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E1AF promotes breast cancer cell cycle progression via upregulation of Cyclin D3 transcription

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Abstract

E1AF transcription factor, a member of Ets family, is deregulated in many tumors and widely known to play critical roles in tumor metastasis via directly binding to the promoter of genes involved in tumor migration and invasion. Here, we found that E1AF overexpression promoted breast cancer cell cycle progression and growth *in vivo* as well as the transcription of cell cycle-related protein Cyclin D3. And, the interference of Cyclin D3 expression by transfecting with Cyclin D3 RNAi inhibited the positive role of E1AF in cell cycle progression. We further showed that decreasing the expression of E1AF by E1AF RNAi reduced Cyclin D3 transcription and expression, and inhibited cell cycle progression that was abrogated by Cyclin D3 overexpression. Taken together, E1AF increases cell cycle progression via upregulation of Cyclin D3 transcription, which elicits a new mechanism of breast cancer growth and a new mechanism of Cyclin D3 transcription.

Keywords: E1AF; Cyclin D3; MDA231; Transcription; Cell cycle

The Ets family of transcription factors characterized by an evolutionarily conserved DNA-binding domain regulate expression of a variety of viral and cellular genes by binding to a purine-rich GGAA/T core sequence in cooperation with other transcriptional factors and cofactor and play critical roles in regulation of cellular function including growth, apoptosis, development, differentiation, and oncogenic transformation [1,2].

One member of Ets-related family member, E1AF, a human homologue of mouse PEA3, was isolated by its ability to bind adenovirus E1A enhancer elements [3]. Biologically, E1AF contributes in a number of processes including neuronal pathfinding, mammary gland development, and male sexual function [4–6]. Pathologically, expression of E1AF is correlated with the metastasis phenotype of breast cancer and invasive phenotype of neuroblastoma, oral squamous cell carcinoma, and non-small-cell lung cancers [3,7–9]. And, E1AF plays an important role in HER2/Neu-mediated mammary oncogenesis and hepatocyte growth factor-induced cancer invasiveness and metastasis via directly binding to the promoters of genes involved tumor migration and invasion [10–12], suggesting the contribution of E1AF in various malignant phenotypes of cancers cells. In spite of this knowledge, the contribution and mechanisms of E1AF in tumor development remains largely unknown.

Here, we found that E1AF overexpression promoted breast cancer cell cycle progression and growth *in vivo* in a Cyclin D3-dependent manner. Furthermore, decreasing the expression of E1AF by E1AF RNAi reduced Cyclin D3 transcription and expression, and inhibited cell cycle progression that was abrogated by Cyclin D3 overexpression. Taken together, E1AF increases cell cycle progression via upregulation of Cyclin D3 transcription which elicits a new growth signal in breast cancer.

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Experimental procedures

Materials. Restriction enzymes, Bovine calf serum, RPMI-1640 medium, Trizol Reagent, and the mammalian expression vector pcDNA3 were from Invitrogen. G418, PMSF, aprotinin, and pepstatin were from Sigma Chemical Co. The enhanced chemiluminescence (ECL) assay and [γ-^{32}P]dATP were from Amersham Pharmacia Biotech. TaKaRa RNA PCR Kit (AMV Ver.2.1) and TakaRa MutanBEST kit was from TaKaRa. Anti-human-E1AF Ab (sc-113, sc-113X), anti-human-Cyclin D1, anti-human-Cyclin D2, anti-human-Cyclin D3, antibody-human-CDK4, anti-human-CDK6 antibodies were purchased from Santa Cruz Biotechnology. Anti-myc and anti-GAPDH antibodies were purchased from Oncogene. Anti-mouse-HRP secondary antibody and anti-rabbit-HRP secondary antibody were purchased from New England Biology. Other reagents were commercially available in China.

Cell culture and transfection and implantation of tumor cells in mice. MDA231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Cell transfection was performed with Lipofectamine (Invitrogen) according to the manufacturer's instructions. For stable transfection, 72 h after transfection, the cells were selected in the DMEM containing G418 (400 μg/ml). After 2–3 week growth in G418-containing medium, the individual G418-resistant clones were selected and expanded. Tumorigenicity assay was performed as described previously [13].

