Mutant p53 Promotes Tumor Cell Malignancy by Both Positive and Negative Regulation of the Transforming Growth Factor β (TGF-β) Pathway*

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Background: The molecular mechanisms by which mutant p53 deregulates the TGF-β pathway remain obscure.

Results: Mutant p53 disrupts the Smad3/Smad4 complex by occupying the MH2 domain in Smad3 upon ERK activation.

Conclusion: Mutant p53 achieves gain of function by attenuating and cooperating with the TGF-β pathway via targeting Smad3.

Significance: We discovered a new mechanistic mode explaining the cross-talks among the mutant p53, TGF-β, and ERK pathways.

Specific p53 mutations abrogate tumor-suppressive functions by gaining new abilities to promote tumorigenesis. Inactivation of p53 is known to distort TGF-β signaling, which paradoxically displays both tumor-suppressive and pro-oncogenic functions. The molecular mechanisms of how mutant p53 simultaneously antagonizes the tumor-suppressive and synergizes the tumor-promoting function of the TGF-β pathway remain elusive. Here we demonstrate that mutant p53 differentially regulates subsets of TGF-β target genes by enhanced binding to the MH2 domain in Smad3 upon the integration of ERK signaling, therefore disrupting Smad3/Smad4 complex formation. Silencing Smad2, inhibition of ERK, or introducing a phosphorylation-defective mutation at Ser-392 in p53 abrogates the R175H mutant p53-dependent regulation of these TGF-β target genes. Our study shows a mechanism to reconcile the seemingly contradictory observations that mutant p53 can both attenuate and cooperate with the TGF-β pathway to promote cancer cell malignancy in the same cell type.

Wild-type p53, a well known tumor suppressor gene, is activated in response to oncogenic stress to prevent cancer development (1). The p53 gene is mutated in around 50% of all human cancers, and more than 75% of p53 alterations are missense mutations that lead to the synthesis of a stable but functionally aberrant protein (2–4). Mainly clustered at six hot spots, p53 mutations occur with unusually high frequency and result in loss of DNA binding. Most p53 mutants fall into two subclasses, DNA contact mutants and DNA-binding domain structural mutants, exemplified by R273H and R175H, respectively (5). It has been suggested that mutant p53 exerts its influence by either a dominant-negative or a “gain of function” effect on cells. The dominant-negative effect is well supported by the observation that mutant p53 oligomerizes with wild-type p53 and inhibits its function (6). The gain of function hypothesis states that mutant p53 not only simply loses wild-type p53 function but gains multiple new activities, contributing to tumorigenesis (7, 8). Recent years, compelling evidence has been accumulated in support of the mutant p53 gain of function theory (9–11). Expression of mutant p53 in human cancers has been linked to a poorer prognosis (12).

Many members of the TGF-β superfamily play a role in tumorigenesis (13). TGF-β signaling is mediated through its binding to type I and type II receptors, and the activated ligand-receptor complex typically activates Smad-dependent signal transduction (14). The canonical Smad signaling cascade is initiated by phosphorylation of R-Smad (Smad2 and/or Smad3) by activated ALK5. This allows R-Smad binding to Smad4 and translocation of the complex to the nucleus, where it can recruit transcriptional coactivators or corepressors to Smad binding elements (SBEs) in the promoters of TGF-β target genes (15).

It is known that Smad2 and Smad3 interact physically and are structurally similar to 90% homology in their amino acid sequences (16), but the distinct functions of these two genes in embryonic development have been noted (17). Experimental data indicate that TGF-β can induce different functions in the same cell lines, such as growth arrest and epithelial-to-mesenchymal transition. Even though both play important roles in tumorigenesis, intriguingly, there is ample evidence to suggest that Smad2 and Smad3 have distinct and non-overlapping roles in TGF-β signaling (18). It is known that Smad2 and Smad3 require interactions with a number of common transcriptional

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regulators for their actions (19, 20). Some of these “cofactors” can act to alter the balance of Smad2 versus Smad3-mediated TGF-β signaling in the nucleus (21, 22). However, the mechanisms for selective activation of Smad2 versus Smad3 are still largely unknown.

A convergence of p53 and Smad signaling pathways has been established (23), but fully understanding the cross-talk between TGF-β and mutant p53 is complicated by the fact that TGF-β can act both as a tumor suppressor at early stages in carcinogenesis and as a prometastatic signal transducer at advanced stages (24). The studies by Adorno and colleagues suggest that mutant p53 can bind to the Smad2-p63 complex but not to Smad2 itself and cooperate with TGF-β, leading to suppression of the p63 growth-inhibitory signal and induction of promigratory events (25). Prior studies in a different laboratory found that mutant p53 can attenuate TGF-β induced migration through the suppression of a range of TGF-β-dependent genes, including the receptor gene TGFBR2 (26). It is still unclear how to reconcile the seemingly contradictory observations.

In this study, we show that mutant p53 breaks the balance between Smad2- and Smad3-mediated signal transduction by occupying the MH2 domain in Smad3 and disrupting Smad3-Smad4 complex formation. This action of mutant p53 hijacks the ERK signaling that is required for formation of the mutant p53-Smad3 complex. Our study provides insights into how mutant p53 differentially regulates subsets of TGF-β target genes for tumor suppressors and tumor promoters in the same cell type.

