Cell Density-dependent Nuclear Accumulation of ELK3 is Involved in Suppression of PAI-1 Expression

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ABSTRACT. Cell-cell contact regulates the proliferation and differentiation of non-transformed cells, e.g., NIH/3T3 cells show growth arrest at high cell density. However, only a few reports described the dynamic behavior of transcription factors involved in this process. In this study, we showed that the mRNA levels of plasminogen activator inhibitor type 1 (*PAI-1*) decreased drastically at high cell density, and that ELK3, a member of the Ets transcription factor family, repressed *PAI-1* expression. We also demonstrated that while ELK3 was distributed evenly throughout the cell at low cell density, it accumulated in the nucleus at high cell density, and that binding of DNA by ELK3 at the A domain facilitated its nuclear accumulation. Furthermore, we found that ETS1, a *PAI-1* activator, occupied the ELK3-binding site within the *PAI-1* promoter at low cell density, while it was released at high cell density. These results suggest that at high cell density, the switching of binding of transcription factors from ETS1 to ELK3 occurs at a specific binding site of the *PAI-1* promoter, leading to the cell-density dependent suppression of *PAI-1* expression.

Key words: High cell density, transcription factors, nucleocytoplasmic transport

Introduction

Cell-cell contact plays an essential role in the survival of non-transformed cells. Several studies have shown that fibroblasts, which are typical non-transformed cells, exhibit growth arrest at high cell density (Levine *et al.*, 1965; Eagle and Levine, 1967). For example, at high cell density, NIH/3T3 cells, which are mouse embryonic fibroblasts, exhibit limited proliferation and migration at high cell density, coupled with the activation of anti-proliferative signals and subsequent cell cycle arrest at the G0/G1 phases (Holley and Kiernan, 1968; Küppers *et al.*, 2010). Vascular endothelial cells, upon contact with neighboring cells, impair cell proliferation and cell differentiation by the activation of Notch signaling during angiogenesis and embryogenesis

(Augustin *et al.*, 1994; Eiraku *et al.*, 2005). In addition, *in vivo* homeostasis, e.g., tissue formation and control of organ size, is maintained by cell-cell contact at high cell density (Zeng and Hong, 2005). Conversely, inappropriate proliferative signals usually lead to unregulated and unrestricted cell proliferation, and may even give rise to transformation and tumor progression (Fagotto and Gumbiner, 1996; Lozano *et al.*, 2003; Kim *et al.*, 2011). However, it is not known how cell-cell contact at high cell density spatially regulates anti-proliferative and proliferative signals in cells.

Plasminogen activator inhibitor type 1 (PAI-1) is a member of the serine protease inhibitor superfamily and is involved in fibrinolysis as a secretory protein. PAI-1 inhibits the functions of urokinase-type plasminogen activator and tissue-type plasminogen activator, which convert plasminogen to plasmin, upon binding to their receptors, thereby inhibiting subsequent PAI-1-regulated cell migration by degradation of the extracellular matrix (Strickland, 2001; Ploug, 2003; Dellas and Loskutoff, 2005). These effects are compounded by suppressing the expression of PAI-1 at the G0 phase, which is re-activated during transition to the G1 phase (Qi *et al.*, 2006). In addition, the expression level of PAI-1 has been reported to be associated with tumor invasion. For example, when malignant keratino-

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Abbreviations: CRM1, chromosome region maintenance protein 1; DAPI, 4',6-diamidino-2-phenylindole; Ets, E-26 transformation-specific sequence; EBS, Ets transcription factors binding site; EMSA, electrophoretic mobility shift assay; NES, nuclear export signal; NLS, nuclear localization signal; PAI-1, plasminogen activator inhibitor type 1.

cytes are transplanted into PAI-1-deficient mice, the cells fail to invade the host tissue (Bajou *et al.*, 1998). Furthermore, knockdown of PAI-1 by RNA interference represses the metastasis of human scirrhous gastric cancer cells (Nishioka *et al.*, 2012).

