Convergence of p53 and Transforming Growth Factor β (TGFβ) Signaling on Activating Expression of the Tumor Suppressor Gene maspin in Mammary Epithelial Cells*

Using two-dimensional difference gel electrophoresis, we identified the tumor suppressor gene maspin as a transforming growth factor β (TGFβ) target gene in human mammary epithelial cells. TGFβ up-regulates Maspin expression both at the RNA and protein levels. This up-regulation required Smad2/3 function and intact p53-binding elements in the Maspin promoter. DNA affinity immunoblot and chromatin immunoprecipitation revealed the presence of both Smads and p53 at the Maspin promoter in TGFβ-treated cells, suggesting that both transcription factors cooperate to induce Maspin transcription. TGFβ did not activate Maspin-luciferase reporter in p53-mutant MDA-MB-231 breast cancer cells, which exhibit methylation of the endogenous Maspin promoter. Expression of ectopic p53, however, restored ligand-induced association of Smad2/3 with a transfected Maspin promoter. Stable transfection of Maspin inhibited basal and TGFβ-stimulated MDA-MB-231 cell motility. Finally, knockdown of endogenous Maspin in p53 wild-type MCF10A/HER2 cells enhanced basal and TGFβ-stimulated motility. Taken together, these data support cooperation between the p53 and TGFβ tumor suppressor pathways in the induction of Maspin expression, thus leading to inhibition of cell migration.

Members of the transforming growth factor β (TGFβ) family play an important role in the regulation of cellular fate both in embryonic development and adult tissue homeostasis (1). It is generally accepted that they have both tumor suppressor and tumor promoter functions (reviewed in Refs. 2 and 3). The TGFβ ligands bind to cognate serine/threonine kinase transmembrane receptors, which, in turn, phosphorylate and activate the Smad family of signal transducers. Once activated, Smad2 and Smad3 translocate to the nucleus where they control gene expression in association with Smad4 and transcriptional coregulators (4). The intrinsic DNA binding activity of Smads is of relative low affinity and specificity. While this activity is sufficient to drive Smad-regulated transcription of artificial reporter constructs, tissue-specific regulation of transcription by Smads in vivo is thought to depend on interactions with additional site-specific DNA binding factors. The most studied Smad-dependent transcriptional responses are those involved in cell cycle arrest and apoptosis, essential for the tumor suppressor role of the TGFβs. Mutational inactivation or loss of TGFβ receptors and/or Smads is permissive for epithelial cell transformation and carcinogenesis (1, 2). On the other hand, introduction of dominant negative TGFβ receptors into metastatic cancer cells inhibits epithelial-to-mesenchymal transdifferentiation, motility, invasiveness, and survival, supporting the tumor promoter role in TGFβ in fully transformed cells (reviewed in Ref. 5). In addition, excess production and/or activation of TGFβ by cancer cells contributes to tumor progression by paracrine mechanisms that modulate the tumor microenvironment (6).

Most carcinomas attenuate or lose the Smad-dependent anti-mitogenic effect but gain pro-metastatic abilities in response to TGFβ. In most tumors, this change occurs without acquiring genetic defects involving Smads or TGFβ receptors, suggesting that alterations in other regulatory molecules can have a profound influence of the cellular response to TGFβ. One possible explanation is the presence of different Smad partners in different cell types, which converge with Smads at the level of target gene expression, thus modifying the biological output of TGFβ signaling.

TGFβ signaling synergizes with transformation induced by ErbB receptor tyrosine kinases. For example, overexpression of active TGFβ1 or active mutants of the type I TGFβ receptor (ALK5) in the mammary gland of transgenic mice that also express MMTV/Neu (ErbB2) accelerates mammary tumor metastases (7–9). Treatment with exogenous TGFβ or transduction of TGFβ1 or TGFβ3 into non-tumorigenic human...
mammary epithelial cells transfected with HER2 (ErbB2) induces cell motility and invasion (10, 11).

In an effort to understand the molecular mediators of cross-talk between TGFβ2 and ErbB receptor signaling, we performed two-dimensional multi-variable difference gel electrophoresis (DIGE) coupled with mass spectrometry in MCF10A/HER2 cells treated with TGFβ2. In these cells, TGFβ2 induces signaling programs associated with enhanced motility and survival (12, 13). One of the proteins induced by TGFβ2 was Maspin, a tumor suppressor related to the serpin (serine protein inhibitor) family of protease inhibitors (14). Maspin was equally induced in MCF10A cells not overexpressing HER2 (controls), suggesting it was not involved in the transformed phenotype induced by TGFβ2 in cells overexpressing the oncogene. Indeed, knock-down of Maspin with RNA interference in MCF10A/HER2 cells was permissive for TGFβ2-induced motility, suggesting Maspin had retained its tumor suppressor function in these cells. Because of the known regulation of maspin gene transcription by members of the p53 family (15, 16), we examined the coregulation of Maspin expression by p53 and Smads. Thus representing a point of convergence of two tumor suppressor pathways.

