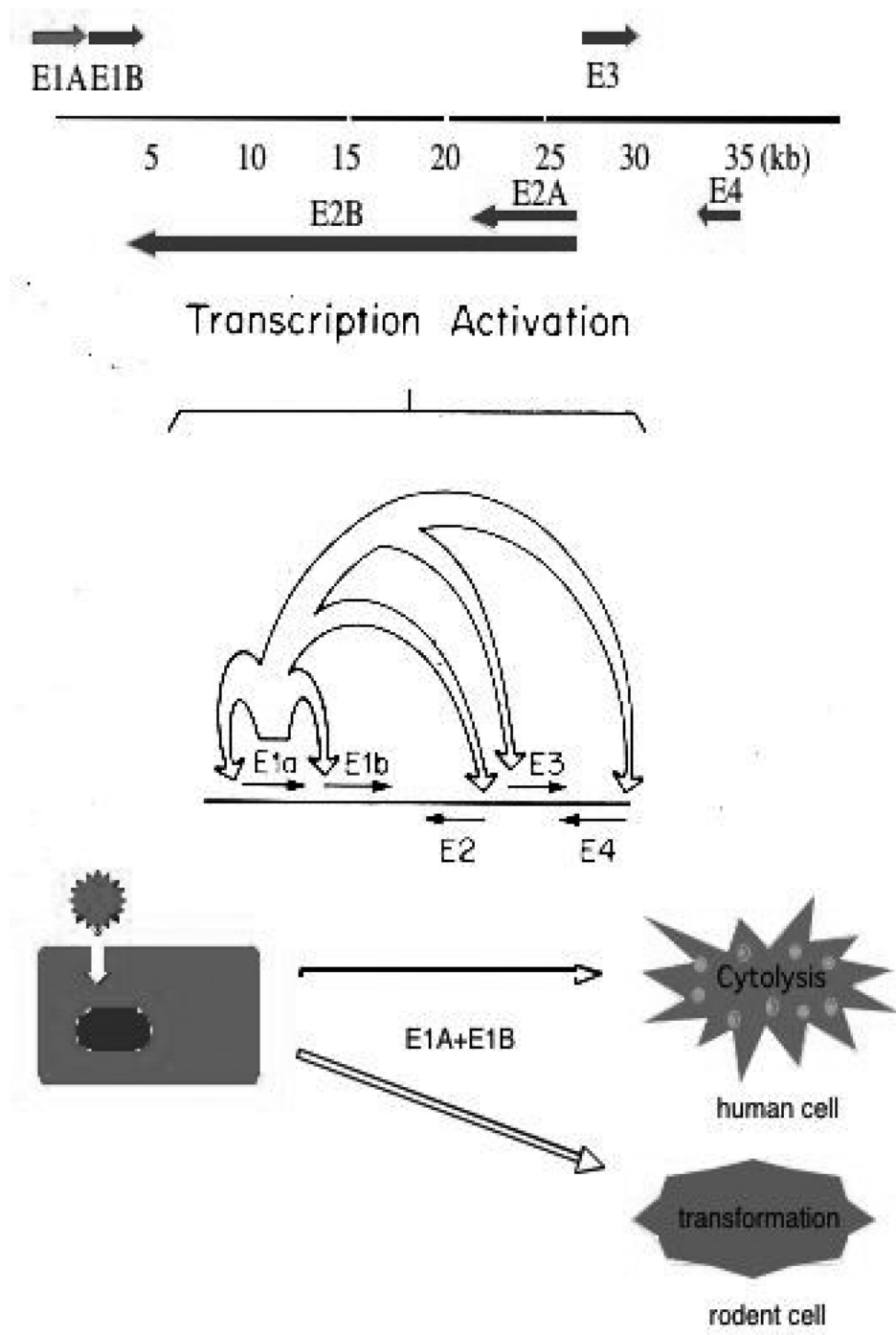
### **Abstract**

E1AF is an ets-oncogene family transcription factor. E1AF was shown to upregulate multiple matrix metalloproteinase (MMP) genes and contribute to the malignant phenotype of cancer cells by inducing invasive and metastatic activities. E1AF is upregulated by hepatocyte growth factor (HGF) stimulation, which indicates that E1AF would participate in cell motility by HGF/scatter factor. On the other hand, E1AF upregulates p21<sup>waf1/cip1</sup> to induce cell cycle arrest when cells are exposed to stress. EWS/ETS fusions are frequently observed in Ewing's sarcoma, and we have revealed that EWS/ETS chimeric protein activates telomerase activity by upregulating hTERT. However, substitution ets binding site (EBS) mutants did not affect the responsiveness to EWS/E1AF. DNA-IP assay showed that the complexes contained EWS/E1AF bound to the hTERT promoter, which suggested that EWS/ETS functions as a co-activator for TERT transcription. Our findings that EWS/ETS acts as a transcriptional co-factor may imply that the transcription pathway is regulated by the interaction of transcription factors.

### **1. Introduction**

Adenoviruses are double-stranded DNA viruses that have been investigated as a model of carcinogenesis. E1A and E1B were identified as potent viral oncogenes that have the ability to inhibit the anti-oncogene function of Rb and p53, respectively [1]. Adenoviruses have been shown to be oncogenic viruses in rodent cells; however, adenoviruses cause infectious lesions in human beings by cytolytic activity with proliferation. The adenovirus E1A gene is the first transcribed unit in productive infection, and E1A was shown