Requirement of Smad3 and CREB-1 in Mediating Transforming Growth Factor-β (TGFβ) Induction of TGFβ3 Secretion*

Guangming Liu, Wei Ding, Jill Neiman, and Kathleen M. Mulder

From the Department of Pharmacology, Penn State College of Medicine, Hershey, Pennsylvania 17033

Because increased transforming growth factor-β (TGFβ) production by tumor cells contributes to cancer progression through paracrine mechanisms, identification of critical points that can be targeted to block TGFβ production is important. Previous studies have identified the precise signaling components and promoter elements required for TGFβ induction of TGFβ1 expression in epithelial cells (Yue, J., and Mulder, K. M. (2000) J. Biol. Chem. 275, 30765–30773). To determine how regulation of TGFβ3 expression differs from that of TGFβ1, we identified the precise signaling pathways and transcription factor-binding sites that are required for TGFβ3 gene expression. By using mutational analysis in electrophoresis mobility shift assays (EMSAs), we demonstrated that the c-AMP-responsive element (CRE) site in the TGFβ3 promoter was required for TGFβ-inducible TGFβ3 expression. Electrophoresis mobility supershift assays indicated that CRE-binding protein 1 (CREB1) and Smad3 were the major components present in this TGFβ-inducible complex. Furthermore, by using chromatin immunoprecipitation assays, we demonstrated that CREB-1, ATF-2, and c-Jun bound constitutively at the TGFβ3 promoter (−100 to +1), whereas Smad3 bound at this site only after TGFβ stimulation. In addition, inhibition of JNK and p38 suppressed TGFβ induction of TGFβ3 transactivation, whereas inhibition of ERK and protein kinase A had no effect. Small interfering RNA-CREB1 and small interfering RNA-Smad3 significantly inhibited TGFβ stimulation of TGFβ3 promoter reporter activity and TGFβ3 production. Our results indicate that TGFβ activation of the TGFβ3 promoter CRE site, which leads to TGFβ3 production, is required for TGFβRII, JNK, p38, and Smad3 but was independent of protein kinase A, ERK, and Smad4.

TGFβ is the prototype of a large superfamily of multifunctional cytokines that are differentially expressed and function in a wide range of target cells (1). TGFβ suppresses the proliferation of normal cells. However, in malignant cells TGFβ production often enhances tumor progression, especially once the cells have lost the negative growth control signals imparted by TGFβ. Therefore, identification of critical points that can be targeted to block TGFβ production in cancer cells is important (2, 3). A better understanding of the mechanisms underlying TGFβ auto-regulated secretion will assist in the identification of these critical branch points.

The TGFβ family has the following three homologous forms: TGFβ1, TGFβ2, and TGFβ3. Although the three TGFβs share 60–80% identity, they are encoded by distinct genes, and their expression is controlled by a different regulatory sequence or promoter (4–6). They also show different physiological and pathological activities in certain cell types and systems (7–17). Because the expression of TGFβ is controlled by an auto-feedback loop (18), TGFβ is an important mechanism for amplifying its own expression and production. Because of the critical role that TGFβ plays in regulating a broad range of physiological and pathological effects, such as proliferation, cell cycle arrest, apoptosis, angiogenesis, metastasis, and invasiveness in cells and tissues, any changes in the signaling pathways mediating TGFβ autoregulation may lead to severe abnormalities. It is therefore important to understand the mechanisms underlying TGFβ autoregulation, as well as the signal transduction pathways mediating TGFβ-induced TGFβ3 expression.

The autoregulation of TGFβ appears to be mediated through specific sites in the promoter regions of the distinct isoforms (19). Although these sites have been examined for the TGFβ1 promoter (19), little is known about the relevant regions in the TGFβ3 promoter. The promoter area of the TGFβ3 gene has little structural or functional similarity to the TGFβ1 promoter. Similarly, transcriptional regulation of this gene has significantly diverged from that of the other TGFβs (6). It has been reported that a region proximal to the TATA box in the 5′-flanking region of the TGFβ3 gene might be important in regulating TGFβ3 expression (6), but no conclusive studies have defined the critical sites required for TGFβ induction of TGFβ3 expression.

Here we have determined that the TGFβ-inducible region of the TGFβ3 promoter contains three transcription factor binding consensus sites: a CRE at −45 to −39 (GACGTCGA), an SBE at −49 to −46 (CAGA), and an activator protein-2 (AP-2) site at −57 to −50 (CCCCAGGC). We have investigated the transcriptional regulation of each of these TGFβ3 gene promoter regions in response to TGFβ exposure, and we have demon-
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strated that the CRE site is the critical site. We have also defined the signal transduction pathways mediating TGFβ induction of TGFβ3 secretion, and we demonstrate that Smad3 and CREB-1 are the critical transcription factors involved. Although we have shown previously that JNKs and ERKs were the critical mediators of TGFβ1 production in untransformed epithelial cells (19), here we show that JNKs and p38, but not PKA and ERKs, are the critical upstream mediators of TGFβ3 production. Our studies provide new insights into the TGFβ auto-negative feedback regulatory mechanisms that are required for one of the key biological responses to TGFβ, namely stimulation of its own secretion. In this case, however, the results are specific for production of TGFβ3. Furthermore, our results demonstrate that blocking these critical points reduces TGFβ3 production, and as such, our findings suggest important intervention strategies for controlling tumor growth mediated by excessive tumor cell-secreted TGFβ3 in the tumor microenvironment.

MATERIALS AND METHODS

Cell Culture—The untransformed rat intestinal epithelial cell line 4-1 (IEC4-1, TGFβ-sensitive) was isolated as described previously (20, 21). Cells were routinely maintained in SMIGS medium, consisting of McCoy’s 5A (Invitrogen), supplemented with amino acids, pyruvate, antibiotics (streptomycin, penicillin), insulin (4 μg/ml), glucose (4.5 mg/ml), and 5% fetal bovine serum. IEC4-1 cells, stably transfected with dominant-negative Smad3 and CREB-1 Mediate TGFβ3 Expression in SMIGS medium in the presence of 5 mM KCl, 0.5% Nonidet P-40, 100 μM dithiothreitol, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After a 1-min centrifugation (16,000 × g, 4 °C), the pellet containing the nuclei was washed once with 500 μl of buffer B (buffer A without Nonidet P-40), resuspended in 120 μl of extraction buffer (buffer B but with 500 mM KCl and 10% glycerol), and incubated with shaking at 4 °C for 30 min. The nuclear extracts were stored at −70 °C until analysis. The DNA binding reaction (for EMSA) was carried out at room temperature for 30 min in a mixture containing 4 μg of nuclear protein, 1 μg of poly(dI-dC), and 15,000 cpm of 32P-labeled double-stranded oligonucleotide. The samples were fractionated through a 5% polyacrylamide gel. Gels were dried and exposed to x-ray film for further analysis.