**EXPERIMENTAL PROCEDURES**

**Cells, Plasmids, Viruses, and shRNA**—MCF10A and MCF10A/HER2 cells were generated and grown as described (11). MDA-MB-231 and Phoenix-Ampho cells were grown in Dulbecco’s modified Eagle’s medium (Cambrex) containing 10% fetal bovine serum (Hyclone) in a humidified 5% CO2 incubator at 37 °C. NMuMG cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 10 μg/ml insulin. Human primary mammary epithelial cells (HMECs) were provided by Dr. Jennifer Pietenpol (Vanderbilt University) and cultured in supplemented mammary epithelium basal medium (Cambrex). The following reagents were used: human recombinant TGFβ1 (R&D Systems), puromycin (Calbiochem), and G418 (Research Products International Corp.). Maspin promoter-luciferase reporter plasmids (−154), (−154mtp53-II), (−154mtp53-IIS), (−154mt-SBE-I), (−116), (−116mtp53-I), (−116mt-SBE-I), and (−116mt-SBE-I) were generated by PCR using pm-Luc (−759) (16) as a template and are summarized in Fig. 4B. Primers used to introduce point mutations are: 5’-ctggtagctgctATAGATcgtag (for −154mtp53-II), 5’-ctagctgatATAGATcgtagtag (for −154mtp53-IIS), 5’-cta-
matched nucleotides compared with the human maspin gene are shown in caps) were annealed and inserted into the same vector. The resulting plasmids were transplanted into Phoenix-Ampho cells (19) to produce human and mouse si-msp virions, which were harvested 48 h after transfection. Human Maspin siRNA (si-msp) or mouse Maspin siRNA (control siRNA) were used to infect cells. Stably transduced colonies were selected in puromycin (1 μg/ml).

**Two-dimensional DIGE Mass Spectrometry (MS)---** MCF10A/HER2 cells were treated with TGFβ for different times (0, 8, 24, and 40 h), washed with phosphate-buffered saline, and harvested with ice-cold Nonidet P-40 lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.1 mM EDTA, plus protease and phosphatase inhibitors). After sonication for 10 s and centrifugation (14,000 rpm), protein concentration in the supernatants was measured using the BCA protein assay reagent (Pierce). The treatment timecourse was repeated independently in triplicate, and the global protein expression profiles of over 1,000 resolved proteins (including charged isofoms and processed proteins) from the resulting 12 samples were quantitatively analyzed and identified using minimal labeling two-dimensional DIGE and MS as described (20, 21). The resulting mass spectral data were used to interrogate sequences present in the Swiss-Prot and NCBI databases to generate statistically significant candidate identifications using GPS Explorer software (Applied Biosystems) running the MASCOT search algorithm. Maspin was identified from 16 peptide ions, 8 of which provided independent MS/MS matches to provide a combined MOWSE score of 591 and 49% sequence coverage (21).

**Extraction of Total RNA and RT-PCR---** Total RNA was extracted using the RNasy mini-kit (Qiagen). RT-PCR was carried out using the titanium one-step RT-PCR kit (BD Biosciences). For each RT-PCR reaction, 100 ng RNA were added to a 50-μl reaction system according to the manufacturer’s protocol (50 °C for 1 h followed by 30 cycles of PCR amplification) using the same primers for Maspin cDNA cloning. The PCR products were analyzed in 1.2% agarose gels.

**Transfection and Luciferase Reporter Assays---** Cells were seeded in 6-well plates and transfected with 0.5 μg of luciferase reporter plasmid with or without 1 μg of p53 expressing plasmid pCEP4-p53 or empty vector, along with 0.01 μg of pCMV-Renilla using FuGENE 6 according to the manufacturer’s protocol. Firefly and Renilla reniformis luciferase activities were measured using the dual luciferase assay system (Promega) as reported previously (22).