Experimental Procedures

Antibodies, Reagents, and Plasmids—Rabbit anti-p53, HSP90, GFP and mouse anti-p53, GAPDH, and Smad4 antibodies were obtained from Santa Cruz Biotechnology. Rabbit anti-p53, HSP90, GFP and mouse anti-p53, GAPDH, and Smad4 antibodies were purchased from Sigma. Mouse anti-p21 antibody was purchased from Cell Signaling Technology and was dissolved in PBS containing 0.5% BSA. Rabbit anti-p53 (Ser-392), and Slug antibodies were obtained from Cell Signaling Technology and was reconstituted to a final concentration of 5 μg/ml. All reagents and antibodies were purchased from Santa Cruz Biotechnology, and were purchased from Cell Signaling Technology and were dissolved in PBS containing 0.5% BSA. The CaMEK1 and HA-ERK1 were provided by Dr. Ping Wang (East China Normal University). The TGF-β1 was obtained from PeproTech (Baylor College of Medicine). Different p53 point mutations and truncations were generated by PCR and cloned into the pCDNA3.1 vector. The Smad2 and Smad3 truncations were amplified from pRK5-Smad2 and pRK5-Smad3 by PCR and constructed into the pCDNA3.1 vector. caMEK1 and HA-ERK1 were provided by Dr. Ping Wang (East China Normal University).

Cell Cultures—The mouse oral cancer derived cell lines J4708 (p53−/−) and J4705 (R172H p53) were a gift from Dr. Carlous Caulin (MD Anderson Cancer Center). The Detroit 562 Lenti and shp53 stable cell lines were provided by Dr. Jeffery N. Myers (MD Anderson Cancer Center). The Smad3 knockout MEF cells were provided by Dr. Xin Hua Feng. H1299 cells stably expressing empty vector and R175H p53 have been generated previously (27).

Time-lapse Imaging—Time-lapse phase-contrast microscopy was performed on a Nikon inverted microscope equipped with a phase-contrast ×10 objective, a sample heater (37 °C), and a home-made CO2 incubation chamber. Images were obtained every 10 min for 12 h.

Quantitative RT-PCR—To assess mRNA levels, RNA was isolated from cells using TRIzol reagent (Takara), and cDNA was synthesized using MLV reserve transcriptase from Promega. For the quantitative RT-PCR analysis, the reverse-transcribed cDNA was subjected to RT-PCR using SYBR Green master mix (Toyobo) and the Mx3005P quantitative RT-PCR system (Stratagene). Each experiment was performed in duplicates and was repeated three times. The primers used for quantitative PCR were as follows. For the human version: p21 (5'-GCCAGACCAGCATGACATT-3' and 5'-GGGATTAAGGGGCACTG-3'), p15 (5'-CGTTAAGTTTTACGGCACAACG-3' and 5'-GGTGAAGTGAGGGCAAGTT-3'), TBCD15 (5'-GCCGCTACATCATCAC-3' and 5'-CATCGGCTGCTGAA-3'), p53-Smad3 complex. Our study provides insights into how mutant p53 hijacks the ERK signaling that is required for formation of the mutant p53-Smad3 complex. Our study provides insights into how mutant p53 hijacks the ERK signaling that is required for formation of the mutant p53-Smad3 complex. Our study provides insights into how mutant p53 hijacks the ERK signaling that is required for formation of the mutant p53-Smad3 complex. Our study provides insights into how mutant p53 hijacks the ERK signaling that is required for formation of the mutant p53-Smad3 complex.
gos (synthesized by Shanghai GenePharma Co., Ltd.) were transfected by Hilymax (Dojindo Molecular Technologies) and Lipofectamine 2000 (Invitrogen), respectively (siSmad2 target sequence, GGATGAAGTAGTGTAAC (28); siSmad3 target sequence, GGATTGAGCTGCACCTGAATG (29); siSlug target sequence, GGACCAGTGCTCTCAGAA (30); and si53 target sequence, CCAGGCATGCCGCTACATA (25)). Lentiviral empty vectors, a vector coding for wild-type-p53, or mutant p53 were transfected into 293T cells in combination with pMD2G and pSPAX2 to produce the viral particles needed for H1299 cell infection.

**Coimmunoprecipitation**—H1299 or generated stable cells were transfected with constructs or treated as explained in the figure. Cells were then scraped into ice-cold PBS and lysed with lysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% Nonidet P-40, 150 mM NaCl, 10% glycerol, and protease inhibitors) 24 h after transfection or after treatment with TGF-β or ERK inhibitor for the indicated periods. Specific proteins were immunoprecipitated, followed by three washes with wash buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% Nonidet P-40, 150 mM NaCl, 10% glycerol and protease inhibitors). The pellet was then resuspended in SDS sample buffer and analyzed by the indicated antibodies as described in the figure legends.