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were performed as described (24). Nuclear protein extracts were prepared from either IEC4-1 or CCL64 cells by a method described previously (24). Briefly, the cells were cultured in 10-cm dishes and treated with TGFβ3 (10 ng/ml) for 1 h. The cells were then cross-linked with formaldehyde. Nuclear extracts were sonicated to obtain −250-bp fragments of DNA. The resulting DNA was immunoprecipitated overnight with antibodies to CREB-1, CREB-2, ATF-2, c-Jun, JunD, Smad3, or Smad4 (Santa Cruz Biotechnology) and salmon sperm/agarose beads (catalog number 16-157; Upstate Biotechnology, Inc.). The resulting DNA sample incubated with IgG or with no antibody was used as a negative control. The supernatant from a sample was used as total DNA input. The DNA was dissolved in 30 μl of water, and 2 μl was employed for PCR analysis using the primers for the TGFβ3 promoter region containing AP-2, CRE, and SBE sites (5′-TGTCAAGCAGAATGCCGTTGCGTG-3′ and 5′-TCTATTTCTCTCTGCTGAA-3′). The products were resolved in a 3% agarose gel and visualized with ethidium bromide.

siRNA Transfections—siRNA-CREB-1 was purchased from Santa Cruz Biotechnology. siRNA-Smad3 was purchased from Cellogenetics, Inc. (Baltimore). The siRNA products were annealed duplexes of RNA. Transfection of siRNAs into cells was performed according to the manufacturer’s instructions. A nontargeting siRNA was used as a control for nonsequence-specific effects of the transfected siRNAs (Dharmacon, Inc., Dallas).

Construction of TGFβ3 Promoter Luciferase Reporter Plasmid—The TGFβ3-P221-110-Luc encodes a TGFβ3 promoter region from −221 to +110. The nucleotide sequence was PCR-amplified. The primers used were as follows: sense, 5′-acttcgaACGGCTTtgtgcaagagtcaacttaaga-3′ (the capital letters indicate an MluI restriction enzyme digestion site), and antisense, 5′-gcagcCAATCTTcttgacttgactctgcc-3′ (the capital letters indicate a BglII restriction enzyme digestion site). The PCR products and the basic pGL3 luciferase reporter vector (Promega, Madison, WI) were digested using MluI and BglII, and the products were gel-purified and inserted into the vector using Quick Ligase (New England Biolabs, Beverly, MA). The sequence of the resulting TGFβ3-P221-110-Luc luciferase reporter plasmid was confirmed by sequencing in both directions. The
CRE-mutated Tβ3-P221/110-Luc luciferase reporter plasmid (Mut-Tβ3-P221/110-Luc) was constructed using QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used were as follows: sense, 5’-CCACCCCAAGCCAGAAGACGCTATGGAGGGAGGTATA-3’, and antisense, 5’-TATACCTCCCTCCCATagtGTCTCTGGGCTGGGTGG-3’ (the lowercase letters indicate mutated CRE site).

Luciferase Reporter Assays—IEC-1 and CCL64 cells were seeded in 24-well plates and grown to 70–80% confluence. Cells were transfected with 0.4 μg of Tβ3-P221/110-Luc, siRNA plasmids at the indicated concentrations, and 0.1 μg of Renilla luciferase control reporter (pRL-SV40) per well, using Lipofectamine™ 2000 (Invitrogen), as described in the user manual. TGFβ3 (10 ng/ml) was added 24 h after transfections, and luciferase activities were measured at 24 h after TGFβ3 treatment. Transfection efficiencies were determined by co-transfecting with Renilla luciferase. IEC-1 cells were also co-transfected with Tβ3-P221/110-Luc and a dominant-negative Smad4 plasmid (19) at the concentrations indicated.

Real Time Quantitative RT-PCR—RNA was isolated from HaCaT cells and their siRNA-CREB-1 and siRNA-Smad3 transfectants as described above using TRIzol reagent by Invitrogen. Total cDNA was prepared by RT-PCR using random hexamer primers purchased from Invitrogen and the iso- lated RNA as the template. Real time RT-PCR was performed using SYBR Green I agent from Qiagen Inc. (Valencia, CA), with the cDNA prepared above as the template. 18 S rRNA was used as an internal control. The TGFβ3 primers used were as follows: sense, 5’-TGCGCAGCCCGTCTCTA-3’, antisense, 5’-GGTTGAAGCAGCCACGTCTTCA-3’.

Quantitation of TGFβ3—The quantitative determination of TGFβ3 was performed. IEC-4 cells, and their siRNA-CREB-1 or siRNA-Smad3 transfectants, were seeded in 12-well plates and grown in the absence or presence of TGFβ1 for 72 h. Conditioned medium (CM) was then collected for quantitative determination of TGFβ3 using an ELISA kit (DuoSet ELISA Development System, R & D Systems, Inc., Minneapolis, MN). The system specifically detects TGFβ3 with no cross-reactivity or interference with TGFβ1. The cells were trypsinized and counted. “Total” TGFβ3 was quantitated according to the manufacturer’s instructions. Briefly, the samples were acid-activated using 1 N HCl to activate latent TGFβ3 and then neutralized by addition of 1.2 N NaOH, 0.5 M HEPES solution to convert all TGFβ3 to the form detectable using the antibody in the ELISA kit.

Western Blotting—IEC-4 cells were transfected with the siRNA plasmids indicated, and the indicated cells were lysed and prepared 36 h after transfection. Western blotting for CREB-1, Smad3, and phosphorylated CREB-1 proteins was carried out using specific antibodies, according to the supplier’s recommendations. Anti-CREB-1 antibody (9192) and anti-phospho- CREB-1 antibody (9191) were from Cell Signaling Technology (Beverly, MA). Anti-Smad3 antibody (51-1500) was Zymed Laboratories Inc.