**DNA Affinity Immunoblotting (DAI) and Chromatin Immunoprecipitation (ChIP)---** DAI and ChIP assays were performed as described (12). For DAI assay, the wild-type Maspin promoter DNA and a promoter with a mutation in its p53 site I were released from pm-Luc (−297) and pm-Luc (−297mt1), respectively, by restriction enzyme digestion and subsequently labeled with biotin. Labeled probes were incubated with 200 μg of nuclear extract prepared from cells that had been treated or not with TGFβ. Streptavidin magnetic beads (0.1 mg) were added for 1 h and then washed with DNA-binding buffer 5 times, boiled in 20 μl of 2× SDS-gel loading buffer before separation by SDS-PAGE followed by immunoblot. For ChIP

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**FIGURE 2. TGFβ up-regulates Maspin expression via the Smad pathway.** MCF10A cells growing on 6-well plates were infected with adenoviruses encoding Smad7 or β-Gal (multiplicity of infection: 15). Twenty-four h after infection, cells were treated with TGFβ, harvested at the indicated times, and then subjected to immunoblot analysis with the indicated antibodies (A) or RNA extraction and RT-PCR (B) as indicated under “Experimental Procedures.” Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified and used as an internal control in the RT-PCR. The protein and cDNA bands of Maspin were quantified and shown as their ratio to untreated control samples (below first row in both A and B).
FIGURE 3. p53 and Smads cooperate to activate Maspin transcription. A, 293 cells were transiently transfected with Maspin promoter luciferase reporters and 8 h later treated or not with TGFβ. Twenty-four h after the addition of TGFβ, the cells were harvested and tested for luciferase expression as indicated under “Experimental Procedures.” Each bar represents the mean normalized luciferase activity ± S.D. calculated from three wells. The TGFβ-responsive p3TP-Luc reporter was used as a control. B, for DAI assay, biotin-labeled Maspin promoter (lanes 1–4) or biotin-labeled Maspin promoter with a mutation at the p53-binding site (p53-I; lanes 5–8) were incubated with nuclear extracts from MCF10A cells that had been treated or not with TGFβ for 1 h. Where indicated, a 10-fold excess of unlabeled (CAGA)12 competitor DNA (lanes 3, 4, 7, and 8) was added to the incubation mix. Input nuclear extracts were also loaded (lanes 9 and 10). The presence of Smads and p53 on Maspin promoter DNA was detected in Streptavidin pulldowns subjected to immunoblot analysis using specific antibodies. A c-Jun antibody was used as a negative control. C, MCF10A cells were treated with TGFβ at the indicated times prior to ChIP. After precipitation with the antibodies indicated to the left, primers encompassing Maspin promoter region from −514 to +87 were used for PCR amplification. The c-Jun antibody was used as a negative control. To control for equal chromatin input, cell lysates before immunoprecipitation were used as templates for PCR (top four rows). When primers encompassing a 194-bp Maspin coding region were used for PCR, an amplified PCR product was not detectable in any of the ChIP samples (bottom three rows).

Coat growth factor–reduced Matrigel invasion chambers (BD Biosciences) according to the manufacturer’s protocol.

Indirect Immunofluorescence Assay (IFA)—Cell IFA were performed as described previously (24). Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axioshot upright microscope.

RESULTS

Characterization of maspin as a TGFβ Target Gene—We used two-dimensional DIGE to identify TGFβ-induced changes in protein abundance in MCF10A/HER2 human mammary epithelial cells (21). Two species of Maspin resolved by isoelectric focusing, i.e. the acidic and basic forms, were found as proteins regulated by TGFβ. Both forms of Maspin, which may indicate post-translational modifications, were upregulated by TGFβ treatment starting at 8 h and continuing to increase at 24 and 40 h. At 40 h, there was a 65% (1.65-fold average increase; $p = 0.003$) and 67% (1.67-fold average increase; $n = 3, p = 0.0069$) increase in the acidic and basic forms of Maspin, respectively (Fig. 1A). This time-dependent up-regulation was confirmed in immunoblots of MCF10A cells.
TGFβ Regulates maspin Expression through p53

FIGURE 4. Mutations of p53 sites in the Maspin promoter impair TGFβ-induced transcription. A, sequence of the Maspin promoter region from −297 to +87. Potential SBEs are marked with the solid line boxes; the two p53-binding sites p53-I (reported by Zou et al. (16)) and p53-II (first identified in this paper) are indicated by the dotted lines, B, EMSA showing that p53 and Smads bind to the p53-II/SBE-II site in the Maspin promoter. Annealed p53-II, mtp53-II, and mtp53-III probes were end-labeled with [32P]ATP and incubated with the nuclear extracts of MCF10A cells (−TGFβ for 1 h). No nuclear extract was added in the negative control (lanes 1, 6, and 9). Antibody against p53 or Smad4 was used to generate the supershifted bands (lanes 4 and 5), specific band; SS, supershifted band. C, schematic representations of the Maspin promoter luciferase reporters and their response to TGFβ. All of the Maspin promoter reporters have the common 3′ ends (−87) and different truncated 5′ ends. MCF10A cells were transiently transfected with Maspin promoter reporters and 8 h later treated or not with TGFβ. At 24 h, the cells were harvested and tested for luciferase reporter activity as indicated under “Experimental Procedures.” Each bar represents the mean normalized luciferase activity ± S.D. calculated from three wells.

stably transfected with HER2 or vector alone (Fig. 1B), as well as in HMECs and NMuMG mouse mammary epithelial cells (Fig. 1C).

