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STAT1 and Nmi are downstream targets of Ets-1 transcription factor in MCF-7 human breast cancer cell

Hae Hyun Jung[a], Jeeyun Lee[a,b], Joo Hyun Kim[a], Kyoung-Ju Ryu[a], Sun-A Kang[a], Chaehwa Park[a], Kiwoong Sung[c], Do-Hyun Nam[d], Won Ki Kang[a,b], Keunchil Park[a,b], Young-Hyuck Im[a,b,*]

[a] *Biomedical Research Institute, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-dong Kangnam-gu, Seoul 135-710, Korea*
[b] *Division of Hematology-Oncology, Departments of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-dong Kangnam-gu, Seoul 135-710, Korea*
[c] *Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-dong Kangnam-gu, Seoul 135-710, Korea*
[d] *Neurosurgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-dong Kangnam-gu, Seoul 135-710, Korea*

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Abstract **Ets-1 is a cellular homologue of the product of the viral ets oncogene of the E26 virus, and it functions as a tissue-specific transcription factor. It plays an important role in cell proliferation, differentiation, lymphoid cell development, transformation, angiogenesis, and apoptosis. Ets-1 controls the expression of critical genes involved in these processes by binding to ets binding sites present in the transcriptional regulatory regions. Here, we transiently overexpressed Ets-1 in MCF-7 and comprehensively searched for potential downstream targets of Ets-1 by cDNA microarray analysis. The expressions of several interferon-related genes including STAT1 and Nmi were augmented by the overexpression of Ets-1. RT-PCR and Western blotting confirmed the increase in the levels of STAT1 and Nmi mRNA and protein. In contrast, Ets-1 siRNA decreased the expression of STAT1 and Nmi proteins. As in our transient transfection experiments, stable overexpression of Ets-1, also increased the protein expression of STAT1 and Nmi in MCF-7 cells. Taken together, our results indicate that STAT1 and Nmi are downstream targets of Ets-1 in MCF-7 human breast cancer cells.**

Keywords: Ets-1; STAT1; Nmi; cDNA microarray

1. Introduction

The Ets-1 protein is a member of the Ets family of transcription factors that share a unique DNA binding domain, the Ets domain [1]. The DNA binding domain allows these proteins to specifically bind to promoter elements that contain a GGAA/T motif, and it has been a challenge to differentiate redundant from specific functions of various Ets proteins in vivo. However, it has been suggested that such functional redundancy may be a central component of a network of differentially regulated specific Ets factors, resulting in distinct biological and pathological consequences [2]. The Ets proteins transcriptionally regulate a number of genes involved in cellular proliferation, differentiation, development, hematopoiesis, apoptosis, metastasis, tissue remodeling, angiogenesis and malignant transformation of cell. The Ets transcription factors have been implicated in tumorigenesis both through formation of gain-of-function oncoproteins in acute leukemia and Ewing sarcoma and through overexpression in tumors of epithelial origin, including breast cancer [3,4]. Fusion proteins formed between the pointed domain of the Ets transcriptional repressor TEL and certain kinases or between TEL and the transcription factor AML-1 are known to be responsible for the development of leukemia by rendering constitutively active kinases [5,6]. In Ewing's tumor, the Ets domain of Fli-1, Erg-1, ETV-1, E1AF or FEV is fused to the Ewing sarcoma protein generating a transforming transcription factor that deregulates transcription [7].

