Smad3 interacts with JunB and Cbfa1/Runx2 for Transforming Growth Factor-β1-Stimulated Collagenase-3 Expression in Human Breast Cancer Cells®

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SUMMARY

We have previously shown that TGF (transforming growth factor)-β1, a crucial molecule in metastatic bone cancer, stimulates collagenase-3 expression in the human breast cancer cell line, MDA-MB231. To understand the molecular mechanisms responsible for TGF-β1-response on collagenase-3 promoter activity, a functional analysis of the promoter region of the collagenase-3 gene was carried out and we identified the distal RD (runt domain) and proximal RD/AP-1 (activator protein-1) sites as necessary for full TGF-β1-stimulated collagenase-3 promoter activity. Gel shift, real time RT-PCR and Western blot analyses showed increased levels of c-Jun, JunB, and Cbfa1/Runx2 upon TGF-β1 treatment in MDA-MB231 cells. Co-immunoprecipitation in vitro studies identified no physical interaction between JunB and Cbfa1/Runx2; whereas Smad3 interacted with both. Chromatin immunoprecipitation experiments confirmed interaction of Smad3 with JunB and Cbfa1/Runx2. Under basal conditions, Cbfa1/Runx2 bound to both the proximal RD/AP-1 and distal RD sites. In response to TGF-β1, Cbfa1/Runx2 was seen only at the distal RD site; whereas JunB occupied the proximal RD/AP-1 site. An assemblage of Smad3, JunB, and Cbfa1/Runx2 at the distal RD site of the collagenase-3 promoter occurred in response to TGF-β1 in MDA-MB231 cells. Co-transfection of Smad3, JunB, and Cbfa1/Runx2 constructs along with a constitutively active TGF-β type I receptor construct identified functional interaction of these proteins and transcriptional activation of collagenase-3 gene by TGF-β1. Taken together, our results suggest that TGF-β1 stimulated JunB and Cbfa1/Runx2 to bind to their respective DNA consensus sites and Smad3 is likely to stabilize their interaction to confer functional TGF-β1-stimulation of collagenase-3 expression in MDA-MB231 cells.
INTRODUCTION

Matrix metalloproteinases (MMPs), a family of secreted or transmembrane proteinases (1) play an integral role in tumor invasion that is characterized by increased motility of epithelial cells and growth of metastasized tumor cells (2). Since MMPs are important contributors to tumor progression and metastasis, they provide a rationale for developing new cancer drugs that target MMP activity (3). An intensive drug discovery program led to many clinical trials of MMP inhibitors for cancer therapy. However, these trials have largely been disappointing (4, 5). A greater understanding of the regulatory mechanisms that control MMP transcription, activation and inhibition will provide several new avenues for therapeutic intervention.

Recent studies have shown that collagenase-3, one of the MMPs (MMP-13) is overexpressed in a variety of malignant tumors. Human collagenase-3 was originally identified in human breast carcinoma cells (6) and is homologous to rat collagenase-3 (7). Apparently due to the wide substrate specificity of collagenase-3, its physiological expression is limited to situations in which rapid and effective extracellular matrix (ECM) remodeling is required. Also, the expression of collagenase-3 is very restricted and appears to be subjected to stringent regulatory mechanisms. During tumor growth, these controls are lost and tumor cells acquire the ability to produce this protease under stimulation by different factors, including cytokines, growth factors, and tumor promoters. Among them, IL-1α and IL-1β are potential candidates for inducing the expression of this MMP gene in breast carcinomas (8, 9).

Bone is one of the major sites for formation of breast cancer metastases and bone matrix is enriched with several growth factors and cytokines (e.g. TGF-β1). Alterations in
TGF-β-signaling can result in various fibrotic as well as malignant diseases (10-12). Smad proteins have been demonstrated to be downstream mediators of the TGF-β-signaling pathway from the serine/threonine kinase receptors to the nucleus (13, 14). A typical resistance to the growth inhibitory effect of TGF-β1 in highly invasive and metastatic human breast cancer cells (MDA-MB231) is due to selective loss of the c-myc down-regulation response (15). Moreover, with breast cancer cells, TGF-β1 stimulates invasion (16) and formation of TGF-β1-dependent bone metastases in an animal model system (17).

TGF-β1 could promote breast cancer metastasis by acting directly on the tumor cells (17) via production of PTHrP that is the critical mediator of bone destruction. The molecular mechanisms responsible for osteolytic metastases are complex and involve bi-directional interactions between tumor cells and bone. One of the possible ways that TGF-β1 is involved in bone destruction is via regulation of MMP activity in metastasizing bone cancer cells. Recently we have shown that TGF-β1 stimulated collagenase-3 expression in the human breast cancer cell line, MDA-MB231 (18). Since collagenase-3 is characterized by its ability to degrade the ECM and is stimulated by TGF-β1 in MDA-MB231 cells, collagenase-3-driven ECM proteolysis may support cancer cell growth both biochemically, by exposing mitogenic factors, and physically, by providing space for the proliferating cells.

To delineate the molecular mechanisms responsible for this stimulation of collagenase-3 by TGF-β1 in these cells, a functional analysis of the collagenase-3 promoter was carried out and we have identified that the distal RD (runt domain) and proximal RD/AP-1 (activator protein-1) sites are necessary for TGF-β1-stimulated collagenase-3 promoter
activity. The RD site is identical to that previously reported as AML/Cbfa/Runx binding
site (18-23). The proteins binding to the AP-1 site are AP-1 dimers that consist of two
subunits formed either by heterodimerization of a Fos family protein (c-Fos, FosB, Fra1,
Fra2) with a Jun family protein (c-Jun, JunB, JunD) or homodimerization of the Jun
family members. Similarly, the RD site is bound by members of the core binding factor
alpha (Cbfa) family (Cbfa1, Cbfa2, and Cbfa3, now renamed Runx-2, -1, -3). Dominant-
negative inhibitors of Cbfa/Runx defined its role in TGF-β1-stimulated collagenase-3
promoter activity in MDA-MB231 cells. Smad3, an intracellular mediator of TGF-β
signaling interacts with both JunB and Cbfa1/Runx2 in vitro and in vivo and the
assemblage of this protein complex on human collagenase-3 promoter may confer
functional TGF-β1-stimulation of collagenase-3 expression in MDA-MB231 cells.
MATERIALS AND METHODS

Materials - TGF-β1 was purchased from Promega, Madison, WI. Synthetic oligonucleotides were synthesized by Invitrogen, Gaithersburg, MD. Tissue culture media and reagents were also obtained from Invitrogen. Anti-Cbfa1, -Smad3, -c-Myc, -Fos, and -Jun antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Monoclonal anti-Flag antibody was obtained from Sigma Chemical Co., St. Louis, MO. Polyclonal anti-Smad4 antibody was purchased from Zymed Laboratories, Inc., San Francisco, CA. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Cell culture - MDA-MB231 and Cos-7 cells were obtained from ATCC (American Type Culture Collection). The cells were maintained in culture in Dulbecco’s modified essential medium-(DMEM-F12) or in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. MDA-MB231 cells were serum starved in 0.1% DMEM-F12 medium for 16 h before TGF-β1 (2 ng/ml)-treatment.