Statistical Analysis—Data were analyzed and statistically significant differences determined using the Student’s t test. The results are expressed as the mean ± S.E.

RESULTS

The CRE Consensus Site in Tβ3-P61/35 Is Critical for TGFβ3 Responsiveness—It has been reported that a sequence from −490 to +100 in the 5’-flanking region of the TGFβ3 gene might be important in regulating TGFβ3 expression (6). This region contains two ubiquitously expressed Sp-1 transcription factor DNA-binding sites (−420 to −415 and −363 to −358), an AP-2 site (−57 to −50), and a CRE site (−45 to −39). The CRE site has been shown to be involved in TGFβ3 transcription, although its TGFβ3-inducible expression was not examined (6). In addition to the AP-2 and CRE sites, we also identified an SBE site (−49 to −46), between the AP-2 and CRE sites, which can also be induced by TGFβ3 (27). We presumed that the SBE and/or CRE sites, but not the Sp-1 sites, in the TGFβ3 promoter region might be important for TGFβ3 induction of TGFβ3 gene expression because Sp-1 has relatively little effect on the activation of the promoter (6). To address this issue, we constructed a Tβ3-P221/110-Luc luciferase reporter plasmid using the DNA sequence from the TGFβ3 5’-flanking region (−221 to +110) of the promoter. This sequence contains the AP-2, SBE, and CRE sites. We treated IEC-4 cells with TGFβ3 to stimulate the auto-induction loop, and we measured Tβ3-P221/110-Luc reporter activity. Here we show that TGFβ3 affected a time-dependent increase in Tβ3-P221/110-Luc activity. The TGFβ3 stimulation of Tβ3-P221/110-Luc activity reached a peak 24 h after TGFβ3 treatment (Fig. 1A). These results indicate that Tβ3-P221/110 is important in TGFβ3 auto-induction of TGFβ3 gene transcription.

The IEC-4 cells were used for these studies because they represent a good model for TGFβ regulation in untransformed epithelial cells because of their high level of TGFβ sensitivity and their well characterized TGFβ pathways (21, 22, 28, 29). In addition, these cells have been shown to produce high levels of endogenous TGFβ (20).

To determine whether the AP-2 (−57 to −50), SBE (−49 to −46), and/or CRE (−45 to −39) sites within the −221 to +110 region of the TGFβ3 promoter were involved in TGFβ3 induc- tion of TGFβ3 gene transcription, we examined the DNA bind- ing activity of a sequence containing these three sites (T3-P61/35) in response to TGFβ3. As shown in Fig. 1B, TGFβ3 significantly increased complex formation at Tβ3-P61/35 in a time-dependent manner, with peak levels occurring 1 h after TGFβ3 treatment. Thus, our results indicate that a TGFβ3-inducible complex formed at Tβ3-P61/35 in response to TGFβ3 treatment.

During the TGFβ signaling process, TGFβ binds to RII, which then recruits RII into the active complex (30). To investigate whether the TGFβ receptors were required for auto-regulation of TGFβ3 expression, we performed EMSAs with Tβ3-P61/35 after expression of a dominant-negative TGFβ receptor II (DN RII) (22). As shown in Fig. 2A, the Tβ3-P61/35 DNA binding activity stimulated by either TGFβ1 or TGFβ3 was significantly suppressed in the DN RII-expressing IEC-4 cells (Fig. 2A, lanes 6 and 7 versus lanes 2 and 3). In contrast, DN RII had no effect in the PMA-induced cells (Fig. 2A, lane 8 versus lane 4). These results indicate that RII is required for TGFβ3 auto-loop regulation.
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To determine which DNA-binding sites are required for TGFβ production, we performed site-directed mutagenesis at the relevant sites, and we then repeated the EMSAs using labeled versions of these as probes. As shown in Fig. 2B, mutation of the CRE site completely abolished DNA binding activity at Tβ3-P61/35 (lanes 10–12), whereas mutation of the AP-2 (lanes 4–6) or SBE (lanes 7–9) sites had no effect. The TGFβ-inducible complex formed at the wild-type Tβ3-P61/35 is shown for comparison (Fig. 2B, lanes 2 and 3). These results indicate that DNA binding activity at the CRE site is critical for mediating the TGFβ3 auto-induction loop. As shown in Fig. 2B (lanes 1, 4, 8, and 10), a 10-fold excess of unlabeled probe completely competed for DNA binding of the radioisotope-labeled probe, indicating that the bands observed represent specific binding to Tβ3-P61/35.

To further confirm that the CRE site in the TGFβ3 promoter is indeed required for the TGFβ3 gene transcription, we constructed Mut-Tβ3-P221/110-Luc, a CRE site-mutated Tβ3-P221/110-Luc luciferase reporter plasmid. We transfected IEC4-1 cells with Mut-Tβ3-P221/110-Luc, followed by treatment of the IEC4-1 transfectants with TGFβ. As shown in Fig. 2C, CRE mutation completely abolished the luciferase activity induced by TGFβ. This result confirms that this CRE site in the TGFβ3 promoter region is critical for TGFβ3 gene transcription.

CREB-1 and Smad3, but Not Smad4, Are Bound at the Tβ3-P61/35—It is known that CREB-1 can recognize and bind to CRE consensus sites in various gene promoters. As we have demonstrated that the CRE site in Tβ3-P61/35 is critical for TGFβ auto-induction of TGFβ3, we examined whether the CRE-binding protein was a component of the transcription factor complex bound at this Tβ3-P61/35. As shown in Fig. 3A by EMSAs, TGFβ-inducible complex formation (lanes 1 and 2) was reduced when the CRE-1 antibody was present (lane 4) but not when rabbit IgG was present (lane 3). A supershift was also observed using the CRE-1 antibody (Fig. 3A, lane 4). Thus, CREB-1 was in the transcription factor complex bound to the Tβ3-P61/35 region.

