Mutant p53 Disrupts Role of ShcA Protein in Balancing Smad Protein-dependent and -independent Signaling Activity of Transforming Growth Factor-β (TGF-β)*\S

Shu Lin†, Lan Yu‡, Junhua Yang‡, Zhao Liu§, Bijal Karia‡§, Alexander J. R. Bishop‡¶, James Jackson†, Guillermina Lozano†, John A. Copland***, Xiaoxin Mu†¶, Beicheng Sun†‡, and Lu-Zhe Sun†‡‡

From the *Department of Cellular and Structural Biology, ‡Greehey Children’s Cancer Research Institute, and §Department of Cancer Biology, Mayo Clinic, Jacksonville, Florida 32224, and ‡‡ Key Laboratory of Living Donor Liver Transplantation, First Affiliated Hospital of Nanjing Medical University, Nanjing, China 210009

Background: Biomarkers driving TGF-β from tumor-suppressing to tumor-promoting remain elusive.

Results: p53 mutation inhibits TGF-β-induced ShcA/ERK signaling and enhances Smad signaling. Elevated p-p52ShcA levels shift the role of TGF-β from growth suppression to migration promotion.

Conclusion: Mutant p53 disrupts the role of ShcA in balancing Smad-dependent and -independent signaling activity of TGF-β.

Significance: Elevated p-p52ShcA levels are a promising biomarker for TGF-β as a tumor promoter.

Biomarkers are lacking for identifying the switch of transforming growth factor-β (TGF-β) from tumor-suppressing to tumor-promoting. Mutated p53 (mp53) has been suggested to switch TGF-β to a tumor promoter. However, we found that mp53 does not always promote the oncogenic role of TGF-β. Here, we show that endogenous mp53 knockdown enhanced cell migration and phosphorylation of ERK in DU145 prostate cancer cells. Furthermore, ectopic expression of mp53 in p53-null PC-3 prostate cancer cells enhanced Smad-dependent signaling but inhibited TGF-β-induced cell migration by downregulating activated ERK. Reactivation of ERK by the expression of its activator, MEK-1, restored TGF-β-induced cell migration. Because TGF-β is known to activate the MAPK/ERK pathway through direct phosphorylation of the adaptor protein ShcA and MAPK/ERK signaling is pivotal to tumor progression, we investigated whether ShcA contributed to mp53-induced ERK inhibition and the conversion of the role of TGF-β during carcinogenesis. We found that mp53 expression led to a decrease of phosphorylated p52ShcA/ERK levels and an increase of phosphorylated Smad levels in a panel of mp53-expressing cancer cell lines and in mammary glands and tumors from mp53 knock-in mice. By manipulating ShcA levels to regulate ERK and Smad signaling in human untransformed and cancer cell lines, we showed that the role of TGF-β in regulating anchorage-dependent and -independent growth and migration can be shifted between growth suppression and migration promotion.

Thus, our results for the first time suggest that mp53 disrupts the role of ShcA in balancing the Smad-dependent and -independent signaling activity of TGF-β and that ShcA/ERK signaling is a major pathway regulating the tumor-promoting activity of TGF-β.

Transforming growth factor-β (TGF-β) is a tumor suppressor during early tumor outgrowth. However, carcinogensis-mediated elevation of TGF-β production and signaling is often tumor-promoting at later stages, leading to enhanced tumor cell migration, invasion, and metastasis (1). Increased TGF-β is often associated with the loss of the growth-inhibitory activity of TGF-β and its conversion to promote malignant progression of cancers (2), making TGF-β a potential therapeutic target. Indeed, many preclinical studies have shown the efficacy of various types of TGF-β inhibitors in blocking tumor growth, angiogenesis, and metastasis in animal models of human and rodent cancer (3–5). However, biomarkers are lacking for signifying the complicated molecular alterations mediating the switch of TGF-β from tumor suppression to tumor promotion and for identifying appropriate cancer patients for therapy with TGF-β inhibitors.

As a homodimeric polypeptide in humans and mice, TGF-β signals through cell surface receptors called TGF-β type I (RI) and type II (RII) receptors to regulate multiple cellular functions including cell proliferation, differentiation, migration, and wound healing (1). RI and RII are transmembrane serine/threonine kinase receptors that also contain tyrosine kinase activity (6). Active TGF-β ligands bind first to RII, which then

* This work was supported, in whole or in part, by National Institutes of Health Grants R01CA075253 (to L.-Z. S.), R01CA079683 (to L.-Z. S.), R01CA104505 (to J. A. C.), and P30CA054174-17, an NCI cancer center support grant (to the Cancer Therapy and Research Center at San Antonio).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

1 To whom correspondence should be addressed: Dept. of Cellular and Structural Biology, University of Texas Health Science Center, 7703 Floyd Curl Dr., Mail Code 7762, San Antonio, TX 78229-3900. Tel: 210-567-5746; Fax: 210-567-3803; E-mail: sunl@uthscsa.edu.

2 The abbreviations used are: RI, TGF-β type I receptor; RII, TGF-β type II receptor; mp53, mutant p53; SBE, Smad-binding element; R-Smad, receptor-regulated class of Smads; DNRII, dominant-negative RII; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MMTV, murine mammary tumor virus; p-ERK, phosphorylated ERK; *MEK-1, constitutively active form of MEK-1.
recruits RI, leading to the phosphorylation and activation of RI. Active RI directly phosphorylates the receptor-regulated class of Smads (R-Smad), Smad2/3. Phosphorylated R-Smads in turn associate with Smad4 and translocate into the nucleus to regulate the transcription of TGF-β-responsive target genes (1).

Besides Smad-mediated canonical signaling, TGF-β also signals through Smad-independent pathways including the Ras/ Raf/ERK pathway (7). The integration of TGF-β-mediated Smad-dependent and -independent signaling is believed to contribute to the key events of TGF-β-induced tumor progression including ERK signaling-mediated cell migration (8, 9). TGF-β activates Ras/ERK signaling through the direct phosphorylation of the adaptor protein ShcA (10). ShcA belongs to the family of Shc adaptor proteins, which are substrates of receptor tyrosine kinases (11). ShcA consists of three isoforms, p46, p52, and p66. They are derived from two different transcripts, called p66 and p52/p46 mRNAs (12). Compared with p52ShcA, p46ShcA results from a different in-frame ATG transcript and is predominantly expressed in mitochondria with an elusive role (13). Following the TGF-β engagement, tyrosine-phosphorylated RI recruits and directly phosphorylates p52/46ShcA proteins on tyrosine sites, leading to their association with Grb2 adaptor protein and Sos GTP exchange factor (14). The ShcA-Grb2-Sos complex activates Ras (15), thereby initiating the sequential activation of c-Raf, MEK, and ERK1/2. Among the three tyrosine phosphorylation sites at residues 239/240 (Tyr-239/240) and 317 (Tyr-317) in the CH1 domain of p52/46ShcA, Tyr-317 plays the major role in Ras/ERK signaling activation (11, 16). Clinical studies have shown that a high amount of Tyr-317-phosphorylated p52/46ShcA alone with a low amount of p66ShcA serves as an efficient predictor for identifying aggressive breast tumors with a high risk of recurrence (17, 18). High levels of TGF-β are also associated with poor outcome of human cancer (1). However, it is unclear whether ShcA-mediated activation of ERK signaling contributes to the switch of TGF-β function during carcinogenesis.