ETS1 and ELK3 are members of Ets transcription factor family. ETS1 is involved in shear stress-induced PAI-1 expression, while ELK3 functions as a PAI-1 repressor (Buchwalter *et al.*, 2005; Nakatsuka *et al.*, 2006). It is well known that transcription factors have to pass through the nuclear pore complex to function in the nucleus, although it remains unclear how the functions of ETS1 and ELK3 are spatio-temporally regulated in a cell density-dependent manner. After translocation into the nucleus, both ETS1 and ELK3 recognize the Ets transcription factor binding site (EBS), which has the consensus binding core sequence 5'-GGA(A/T)-3' within the promoter region of target genes (Szymczyna and Arrowsmith, 2000).

In this study, we characterized the dynamic behavior of transcription factors involved in cell density. We demonstrated that PAI-1 mRNA levels decreased drastically in NIH/3T3 cells at high cell density. We also found that ELK3 had a role in the suppression of *PAI-1* expression at high cell density and that ELK3 accumulated in the nucleus at high cell density. Further analysis revealed that the binding of ELK3 to DNA facilitated its nuclear accumulation. Importantly, ETS1 and ELK3 bound to the same EBS within the *PAI-1* promoter, strongly suggesting that they

function antagonistically to regulate *PAI-1* expression in a cell density-dependent manner. Taken together, our findings indicate that high cell density induces the binding of ELK3 to EBS, followed by the suppression of *PAI-1* expression.

Results

PAI-1 mRNA levels at high cell density

To identify genes whose expression levels change in a cell-density dependent manner, we performed DNA microarray analysis using NIH/3T3 cells cultured at low or high cell density. In agreement with a previous report (Comi *et al.*, 1995), we found that *PAI-1* expression was significantly down-regulated at high cell density (Supplemental Table SII). Northern blotting analysis verified that while *PAI-1* mRNA levels remained unchanged when the cells were cultured at low cell density for over 4 h (Fig. 1A), they decreased drastically when they were cultured at high cell density (Fig. 1B).

Next, to exclude the possibility that factors in extracellular fluids may contribute to the decrease in *PAI-1* mRNA levels, we incubated NIH/3T3 cells cultured at low cell density in conditioned medium from cells cultured at low or high cell density. Whereas there was no difference in *PAI-1* mRNA levels when the cells were incubated in conditioned medium from cells at low cell density, incubation in condi-

Fig. 1. *PAI-1* mRNA levels decrease drastically at high cell density. (A, B) Northern blotting of *PAI-1* mRNA levels over 4 h. NIH/3T3 cells were plated at low and high cell density (0 h), and cultured for 4 h, followed by extraction of total RNA every 1 h. *PAI-1* mRNA levels over 4 h at high cell density. (C) Conditioned medium was prepared using NIH/3T3 cells that were plated at low and high cell density. Newly prepared cells were then plated at low cell density, and cultured with the conditioned medium for 4 h. (D) NIH/3T3 cells were plated at 60% confluence and treated with 1 μ g/mL actinomycin D, an RNA polymerase I inhibitor. Actinomycin D treatment decreased *PAI-1* mRNA levels over time, independent of GAPDH.

tioned medium from cells at high cell density resulted in only a slight decrease of *PAI-1* mRNA levels (Fig. 1C). These results suggest that although it is possible that extracellular factors affect *PAI-1* expression to some extent, *PAI-1* expression was suppressed in a cell autonomous manner at high cell density.

Then, to confirm that *PAI-1* mRNA levels were regulated at the transcriptional level, we treated the cells with the transcriptional inhibitor actinomycin D. Treatment with actinomycin D reduced *PAI-1* mRNA levels in a similar manner as was observed in cells cultured at high cell density (Fig. 1D). This finding indicates that high cell density leads to the decrease of *PAI-1* mRNA levels mainly at the transcriptional level.