**GST Pulldown Assays**—Plasmid DNA constructs encoding GST fusion proteins were transformed into BL21 E. coli and purified using a standard purification protocol with glutathione beads (GE Healthcare). Purified GST fusion proteins adsorbed to glutathione beads were eluted and stored at −80 °C in small aliquots. Different p53 derivatives were in vitro-translated and labeled with [35S]methionine using the Tnt Quick translation system (Promega). Translated protein or overexpression mutant p53 cell lysate was incubated with recombinant Smad2, Smad3ΔExon3, Smad3, or their truncation mutants at 4 °C for 2 h. Beads were then washed four times with Triton X-100 lysis buffer (50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol). Samples were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue staining or specific antibody incubation. Binding of labeled protein to GST fusion proteins was analyzed by autoradiography or Western blotting.

**Migration Assays**—Transwell assays were performed in 24-well PET inserts (Millipore, 8.0-μm pore size) for cell migration. Cells plated in 6-cm dishes were transfected with the indicated siRNA for 48 h before the overnight serum starvation. 5 × 10⁴ cells in serum-free media were plated in the upper chamber of transwell inserts (two replicates for each sample). The inserts were then placed into 10% serum media for 6 h of migration. Cells in the upper chambers were removed with a cotton swab, and migrated cells were fixed in paraformaldehyde 4% and stained with crystal violet 0.5%. Filters were photographed, and the total migrated cells were counted. Every experiment was repeated independently three times.

**Electrophoretic Mobility Shift Assay**—DNA-binding assays for Smad3/Smad4 were performed following protocols described previously (27, 31, 32). Briefly, ³²P-labeled double-stranded SBE oligonucleotides from the p21waf1 gene promoter region were used as probes, and competition assays were performed with a 100-fold excess of unlabeled wild-type or mutant SBE oligonucleotides (31).

In Vitro Kinase Assay—Purified GST-R175H p53 protein was incubated with HA-ERK enriched from ERK-transfected cell lysate in kinase buffer with 50 μM ATP at 30°C for 30 min (33). The reactions were terminated with SDS sample buffer. The phosphorylation of R175H p53 was detected by the site-specific antibody for Ser-392.

**RESULTS**

**Mutant p53 Enhances the Motility of Lung Cancer Cells**—To pursue mechanistic studies, we generated stable cells expressing mutant p53 (R175H) in the non-small-cell lung carcinoma cell line H1299, which lacks p53 expression and, therefore, precludes the interference of wild-type p53 (3). Using the H1299 stable cells, we analyzed the interference on cytostatic effects of TGF-β by tumor-derived mutant p53. By BrdU incorporation cell proliferation assays, H1299 cells expressing mutant p53 (mutp53-H1299) were less responsive than control H1299 cells (expressing vector only) to TGF-β induced growth inhibition (Fig. 1A). Western blotting showed that mutant p53 attenuated TGF-β-induced expression of p21WAF1 without influencing the phosphorylation of R-Smads (Fig. 1B), indicating that the mutant p53 may attenuate TGF-β signal transduction by a previously unidentified mechanism.

Using time-lapse microscopy and software to track single cell movement, we successfully recorded the migratory behaviors of the mutp53-H1299 and control H1299 cells. In agreement with previous studies (3), the paths extracted from each single cell demonstrated that the mutp53-H1299 cells moved faster but with less orientation than the control cells (Fig. 1C). Then the migration abilities of these cells in the presence or absence of TGF-β were examined by transwell assays. Interestingly, mutant p53-harboring cells migrated faster than control cells, whereas the mutp53-H1299 cells displayed faster migration than the control cells upon TGF-β stimulation (Fig. 1D), indicating that mutant p53 could enhance the response of the cells to TGF-β-dependent migration. Taken together, we speculate that mutant p53 attenuates the anti-tumor functions of TGF-β and cooperates with its tumorigenic functions.