A recently published study has shown that the presence of mutated p53 (mp53) in the DNA-binding domain in certain cells together with additional Ras activation can switch TGF-β activity to that of a tumor promoter in the MDA-MB-231 breast cancer cell line and H1299 non-small cell lung cancer cell line (19). Somatic mutation-induced inactivation of p53 occurs in about 50% of human cancers including breast and prostate cancer (20). p53 mutation is considered a biomarker of advanced prostate cancer in which prostate cancer cells lose differentiated phenotypes and transit from androgen-dependent to androgen-independent growth (21). Rather than losing the wild-type p53 (WTp53), the tumor with retention of mp53 has been shown to be more aggressive and associated with poor outcome in certain cancer types (22). For example, mp53 was shown to enhance the cell migration and invasion in breast and lung cancer cell lines (23). However, the role of mp53 in mediating the key steps of cancer progression including cell migration and invasion is largely cell context-dependent and controversial (24). For example, in human endometrial cancer cells, the p53 R213Q mutation does not promote cell migration (25). As shown in another study using the H1299 cell line, p53 R175H negatively regulates cell migration when TGF-β/Smad signaling is repressed (26). At present, the cellular context for mp53 to exert its oncogenic activity is underexplored. In addition, little is known about whether mp53 alone is sufficient to activate the switch of TGF-β during tumor progression.

To gain a more thorough understanding of the effect of mp53 on tumor progression, particularly with respect to the role of TGF-β signaling, we investigated whether mp53 contributes to the conversion of the role of TGF-β in the regulation of cell growth and migration. Here, we show that mp53 alone is not sufficient to promote the oncogenic role of TGF-β. We further demonstrate that mp53 disrupts the role of ShcA in altering the signaling strength of TGF-β through ERK and Smad pathways in certain human untransformed and cancer cell lines and mice with mp53 knock-in. ShcA-mediated ERK signaling appears to play a more dominant role in conferring the tumor-promoting activity of TGF-β in the regulation of cell growth and migration. Our finding provides novel insight into the role of ShcA as a promising biomarker in driving TGF-β signaling toward tumor promotion.

**EXPERIMENTAL PROCEDURES**

*Ethics Statement—*All animal experiments were conducted following appropriate guidelines. They were approved by the Institutional Animal Care and Use Committee and monitored by the Department of Laboratory Animal Resources at the University of Texas Health Science Center at San Antonio (protocol identification numbers 99142-34-11-A and 05054-34-01-A) and the M. D. Anderson Institutional Animal Care and Use Committee (protocol identification number 079906634).

*Cell Culture—*Human untransformed mammary epithelial cell line MCF-10A was obtained from the Michigan Cancer Foundation. These cells were grown in DMEM/F-12 supplemented with 5% horse serum, EGF, NaHCO3, hydrocortisone, insulin, Fungizone, CaCl2, cholera toxin, and antibiotics. Human prostate carcinoma cell lines PC-3, DU145, and 22Rv-1 and human breast cancer cell line BT20 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The human breast cancer MCF-7 control cell line and the dominant-negative RI (DNRII)-transfected cell line were provided by Dr. Michael G. Brattain (27). All these cells were cultured in McCoy’s 5A medium with 10% fetal bovine serum (FBS) and other supplements as described previously (28). The human breast cancer cell line BT474 was obtained from ATCC and cultured in DMEM (low glucose) with 10% FBS. Cells were maintained at 37 °C in a 5% CO2 humidified incubator.

*Chemicals—*The small RI kinase inhibitor HTS466284 reported previously to be an ATP-competitive inhibitor of RI kinase (29) was synthesized by the Chemical Synthesis Core of Vanderbilt University. U0126 is an MEK-1/2 inhibitor from Calbiochem.

*Plasmids and Transfection—*p52/46ShcA plasmid was purchased from Origene. p53 R175H-pCMV-Neo-Bam plasmid was provided by Dr. Harikrishna Nakshatri. p53 R273H-pCDNA3 plasmid was provided by Dr. Zhi-Min Yuan. Human WTp53 expression plasmid pRC/CMV hp53 was obtained from Dr. Arnold Levine. Constitutively active MEK-1 plasmid was provided by Dr. Kun-Liang Guan. Stable transfection of p53 R175H into PC-3 cells was performed by using Lipo-
fectamine 2000 (Invitrogen). Forty-eight hours after the transfection, G418 selection of neomycin-resistant cells was conducted for 1 week. The sequence of p53 siRNA 1 is 5’-CCG GAC GAU AUU GAA CAA UGG UUC A-3’, and the sequence of p53 siRNA 2 is 5’-GCU-UCG AGA UGU UCC GAG AGC UGA A-3’ (Invitrogen). The sequence of ShcA siRNA is 5’-GAC UAA GGA UCA CCG CUU U-3’ (Dharmacon, Lafayette, CO). All transient transfections were performed by using Lipofectamine 2000 according to the manufacturer’s protocol. The pLKO.1-puro lentiviral RII shRNA and control shRNA were purchased from Sigma. The sequence of RII shRNA is TGA CTA GCA ACA AGT CAG GTT TTG-3’. The process of generating MCF-10A cells with stable knockdown of RII was conducted according to the manufacturer’s protocol.

Immunoblotting Analysis—Immunoblotting analyses were performed as described previously (30). Primary antibodies were obtained from the following sources: p-Smad2, p-ERK (Thr-202/Tyr-204), p-ShcA (Tyr-317), total-ERK, and MEK-1/2 from Cell Signaling Technology (Danvers, MA); p-Smad3 from Epitomics (Burlingame, CA); total Smad2/3 from BD Transduction Laboratories; p53 from Santa Cruz Biotechnology (Santa Cruz, CA); total ShcA from BD Biosciences; and RII from Abcam (Cambridge, MA). Relative expression levels of the indicated genes were quantified with ImageJ software (National Institutes of Health).