to activate other viral early and late genes (Fig.1). Thus, E1A has a critical role in adenovirus infection in human cells[1]. E1AF is an ets-oncogene family transcription factor that was cloned by the ability to bind the adenovirus E1A enhancer element [2]. The deduced amino acid sequence has revealed that E1AF has a functional acidic domain, glutamine-rich domain and ets domain. The ets domain is a unique amino acid sequence that is a feature of the ets-oncogene family. The ets domain of E1AF is situated in its C-terminal with 85 amino acids that have a helix-loop-helix structure with a tryptophane repeat and it has the

ability to bind the enhancer region of target genes as a GGAA/T core motif. The acidic domain and glutamine-rich domain are thought to activate transcriptional ability [Higashino, 1993 #12]. Over 30 species of the ets-family have been identified, and E1AF is categorized as a PEA 3 member with ER81 and ERK [3]. E1AF was shown to be located on 17q21, and chromosomal translocation of E1AF was identified in Ewing's sarcoma and undifferentiated sarcoma that had a chimera gene between EWS and E1AF [4, 5]; thus an oncogenic property of E1AF is proposed. We have been investigating the role and function of E1AF.



**Fig. 1** Structure and function of adenovirus. Adenovirus encodes the early transcribed genes of E1A, E1B, E2A, E2B, E3 and E4 . E1A regulates other early region gene transcription.

E1A and E1B co-operate to transform rodent cells whereas adenovirus causes cytolysis in infected human cells.

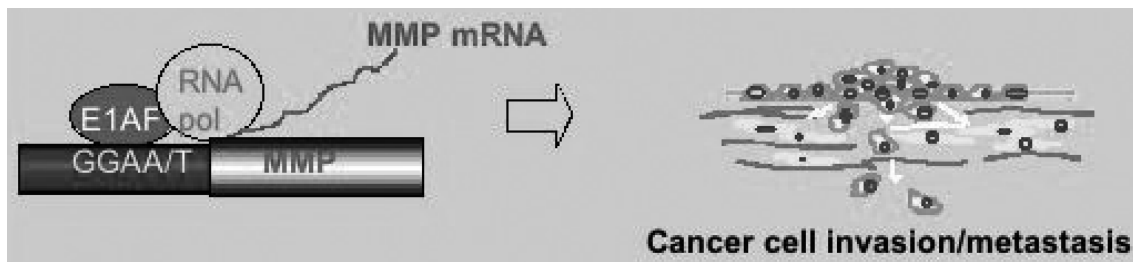
## 2. E1AF can upregulate matrix metalloproteinase genes that contribute to the invasive/metastatic phenotype of cancer cells.