**Mutant p53 Deregulates the TGF-β Pathway**

Mutant p53 Deregulates the TGF-β Pathway—To investigate how mutant p53 deregulates TGF-β-dependent transcription, pooled mutp53-H1299 and control H1299 cells were treated with TGF-β before being harvested for quantitative RT-PCR. Expression of p21 and p15, two of the canonical tumor suppressor genes responsive to TGF-β, was significantly attenuated in R175H p53 cells compared with expression in their p53 null counterpart controls. Given a recent finding that mutant p53 can inhibit the function of the tumor suppressor DAB2IP (34), we speculated that TGF-β and mutant p53 might be involved in the regulation of a group of GTPase-activating proteins. As expected, both DAB2IP and TBC1D16 GTPase-activating protein genes had reduced expression in mutant p53 cells. However, some TGF-β-responsive genes involved in the regulation of cell migration and invasion, including MMPs and Slug, were further up-regulated after TGF-β stimulation in mutant p53 expressing cells (Fig. 2A).
To ensure that the expression of these genes was regulated upon activation of TGF-β signaling, SB431542, an inhibitor for TGF-β receptor, was applied to cells before TGF-β treatment (Fig. 2A). The inhibitor blunted expression of all these target genes in response to TGF-β in H1299 cells regardless of mutant p53 status. To preclude potential artifacts because of exogenous expression of R175H p53 in H1299 cells, a pharynx carcinoma cell line, Detroit562 which harbors an endogenous R175H p53 mutant, was employed for further analysis. A stable cell line silencing mutant p53 in Detroit562 was generated using a specific p53 shRNA established before (35). Consistent with our observation in mutp53-H1299 and control cells, genes controlling cell migration (MMPs and Slug) had a better response to TGF-β in the mutant p53-containing Detroit562 cells (with integration of only a lentiviral vector, therefore named “Detroit Lenti”) than in the shp53 Detroit562 cells (Fig. 2B). However, the tumor-suppressive genes (p21 and p15) had less of a response to TGF-β in the Detroit562 Lenti cells than in the shp53 Detroit562 cells. A similar expression pattern was observed in J4705 and J4708, two oral cancer cell lines derived from mutant p53 (R172H) knockin and p53 knockout mice, respectively (Fig. 2C) To our surprise, we did not observe any difference in the phosphorylation of Smad2 and Smad3 between control and mut-p53 H1299 cells or Detroit Lenti and shp53 cells (Fig. 1B and data not shown). In addition, we checked changes in phosphorylation of R-Smads in J4705 and J4708 or in H1299 cell-transfected viral vectors expressing wild-type p53, R175H p53, or control vector. Consistent results were observed in these cells, showing that mutant p53 did not induce differences in phosphorylation of R-Smads (Fig. 2, D and E). As shown in Fig. 2F, TGF-β treatment for 30 min induced the translocation of Smad3 into the nucleus in H1299 cells expressing either wild-type or mutant p53, suggesting that mutant p53 had no influences on the nuclear translocation of Smad3’s. We conclude, on the basis of these results, that mutant p53 may achieve its gain of function by attenuating and enhancing subsets of TGF-β-responsive genes without affecting phosphorylation of Smad2 or Smad3, suggesting an effect downstream of R-Smad translocation to the nuclei.

### FIGURE 1. Mutant p53 Enhances Lung Cancer Cell Malignancy

A. mutant p53 attenuates the cytostatic effect by TGF-β. H1299 EV and R175H cells were cultured with TGF-β at a concentration of 5 ng/ml for the indicated days. Cell growth was measured by BrdU incorporation cell proliferation assays. Results represent the mean ± S.D. of three experiments performed in triplicate. *, p < 0.05 and **, p < 0.01, as determined by Student’s t test. B, mutant p53 attenuates p21 expression. H1299 EV and R175H cells were starved for 24 h before being treated with TGF-β for various times. The indicated proteins were detected by Western blotting (left panel), and quantification of p21 levels from three independent experiments is shown (right panel). C, mutant p53 promotes cell migration. H1299 cells stably expressing mutant p53 or a control vector were monitored by time-lapse video microscopy. The movement of individual cells was recorded using ImageJ cell tracking software. The speed and persistence of migration were extracted from the track plots. Directionality (persistence) is defined as the ratio of the vectorial distance to the total path length traveled by the cells. Values are means ± S.E. of 90 track plots from three independent experiments. *, p < 0.05 and ***, p < 0.01, as determined by Student’s t test. D, H1299 cells were seeded on transwell membranes where the cells were treated with or without TGF-β. The migrated cells were fixed and stained with crystal violet (left panel). The percentage of migrated cell was quantified (right panel). Con, control. *, p < 0.05, as determined by Student’s t test.