Cell Migration Assay—Cell migration assays were performed in 24-well Boyden chambers with 8-μm pore polycarbonate membranes (BD Biosciences). Cells at the indicated number in serum-free medium were seeded in the upper insert. Complete medium with or without treatment was added in the lower chamber. After 18 h, cells that had migrated through the membrane were stained with the Hema 3 Stain 18 kit (Fisher Scientific) according to the manufacturer’s protocol. Migrated cells were counted under a microscope with 100× magnification.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay—Cells were plated in a 96-well plate at 2,500 cells/well with or without TGF-β1 treatment. Two hours before each time point, 50 μl of MTT (2 mg/ml in PBS) was added into each well, and cells were incubated at 37 °C for another 2 h. DMSO (100 μl) was added into each well after the medium was removed. For dissolving the precipitate, 1 ml of 0.4% low melting agarose (Invitrogen) with culture medium were plated at 6,000 cells/well on top of existing 0.8% agarose in 6-well plates. The wells were covered with 1 ml of culture medium before each time point, 50 μg of luciferase reporter gene (pSBE4-Luc) (Invitrogen) was added into each well, and cells were incubated at 37 °C for 18 h. The wells were covered with 1 ml of culture medium. Visualized luciferase activity was normalized to β-galactosidase activity.

Animal Tissue Protein Extraction—C57BL/6 mice with a heterozygous p53R172H mutation were used (33). Heterozygous p53R172P mice were generated in a similar way (34). They were crossed to C57BL/6 p53+/+ mice (The Jackson Laboratory, Bar Harbor, ME) (35). Heterozygous breeding cohorts of p53R172P+/+ p53R172H+/+ and p53R172H+/+ were intercrossed to produce the p53R172P+/+ p53R172H+/+ and p53R172H+/+ mice. MMTV-Wnt1 mice were bred with p53R172H+/+ or p53R172H+/+ mice to generate MMTV-Wnt1 mice in p53R172H+/+, p53R172H+/+, and p53R172H+/+ backgrounds. A fraction of tumors in the p53R172H+/+ background underwent loss of heterozygosity (herein referred to as p53R172H/O) and thus are functionally p53 mutants. Loss of heterozygosity analysis was performed as described previously (36). The tumor sizes were measured regularly with a caliper in two dimensions. Tumor volumes (V) were calculated with the equation V = (L × W²) × 0.5 where L is length and W is width. When tumors reached a volume of ~500 mm³, mice were sacrificed, and the tumors were collected.

RESULTS

Mutant p53 Inhibits Cell Migration and Down-regulates ERK Signaling in Prostate Cancer Cell Lines—To investigate whether mp53 alone can promote tumor migration, we knocked down mp53 in the human prostate cancer cell line DU145 containing inactive endogenous p53 P223L and V274F mutations in its DNA-binding domain (37). We found that instead of making cells less migratory mp53 knockdown significantly enhanced cell migration accompanied by the activation of ERK via phosphorylation (p-ERK) (Fig. 1, A and B). Active ERK signaling is reported to be essential for tumor metastasis progression including cell migration (9), suggesting that the mp53 depletion-induced migration was likely caused by the up-regulated ERK signaling. We further stably introduced mutant p53 R175H with a mutation in its DNA-binding domain into the p53-null human prostate cancer cell line PC-3 (PC-3/mp53). This resulted in the repression of cell migration as well as the down-regulation of ERK signaling (Fig. 1, C and D).
Mutant p53 and ShcA Alter Oncogenic Role of TGF-β

FIGURE 1. Mutant p53 inhibits cell migration and ERK signaling in prostate cancer cell lines. A, immunoblotting analysis of the indicated gene expression in DU145 cells, which were transfected with control or p53 siRNA for 72 h. B, cell migration was assessed in DU145 cells 48 h after the transfection. Cells were plated at 40,000 cells/insert and incubated for 18 h as described under "Experimental Procedures." Data represent mean ± S.E. from three inserts. *, p < 0.05. C, immunoblotting analysis of the indicated gene expression in PC-3 cells with stable introduction of p53 R175H expression plasmid (PC-3/mp53) or empty vector. D, cell migration was assessed in PC-3/mp53 and control cells plated at 70,000 cells/insert and incubated for 18 h. Data represent mean ± S.E. from three inserts. *, p < 0.05. T-ERK, total ERK. Error bars represent S.E.

These data indicate that mp53 in certain prostate cancer cell lines attenuates cell migration, which is a key step of pro-cancer metastasis.

Mutant p53 Represses Oncogenic Role of TGF-β and Enhances TGF-β/Smad Signaling in Prostate Cancer Cell Line—The presence of mp53 has been shown to facilitate the tumor-promoting activity of TGF-β in some breast cancer models (19). Because mp53 was found not to enhance cell migration in our studies with two prostate cancer cells, we explored whether mp53 showed a different effect on the oncogenic role of TGF-β. We observed that TGF-β significantly increased migration of the control PC-3 cells, whereas it suppressed migration in the PC-3/mp53 cells, suggesting that the activity of TGF-β is affected by the presence of mp53 (Fig. 2A). Because PC-3 is a tumorigenic cell line, we next performed a soft agar colony formation assay to assess the effect of p53 R175H on the growth of PC-3 cells in an anchorage-independent manner. We found that the presence of p53 R175H repressed the anchorage-independent growth ability of PC-3/mp53 cells compared with cells without mp53. Interestingly, the colony formation was more dramatically inhibited by TGF-β, and the treatment with HTS466284, an RI kinase inhibitor (29), significantly stimulated colony formation in PC-3/mp53 cells (Fig. 2, B and C). To rule out the possibility that the p53 R175H-reduced cell migration in PC-3 cells was due to an altered cell growth rate, we verified that the presence of p53 R175H in PC-3 cells showed little effect on cell growth but that TGF-β treatment induced a moderate growth inhibition in a dose-dependent manner (Fig. 2D). Thus, these results indicate that p53 R175H alone is not sufficient to switch TGF-β to be more tumor-promoting in the PC-3 cell line. Considering that TGF-β-inhibited anchorage-dependent and -independent cell growth is mainly due to Smad-dependent signaling (38, 39), we investigated whether p53 R175H could alter the activation of TGF-β/Smad signaling in PC-3/mp53 cells. We found that p53 R175H made PC-3 cells more sensitive to TGF-β in a dose-dependent manner as evidenced by higher levels of TGF-β-induced phosphorylation of Smad2/3 (Fig. 2E). Additionally, the transcriptional activity of TGF-β was also increased in PC-3/mp53 cells when compared with the control cells as detected with transfection of a Smad-responsive promoter-luciferase reporter plasmid (SBE-Luc) (Fig. 2F), further indicating that the presence of p53 R175H enhanced the Smad-dependent signaling. These results revealed that the addition of p53 R175H to PC-3 cells enhanced TGF-β/Smad-signaling while inhibiting TGF-β-induced cell migration.