EBS2 serves as a critical site for the suppression of PAI-1 expression at high cell density

Next, we performed a luciferase assay to identify the promoter essential for the transcriptional repression of *PAI-1* at high cell density. Transient transfection studies were performed using deletion fragments of nucleotides –3000, –1000, or –190 to +50 relative to the *PAI-1* transcription start site, which were inserted into the luciferase reporter gene. At high cell density, the luciferase activity of the nucleotides –3000 fragment was significantly reduced as compared to that at low cell density. Deletion analysis also revealed that the luciferase activity of the nucleotides –190 and –1000 fragments increased gradually, but was still lower than that at low cell density. These results indicate that the region of the nucleotides –3000 to +50 contains several transcription factor binding sites required for the suppression of *PAI-1* expression at high cell density (Fig. 2A). Then, by conducting a MOTIF search, we identified 4 transcription factor binding sites within the nucleotides –190 of the *PAI-1* promoter, namely, the myeloid zinc finger protein 1 binding site (MZF1), activator protein 2 binding site (AP2), EBS1, and EBS2 (Fig. 2B).

An electrophoretic mobility shift assay (EMSA) was performed to characterize the functional specificity of the respective motifs within *PAI-1* promoter. We identified two distinct protein bands bound to the ³²P-labeled MZF1 probe (Fig. 2C); however, the intensity of these bands did not change at low and high cell density (Fig. 2C, lanes 2–3). No band was detected for AP2 or EBS1 (Fig. 2C, lanes 5–6 and lanes 8–9, respectively). In contrast, we found that ³²P-labeled EBS2, which is located at the nucleotides –71 to –64 of the *PAI-1* promoter, formed complexes with proteins derived from cells cultured at low and high cell density, and that complex formation increased at high cell density (Fig. 2C, lanes 11–12).

To confirm the specificity of complex formation with EBS2, we carried out a competition assay using a 200-fold excess of unlabeled EBS2 and EBS2mt, in which the core binding sequence 5'-TTCC-3' was mutated to 5'-TCTT-3'.

Unlabeled EBS2 dramatically decreased complex formation, whereas EBS2mt had no effect (Fig. 2D). Furthermore, when the assay was performed using pGL3-PAI-1(EBSmt), which contained the EBS2mt sequence, luciferase activity did not decrease at high cell density. These results indicate that EBS2 within the *PAI-1* promoter is essential for the optimal binding of proteins, which subsequently facilitates the suppression of *PAI-1* expression at high cell density.

Nuclear accumulation of ELK3 and suppression of PAI-1 expression at high cell density

EBS2 contains a consensus binding site for Ets transcription factors, and it was reported that ELK3, a member of the Ets transcription factor family, functions as a repressor of *PAI-1* (Buchwalter *et al.*, 2005). To test whether ELK3 is involved in the suppression of *PAI-1* expression at high cell density, NIH/3T3 cells were transiently transfected with pGL3-PAI-1(–190) and pRL-tk in combination with an expression plasmid for ELK3 (pFLAG-ELK3). At low cell density, the cells co-transfected with pFLAG-ELK3 exhibited less luciferase activity than the control cells co-transfected with pFLAG. Importantly, at high cell density, the luciferase activity of pFLAG-ELK3 was significantly decreased compared with that of pFLAG (Fig. 3A). Our data indicate that ELK3 indeed suppresses *PAI-1* expression at high cell density.

Next, we compared the expression of *ELK3* at low and high cell density using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). *ELK3* expression was maintained at low and high cell density, although the expression of its target *PAI-1* was suppressed at high cell density (Fig. 3B).

To investigate the cell-density dependent behavior of ELK3, we next observed the subcellular localization of ELK3 (Fig. 3C). We found that while green fluorescent protein (GFP)-ELK3 was distributed evenly throughout the cell at low cell density, it accumulated in the nucleus at high cell density. These results suggest that the nuclear accumulation of ELK3 is involved in the suppression of *PAI-1* expression at high cell density.