Upregulation of expression of the Ets-1 gene, a prototype ets gene, has been documented in many types of human cancers, including epithelial ovarian tumors and breast cancer [8–11]. The degree of Ets-1 expression was correlated to the extent of breast carcinoma invasion and the atypism of carcinoma was significantly correlated with Ets-1 expression [10]. Ets-1 has been reported as an independent prognostic marker for breast relapse-free survival in breast cancer, which was not linked to other tumor markers, such as nodal status, tumor size, histological grade or estrogen receptor status [9]. In addition, the role of Ets-1 protein in breast cancer metastasis has been implicated by finding high Ets-1 protein levels in breast cancer tissues compared to the fibroadenoma specimens and by the correlation with urokinase plasminogen activator (uPA), a matrix-degrading protease [11]. The Ets factors Ets-1, Ets2, Fli1, and Erg transform cells when overexpressed, an effect that is associated with the stimulation of cell proliferation [12–15]. Few other functional roles of Ets-1 in tumorigenesis have also been postulated. Ets-1 regulates the expression of genes encoding for enzymes involved in degradation of the extracellular matrix, such as MMP-1, MMP-3, MMP-7, and MMP-9 [16]. It has been demonstrated that the expression of Ets-1 lacking its activation domain decreased uPA proteolytic activity and cell motility and impaired normal tubulogenesis and cancerous scattering in mammary epithelial cells [17]. These findings imply that Ets-1 is required for mammary oncogenesis by regulating the expression of target genes whose products play critical roles in breast cancer, although an array

*Corresponding author. Fax: +82 2 3410 0041.
E-mail address: imyh@smc.samsung.co.kr (Y.-H. Im).

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of target genes and the interactive cellular mechanism remain undefined.

Over 200 ets target genes were identified and an array of genes has been shown to be regulated via ets binding sites [16]. The identification of downstream cellular target genes of ets proteins is essential. The objective of the present study was to identify potential downstream target genes of Ets-1 in MCF 7 breast cancer line in order to further clarify its role and mechanism in mammary tumorigenesis. We transiently overexpressed Ets-1 gene in Ets-1 deficient MCF-7 breast cancer cell and comprehensively searched for potential downstream targets by cDNA microarray analysis. Transiently transfected Ets-1 in MCF-7 breast cancer cell line induced interferon-related genes including STAT1 and Nmi, which were confirmed in stably ets-1 transfected MCF-7 cell. Results suggest that STAT1 (signal transducers and activators of transcription) and Nmi (N-Myc-interacting protein) are downstream targets of Ets-1 in breast cancer.

2. Materials and methods

2.1. Cell lines

MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured and maintained in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in 5% CO_2.

2.2. Transient and stable transfection of Ets-1 into MCF-7 cells

An expression vector for FLAG-tagged Ets-1 was constructed by subcloning PCR-amplified cDNA into the G418-resistant plasmid pcDNA3.1 vector. MCF-7 cells were transiently transfected using Effectene transfection reagent (QIAGEN Inc., Valencia, CA) according to the manufacture's instructions. MCF-7 cells were stably transfected with either vector (pcDNA3.1-Ets-1) or pcDNA3.1 in the presence of Effectene transfection reagent (QIAGEN Inc.) for 48 h and treated with 500 μg/ml of G418, and G418-resistant colonies were selected for two months.

2.3. RT-PCR analysis

Total cellular RNA was isolated by using Trizol™ (Gibco BRL, Carlsbad, CA) according to the manufacturer's instruction. For reverse transcription (RT)-PCR, 2 μg of RNA was treated with RNase-free DNase, and cDNA was obtained using Moloney murine leukemia virus reverse transcriptase. cDNA (1 μl) was amplified by PCR (denaturation for 1 m at 94 °C, annealing for 1 m at 58 °C, and elongation for 1 m at 72 °C) using Ets-1 (28 cycles), STAT1 (30 cycles), Nmi (30 cycles) or β-actin primers (25 cycles). The primers used in this analysis are as follows: β-actin, 5′-atc tgg cac cac acc ttc tac aat gag ctg ctg cg-3′ and 5′-cgt cat act cct gct tgc tga tcc aca tct g-3′; Ets-1, 5′-aaa cag caa agaaat gat gt-3′ and 5′-gct cga gaa agc agtctt ta-3′; STAT1, 5′-gat act tta gct tta att tta aaa caa aac-3′ and 5′-gtt att agg gtg gta ttt agt cta tta-3′; Nmi, 5′-ata aac aaa act ttt tat tac agt gca ctt-3′ and 5′-gca gtg ctt ctg aca gga gt-3′. The reaction products were visualized by subjecting them to electrophoresis in 1.5% agarose in 1× TBE buffer containing 0.5 μg/ml ethidium bromide. The final normalized results were calculated by dividing the relative transcript levels of the test genes by the relative amounts of β-actin transcripts.