Plasmids. Expression constructs for pSilencer-2.0, Cyclin D3 RNAi, myc-tagged E1AF, Ets1, Ets2, Elk1, Net, ETV1, ETV5, Cyclin D3, pcDNA3.1-myc, Cyclin D3 promoter serious deletion mutation constructs and pGL3Basic have been described previously [13–16]. Cyclin D3 promoter MUT site-directed mutagenesis constructs (MUT1 and MUT2) were derived from pGL3(−300/−1) by PCR amplification using TakaRa MutanBEST mutagenesis kit. Construction of E1AF RNAi was performed using siRNA construction kit (KCsiRNA) according to the manufacture's suggestions [14]. The sequence of E1AF mRNA target oligonucleotide was as follows: 5′-AGGATCTAAGTCACTTCCA-3′, annealed and cloned into pSilencer-2.0 vector.

Analysis of cell cycle by FACS and flow cytometry and Western blot analysis. Cells were harvested and measured by flow cytometry 48 h after transfection, as previously reported [13]. Western blot was performed as previously described [14], using an antibody to GAPDH to ensure equivalent loading.

Dual luciferase assay and reverse transcription (RT)-PCR. Dual luciferase assay and reverse transcription PCR were performed as described [15]. Primers used for PCR were as follows: Cyclin D3-F 5′-AGTTGCGGGACTGGGAGGTG-3′ and Cyclin D3-R 5′-GTAGCA CAGAGGGCCAAAA-3′. The PCR products for Cyclin D3 were 184 bp.

Chromatin immunoprecipitation assay and gel shift assay. The association of E1AF with Cyclin D3 chromatin DNA in MDA231 cells was confirmed using a CHIP assay kit (Upstate Biotechnology) with anti-E1AF antibody as described by the manufacturer. Normal anti-mouse IgG was used as a negative control. The Cyclin D3 promoter region (−300/−1) was amplified by conventional PCR. Gel mobility shift assay was carried out using Gel Shift Assay System (Promega) as previously described [14].

Results

E1AF overexpression induced cell cycle progression and cell growth

To elucidate the biological significance of E1AF in breast cancer cell, myc-tagged E1AF construct was stably transfected into breast cancer cell line MDA231 and the expression of ectopic expression of E1AF in MDA231/Control and MDA231/myc-E1AF was examined by Western blot analysis (Fig. 1A). Cell cycle analysis showed that MDA231/myc-E1AF had a much higher percentage cells in the S-phase (60.66%) and a lower percentage cells in the G1-phase (14.34%), compared to MDA231/Control cells (34.26% and 52.44%, respectively) (Fig. 1B), indicating the contribution of E1AF in breast cancer growth. The same result was obtained from clonogenic assay (data not shown).

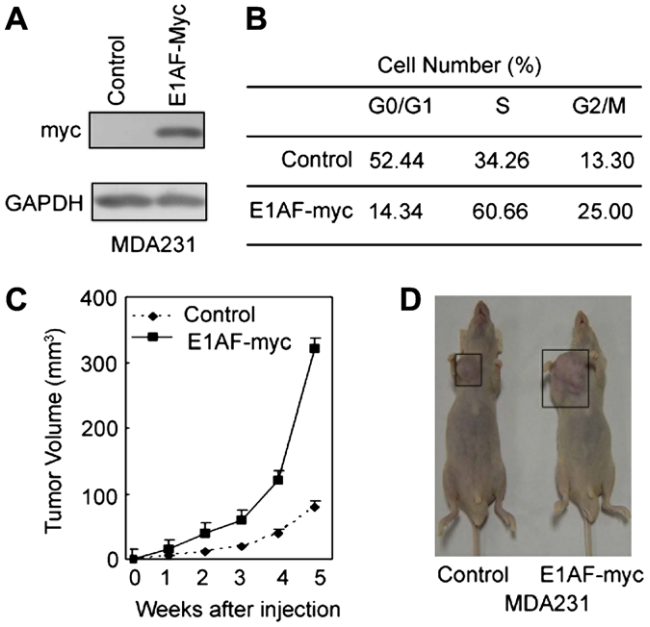


Fig. 1. E1AF overexpression promoted breast cancer cell MDA231 growth. (A) Western blot assay demonstrated E1AF-myc expression in MDA231 cells stably transfected with control or E1AF-myc construct using anti-myc antibody. GAPDH served as a loading control. (B) MDA231/Control or MDA231/E1AF-myc cells were harvested, and cell cycle parameters were determined. (C) Nude mice were injected with MDA231/Control or MDA231/E1AF-myc cells. Tumor volumes were measured every week. Each point represents the mean volume ± SD of eight tumors. (D) MDA231/Control or MDA231/E1AF-myc cells were injected into nude mice. Xenografts grew in nude mice for 5 weeks and photographs were taken.