DNA-binding at the A domain of ELK3 serves as an anchor for its nuclear accumulation

To further elucidate the impact of cell density on the dynamic behavior of ELK3, we examined whether the nuclear accumulation of ELK3 was induced by the inhibition of its nuclear export. We constructed 3 ELK3 mutants; ELK3 Δ21–93, containing its nuclear export signal (NES) (Ducret *et al.*, 1999), but lacking the binding region of the A domain; ELK3 NESmt, in which a leucine at position 16 of ELK3 was mutated to alanine; and ELK3 NESmt-NES, in which an additional ELK3 NES was inserted at the carboxyl terminus of ELK3 NESmt (Fig. 4A). GFP-ELK3 accumu-

Fig. 2. EBS2 is located at the nucleotides -190 of the *PAI-1* promoter. (A) Nucleotides -190 of the *PAI-1* promoter were identified as essential for the suppression of *PAI-1* expression at high cell density. pGL3 luciferase reporter plasmid carrying the *PAI-1* promoter and pRL-tk were co-transfected in NIH/3T3 cells, and the cells were replated at low (black bars) and high cell density (white bars). The relative luciferase activity of the control at low cell density was set at 1.0. * $P < 0.05$; *** $P < 0.005$. (B) Schematic representation of nucleotides -190 of the *PAI-1* promoter. MZF1, AP2, EBS1, and EBS2 are transcription factor binding sites predicted using MOTIF (<http://www.genome.jp/tools/motif/>). MZF1, myeloid zinc finger protein 1 binding site; AP2, activator protein 2 binding site; EBS1 and EBS2, Ets transcription factor binding sites 1 and 2, respectively. (C) EMSA using probes targeting the *PAI-1* promoter. The arrowheads indicate the binding of proteins to the 32 P-labeled probes. Complex formation with EBS2 increased at high cell density, compared to low cell density. (D) EBS2 contains the core binding sequence 5'-TTCC-3' of Ets transcription factor family. EBS2mt has this core binding sequence mutated to 5'-TCTT-3'. EBS2 and EBS2mt were used in an EMSA competition assay. EBS2mt did not inhibit the binding of proteins to EBS2.

Fig. 3. ELK3 suppresses *PAI-1* expression and accumulates in the nucleus at high cell density. (A) ELK3 played a role in the suppression of *PAI-1* expression at high cell density. NIH/3T3 cells were co-transfected with pFLAG (black bars), pGL3-PAI-1(-190), and pRL-tk, or with pFLAG-ELK3 (white bars), pGL3-PAI-1(-190), and pRL-tk. The relative luciferase activity of FLAG was set at 1.0 as the control. * $P < 0.05$; ** $P < 0.01$. (B) Semi-quantitative RT-PCR showed that the expression levels of *ELK3* remained similar at low and high cell density. Hypoxanthine phosphoribosyl transferase (*HPRT*) was used as an internal control. (C) NIH/3T3 cells after GFP-ELK3 transient transfection. ELK3 was distributed evenly throughout the cell at low cell density, while it accumulated in the nucleus at high cell density. Green fluorescence indicates the subcellular localization of ELK3 and blue fluorescence indicates DAPI staining of the nuclei.

lated in the nucleus at high cell density, and the nuclear localized GFP-ELK3 was 83% of GFP positive cells at high cell density, compared to 9% at low cell density. We also observed that GFP-ELK3 NESmt was localized to the nucleus even at low cell density. In contrast, GFP-ELK3 NESmt-NES did not accumulate in the nucleus even at high cell density. Importantly, GFP-ELK3 $\Delta 21-93$ was mainly localized to the cytoplasm at low and high cell density (Fig. 4B). These results suggest that the nuclear accumulation of ELK3 requires both the suppression of ELK3 nuclear export and the DNA-binding of ELK3 at the A domain.