TGFβ Up-regulates Maspin Expression via Smad Pathway—To determine whether Smad signaling is required for TGFβ-induced Maspin expression, we tested this induction over a time course in MCF10A cells infected with an adenovirus encoding the inhibitory Smad7 or β-galactosidase. Expression of Smad7 abolished TGFβ-induced Smad2 phosphorylation and attenuated the induction of Maspin protein (Fig. 2A). At the mRNA level, induction of Maspin mRNA by TGFβ occurred as early as 1 h, and this was also attenuated by forced expression of Smad7 (Fig. 2B).

p53 and Smads Cooperate at the Maspin Promoter to Activate maspin Transcription—It has been reported that the tumor suppressor p53 can physically interact with Smad2 and therefore plays a key role in the cellular response to TGFβ (25). Since the Maspin promoter contains a p53-binding site that can be transactivated by p53 (16), we speculated that TGFβ activates maspin transcription by inducing an interaction between Smads and p53 at the Maspin promoter. To test this, we transiently transfected 293 cells with luciferase reporter genes driven by various Maspin promoter regions (Fig. 3A). Reporters containing Maspin promoter regions starting from −759 and −297 were activated by TGFβ−8-fold. However, a reporter containing a mutation in the p53-binding site (designated as p53-I) exhibited a markedly impaired response to TGFβ (Fig. 3A), suggesting that the p53-I site is required for TGFβ-induced maspin gene transcription.

We next confirmed the interaction between Maspin promoter regions and Smads by DAI using biotin-labeled Maspin promoters released from pm-Luc(−297) and pm-Luc(−297/mtp53) containing a p53 wild-type (wt)-binding site or a mutation at this site (p53-I), respectively. After incubating the labeled probes with nuclear extracts from MCF10A cells, the wt Maspin promoter showed increased binding to Smads and p53 after treatment with TGFβ and p53 after treatment with TGFβ (Fig. 3B). TGFβ did not affect p53 levels in nuclear extracts (Fig. 3B, lanes 9 and 10). This suggests that TGFβ increases p53 binding to the Maspin promoter by increasing the binding of Smads to this same promoter. Consistent with this speculation, addition of unlabeled competitor DNA, (CAGA)12, which specifically binds to Smads, decreased both Smads and p53 binding. Compared with the wt Maspin promoter, less p53 and Smads were bound to the probe mutated in the p53-I site, suggesting that binding of p53 to this site may also stabilize Smads binding. Interestingly, the mutation in the p53-I site decreased but did not completely abolish p53 binding to the Maspin promoter (Fig. 3B, lanes 5 and 6). This suggested that other p53-binding sites, besides p53-I, may be present in Maspin promoter. Indeed, a second p53-binding site (designated as p53-II) was found in subsequent experiments (Fig. 4).

Finally, to prove that TGFβ induces binding of p53 to the Maspin promoter in vivo through Smads, ChIP over a time course was performed. TGFβ induced binding of p53 to the Maspin promoter as early as 15 min reaching a maximum at 30 min and 1 h. Detectable maximal binding of Smads to this promoter, as indicated by recovery of the Maspin promoter PCR product in Smad2/3 antibody pulldowns, reached a maximum at 1 h after treatment with exogenous ligand (Fig. 3C, top two rows). When primers encompassing a 194-bp Maspin coding region were used for PCR, none of the ChIP samples gave amplified bands (Fig. 3C, bottom panel).
TGFβ Regulates maspin Expression through p53

Mutations of p53 Sites in the maspin Promoter Impair TGFβ-induced Transcription—By sequence analysis we identified four potential SBEs and two p53-binding sites in the Maspin promoter region from −297 to +87 (Fig. 4A). To determine how these sites are involved in p53-mediated induction of Maspin by TGFβ, we constructed a series of Maspin promoter-luciferase reporters with various truncated 5′ ends and point mutations to eliminate the p53 and SBEs (Fig. 4C). The function of the new p53 site (p53-II) that overlaps with the SBE-I sites was confirmed by EMSA and transient transfection assays. Incubation of a [32P]-labeled p53-II probe with nuclear extracts from TGFβ-treated MCF10A cells resulted in the formation of a shifted band which underwent supershift upon the addition of p53 or Smad4 antibodies, suggesting that both p53 and Smads directly bind to this promoter site (Fig. 4B, lanes 1–5). In contrast, no shifted bands were observed when probe mtp53-II, with mutations at both p53-II and SBE-II sites, or probe mtp53-IIS carrying mutant p53-II but wild-type SBE-II were used (Fig. 4B, lanes 6–11).