2.4. Western blot analysis and antibodies

Total cell extracts were obtained using ice-cold RIPA buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% SDS, and 1 mM PMSF). After 20-min incubation of the cell extracts on ice, they were centrifuged for 20 min at $12000 \times g$ at 4 μg and the supernatant collected. Proteins were quantified with the BCA protein assay kit (Pierce, Rockford, IL, USA). Lysates containing equal amount of total protein were separated by SDS–PAGE under reducing conditions and transferred to nitrocellulose membrane. The membrane was incubated for 2 h in blocking solution containing 5% non-fat dry milk to inhibit non-specific binding. The membrane was incubated with the primary antibody for 2 h. After several washes in PBS, the membrane was incubated with 1:3000 HRP-conjugated secondary antibodies (Zymed). The blots were developed using the ECL chemiluminescent kit (Amersham, Arlington Heights, IL). Anti-Ets-1 (dilution factor 1:1000), STAT1 (Stat1 alpha p91 (C-111), 1:1000), phospho-STAT1 (1:1000) and Nmi (1:1000) antibodies were used, which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin FLAG was purchased from Sigma (St. Louis, MO).

2.5. cDNA microarray analysis

The cDNA microarray containing a set of 17448 sequence-verified human cDNA clone was provided by GenomicTree Inc. (Korea). The synthesis of target cDNA probes and hybridization were performed as previously described [18]. Each 100 μg total RNA was reverse transcribed in the presence of Cy3 or Cy5-dUTP (NEN Life Sciences, Boston, MA) at 42 °C for 2 h. The control RNA was labeled with fluorescent Cy3-dUTP and the testing RNA was labeled with fluorescent Cy5-dUTP. Both the Cy3 and Cy5-labeled cDNAs were purified using PCR purification kit (QIAGEN Inc.) as recommended by the manufacturer. The purified cDNA was resuspended in 80 μl of hybridization solution containing 3.5× SSC, 0.3% SDS, 20 μg of human Cot-1 DNA, 20 μg of poly A RNA and 20 μg of the yeast tRNA (Invitrogen, Carlsbad, CA). The hybridization mixtures were heated at 100 °C for 2–3 min and directly pipetted onto microarrays. The arrays hybridized at 65 °C for 12–16 h in the humidified hybridization chamber (GenomicTree Inc.). The hybridized microarrays were washed with 2× SSC for 2 min, 0.1× SSC/0.1% SDS for 5 min, and 0.1× SSC for 5 min. The washed microarrays were immediately dried using the microarray centrifuge (GenomicTree Inc.). All microarray hybridizations were performed in duplicates and we collected the average data.

2.6. Data acquisition and analysis

The hybridization images were analyzed by GenePix Pro 4.0 (Axon Instruments, CA). The average fluorescence intensity for each spot was calculated and local background was subtracted. All data normalization and statistical analysis were performed using GeneSpring 6.1 (Silicon Genetics, USA). Genes were filtered according to the two-component model for estimating variation from control strength [19]. Intensity-dependent normalization (Lowess) was performed, where the ratio was reduced to the residual of the Lowess fit of the intensity vs. ratio curve. The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity. Hierarchical clustering was performed by similarity measurements based on Pearson correlations around 0. Functional annotation of genes was performed according to Gene Ontology™ Consortium (http://www.geneontology.org/index.shtml) by GeneSpring 6.1.

2.7. siRNA synthesis

Small interfering RNA (siRNA) were synthesized and high-performance purified (Qiagen-Xeragon, Germantown, MD). The sequence of siRNA Ets-1 targeting nucleotides was $^{5'}$r(GGUGUGCUGUUUGGAGUUC)d(TT)$^{3'}$ and its corresponding complementary strand $^{5'}$r(GAACUCCAAACAGCACACC)d(TT)$^{3'}$. siRNAs were dissolved in buffer (100 mM potassium acetate, 30 mM HEPES–potassium hydroxide, and 2 mM magnesium acetate, pH 7.4) to a final concentration of 20 μM. The siRNA solutions were heated to 90 °C for 60 s and incubated at 37 °C for 60 min prior to use to disrupt any higher order aggregates formed during synthesis.