Matrix metalloproteinase (MMP) has been shown to have a potent role in helping cancer cells to invade through extracellular matrix and to form metastatic lesions. MMP is a family of proteinases that have the structural features of an N-terminal propeptide domain, a zinc-coordinating active site and a C-terminal hemopexin-like domain. MMP is comprised of over 20 types and divided into subgroups as archetypal MMPs (collagenases: MMP-1, -8, -13, stromelysins: MMP-3, -10, other MMPs: MMP-12, -19, -20, -27), matrilysins (MMP-7, -26), gelatinases (MMP-2, -9) and convertase-activatable MMPs (secreted: MMP-11, -21, -28, membrane-types: MT1, MT2, MT3, MT4, MT5, MT6, MMP-23) [6]. Generally, MMPs are synthesized as a proform, and enzyme activation is achieved by removal of the N-terminal propeptide domain through exogenous or autocatalytic cleavage [6, 7]. The transcription regulatory regions of MMP genes often contain *ets* binding motifs, in addition to other transcription factors such as AP1, SP1 and NFkB [8]. It has been reported that *c-ets-1* and *c-ets-2* activate the rat stromelysin promoter through two different elements [9]. Ets-binding site/PEA3 (polyomavirus enhancer activator 3) acts synergistically with AP-1-binding site/TRE (TPA responsive element) to achieve induction of MMP-1 and MMP-3 transcription by TPA (12-O-tetradecanoyl-phorbol-13-acetate) and several oncogenes [10].

We investigated the transcriptional activity of E1AF on MMP-1, -3, and -9 genes in transient expression assays. E1AF can up-regulate promoter activities of MMP-1, -3 and -9 in a dose-dependent manner [11]. HSC3, a highly invasive oral squamous cell carcinoma-derived cell line, was shown to have correlative E1AF and MMP-1, -9 expression [12], and transfection of the E1AF expression vector into MCF7, a weakly invasive human breast cancer cell line, results in induction of invasive and motile activities accompanied by an increase of 92 kDa type IV collagenase (MMP-9) gene expression [13]. We reconstructed an E1AF antisense expression vector, transfected HSC3 cells with the vector and obtained HSC3AS cells that expressed E1AF antisense RNA. HSC3AS showed decreasing mRNA and protein levels of MMP-1, -3 and -9. Moreover, HSC3AS showed lower invasive potential in *in vitro* three-dimensional raft culture and *in vivo* implantation into nude mice [14]. These results imply that E1AF positively participates in epithelial cancer cell invasion by upregulating MMP genes.

The correlation of E1AF expression and cancer cell malignancies in *in vivo* tumors was also shown. E1AF expression in resected tumors of non-small cell lung carcinomas (NSCLCs) was analyzed by Northern blot and *in situ* hybridization and it was found that 12 of 19 tumors expressed E1AF mRNA while normal lung tissue and concomitant normal cells within tumors did not [15]. Twenty-seven oral squamous cell carcinoma (SCC) patients were examined using RT-PCR, Southern blot hybridization and *in situ* hybridization (ISH). E1AF mRNA was detected in 15 of the 27, and 13 of 17 E1AF positive SCCs showed invasive phenotype, whereas the majority of SCCs not expressing E1AF showed an expansive growth pattern. Increased prevalence of E1AF-positive oral SCC was observed in cases with nodal metastasis [16]. These

results indicate that E1AF may be involved in cancer cell malignancies through its ability to promote invasive potential (Fig. 2).



**Fig. 2** E1AF correlates with cancer cell invasion/matastasis with matrix metalloproteinases (MMPs) upregulation.

### **3. E1AF participates in HGF (hepatocyte growth factor) signaling.**

HGF is known as the scatter factor, and is thought to play a role in cell motility and invasion [17]. We examined the effect of HGF on E1AF and MMP gene expression in terms of the invasive potential of the oral squamous cell carcinoma cell line HSC3. HGF stimulated expression of the E1AF gene. The levels of MMP-1, MMP-3 and MMP-9 mRNAs increased in cells treated with HGF and correlated with E1AF upregulation. In contrast, no obvious upregulation of MMP-1 and MMP-9 mRNA was observed in ASE1AFHSC3 cells transfected with the antisense E1AF expression vector into parental HSC3 cells. The wild-type MMP-9 gene promoter was activated by endogenous E1AF in HSC3 cells, and chloramphenicol acetyltransferase (CAT) activity increased when HGF was added to transfected cells. On the other hand, CAT activity was reduced to almost two-thirds of the wild-type activity when HSC3 cells were transfected with a CAT reporter plasmid driven by a mutant MMP-9 promoter lacking the Ets-binding site, and induction of CAT activity was not observed upon addition of HGF [18]. These results suggest that HGF induces expression of the Ets-related E1AF transcription factor gene whose product in turn activates MMP genes and leads to oral cancer cell invasion.

