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Transforming Growth Factor-β1 Regulation of ATF-3 and Identification of ATF-3 Target Genes in Breast Cancer Cells

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ABSTRACT

Transforming growth factor-β1 (TGF-β1) is a crucial molecule for stimulation of breast cancer invasion and formation of bone metastases. The molecular mechanisms of how TGF-β1 mediates these effects have yet to be completely determined. We have found that activating transcription factor-3 (ATF-3) is strongly stimulated and its level is sustained by TGF-β1 in highly invasive and metastatic human breast cancer (MDA-MB231) and in mouse mammary pad tumor cells (r3T). ATF-3 is also overexpressed in human primary breast cancer tissue. Overexpression of ATF-3 increased normal human mammary epithelial cell number and DNA synthesis suggesting a role for ATF-3 in cell proliferation. The functional role of ATF-3 in breast cancer progression was determined by the RNA interference technique. Knockdown of ATF-3 by ATF-3 shRNA in MDA-MB231 cells decreased expression of cell cycle gene, cyclin A1 in MDA-MB231 cells. ATF-3 shRNA also decreased expression of an invasive and metastatic gene, matrix metalloproteinase-13 (MMP-13; collagenase-3) in these cells. Chromatin immunoprecipitation experiments identified the direct physical interaction of ATF-3 protein on the human MMP-13 promoter. Thus, the dysregulation of ATF-3 by TGF-β1 is likely to activate cyclin A1 and MMP-13 genes in breast cancer cells and that would be key to the subsequent cancer cell invasion and metastasis. J. Cell. Biochem. 108: 408–414, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: TGF-B; ATF-3; CYCLIN A1; MMP-13

Transforming growth factor-β1 (TGF-β1), a multipotent cytokine, has a wide range of physiological and pathological effects [Piek et al., 1999; Massague and Wotton, 2000]. TGF-β is the most potent known growth inhibitor for epithelial cells [Piek and Roberts, 2001] and targeted disruption of the *Tgfb1* gene in mice results in carcinomas [Engle et al., 1999]. TGF-β signaling involves the type I receptor TβR-I, the type II receptor TβR-II, the regulatory Smads (Smad2 and Smad3), and Smad4 [Massague, 2000]. Most of these components are mutated in several human cancers, yet mutations in TGF-β receptors or Smads are rare in breast cancer [Anbazhagan et al., 1999; Tomita et al., 1999]. Indeed, for breast cancer cells, TGF-β1 is a crucial molecule in stimulating metastatic breast cancer invasion [Welch et al., 1990; Oft et al., 1996] and formation of TGF-β-dependent bone metastases in model systems [Yin et al., 1999].

Independent observations over the years have defined a small group of immediate TGF-β target genes that contribute to the effect of TGF-β on epithelial cell homeostasis and growth inhibition [Turner et al., 1998; Yin et al., 1999; Massague, 2000; Chen et al., 2001]. There is growing evidence indicating that transcription

Abbreviations used: TGF-β, transforming growth factor-beta; ATF-3, activating transcription factor-3; shRNA, short hairpin RNA; MMP-13, matrix metalloproteinase-13; AP-1, activator protein-1; RD, runt domain; CAT, chloramphenicol acetyl transferase; RT-PCR, reverse transcriptase polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; ChIP, chromatin immunoprecipitation.

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factors such as GADD153, Twist, Runx2, Stat3, NRIF3, TBX3, NF kappaB, DEC1 have the ability to alter the progression of breast cancer growth and metastasis. ATF-3 (activating transcription factor-3), a member of the ATF/CREB subfamily is a bZip transcription factor [Hsu et al., 1992, 1993; Chen et al., 1994; Kang et al., 2003]. ATF-3 is expressed at very low levels in normal, quiescent cells but can be rapidly and highly induced in different cell types by multiple and diverse extracellular signals [Wolfgang et al., 1997; Hai et al., 1999]. ATF-3 is a common target of TGF-β1 and stress signals and serves to inhibit cell growth in normal epithelial cells [Kang et al., 2003]. Thus, there is strong circumstantial evidence that this transcription factor plays an important role in the regulation of normal and neoplastic growth responses.