ETS1 prevents the binding of ELK3 to EBS2 at low cell density

Next, we examined the dynamic behavior of ETS1, another member of the Ets transcription factor family that is known to activate the transcription of *PAI-1* during shear stress (Nakatsuka *et al.*, 2006). We first investigated whether ETS1 plays a role in the activation of *PAI-1* expression using a luciferase assay. When pFLAG-ETS1 was co-transfected with the luciferase reporter gene at low cell density, 3.37-fold more luciferase activity was observed compared with the control cells co-transfected with pFLAG. In contrast, there was no significant change in relative luciferase activity at high cell density (Fig. 5A), even though GFP-ETS1 was localized to the nucleus at low and high cell density (Fig. 5B).

We then carried out an EMSA to investigate the cell density-dependent DNA-binding modes of ELK3 and ETS1. The cells were transfected with pFLAG-ELK3 or pFLAG-ETS1 and then cultured at low or high cell density. When the nuclear extracts were prepared from the cells cultured at high cell density and mixed with 32 P-labeled EBS2, the intensity of the FLAG-ELK3 protein band increased sharply (Fig. 5C, lanes 2–3). Conversely, the intensity of the FLAG-ETS1 band decreased at high cell density (Fig. 5, lanes 11–12). Supershift assays using an anti-FLAG antibody to confirm the components of the complexes revealed the presence of super mobility-shifted bands, indicating the specific binding of ELK3 and ETS1 to EBS2 (Fig. 5C, lanes 6–7 and lanes 15–16, respectively). Control IgG did not recognize FLAG-ELK3 or FLAG-ETS1 (Fig. 5C, lanes 4–5 and lanes 13–14, respectively). Moreover, competition assays with unlabeled EBS2 showed that complex formation with ELK3 or ETS1 decreased dramatically (Fig. 5C, lanes 8–9 and lanes 17–18, respectively). Taken together, these results suggest that ETS1 mainly binds to EBS2 at low cell density, leading to the inhibition of the DNA-binding activity of ELK3 and that the release of ETS1 from EBS2 at high cell density allows the subsequent binding of ELK3 to EBS2. Such switching of the binding of these transcription factors at EBS2 may cause the apparent nuclear accumulation of ELK3 to suppress *PAI-1* expression.

A **B**

Fig. 4. DNA-binding of ELK3 facilitates its own nuclear accumulation. (A) GFP-ELK3 Δ 21–93 lacks the DNA-binding region within the A domain; ELK3 NESmt has its leucine 16 mutated to alanine; ELK3 NESmt-NES contains the NES of ELK3 (LWQFLHLLLD) at the carboxyl terminus of ELK3 NESmt. (B) NIH/3T3 cells after GFP-ELK3, GFP-ELK3 Δ 21–93, GFP-ELK3 NESmt, and GFP-ELK3 NESmt-NES transient transfection. GFP-ELK3 Δ 21–93 was localized to the cytoplasm. GFP-ELK3 NESmt was localized to the nucleus. GFP-ELK3 NESmt-NES did not accumulate in the nucleus at high cell density. The subcellular localization of GFP fusion proteins was classified as nucleus (N, black bars), cytoplasm (C, white bars), or both (N/C, ash bars). *** $P < 0.005$.

Fig. 5. ETS1 is released from EBS2 at high cell density. (A) ETS1 played a role in the activation of *PAI-1* expression at low cell density. NIH/3T3 cells were co-transfected with pFLAG (black bars), pGL3-PAI-1(-190), and pRL-tk, or with pFLAG-ETS1 (white bars), pGL3-PAI-1(-190), and pRL-tk. * $P<0.05$. (B) NIH/3T3 cells after GFP-ETS1 transient transfection. GFP-ETS1 was localized to the nucleus at low and high cell density. The subcellular localization of GFP-ETS1 was classified as nucleus (N, black bars) or nucleus/cytoplasm (N/C, ash bars). (C) EMSA experiment using nuclear extracts prepared from FLAG-ELK3- and FLAG-ETS1-overexpressing cells that were incubated with 32 P-labeled EBS2. Complex formation of FLAG-ELK3 with EBS2 increased at high cell density, whereas that of FLAG-ETS1 with EBS2 decreased at high cell density. For the supershift assay, antibodies against FLAG or normal rabbit IgG were incubated with the prepared nuclear extracts. Competition was carried out using unlabeled-EBS2 (cold).