On the basis of our *in vitro* studies indicating the contribution of E1AF in breast carcinoma cell proliferation, we examined the role of E1AF in the ability of breast carcinoma cell to form tumors *in vivo* in nude mice. These cells were s.c. injected into 8-week-old nude mice, and tumor growth was measured once a week. Compared to the control cells, MDA231/myc-E1AF cells grew significantly faster at all time points examined (Fig. 1C), and developed tumors with a markedly larger size during the 5 weeks of observation (Fig. 1D).

E1AF overexpression upregulated Cyclin D3 expression via transcriptional activation

To address the mechanisms of E1AF-enhanced cell growth, we investigate the effect of E1AF overexpression on the expression of Cyclin D family protein (Cyclin D1, D2, and D3) and their corresponding kinases (CDK4 and CDK6), which are believed to be involved in the regulation

of cell cycle progression and mitogenic signals [17]. As depicted in Fig. 2A, E1AF overexpression significantly induced the expression of Cyclin D3, without significantly changing the other proteins examined. Consistent with previous report [18], the expression of Cyclin D2 protein was rarely detected in MDA231 cells.

To clarify the role of E1AF in regulation of Cyclin D3 expression, the contribution of E1AF in Cyclin D3 mRNA expression level was measured by semiquantitative RT-PCR assay. As shown in Fig. 2B, the ectopic expression of E1AF dramatically increased Cyclin D3 mRNA expression level. To determine the role of E1AF in Cyclin D3 transcription, we constructed Cyclin D3 promoter containing a region from nucleotide position −2064 to −1 relative to the ATG start codon. Transfection studies using the Cyclin D3 promoter construct pGL3(−2064/−1) and increasing amounts of E1AF expression plasmids showed that the forced expression of E1AF stimulated the activity of Cyclin D3 promoter in a dose-dependent manner in MDA231 cells (Fig. 2C). To assess the specificity of the members of the Ets transcription factor family in the regulation of the Cyclin D3 promoter activity, pGL3(−2064/−1) was co-transfected into MDA231 cells along with Ets family expression plasmid, such as Ets1, Ets2, E1AF, Elk1, ETV1, ETV5, Net or the empty control. The highest activation of the Cyclin D3 promoter was obtained by E1AF (Fig. 3A).