To date, only a few target promoters for ATF-3 (gadd153/CHOP10, cyclin D1 and ATF-3 itself) [Wolfgang et al., 1997; Hai et al., 1999; Wolfgang et al., 2000; Allan et al., 2001] have been identified. The presence of potential ATF-3 binding sites in the promoter regions of other cyclins [Yoshizumi et al., 1995] and of Rb [Sakai et al., 1991] suggests that several additional cell cycle-related genes may be subject to regulation by ATF-3. To our knowledge, no study has been reported investigating the functional role of ATF-3 in breast cancer progression. Since breast cancer progression is a multi-step process, we addressed the functional role of ATF-3 and its target genes in breast cancer cell growth (in terms of cell proliferation, invasion and metastatic ability) at molecular level in vitro by utilizing RNA interference. We have identified for the first time the requirement of ATF-3 for expression of cyclin A1 (cell cycle gene) and matrix metalloproteinase-13 (MMP-13; collagenase-3; invasive and metastasis gene) genes in breast cancer cells which may suggest a role for ATF-3 in breast cancer cell growth and metastasis.

MATERIALS AND METHODS

MATERIALS

Human TGF-β1 was purchased from R&D Systems (MN). Tissue culture media and reagents were obtained from Invitrogen. The c-Jun, JunB, ATF-3, and α-tubulin antibodies were purchased from Santa Cruz Biotechnology (CA). Other chemicals were obtained from Sigma (St. Louis).

CELL CULTURE

MDA-MB231 and MCF-10A cells were obtained from American Type Culture Collection (ATCC). The cells are maintained in culture in Dulbecco's modified essential medium-(DMEM-F12) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The r3T cells are maintained in α-MEM supplemented with 10% FBS, EGF, insulin, and hydrocortisone at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

ATF-3 SHRNA CONSTRUCTION

Targeted gene silencing through RNA interference (RNAi) utilizes vectors expressing short hairpin RNA (shRNA), which is processed in the cells to siRNA. The human ATF-3 shRNA has been cloned into siSTRIKE U6 hairpin system (Promega, WI). Two hairpin oligonucleotides (Oligo A and Oligo B) were synthesized (Invitrogen). Oligonucleotide A hairpin sequence contained the ATF-3 target sequence, the loop sequence and the reversed target sequence. Oligonucleotide B contained the complementary sequence of the oligonucleotide A hairpin sequence. The oligonucleotides A and B were annealed to form a double-stranded DNA fragment for ligation into the psiSTRIKE vector. Nucleotide sequences of ATF-3 shRNA were selected using the siRNA tool at: www.promega.com/techserv/tools/. The human ATF-3 target sequences used in the RNA interference study are as follows: 5′ GGTTTGCCATCCAGAACA 3′; 5′ GTCTCTGCCTCGGAAGTG 3′. The scrambled sequence used in this study is as follows: 5′ TGACCACCCTGACCTACG 3′.

TRANSIENT TRANSFECTION

The plasmid DNAs were transiently transfected into cells using GeneJammer, according to the manufacturer's (Stratagene) instructions. Briefly, cells were plated at $1\text{–}2 \times 10^5$ in 6-well plates in DMEM-F12 containing 10% FBS. The following day, the cells were transfected with 1 μg DNA and 5 μl GeneJammer per well in 1 ml of serum-free DMEM-F12. After 3 h, 1 ml of DMEM-F12 containing 10% FBS was added. After 24 h, the cells were treated with either control or TGF-β1 (1 ng/ml)-containing media for 24 h. CAT activity was measured by reacting 50 μl of cell lysate in duplicate in a 100 μl reaction volume consisting of final concentrations of 250 μM *n*-butyryl-coenzyme A and 23 mM [^{14}C]-chloramphenicol (0.125 μCi/assay) as described previously [Selvamurugan et al., 1998]. A standard curve using purified CAT was performed for every experiment to determine the linear range of the enzyme assay. The normalization of transfection was also carried out by cotransfection with a Renilla luciferase reporter gene [Selvamurugan et al., 2004].

WESTERN BLOT ANALYSIS

Whole cell extracts were prepared as described previously [Selvamurugan et al., 2004]. The proteins were resolved by 12% SDS–PAGE. The proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad). After blocking in Tris-buffered saline containing 5% (w/v) nonfat dry milk, the membrane was exposed to primary antibody overnight at 4°C. The membrane was washed and exposed to horseradish peroxidase-conjugated secondary antibody. The immunoreactive signals were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences). The experiments were carried out at least three times.