2.8. Small interfering RNA

The 2×10^5 cells were plated into 35 mm 6-well plate and allowed to adhere for 24 h. Eight microliters of RNAiFect transfection reagent (QIAGEN Inc.) and two microliters of siRNA solution per well was added to Buffer EC-R for a final complexing volume of 100 μl. This complex was gently mixed and incubated at room temperature for 20 min. The transfection agent/siRNA complex was added into the wells containing 1900 μl DMEM with 10% FBS and incubated in normal cell culture conditions.

Table 1
List of augmented genes in cDNA microarray analysis

Type of gene	Fold induction
IFN-induced transmembrane protein 2 (1-8D)	6.973
Nmi	12.6
IFN-α inducible protein (clone IFI-15K)	29.32
IFN-α inducible protein (clone IFI-6-16)	18.364
IFN-induced transmembrane protein 2 (1-8D)	10.003
IFN-stimulated gene 20 kDa	8.90
IFN-stimulated transcription factor 3, gamma 48 kDa	8.652
STAT1	5.17
B-factor, properdin	4.631
Nerve growth factor receptor (TNFR superfamily, member 16)	4.25
Major histocompatibility complex, class I, C	4.25
Nuclear transport factor 2	3.02
Bone marrow stromal cell antigen 2	16.18
Leucine aminopeptidase 3	6.191
Lectin, galactoside-binding, soluble, 3 binding protein	15.807
Cullin 5	8.61
Apolipoprotein L, 3	10.49
Adrenergic, alpha-1D-, receptor	5.312
Myeloid differentiation primary response gene	3.232
Metallothionein 1B	2.94
Proteasome (prosome, macropain) subunit, beta type	4.897
Major histocompatibility complex, class I, A	4.13

3. Results

3.1. cDNA microarray results in ets-1 transiently transfected MCF-7 cell line

To identify genes transcriptionally regulated by Ets-1 in breast cancer cells, we transfected MCF-7 cell line with Ets-1 and compared the expression profile with that of MCF-7 infected with G418-resistant plasmid pcDNA3.1 vector. In order to minimize the interaction with endogenous Ets-1, MCF-7, MDA-MB-231, SKBR-3 breast cancer cell lines were prescreened for the level of endogenous Ets-1 expression and found that MCF-7 cell line expressed less than 10% compared to other cell lines (data not shown). After 24 h incubation, we analyzed the expression profile with a cDNA microarray. Among the 17448 human genes in the microarray, 286 of them were augmented in their expression more than 2-fold and 42 of them were augmented in *t* test. Of the 42 augmented genes in cDNA microarray analysis, relevant genes are listed in Table 1. In particular, genes coding for proteins involved in interferon (IFN) signaling pathway, such as IFN-α inducible protein (clone IFI-15K, IFI-6-16), IFN-induced transmembrane protein 2 (1-8D), IFN-γ induced protein, IFN-stimulated gene 20 kDa, IFN-stimulated transcription factor 3, gamma 48 kDa, STAT1 and Nmi were markedly augmented in their expression level.

Of these proteins, we were interested in STAT1 and Nmi in relation to Ets-1 overexpression in breast cancer cells. STAT1, a member of the STAT family, plays an essential role in regulating the growth, differentiation, death of normal and tumor cells in response to various stimuli, including cytokines and growth factors [20]. Nmi has been shown to augment STAT-mediated transcription in response to IL-2 and IFN-γ [21]. Because there were significantly augmented expressions of IFN-related protein after transfection with Ets-1 construct, we investigated the transcriptional regulation of STAT1 and Nmi by Ets-1. As shown in Fig. 1A, transient overexpression of Ets-1 gene led to an enhancement of STAT1 and Nmi mRNA expression levels. A time course experiment revealed that an increase in the level of STAT1 and Nmi proteins were evident as early as 24 h after the Ets-1 transfection and continued to increase until 48 h after transfection (Fig. 1B). The increase in expression level of phospho-STAT1 was observed at 24 h after the Ets-1 transfection by Western analysis (Fig. 1C).