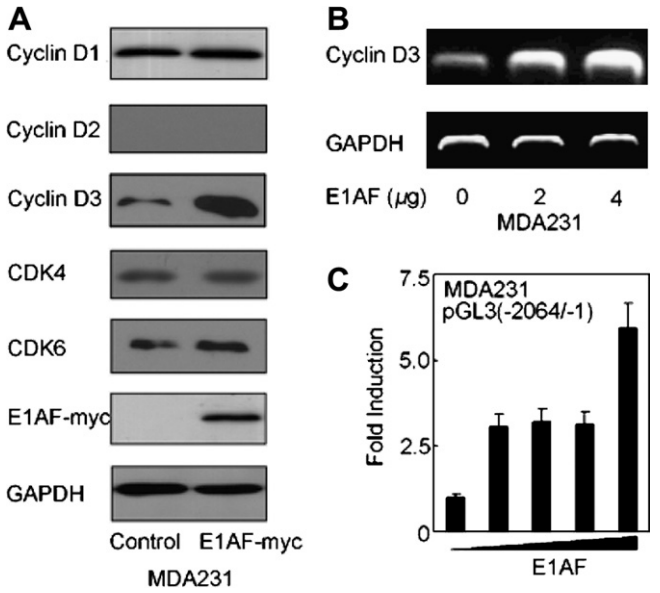


Fig. 2. E1AF overexpression increased Cyclin D3 levels. (A) Equal amounts of proteins from MDA231/Control or MDA231/E1AF-myc cells were immunoblotted with the indicated antibodies. (B) MDA231 cells were transiently transfected with vector or E1AF and the level of Cyclin D3 mRNA expression was analyzed by RT-PCR. (C) E1AF in dose dependence of Cyclin D3 promoter activation. Increasing amounts of E1AF expression plasmids were transiently co-transfected into MDA231 cells with Cyclin D3 promoter construct pGL3(−2064/−1). Luciferase activities were measured as described above. Normalized luciferase activity was standardized to pGL3(−2064/−1) with vector alone. Each value is the means ± SD of at least three independent experiments.

E1AF bound to Cyclin D3 promoter in vitro and in vivo

To identify the *cis*-element responsible for the effect of E1AF, luciferase reporter constructs containing progressive deletion of the 2064-bp genomic DNA fragment were generated, and co-transfected into MDA231 cells along with empty control vector or E1AF expression vector. The luciferase assay showed that a deletion from −300 to −162 resulted in a loss of E1AF activation (Fig. 3B). Inspection of this region between −251 and −248 revealed one potential Ets-binding site. Next, EMSA was performed to determine whether E1AF recognized the Cyclin D3 promoter. Nuclear extracts from MDA231 cells formed the complex with the E1AF-consensus sequence probe (Fig. 3C, lane 2), and the complex was disrupted by treatment with the unlabeled double-strand oligonucleotides spanning the region between nucleotide positions −262 to −240 of the Cyclin D3 promoter (Fig. 3C, lane 3). Furthermore, incubation of the double-stranded 23-mer oligo probe between nt −262 and −240 with MDA231 nuclear extracts formed specific protein–DNA complex (Fig. 3C, lane 4), which was markedly disrupted by incubation with the unlabeled E1AF-consensus oligonucleotides in a dose-dependent manner (Fig. 3C, lanes 5 and 6). To identify specific proteins that bind to the Ets-binding site, we used antibodies against E1AF. It was found that antibody against E1AF supershifted protein–DNA complexes (Fig. 3C, lane 7), indicating the binding of E1AF to Cyclin D3 promoter.

CHIP assay was performed on MDA231 cells to ascertain whether endogenous E1AF binds to the Cyclin D3 promoter *in vivo*. As shown in Fig. 3D, PCR primers that span the region of −300 to −1 of the Cyclin D3 promoter clearly detected Cyclin D3 promoter DNA in CHIP samples generated using an E1AF antibody. These data demonstrated that E1AF associated with the Cyclin D3 promoter under physiological conditions. To determine whether this Ets-binding site was responsible for the positive effect of E1AF on Cyclin D3 transcription, we introduced site-directed mutagenesis into this Ets-binding site or the bases in its vicinity (Fig. 3E, upper panel). It was found that mutation of this Ets-binding site deprived E1AF of responsiveness (Fig. 3E, lower panel). Taken together, these data identified Cyclin D3 as a direct target gene of E1AF transcription factor.

E1AF promoted MDA231 cell cycle progression in a Cyclin D3-dependent manner

If increased Cyclin D3 expression was required for E1AF-induced cell cycle progression, then downregulation of Cyclin D3 might overcome E1AF-increased cell growth. To address this point, siRNA targeted to Cyclin D3 was transiently transfected into MDA231 cells alone or with E1AF expression vector, following FACS analysis. As expected, downregulation of Cyclin D3 mediated by siRNA targeted to Cyclin D3 decreased the proportion of cells in S-phase with a concomitant increase in the number of