We further explored whether Smad3 and Smad4 might be present in the complex at Tβ3-P61/35, because Smads are known to play important roles in TGFβ signaling and because an SBE site is present in Tβ3-P61/35. As shown in Fig. 3A, lane 5, the anti-Smad3 antibody reduced the TGFβ-inducible DNA binding, indicating that Smad3 was present in the transcription factor complex bound to Tβ3-P61/35. This did not occur when phorbol myristate acetate was used as the inducer (data not shown). Because activated Smad2 or Smad3 can form a dimer with Smad4 prior to stimulation of transcriptional activation of target genes through SBE sites, it was of interest to determine whether Smad4 was also in this complex bound to Tβ3-P61/35. As shown in Fig. 3A, lane 6, the anti-Smad4 antibody had no effect on DNA binding of Tβ3-P61/35, suggesting that Smad4 may not be involved in the transcription factor complex bound to Tβ3-P61/35. To verify the specificity of the anti-Smad3 and anti-Smad4 antibodies used, a full palindromic SBE sequence was used in the supershift EMSAs. As shown in Fig. 3B, a 10-fold excess of unlabeled cold probe completely competed for TGFβ induction of the DNA binding of the radioisotope-labeled probe, indicating that the band observed was specific for the consensus palindromic SBE site (lane 1). Both Smad3 and Smad4 were present in the transcription factor complex bound to the consensus SBE site (Fig. 3A, lanes 5 and 6). The results in Fig. 3A, lane 2, indicate that in the absence of TGFβ, there was no binding to the consensus palindromic SBE site in IEC4-1 cells, whereas TGFβ could induce DNA binding at this site, as shown in lane 3 (Fig. 3B). As shown in Fig. 3B (lane 4), addition of rabbit IgG in the EMSAs had no effect on TGFβ induction of DNA binding at the consensus palindromic SBE site. Overall, our results indicate that the anti-Smad3 and anti-Smad4 antibodies used (Fig. 3A) were capable of competing effectively for DNA binding at the consensus SBE site in the EMSA supershift assays.

Smad3 Is Bound at the Tβ3-P61/35 Only in Response to TGFβ Stimulation—Because we have demonstrated that both CREB-1 and Smad3 were required for DNA binding at Tβ3-P61/35 in response to TGFβ treatment, we performed ChIP analyses to confirm these findings, as well as to explore which other potential transcription factors bind to this TGFβ3 promoter region. It has been reported that CREB-1, ATF-2, and c-Jun are able to bind to consensus CREs (20, 31, 32). In addition, CREB-2 is a member of the CREB family, and JunD is a close homologue of c-Jun, but it was not clear whether these transcription factors were effective in TGFβ induction of DNA
binding at the CRE site in the TGFβ3 promoter (−45 to −39). Therefore, we examined whether CREB-1, CREB-2, ATF-2, c-Jun, JunD, Smad3, and/or Smad4 could bind to this TGFβ3 promoter region (−45 to −39). To determine the transcription factors bound to the endogenous TGFβ3 promoter, we performed ChIP analyses using noncancerous TGFβ3-responsive HaCaT human keratinocytes. As shown in Fig. 3C, in the absence of TGFβ, the transcription factors that bound to the CRE site in the TGFβ3 promoter (−45 to −39) were CREB-1, ATF-2, and c-Jun. In contrast, CREB-2, JunD, Smad3, and Smad4 were not present in the complex bound to this CRE site under these conditions (Fig. 3C, upper panel). After addition of TGFβ, however, the DNA binding activity of CREB-1 and ATF-2, but not that of c-Jun, was increased (Fig. 3C, lower panel). Most significantly, Smad3 was detectable in the complex bound at the CRE site in the TGFβ3 promoter (−45 to −39) only in response to TGFβ treatment (Fig. 3C, lower panel). Thus, although CREB-1, ATF-2, and c-Jun all bound constitutively to the CRE site in the TGFβ3 promoter (−45 to −39), CREB-1 and ATF-2 binding was also inducible by TGFβ, and Smad3 only bound to the TGFβ3 promoter CRE after TGFβ treatment. These results suggest that Smad3, as well as the CREB/ATF family members CREB-1 and ATF-2, may play a role in TGFβ induction of TGFβ3 promoter DNA binding activity.

**Smad3 Is Required for TGFβ Induction of TGFβ3 Promoter DNA Binding Activity**—We have demonstrated above that Smad3 is present in the transcription factor complex bound to Tβ3-P61/35 after induction by TGFβ. Because there is an SBE site upstream of the CRE site in Tβ3-P61/35, which was not required for TGFβ-inducible DNA binding to Tβ3-P61/35 (Fig. 2B), we employed CCL64-Smad3C cells, and their parental CCL64-L20 cells, in order to confirm whether Smad3 was required for TGFβ-inducible complex formation at the Tβ3-P61/35. CCL64-Smad3C cells stably express a dominant-negative form of Smad3 (33). In this cell line, TGFβ is unable to induce phosphorylation of Smad3, and the inhibitory effect of TGFβ on cell growth is blocked (33). Thus, normal Smad3 function in this cell line is lost. As shown in Fig. 4A by EMSAs, TGFβ induction of complex formation at Tβ3-P61/35 was blocked in the CCL64-Smad3C cells, compared with control L20 cells (lane 5 versus lane 2). In contrast, the PMA-induced complex formation at Tβ3-P61/35 was not diminished, when compared with control L20 cells (Fig. 4A, lane 6 versus lane 3). Thus, Smad3 is required for TGFβ induction of DNA binding at the CRE site of the TGFβ3 promoter region.

We also examined TGFβ induction of Tβ3-P221/110-Luc luciferase activity after transfection of L20 or Smad3C cells with Tβ3-P221/110-Luc. As shown in Fig. 4B, TGFβ induced a 5.9-fold increase of Tβ3-P221/110-Luc luciferase activity in the L20 cells, but only a 1.6-fold increase in the Smad3C cells. Statistic

Mut-Tβ3-P221/110-Luc and 0.1 μg of Renilla luciferase control reporter (pRL-SV40) per well using Lipofectamine™ 2000 (Invitrogen), as described in the user manual. 24 h after transfection, the cells of the TGFβ3 group were exposed to TGFβ (10 ng/ml), whereas the control group was not treated. The cells were harvested another 24 h later, and luciferase assays were performed as described under the “Materials and Methods.” Data are plotted as mean ± S.E. of triplicate samples for each of three independent experiments.
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A