Transient Transfection and CAT activity - Previously we cloned and characterized the rat genomic clone 600-10, which contains the 5’ end of the gene (7, 19). From this, the –148 collagenase-3 promoter construct was subcloned upstream of a chloramphenicol acetyl transferase (CAT) reporter gene in pSV0 (Promega). The mutations in the rat collagenase-3 promoter constructs were carried out by Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The constructs were verified by sequencing at UMDNJ-RWJMS-core DNA automatic sequencing facility. The plasmid DNAs were transiently transfected into cells using Lipofectamine 2000 (Invitrogen). Briefly, cells
were plated at 4 X 10^5/well in six-well plates in either DMEM-F12 or DMEM containing 10% FBS. The following day, the cells were transfected with 1 µg DNA and 5 µl Lipofectamine 2000 per well in 1 ml of serum-free DMEM-F12. After 16 h, 1 ml of DMEM-F12 containing 10% FBS was added. After 24 h, the cells were treated with either control or TGF-β1-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 [14C]-chloramphenicol (0.125 µCi/assay). The values were normalized to protein as determined by the Bradford dye binding (BioRad, Hercules, CA) method. A standard curve using purified CAT was performed every experiment to determine the linear range of the enzyme assay.

**Gel Mobility Shift Assay** - Nuclear extracts were prepared as described (19). Approximately 5 µg of extracts were incubated in a volume of 20 µl containing binding buffer (final concentrations: 4% glycerol, 1 mM MgCl₂, 0.5 mM DTT, 50 mM KCl, 10 mM Tris-HCl, pH 7.5), 100 ng/µl poly (dI-dC), and antisera or competitor DNA at room temperature for 30 min. [32P]-labeled double stranded oligonucleotide was added to the reaction immediately following the above reagents. The incubation was carried out for 15 min at room temperature. The reaction was stopped by the addition of 2 µl of 10X gel loading dye. Electrophoresis was performed at 4° C on a 6% non-denaturing polyacrylamide gel in TGE buffer (25 mM Tris, 190 mM glycine and 1.1 mM EDTA, pH 8.5). The protein-DNA complexes were visualized by autoradiography. The sequences of the oligonucleotides used in this study were as follows: wRD/wAP-1, 5’-TAAGTGATGACTCACCATTGC-3’; wRD/mAP-1, AAGTGATGCGGCACCATTGC-
3’; mRD/wAP-1, 5’-TAAGTGATGACTCTGATTGC-3’. The mutated nucleotides are in bold.

Real Time Quantitative RT-PCR – Total RNA was prepared using the Qiagen RNeasy kit. The reverse transcriptase reaction was carried out using the Taqman Reverse Transcription reagents (Roche, Indianapolis, IN). PCR reactions were performed according to the real time PCR machine manufacturer’s instructions (DNA Engine Opticon, MJ Research Inc, MA), which allow real time quantitative detection of the PCR product by measuring the increase in SYBR green fluorescence caused by binding of SYBR green to double-stranded DNA. The SYBR kit for PCR reactions was purchased from Perkin Elmer Applied Biosystems. Primers for human AP-1, Cbfa1/Runx2, and β-actin were designed using the PrimerExpress software (Perkin Elmer Applied Biosystems).

The primer sequences used were as follows:

| Gene   | Primers                                                                 |
|--------|-------------------------------------------------------------------------|
| c-Fos  | sense, 5’-TGCCCTCTCCTCAATGACCCTGA-3’                                   |
|        | antisense, 5’-ATAGGTCCATGTCTGGCACGGA-3’                                 |
| FosB   | sense, 5’-AGCAGCAGCTAAATGCAGGA-3’                                      |
|        | antisense, 5’-TTT TTGGAGCTCGGCGATCT-3’                                 |
| Fra1   | sense, 5’-ATCTGCAAATCCCGGAAGG-3’                                       |
|        | antisense, 5’-AGTGCCTCAGGTTCAAGCAGAC-3’                                |
| Fra2   | sense, 5’-TAGATATGCTGCTCAGGCAG-3’                                      |
|        | antisense, 5’-GGTTGGACATGGAGTGATCAC-3’                                 |
| c-Jun  | sense, 5’-CTCCAAGTGCGCCGAAAAAGGAAG-3’                                  |
|        | antisense, 5’-CACCTGTTCCCTGAGCATGTTG-3’                                |
**DNA-affinity immunoprecipitation** - Nuclear extracts from control and TGF-β1-treated MDA-MB231 cells were diluted with HKMG buffer (10 mM Hepes, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM DTT/0.1% Nonidet P-40) containing phosphatase inhibitors (Sigma) and a mixture of protease inhibitors (Roche, Indianapolis, IN) and incubated with 1 µg of biotinylated double-stranded oligonucleotides for 16 h. DNA–protein complexes were collected by precipitation with streptavidin-agarose beads (Pierce, Rockford, IL) for 2 h, washed three times with HKMG buffer, and subjected to Western blot analysis. The sequences of the human biotinylated oligonucleotides used in this study were as follows:

| Oligonucleotide | Sense Sequence | Antisense Sequence |
|-----------------|----------------|--------------------|
| JunB            | 5'-ATGGAACAGCCCTTCTACCACG-3' | 5'-AGGCTCGGTTTCAGGAGTTTG-3' |
| JunD            | 5'-GTCTACGCGAACCTGAGCAGCTA-3' | 5'-CTCGTCCTTGAGCGCAGCCAGGC-3' |
| Cbfa1/Runx2     | 5'-GGAGTGGACGGAGCAAGAGTTT-3' | 5'-AGCTTCTGTCTGTGCCTTCTGG-3' |
| β-actin         | 5'-ATTGCCGACAGGATGCGCGAGA-3' | 5'-ACATCTGCTGGAAGGTGGACAG-3' |

**Proximal wild RD/wild AP-1**

Sense, 5’-TAAGTGATGACTCACCATTGCTTAAGTGATGACTCACCATTGCTAAGTGATGACTCACCATTGCTAAGTGATGACTCACCATTGCTAAGTGATGACTCACCATTGCTAAGTGATGACTCACCATTGCTAAGTGATGACTCACCATTGCT
The DNA consensus sites are in bold and the mutated nucleotides are in bold and in italicized. Only the sense strand sequences were represented above.

**Western blot** – The lysates containing 50 µg of total protein were electrophoresed on 12% SDS-polyacrylamide gels. The proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking in Tween-Tris-buffered saline (0.1% Tween 20, 138 mM NaCl, 5 mM KCl, and 25 mM Tris-HCl (pH 8.0)) 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 goat anti-rabbit or anti-mouse secondary antibodies. The antigen-antibody complexes were detected by enhanced chemiluminescence (ECL) (Amersham Biosciences).

**Co-immunoprecipitation** – Cos-7 cells were transiently transfected with appropriate eukaryotic expression vectors. Then, cells were washed with 1X PBS and lysed in lysis buffer containing 0.5% NP40, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, protease inhibitors, and 0.1 mM PMSF. Cells were scraped and were incubated at 4°C for 20 min. Cellular debris was pelleted by centrifugation. The supernatant was precleared by addition of either 1 µg of rabbit IgG or 1 µg of mouse IgG and 20 µl protein
AG-agarose, incubating for one h at 4º C, and centrifuging to pellet the agarose. Equal amounts of lysates were mixed with either 1 µg of monoclonal or 1 µg polyclonal antibodies and 20 µl protein AG-agarose. The samples were incubated overnight at 4º C and washed three times with lysis buffer. Next, the samples were resuspended in 2X SDS sample buffer and lysis buffer and boiled for 5 min. Both precleared lysates and immunoprecipitated samples were resolved by 12% SDS-polyacrylamide gels. The proteins were transferred electrophoretically to PVDF membranes. The membranes were immunoblotted with either monoclonal or polyclonal antibodies as described above.