Fig. 6. Model for the suppression of *PAI-1* and nuclear accumulation of ELK3 in a cell density-dependent manner. At low cell density, importin $\alpha 5$ recognizes the NLS of ELK3, and ELK3 then translocates into the nucleus. However, ETS-1 occupies the Ets transcription factor binding site of EBS2 within the *PAI-1* promoter. Consequently, CRM1 recognizes the NES of ELK3, and ELK3 shuttles between the cytoplasm and nucleus. Conversely, in response to cell-cell contact at high cell density, ETS-1 is released from EBS2, allowing the binding of ELK3 to EBS2. The subsequent binding of ELK3 to DNA induces the suppression of *PAI-1* expression and its own nuclear accumulation.

Discussion

We identified *PAI-1*, whose product is known to regulate cell migration, as one of the genes that are differentially expressed in a cell density-dependent manner. We demonstrated that at high cell density, ELK3, a member of the Ets transcription factor family, bound to EBS2, which is located between nucleotides -190 and $+50$ within the *PAI-1* promoter. Moreover, we found that while ELK3 was distributed evenly throughout the cell at low cell density, it accumulated in the nucleus at high cell density. ELK3 has a classical nuclear localization signal (NLS) located in its D domain (Supplemental Fig. S1), and importin $\alpha 5$ specifically recognized the NLS of ELK3 (Supplemental Fig. S2). In addition, it was shown that the nuclear export of ELK3 occurs in a chromosome region maintenance protein 1 (CRM1)-dependent manner (Ducret *et al.*, 1999; Supplemental Fig. S3). These results indicate that ELK3 shuttles between the cytoplasm and nucleus, consistent with its even distribution throughout the cell at low cell density.

Conversely, at high cell density, ELK3 apparently accumulated in the nucleus, although the expression levels of importin $\alpha 5$ and CRM1 remained unchanged (Supplemental Fig. S4). Therefore, there are two possibilities to explain the apparent nuclear accumulation of ELK3. One is that its nuclear export efficiency is suppressed at high cell density. The binding of STAT1 to DNA has been reported to facili-

tate the masking of its own NES, which is located near its DNA-binding region (McBride *et al.*, 2000). Thus, the binding of ELK3 to DNA may also mask its own NES to suppress its nuclear export. The other possibility is that ELK3 accumulates in the nucleus due to its nuclear retention, e.g., via DNA binding. As we observed, ELK3 $\Delta 21-93$, which lacks the DNA-binding region at the A domain, was localized to the cytoplasm. Consistently, ELK3d, an ELK3 splice variant, which also lacks a partial DNA-binding region at the A domain, is also known to localize to the cytoplasm (Kerr *et al.*, 2010). Similarly, although STAT1 localizes to the nucleus in response to interferon- γ (Darnell *et al.*, 1994; Ihle and Kerr, 1995), a STAT1 mutant, which contains a mutated DNA-binding region, localizes to the cytoplasm, even in the presence of interferon- γ . In either case, the DNA-binding activity of ELK3 via its A domain plays an indispensable role in its nuclear accumulation.

