Gain of Oncogenic Function of p53 Mutants Induces Invasive Phenotypes in Human Breast Cancer Cells by Silencing *CCN5/WISP-2*

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Abstract

CCN5/WISP-2 is overexpressed in noninvasive breast cancer cells and tissue samples, whereas its expression is minimal or undetected in invasive conditions. *CCN5/WISP-2* has been considered as an antiinvasive gene because CCN5/WISP-2 silencing augments the invasive phenotypes *in vitro*. However, the mechanism of silencing of CCN5 during the progression of the disease has been elusive. Because p53 mutations are associated with breast cancer progression and have been shown to correlate inversely with CCN5/WISP-2 expression in other cancer cell types, the objective of this study was to explore whether p53 mutants suppress CCN5 expression in breast tumor cells resulting in the progression of this disease. We found CCN5 expression is inversely correlated with the mutational activation of p53 in human breast tumor cells. The ectopic expression of p53 mutants in ER-positive noninvasive breast tumor cells silenced the CCN5/WISP-2 expression and enhanced invasive phenotypes, including the induction of morphologic changes from the epithelial-to-mesenchymal type along with the alterations of hallmark proteins of these cell types and an augmentation of the migration of these cells. The suppression of CCN5 by the p53 mutants can be nullified by estrogen signaling in these cells through the transcriptional activation of the *CCN5* gene. Moreover, the invasive changes can be imitated by blocking the CCN5/WISP-2 expression through RNA interference or can be reversed by the addition of CCN5/WISP-2 recombinant protein in the culture. Thus, these studies suggest that *CCN5* inactivation could be an essential molecular event for p53 mutant–induced invasive phenotypes. [Cancer Res 2008;68(12):4580–7]

Introduction

A strict regulation of the gene expression profile is crucial for cellular architecture, their controlled proliferation and differentiation during the development, and maintenance of a eukaryotic organism. Malfunctions in these cascades of events seem to play a vital role in the carcinogenic switch in different organs, including breast. Multiple signaling proteins, with their stage-specific expressions and functions, are emerging as key players in the development of human breast cancers. One such signaling protein is CCN5 (previously know as WISP-2), a member of the cysteine-rich 61/connective tissue growth factor/nephroblastoma-overexpressed (CCN) family of growth factors (1, 2). CCN5/WISP-2 is a 29-kDa secreted protein and is very structurally similar to the other members of the CCN family, except for the cysteine knot (CT) domain, which is absent in this protein (2–4). CCN5/WISP-2 is overexpressed in preneoplastic disorders in human breast, including atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS) compared with adjacent invasive cancer cells where expression levels were either undetected, minimally detected, or only sporadically detected (5). Consistent with *in vivo* results, further studies have shown that CCN5/WISP-2 is differentially expressed in various breast tumor cell lines and its expression profile is varied depending on the microenvironment and the aggressive nature of the cells (1, 6). For example, CCN5/WISP-2 is constitutively expressed in less aggressive human breast cancer cells (i.e., MCF-7 and ZR-75-1), whereas its expression was minimally detected in the moderately aggressive breast cancer cell line (i.e., SKBR-3) and undetected in the highly aggressive breast cancer cell line (i.e., MDA-MB-231; refs. 6, 7). Notably, our studies have established that CCN5 is a two-faced signaling molecule, and one of the major functions of the CCN5 protein is to block aggressive behavior, such as the mesenchymal-to-epithelial transition (MET), of cancer cells (8). These findings were further supported by a recent study (9). However, the pathophysiologic implication, as well as the molecular mechanism, of the down-regulation of CCN5 during the course of progression of this disease remains elusive. CCN5 protein expression is inversely correlated with the mutant p53 overproduction in pancreatic adenocarcinoma samples (8). The gene array and mathematical model studies have suggested that CCN5 expression can be abrogated by p53 protein (10). It was, therefore, anticipated that overexpression of oncogenically mutated forms of *p53* gene may be associated with the silencing of CCN5 during the progression of cancer.