3.2. Ets-1 enhances STAT1 and Nmi expression in a stable MCF-7 cell line

In order to investigate the effect of an intracellular accumulation of Ets-1, we established a stable MCF-7 cell line transfected with either Ets-1 or a vector. Nine G418-resistant clones were isolated and screened for expression of Ets-1 by RT-PCR and Western blot analysis. As shown in Fig. 2, clone #10 and #14 stably expressed Ets-1 protein and showed increased expression of STAT1 and Nmi, whereas clone #1 (without Ets-1 expression) did not show STAT1 or Nmi expression in Western blot or RT-PCR analysis. These data demonstrated that the overexpression of Ets-1 has led to an enhancement of intracellular STAT1 and Nmi expression.

3.3. STAT1 and Nmi induction are blocked by Ets-1 siRNA in MCF-7 cell

siRNA to Ets-1 provide further evidence for the positive regulatory role of Ets-1 in the regulation of STAT1 and Nmi (Fig. 3). The ability of Ets-1 siRNA to suppress Ets-1 expression was confirmed by Western blot analysis. Up to 50% suppression of Ets-1 protein expression was observed within 24 h of transfection. Actin expression was unaffected by either control or Ets-1 siRNA treatment, indicating that non-specific downregulation of protein expression did not occur (data not shown). Next, we determined the effect of Ets-1 siRNA on STAT1 and Nmi expression in MDA-MB-231, which expresses endogenous Ets-1 in higher level than MCF-7. STAT1 and Nmi were both downregulated by the addition of Ets-1 siRNA in MDA-MB-231 cell line.

4. Discussion

Recently, the association of Ets-1 with invasiveness and metastasis has been implicated in breast cancer cells. The detailed mechanism of how Ets-1 modulate the downstream gene transcription during tumorigenesis remains uncertain. The goal of this study was to identify downstream cellular targets that are either directly or indirectly regulated by the Ets-1 proto-oncogene. The cDNA microarray analysis revealed that Ets-1 augmented the expression of 9 IFN-related genes in breast cancer cells. None of these genes have been previously recognized as putative targets of Ets-1 in breast cancer. We further clarified two of these genes, STAT1 and Nmi.

STAT protein usually exists in the cytoplasm as a monomer in unstimulated cells and forms a dimer upon activation by tyrosine phosphorylation in response to ligand stimulation. The STAT dimer then translocates into the nucleus to activate transcription of various genes. Initially identified as playing a key role in hematologic and immune cells, STATs are increasingly recognized as an important factor in a wide array of human cancers. A number of proteins have been identified to