**Chromatin immunoprecipitation (ChIP)** – ChIP assay was carried out using the ChIP kit from Upstate Biotechnology. Cells were incubated for 10 min at room temperature with medium containing 1% formaldehyde. Cells were then washed in ice-cold PBS containing protease inhibitors and 1 mM phenylmethylsulfonyl fluoride, and resuspended in SDS lysis buffer containing protease inhibitors for 10 min on ice. Samples were sonicated to reduce the DNA length to 0.1-0.6 kbp, and cellular debris was removed by centrifugation and the supernatant diluted 10-fold in dilution buffer supplemented with protease inhibitors. Protein concentrations were determined using BioRad reagent. Prior to chromatin immunoprecipitations, the samples were pre-cleared with 100 µl of a 25% (v/v) suspension of DNA-coated protein A/G-agarose. The supernatant was recovered and used directly for immunoprecipitation experiments with appropriate antibody overnight at 4 ºC. Immune complexes were mixed with 100 µl of a 25% precoated protein A/G-agarose suspension followed by incubation for 1 h at 4 ºC. The supernatant was saved and used for re-chromatin immunoprecipitations. Beads were collected and sequentially washed with 1 ml each of the following buffers: low salt wash buffer, high salt wash buffer, and LiCl wash buffer. The
beads were then washed twice using 1 ml of TE buffer. The proteins from the immunocomplexes were eluted with SDS-protein loading dye, boiled, and loaded onto 10% SDS-PAGE.

For PCR analysis, aliquots (1/100) of total chromatin DNA before immunoprecipitation were saved (input). After immunoprecipitation, the beads were sequentially washed with 1 ml each of the following buffers: low salt wash buffer, high salt wash buffer, and LiCl wash buffer as mentioned above. The beads were then washed twice using 1 ml of TE buffer. The immunocomplexes were eluted two times by adding a 250 µl aliquot of a freshly prepared solution of 1% SDS, 0.1 M NaHCO₃. The samples were added with 20 µl of 5M NaCl and cross-linking reaction was reversed by 6 h incubation at 65 °C. Further, the samples were digested with proteinase K (10 mg/ml) at 42 °C for 1 h and DNA was recovered by phenol/chloroform extractions. The DNA was precipitated with two volumes of ethanol using 1 µl of 20 mg/ml glycogen as carrier. The input lysates were also processed as above. The DNA was resuspended in water and used for semi quantitative PCR. The sequences of the oligonucleotides of the human collagenase-3 promoter used in this study were as follows: proximal RD/AP-1, Sense, 5’-GTCGCCACGTAAGCATGTTT-3’; proximal RD/AP-1, Antisense, 5’-CATCTTGAATGGTGGTGCCT-3’; distal RD, Sense, 5’-CCATAAATATGCTGAGGCCG-3’, and distal RD, Antisense, 5’-GGTTTCCACTTCCTAGTCAC-3’.
RESULTS

The RD and AP-1 sites are necessary for TGF-β1-stimulated collagenase-3 promoter activity – We have previously shown that TGF-β1 stimulates collagenase-3 expression in MDA-MB231 cells and that this also requires de novo protein synthesis (18). Here we show the molecular mechanisms responsible for TGF-β1-stimulated collagenase-3 promoter activity in these cells. The collagenase-3 promoter region includes consensus binding sites for several DNA-binding proteins, C/EBP, SBE, RD, p53, PEA-3, AP-2, and AP-1. Previously, we deleted regions of the rat collagenase-3 promoter from the 5’-end and placed the resulting promoter sequences 5’ of the CAT gene (19). In order to analyze the TGF-β1-responsive region in the collagenase-3 promoter, we have transiently transfected longer collagenase-3 promoter constructs into MDA-MB231 cells and CAT assays were carried out. We have found that the TGF-β1-response region was retained only within the 148 base pairs upstream of the transcriptional site (18). In the -148 collagenase-3 promoter region, there are four consensus sites, namely a RD site, a p53 site, a PEA-3 site, and an AP-1 site, which are highly conserved both in sequence and location in both the human and rat collagenase-3 promoters. The RD binding site is identical to a Cbfa/Runx binding site and the Cbfa/Runx transcription factors can bind to this site (18-23). The collagenase-3 promoter also contains another RD site (proximal) overlapping with the AP-1 site and its sequences (ACCAC) are similar to the Cbfa/Runx consensus site, ACC(A/G)CA. Even though the AP-1 site is conserved among the human and rat collagenase-3 genes, the proximal RD site overlapping with the AP-1 site is located in the opposite orientation and on the opposite strand (Fig. 1A). Since the RD site
acts as an enhancer (23), the proximal RD site could also be a functional element in mediating the TGF-β1-response.

In order to analyze the specific response elements involved in TGF-β1-stimulated collagenase-3 promoter activity in MDA-MB231 cells, we have made collagenase-3 promoter constructs having mutations at either the distal RD or the proximal RD or the AP-1 sites. These promoter constructs were transiently transfected into MDA-MB231 cells and CAT activity was assessed in these cells. Mutation of both the proximal RD and AP-1 sites inhibited basal expression; whereas mutation of any one of the sites (the distal RD site or the proximal RD site or the AP-1 site) was enough to cause a significant loss of TGF-β1-response for collagenase-3 promoter activity in the breast cancer cells (Fig. 1B). Hence, these results suggest that the distal RD and the proximal RD/AP-1 sites are necessary for full TGF-β1-stimulated collagenase-3 promoter activity in MDA-MB231 cell. It is also interesting to note from the transfection experiments that mutation of any one of the sites (distal RD, proximal RD, and AP-1) abolished TGF-β1-response suggesting that TGF-β1 stimulates differential gene expression and formation of a stable protein complex between these sites.

*TGF-β1 stimulates differential expression of the AP-1 factors at the proximal RD/AP-1 site* - To define the contributions of transcription factors binding to the proximal RD/AP-1 site for TGF-β1-stimulated-collagenase-3 promoter activity, nuclear extracts were prepared from control or TGF-β1-treated (1 h) MDA-MB231 cells and gel mobility assays performed with labeled human proximal RD/AP-1 site probe. Fig. 2A demonstrates that proteins in both control and TGF-β1-treated nuclear extracts were able to bind to the human collagenase-3 RD/AP-1 site, but significantly more protein-DNA
complex was produced when extract from TGF-β1-treated cells was used, as evidenced by the greater intensity of the two shifted complexes. To investigate differential expression of Fos and Jun family members bound to the RD/AP-1 site, nuclear extracts were preincubated with an antibody specific to either Fos or Jun family members. Among the Fos family members, c-Fos, Fra1, and Fra2 were neither significantly down regulated or up regulated by TGF-β1, as evidenced by no significant changes in the level of shifted complexes. There was a marginal decrease in the amount of shifted complexes by FosB antibody in TGF-β1-treated nuclear extracts. The Fra1 antibody increased the amount of shifted complexes only in the control nuclear extracts. Among the Jun family members, the antibody specific to c-Jun caused a supershift complex in both control and TGF-β1-treated conditions and there was also a marginal increase in the amount of supershifted complex in TGF-β1-treated nuclear extract. The antibody specific to JunD caused a supershifted complex in both control and TGF-β1-treated conditions with no significant difference in abundance between the two conditions. The antibody specific to JunB caused a significant abolition of these shifted complexes upon TGF-β1-treatment suggesting the upregulation of JunB for collagenase-3 promoter activation. These two shifted complexes contain either Fos/Jun family heterodimers or Jun/Jun family homodimers or both. We included normal rabbit IgG as a negative control. The increased level of TGF-β1-stimulated shifted complexes bound to the proximal RD/AP-1 was specific because there was no change in the level of shifted complexes when a labeled Oct1 (an ubiquitous factor) probe was used (Fig. 2B).