In cells co-transfected with Maspin reporters and a plasmid encoding p53 cDNA, mutation of the p53-II/SBE-II site (−154mtp53-II) or p53-II site alone (−154mtp53-IIS) resulted in 60–75% reduction of p53-induced reporter activity (Fig. 4D). Transfection of a Maspin promoter construct with a mutation in the p53-I site (−297mtp53-I compared with −297; shown in Fig. 4C) into MCF10A cells partially abrogated TGFβ-mediated induction (from 6.7- to 4.0-fold; Fig. 4C), consistent with the experiment shown in Fig. 3A in 293 cells. Mutation of p53-II/SBE-II site (−154mtp53-II compared with −154) also reduced the response to TGFβ from 6.9- to 3.2-fold (Fig. 4C). The mutant reporter pm-Luc (−154mtp53-IIS) with mutant p53-II but wt SBE-II also exhibited a lower response (3.1-fold) to TGFβ, suggesting that p53 binding to this site is required. Deletion of the two distal SBEs (−154 compared with −297) did not impair the promoter response to TGFβ, whereas the two proximal SBEs, one overlapping with p53-II and the other one adjacent to p53-I, were required for Maspin reporter transcription. Mutation or deletion of either SBE-I or SBE-II (−154mtSBE-I, −116, −116mtSBE-I) also impaired the response to TGFβ (Fig. 4C).

TGFβ Fails to Activate Maspin Promoter in p53-mutant Cells—MDA-MB-231 human breast cancer cells harbor a point mutation in codon 280 (G/A) of the p53 gene. We transiently transfected these cells with a Maspin promoter luciferase reporter. Treatment with TGFβ did not induce Maspin promoter activity but still induced Smad reporter p(CAGA)12-Luc (Fig. 5A). Co-transfection with a wt p53 plasmid restored TGFβ-induced Maspin promoter activity (Fig. 5A), suggesting that p53 function is necessary for the induction of Maspin transcription by TGFβ. ChIP in cells transfected with a Maspin promoter and wt p53 showed that both p53 and Smads bound to the Maspin promoter upon treatment with TGFβ.

Due to the p53 mutation and the reported methylation of endogenous Maspin promoter in MDA-MB-231 (26), detectable Maspin protein levels are very low in these cells and are not up-regulated by TGFβ (Fig. 5C, lanes 1 and 2). To determine the possible significance of low Maspin expression, we stably transfected a Maspin-encoding plasmid into MDA-MB-231 (26), together with 1 μg of wt p53 plasmid pCEP4-P53 or empty vector DNA, and 8 h later treated or not with TGFβ. The Smad reporter plasmid p(CAGA)12-Luc was used as a positive control. After 24 h, the cells were harvested and then tested in a luciferase reporter assay. Each bar represents the mean normalized luciferase activity ± S.D. calculated from 3 wells. B, MDA-MB-231 cells grown on 100-mm dishes were co-transfected with 2 μg of pm-Luc (−759) and 5 μg of pCEP4-P53 (lanes 4–6) or empty vector DNA (lanes 1–3). Cells were treated with TGFβ for 0 min, 30 min, or 1 h prior to ChIP. DNA-protein complexes were precipitated with the antibodies indicated to the left of each panel followed by PCR amplification using primers specific to the maspin promoter as indicated under “Experimental Procedures.” To control for chromatin input, whole cell lysates (before the antibody pulldown) were used as templates for the PCR (bottom row). C, MDA-MB-231 cells stably transfected with Maspin or control vector were treated or not with TGFβ for 24 h. Cell lysates were separated by SDS-PAGE followed by immunoblot analysis with the indicated antibodies. D, close-to-confluent monolayers of MDA-MB-231 cells stably expressing Maspin or control vector were serum-starved for 24 h before wounding. Serum-free medium ± TGFβ was replenished and wound closure was followed 20 h later as indicated under “Experimental Procedures.” E, MDA-MB-231 cells stably expressing maspin or control vector were seeded (2.5 × 10⁴ cells/well) on Matrigel-coated transwells and allowed to invade toward growth medium ± TGFβ. At 24 h, the cells invading into the Matrigel were counted; each bar represents the mean ± S.D. of three wells.
Knockdown of Maspin Facilitates TGFβ-induced motility—We have previously reported that TGFβ induces the motility of MCF10A/HER2 cells via PI3K-dependent activation of the Rac GTPase and its substrate Pak1 (11, 13). Using siRNA against human maspin gene, we knocked down both basal and TGFβ-induced Maspin expression in MCF10A/HER2 cells (Fig. 6A). In wound closure assays, TGFβ-induced motility was accelerated in cells expressing Maspin siRNA compared with serum-starved cells expressing control siRNA (Fig. 6B). Meanwhile, the number of focal adhesions was significantly decreased in cells expressing Maspin siRNA as detected by immunofluorescence using an antibody against Vinculin (Fig. 6C). In addition, both basal and TGFβ-induced cell invasion in Matrigel were enhanced in cells expressing Maspin siRNA (Fig. 6D). This result further suggests that low levels or absence of the Maspin tumor suppressor protein enhance the tumor promoting effects of TGFβ in transformed cells.