The p53 transcriptional factor is a tumor suppressor protein and a vital competent for the regulation of normal cellular physiology (11–14). It responds to multiple cellular stresses, including DNA damage, hypoxia, heat shock, ionizing radiation as well as oncogenic stimulation by regulating genes linked to cell cycle, apoptosis, and other essential molecular pathways associated with the cell fate (11, 12, 15, 16). Functions of normal p53 protein are frequently mislaid in various human cancers, including breast tumors primarily through the mutations or deletions of this gene (15, 17, 18). Accumulating evidence indicate that the mutational changes, which prolonged the half-life of this protein, not only perturb the normal tumor suppressor function of this gene, but

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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gain new tumor-promoting functions through the inactivation of DNA damage response genes or transactivation of target genes associated with the cell proliferation, apoptosis, tumorigenesis, and tissue invasiveness (19–26). Therefore, *p53* has been considered as a two-faced cancer gene (22).

Given the importance of the significant role of the gain of oncogenic function of mutant p53 protein in the progression of cancer, in this study, we examined whether ectopically expressed mutant p53 is capable of silencing the *CCN5* gene in noninvasive breast cancer cells, and if so, then, whether this alteration is able to enhance the aggressive phenotypes of these cells.

Materials and Methods

Tissue samples. Breast cancer tissue samples were obtained from University of Kansas Medical Center core facility, and breast cancer tissue array slides were obtained from Imgenex.

Cell lines and culture conditions. Noninvasive MCF-7, ZR-75-1 (wild-type p53), and T-47D (p53 heterozygous) cell lines and p53 mutant invasive breast carcinoma cell lines, including SKBR-3, MDA-MB-231, HCC-70, and HTB-19, were obtained from American Type Culture Collection. These cells were grown in DMEM containing penicillin and streptomycin (100 units/mL) and supplemented with 10% fetal bovine serum. In each experiment, cells were initially grown in complete media until the culture became ~60% confluent short hairpin RNA (shRNA).

Synthesis and cloning in pSilencer vectors. CCN5/WISP-2 specific shRNAs or mismatched shRNA were designed, synthesized, and cloned into the pSilencer vectors according to our previous method (7, 27). RNA interference sequences for CCN5/WISP-2 were described in details earlier (6–8).

Transfection. For transient transfection experiments, breast tumor cells were transfected with shRNAs or expression vectors (i.e., pCMV) containing missense mutant *p53* genes (R-175H or R-273H; kindly provided by Bert Vogelstein) using Lipofectin reagent (Invitrogen) according to our previous method (7, 27).

RNA extraction, cDNA synthesis, probe preparation and Northern blot analysis. Cytoplasmic RNA was extracted from cells using Trizol (Invitrogen) extraction procedure as described previously (7, 27, 28). cDNA preparation, reverse transcription–PCR analysis, and nonradioactive probe synthesis were performed essentially as reported (7, 29).

The nonradioactive Northern blot procedures are described elsewhere (7). Briefly, 10 μg of total RNA was fractionated by electrophoresis in 1% agarose gels containing formaldehyde and transferred to Supercharge nylon membrane (Schleicher & Schuell). Membranes were hybridized with DIG-labeled *WISP-2/CCN5* or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene specific probe. Relative expression of WISP-2/CCN5 mRNAs was calculated by densitometric analysis using one-dimensional image analysis software version 3.6 (Kodak Image Station). The signal intensity of WISP-2/CCN5 bands were normalized to that obtained with GAPDH bands.

Western immunobloting. Whole-cell protein extraction and immuno-Western blotting were performed as described previously (7). Briefly, equal amounts of proteins were separated by SDS-PAGE, transferred to the nitrocellulose membranes, and blocked with SuperBlock blocking buffer (Pierce). The protein bands were detected by specific antibodies using enhanced chemiluminescence kits (Pierce). The intensity of the band was measured by densitometric analysis using one-dimensional image analysis software version 3.6.

Colocalization studies. Colocalization of CCN5/WISP-2 mRNA and p53 protein in the cells was performed by simultaneous *in situ* hybridization and immunohistochemical analysis. *In situ* hybridization and immunohistochemistry were carried out essentially as described previously (5, 8, 30).

Migration assays. Transwell migration assays were performed as described earlier (31). Briefly, MCF-7 cells (20,000 per well) were seeded on 8-μm pore transwell filter insert (Becton Dickinson) coated with fibronectin (10 μg/mL). The cells were incubated for 24 h at 37°C with 5% CO_2. Cells adherent to the upper surface were removed, and migrated cells to the bottom surface were fixed with methanol, stained with Giemsa, and counted.