Since the proximal RD site overlaps with the AP-1 site (22), we wanted to determine the association of Cbfa1/Runx2 in these shifted complexes. Control and TGF-β1-treated
MDA-MB231 cells were preincubated with either anti-Cbfa1 antibody or normal rabbit IgG followed by incubation with the labeled wild type proximal RD/wild type AP-1. Apparently anti-Cbfa1 caused no significant changes in the intensity of shifted complexes in control and TGF-β1-treated nuclear extracts suggesting the absence of Cbfa1/Runx2 in these DNA-protein complexes (Fig. 2C). We identified the specificity of the shifted complexes by unlabeled competition experiments using the wild type RD/wild type AP-1, mutant RD/wild type AP-1, or wild type RD/mutant AP-1 site oligonucleotides in 50-fold molar excess (Fig. 2D). All three unlabeled oligonucleotide probes significantly competed the two shifted complexes indicating the specificity of proteins binding to this site.

There is increased FosB, Fra2, c-jun, junB and Cbfa1/Runx2 mRNAs in response to TGF-β1 – Changes in the mRNA expression of AP-1 factors and Cbfa1/Runx2 were also measured by real time quantitative PCR methods. The RNA samples obtained from control and TGF-β1-treated MDA-MB231 cells at different time periods (0, 15 min, 1 h, and 2 h) were subjected to cDNA synthesis followed by quantitative PCR assays. Figure 3A shows the changes in the expression of Fos family members. Maximal mRNA levels for fra2 and fosB were observed at 15 min and 1 h, respectively (2.9 ± 0.9 and 2.8 ± 0.7, respectively) in MDA-MB231 cells treated with TGF-β1. The fold increase is represented as mean ± SEM (n = 3). c-fos mRNA level was neither induced nor reduced upon TGF-β1-treatment. Among Jun family members, c-jun and junB mRNA levels were increased after TGF-β1-treatment (Fig. 3B). The fold increase in c-jun mRNA by TGF-β1 was 1.2 ± 0.2, 2.6 ± 0.3, and 3.1 ± 0.2 for 15 min, 1h, and 2h, respectively. The fold induction of junB mRNA by TGF-β1 was substantially higher than the other members, being 2.7 ±
0.4, 17.7 ± 1.1, and 14.7 ± 1.2 for 15 min, 1h, and 2h, respectively. Notably, TGF-β1 increased Cbfa1/Runx2 mRNA in MDA-MB231 cells up to 6-fold at 2h (Fig. 3C).

There is increased c-Jun, JunB, and Cbfa1/Runx2 proteins in response to TGF-β1- To determine whether the increased mRNA level in response to TGF-β1 is correlated with protein expression, cell lysates from MDA-MB231 cells treated with or without TGF-β1 were carried out for Western blot analysis (Fig. 4A, B, C). The relative levels of c-Jun, JunB, and Cbfa1/Runx2 proteins were quantitated by scanning densitometry and corrected for the levels of α-tubulin. The fold stimulation was calculated over control (0 min). TGF-β1 maximally stimulated only c-Jun (2-fold), JunB (9-fold), and Cbfa1/Runx2 (1.5 fold) expression at 2 h. There was no detectable significant change in the levels of other Fos and Jun family members (data not shown). Since Cbfa1/Runx2 is a bone specific transcription factor (21, 24-26) and we have found its RNA and protein expressed in mammary epithelial cells (MDA-MB231), we wanted to confirm the presence of Cbfa1/Runx2 protein in MDA-MB231 cells. Whole lysates prepared from HeLa, MDA-MB231, and Saos2 cells were subjected to Western blot analysis (Fig. 4D). HeLa cells (human fibroblast cells) do not express Cbfa1/Runx2 (27). Blots were incubated with either IgG or anti-Cbfa1 or anti-Cbfa1 that had been preadsorbed with Cbfa1/Runx2 peptide. Cbfa1/Runx2 with a mass of 60 kDa was expressed in MDA-MB231 as well as the human osteosarcoma, Saos2.

Functional requirement of the RD sites for TGF-β1-stimulated collagenase-3 promoter activity - To determine the functional role of the RD sites and its transcription factor family, Cbfa/Runx, for TGF-β1-stimulated collagenase-3 promoter activity in MDA-MB231 cells, we used AML/ETO, a chimeric protein caused by chromosomal
translocation t(8;21) that lacks a transactivation domain at the carboxyterminal portion of AML1 (19). Since mutations either at the distal or the proximal RD sites in the collagenase-3 promoter constructs inhibit the TGF-β1-response (Fig. 1B), the wild type collagenase-3 promoter construct (-148) was used in these studies. The collagenase-3 promoter construct was transiently co-transfected into MDA-MB231 cells with an AML/ETO expression plasmid (Fig. 5). The result demonstrates that the basal and the TGF-β1-response were greatly reduced by overexpression of AML/ETO, suggesting that TGF-β1 requires the RD site and its transcription factor family, Cbfa/Runx, for collagenase-3 promoter activity in MDA-MB231 cells.

Mutation of the proximal RD site does not completely prevent binding of the AP-1 factors to the AP-1 site and Cbfa1/Runx2 is directly bound to the distal RD site – From the gel shift experiments (Fig. 2C), it is not completely clear whether Cbfa1/Runx2 protein physically interacts with the proximal RD/AP-1 site. Since the proximal RD and AP-1 sites overlap, we also wanted to address whether mutation of the proximal RD site prevents binding of the AP-1 factors to the human proximal RD/AP-1 site. We used biotinylated double-stranded oligonucleotides corresponding to the proximal RD/AP-1 sequences to precipitate binding proteins from nuclear extracts. As an analytical technique, biotin/streptavidin DNA-affinity purification permits the direct identification of sequence-specific DNA-binding proteins (15). Nuclear extracts from control and TGF-β1-treated MDA-MB231 cells were incubated with biotinylated double-strand oligonucleotides corresponding to the proximal wild type RD/wild type AP-1 or mutant RD/wild type AP-1. DNA–protein complexes were collected by precipitation with streptavidin-agarose beads, and subjected to Western blot analysis using rabbit polyclonal
antiserum for either JunB or Cbfa1/Runx2. Both wild type RD/wild type AP-1 and mutant RD/wild type AP-1 biotinylated probes yielded protein complexes containing JunB from control and TGF-β1 treated nuclear extracts (Fig. 6A). Mutation of the RD site at the proximal RD/AP-1 site decreased but did not completely prevent binding of JunB to the AP-1 site. A similar result was also obtained with anti-c-Jun antibody (data not shown). In contrast, no Cbfa1/Runx2 was bound with either the wild or the mutant RD sites of the proximal RD/AP-1 biotinylated oligonucleotides. It is evident that TGF-β1 induced both JunB and Cbfa1/Runx2 proteins, which are directly detected from nuclear extracts by Western blot. The blot was stripped and reprobed with anti-c-Fos antibody. Since c-Fos expression was very low and was not induced by TGF-β1 in MDA-MB231 cells (Fig. 2A), we used this antibody as a negative control. No c-Fos expression was detected confirming the specificity of antibody recognizing its own protein in the complexes and the specificity of proteins binding to the biotinylated probes.