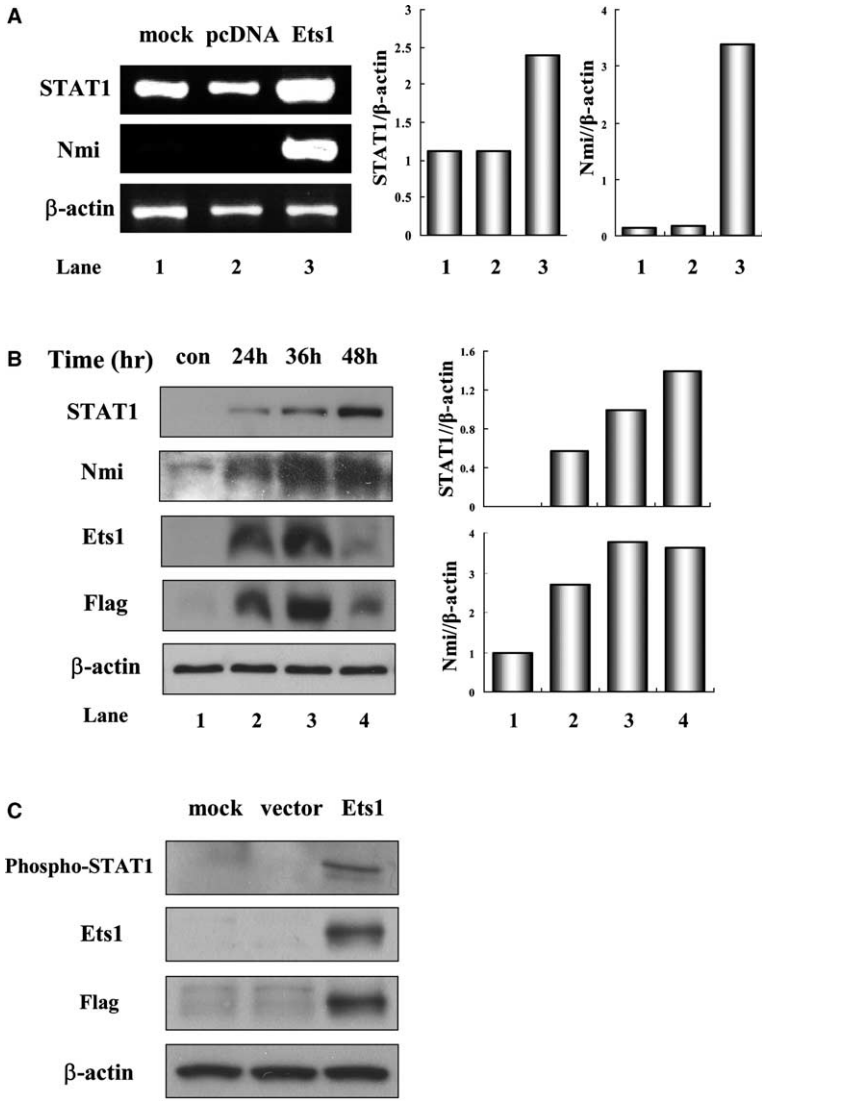

Fig. 1. Effect of exogenous Ets-1 on the expression of STAT and Nmi. Characterization of MCF-7 cells transiently transfected with an Ets-1 expression plasmid. (A) MCF-7 cells were transfected with Ets-1-FLAG construct and total RNA was extracted from the cells. STAT1 and Nmi mRNA level was assessed by RT-PCR. The RNA amount was quantified by densitometric analysis, and the ratio of STAT, Nmi/β-actin in each lane was calculated. (B) MCF-7 cells were transfected with Ets-1-FLAG construct and were harvested at the indicated time. Ets-1, FLAG, STAT1 and Nmi protein level were assessed by Western blot. The protein amount was quantified by densitometric analysis, and the ratio of STAT, Nmi/β-actin in each lane was calculated.

interact with STATs and modulate the activity of STATs at various steps of the activation–inactivation cycle [22]. Activated STAT1, in particular, has been found in nuclear extracts from breast cancer specimens [23]. The role of STAT1 activation is not clear in mammary tumorigenesis, however. Breast cancer patients with high STAT1 activation were reported to have a longer overall and relapse-free survival, although the STAT1 activation and expression of the STAT1 protein were not linked [24]. Few studies have suggested a direct binding of Ets-1 and STAT proteins. Travagli et al has demonstrated that Ets-1 physically interacts with STAT6, which is implicated in the regulation of Socs-1 expression in human keratinocytes [25]. The involvement of STAT1 and Ets elements such as PU.1 and Spi-B in interferon-γ induction of CD40 transcription in macrophages has recently been proposed [26]. The cellular implication of the physical binding between STAT1 and Ets-1 in breast cancer cells needs to be defined.