We introduced the E1AF gene into VMRC-LCD and NCI-H226, non-small cell lung carcinoma (NSCLC) cell lines lacking E1AF expression, and examined the role of E1AF in HGF stimulation. HGF stimulated the motile and invasive activities in E1AF-transfected VMRC-LCD and NCI-H226 cells but not in their parental or vector-transfected control cells. HGF induced expression of urokinase-type plasminogen activator (uPA) genes specifically in E1AF-transfected cells [15].

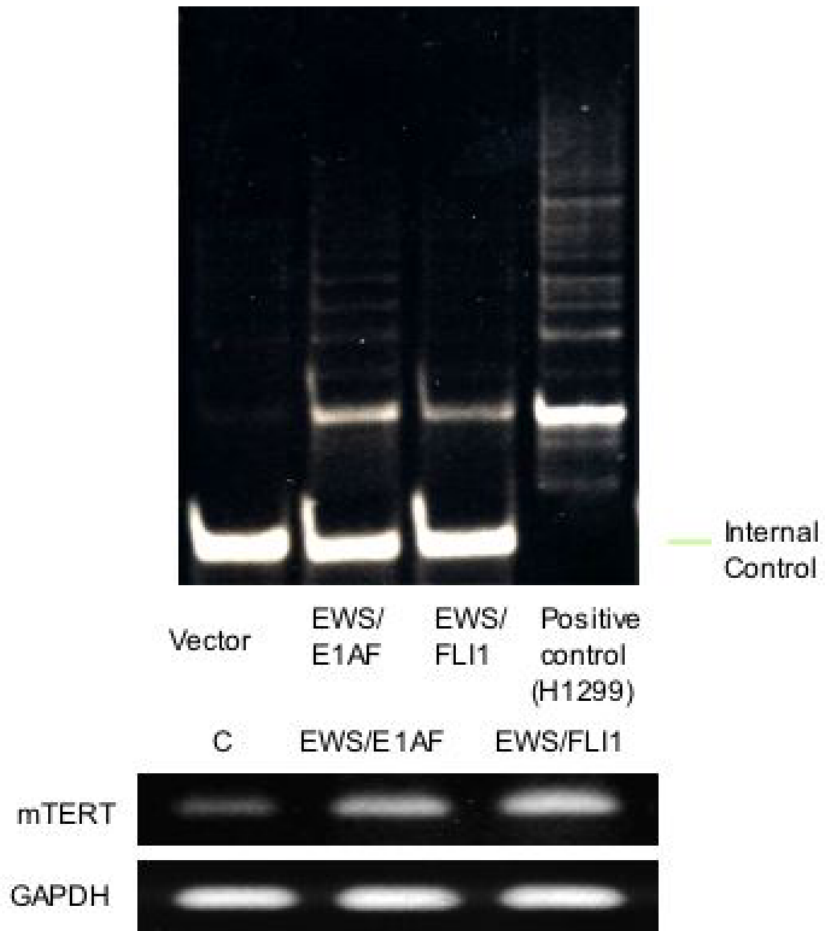
These findings suggest that E1AF plays a substantial role in the cell motility and invasion of oral SCCs and NSCLCs.