Chloramphenicol acetyltransferase assays. Chloramphenicol acetyltransferase (CAT) assay, using CAT-ELISA kits (Roche Applied Science, Inc.), were essential, as described (32). Briefly, *CCN5/WISP-2*-CAT promoter constructs (cloned into the pCAT-3-Basic Vector, Promega) containing the 1.9-kb human *CCN5* gene promoter sequence (−1,919 to +13) were transiently transfected alone or cotransfected with expression vectors for R-273H-p53 mutant into the MCF-7 cells using the Lipofectin (20 μg/mL) method (7). Transfected cells were harvested, and cellular extracts were prepared for CAT assays according to the manufacturer's instructions. CAT activity was measured at 405 nm using a microplate (ELISA) reader (Spectramax 340, Molecular Devices).

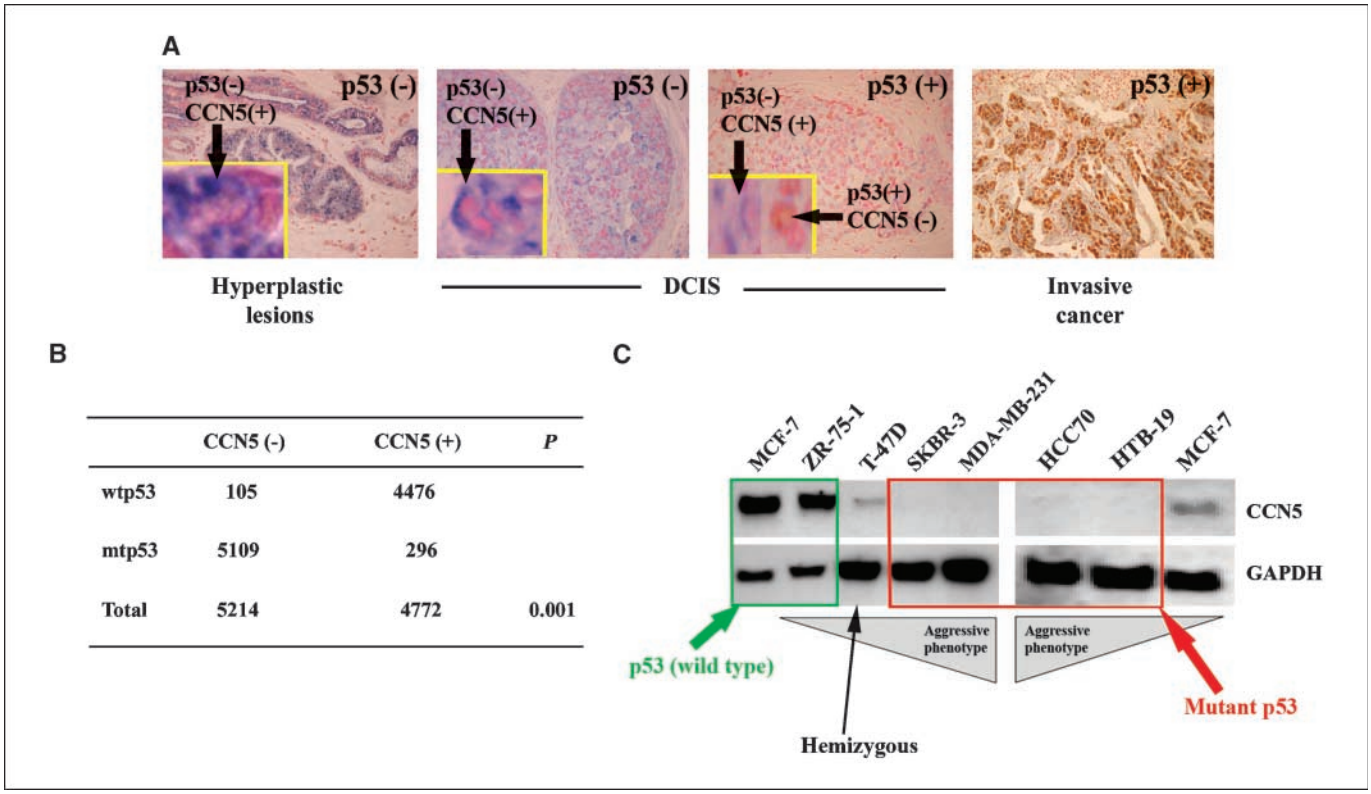
Statistical analysis. Each experiment was performed in triplicate. Results are expressed as mean ± SD, and effects were compared with untreated control cells on same experimental conditions. Paired Student's *t* tests or logistic regression analysis were used. A P value of <0.05 was considered significant.