CELL PROLIFERATION

Cells were seeded at 1×10^5 cells/well in 6-well plates. Cells were transiently transfected with empty vector (pCMV) or vector containing ATF-3 (pCMV-ATF-3) for 24 h using GeneJammer as described above. On day 6, cells were detached from the plates with trypsin/EDTA, and the cell numbers were counted using a haemocytometer.

Cells were seeded at 1×10^5 cells/well in 6-well plates. Cells were transiently transfected with empty vector (pCMV) or vector containing ATF-3 (pCMV-ATF-3) for 24 h. Cells were switched to

0.1% serum containing medium for 1 day followed by incubation with [^{3}H]thymidine (1 μCi/ml) for 3 h. DNA-containing incorporated radiolabel was collected onto glass fiber filters using a semiautomatic cell harvester (Skatron), whereas unincorporated [^{3}H]thymidine was removed by exhaustive water elution. The incorporation was assayed by liquid scintillation counting.

SEMIQUANTITATIVE RT-PCR

Total RNA was prepared using the Trizol Reagent method (Sigma) and RNA was then used for reverse transcription polymerase chain reaction (RT-PCR) with the SuperScript III First Strand cDNA synthesis kit (Invitrogen) and the Taq PCR Master Mix kit (Qiagen). The oligonucleotides (sense 5′-CCTCCTGGGCCAAATTATGGAG-3′ and antisense 5′-CAGCTCCGCATCAACCTGCTG-3′) corresponding to human MMP-13 were used for specific amplification of a 392 bp fragment of MMP-13 mRNA as described previously [Selvamurugan et al., 2002].

CHROMATIN IMMUNOPRECIPITATION (CHIP)

ChIP assays were carried out using the ChIP kit from Upstate Biotechnology as described previously [Selvamurugan et al., 2004]. Soluble chromatin was prepared. Aliquots (1/100) of total chromatin DNA before immunoprecipitation were saved (input). Pre-cleared lysates were used for immunoprecipitation experiments with either IgG or ATF-3 antibody overnight at 4°C. The immunocomplexes were eluted by adding a 250-μl aliquot of a freshly prepared solution of 1% SDS, 0.1 M $NaHCO_3$. Samples were sequentially digested with RNase A (10 mg/ml) at 37°C for 1 h and proteinase K (20 mg/ml) at 42°C for 2 h to remove RNA and protein. The cross-linking reaction was reversed by 4 h incubation of the sample at 68°C, and the DNA was recovered. An aliquot of each DNA fraction was used for semiquantitative PCR to detect the presence of specific DNA segments.

The sequences of the human MMP-13 promoter oligonucleotide probes used in this study are as follows:

Proximal AP-1 site

5′ GTCGCCACGTAAGCATGTTT 3′ (sense);
5′ CATCTTGAATGGTGGTGCCT 3′ (antisense).

Distal RD/Runx site

5′ CCATAAATATGCTGAGGCCG 3′ (sense);
5′ GGTTTCCACTTCCTAGTCAC 3′ (antisense).

RESULTS

In order to investigate the role played by ATF-3 in breast cancer cell growth and metastasis, we first examined TGF-β1 regulation of ATF-3 in MCF-10A (normal human mammary epithelial cells) and MDA-MB-231 cells (invasive and bone metastatic human breast cancer cells). The cells were treated with TGF-β1 at different time periods, the whole cell lysates were prepared and subjected to Western blot analysis. TGF-β1 stimulated expression of ATF-3, c-Jun, and JunB in both MCF-10A and MDA-MB231 cells but ATF-3 and c-Jun levels were sustained in MDA-MB231 cells (Fig. 1A,B). The TGF-β1-stimulation of ATF-3 expression was found even at 24 h in MDA-MB231 cells (data not shown). There was no change in the level of JunD expression after TGF-β1 treatment following normalization with α-tubulin expression in MCF-10A and MDA-MB231 cells.

We next generated a series of metastatic murine mammary epithelial cell lines using normal mice rather than nude mice. Cardiac injection of mouse mammary pad tumor cell line r3T into 129 strain female mice leads to development of bone metastases [Chen and Rittling, 2003]. In order to identify the expression pattern of ATF-3, r3T cells were treated with TGF-β1 and subjected to Western blot analysis. TGF-β1 stimulated expression of ATF-3 and its level was sustained even at 24 h in r3T cells (Fig. 2A). The observation of a low basal expression of these proteins in these cells could be due to either the nature of treatment, that is, medium containing 0.1% serum, or the secreted factors (autocrine) from the cells (Figs. 1 and 2).