IEC 4-1

TGFβ

Rabbit IgG

CREB-1 Ab

C-Jun Ab

Smad3 Ab

Smad4 Ab

Tβ3-P61/35

1 2 3 4 5 6

B

Cold probe

TGFβ

Rabbit IgG

Smad3 Ab

Smad4 Ab

Consensus palindromic SBE

Non-specific binding

IEC4-1

1 2 3 4 5 6

C

TGFβ-

No Antibody

IgG

CREB-1

CREB-2

ATF-2

C-Jun

JunD

Smad3

Smad4

TGFβ

-100/+1

DNA Input

100

HaCaT

DNA Input

100

FIGURE 3. CREB-1 and Smad3 are the primary components present in the transcription factor complex binding at Tβ3-P61/35. Nuclear protein was extracted from IEC4-1 cells, and EMSA supershift assays using the antibodies (Ab) indicated were performed as described under the “Materials and Methods.” A, cells were incubated in the absence or presence of TGFβ3 (10 ng/ml). The probe used was Tβ3-P61/35, and the arrow indicates the supershifted band. B, cells were incubated in the absence or presence of TGFβ3 (10 ng/ml). The probe used was the full palindromic SBE sequence as described under the “Materials and Methods.” The antibodies indicated were incubated with cross-linked DNA isolated from HaCaT cells that had been treated in the absence (upper panel) or presence (lower panel) of TGFβ. The TGFβ3 promoter region -100 to +1 was amplified using the template ChIP DNA obtained with the antibodies indicated. Analyses indicated a significant difference between the fold changes of the two cell groups (p < 0.01). Collectively, our results demonstrate that Smad3 not only binds to Tβ3-P61/35 but is also functionally required for TGFβ3 autoregulation.

Because Smad4 plays an important role in mediating Smad-dependent TGFβ responses, usually by forming dimers with Smad3 or Smad2, it was conceivable that Smad4 might be involved in the TGFβ induction of Tβ3-P221/110-Luc luciferase activity. Accordingly, we co-transfected a DN-Smad4 plasmid with Tβ3-P221/110-Luc into IEC4-1 cells. As shown in Fig. 4C, DN-Smad4 expression did not block Tβ3-P221/110-Luc activity either in the absence or presence of TGFβ. Thus, Smad4 is not required for TGFβ induction of Tβ3-P221/110-Luc luciferase activity, suggesting that Smad4 is not required for the TGFβ induction of TGFβ3 transcription mediated through Smad3.

JNK and p38, but Not PKA or the ERKs, Are Required for TGFβ3 Autoregulation—We have demonstrated previously that TGFβ induction of DNA binding to Tβ3-P61/35 occurs through the TGFβ receptors (Fig. 2A). Accordingly, it was of interest to explore whether downstream signaling events were also involved in TGFβ induction of TGFβ3 transcription. Furthermore, it is known that mitogen-activated protein kinases (MAPks) and PKA are upstream kinases of CREB-1 (34–36). Upon activation, these kinases translocate to the nucleus where they phosphorylate CREB-1 and initiate its DNA binding to the consensus CRE site. To determine whether JNK, ERK, p38, or PKA were required for TGFβ3 auto-regulation, we employed the following specific inhibitors: SP600125 as a selective JNK inhibitor, SB203580 as a selective p38 inhibitor, PD98059 as a selective MEK inhibitor, and H89 as a selective inhibitor of PKA. Our results indicate that SP600125 and SB203580 effectively suppressed TGFβ induction of Tβ3-P61/35 DNA binding activity (Fig. 5A) and transactivation (Fig. 5B) in a dose-dependent manner. However, PD98059 affected neither DNA binding to Tβ3-P61/35 nor Tβ3-P221/110-Luc activity (Fig. 5, A and B) at concentrations previously demonstrated to be effective in blocking TGFβ activation of ERKs in IEC4-1 cells (37). All three inhibitors had little effect on basal Tβ3-P61/35 DNA binding activity (data not shown).

Most unexpectedly, H89 (10 μM) had no effect on either DNA binding to Tβ3-P61/35 or Tβ3-P221/110-Luc luciferase activity, either in the absence or presence of TGFβ (Fig. 5, C and D). H89 has been shown previously to be effective in selectively blocking PKA in several cell types over the concentration range of 5–20 μM (38–42). Thus, a concentration of 10 μM H89 would be expected to completely block PKA activity in our system. Therefore, our results suggest that the signaling pathways mediating TGFβ3 auto-regulation are independent of PKA.

siRNA-CREB-1 and siRNA-Smad3 Both Suppress TGFβ Induction of Tβ3-P61/35 DNA Binding and Tβ3-P221/110-Luc Luciferase Activity—Because we have shown that CREB-1 and Smad3 were both present in the complex at Tβ3-P61/35, it was
of interest to confirm our results using siRNA-CREB-1 and siRNA-Smad3 transfectants in the EMSA and luciferase reporter experiments. Commercially available double-stranded siRNA was purchased and transfected into IEC4-1 cells to block target gene protein expression. As shown in Fig. 6A, both CREB-1 and Smad3 were detectable using either an anti-CREB-1 (lane 1, 1st panel) or an anti-Smad3 antibody (lane 1, 3rd panel). Fig. 6A, lane 2 of the 1st and 3rd panels, indicates that the scrambled control siRNAs had no effect on expression levels of either CREB-1 (lane 2, 1st panel) or Smad3 (lane 2, 3rd panel). Fig. 6A (lane 3, 1st panel) also indicates that the expression levels of CREB-1 in IEC4-1 cells transfected with siRNA-CREB-1 were significantly suppressed compared with untransfected IEC4-1 cells (Fig. 6A, lane 1) or to IEC4-1 cells transfected with the scrambled control siRNA (lane 2). In contrast, as expected, siRNA-Smad3 did not suppress CREB-1 levels (Fig. 6A, lane 4, panel 1). Fig. 6A (lane 3) also demonstrates that siRNA-Smad3 completely blocked Smad3 expression (lane 4), whereas siRNA-CREB-1 (lane 3) was ineffective. β-Actin was used as a loading control (Fig. 6A, 2nd and 4th panels). Thus, siRNA-CREB-1 and siRNA-Smad3 could effectively block the expression of their respective target proteins.