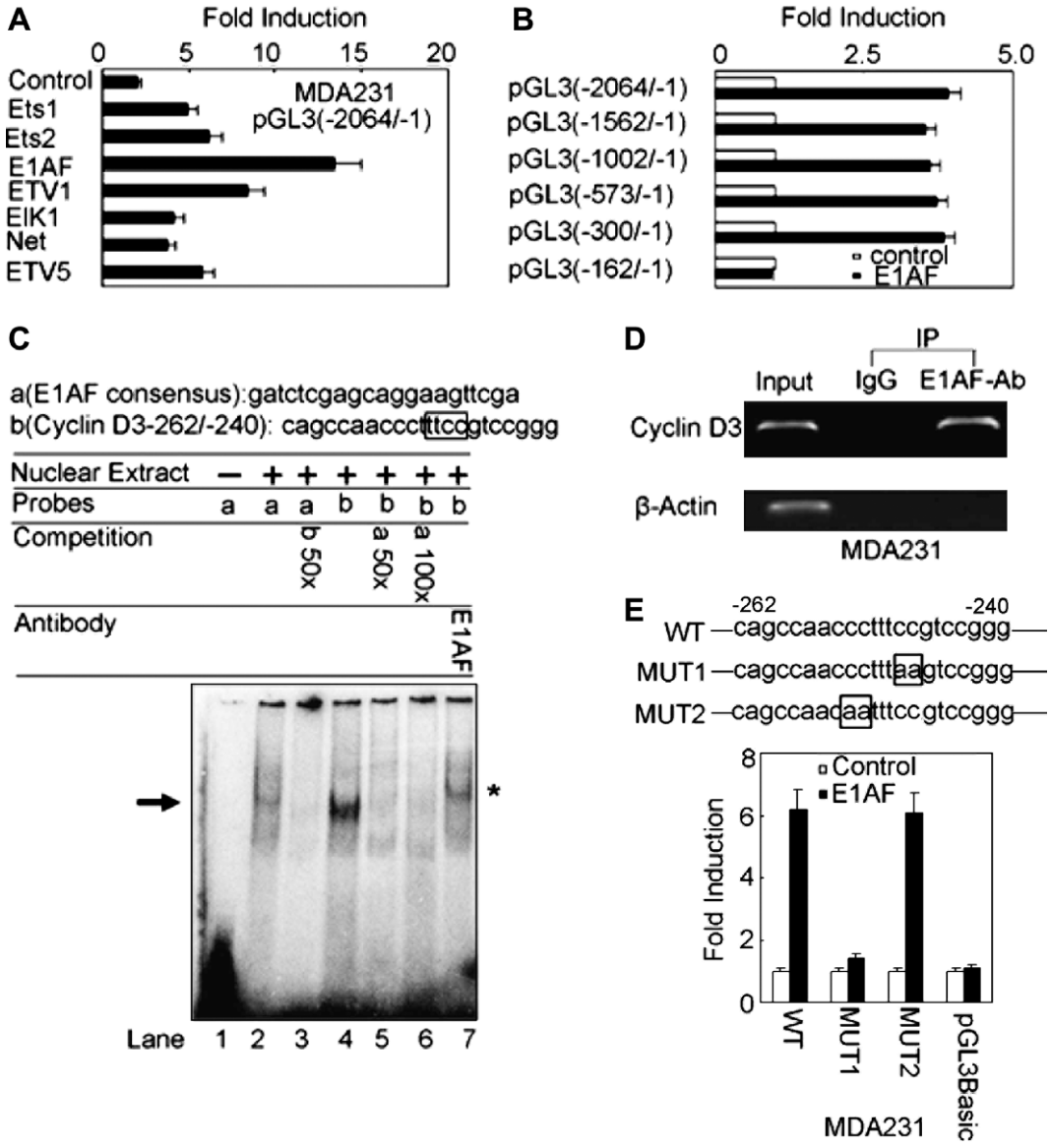
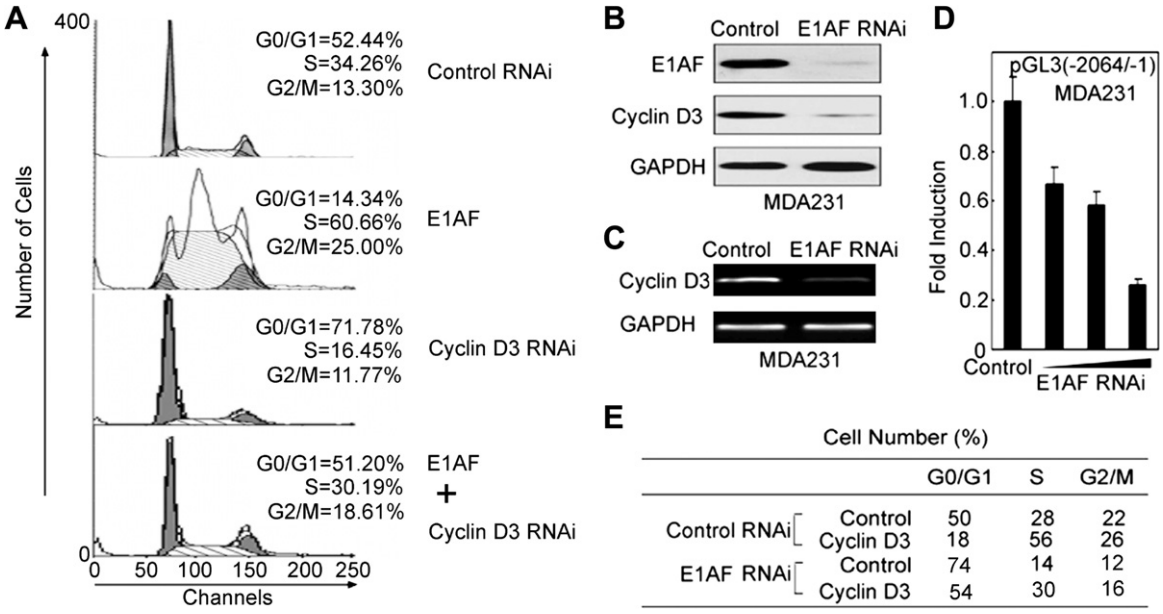