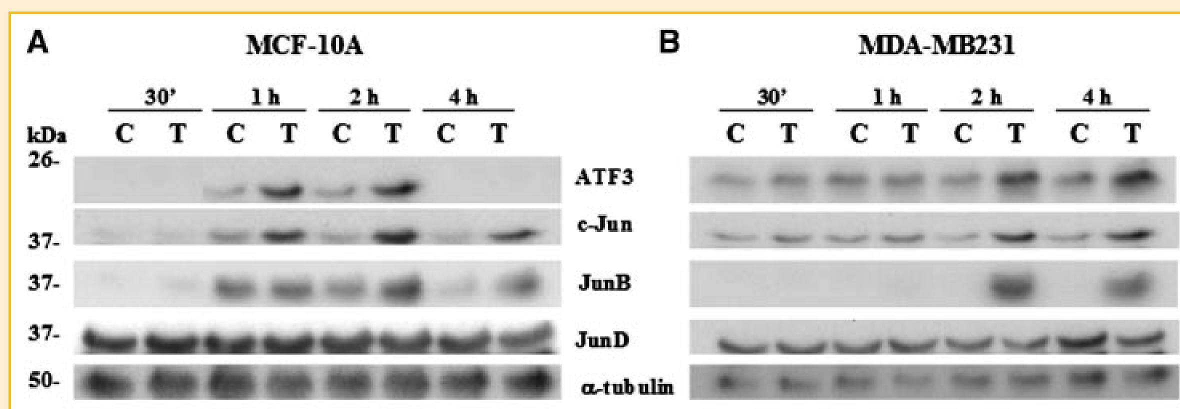

Fig. 1. MCF-10A (A) and MDA-MB231 (B) cells were treated with control or TGF-β1 (1 ng/ml)-containing media for the indicated times. Whole cell lysates were prepared and subjected to Western blot analysis using the antibodies shown in the figure. α-Tubulin was used as a loading control (C: control; T: TGF-β1).

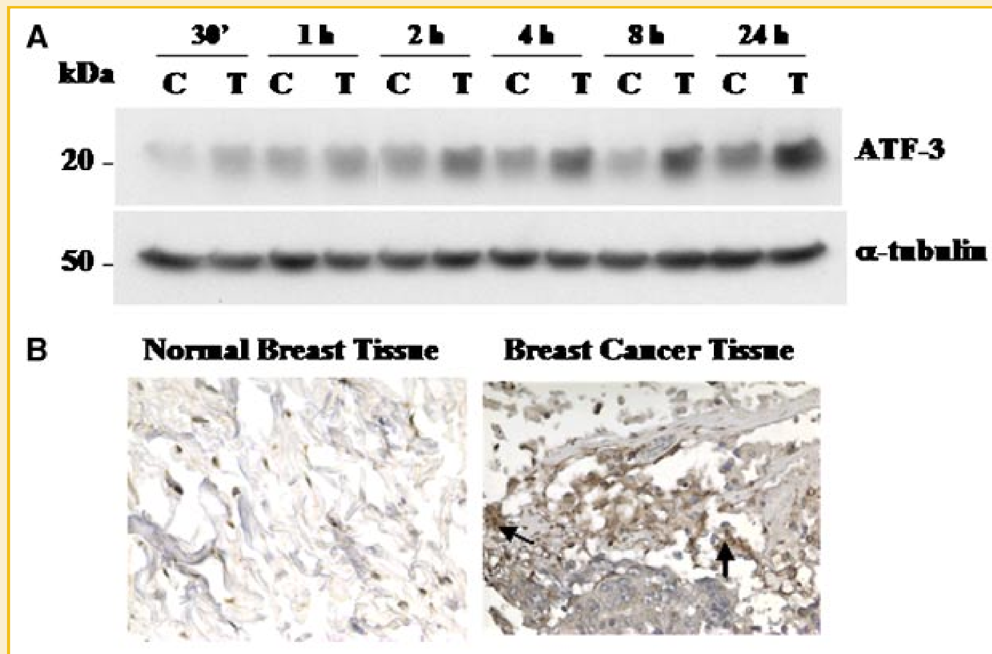

Fig. 2. A: Mouse mammary pad tumor cells (r3T) were treated with control or TGF-β1 (1 ng/ml) containing media for the indicated times. Whole cell lysates were prepared and subjected to Western blot analysis using the antibodies shown in the figure. α-Tubulin was used as a loading control (C: control; T: TGF-β1). B: Histospot staining in tissue microarrays shows low level staining in normal breast tissue and strong staining in breast cancer tissue (40× magnification). ATF-3 protein is represented by brown staining (arrows).