Mutant p53 Represses Activation of ShcA/ERK Signaling—It is suggested that Smad-dependent and -independent signaling pathways work together to drive the key events of TGF-β-induced cell migration and metastasis (8). However, our observations indicate that Smad-dependent signaling is not responsible for the loss of TGF-β-induced migration in PC-3/mp53 cells as reflected by the enhanced TGF-β/Smad signaling. Consequently, we speculated that MAPK/ERK signaling might be involved in the loss of TGF-β-induced cell migration of PC-3/mp53 cells. Consistent with our observation above (Fig. 1C), we found that the basal level of p-ERK was markedly reduced in the PC-3/mp53 cells when compared with control cells (Fig. 3A). Additionally, TGF-β was unable to further activate ERK in PC-3/mp53 cells (Fig. 3A). To confirm that this observation was specifically due to mp53 expression and not an aberrant selection of a pool of antibiotic-resistant clones, we transiently introduced p53 R175H into PC-3 cells and found that p-ERK was greatly reduced when compared with PC-3 cells transfected with a control plasmid or a WT-p53 expression plasmid (Fig. 3B). In parallel, p53 R175H knockdown in PC-3/mp53 cells with p53 siRNA restored the p-ERK level (Fig. 3C). We additionally used another p53 mutation in the DNA-binding domain, p53 R273H, which also enhanced TGF-β-stimulated phosphorylation of Smad2/3 and inhibited the basal and TGF-β-stimulated p-ERK levels. Consistently, p53 R273H also significantly attenuated TGF-β-induced cell migration of PC-3 cells in comparison with the control cells (Fig. 3D and supplemental Fig. 1A). However, exogenous human WT-p53 expression in PC-3 cells showed no effect on the level of TGF-β-induced cell migration, TGF-β-induced activation of ERK, or Smad signaling (Fig. 3E and supplemental Fig. 1B). Because tyrosine-phosphorylated p52ShcA has been shown to positively mediate TGF-β-activated Ras/ERK signaling (10), we next examined whether the mp53-inhibited phosphorylation of ERK was linked to the down-regulation of p-p52ShcA. Indeed, the expression of p53 R175H and p53 R273H, but not WT-p53, in PC-3 cells inhibited TGF-β-induced phosphorylation of p52ShcA.
compared with control cells (Fig. 3F). Thus, these data revealed that mp53, but not WTp53, has the ability to down-regulate ShcA-mediated ERK signaling.

Expression of MEK-1 or ShcA Restores Active ERK Level and TGF-β-induced Cell Migration—To determine whether the attenuated ERK signaling led to the loss of TGF-β-induced cell migration in PC-3/mp53 cells, we examined whether TGF-β-induced cell migration could be rescued by the reactivation of ERK. As shown in Fig. 4A, ectopic expression of the ERK activator, a constitutively active form of MEK-1 (*MEK-1), was able to restore the level of p-ERK in PC-3/mp53 cells. More importantly, compared with control plasmid-transfected cells, PC-3/mp53 cells with *MEK-1 expression-rescued p-ERK became responsive again to TGF-β in cell migration (Fig. 4B). These results suggest the need for active ERK signaling to mediate the migration-promoting activity of TGF-β in the presence of mp53. To further confirm our results, we used DU145 cells to test whether manipulating the active ERK by elevated ShcA activation also affected TGF-β-induced cell migration in a different mp53-containing model system. We found that TGF-β slightly induced the phosphorylation of ShcA and did not induce the phosphorylation of ERK (Fig. 4C). Neither was TGF-β able to induce DU145 cell migration (Fig. 4D), recapitulating the results with PC-3/mp53 (Fig. 2A). Conversely, ectopic overexpression of p52/46ShcA in the presence of TGF-β expression resulted in the clear activation of p52ShcA and ERK as well as the stimulation of cell migration. These findings are highly consistent with the findings from PC-3/mp53 cells with reactivated ERK (Fig. 4B). Our observations thus far have demonstrated that TGF-β is able to induce cell migration in the context of mp53 when p-p52ShcA is elevated and that this correlates with activation of ERK. To verify that the increase of TGF-β-induced cell migration after overexpression of p52/46ShcA was in fact mediated by the activation of ERK, we treated the PC-3 cells with an ERK signaling inhibitor, U0126, and found that TGF-β-induced cell migration was totally abolished (Fig. 4E). Thus, these findings indicate that TGF-β-induced cell migration is dependent on its activation of ERK signaling in prostate cancer cells in which mp53 tends to attenuate TGF-β-induced cancer malignancy.
Mutant p53 and ShcA Alter Oncogenic Role of TGF-β

ShcA Alters Role of TGF-β in Cellular Migration and Anchor-age-dependent and -independent Growth in Transformed Cells—To this point, we have demonstrated that mp53-induced alteration of Smad-dependent and -independent TGF-β signaling is due to altered ShcA activation. Therefore, we next investigated whether ShcA could serve as a biomarker in converting the role of TGF-β from growth suppression to migration promotion in cancer cells. To this end, we examined the effect of manipulating the expression level of ShcA in PC-3 cells on growth and migration. Interestingly, we found that knockdown of ShcA isoforms by pan-ShcA siRNA decreased TGF-β-induced phosphorylation of Smad2/3 (Fig. 5A). Conversely, the ectopic overexpression of p52/46ShcA raised TGF-β-induced phosphorylated ERK levels and reduced TGF-β-induced phosphorylated Smad2/3 levels (Fig. 5B). Consistently, we found that knockdown of p52/46ShcA enhanced, but ectopic overexpression of p52/46ShcA reduced, TGF-β-inhibited anchorage-dependent (Fig. 5, C and D) and -independent cell growth (Fig. 5, E and F). TGF-β-induced cell migration was significantly diminished in ShcA-depleted cells but significantly increased in p52/46ShcA-overexpressing cells (Fig. 5, G and H). Because anchorage-independent growth ability is associated with tumorigenicity in vivo and cell migration is a key step of tumor progression, our results suggest that ShcA attenuates the tumor suppressor activity of TGF-β while enhancing its tumor promoter activity.