Fig. 3. E1AF bound to Cyclin D3 promoter *in vitro* and *in vivo*. (A) Cyclin D3-Luc construct pGL3(−2064/−1) and expression plasmids for E1AF, Ets1, Ets2, ETV1, Elk1, Net or the empty control vector were transiently co-transfected into the MDA231 cells. Normalized luciferase activity was standardized to pGL3(−2064/−1) with vector alone. Each value is the means ± SD of at least three independent experiments. (B) Cyclin D3-luc constructs containing various lengths of Cyclin D3 promoter regions were transiently co-transfected with control vector or E1AF into MDA231 cells. Luciferase activities were measured as described above. Normalized luciferase activity was standardized to that with vector alone. (C) Two oligo sequences corresponding to E1AF-binding consensus and the probes containing human Cyclin D3 promoter sequence −262 to −240 used in the following EMSA studies were listed and the potential Ets-binding site was shown in black plane (upper panel). Nuclear extracts from MDA231 cells were incubated with ^{32}P-labeled probe of E1AF-binding consensus sequence or Cyclin D3 promoter sequence −262 to −240 untreated or treated with the indicated cold competitors stranded oligonucleotides or an antibody specific to E1AF. The arrowhead indicated the DNA-protein complexes and the supershifted complex was indicated by asterisk. (D) Chromatin immunoprecipitation (CHIP) assays were performed using an antibody against E1AF or normal mouse IgG. PCR primers for the Cyclin D3 promoter (upper panel) or the actin promoter (lower panel) were used to detect promoter fragments in immunoprecipitates. (E) Schematic representation of the Cyclin D3 promoter pGL3(−300/−1) with the wild type (WT), Ets-binding site mutated (MUT1) or the bases in its vicinity mutated (MUT2) and mutated sites were indicated using pane (upper panel). Luciferase reporter vectors were transiently co-transfected along with control vector or E1AF expression vector into MDA231 cells and luciferase activities were measured as described above (lower panel).

cells in G0/G1 and blocked the positive role of E1AF in cell cycle progression (Fig. 4A). To further investigate the role of E1AF-induced Cyclin D3 expression in cell cycle progression, siRNA construct targeted to E1AF was constructed and transiently transfected into MDA231 cells and the expression of E1AF and Cyclin D3 was investigated. As depicted in Fig. 4B, decreasing the expression of E1AF by E1AF RNAi inhibited the level of Cyclin D3 protein expression. Consistent with this, decreasing the expression of E1AF by E1AF RNAi reduced the level of Cyclin D3 mRNA expression (Fig. 4C), and inhibited the activity of Cyclin D3 promoter in a dose-dependent manner in MDA231 cells (Fig. 4D). Furthermore, downregulation of E1AF mediated by E1AF RNAi decreased the