We wanted to further determine the relative expression level of ATF-3 in human primary breast tumors. Slides containing normal human breast tissue and human primary breast cancer tissue were obtained from Imgenex, CA and were processed for immunohistochemical staining with ATF-3 antibody. The results clearly indicate that ATF-3 expression was very low in normal breast tissue and was high in breast cancer tissue (Fig. 2B).

Since ATF-3 expression is prolonged and sustained in human and mouse mammary cancer cells (Figs. 1 and 2A) and is highly expressed in human primary breast cancer (Fig. 2B), we wanted to first determine whether ATF-3 expression is sufficient to induce cellular proliferation in vitro. MCF-10A cells were transiently transfected with the ATF-3 eukaryotic expression plasmid (pCMV-ATF-3). The empty eukaryotic expression plasmid (pCMV) was also transfected as a control for transfection effects. The expression level of ATF-3 protein was confirmed by Western blot analysis (data not shown). The cells were counted using a haemocytometer on day 6. To determine DNA synthesis, cells were pulsed with 0.5 μCi/ml [^{3}H]thymidine for 3 h before harvesting and assessing incorporated radioactivity. The results indicated that overexpression of ATF-3 increases normal human mammary epithelial cell number (Fig. 3A) and DNA synthesis (Fig. 3B) over control (empty vector).

To determine the functional role of ATF-3 in breast cancer metastasis, we used the RNA interference technique for in vivo depletion of a gene product. The hairpin oligonucleotides that target ATF-3 (ATF-3 shRNA) or nonspecific sequences (scrambled shRNA) were cloned into the psiSTRIKE U6 hairpin vector (Promega, WI). The vectors containing either the scrambled shRNA or ATF-3 shRNA were transiently transfected into MDA-MB231 cells. The cells were then treated with TGF-β1. The lysates were prepared and subjected to Western blot analysis. Transient transfection of MDA-MB231 cells with the psiSTRIKE vector that contained hairpin oligonucleotides with a human ATF-3 target sequence decreased both the basal and TGF-β1-stimulated ATF-3 expression, compared with the

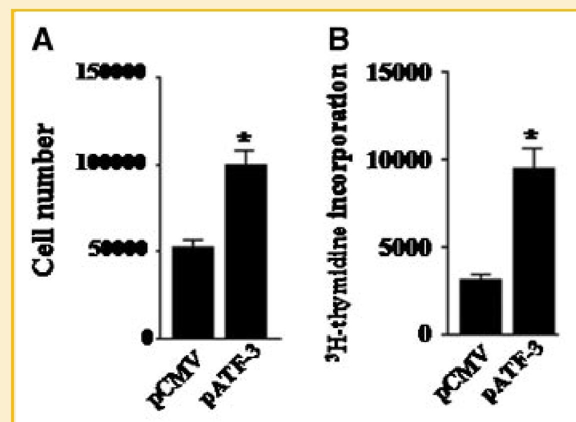

Fig. 3. MCF-10A cells were transiently transfected with either pCMV or pCMV-ATF-3 vectors for 24 h. A: On day 6, cells were trypsinized, harvested, and counted by haemocytometer. B: Cell proliferation in terms of DNA synthesis was assessed by measuring the incorporation of ^{3}H-thymidine into DNA for a 3 h period. Cells were harvested onto glass fiber filters using an automated cell harvester, and counted in a Packard liquid scintillation counter. Data represent mean ± SE of three experiments. * Significant difference compared with empty vector, pCMV ($P < 0.05$).