DISCUSSION

We performed two-dimensional DIGE in MCF10A mammary epithelial cells overexpressing HER2. Treatment with TGFβ resulted in a time-dependent change in the abundance of over 60 protein species. Among these, two forms of Maspin protein with the same molecular weight but slight variations in their isoelectric points (pI), were characterized. The maspin gene encodes a protein related to the serpin (serine protease inhibitor) family of protease inhibitors. It was first identified as a tumor suppressor gene with a potential role in human breast cancer (14). Maspin protein is expressed in non-tumor mammary epithelial cells but is absent in most breast cancer cell lines (14). In MMTV/TGFα transgenic mice, Maspin expression inversely correlates with mammary tumor progression (27). Restoration of Maspin protein expression in MDA-MB-435 breast cancer cells reduces the abilities of these cells to form tumors and metastasize in nude mice (14). Other studies have shown that Maspin acts at the cell membrane to inhibit the invasion and motility of prostate and breast cancer cell lines (28). Treatment of breast cancer cells with exogenous Maspin increases adhesion to fibronectin and inhibits Rac GTPase activity (29). Maspin can be tyrosine-phosphorylated in both normal and tumor mammary cells; recombinant Maspin protein can be phosphorylated by the epidermal growth factor receptor tyrosine kinase in vitro (30), although the biological significance of this modification is unknown. Interestingly, another serpin family member, plasminogen-activator inhibitor (PAI)-1, is strongly induced by TGFβ. In contrast to Maspin, however, both PAI-1 and PAI-2 are overexpressed in malignant tumors (28, 31–33).

Two major mechanisms regulate Maspin expression: transcription by p53 family members and promoter methylation. Adenovirus-encoded wild-type p53 strongly up-regulates Maspin gene transcription and protein levels in breast and prostate cancer cells as a result of binding of p53 to a p53 consensus site (i.e. the p53-I site shown in Fig. 4A) in the Maspin promoter (16). Consistent with this finding, tissue microarray analyses have shown that Maspin expression is down-regulated in cancers with a high histological grade and low expression correlates with presence of p53 mutations in various tumor types (34). Interestingly, Maspin is overexpressed in lung cancer, suggesting it may have a role other than tumor suppressor (35). p63, a homolog of p53, transactivates the Maspin promoter via directly binding to the p53 site in cancer cells (15). In addition, aberrant cytosine methylation of the Maspin promoter resulting in gene silencing has been found in mammary cell lines including MDA-MB-231 cancer cells but not in the non-tumorigenic MCF10A cells (26). Therefore, p53 mutation and promoter methylation can cooperate to silence Maspin expression in cancer cells. Finally, transactivation through an Ets and Ap1 sites as well as transcriptional repression through a negative hormonal responsive element recognized by the androgen receptor are also found in the Maspin promoter (36,
TGFβ Regulates maspin Expression through p53

37), providing additional mechanisms to regulate Maspin expression.