ShcA Alters Role of TGF-β in Cellular Migration and Cell Growth in Untransformed Cells—To further investigate the effect of ShcA on TGF-β-mediated cellular growth and migration, we introduced pan-ShcA siRNA or p52/46ShcA cDNA into untransformed MCF-10A cells, which lack complicated alterations of tumor suppressor genes and oncogenes. By using this spontaneously immortalized and non-tumorigenic human mammary epithelial cell line, we were able to examine the transition of the role of TGF-β in the early stage of transformation. We essentially observed the same phenotypic changes as in the transformed PC-3 cells. Specifically, knockdown of ShcA isoforms down-regulated TGF-β-induced phosphorylation of ERK and up-regulated TGF-β-induced phosphorylation of Smad2/3 (Fig. 6A). In cells with knockdown of ShcA, we found that TGF-β-inhibited cell growth was significantly enhanced (Fig. 6C) and that TGF-β-inhibited cell migration was significantly reduced (Fig. 6E) in comparison with the control siRNA-transfected cells. In contrast, when compared with vehicle-transfected cells, ectopic overexpression of p52/46ShcA augmented TGF-β-induced phosphorylation of ERK (Fig. 6B) and TGF-β-induced cell migration (Fig. 6F) but repressed TGF-β-induced phosphorylation of Smad2/3 (Fig. 6B) and significantly attenuated TGF-β-inhibited cell growth (Fig. 6D). Thus, our data suggest that ShcA can alter the role of TGF-β in controlling cellular growth and migration by balancing its signaling between the ERK and Smad pathways. We next asked whether TGF-β-induced tyrosine phosphorylation of p52ShcA by RII requires RII. Therefore, we knocked down RII in MCF-10A cells by using an RII shRNA and found that the depletion of RII led to the attenuation of TGF-β-induced phosphorylation of p52ShcA and ERK (Fig. 6G).

Additionally, we have previously shown that ectopic expression of a DNRII blocked TGF-β signaling and reduced the level of active ERK in the human MCF-7 breast cancer cell line, which contains a high level of autocrine TGF-β activity (40). Interestingly, we found that this DNRII-expressing MCF-7 cell line also has a lower level of the tyrosine-phosphorylated p52ShcA than the control vector-transfected cells (Fig. 6H). Thus, our observations suggest that RII is required for the activation of p52ShcA by TGF-β.

Mutant p53 Positively Correlates with Activation of Smad-dependent Signaling and Negatively Correlates with Active ShcA/ERK Signaling in Human Cancer Cell Lines and Transgenic Mouse Models—To further generalize our findings, we tested the correlation among the presence of mp53, activation of ShcA/ERK signaling, and Smad-dependent signaling in a
panel of human breast and prostate cancer cell lines, each expressing varying p53 mutations, although all mutations were within the DNA-binding domain. Human breast cancer cell lines BT20 with p53 K132Q mutation, BT474 with p53 E285K mutation (41), and DU145 were used in our study. We also included human prostate cancer cell line 22Rv1 with p53 Q331R mutation in the dimerization domain (42). mp53 knock-down in those cell lines increased the phosphorylation of p52ShcA and ERK, whereas it decreased the phosphorylation of Smad2 or Smad3 (Fig. 7A). In mammary gland tissues from WTP53+/+, p53R172H/+R172H mutant, or p53R172P/+R172P mutant (equivalent to human p53 R175H and R175P mutations, respectively) female mice on a C57BL/6 genetic background, we found that the total ERK-normalized phosphorylation of ERK was down-regulated, whereas the total Smad2/3-normalized phosphorylation of Smad3 was up-regulated in the tissues bearing the mp53 when compared with WTP53-expressing tissues (Fig. 7B). We also normalized the p-ERK and p-Smad3 expression levels with GAPDH protein levels and obtained a similar outcome (supplemental Fig. 2A). The p-ShcA expression levels were undetectable, and we speculated that the normal müllerian mammary tissues might express low levels of p-ShcA (data not shown). Furthermore, to evaluate the correlation among mp53, ShcA/ERK signaling, and Smad signaling in a more clinically relevant setting, we collected mammary tumor tissues with WTP53+/+, p53R172H/+R172H mutation, or p53R172H/+ (p53 R172H heterozygous with loss of heterozygosity) on the background of the MMTV-Wnt1 transgenic mouse, a well established model of breast cancer development and progression. The presence of mp53 showed negative correlation with the active ShcA/ERK signaling and positive correlation with the active Smad-dependent signaling (Fig. 7C and supplemental Fig. 2B). These observations further support the conclusion that p53 mutation disrupts the role of ShcA in balancing the Smad-dependent and Smad-independent signaling activity of TGF-β.

**DISCUSSION**

The cross-talk between p53 mutation and oncogenic Ras/ERK signaling has been demonstrated to promote TGF-β-induced cell migration and metastasis in certain breast cancer models (19). However, it is still unclear whether mp53 alone can act as a tumor promoter and cause TGF-β signaling to become oncogenic. Several studies have revealed that the malignancy gained from mp53 is cell context-dependent and controversial (24). For example, mp53 can enhance the cell migration and invasion via up-regulation of the epithelial-mesenchymal transition factor Slug in human non-small cell lung cancer and breast cancer cell lines (23). In contrast, p53 R312Q mutation does not positively regulate migration of human endometrial cancer cells (25). p53 R175H mutation was shown to inhibit the migration of the H1299 lung cancer cell line when its RII was down-regulated and TGF-β/Smad signaling was repressed (26). Here, we focused on the p53 DNA-binding domain mutations,
which have been reported as the majority of p53 mutations in human breast and prostate cancer cell lines (43). We found that in DU145 and PC-3 human prostate cancer cells the endogenous or ectopic expression of p53 with a point mutation in its DNA-binding domain does not enhance the cell migration or cause TGF-β to be more migration-promoting. Hence, our results from these models indicate that mp53 alone does not always function as a promoter of migration in already transformed cells, and its function is likely context-dependent. Indeed, p53 mutation has been shown to occur relatively late during multistage oncogenic progression and often follows Ras mutation-induced ERK signaling (44). It has been shown that mp53 works together with oncogenic Ras to induce the expression of several protumorigenesis and prometastasis genes in gene expression profiling studies (45). Our finding that mp53 repressed the oncogenic role of TGF-β in the model systems we used suggests that additional signaling activation and genetic alterations such as ERK signaling activation and attenuation of TGF-β-induced growth inhibition appear necessary to collaboratively confer the tumor-promoting activity of mp53 and TGF-β during cancer progression. Although the sequestration of metastasis suppressor p63 by the formation of an mp53-Smad-p63 ternary complex has been shown to enhance the oncogenic activity of TGF-β (19), an additional mechanism involving mp53 but not the formation of the ternary complex has also been shown to drive TGF-β to become more tumor-promoting (46). DU145 cells are negative for p63 expression (47). Thus, the conversion of TGF-β is independent of the ternary complex formation in DU145 cells with restored ShcA/ERK signaling. The presence of mp53 repressed the basal level of phosphorylated p52ShcA and also abolished the activation of p52ShcA by TGF-β, suggesting that mp53 could be the upstream regulator of p52ShcA. We speculate that the mechanism by which mp53 down-regulates the phosphorylation of p52ShcA is perhaps through an aberrant or dysregulated protein interaction. However, further investigation is needed to elucidate the mechanism.