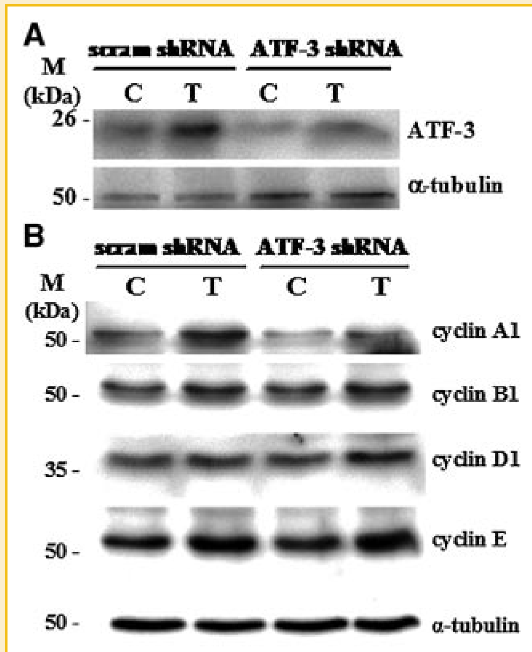

Fig. 4. A: MDA-MB231 cells were transiently transfected with either scrambled shRNA or ATF-3 shRNA constructs for 24 h and then treated with control or TGF-β1 (1 ng/ml)-containing media for 4 h. Whole cell lysates were prepared and subjected to Western blot analysis using the ATF-3 and α-tubulin (loading control) antibodies. B: MDA-MB231 cells were transiently transfected with either scrambled shRNA or ATF-3 shRNA constructs for 24 h and then treated with control or TGF-β1 (1 ng/ml)-containing media for 24 h. Whole cell lysates were prepared and subjected to Western blot analysis using the antibodies as indicated. α-Tubulin was used as a loading control.

nonspecific target sequences (Fig. 4A). Since TGF-β1 stimulates and sustains expression of ATF-3 in MDA-MB231 and r3T cells (Figs. 1 and 2A) and ATF-3 increases cell proliferation in normal human mammary epithelial cells, MCF-10A (Fig. 3), we wanted to determine whether knockdown of ATF-3 expression has any effect on expression of cell cycle genes. MDA-MB231 cells were transiently transfected with either scrambled shRNA or ATF-3 shRNA constructs. The cells were then treated with TGF-β1, followed by preparation of lysates and Western blot analysis. As shown in Figure 4B, TGF-β1 stimulated expression of cyclin-A1, -B1, and -E and to a lesser extent, -D1 in these cells while ATF-3 shRNA only decreased expression of cyclin A1 in both control and TGF-β1-stimulated MDA-MB231 cells. Thus, ATF-3 must be involved in mediating TGF-β1-stimulation of cyclin A1, and cyclin A1 is possibly an ATF-3 target gene.

Since MDA-MB231 cells are highly invasive and bone metastatic in nature and TGF-β1 stimulates expression of MMP-13 (an invasive and metastatic gene) [Selvamurugan et al., 2002, 2004] and ATF-3 genes in these cells (Fig. 1), we next determined whether MMP-13 is a target gene for ATF-3. MDA-MB231 cells were transiently transfected with either scrambled shRNA or ATF-3 shRNA constructs. As shown in Figure 5A, cells transiently transfected with scrambled shRNA expressed MMP-13 mRNA and this mRNA expression level was increased by TGF-β1 treatment. When cells were transiently transfected with ATF-3 shRNA, there was decreased expression of MMP-13 mRNA under both control and TGF-β1-treated conditions. Thus, ATF-3 must be involved in mediating TGF-β1-stimulation of MMP-13 gene expression in human breast cancer cells.

We further determined the functional role of ATF-3 for MMP-13 expression by reporter gene analysis. In our laboratory we previously identified and characterized the TGF-β-responsive regions present in the MMP-13 promoter. The −148 MMP-13 promoter that contains 148 base pairs upstream of the transcription initiation site retains the TGF-β-responsive region [Selvamurugan et al., 2004]. The −148 MMP-13 promoter fused with a chloramphenicol acetyl transferase (CAT) reporter gene was transiently transfected into MDA-MB231 cells along with either scrambled shRNA or ATF-3 shRNA constructs. As shown in Figure 5B, TGF-β1 stimulated MMP-13 promoter activity and ATF-3 shRNA reduced both the control and TGF-β1-stimulated MMP-13 promoter activity in these cells. Hence, the MMP-13 gene (another potential ATF-3 target gene) is regulated by TGF-β1 via ATF-3.