Mutant p53 Fulfills Its Gain of Function by Occupying the MH2 Domain of Smad3—To understand the molecular details by which mutant p53 affects transcription of TGF-β-responsive genes, we tested physical interactions between mutant p53 and Smad proteins by GST pulldown or immunoprecipitation assays. When we used GST-Smad2 or GST-Smad3 to pull down 35S-labeled wild-type or mutant R175H p53 (Fig. 3A), both
wild-type and mutant p53 exhibited a higher affinity to Smad3 than Smad2, whereas mutant p53 had no interaction with Smad4 (data not shown). Interestingly, R175H p53 and several other p53 mutants all had a higher affinity to Smad3 than Smad2 (Fig. 3B), which was consistent with the previous observation that mutant p53 barely interacted with Smad2 by itself (25). The differential affinity for Smad3 by wild-type and mutant p53 encouraged us to analyze whether the binding regions in Smad3 to p53 are the same or different. Consistent with a previous report showing that wild-type p53 binds to the
FIGURE 3. Mutant p53 fulfills its gain of function by occupying the MH2 domain of Smad3. A, preferential interaction with Smad3 by mutant p53. Pulldown assays were performed with GST, GST-Smad2, or GST-Smad3 together with S35-labeled wild-type and mutant p53. B, mutant p53 showed a higher affinity for Smad3. Pulldown assays were performed with GST-Smad2 or GST-Smad3 and lysate from H1299-overexpressed mutant p53. C and D, distinct domains in Smad3 bind to WT p53 and mutant p53. C, FLAG-tagged human Smad3 and its truncation constructs expressing the MH1 domain, Linker region, or MH2 domain. Fragments a–e refer to constructs expressing distinct domains, and v means control vector. D, H1299 cells were transfected with FLAG-tagged full-length Smad3, MH1, MH2 together with WT p53, or R175H p53, and the cells were harvested for coimmunoprecipitation (IP) assays 24 h later. E, R175H p53 binds to the MH2 domain in Smad3. Coimmunoprecipitation assays were performed to determine the binding of R175H p53 to specific domain(s) in FLAG-Smad3. F, different p53 mutants bind to the MH2 domain in Smad3. FLAG-MH1, FLAG-MH2, and mutant p53s were transiently transfected to H1299 for coimmunoprecipitation assays. G, 30 amino acid residues coded by exon3 of the Smad2 gene attenuates its interaction with p53. GST, GST-Smad2ΔExon3 (short for GST-S2ΔE3), and GST-Smad3 were incubated with in vitro-translated WT p53 or R175H p53, respectively, followed by pulldown assays. H, mutant p53 attenuates endogenous Smad3-Smad4 interactions. Cell lysates from H1299 EV and H1299 R175H were immunoprecipitated with a mouse anti-Smad4 antibody before and after TGF-β treatment, and the precipitates were analyzed with rabbit anti-Smad3 antibody. I, mutant p53 attenuates Smad3-Smad4 complex affinity for the SBE probe. H1299 cells were transfected with the indicated constructs and treated with or without TGF-β, and EMSA assays were performed with the double-stranded oligonucleotides containing the SBE box from the p21 promoter. WT comp, wild type competitor; Mut comp, mutant competitor. J, H1299 cells were transfected with SBE-Luc together with R175H p53 or wild-type p53 expression plasmids, and luciferase activity was measured following TGF-β treatment. **, p < 0.01, as determined by Student’s t test. K—M, mutant p53 attenuates Smad3-dependent signal transduction. J4708 and J4705 ARO cells with transient depletion of R273H p53 and H1299 EV and R175H were treated with TGF-β for 6 h. The RNA was extracted, and specific gene expression was analyzed. *, p < 0.05 and **, p < 0.01, as determined by Student’s t test.
MH1 domain in Smad3 (36), we found that wild-type p53 interacted with Smad3 through the MH1 domain only (Fig. 3, C and D). However, R175H p53 bound exclusively to MH2 rather than the MH1 domain in Smad3 (Fig. 3, D and E). To test whether other mutant p53 may bind to Smad3 in a similar fashion, constructs encoding R273H, R248W, or R282W p53 were transfected together with FLAG-MH1 or MH2 from Smad3 to H1299 for the interaction assays. All tested mutant p53s were coimmunoprecipitated with FLAG-MH2 but not the MH1 domain in Smad3 (Fig. 3F). Given that Smad2 and Smad3 are 91% identical in full-length amino acid sequence and 96% identical in their MH2 domains (37), we found no differences in the binding of mutant p53 to isolated MH2 domains from Smad2 or Smad3 (data not shown). This prompted us to explore why Smad2 had a reduced affinity to mutant p53. Previous research has shown that the MH1 domain can interfere with the function of the MH2 domain by direct interactions (16). The Smad2 protein has a 30-amino acid region in the middle of the MH1 domain coded by exon3, which does not exist in Smad3 or other Smads in mammals (38). It has been suggested that Smad2ΔExon3 may function as a Smad3-like molecule (16). By comparing the interactions between p53 and purified Smad2, Smad2ΔExon3, or Smad3 proteins, we found that the extra 30 amino acid residues in Smad2 attenuate its interaction with both wild-type and mutant p53 (Fig. 3G).

Given that the MH2 domain of Smad3 is responsible for receptor interaction and the formation of homomeric as well as heteromeric Smad complexes (39), we predicted that mutant p53 might disrupt the Smad3-Smad4 complex by occupying the MH2 domain in Smad3. We indeed observed attenuated interactions between endogenous Smad3 and Smad4 in cells expressing mutant p53 upon TGF-β treatment (Fig. 3H). Disruption of the Smad3-Smad4 complex by mutant p53 resulted in reduced affinity to the SBE probe, as observed in the EMSA assay (Fig. 3I). In addition, the SBE-Luc activity was attenuated by mutant p53 overexpression in H1299 cells (Fig. 3J). These results suggest that mutant p53 may disrupt Smad3-dependent TGF-β signal transduction. Expression profiles in additional cell lines, including naturally occurring mutant p53 ARO cells, were supportive of mutant p53 GOF (Fig. 2, K and L). Despite the fact that mutant p53 may directly influence transcription of some target genes, we found several classical and newly defined Smad3-dependent genes (40) that are not affected by mutant p53 (Fig. 2M). Taken together, the above results indicate that mutant p53 may achieve its gain of function by preventing Smad3-Smad4 complex formation and altering TGF-β signal transduction.

The ERK Signal Is Required for the Interaction between Mutant p53 and Smad3—To determine the mechanisms for the mutant p53 and Smad3 interactions, we decided to define the domains required for this interaction on mutant p53. Using in vitro-translated deletion mutants and purified GST-Smad3, we localized the Smad3-binding region to the C terminus of mutant p53 because the mutant p53 lacking amino acids 301–393 lost binding to Smad3 (Fig. 4A). To validate this, we tested whether overexpression of MH2 or the mutant p53 C-terminal domain would block the interaction. By cotransfecting a GFP control vector, the GFP tagged C-terminal domain of mutant p53 (GFP301–393), or the GFP-MH2 domain of Smad3 together with FLAG-Smad3 and R175H p53 to H1299 cells, we found that both GFP tagged MH2 and GFP301–393 fragments, but not GFP itself, blunted the interaction between mutant p53 and Smad3 in a dominant negative fashion (Fig. 4B).