		G0/G1	S	G2/M
Control RNAi	Control	50	28	22
	Cyclin D3	18	56	26
E1AF RNAi	Control	74	14	12
	Cyclin D3	54	30	16

Fig. 4. The contribution of E1AF-induced Cyclin D3 expression in cell cycle progression. (A) MDA231 cells were transiently transfected with control RNAi, and/or Cyclin D3 RNAi, and/or E1AF. Forty-eight hours after transfection, cell cycle parameters were determined. (B) Equal amounts of proteins from MDA231 transiently transfected with control RNAi or E1AF RNAi were immunoblotted with the indicated antibodies. (C) MDA231 cells were transiently transfected with control RNAi or E1AF RNAi and the level of Cyclin D3 mRNA expression was analyzed by RT-PCR. (D) Increasing amounts of E1AF RNAi plasmids were transiently co-transfected into MDA231 cells with Cyclin D3 promoter construct pGL3(−2064/−1). Luciferase activities were measured as described above. (E) MDA231 cells were transiently transfected with control RNAi, and/or E1AF RNAi, and/or Cyclin D3. Forty-eight hours after transfection, cell cycle parameters were determined.

proportion of cells in S-phase with a concomitant increase in the number of cells in G0/G1 by FACS analysis, which was blocked by Cyclin D3 overexpression (Fig. 4E).

Discussion

One of the main properties of cancer cells is their increased and deregulated proliferative activity. It is now well known that abnormalities in many positive and negative modulators of the cell cycle are frequent in many cancer types, including breast carcinomas [19,20]. In this study, we provided evidence that E1AF-induced Cyclin D3 expression was necessary for breast cancer cell growth.

E1AF is invariably overexpressed in mammary tumors of transgenic mice that expression the wide-type rat neu cDNA and overexpressed in 76% of all human breast tumors [21,22]. Accumulated evidences indicated that E1AF plays key regulatory roles in both mammary gland development and tumor invasiveness and metastasis through transcriptions of metastasis-related genes [3,6,23]. However, the mechanism of how E1AF influences the tumor cell development remained largely unknown. Here, we found that E1AF overexpression induced cell cycle transition from G1- to S-phase, promoted cell growth *in vivo* and induced the expression of Cyclin D3. Consistent with this, decreasing the expression of E1AF by E1AF RNAi reduced Cyclin D3 transcription and expression as well as cell cycle progression in a Cyclin D3-dependent manner. Furthermore, the interfering of expression of Cyclin D3 inhibited the positive role of E1AF in cell cycle progression. Taken together, these data suggested that E1AF-induced Cyclin D3 expression might contribute in breast cancer development.

Cyclin D3, a member of the Cyclin D family, regulates the initial G1 to S transition by inhibiting Rb and activating E2F proteins and contributes in cell apoptosis, proliferation and differentiation [24–28]. Cyclin D3 is amplified and/or overexpressed in a number of human cancers, including breast carcinomas, B cell and T cell malignancies and contributed in tumor cell growth [24,27–31]. Accumulated evidences indicated that there is extensive cross talk between the transcriptional and posttranscriptional regulation of Cyclin D3 expression and its biological functions [32–35]. Here, we described several observations to identify Cyclin D3 as a target gene of E1AF transcription factor. Co-transfection with E1AF resulted in 15-fold increase in luciferase activity as compared with vector alone and the consensus EBS in the region between nt −262 and −240 in Cyclin D3 promoter was critical for activation by E1AF. EMSA and CHIP analysis showed specific binding of E1AF to this EBS within Cyclin D3 promoter in MDA231 cells. To the best of our knowledge, this is the first report of E1AF-targeted growth-related gene and Ets family medicating Cyclin D3 transcription regulation, which provided a new regulation mechanism of Cyclin D3 transcription.

In a summary, E1AF increases cell cycle progression via upregulation of Cyclin D3 transcription, which elicits a new growth signal in breast cancer and a new mechanism

of Cyclin D3 transcription. And, the contribution of E1AF-induced Cyclin D3 expression in other tumor growth should be further investigated.

Acknowledgments

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