CREB-1 is activated by phosphorylation at Ser-133, which is critical for its biological function. Although we have demonstrated that siRNA-CREB-1 effectively suppressed CREB-1 protein expression (Fig. 6A), it was of interest to further investigate whether siRNA-CREB-1 suppressed TGFβ1 induction of CREB-1 phosphorylation specifically at Ser-133. Accordingly, we incubated IEC4-1 cells and their respective transfectants with or without TGFβ and performed Western immunoblotting by using an antibody specific for the activated form of phospho-CREB-1 (Fig. 6B, upper panel). As shown in Fig. 6B, siRNA-CREB-1 significantly blocked both basal and TGFβ-induced phosphorylation of CREB-1 at Ser-133. Densitometric scanning of the phospho-CREB-1 bands from Western analysis demonstrated that TGFβ could induce a 3-fold increase in phosphorylation of CREB-1 in IEC4-1 cells or IEC4-1 cells transfected with the siRNA control (Fig. 6B, lower panel). However, TGFβ only induced a 1.4-fold increase of phosphorylation in CREB-1 in IEC4-1 cells transfected with siRNA-CREB-1, indicating that siRNA-CREB-1 suppressed TGFβ induction of phosphorylation of the protein. Interestingly, siRNA-Smad3 also suppressed TGFβ induction of CREB-1 phosphorylation, with only a 1.6-fold increase being observed. Thus, Smad3 might also be involved in the pathway mediating TGFβ induction of CREB-1 phosphorylation.

As we have demonstrated that both CREB-1 and Smad3 are involved in the transcription factor complex formed at the CRE site in Tβ3-P61/35 in response to TGFβ treatment, we performed EMSAs using siRNA-CREB-1 and siRNA-Smad3 to
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A. IEC-4-1 cells were pretreated with either SB203580, PD98059, or SP600125 (Calbiochem) at the concentrations indicated for 30 min. Cells were then incubated in the absence or presence of TGFβ (10 ng/ml) for 1 h. Nuclear protein was harvested, and EMSAs were performed as described under the “Materials and Methods.”

B. IEC-4-1 cells were transfected with Tβ3-P221/110-Luc. 24 h after transfection, the cells were pretreated with either SB203580, PD98059, or SP600125 at the concentrations indicated for 30 min and then incubated in the absence or presence of TGFβ (10 ng/ml) for an additional 24 h. The cells were harvested, and luciferase assays were performed as described under the “Materials and Methods.”

C. IEC-4-1 cells were transfected with Tβ3-P61/35. 24 h after transfection, the cells were pretreated with H89 at the concentrations indicated for 30 min and were then incubated in the absence or presence of TGFβ (10 ng/ml) for an additional 24 h. The cells were harvested, and luciferase assays were performed as described under the “Materials and Methods.”

D. IEC-4-1 cells were transfected with Tβ3-P221/110-Luc. 24 h after transfection, the cells were pretreated with H89 at the concentrations indicated for 30 min and were then incubated in the absence or presence of TGFβ (10 ng/ml) for an additional 24 h. The cells were harvested, and luciferase assays were performed as described under the “Materials and Methods.”

FIGURE 5. JNKs and p38, but not PKA or ERKs, are required for TGFβ induction of Tβ3-P61/35 DNA binding and Tβ3-P221/110-Luc luciferase reporter activity. A. IEC-4-1 cells were pretreated with either SB203580, PD98059, or SP600125 (Calbiochem) at the concentrations indicated for 30 min. Cells were then incubated in the absence or presence of TGFβ (10 ng/ml) for 1 h. Nuclear protein was harvested, and EMSAs were performed as described under the “Materials and Methods.” Three independent experiments were performed, and a representative figure is shown.

B. IEC-4-1 cells were transfected with Tβ3-P221/110-Luc reporter construct. 24 h after transfection, the cells were pretreated with TGFβ (10 ng/ml) for an additional 24 h. The cells were harvested, and luciferase assays were performed as described under the “Materials and Methods.” Data plotted are the mean ± S.E. of triplicate samples from one of three independent experiments. Asterisks indicate a statistically significant difference (p < 0.05) in fold changes between the absence and presence of TGFβ in the inhibitor-treated IEC4-1 cells compared with the control IEC4-1 cells (no inhibitors). C. IEC-4-1 cells were transfected with H89 at the concentrations indicated for 30 min and were then incubated in the absence or presence of TGFβ (10 ng/ml) for 1 h. Nuclear protein was harvested, and EMSAs were performed as described under the “Materials and Methods.” Three independent experiments were performed, and a representative figure is shown. D. IEC-4-1 cells were transfected with Tβ3-P221/110-Luc. 24 h after transfection, the cells were pretreated with H89 at the concentrations indicated for 30 min and were then incubated in the absence or presence of TGFβ (10 ng/ml) for an additional 24 h. The cells were harvested, and luciferase assays were performed as described under the “Materials and Methods.” Data plotted are the mean ± S.E. of triplicate samples from one of three independent experiments.

Because we have demonstrated that both CREB-1 and Smad3 were required for DNA binding at Tβ3-P61/35 in response to TGFβ treatment, we performed Tβ3-P221/110-Luc luciferase activity assays to examine whether CREB-1 and Smad3 were functionally required for initiating promoter transactivation. As shown in Fig. 6D, TGFβ induced a 4.7-fold increase of Tβ3-P221/110-Luc activity in control IEC4-1 cells (Control). TGFβ also induced a 5.6-fold increase of Tβ3-P221/110-Luc activity in IEC4-1 cells transfected with siRNA scramble control (siRNA-Ctrl) and to 2.3-fold in siRNA-CREB-1 transfected IEC4-1 cells (siRNA-CREB-1) and to 2.4-fold in siRNA-Smad3 transfected IEC4-1 cells (siRNA-Smad3). These results established that both CREB-1 and Smad3 were not only present in the transcription factor complex bound at Tβ3-P61/35 but were also functionally required for promoter transactivation in response to TGFβ treatment.