TGF-β receptors have been shown to possess dual tyrosine and serine kinase activity and can directly tyrosine phosphorylate ShcA to activate Ras/MAPK signaling or serine phosphorylate ShcA with an unclear consequence (10). The same study suggests that tyrosine phosphorylation of p66Shc, the inhibitory isoform of ShcA, is more dependent on RI in untransformed cells. On the other hand, it is unclear whether RI alone is sufficient to induce tyrosine phosphorylation of p52ShcA. In our study, the depletion of RII in the untransformed human breast cancer cell line that has autocrine TGF-β activity, we found that the dysfunctional RII caused the inhibition of ShcA/ERK activation. Our observations suggest that RII is required for TGF-β to activate the p52ShcA/ERK pathway. It is worth mentioning that other tyrosine kinase receptors such as epidermal growth factor receptor and insulin receptor also have the ability to tyrosine phosphorylate p52/46ShcA and consequently activate Ras/
ERK signaling (48). We speculate that RI and other tyrosine receptors may compete for available ShcA. This competition may further influence the signaling strength of TGF-β between Smad and ERK signaling. Currently, how those pathways may affect the Smad-dependent and -independent signaling of TGF-β via p52/46ShcA is also under investigation.

The activation of TGF-β/Smad-dependent signaling requires the serine/threonine kinase activity of RII and RI. Tyrosine kinases, instead of serine/threonine kinases, have been reported as the catalytic center of RI in certain structural features (49). Conceivably, R-Smads and ShcA may compete for RI kinase, and the balance between the tyrosine phosphorylation of ShcA and serine phosphorylation of R-Smads could determine the signaling strength of TGF-β through the Smad-independent and -dependent pathways, respectively.

It is well known that TGF-β-induced cell growth inhibition in epithelial cells depends on Smad-dependent signaling (38, 39). Loss of Smad4 during cancer progression has been shown to contribute to the resistance of TGF-β-inhibited cell growth, which is considered one mechanism for the TGF-β to be tumor-promoting (1). However, it is less clear what other mechanistic biomarkers can indicate the change of TGF-β signaling in favor of tumor progression during tumorigenesis. Our study showed that knockdown of ShcA isoforms enhanced, whereas overexpression of p52/46ShcA attenuated, TGF-β-inhibited anchorage-dependent and independent-growth of tumor cells, implicating ShcA as a negative mediator of TGF-β-induced tumor suppression. For the Smad-dependent TGF-β signaling, TGF-β-mediated cell cycle arrest involves a Smad-dependent induction of cyclin-dependent kinase inhibitors p15 and p21. On the other hand, the activation of MAPK/ERK signaling is pivotal for the TGF-β-induced epithelial-mesenchymal transition and cell migration, both of which are considered important steps of prometastatic progression (1). Thus, it is conceivable that the mechanism by which p52/46ShcA regulates TGF-β-induced cell migration or growth inhibition is cell context-dependent. For DU145, PC-3, and MCF-10A cells, we found that the cells are sensitive to TGF-β as evidenced by the increased p-Smad2/3 and that manipulating ShcA enhanced TGF-β-induced cell migration accompanied by increased TGF-β/ShcA/ERK signaling and decreased TGF-β/Smad signaling. Because TGF-β/Smad-dependent stimulation of p21 expression has been observed in these cells and p21 was shown to be up-regulated by the Smad3-FoxO Forkhead transcription factor transcriptional complex (50), it is likely that the altered cell growth we observed was due to the manipulation of p52/46ShcA and altered Smad/FoxO/p21 cascade. On the other hand, in cancer cells with Smad4 mutation or aberrant regulation of p21 and/or p15, the alteration of Smad signaling due to a change in ShcA activity may not result in TGF-β-inhibited cell growth.

It is believed that p66ShcA is not involved in the activation of MAPK signaling (51). In fact, p66ShcA was shown to expression was performed in MCF-10A cells with or without RII knockdown after TGF-β1 treatment for 40 min. H, immunoblotting analysis for the indicated gene expression was performed in MCF-7 cells with or without DNRII expression. T-ERK, total ERK; T-Smad2/3, total Smad2/3; T-p52ShcA, total p52ShcA; con, control. Error bars represent S.E.

FIGURE 6. ShcA alters TGF-β-mediated Smad and ERK signaling and activity in untransformed human cells. MCF-10A cells were transfected with a pan-ShcA siRNA or a p52/46ShcA expression plasmid as shown in A and B, respectively. Seventy-two hours post-transfection, immunoblotting analysis for the indicated gene expression was performed using the transfected cells treated with or without 2 ng/ml TGF-β1 for 40 min. C and D, TGF-β1 (2 ng/ml)-induced cell growth inhibition was measured with the MTT assay in cells 72 h after transfection. Data are presented as the percentage of TGF-β1-induced cell growth inhibition relative to the untreated cells. Data represent mean ± S.E. from four wells. E and F, cell migration was assayed in cells 48 h after transfection. Cells were plated at 70,000 cells/insert with or without TGF-β1 (2 ng/ml) for 18 h. Data are presented as the fold change of TGF-β-induced migration relative to untreated cells. Data represent mean ± S.E. from three inserts. *, p < 0.05; **, p < 0.01. G, immunoblotting analysis of the indicated gene
Mutant p53 and ShcA Alter Oncogenic Role of TGF-β

Consistent with a previous study demonstrating that knockdown of ShcA or a dominant-negative form of ShcA inhibited TGF-β-induced cell migration and invasion of murine breast cancer cells bearing activated Neu/ErbB-2 receptor (56), our study showed that ShcA depletion-caused down-regulation of ERK signaling resulted in the attenuation of TGF-β-induced cell migration in human untransformed and transformed cells. On the other hand, we found that increased ShcA/ERK signaling augmented TGF-β-induced cell migration. We further showed that in DU145 cells the restored activation of p52/46ShcA/ERK signaling completely switched the role of TGF-β from suppression to promotion of cell migration. ERK signaling-induced cell migration is a key sign of tumor progression (9); therefore, our results provide a novel concept that the ShcA/ERK pathway acts as a pivotal driver of the oncogenic role of TGF-β. In an unpublished study,3 we found that knockdown of Smad4 in MCF-10A cells moderately reduced TGF-β-induced cell migration in comparison with control siRNA-transfected cells, indicating that Smad-dependent signaling partially contributes to TGF-β-induced cell migration. Here, we showed that the ShcA-mediated increase of TGF-β-induced migration was in the presence of down-regulated Smad signaling. These observations suggest that ShcA-enhanced ERK signaling appears to play a dominant role in TGF-β-induced cell migration. Thus, the down-regulation of p-Smad2/3 is not likely necessary for the ShcA/ERK signaling-mediated increase of TGF-β-induced migration.