ATF-3 could activate the MMP-13 promoter by either directly binding to the promoter or by sequestering specific repressors away from the promoter. MMP-13 promoter activation has been well studied in our laboratory [Selvamurugan et al., 2002, 2004]. We previously identified the TGF-β-responsive region containing the RD/Runx site and the AP-1 site responsible for MMP-13 promoter activation [Selvamurugan et al., 2004]. Hence, to examine whether TGF-β1-stimulated ATF-3 protein is associated with the promoter or not, we performed ChIP assays using a specific antibody against ATF-3. Control and TGF-β1-treated whole cell lysates obtained after formaldehyde cross-linking of DNA to protein and protein to protein in intact MDA-MB231 cells were immunoprecipitated with either IgG or anti-ATF-3 antibody. After immunoprecipitation, DNA was extracted from control and TGF-β1-treated lysates from the beads as described under Materials and Methods Section and used for semiquantitative PCR with primers that amplify the regions of the proximal AP-1 site and the distal RD/Runx site of the human MMP-13 promoter. The PCR amplification of the DNA fragments indicated that ATF-3 was associated only with the proximal AP-1 site under control conditions; whereas ATF-3 was associated with both the proximal AP-1 and the distal RD/Runx sites of the human MMP-13 promoter after TGF-β1-treatment (Fig. 5C). Thus, this result shows in vivo presence of ATF-3 protein on the human MMP-13 promoter and also increased binding of this protein to the distal RD/Runx site after TGF-β1 treatment in MDA-MB231 cells.

DISCUSSION

In most of the systems examined thus far, ATF-3 mRNA increases shortly after the exposure of cells to the signals [Chen et al., 1994; Wolfgang et al., 1997]. The kinetics of ATF-3 mRNA induction are usually immediate and transient. We report here that ATF-3 is strongly stimulated and its level is sustained by TGF-β1 in highly invasive and metastatic human breast cancer cells and in mouse mammary pad tumor cells, in contrast to the normal transient ATF-3 expression stimulated by TGF-β1 in non-cancerous cells (Figs. 1

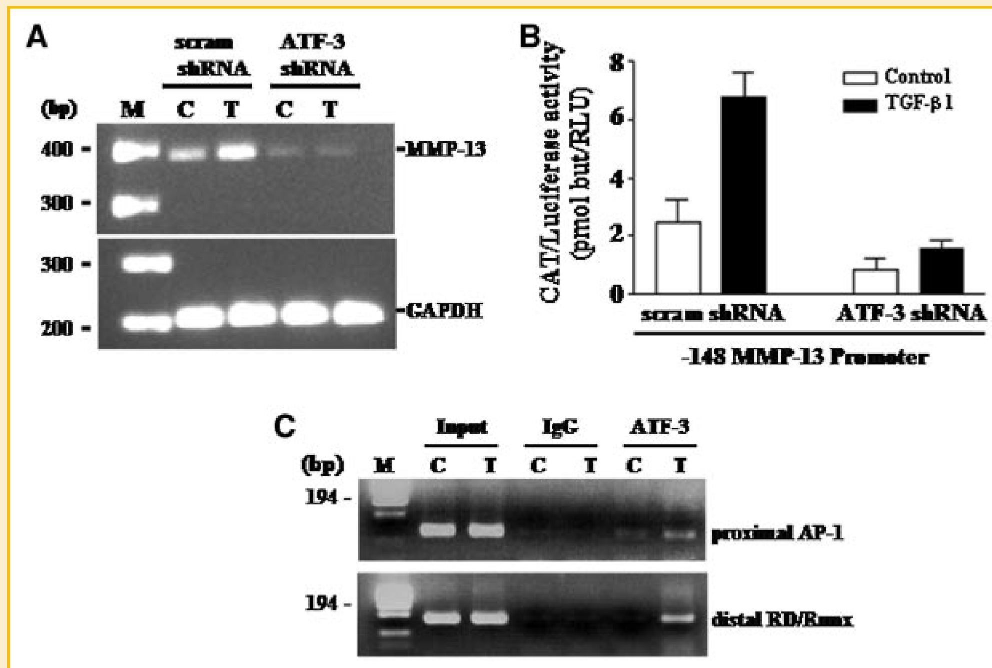

Fig. 5. A: MDA-MB231 cells were transiently transfected with either scrambled shRNA or ATF-3 shRNA constructs for 24 h and then treated with control or TGF-β1 (1 ng/ml)-containing media for 24 h. Total RNA was isolated and subjected to semiquantitative RT-PCR using specific primers for human MMP-13 and GAPDH. The PCR products were separated onto a 2% agarose gel. B: The wild type MMP-13 promoter construct (−148) was transiently cotransfected with either scrambled shRNA or ATF-3 shRNA constructs into MDA-MB231 cells for 24 h and then treated with control or TGF-β1 (1 ng/ml)-containing media for 24 h. Lysates were prepared and assayed for CAT activity. Rinella luciferase was used to normalize the transfection efficiency. Data represent mean ± SE of three experiments. C: MDA-MB231 cells were treated with control (C) or TGF-β1 (1 ng/ml)-containing media (T) for 4 h. Cells were fixed with formaldehyde and lysates were prepared as described in Materials and Methods Section. After immunoprecipitation of the cross-linked lysates with either IgG or ATF-3 antibody, the DNA was subjected to PCR with primers that amplify the proximal AP-1 region and the distal RD/Runx region of the human MMP-13 promoter. Input DNA (1/100) is the positive control for the assay. M, DNA marker; bp, base pairs.