### **4. EWS/ETS fusions activate telomerase activity to induce cell immortalization.**

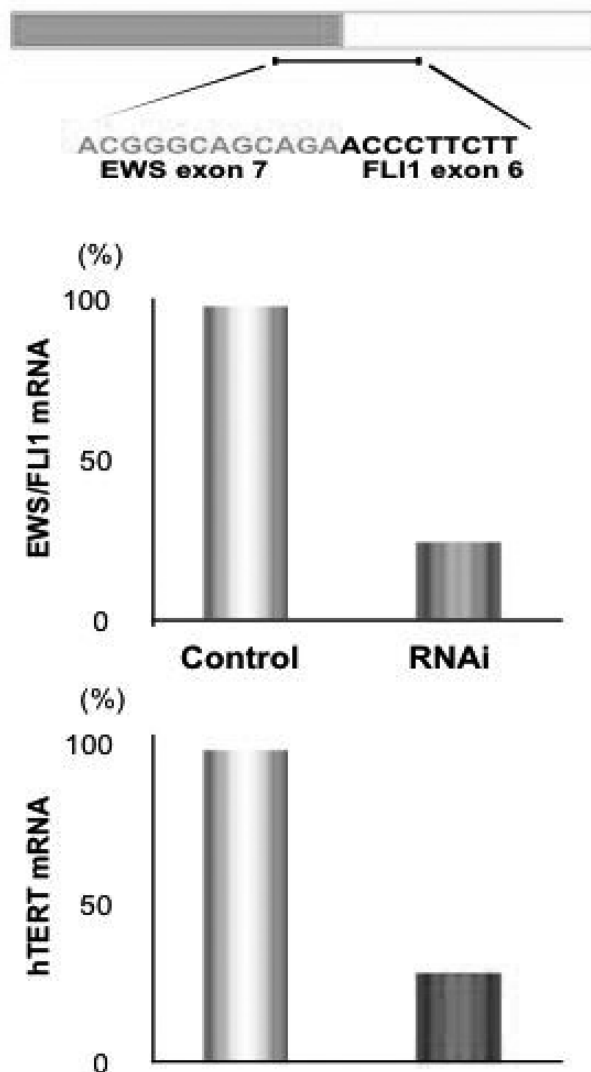
Ewing's sarcoma is an aggressive bone neoplasia mostly occurring in young adults and adolescents. It appears as undifferentiated small round tumor cells that are thought to derive from neural crest progenitors. These tumors are

characterized by specific chromosomal translocations wherein the EWS gene on chromosome 22 is fused to one of five members of the ETS gene family (FLI1, ERG, ETV1/ER81, E1AF/PEA3, FEV) [19]. These translocations produce five chimeric proteins that include the N-terminal transactivation domain of EWS and the C-terminal DNA binding domain of ETS family transcription factors [20]. EWS/E1AF was found to be the gene responsible for tumorigenesis in Ewing's sarcoma; however, the transforming activity and the tumorigenic mechanism of EWS/E1AF have remained unclear.