In this study we show that 1) TGFβ induces Maspin expression through Smads, and 2) wt p53 binding to the Maspin promoter is required for TGFβ-induced transcription. p53 promotes the activation of other TGFβ target genes during Xenopus embryonic development. p53-deficient mammalian cells display impaired responses to TGFβ including the induction of p21WAF1, PAI-1, and MMP2 as well as growth arrest (25). Based on these findings, a model for cooperation between p53 and Smads has been proposed: p53 associates with Smad2/3 and Smads has been proposed: p53 associates with Smad2/3 (25). Based on these findings, a model for cooperation between p53 and Smads to facilitate and/or enhance TGFβ-induced transcription of (25, 38). In this model, Smad2/3 enter the nucleus where they associate with Smad4 and specific cofactors to bind target sequences at gene promoters. The presence of a nearby p53-binding site and p53 binding to DNA are both necessary for the synergistic transcriptional activation by p53 and Smads. The convergence between p53 and TGFβ signaling can also co-repress target gene expression such as α-fetoprotein. The α-fetoprotein promoter contains an overlapping Smad binding and p53 regulatory element (SBE/p53RE), where p53 and Smads cooperate to induce histone deacetylation and DNA methylation to inhibit TGFβ-induced promoter activity (39).

We identified two p53-binding sites in the human Maspin promoter, both required for full response to TGFβ stimulation. The previously reported site p53-I is only 9 bp away from a downstream SBE, which when mutated will impair the response to TGFβ (as in p53-Luc(−154mtSBE-I) and pM-Luc(−116mtSBE-I); Fig. 4C). A new p53 site identified in this study, designated as p53-II, also overlaps with an SBE and, therefore, allowed the p53:Smad2 interaction. ChIP in MCF10A cells indicated rapid recruitment of p53 and Smads to the Maspin promoter as early as 15 min after the addition of TGFβ, with p53 reaching maximal binding at 30 min and Smad2/3 at 1 h (Fig. 3C). Interestingly, the amount of p53 that binds to the Maspin promoter was almost undetectable 4 h after the addition of TGFβ (Fig. 3); this was followed by a second increase at 8 h. This second increase in promoter binding was not observed for Smad2/3, suggesting the possibility of another TGFβ-induced signaling pathway contributing to this secondary, p53-dependent activation of Maspin transcription.

The results herein suggest that p53 is required for the cellular response to TGFβ. Data in support of the opposite have also been reported. For example, TGFβ depletion results in decreased p53 phosphorylation in Ser-18 and an altered cellular response to DNA damage (40), implying that TGFβ maybe essential for p53-mediated cell fate decisions in situ. Thus, we speculate that convergence between p53 and TGFβ signaling on the co-regulation of gene expression, such as maspin in this report, may represent a common pattern of cooperation between two tumor suppressive pathways that affect a broader set of targets. These gene targets may not only be related to an antiproliferative response but also, in the case of Maspin, to the suppression of cell migration and invasion, thus linking the tumor suppressor function of p53 with TGFβ-regulated extra-cellular cues at the cell surface during transformation.