and 2A). Since ATF-3 expression is prolonged and sustained, ATF-3 might dimerize with Fos, Jun, and other ATF family members and the dimerization partners may be different in MCF-10A and MDA-MB231 cells. It has been shown that ATF-3 forms a homodimer that represses transcription from the ATF-3 gene itself [Chen et al., 1994; Wolfgang et al., 1997], TNF-α-induced E-selectin gene expression [Eferl and Wagner, 2003], and arsenite-responsive activation of the GADD153 gene [Fawcett et al., 1999]; whereas heterodimers of ATF-3 with c-Jun and JunB activate transcription in transient transfection assays [Hsu et al., 1992, 1993]. Thus, the change in alterations of either homo- or hetero dimerization partners of ATF-3 along with AP-1 proteins and other co-factors could activate genes which participate in breast cancer progression.

In this study we show that overexpression of ATF-3 in normal human mammary epithelial cells induced cellular proliferation in vitro (Fig. 3). Even though it has been reported that transient stimulation and expression of ATF-3 by TGF-β1 mediates the growth suppression function of normal epithelial cells [Kang et al., 2003], we report here that overexpression of ATF-3 can alter the nature of normal mammary epithelial cells in terms of cell proliferation. The presence of potential ATF-3 binding sites in the promoter regions of cyclins [Yoshizumi et al., 1995] and of Rb itself [Sakai et al., 1991; Linardopoulos et al., 1993] suggests that several cell cycle-related genes may be subject to regulation by ATF-3. We have identified that cyclin A1 is a target gene for ATF-3 and knockdown of ATF-3 decreased cyclin A1 expression in human breast cancer cells (Fig. 4B).

MMP-13, an invasive and metastatic gene, is highly expressed in several primary tumors and cancer cell lines. Expression and regulation of the MMP-13 gene by TGF-β1 in osteoblastic and breast cancer cell lines has been well studied in our laboratory [Selvamurugan et al., 2002, 2004]. We report here that MMP-13 is a target gene for ATF-3 in human breast cancer cells (Fig. 5A,B). Due to the autocrine role played by TGF-β, there is basal expression of ATF-3 protein (Fig. 4A), MMP-13 mRNA (Fig. 5A) and MMP-13 promoter activity (Fig. 5B) in MDA-MB231 cells, and ATF-3 shRNA decreased expression of ATF-3 protein, MMP-13 mRNA and MMP-13 promoter activity in these cells. In this study we demonstrate that ATF-3 occupied both the proximal AP-1 and the distal RD/Runx sites of the human MMP-13 promoter after TGF-β1 treatment (Fig. 5C). We have previously shown that in the human MMP-13 promoter, the proximal AP-1 site is occupied by JunB and the distal RD/Runx site is occupied by Runx2 upon TGF-β1-treatment in MDA-MB231 cells. A role for Runx2 in breast cancer cell invasion and bone metastasis in vivo has also been reported [Barnes et al., 2004; Javed et al., 2005; Pratap et al., 2005, 2006]. Smad3, an intracellular signaling component of the TGF-β-pathway acted as a direct mediator for physical interaction of both JunB and Runx2 proteins on the human MMP-13 promoter [Selvamurugan et al., 2004]. It has been shown that Smad3 directly interacts with ATF-3

[Kang et al., 2003]. Hence, it is most likely that ATF-3 could form different protein complexes in the proximal AP-1 site and the distal RD/Runx site for TGF-β1-stimulation of MMP-13 promoter activation in MDA-MB231 cells.

Overall, our studies indicate that TGF-β1 stimulates ATF-3 expression in a sustained and prolonged manner in breast cancer cells and that may be required for activation of cyclin A1 and MMP-13 genes resulting in breast cancer progression. Understanding ATF-3 function in breast cancer cell growth and metastasis in vivo will undoubtedly be used for future therapeutic targeting and for early diagnosis of aggressive primary breast tumors.

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