The ERK signal is known to synergize with mutant p53 in certain gain of function manifestations (25). Phosphorylation of Ser-6 and Ser-9 on the N terminus of wild-type p53 mediates the interactions between p53 and Smad3 (36). We speculated that kinase signals might affect mutant p53 interaction with Smad3. Kinase inhibitors for ERK, PI3K, and p38 were tested for their influence on the interaction between mutant p53 and Smad3. Interestingly, both the MEK inhibitor PD98059 and the ERK inhibitor U0126, but not other kinase inhibitors, blocked the interaction between mutant p53 and Smad3 (Fig. 4C). Even in the presence of TGF-β, ERK inhibitors could prevent the interaction between R175H p53 and Smad3 (Fig. 4E), indicating that the MEK-ERK signal plays a vital role in mediating the interaction. As predicted, silencing ERK or the presence of U0126 in R175H-expressing cells significantly attenuated the interaction between R175H p53 and Smad3 (Fig. 4, D and E). To validate that protein modification is essential for R175H p53 and Smad3 interaction, we immunoprecipitated FLAG R175H p53 or HA Smad3, followed by λ-phosphatase treatment to erase phosphorylation and another round of HA cross-immunoprecipitation. Phosphatase efficiency was examined together with the utilization of a site-specific phosphorylation antibody (Fig. 4F, bottom panel). The results demonstrated that, when phosphorylation on FLAG-R175H p53 was removed, R175H p53 and Smad3 interaction was attenuated (Fig. 4F, top panel, left), suggesting the requirement of a kinase signal in promoting mutant p53 actions. As controls, λ-phosphatase-treated or untreated HA Smad3 beads could equally precipitate FLAG R175H p53 (Fig. 4F, top panel, right).

To further define the phosphorylation region/site in mutant p53, we performed GST pulldown assays using purified GST-Smad3 together with a series of in vitro-translated C-terminal truncation mutants of R175H p53. GST-Smad3 failed to interact with mutant p53 truncations lacking amino acids 320–342 or 367–393 (Fig. 4G). Sequence analysis indicated that these mutants lacked the tetramerization domain or the C-terminal regulatory domain, consistent with a previous finding showing that these domains might be required for the mutant p53 gain of function (41). Through a series of site-directed mutagenesis and immunoprecipitation analysis, we found that phosphorylation at Ser-392 was important for the interaction between R175H p53 and Smad3 (Fig. 4H). Although phosphorylation at N terminus serines (S6A/S9A) has been known to mediate the interaction between wild-type p53 and Smad3, mutations at these serines had no effect on mutant p53 actions (Fig. 4H). Furthermore, U0126 repressed the phosphorylation on Ser-392 in R175H p53 (Fig. 4I). An in vitro kinase assay defined the phosphorylation of mutant p53 at Ser-392 by activated ERK1 (Fig. 4J). Together, these data suggest that the MEK-ERK signal is important for the mutant p53 gain of function by promoting phosphorylation at Ser-392 and formation of the mutant p53-Smad3 complex.
FIGURE 4. The ERK signal promotes the interaction between mutant p53 and Smad3. A, the C terminus of mutant p53 binds to Smad3. Pulldown assays with GST-Smad3 and full-length (FL), N-terminal, or C-terminal truncations of R175H p53 were performed. B, dominant negative effect of the mutant p53 C-terminal fragment and the MH2 domain. GFP 301–393 of R175H p53 or GFP-MH2 of Smad3 was cotransfected to H1299 together with FLAG Smad3 and R175H p53 to test interactions. IP, immunoprecipitation. C, inhibition of MEK-ERK signaling prevents mutant p53 interactions with Smad3. Following cotransfection of FLAG Smad3 and R175H p53 to H1299 for 24 h, different kinase inhibitors were applied to the cells for an additional 6 h before harvesting for coimmunoprecipitation assays. D, silencing endogenous ERK1/2 in H1299 cells significantly attenuated mutant p53 and FLAG Smad3 interactions in coimmunoprecipitation assays. siN, scrambled RNA. E, the ERK inhibitor antagonizes the TGF-β/H9252-induced R175H-Smad3 interaction. R175H-expressing H1299 cells treated with TGF-β/H9252, U0126, or in combination were lysed for coimmunoprecipitation of Smad3 by an anti-p53 antibody. F, phosphorylation on mutant p53 is required for R175H-Smad3 interaction. H1299 cells transfected with FLAG R175H p53 or HA Smad3 were immunoprecipitated with anti-FLAG or anti-HA beads, followed by treatment of λ-phosphatase (APP) or a vehicle. Then λ-phosphatase-treated or untreated FLAG proteins were eluted by FLAG peptide for another round of immunoprecipitation by HA bead-bound Smad3. G, R175H p53 deletion mutant constructs (A–F). Following in vitro translation 35S-labeled R175H p53 derivatives were incubated with purified GST-Smad3 for pulldown assays. TA, transcription activation domain; DBD, DNA binding domain; TET, tetramerization domain; REG, regulation domain. H, phosphorylation at Ser-392 on mutant p53 is important for R175H-Smad3 interaction. H1299 cells were cotransfected with FLAG Smad3 and a series of site-directed R175H p53 mutants, followed by coimmunoprecipitation assays. C-2A, S315A/S392A R175H p53; C-3A, S303A/S315A/S392A R175H p53. I, U0126 inhibited phosphorylation of mutant p53 at Ser-392. H1299 cells were transfected with R175H p53 and treated with U0126 (10 μM for 6 h). Changes in phosphorylation at Ser-392 were detected by a specific phospho-antibody. J, the Ser-392 in mutant p53 is a directly target of ERK1. Following cotransfection of caMEK1 and HA-ERK1 to H1299 cells for 24 h, activated HA-ERK1 was purified and incubated with purified GST-R175H p53 in vitro at 30 °C for 30 min. The phosphorylation levels at Ser-392 were detected by a site-specific phosphorylation antibody.
Mutant p53 Deregulates the TGF-β Pathway