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REFERENCES

1. Massague, J., Blain, S. W., and Lo, R. S. (2000) Cell 103, 295–309
2. Siegel, P. M., and Massague, J. (2003) Nat. Rev. Cancer 3, 807–820
3. Wakefield, L. M., and Roberts, A. B. (2002) Curr. Opin. Genet. Dev. 12, 22–29
4. Massague, J., Seoane, J., and Wotton, D. (2005) Genes Dev. 19, 2783–2810
5. Arteaga, C. L. (2006) Curr. Opin. Genet. Dev. 16, 30–37
6. Bierie, B., and Moses, H. L. (2006) Nat. Rev. Cancer 6, 506–520
7. Siegel, P. M., Shu, W., Cardiff, R. D., Muller, W. J., and Massague, J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8430–8435
8. Muraoka, R. S., Koh, Y., Roebeck, L. R., Sanders, M. E., Brantley-Sieders, D., Gorska, A. E., Moses, H. L., and Arteaga, C. L. (2003) Mol. Cell. Biol. 23, 8691–8703
9. Muraoka-Cook, R. S., Shin, I., Yi, J. Y., Easterly, E., Barcellos-Hoff, M. H., Yingling, J. M., Zent, R., and Arteaga, C. L. (2005) Oncogene 24, 3408–3423
10. Seton-Rogers, S. E., Lu, Y., Hines, L. M., Koundinya, M., LaBaer, J., Muthuswamy, S. K., and Brugge, J. S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 1257–1262
11. Ueda, Y., Wang, S., Dumont, N., Yi, J. Y., Koh, Y., and Arteaga, C. L. (2004) J. Biol. Chem. 279, 24505–24513
12. Wang, S. E., Wu, F. Y., Shin, I., Qu, S., and Arteaga, C. L. (2005) Mol. Cell. Biol. 25, 4703–4715
13. Wang, S. E., Shin, I., Wu, F. Y., Friedman, D. B., and Arteaga, C. L. (2006) Cancer Res. 66, 9591–9600
14. Zou, S., Anisowicz, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. (1994) Science 263, 526–529
15. Kim, S., Han, J., and Park, C. (2004) Cancer Res. 64, 6900–6905
16. Zou, S., Gao, C., Nagaich, A. K., Connell, T., Saito, S., Moul, J. W., Seth, P., Appella, E., and Srivastava, S. (2000) J. Biol. Chem. 275, 6505–6504
17. Fujii, M., Takeda, K., Imamura, T., Aoki, H., Sampath, T. K., Enomoto, S., Kawabata, M., Kato, M., Ichijo, H., and Miyazono, K. (1999) Mol. Biol. Cell 10, 3801–3813
18. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) Science 296, 550–553
19. Oliff, A., McKinney, M. D., and Agranovsky, O. (1985) J. Virol. 54, 864–868
20. Friedman, D. B., Stauff, D. L., Pichchany, G., Whitwell, C. W., Torres, V. J., and Skaar, E. P. (2006) PLoS Pathogens 2, 777–789
21. Friedman, D. B., Wang, S. E., Whitwell, C. W., Capriolo, R. M., and Arteaga, C. L. (2007) Mol. Cell. Proteomics 6, 150–169
22. Dumont, N., Bakin, A. V., and Arteaga, C. L. (2003) J. Biol. Chem. 278, 3275–3285
23. Wang, S. E., Wu, F. Y., Fujimuro, M., Zong, J., Hayward, S. D., and Hayward, G. S. (2003) J. Virol. 77, 600–623
24. Bakin, A. V., Rinehart, C., Tomlinson, A. K., and Arteaga, C. L. (2002) J. Cell Sci. 115, 3193–3206
25. Cordenonisi, M., Dumont, S., Maretto, S., Insigna, A., Imbriano, C., and Picollo, S. (2003) Cell 113, 301–314
26. Oshiro, M. M., Watts, G. S., Wozniak, R. J., Junk, D. J., Munoz-Rodriguez, J. L., Domann, F. E., and Futschek, B. W. (2003) Oncogene 22, 3624–3634
27. Reddy, K. B., McGowen, R., Schuger, L., Visscher, D., and Sheng, S. (2001) Oncogene 20, 6538–6543
28. Sheng, S., Carey, J., Seftor, E. A., Dias, L., Hendrix, M. J., and Sager, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11669–11674
29. Odero-Marah, V. A., Khalikai-Ellis, Z., Chunhafong, A., Amir, S., Seftor,
30. Odaro-Marah, V. A., Khalkhali-Ellis, Z., Schneider, G. B., Seftor, E. A., Seftor, R. E., Koland, J. G., and Hendrix, M. J. (2002) Biochem. Biophys. Res. Commun. 295, 800–805
31. Foekens, J. A., Schmitt, M., van Putten, W. L., Peters, H. A., Kramer, M. D., Janicke, F., and Klijn, J. G. (1994) J. Clin. Oncol. 12, 1648–1658
32. Foekens, J. A., Buessecker, F., Peters, H. A., Krainick, U., van Putten, W. L., Look, M. P., Klijn, J. G., and Kramer, M. D. (1995) Cancer Res. 55, 1423–1427
33. Hamilton, J. A., Wojta, J., Gallichio, M., McGrath, K., and Filonzi, E. L. (1993) J. Immunol. 151, 5154–5161
34. Zhang, W., and Zhang, M. (2002) Int. J. Oncol. 20, 1145–1150
35. Smith, S. L., Watson, S. G., Ratschiller, D., Gugger, M., Betticher, D. C., and Heighway, J. (2003) Oncogene 22, 8677–8687
36. Zhang, M., Magit, D., and Sager, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5673–5678
37. Zhang, M., Maass, N., Magit, D., and Sager, R. (1997) Cell Growth Differ. 8, 179–186
38. Dupont, S., Zacchigna, L., Adorno, M., Soligo, S., Volpin, D., Piccolo, S., and Cordenonsi, M. (2004) Cancer Lett. 213, 129–138
39. Wilkinson, D. S., Ogden, S. K., Stratton, S. A., Piechan, J. L., Nguyen, T. T., Smulian, G. A., and Barton, M. C. (2005) Mol. Cell. Biol. 25, 1200–1212
40. Ewan, K. B., Henshall-Powell, R. L., Ravani, S. A., Pajares, M. J., Arteaga, C., Warters, R., Akhurst, R. J., and Barcellos-Hoff, M. H. (2002) Cancer Res. 62, 5627–5631

TGFβ Regulates maspin Expression through p53