siRNA-CREB-1 and siRNA-Smad3 Both Suppress TGFβ Induction of TGFβ Expression—Although we have demonstrated that CREB-1 and Smad3 were involved in TGFβ induction of TGFβ3 promoter DNA binding and transcriptional activities, it was of interest to extend our observations to exam-
FIGURE 6. siRNA-CREB-1 and siRNA-Smad3 both suppress TGFβ induction of Tβ3-P61/35 DNA binding and Tβ3-P221/110-Luc luciferase reporter activity. A, IEC4-1 cells transfected with the indicated siRNA plasmids were harvested. Cell lysates were subjected to Western blotting using the CREB-1 and Smad3 antibodies described under the "Materials and Methods." As a loading control, β-actin was also detected in the same membrane using an anti-β-actin antibody. The siRNA-Control (siRNA-Ctrl) used was an siRNA-scrambled sequence from Ambion. B, upper panel, siRNA-transfected IEC4-1 cells as described in A were incubated in the absence or presence of TGFβ3 (10 ng/ml) for 30 min. The cells were then harvested, and lysates were prepared, and Western blotting was performed with phospho-CREB-1 and phospho-Smad3 antibodies, as described under the "Materials and Methods." As a loading control, β-actin was also detected in the same membrane using an anti-β-actin antibody. The siRNA-control used was an siRNA-scrambled sequence from Ambion. Lower panel, quantitative analysis of the bands in the upper panel. The numbers on top of the bars indicate fold increases compared with control (Ctrl). C, the siRNA-transfected IEC4-1 cells described in A were incubated in the absence or presence of TGFβ3 (10 ng/ml) for 1 h. Nuclear protein was harvested, and EMSAs were performed as described under the "Materials and Methods." The arrow indicates the relevant DNA complex. Three independent experiments were performed, and a representative figure is shown. D, IEC4-1 cells were co-transfected with Tβ3-P221/110-Luc and the indicated siRNA plasmids. 24 h after transfection, the cells were incubated in the absence or presence of TGFβ3 (10 ng/ml). The cells were harvested after an additional 24 h, and luciferase assays were performed as described under the "Materials and Methods." Data plotted are the mean ± S.E. of triplicate samples from one of three independent experiments. Asterisks indicate a statistically significant difference (p < 0.05) in the fold changes between the absence and presence of TGFβ3 in the siRNA-transfected IEC4-1 cells compared with siRNA-Ctrl IEC4-1 cells. E, real time RT-PCR was performed as described under the "Materials and Methods." Data plotted are the mean ± S.E. of triplicate samples from one of two independent experiments. Asterisks indicate a statistically significant difference (p < 0.05) of the siRNA-transfected HaCaT cells compared with siRNA-Ctrl HaCaT cells for their fold changes between the absence and presence of TGFβ3. F, quantitative determination of TGFβ3 in CM from IEC4-1 cells and their siRNA transfectants was performed as described under the "Materials and Methods." Results were normalized for cell number, and secreted TGFβ3 was expressed as pg/10^6 cells. Data represent the mean ± S.E. of triplicate wells from a representative experiment (n = 3). Asterisk indicates p < 0.01.
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...either CREB-1 and Smad3 were necessary for TGFβ induction of TGFβ3 expression. We therefore performed real time RT-PCR to examine TGFβ3 mRNA expression levels after knockdown of CREB-1 or Smad3. As shown in Fig. 6E, TGFβ treatment induced a 5.7-fold increase in TGFβ3 mRNA expression in control cells, confirming the presence of an active TGFβ3 auto-loop. Furthermore, siRNA-CREB-1 or siRNA-Smad3 decreased the TGFβ induction of TGFβ3 mRNA expression to levels of only 3.1-fold, a statistically significant decrease compared with that for siRNA-Ctrl ($p < 0.01$). These results indicate that CREB-1 and Smad3 are required for TGFβ induction of TGFβ3 mRNA expression.

siRNA-CREB-1 and siRNA-Smad3 Both Suppress Endogenous TGFβ3 Production—To measure endogenous TGFβ3 production in response to TGFβ3 stimulation, we performed ELISA quantitative analyses on CM from IEC4-1 cells, which produce high levels of TGFβ3 (20). Because the TGFβ3 ELISA is specific for TGFβ3, with no cross-reactivity with TGFβ1 or TGFβ2, we used TGFβ1 to treat the cells and performed ELISAs to evaluate TGFβ3 concentrations in CM collected from IEC4-1 cells. As shown in Fig. 6F, basal TGFβ3 production was reduced in siRNA-CREB-1 transfected IEC4-1 cells. Upon TGFβ1 stimulation, TGFβ3 production in IEC4-1 cells was increased by more than 6-fold. This induction of TGFβ3 by TGFβ1 was significantly suppressed in siRNA-CREB-1 and siRNA-Smad3-transfected IEC4-1 cells. The results are consistent with our previous observations in EMSAs and reporter assays. Furthermore, the results indicate that a TGFβ3 autocrine loop exists in IEC4-1 cells and that both CREB-1 and Smad3 binding at the CRE site of the TGFβ3 promoter are critical for the physiological production of TGFβ3 in these cells.

**Schematic Model for TGFβ3 Autoregulation**—Based upon the results in the preceding figures, we have defined the signal transduction pathways that mediate TGFβ3 autoregulation, specifically focusing on TGFβ3 induction of TGFβ3 secretion, as depicted in the schematic diagram in Fig. 7. According to this model, CREB-1, ATF-2, and c-Jun constitutively bind at the TGFβ3 promoter CRE site. TGFβ activates Smad3 as well as JNKs and p38 through the TGFβ receptors (43–45). JNKs and p38 activate CREB-1 and ATF-2 (45–47), which thereafter bind at the CRE site (−45 to −39) in the TGFβ3 promoter region. Phospho-Smad3 is also bound at this CRE site. Based upon our results, the activation of Smad3, CREB-1, and ATF-2 by TGFβ results in an increase in TGFβ3 transcriptional activity and a subsequent increase in TGFβ3 production. c-Jun is also constitutively bound to this TGFβ3 promoter CRE site. However, the DNA binding activity of c-Jun is not TGFβ-inducible, suggesting that c-Jun may play a role in basal TGFβ3 production in response to stimuli other than TGFβ.