To determine whether Cbfa1/Runx2 binds to the distal RD site of the human collagenase-3 promoter, nuclear extracts from control and TGF-β1-treated MDA-MB231 cells were incubated with biotinylated double-strand oligonucleotides corresponding to the distal wild type RD or mutant RD, followed by precipitation with streptavidin-agarose beads, and subjected to Western blot analysis using rabbit polyclonal antiserum for Cbfa1/Runx2. The biotinylated distal wild type RD probe yielded a protein complex containing Cbfa1/Runx2 from control and TGF-β1 treated nuclear extracts (Fig. 6B). Mutation of the distal RD site completely prevented binding of Cbfa1/Runx2 to this site showing the binding specificity of Cbfa1/Runx2 proteins. Hence, these results (Figs. 6A
& 6B) indicate that Cbfa1/Runx2 may bind only to the distal RD site of the human collagenase-3 promoter and not the proximal RD site.

**Smad3 physically interacts with both JunB and Cbfa1/Runx2** - From the above results, it is evident that there are increased levels of c-Jun, JunB, and Cbfa1/Runx2 in response to TGF-β1 in metastatic breast cancer cells. c-Jun and JunB bind to the proximal AP-1/RD site and Cbfa1/Runx2 binds to the distal RD site. To determine whether there is physical interaction between these proteins, a co-immunoprecipitation assay was used. Cos-7 cells were transiently transfected with eukaryotic expression constructs of JunB and c-Myc-tagged Cbfa1. Since TGF-β1 increased JunB (Fig. 4B) substantially more than c-Jun (Fig. 4A), we used only the JunB expression construct. Total cellular lysates were prepared and incubated with IgG, anti-JunB, or anti-c-Myc. The immunoprecipitates were probed with either anti-JunB or anti-c-Myc antibodies. Fig. 7A shows that JunB and Cbfa1/Runx2 do not interact with each other.

Recently we have shown that Smad3 is necessary for TGF-β1-stimulated promoter activity in MDA-MB231 cells (18). Since JunB and Cbfa1/Runx2 do not physically interact, we speculated that Smad3 could mediate this interaction. In order to identify this interaction, JunB, c-Myc-Cbfa1, Flag-Smad3, and Flag-Smad4 eukaryotic expression constructs were transiently transfected into Cos-7 cells. Total lysates were subjected to immunoprecipitation with IgG, anti-JunB, or anti-c-Myc antibodies followed by immunoblot with anti-Flag antibody (Fig. 7B). It is evident Smad3, not Smad4 interacts with both JunB and Cbfa1/Runx2. Similarly, lysates were also immunoprecipitated with either IgG or anti-Flag antibody and immunoblotted with either anti-JunB or anti-c-Myc antibodies (Fig. 7C). This further confirms that Smad3 interacts with both JunB and...
Cbfa1/Runx2. In order to verify that Smad4 does not interact with JunB and Cbfa1/Runx2, lysates prepared from Cos-7 cells that had been transfected with JunB, c-Myc-Cbfa1, Flag-Smad3, and Flag-Smad4 were immunoprecipitated with mouse IgG, rabbit IgG, anti-JunB, anti-c-Myc, and anti-Flag antibodies. The blot was probed with anti-Smad4 antibody (Fig. 7D). The results indicate that Smad4 does not interact with either JunB or Cbfa1/Runx2. We have also co-transfected c-Myc or Flag tagged vectors as controls and there was no interaction contributed by these tags (data not shown).

**TGF-β-mediated interaction of JunB and Cbfa1/Runx2 proteins with Smad3 on collagenase-3 promoter in vivo** – To assess that TGF-β1-stimulated JunB and Cbfa1/Runx2 proteins interact with Smad3 in vivo, we performed ChIP assays using specific antibodies against JunB and Cbfa1/Runx2. Control and TGF-β1-treated (2 h) whole cell lysates obtained after formaldehyde cross-linking of DNA to protein and protein to protein in intact MDA-MB231 cells were immunoprecipitated with IgG, anti-JunB or anti-Cbfa1 antibodies, followed by immunoblot analysis with anti-Smad3 antibody. It is clear from Fig. 8A that JunB interacted with Smad3 in vivo, and this interaction occurred in the presence of TGF-β1 due to stimulation of JunB by its treatment. Similarly, immunoprecipitation of lysates with anti-Cbfa1 antibody and immunoblot analysis with anti-Smad3 antibody shows that Cbfa1/Runx2 interacted with Smad3 in vivo, and this interaction occurred in the absence of TGF-β1 (Fig. 8B). Further, this interaction was enhanced in the presence of TGF-β1 likely due to an increase in Cbfa1/Runx2 protein after TGF-β1 treatment. Next, we wanted to determine whether Smad3 directly mediates the interaction of both JunB and Cbfa1/Runx2 proteins. After immunoprecipitation with either JunB or Cbfa1 antibody, the lysates were re-
immunoprecipitated with either Cbfa1 or JunB antibody, respectively. The specificity of the recognition by the antibody was controlled by including samples immunoprecipitated with purified IgG. When re-immunoprecipitation was carried out with Cbfa1 antibody from the lysates after immunoprecipitation with JunB antibody, followed by immunoblot analysis with Smad3 antibody, it is evident that there was no Smad3 protein available in TGF-β1-treated lysates (Fig. 8C); whereas Smad3 was available in control lysates due to no basal expression of JunB. Since immunoprecipitation of TGF-β1-treated lysates with JunB antibody resulted in pull down of JunB, Smad3, and Cbfa1/Runx2 complex, re-immunoprecipitation of the TGF-β1-treated lysates with Cbfa1 antibody, followed by immunoblot analysis with Smad3 antibody showed no signal. When re-immunoprecipitation was carried out with JunB antibody from the lysates after immunoprecipitation with Cbfa1 antibody, followed by immunoblot analysis with Smad3 antibody, it is clear that there was no Smad3 protein available in both control and TGF-β1-treated lysates (Fig. 8C). Since Cbfa1/Runx2 is expressed under both control and TGF-β1-treated conditions, immunoprecipitation of lysates with Cbfa1 antibody resulted in no availability of Smad3 protein for re-immunoprecipitation with JunB antibody. Thus, these results clearly provide in vivo evidence that Smad3 acts as a direct mediator for physical interaction of both JunB and Cbfa1/Runx2 proteins.

To further assess interaction and distribution of Smad3, JunB and Cbfa1/Runx2 proteins within the TGF-β-responsive region of the human collagenase-3 promoter in vivo, we continued the ChIP assays using specific antibodies against Smad3, JunB, and Cbfa1/Runx2. After immunoprecipitation, DNA was extracted from control and TGF-β1-treated lysates (6 h) from the beads as described in the methods section and used for semi
quantitative PCR with primers that amplify the regions of the proximal RD/AP-1 site and the distal RD site of the human collagenase-3 promoter. The PCR amplification of the DNA fragments corresponding to the proximal RD/AP-1 region showed the presence of Cbfa1/Runx2 under basal conditions, and JunB in response to TGF-β1 treatment (Fig. 8D). The PCR amplification of the DNA fragments corresponding to the distal RD region indicated the presence of Cbfa1/Runx2 in both control and TGF-β1 treatments. In addition, there was increased Smad3 and JunB bound to the distal RD site by TGF-β1-treatment (Fig. 8A, B, C, & D). Thus, these results clearly show in vivo evidence of the interaction of Smad3, JunB, and Cbfa1/Runx2 proteins in response to TGF-β1 and their assemblage on the collagenase-3 promoter in MDA-MB231 cells.