In our study, we found that mp53 positively correlates with phosphorylation of R-Smads, whereas it negatively correlates with phosphorylation of p52ShcA and ERK in a panel of mp53-expressing breast and prostate cancer cell lines as well as in mp53 mouse models. We demonstrated that ShcA depletion-induced cell migration in the presence of Smad2/3 knockdown of Smad4 in MCF-10A cells moderately reduced TGF-β-induced cell migration in comparison with control siRNA-transfected cells, indicating that Smad-dependent signaling partially contributes to TGF-β-induced cell migration. Here, we showed that the ShcA-mediated increase of TGF-β-induced migration was in the presence of down-regulated Smad signaling. These observations suggest that ShcA-enhanced ERK signaling appears to play a dominant role in TGF-β-induced cell migration. Thus, the down-regulation of p-Smad2/3 is not likely necessary for the ShcA/ERK signaling-mediated increase of TGF-β-induced migration.

In our study, we found that mp53 positively correlates with phosphorylation of R-Smads, whereas it negatively correlates with phosphorylation of p52ShcA and ERK in a panel of mp53-expressing breast and prostate cancer cell lines as well as in mp53 mouse models. We demonstrated that ShcA depletion-induced cell migration in the presence of down-regulated Smad signaling. These observations suggest that ShcA-enhanced ERK signaling appears to play a dominant role in TGF-β-induced cell migration. Thus, the down-regulation of p-Smad2/3 is not likely necessary for the ShcA/ERK signaling-mediated increase of TGF-β-induced migration.

FIGURE 7. Mutant p53 correlates with higher level of Smad-dependent signaling and lower level of ShcA/ERK signaling in human cancer cell lines and genetically altered mouse models. A, immunoblotting analysis for the indicated gene expression was performed in BT-20, BT474, 22RV1, and DU145 cells 72 h after transfection of p53 siRNA 1 or control siRNA. B, immunoblotting analysis for the indicated gene expression was performed in the tissue lysates from mammary glands of p53R172H/H11001; p53R172P; p53R172P/H11005, p53R172H/H11005, and p53R172P/H11005. The scatter plot figures under the immunoblots are the total p52ShcA-normalized p-Smad3 levels, total ERK-normalized p-ERK levels, and total Smad2/3-normalized p-Smad3 levels, quantified with ImageJ software. *p < 0.05. C, immunoblotting analysis for the indicated gene expression was performed in the tissue lysates from mammary tumors of MMTV-Wnt1 (WTp53; n = 8), MMTV-Wnt1/p53R172H/H11005 (p53R172H/H; n = 2), and MMTV-Wnt1/p53R172P/H11005 (p53R172P; n = 1) mice and the littermate (WTp53; n = 5). The scatter plot figures under the immunoblots are the total p52ShcA-normalized p-Smad3 levels, total ERK-normalized p-ERK levels, and total Smad2/3-normalized p-Smad3 levels for each sample quantified with ImageJ software. *p < 0.05. 3 S. Lin, L. Yu, J. Yang, and L.-Z. Sun, unpublished data.
obstacle for the utility of TGF-β antagonists for treating metastatic carcinomas. Thus, our study indicates that the TGF-β-dependent increase of tyrosine-phosphorylated p52/46ShcA may be a promising biomarker for an effective anti-TGF-β cancer therapy. Biomarkers identified by this type of research may help cancer patients benefit from anti-TGF-β therapy in blocking tumor progression in the future.

Acknowledgments—We thank Dr. Andrew Hinck (University of Texas Health Science Center at San Antonio, UTHSCSA) for the recombinant TGF-β1, Dr. Michael G. Brattain (University of Nebraska, Omaha) for the MCF-7 cell lines, Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, Maryland) for the p53BE4-Luc plasmid, Dr. Harirkrsn Naokhatri (Indiana University, Indianapolis, IN) for the p53 R175H plasmid, Dr. Lung-Min Yuan (UTHSCSA) for the p53 R273H plasmid, Dr. Arnold Levine (Institute for Advanced Study, Princeton, NJ) for the human WTp53 plasmid and Dr. Kun-Liang Guan (University of California, San Diego) for the constitutively active MEK1 plasmid.