**DISCUSSION**

Although three mammalian isoforms of TGFβ, TGFβ1, TGFβ2, and TGFβ3, share 60–80% identity at the amino acid level, the promoter regions of these isoforms are highly variable, suggesting that their expression is regulated by distinct mechanisms. Our previous results indicated that the proximal AP-1 site in the TGFβ1 promoter region was critical for TGFβ1 auto-induction and that the JNK and ERK cascades, as well as Smad3 and Smad4, were required for TGFβ1 auto-induction in untransformed epithelial cells (19). However, the signaling pathways mediating TGFβ3 regulation of TGFβ3 production have not been investigated previously. In this study, we found that TGFβ3 auto-induction was mediated by the CRE site located between −61 and −35 in the TGFβ3 promoter region and that CREB-1 and Smad3, as well as JNKs and p38, but not p38, were the critical activators of TGFβ3 expression. In addition, we found that CREB-1, ATF-2, and c-Jun are constitutively bound at this CRE site. In contrast, Smad3 binding at the TGFβ3 promoter CRE site was only observed after TGFβ3 stimulation. siRNA-CREB-1 and siRNA-Smad3 significantly inhibited the TGFβ-inducible effects on complex formation at the TGFβ3 promoter CRE, TGFβ3-P221/110-Luc activity, TGFβ3 mRNA expression, and TGFβ3 secretion. Because these data demonstrate that both CREB-1 and Smad3 are critical for mediating TGFβ3 auto-induction, our results provide the first evidence of the physiological relevance of these transcriptional factors, as well as of the CRE site, in mediating this critical biological response to TGFβ. Furthermore, DN RII or DN Smad3, but not DN Smad4, effectively blocked the DNA binding activity induced by TGFβ, confirming that RII and Smad3 are required for this TGFβ3 auto-loop regulation.

In this study, we have shown for the first time that Smad3 does not bind to the SBE site in the TGFβ3-P61/35 region upon TGFβ stimulation, but instead it binds to the CRE site, where...
CREB-1 also binds. Moreover, Smad4 was not present in this binding complex. Because Smad3 knockdown significantly blocked TGFβ induction of Tβ3-P61/35 DNA binding, Tβ3-P221/110-Luc activity, and TGFβ3 secretion, Smad3 is not only present in the complex bound at Tβ3-P61/35 CRE, but it is also functionally required for TGFβ-inducible TGFβ3 secretion. There are several reports that indicate that Smad3, but not Smad4, associates with both the CREB-1-binding protein, as well as the structurally related p300 protein, in response to TGFβ exposure (48–50). In keeping with these previous findings, and our results herein, it is conceivable that Smad3 may actually form a complex with CREB-1 in response to TGFβ and activate TGFβ3 secretion in the absence of Smad4.

We also found that CREB-1 and ATF-2, but not CREB-2, were constitutively bound at the TGFβ3 promoter CRE site. Although CREB-1, CREB-2 (also termed ATF-4), and ATF-2 all belong to the ATF/CREB family, they do not share much similarity other than a basic region-leucine zipper (bZip) motif (51). Our data indicate that CREB1, but not CREB-2, is involved in TGFβ3 secretion mediated by TGFβ3.

It has been shown that the ATF/CREB family of proteins can form heterodimers with AP-1 proteins to activate target gene transcription (51). Similarly, previous reports have indicated that c-Jun can bind to a consensus CRE site. For example, it has been reported that TGFβ3 could stimulate CREB-dependent transcription by increasing the amount of c-Jun present in the CREB-1-binding protein-containing complex bound at a consensus CRE site (52). In addition, overexpression of c-Jun not only activated a reporter gene containing a consensus CRE but also activated a portion of the c-Fos promoter only containing a CRE site (52). Our results also suggest that c-Jun might be involved in TGFβ3 regulation.

It should be pointed out that although Smads are critical for TGFβ signaling, TGFβ3 also stimulates other intracellular signaling pathways, such as JNKs, ERKs, and p38 (19, 43, 44, 53–56). Such MAPks are required for the signaling of TGFβ effects on growth, apoptosis, and gene expression (53–56). MAPks are also kinases that can function upstream of CREB (25). In this study, selective inhibitors of JNKs and p38 effectively blocked DNA binding to the TGFβ3 promoter region Tβ3-P61/35, indicating that JNKs and p38 were required for TGFβ activation of complex formation at this site. Most notably, the selective MEK1 inhibitor failed to suppress the DNA binding to Tβ3-P61/35, although there are reports that ERKs are involved in CREB activation, particularly in specific neuronal cell cultures (26, 31). Although others have shown that JNK and p38 are involved in TGFβ signaling, this is the first report of their requirement in TGFβ3 induction of TGFβ3 gene transcription.

It has been reported that ATF-2, which is known to be a nuclear target of p38 and JNKs, was phosphorylated in the N-terminal activation domain in response to TGFβ (47). In addition, TGFβ3-mediated induction of fibronectin requires activation of JNK, which in turn modulates the activity of ATF-2 and c-Jun (57). Moreover, TGFβ3 can induce the phosphorylation of ATF-2 via p38 and TGFβ3-activated kinase-1 (TAK1), followed by ATF-2 complex formation with Smad3 and Smad4 (58). The binding between ATF-2 and Smad3/4 is mediated via the MH1 domain of the Smad proteins and the basic leucine zipper domain of ATF-2 (58). Furthermore, ATF-2 can cooperate with Smad3 to regulate the rate of chondrocyte maturation in response to TGFβ3 (59). In our report, ATF-2 displayed TGFβ-inducible DNA binding activity at the TGFβ3 promoter CRE site, suggesting that ATF-2 may also play a role in TGFβ3 induction of TGFβ3 production through the signaling pathways described above. Thus, our findings provide evidence of co-regulation by the MAPK and Smad signaling pathway components in mediating this biological response to TGFβ3, similar to the synergistic contribution of these signaling pathways in mediating other TGFβ responses (53, 56, 60).

It is well documented that the autocrine and/or paracrine effects of TGFβ1 play a crucial role in tumor invasion and progression. However, although increased expression of TGFβ3 has been observed in a variety of late stage carcinomas, reports regarding the paracrine effects of TGFβ3 in modulating the tumor microenvironment and the immune system of the host are scarce. It has been reported that expression of TGFβ correlates with the progression of osteosarcomas (61). In addition, TGFβ3 has been shown to contribute to the formation of tumor stroma, induction of angiogenesis, and modulation of extracellular matrix, suggesting that excessive secretion of TGFβ3 by the tumor and/or stromal cells would also foster cancer progression (62). Identification of the factors that are required for TGFβ auto-regulation improves our understanding of the mechanisms underlying TGFβ3 secretion. Our results may enable the design of novel strategies to regulate TGFβ3 secretion in pathological conditions by manipulating factors that are involved in this pathway. For example, blockade of TGFβ3 production in late stage solid cancers using siRNA approaches may prevent the invasiveness and metastatic nature of such cancers. Future studies can address such potential therapeutic strategies.

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