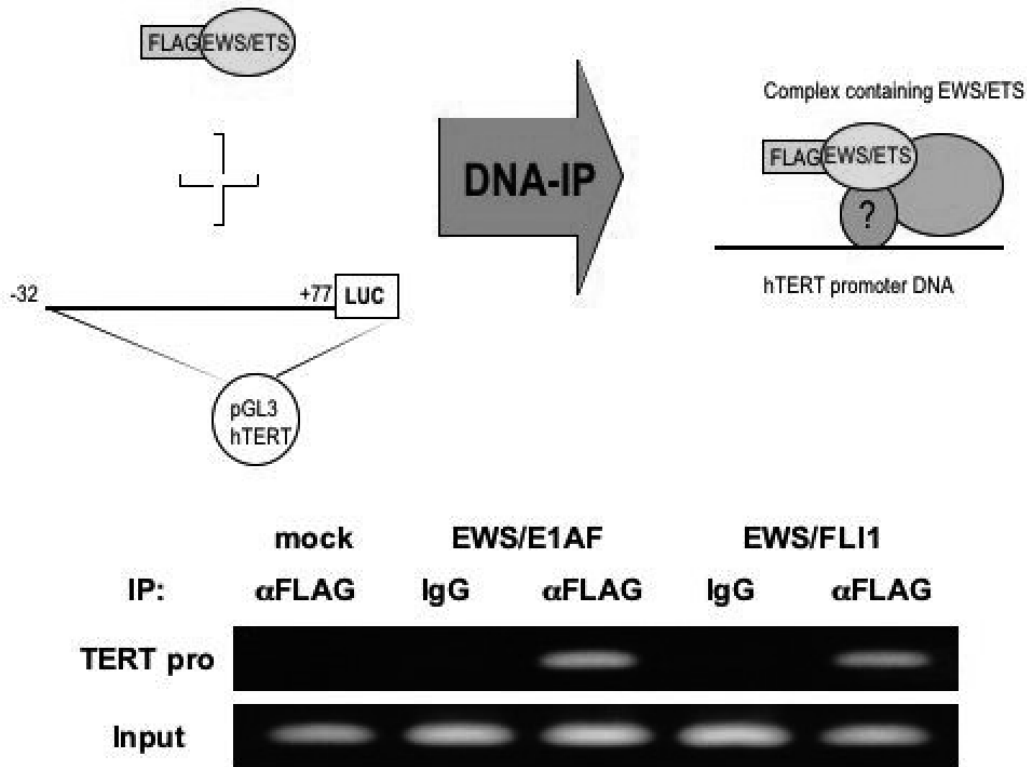
Mammalian telomeres are composed of the sequence 5'-TTAGGG-3' that caps the ends of linear chromosomes. Telomere shortening causes replicative senescence in most somatic cells, and increases genetic instability and tumor formation in mice [21]. Telomeres in most tumor cells maintain a certain length, probably as a result of the function of telomerase, but the relation between telomerase and Ewing's family tumors is still unknown. Telomerase is able to add telomeric repeats to the chromosomes through its enzymatic activity. hTERT, in particular, is a catalytic subunit of telomerase capable of inducing in vitro and in vivo telomerase activities and immortalizing cells [22]. Telomerase activity is tightly regulated at the transcriptional level of hTERT and the expression depends on the proximal 181bp region of the promoter in cancer cells [23]. This promoter region contains E-boxes, GC-boxes and an ETS binding site (EBS), putative binding sites of Myc, Sp1 and ETS transcription factors respectively, which are highly conserved between the human and mouse promoters. Myc and other transcription factors were reported to regulate hTERT expression [24], but the ETS oncogene involvement in hTERT was obscure. We found that EWS/ETS fusion activated telomerase in Ewing's sarcoma cells (Fig.3). EWS/ETS appears to activate the transcription of hTERT and knock down of EWS/FLI1 by RNAi leads to the reduction of hTERT mRNA and telomerase activity (Fig.4). However, substitution ets binding site (EBS) mutants did not affect the responsiveness to EWS/E1AF. These data indicated that EBS was not the direct target of EWS/ETS. To confirm that EWS/ETS was included in the transcriptional initiation complex, we performed DNA-IP assay and found that the complexes contained EWS/E1AF that bound to the hTERT promoter (Fig.5). These results strongly suggested that TERT is one of the targets of EWS/ETS fusions for its oncogenic activity, and that EWS/ETS functions as a co-activator for TERT transcription [25].



**Fig. 3** EWS/ETS activates telomerase activity. NIH3T3 cells expressing EWS/E1AF and EWS/FLI1 showed higher telomerase activity by TRAP assay, and corresponding induction of TERT mRNA was observed.



**Fig. 4** Knock down of EWS/FLI1 by dsRNA reduced the expression of hTERT mRNA. SCCH196 Ewing's sarcoma cell line was transfected with dsRNA. The expression level of EWS/FLI1 reduced about 1/4 and corresponding reduction of hTERT mRNA was observed.



**Fig. 5** Complexes containing EWS/ETS bound to the hTERT promoter as a transcriptional activator. DNA-IP assays were performed in H1299 cells transfected with hTERT luciferase reporter plasmid and FLAG-EWS/E1AF or FLAG-EWS/FLI1 expression vectors. Immunoprecipitated DNA was analyzed by PCR.