REFERENCES
1. Massagué, J. (2008) Cell 134, 215–230
2. Sun, L. (2004) Front. Biosci. 9, 1925–1935
3. Bandyopadhyay, A., Aguin, J. K., Wang, L., Tang, Y., Lei, X., Story, B. M., Cornell, J. E., Pollock, B. H., Mundy, G. R., and Sun, L. Z. (2006) Cancer Res. 66, 6714–6721
4. Muraoka, R. S., Dumont, N., Ritter, C. T., Brantley, D. M., Chen, J., Easterly, E., Roebuck, L. R., Ryan, S., Gotwals, P. J., Koteliansky, V., and Arteaga, C. L. (2002) J. Clin. Investig. 109, 1551–1559
5. Yang, Y. A., Dukhanina, O., Tang, B., Mamura, M., Letterio, J. J., Yang, P. C. (2009) Nat. Cell Biol. 11, 694–704
6. D’Andrea, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hannon, B., Solari, A., Bobis, S., Rondina, M. B., Buzzard, V., Parenti, A. R., Rosato, A., Bicciato, S., Balmain, A., and Piccolo, S. (2009) Cell 137, 87–98
7. Levine, A. J. (1997) Cell 88, 323–331
8. Navone, N. M., Tronocono, P., Pisters, T. L., Goodrow, T. L., Palmer, J. L., Nichols, W. W., von Eschenbach, A. C., and Conti, C. J. (1993) J. Natl. Cancer Inst. 85, 1657–1669
9. Soussi, T., and Béroud, C. (2001) Nat. Rev. Cancer 1, 233–240
10. Wang, S. P., Wang, W. L., Chang, Y. L., Wu, C. T., Chao, Y. C., Kao, S. H., Yuan, A., Lin, C. W., Yang, S. C., Chan, W. K., Li, K. C., Hong, T. M., and Yang, P. C. (2009) Nat. Cell Biol. 11, 694–704
11. Oren, M., and Rotter, V. (2010) Cold Spring Harb. Perspect. Biol. 2, a001107
12. Dong, P., Tada, M., Hamada, J., Nakamura, A., Moriuchi, T., and Sakuragi, N. (2007) Clin. Exp. Metastasis 24, 471–483
13. Kalo, E., Buganim, Y., Shapira, K. E., Bersigrell, H., Goldfinger, N., Weisz, L., Stambolsky, P., Hens, Y. I., and Rotter, V. (2007) Mol. Cell. Biol. 27, 8228–8242
14. Y, K., Koli, K. M., Banerji, S. S., Li, W., Zborowska, E., Willson, J. K., Brattain, M. G., and Arteaga, C. L. (1998) Int. J. Oncol. 12, 87–94
15. Bandyopadhyay, A., Wang, L., López-Casillas, F., Mendoza, V., Yeh, I. T., and Sun, L. (2005) Prostate 63, 81–90
16. Singh, J., Chauqui, C. E., Boriack-Sjodin, P. A., Lee, W. C., Ponz, T., Corbley, M. J., Cheung, H. K., Ardini, M. R., Mead, J. N., Newman, M. N., Papadatos, J. L., Bowes, S., Josiah, S., and Ling, L. E. (2003) Bioorg. Med. Chem. Lett. 13, 4355–4359
17. Lei, X., Bandyopadhyay, A., Le, T., and Sun, L. (2002) Oncogene 21, 7514–7523
18. Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. (1998) Mol. Cell 1, 611–617
19. Chen, C., Wang, X. F., and Sun, L. (1997) J. Biol. Chem. 272, 12862–12867
20. Yang, G. A., Iwakuma, T., Suh, Y. A., Liu, G., Rao, V. A., Parant, J. M., Valentín-Vega, Y. A., Terzian, T., Caldwell, L. C., Strong, L. C., El-Naggar, A. K., and Lozano, G. (2004) Cell 119, 861–872
21. Liu, G., Parant, J. M., Lang, G., Chau, P., Perez-Chvez-Reyes, A., El-Naggar, A. K., Multani, A., Chang, S., and Lozano, G. (2004) Nat. Genet. 36, 63–68
22. Aubrecht, J., Secretan, M. B., Bishop, A. J., and Schiestl, R. H. (1999) Cancer Metastasis Rev. 20, 2229–2236
23. Post, S. M., Quintés-Cardama, A., Terzian, T., Smith, C., Eischen, C. M., and Lozano, G. (2010) Oncogene 29, 1260–1269
24. Isaacs, W. B., Carter, B. S., and Ewing, C. M. (1991) Cancer Res. 51, 4716–4720
25. Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997) Proc. Natl. Acad. Sci. USA. 94, 10669–10674
26. Ramachandra, M., Atencio, I., Rahman, A., Vaillancourt, M., Zou, A., Avanzini, J., Wills, K., Bookstein, R., and Shabram, P. (2002) Cancer Res. 62, 6045–6051
27. Lei, X., Yang, J., Nichols, R. W., and Sun, L. Z. (2007) Exp. Cell Res. 313, 1687–1695
28. Bartek, J., Iggo, R., Gannon, J., and Lane, D. P. (1990) Oncogene 5, 893–899
29. van Bokhoven, A., Varella-Garcia, M., Korch, C., Johannes, W. U., Smith, E. E., Miller, H. L., Nordeen, S. K., Miller, G. J., and Lucia, M. S. (2003) Prostate 57, 205–225
30. Berglind, H., Pawitan, Y., Kato, S., Ishioka, C., and Soussi, T. (2008) Cancer Biol. Ther. 7, 699–708
31. Vogelstein, B., and Kinzler, K. W. (1993) Trends Genet. 9, 138–141
32. Buganim, Y., Solomon, H., Rais, Y., Kistner, D., Nachmany, I., Brait, M., Madar, S., Goldstein, I., Kalo, E., Adam, N., Gordin, M., Rivlin, N., Kogan, L., Brosh, R., Sefadia-Elad, G., Goldfinger, N., Sidransky, D., Kloog, Y., and Rotter, V. (2010) Cancer Res. 70, 2247–2248
33. Muller, P. A., Caswell, P. T. Jr., Boyd, L., Iwanicki, M. P., Tan, E. H., Karim, S., Lukaschuk, N., Gillespie, D. A., Ludwig, R. L., Gosselin, P., Cramer, A., Brugger, J. S., Sansom, O. J., Norman, J. C., and Voutsoud, K. H. (2009) Cell 139, 1327–1341
34. Signoretti, S., Waldegrin, D., Dilks, J., Isaac, B., Lin, D., Garraway, L., Yang, A., Montironi, R., McKeon, F., and Loda, M. (2000) Am. J. Pathol. 157, 1769–1775
35. Okada, S., Yamauchi, K., and Pessin, J. E. (1995) J. Biol. Chem. 270, 20737–20741
36. Huse, M., Chen, Y. G., Massagué, J., and Kuriyan, J. (1999) Cell 96, 425–436
37. Seoane, J., Corbley, M. J., Massagué, J., and Arteaga, C. L. (2004) Cell 117, 211–223
38. Jackson, J. G., Yoneda, T., Clark, G. M., and Yee, D. (2000) Clin. Cancer Res. 6, 1769–1776
52. Migliaccio, E., Mele, S., Salcini, A. E., Pelicci, G., Lai, K. M., Superti-Furga, G., Pawson, T., Di Fiore, P. P., Lanfrancone, L., and Pelicci, P. G. (1997) EMBO J. 16, 706–716
53. Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L., and Pelicci, P. G. (1999) Nature 402, 309–313
54. Ma, Z., Liu, Z., Wu, R. F., and Terada, L. S. (2010) Oncogene 29, 5559–5567
55. Rajendran, M., Thomes, P., Zhang, L., Veeramani, S., and Lin, M. F. (2010) Cancer Metastasis Rev. 29, 207–222
56. Northey, J. J., Chmielecki, J., Ngan, E., Russo, C., Annis, M. G., Muller, W. J., and Siegel, P. M. (2008) Mol. Cell. Biol. 28, 3162–3176