Functional interaction of JunB, Smad3, and Cbfa1/Runx2 proteins - We next analyzed whether physical interaction of the proteins, JunB, Smad3, and Cbfa1/Runx2 is required for functional activation of the collagenase-3 promoter. In order to determine that, the eukaryotic expression plasmids for JunB, Smad3, and Cbfa1/Runx2 were co-transfected with collagenase-3 promoter either individually or all together into Cos-7 cells. As shown in Fig. 9, collagenase-3 promoter activity remained unchanged when each of these constructs was transfected individually. Co-transfection of the three plasmids together resulted in marked enhancement of the collagenase-3 promoter activity, indicating that these factors co-operate with each other. In order to determine whether TGF-β1 further influences transcriptional induction of the collagenase-3 gene, a constitutively active TGF-β type I receptor expression construct along with JunB, Smad3, and Cbfa1/Runx2 expression constructs were co-transfected with collagenase-3 promoter construct into Cos-7 cells. As shown in Fig. 9, the transcriptional induction of collagenase-3 promoter...
was further enhanced in the presence of constitutively active TGF-β type I receptor indicating that post-translational modification(s) of these protein(s) may be responsible for this effect.
DISCUSSION

We have identified two different response elements mediating TGF-β1-stimulated collagenase-3 promoter activity in human breast cancer cells. The first is a distal RD binding site, and the second is a spatially distinct and overlapping proximal RD and AP-1 binding site. Mutation of either of these sites reduces TGF-β1-responsiveness, suggesting that these sites have a functional relationship. The functional interaction between the AP-1 and RD sites and their respective transcription factors could be an important mechanism for mediating both the specificity, and the responsiveness to cross talk, of the TGF-β transcriptional activation signal. The interaction of the AP-1 site with other DNA-consensus sites has been reported for TGF-β1-stimulated collagenase-3 expression in human osteoarthritic chondrocytes and in human fibroblasts (28, 9).

AP-1 complex composition can selectively regulate gene transcription and differential expression of Fos and Jun family members could play a role in regulating the expression of downstream target genes (29, 9). JunD has been reported to be involved in TGF-β1 induction of IL-6, collagenase-1, and collagenase-3 expression (30, 9). Our results indicate that after 1 h treatment with TGF-β1, JunB is a primary component present at the proximal RD/AP-1 site of the collagenase-3 promoter in human breast cancer cells. JunB protein has been shown to play a rate-limiting step in cytokine (IL-17, IL-1β) induced collagenase-3 production (31). In this study, junB mRNA and protein levels were increased by TGF-β1 in MDA-MB231 cells. This result is consistent with an earlier report (15). We previously showed that differential temporal stimulation of the AP-1 family members may be responsible for collagenase-3 expression in osteoblastic and non-osteoblastic cells (32, 33). AP-1 factors directly interact and cooperate with other
transcription factors, such as Ets (34, 35), MyoD (36), Smads (37, 38), Rb (39), and NF-κB (40) to modulate expression of target genes. Overall, it is likely that AP-1 proteins play a pivotal role in the different cytokine responses and in different cell types in terms of collagenase-3 production.

Cbfa1/Runx2, a critical component of bone formation in vivo and a transcriptional regulator of osteoblast differentiation (21, 24-26) is highly restricted to osteoblasts and odontoblasts (21, 41). Cbfa1/Runx2 regulates expression of several genes including alkaline phosphatase (42), type 1 and 2(I) collagen (43), osteopontin (21), RANKL (44, 45), TGF-β type I receptor (46), C/EBPdelta (47), bone sialoprotein (48), and the nuclear matrix associated proteins (49). Collagenase-3 is also a target of the transcriptional activator Cbfa1/Runx2 in vivo (50). Earlier, we reported that Cbfa1/Runx2 is required for collagenase-3 expression in osteoblastic and non-osteoblastic cells (19, 32). Cbfa1/Runx2 also favors expression of the collagenase-3 gene and TGF-β1 is required to achieve a full inducibility of this gene in the osteosarcoma cell line, MG-63 (50). Even though it was uncertain from our gel shift (Fig. 2C) and biotinylated DNA-affinity (Fig. 6A) experiments whether Cbfa1/Runx2 is associated with the proximal RD/AP-1 complexes, the ChIP assays (Fig. 8D) clearly indicated that Cbfa1/Runx2 interacts with the proximal RD/AP-1 and the distal RD sites under basal conditions. In response to TGF-β1, Cbfa1/Runx2 interacts only with the distal RD site. Gel shift and biotinylated DNA-affinity experiments are in vitro assays and they may be less sensitive compared to ChIP assays in detection of protein-DNA interaction in vivo. The reduced TGF-β1-response in the collagenase-3 promoter construct mutated at the proximal RD site (Fig. 1B) could be
due to decreased binding of JunB at this site (Fig. 6A) and this suggests that the flanking sequences of the AP-1 site may be important for maximal AP-1 transactivation.

Smad proteins are the main cytoplasmic signaling pathways in TGF-β1-stimulated collagenase-3 expression in osteoarthritic chondrocytes (28). Smad proteins have been shown to mediate the transcriptional activation of various TGF-β-responsive genes such as collagen (51), the tissue plasminogen activator inhibitor (52), the JunB protooncogene (53), and the p21/WAF1/Cip1 cell cycle inhibitor (54). Even though Smads can bind directly to DNA, this binding is of low affinity and low specificity (55). Smad DNA-binding does not appear to be absolutely required on all promoters (56). Instead Smad-dependent regulation of gene expression requires interactions with specific DNA-binding partners. For example, Smad2 and Smad3 DNA-binding partners include, FAST, Fos/Jun, ATF2, TFE3, VDR, Cbfa, Mixer, LEF1/TCF, NF-κB, and HNF4 (57).

Even though the human collagenase-3 promoter contains several sequences resembling putative SBEs (58), no role for these sequences in human collagenase-3 promoter by TGF-β1 has been reported (59). There are no SBEs present in the collagenase-3 promoter (-148) used in this study. Our recent studies indicated that overexpression of Smad3 mutant inhibits TGF-β1-stimulated collagenase-3 promoter activity in MDA-MB21 cells (18). Hence, it is possible that Smad3 interacts with other transcription factors rather than directly binding to the DNA for promoter activation. Consistent with this, our co-immunoprecipitation experiments support that Smad3 interacts with both JunB and Cbfa1/Runx2. We have observed a stronger interaction of Smad3 protein with Cbfa1/Runx2 than with JunB. This could be a result of lesser expression of JunB under the control of the RSV promoter compared to Cbfa1/Runx2 and Smad3 expression under
CMV promoter control. Also, the poorer anti-JunB antibodies for immunoprecipitation could contribute to differences observed. Nevertheless, Smad3 may have stronger affinity towards to Cbfa1/Runx2 than JunB.