How does ELK3 accumulate in the nucleus only at high cell density? ETS1 and ELK3 redundantly occupy EBS2, and serve as transcriptional regulators of *PAI-1*. Interestingly, genome-wide analyses have suggested that Ets transcription factors exhibit specific or redundant occupancy of EBS within the promoter regions of various genes (Hollenhorst *et al.*, 2007; Odrowaz and Sharrocks, 2012). ETS1, a *PAI-1* activator, binds efficiently to EBS2 at low cell density, while it is released from EBS2 at high cell density, allowing the binding of ELK3 to EBS2. Conse-

quently, ELK3 accumulates in the nucleus to suppress *PAI-1* expression (Fig. 6). It will be interesting to examine further how the binding of these transcription factors to EBS2 switches at high cell density.

Materials and Methods

Cell culture and transient transfection

NIH/3T3 cells were cultured in Dulbecco's modified Eagle Medium (Sigma) supplemented with 10% fetal bovine serum. The cells were maintained in a 37°C incubator with 10% CO₂-humidified air. For low cell density, NIH/3T3 cells were plated at 5.0×10^3 cells/cm²; for high cell density, they were plated at 5.0×10^4 cells/cm² (Holley and Kiernan, 1968) in a 100-mm dish (Cat. 430167; Corning), 6-well plate (Cat. 140675; Nunc), 24-well plate (Cat. 142475; Nunc), or 96-well plate (Cat. 3595; Corning).

NIH/3T3 cells were transiently transfected at 24 h after plating using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Construction of mammalian expression vectors

The promoter regions of *PAI-1* were generated by PCR amplification from the genome of NIH/3T3 cells, and the amplified fragments were digested with *Acc65I* and *HindIII*, and subcloned into the pGL3 firefly reporter plasmid (Promega). GFP-GFP was constructed by subcloning a GFP fragment into pEGFP-C3 (BD Biosciences). Murine ELK3 cDNA was generated from total RNA of NIH/3T3 cells with ELK3-specific oligonucleotide primers using KOD -Plus- (Toyobo), and subcloned into the *HindIII* and *EcoRI* sites of the pFLAG, pEGFP, and pEGFP-GFP expression vectors. GFP-GFP-D domain and GFP-NES were constructed by subcloning the amplified fragments of the D domain and NES of ELK3, respectively, using KOD -Plus- into pEGFP-GFP or pEGFP-C3. GFP-GFP-ELK3 Dmt, GFP-ELK3 $\Delta 21-93$, and GFP-ELK3 NESmt were constructed with pEGFP-ELK3 using site-directed mutagenesis. ELK3 NESmt was produced according to a previous study (Ducret *et al.*, 1999). GFP-ELK3 NESmt-NES was constructed by subcloning the ELK3 NES-mt fragment into the pEGFP-NES expression vector. Murine ETS-1 cDNA was generated from total RNA of NIH/3T3 cells with ETS-1-specific oligonucleotide primers using KOD -Plus-, and subcloned into the *KpnI* and *Sall* sites of the pFLAG and pEGFP expression vectors. All constructs were subjected to DNA sequencing. Supplemental Table SI shows the oligonucleotide sequences used in this study.

Northern blotting

Total RNA extracted from NIH/3T3 cells using TRIzol (Invitrogen) was dissolved in denaturation buffer (1×MOPS, 6.5% formaldehyde, 50% formamide), and incubated at 65°C for 15 min. RNA samples were electrophoresed in a 1% agarose/formaldehyde gel in 1×MOPS and transferred to a Hybond N+ membrane (Amer-

sham Bioscience). The Hybond N+ membrane was crosslinked by UV irradiation, followed by hybridization with ³²P- α CTP labeled mouse PAI-1 cDNA and GAPDH in hybridization buffer (Nacalai Tesque). The hybridized membrane was washed with 2×SSPE/0.1% SDS for 1 h, and 1×SSPE/0.1% SDS for 1 h, and 0.5×SSPE/0.1% SDS for 1 h at 65°C. The bands on the membranes were detected using an imaging plate (Fujifilm).