### 5. E1AF upregulates p21<sup>waf1/cip1</sup> activation

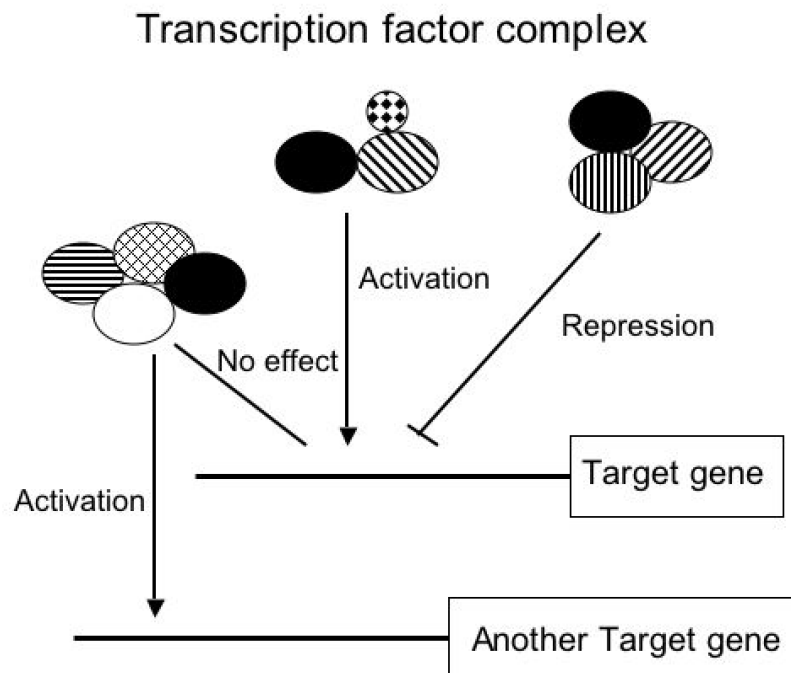
p21<sup>waf1/cip1</sup> is one of the key regulatory proteins in the cell cycle. p21<sup>waf1/cip1</sup> was shown to inhibit the kinase activity of cdks and to play a key role in cell-cycle arrest. Previous reports indicated that p21<sup>waf1/cip1</sup> was overexpressed when cells were under senescence, serum deprivation, contact inhibition, differentiation and exposure to several genotoxic stresses and p21<sup>waf1/cip1</sup> is upregulated by p53 when cells are stressed by various stimuli. Thus, p21<sup>waf1/cip1</sup> acts as a critical regulator of growth control [26].

Northern blot analysis revealed that p21<sup>waf1/cip1</sup> and E1AF were correlatively upregulated in response to cisplatin treatment in HPV18-positive SiHa cells. The p21 promoter was shown to be transactivated by wild-type p53 protein as well as in a p53-independent manner. We identified the *ets*-binding sites located close to the two previously identified p53-responsive elements. Transient expression assays have demonstrated that E1AF can activate the p21<sup>waf1/cip1</sup> promoter-driven luciferase reporter gene in SiHa cells. The p21<sup>waf1/cip1</sup> promoter activity is also increased in p53-null Saos2 osteosarcoma cells, but was markedly reduced when the *ets*-binding sites are deleted. These results indicate that E1AF positively regulates transcription from the p21<sup>waf1/cip1</sup> promoter in response to genotoxic stresses [27]. We examined the role of E1AF using phMT II E1AF, an E1AF expression vector driven by a metallothionein promoter. p21 protein expression was upregulated in ZnCl<sub>2</sub>-stimulated

CA9.22MTF cells with the E1AF expression level synergistically[28]. Our previous *in vitro* result demonstrated by reporter assay that E1AF has the ability to activate transcription of p21 was confirmed by the protein levels *in vivo*.

### Prospects

Transcription factors have functional DNA binding domains that bind to the regulatory regions of target genes and activating domains that have the property to activate transcription. Transcription is also controlled by transcriptional co-factors such as CBP/p300 that assist the activity of transcription factors [9]. Transcription factors have been shown to form complexes, for example, AP-1 is a heterodimer of c-fos and c-myc that has a more powerful effect for transcription [29]. The genome project has been completed and the number of transcribed genes was shown to be smaller than expected [30]. Thus, the importance of protein-protein interaction has been highlighted. Transcription factors are also proteins and research on transcription complexes is now being carried out. PML (promyelocytic leukemia gene product) gene was isolated from promyelocytic leukemia, and SUMO-1-modified PML is known to form complex with p53, Rb, Daxx and CBP, which localize in the nucleus as PODs (PML oncogenic domains) [31]. We found that E1AF also localizes in PODs in association with PML (unpublished data). The precise roles of PODs are still unclear; however, the members in PODs are very attractive. Our findings that EWS/ETS acts as a transcriptional co-factor may imply that the transcription pathway is regulated by the interaction of transcription factors (Fig. 6), and it may contribute to new insights into the transcription mechanism.



**Fig. 6** Possible roles of transcription factor complexes. The members in transcription factor complex may activate or repress the transcription, and choose the direction of transcription .

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