Evidence has shown that Smad proteins directly interact with either AP-1 factors or Cbfa/Runx proteins (53, 60-63). We provide data here that Smad3 could act as a mediator for physical interaction of both JunB and Cbfa1/Runx2 proteins (Figs. 7 & 8). Interestingly, we found that both the proximal RD/AP-1 and distal RD sites of the human collagenase-3 promoter are occupied by Cbfa1/Runx2 but in response to TGF-β-treatment, the bound Cbfa1/Runx2 is displaced from the proximal RD/AP-1 site by induced AP-1 factors like JunB (Fig. 8). The proximal RD/AP-1 site may have greater affinity towards AP-1 factors than Cbfa1/Runx2. We failed to observe interaction of Smad3 at the proximal RD/AP-1 site. Time course kinetics of ChIP assay would reveal more precisely the binding of these transcription factors on proximal RD/AP-1 and distal RD sites. There was increased association of Smad3 and JunB at the distal RD region by TGF-β1. Phosphorylation and nuclear translocation of Smad3 by TGF-β1 could account for increased association of Smad3 at the distal RD region of the human collagenase-3 promoter. The level of Cbfa1/Runx2 associated DNA fragment was not changed by TGF-β1 and this is consistent with our earlier observation that Cbfa1/Runx2 protein levels dropped at later time periods of TGF-β1-treatment (Fig. 4C). Thus, the presence of Smad3, JunB, and Cbfa1/Runx2 proteins at the distal RD region strongly suggests the assemblage of these proteins on the human collagenase-3 promoter for TGF-β-response. Co-transfection of Smad3, JunB, and Cbfa/Runx2 expression constructs stimulated collagenase-3 promoter activity indicating the co-operativity between these proteins.
Further increased collagenase-3 promoter activity with a constitutively active TGF-β type I receptor expression plasmid suggested that post-translational modification(s) of these transcription factor(s) (most likely phosphorylation of Smad3) or repression or induction of other factors may occur in response to TGF-β1 that result in maximal collagenase-3 promoter activity.

Overall, our studies demonstrate transcriptional activation of the collagenase-3 gene by TGF-β1 in human breast cancer cells is mediated by interaction of both the distal RD and proximal RD/AP-1 sites. We also conclude that Cbfa1/Runx2 bound to the distal RD site, and AP-1 factors, JunB at the proximal RD/AP-1 site physically interact through Smad3, as evidenced by both in vitro and in vivo studies and account for TGF-β1-stimulation of collagenase-3 expression in MDA-MB231 cells.
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The abbreviations used are as follows: MMPs, matrix metalloproteinases; TGF-β1, transforming growth factor-beta1; AP-1, activator protein-1; RD, runt domain binding site; Cbfa, core binding factor alpha; SBE, Smad binding element; AML, acute myeloid leukemia; ETO, eight-twenty-one; ECM, extracellular matrix; PTH, parathyroid hormone; PTHrP, parathyroid hormone related protein; RT-PCR, reverse transcriptase-polymerase chain reaction; ChIP, chromatin immunoprecipitation; TβRI, TGF-β type I receptor.
LEGENDS

Figure 1. The RD and AP-1 sites are necessary for TGF-β1-stimulated collagenase-3 promoter activity. (A) Comparison of the nucleotide sequences of the distal RD and the proximal RD/AP-1 sites of the −148 rat and human collagenase-3 promoters (+ indicates the transcription initiation site). The asterisks indicate mutated nucleotides within rat and human collagenase-3 distal RD, AP-1, and proximal RD sites. (B) The wild type and mutant rat collagenase-3 promoter constructs were transiently transfected into MDA-MB231 cells, treated with control or TGF-β1-containing media for 24 h, and then assayed for CAT activity. The TGF-β1-response is shown as -fold stimulation over control. The inset graph shows the basal activity of collagenase-3 promoter constructs. Data represent mean ± S.E. of three experiments. The data was analyzed by ANOVA using Prism 3.0 software. *Significant difference compared with control (promoterless) vector, pSV0 (P< 0.05). (dRD-distal RD site; pRD-proximal RD site; AP-1-activator protein-1 site)

Figure 2. Analysis of the AP-1 factors and Cbfa1/Runx2 binding to the proximal RD/AP-1 site of the human collagenase-3 promoter. (A) MDA-MB231 cells were treated with control (C) or TGF-β1-containing media (T) for 1 h, and nuclear extracts were prepared. Extracts were preincubated for 30 min at 4° C with IgG or antisera to the AP-1 family members, as indicated, before the addition of the labeled human proximal RD/AP-1 site probe. After the addition of the probe, nuclear extracts were incubated for 15 min at 4° C. (B) Control and TGF-β1-treated nuclear extracts prepared from MDA-
MB231 cells were incubated with labeled Oct1 probe. (C) Nuclear extracts from MDA-MB231 cells treated with control or TGF-β1-containing media for 1 h were preincubated for 30 min at 4°C with IgG or antiserum to Cbfa1, as indicated, before the addition of the labeled proximal RD/AP-1 site probe. After the addition of the probe, nuclear extracts were incubated for 15 min at 4°C. (D) TGF-β1-treated nuclear extracts from MDA-MB231 cells were preincubated for 30 min at 4°C with 50-fold molar excesses of unlabeled competitors as indicated, before the addition of the labeled proximal wild type RD/wild type AP-1 site probe. After the addition of the probe, nuclear extracts were incubated for 15 min at 4°C. * indicates non-specific band.

**Figure 3. TGF-β1 stimulates fosB, fra2, c-jun, junB, and Cbfa1/Runx2 mRNAs.** MDA-MB231 cells were treated with control or TGF-β1-containing media for indicated time. Total RNA was isolated and subjected to real time quantitative RT-PCR using specific primers as outlined in methods section. The relative levels of mRNAs were normalized to β-actin and the TGF-β1-fold induction was calculated over control. The data is represented as mean ± SEM (n = 3). (A) quantitative RT-PCR for the detection of Fos family members. (B) quantitative RT-PCR for the detection of Jun family members, and C, quantitative RT-PCR for the detection of Cbfa1/Runx2.

**Figure 4. TGF-β1 stimulates c-Jun, JunB, and Cbfa1/Runx2.** (A) MDA-MB231 cells were treated with control or TGF-β1-containing media for indicated time. Total lysates were prepared and subjected to Western blot analysis using the antibodies as mentioned in the figure. The relative levels of proteins were normalized to α-tubulin and TGF-β1-
fold induction was calculated over control. (B) Whole cell extracts from HeLa, MDA-MB231, and Saos2 cells were subjected to Western blot analysis as described in the methods section. Blots were incubated overnight with either anti-Cbfa1 or IgG or anti-Cbfa1 that had been preadsorbed for 30 min with 100X peptide against which it was raised.

Figure 5. Functional requirement of the RD site and its transcription factors for TGF-β1-stimulated collagenase-3 promoter activity. The wild type collagenase-3 promoter construct (-148) was transiently cotransfected with pCMV-AML-1/ETO construct into MDA-MB231 cells and then treated with control or TGF-β1-containing media for 24 h, and assayed for CAT activity. The total amount of DNA used for all transfection with or without the expression constructs was equalized with pCMV. Data represent mean ± S.E. of three experiments. *Significant difference compared with control (P< 0.001).

Figure 6. Mutation of the proximal RD site does not completely prevent binding of the AP-1 factors to the AP-1 site and Cbfa1/Runx2 is not directly bound to the proximal RD site. (A) Nuclear extracts from MDA-MB231 cells treated with control (C) or TGF-β1-containing media (T) for 1 h were incubated with biotinylated human proximal wild type RD/wild type AP-1 or mutant RD/wild type AP-1 oligonucleotides and streptavidin-agarose beads. Protein-DNA complexes were subjected to Western blotting analysis and immunoblotted with anti-JunB, anti-Cbfa1 and anti-c-Fos polyclonal antibodies. (B) Nuclear extracts from MDA-MB231 cells treated with control
(C) or TGF-β1-containing media (T) for 1 h were incubated with biotinylated distal wild type RD or mutant RD oligonucleotides and streptavidin-agarose beads. Protein-DNA complexes were subjected to Western blotting analysis and immunoblotted with anti-Cbfa1 antibody.