Mutant p53 Deregulates TGF-β Signaling by Antagonizing Smad3-dependent Transcription—Given that disrupting the Smad3 and Smad4 complex underlies the mechanism by which mutant p53 deregulates TGF-β signaling, we asked whether the gene expression patterns in mutant p53-expressing cells could be recapitulated by silencing endogenous Smad3 in control cells or tipping the balance between Smad2 and Smad3. The siRNA for Smad2 or Smad3 has been demonstrated previously to be efficient and specific (28, 29). Interestingly, silencing Smad3 resulted in a similar gene expression pattern as that in cells expressing mutant p53, showing attenuated p21 but increased MMPs and Slug expression in response to TGF-β induction (Fig. 5, A and B). Similarly, mutant p53 had little effect on subsets of TGF-β-responsive genes in Smad3 knock-out MEFs (Fig. 5, C and D). However, silencing Smad2 attenuated mutant p53-mediated up-regulation of MMPs and Slug (Fig. 5, A and B), consistent with the previous observation that the regulation of MMP2 by TGF-β was Smad2-dependent in epithelial cells (17, 42). Our results suggest an inhibitory role for Smad3 in MMPs and Slug expression, whereas Smad2 functions to counterbalance Smad3 effects. Taken together, we conclude that silencing Smad2 or inhibiting the ERK signal pathway in mutant p53 cells can repress the synergy between mutant p53 and TGF-β in their tumor-promoting functions by affecting Smad3-dependent transcriptional regulation of MMPs and Slug.

Mutant p53 Promotes Cell Motility by Enhancing TGF-β-induced Slug Expression—One of the most heavily investigated epithelial-to-mesenchymal regulators in lung cancers is Slug, whose expression is associated with lung cancer metastasis and resistance to target therapy (43). The observation that both mutant p53 and lack of Smad3 can enhance TGF-β induced Slug expression in human lung cancer cells (Fig. 6, A and Fig. 5, A and B) prompted us to test Slug expression in additional cancer cells. Interestingly, we found up-regulation of Slug in the oral cancer cell line J4705 (derived from p53 R172H-knockin mice) and Detroit562 cells compared with control cells with p53 depletion (data not shown and Fig. 6B). To understand expression of R-Smad and Slug in the regulation of cell motility, we silenced Smad3 alone or together with Slug in H1299 cells. Smad3 knockdown clearly enhanced cell motility in a transwell analysis. However, cell movement was slowed down by silencing Smad3 and Slug together (Fig. 6C). Next, we compared migration ability in R175H p53 cells with Smad2 or Smad3

FIGURE 5. Mutant p53 distorts TGF-β signaling by antagonizing Smad3 function. A and B, mutant p53 attenuates p21 but promotes MMPs and Slug expression in a Smad3-dependent manner. Specific siRNA targeting Smad2 or Smad3 was transfected into H1299 EV and R175H cells for 60 h, followed by treatment with TGF-β or vehicle for 6 h. The RNA level was monitored by quantitative PCR (A), and the related protein levels were confirmed by Western blotting (B). siN, scrambled RNA. *, t < 0.05, **, t < 0.01 and ***, t < 0.001, as determined by Student’s t test. C and D, related protein and gene expression in WT MEFs, Smad3 KO MEFs, and Smad3 KO with R175H p53 overexpression MEFs was monitored by Western blotting (C) and quantitative PCR (D), respectively. *, t < 0.05 and **, t < 0.01, as determined by Student’s t test.
knockdown. Silencing Smad3 in R175H p53 cells enhanced cell migration. However, depleting Smad2 reduced TGF-β/H9252-induced chemokinesis (Fig. 6D), consistent with previous observations in a pancreatic ductal adenocarcinoma cell line (44). By single cell migration analysis, we demonstrated that the ability of mutant p53 to drive enhanced migration in H1299 cells was dependent upon Slug expression because depletion of Slug in the mutant p53-expressing cells reversed its migratory behaviors (Fig. 6E). In conclusion, mutant p53 can enhance cancer cell motility by antagonizing Smad3-dependent inhibition of Slug expression.