Nmi was originally identified as a binding partner to the Myc family proteins Max and Mxi [27]. Nmi lacks an intrinsic transcriptional activation domain, but augments transcription in both Il-2-IFN-α-mediated signaling by acting as an adaptor. In a yeast two-hybrid screen using the coiled-coil region of STAT5b as the bait, Nmi was identified as a STAT5-interacting protein [28]. In this study, Nmi has been suggested as a potentiator of transcription that enhances the recruitment of coactivators to STATs in response to interleukin-2 and IFN-γ. Interestingly, they found that Nmi also interacted with all STAT proteins except STAT2. In this study, we found that the expressions of Nmi protein and mRNA were elevated in Ets-1 transfected cells. Although the exact mechanism

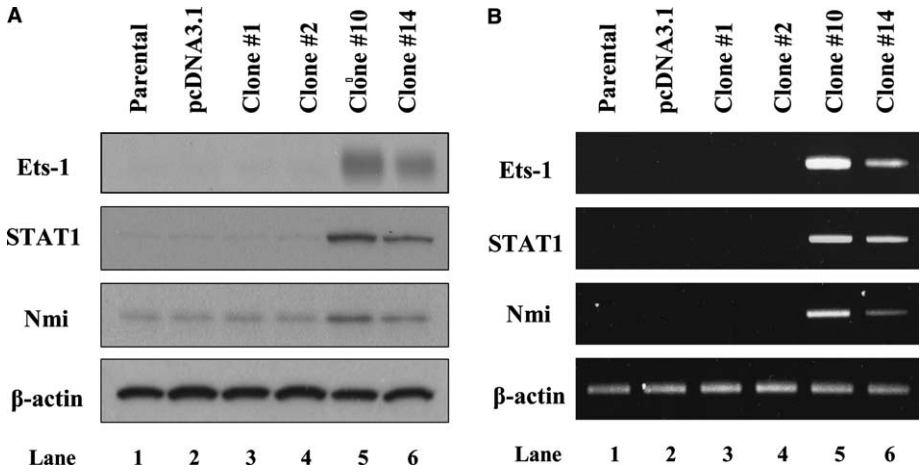

Fig. 2. Effect of endogenous Ets-1 on the expression of STAT and Nmi. Characterization of MCF-7 cells stably transfected with an Ets-1 expression plasmid. (A) Ets-1, FLAG, STAT and Nmi protein levels determined by Western blot in control and Ets-1-FLAG transfected MCF-7 clones. (B) Ets-1, STAT and Nmi RNA levels determined by RT-PCR in control and Ets-1-FLAG transfected MCF-7 clones. Lane 1, parental cell; lane 2, MCF-7 cells only transfected with the plasmid that confers resistance to G418; lanes 3–4, Ets-1-FLAG transfected clones #1, #2; lanes 5–6, cells transfected with the same plasmid as lane 2 but containing also the Ets-1-FLAG construct.

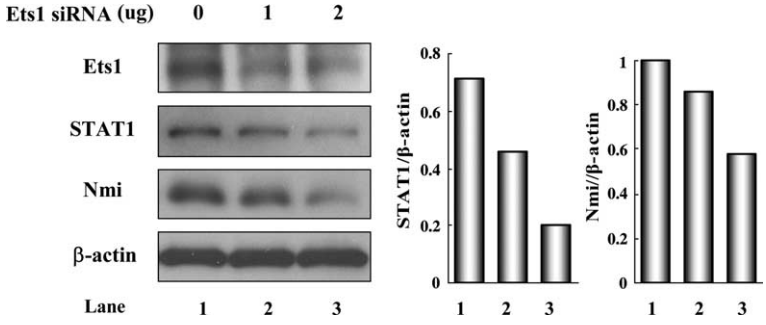

Fig. 3. The downregulation of STAT1 by Ets-1 siRNA. Representative Western blot for Ets-1 and STAT1 following treatment with Ets-1 siRNA. Ets-1 and STAT1 expressions were suppressed by Ets-1-specific siRNA. β-Actin expression was unaffected by siRNA treatment.

underlying Ets-1 induced Nmi expression remains uncertain, it can be speculated that Nmi may interact with STAT1, which is upregulated by Ets-1.

Taken together, cDNA gene expression profiling analysis demonstrated that STAT1 and Nmi are downstream cellular targets of Ets-1 in breast cancer cells. These proteins may be postulated to be potential targets to control tumor-associated Ets-1 activity in breast cancer patients.

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