**Figure 7. Smad3 interacts with both JunB and Cbfa1/Runx2 in vitro.** (A) Cell lysates from transiently transfected Cos-7 cells were subjected to immunoprecipitation with either IgG or anti-JunB or anti-c-Myc antibodies and then immunoblotted using anti-JunB or anti-c-Myc antibody that recognizes Cbfa1/Runx2. (B) Cell lysates from transiently transfected COS-7 cells were subjected to immunoprecipitation with either IgG or anti-JunB or anti-c-Myc antibodies and then immunoblotted using anti-Flag antibody that recognizes Smad3 and Smad4. (C) Cell lysates as mentioned above were subjected to immunoprecipitation with either IgG or anti-JunB antibody and then immunoblotted using anti-JunB and anti-c-Myc antibodies. (HC-heavy chain) (D) Cell lysates as mentioned above were subjected to immunoprecipitation with either IgGs (mouse or rabbit) or anti-JunB or c-Myc or anti-Flag antibodies and then immunoblotted using anti-Smad4. Expression levels of proteins were determined by immunoblotting aliquots of total cell lysates.

**Figure 8. Smad3 interacts with both JunB and Cbfa1/Runx2 and assemblage of these proteins on human collagenase-3 promoter in vivo.** MDA-MB231 cells were treated with control (C) or TGF-β1-containing media (T) for 1 h. Cells were fixed with formaldehyde and lysates were prepared as described in methods section for chromatin
immunoprecipitation (ChIP). (A) Cell lysates were subjected to immunoprecipitation with either IgG or anti-JunB antibody and then immunoblotted using anti-Smad3. (B) Cell lysates were subjected to immunoprecipitation with either IgG or anti-Cbfa1 antibody and then immunoblotted using anti-Smad3. (C) Cell lysates were subjected to immunoprecipitation with either IgG or anti-JunB or anti-Cbfa1 antibodies, followed by re-immunoprecipitation with either IgG or anti-Cbfa1 or anti-JunB antibodies, respectively and then immunoblotted using anti-Smad3. (D) MDA-MB231 cells were treated with control (C) or TGF-β1-containing media (T) for 6 h. Cells were fixed with formaldehyde and lysates were prepared as described in methods section. After immunoprecipitation of the cross-linked lysates with antibodies, as indicated or with IgG as a negative control, the DNA was subjected to PCR with primers that amplify the proximal RD/AP-1 region and the distal RD region of the human collagenase-3 promoter. Input DNA (1/100) is positive control for the assay.

Figure 9. Co-operative interaction of JunB, Cbfa1/Runx2, and Smad3 proteins and transcriptional activation of collagenase-3 promoter by TGF-β1. The collagenase-3 promoter construct (-148) was transiently cotransfected with pCMV-Smad3, pRSV-JunB, pCMV-Cbfa1 individually or all together with or without a constitutively active TGF-β1 type I receptor (TβRI) into Cos-7 cells. CAT activity was assayed after 24 h. The total amount of DNA used for all transfections with or without the expression constructs was equalized with either pCMV or pRSV. *Significant difference compared with collagenase-3 promoter construct cotransfected with pCMV-Smad3, pRSV-JunB, and pCMV-Cbfa1 expression constructs (P<0.049).
Figure 1

(A) Diagram showing the Human and Rat sequences and their interaction with AP-1 and RD/Cbfa.

(B) Bar graph showing the fold stimulation of CAT activity with different constructs.

- **PSV0**
- **148**
- **148dRD**
- **148pRD**
- **148pAP-1**
- **148pRD/AP-1**
- **148p/d/RD/AP-1**

The star (*) indicates significance.
Figure 2

(A) labeled proximal wild RD/wild AP-1 probe

| Probe       | IgG | anti-c-Fos | anti-FosB | anti-Fra1 | anti-Fra2 | anti-c-Jun | anti-JunD | anti-JunB |
|-------------|-----|------------|-----------|-----------|-----------|------------|-----------|-----------|
| C          | T   | C          | T         | C         | T         | C          | T         | T         |

- supershift

- free probe

(B) labeled Oct 1 probe

| Probe | C | T |
|-------|---|---|

- shift

- free probe

(C) labeled proximal wild RD/wild AP-1 probe

| | C | T |
|---|---|---|
| IgG | + | - |
| - | + | - |
| - | + | - |

- extract

- free probe

(D) labeled proximal wild RD/wild AP-1 probe

| | C | T |
|---|---|---|
| IgG | + | - |
| - | + | - |
| - | + | - |

- extract

- free probe
Figure 4

(A) Time (h)  TGF- 
0  1  2  4  c-J un  -tubulin
39 kDa -  -
55 kDa -  -

(B) 55 kDa -  -
39 kDa -  J unB
55 kDa -  -

(C) 60 kDa -  Cbfa1/R unx2
55 kDa -  -

(D) anti-Cbfa1  IgG  anti-Cbfa1 + peptide
kDa  HeLa  MDA  Saos2  HeLa  MDA  Saos2  HeLa  MDA  Saos2  Cbfa1/R unx2
60 -
55 -  -
tubulin
Figure 5

[Bar chart showing CAT activity (pmol chlor but/h/mg ptn) for pSV0, 148, and AML/ETO in Control and TGF-β1 conditions.]

- **Control**
  - pSV0
  - 148
  - AML/ETO

- **TGF-β1**
  - pSV0
  - 148
  - AML/ETO

* indicates a significant difference.
Figure 6

(A) Proximal extracts

|          | C   | T   | C   | T   | C   | T   |
|----------|-----|-----|-----|-----|-----|-----|
| JunB     |     |     |     |     |     |     |
| Cbfa1/Runx2 |  |     |     |     |     |     |
| c-Fos    |     |     |     |     |     |     |

(B) Distal extracts

|          | C   | T   | wRD | mRD |
|----------|-----|-----|-----|-----|
| Cbfa1/Runx2 |  |     |     |     |
Figure 8

(A)  
ChIP:  
- Smad3

IB: Smad3

(B)  
ChIP:  
- Smad3

IB: Smad3

(C)  
Re-ChIP  
ChIP:  
- Smad3

IB: Smad3

(D)  
Input  
- Smad3

IB: Smad3

proximal RD/AP-1

distal RD
Figure 9

CAT activity (pmol chlor but/h/mg)

| Treatment          | -148 | +   | +   | +   | +   | +   | +   | +   |
|--------------------|------|-----|-----|-----|-----|-----|-----|-----|
| pSV0               |      |     |     |     |     |     |     |     |
| Smad3              |      |     |     |     |     |     |     |     |
| JunB               |      |     |     |     |     |     |     |     |
| Cbfa1/Runx2        |      |     |     |     |     |     |     |     |
| TβRI               |      |     |     |     |     |     |     |     |

* Indicates significant difference.
Smad3 interacts with JunB and Cbfa1/Runx2 for transforming growth factor-β 1-stimulated collagenase-3 expression in human breast cancer cells
Nagarajan Selvamurugan, Sukyee Kwok and Nicola C. Partridge

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