DISCUSSION

The TGF-β and p53 signaling networks play a vital role in regulating cell growth and migration. Wild-type p53 is known to be required for full activity of TGF-β-mediated regulation by cooperating with Smads (36). Deregulation in either of these signal pathways may disturb the ability of the cell to maintain normal behavior, eventually leading to the development of cancer.

Despite the fact that mutant p53 has been reported to mediate the repression of TGF-β-signaling by interference with TGF-β receptors (26), a recent study has shown that mutp53 activates TGF-β-induced migration, invasion, and metastasis in breast cancer cells (25). Our study suggests that these seemingly contradictory observations can be partially reconciled. We demonstrate that, in mutant p53-expressing cells, expression of some TGF-β-responsive tumor suppressor genes is attenuated. In contrast, expression of several tumor promoter genes is enhanced. Clearly, mutant p53 can achieve its gain of...
function activity by hijacking Smad3-mediated TGF-β signaling transduction rather than blocking the entire TGF-β-regulated gene network. Identification of preferential interactions between mutant p53 and R-Smads, for example, stronger interactions with Smad3 than Smad2 in our study, provides an alternative mechanism of how mutant p53 suppresses TGF-β signaling. Mutant p53 mainly interacts with the MH2 domain on Smad3 through its C terminus. This explains why mutant p53 attenuated the interaction between Smad3 and Smad4 without influencing the phosphorylation of Smad3. Different from the Smad2/p63/mutant p53 complex formation in breast cancer cells discovered previously (25), our finding of the Smad3-mutant p53 complex in lung cancer cells may reflect a cell-specific and signal-specific mechanism.

The C terminus of mutant p53 contains a tetramerization domain (TD) and a regulatory region. The TD of p53 has long been ignored because it is not a hot spot region often mutated in cancers (41). However, a number of structural and functional analyses have revealed the importance of TD for p53 function, and the TD is also required for some mutant p53 gain of function (45, 46). In this study, we found that mutant p53 deleted the TD or that regulatory domain is unable to interact with Smad3. Intriguingly, when we mutated the serine at 392, whose phosphorylation will stimulate the wild-type p53 tetramerization (47), the interactions between mutant p53 and Smad3 were disrupted. The data presented so far indicate that the Ser-392 phosphorylation may influence mutant p53 gain of function in twisting Smad3-dependent signal transduction, consistent with a previous finding showing that posttranslational modification at the C terminus regulatory domain may affect p53 function (47).

In addition, selective actions of Smad2 and Smad3 on specific target genes may further empower mutant p53 gain of function. In the TGF-β signaling network, how cells selectively activate Smad2 versus Smad3 remains poorly understood. On the basis of large-scale analyses of Smad2 and Smad3-dependent TGF-β target genes in knockout MEFs, Smad2 and Smad3 have distinct roles in TGF-β signaling despite their functional redundancy (17). Mutant p53 may achieve its gain of function by tipping the balance between the Smad2 and Smad3 actions on specific target genes. Previous research has indicated that regulators such as Hic-5 and EID-2 can alter the balance of the Smad3-specific versus the Smad2-specific arm of TGF-β signaling by blocking Smad3 signal transduction through occupying its MH2 domain (22, 48, 49). Here we provide an example showing that mutant p53 can function as a molecular switch to alter the balance between the Smad2- and Smad3-dependent transcriptional output of TGF-β signaling.

Recent research has shown that knocking down Smad3 could increase the migratory response, whereas silencing of Smad2 decreases chemokinesis in pancreatic ductal adenocarcinoma cells (44). Mechanistically, we found that expression of MMPs and Slug is reduced by silencing Smad2 but enhanced by depletion of Smad3, whereas knocking down Smad2 or Slug in mutant p53-expressing cells attenuates motility in lung cancer cells. These findings endorse our hypothesis that mutant p53 enhances TGF-β-induced MMP and Slug expression and cell migration by attenuating Smad3-mediated signal transduction. Consistently, clinical research has found that high p-Smad2 expression in stromal fibroblasts predicted poor survival in patients with clinical stage I to IIIA non-small-cell lung cancer (50), indicating that Smad2 may play an important role in mediating lung cancer metastasis. The association between a high level of p-Smad2 and a malignant phenotype and poor prognosis in patients with advanced carcinoma (51) suggests that targeting mutant p53 may ameliorate the balance between Smad2 and Smad3 signaling for late-stage cancers.

In conclusion, this study provides evidence for a new mechanism by which mutant p53 protein hijacks Smad3-mediated TGF-β signal transduction, in turn unleashing the Smad2-specific pathway. Therefore, mutant p53 may be employed by cancer cells as a molecular switch to alter TGF-β signaling at the R-Smad levels from a tumor-suppressive toward a tumor-promoting pathway. Our findings that physical interactions between mutant p53 and Smad3 are required for the gain of function of mutant p53 propose a potential to develop anticancer drugs able to suppress specific tumors.

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