Luciferase assay

Luciferase assays were performed using the Dual Luciferase Reporter System (Promega) according to the manufacturer's instructions. NIH/3T3 cells (8.5×10^4 cells/well) were plated in 24-well plate, and cultured for 24 h. Using Lipofectamine 2000, the cells were co-transfected with 200 ng pGL3 firefly reporter plasmid, pGL3-PAI-1(-3000), pGL3-PAI-1(-1000), pGL3-PAI-1(-190), or pGL3-PAI-1(EBS2mt), 200 ng pFLAG, pFLAG-ELK3 or pFLAG-ETS1, and 5 ng pRL-tk Renilla reporter plasmid as a control for transfection efficiency. For the preparation of cell lysates for the luciferase assay, the cells were transfected in 24-well plates, and replated at 1.0×10^4 cells/well at 24 h post-transfection in 24-well plates at low cell density and in 96-wells plates at high cell density. The cells were then incubated for 24 h prior to the collection of cell lysates and luciferase activity assay. Relative luciferase activity is represented as the corrected ratio of firefly luciferase to Renilla luciferase activity from 3 independent experiments, with the value of the control set as 1.0 and error bars indicating the mean±SD. Paired two-tailed Student's t-tests performed with Microsoft Office Excel 2010 (Microsoft) were used to determine whether differences were statistically significant.

EMSA

For nuclear extract preparation, 3.0×10^6 NIH/3T3 cells/mL were harvested, and the cell pellet was resuspended in 500 μ L Buffer A (10 mM HEPES at pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μ g/mL leupeptin, 1 μ g/mL aprotinin, and 1 μ g/mL pepstatin A). After a 15 min incubation on ice, 25 μ L of 10% NP-40 were added to the suspension, and the tube was vortexed for 10 s. The mixture was centrifuged at $500 \times g$ for 1 min at 4°C, and the nuclear pellet was resuspended in 60 μ L Buffer C (20 mM HEPES at pH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μ g/mL leupeptin, 1 μ g/mL aprotinin, and 1 μ g/mL pepstatin A). The nuclear suspension was incubated on ice for another 15 min, and centrifuged at $12,000 \times g$ for 10 min at 4°C, and the supernatant was stored at -80°C. Nuclear extracts (2.5 μ g) were incubated with 20,000 cpm ³²P-labeled probe in binding buffer (50 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 5 mM DTT, 5% glycerol, 100 ng poly dI-dC) for 30 min on ice, and complexes of protein with the ³²P-labeled probe were separated from free probe on a 5% non-denaturing polyacrylamide gel. Binding specificity was examined using supershift and competition assay. For the supershift assays, 1 μ g Monoclonal ANTI-FLAG® M2 Antibody (SIGMA) and antibody against normal rabbit IgG (Santa Cruz) were incubated with nuclear extracts for

60 min at 4°C before incubation with the ³²P-labeled probe. We used 5 pmol unlabeled oligonucleotide (cold) in the competition assay.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed using a Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Total RNA (1 µg) extracted from NIH/3T3 cells cultured at low and high cell density was used for reverse transcription. The reactions were carried out at 65°C for 10 min, 55°C for 30 min, and 85°C for 5 min on a GeneAmp® PCR System 9700. First-strand cDNA was amplified with PCR using ELK3-specific oligonucleotide primers. The primer sequences for semi-quantitative RT-PCR were described previously (Kerr et al., 2010).

GFP imaging

GFP fusion proteins were transiently expressed in NIH/3T3 cells in 6-well plates with coverslips. The cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature. After washing with PBS, the cells were treated with 0.5% TritonX-100 in PBS for 5 min at room temperature. Nuclei were stained using 0.1 µg/mL DAPI (Dojindo). 100 GFP positive cells were analyzed to calculate the percentage with the nucleus (N), the cytoplasm (C), or both (N/C) from 3 independent experiments, and error bars indicate the mean±SD.

Acknowledgments. We thank Pui Khuan Liew for critical comments on the manuscript. This work was partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and JST, CREST.

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(Received for publication, March 15, 2013, accepted, May 14, 2013
and published online, May 24, 2013)