Results

CCN5/WISP-2 expression is inversely correlated with p53 mutations in human breast tumor cells. The objective of this work was to evaluate whether overexpression of p53 protein, which is frequently achieved due to the mutational induction of half-life of this protein, has any link with the down-regulation of CCN5 expression in breast cancer samples. The colocalization data of CCN5 mRNA and p53 protein in tissue microarray slides, as well as in archived tissues, had shown an inverse relation between CCN5 and p53 expression (Fig. 1). CCN5 mRNA is highly expressed in the majority of the epithelial cells of ADH and DCIS samples, wherein p53 protein was undetected (Fig. 1*A*). In contrast, p53 immunopositive cells, which were frequently found in p53 mutant breast cancer samples, exhibit no or negligible amount of CCN5/WISP-2 mRNA expression (Fig. 1*A*). Using logistic regression to compare CCN5(−) between normal and mutant p53 samples, we get an odds ratio of 735.77 with corresponding 95% confidence interval of 586.84 to 922.51 and a P value of <0.001 (Fig. 1*B*). Thus, mutant p53 bearing samples are much more likely to have CCN5(−) than normal p53 bearing tissue samples. Note that in p53-positive tumor samples, a separate population of cells was also observed in which both CCN5 and p53 was negative (data not shown).

Subsequent Northern blot analysis was performed in various breast tumor cell lines using a *CCN5/WISP-2* DIG-labeled nonradioactive probe explicit for the above studies and showed that CCN5/WISP-2 mRNA is overexpressed in noninvasive breast cancer cell lines (i.e., MCF-7 and ZR-75) containing a normal *p53* gene, whereas its expression was comparatively less in p53 heterozygous cells T-47D and undetected in p53 mutant invasive breast carcinoma cell lines, including SKBR-3, MDA-MB-231, HCC-70, and HTB-19 (Fig. 1*C*). Together, these studies suggest that CCN5/WISP-2 expression is inversely correlated with the overexpression of mutant p53 proteins in breast cancer cells.

Exogenous overexpression of p53 mutants suppresses CCN5/WISP-2 mRNA expression in noninvasive breast tumor cells. With the aim to confirm the above hypothesis, we transiently transfected two hotspot missense mutants p53 (R-175H or R-273H) into MCF-7 cells or ZR-75-1 cells, which constitutively expressed CCN5/WISP-2 (5, 7). First, the transfection efficiency was assessed by immunocytochemical analysis using a monoclonal antibody against human p53. A significant but differential overexpression of mutant p53 protein was observed in transiently transfected MCF-7



	CCN5 (-)	CCN5 (+)	P
wtp53	105	4476	
mtp53	5109	296	
Total	5214	4772	0.001

Figure 1. Loss of CCN5 mRNA expression is inversely correlated with overexpression of p53 mutants in human breast cancer cells. *A,* representative dual-staining [*in situ* hybridization (*blue*) and immunohistochemistry (*brown*)] sections showing CCN5 mRNA (*blue*) and p53 protein (*brown*) expression in hyperplastic lesions, DCIS lesion, DCIS lesion with intermediate nuclear grade, and invasive cancer. Selected portions of some sections are shown in higher magnification to visualize the inverse relationship adequately. Nuclei were counter-stained with Fast Red. Magnification, 20×. *B,* inverse association between CCN5 mRNA expression and mutant p53 overexpression was scored in individual cell of 20 samples by two investigators including a pathologist (O.T.) in a double-blinded fashion. Logistic regression analysis was used to assess the association between CCN5 mRNA and cell type (normal versus p53 mutant). *C,* total RNA was extracted from human breast cancer cell lines and human mammary epithelial cells with or without p53 mutant background, and CCN5 expression was analyzed by Northern blot and normalized against the levels of GAPDH. The results were confirmed by three different experiments.

cells compared with Lipofectin-treated cells where expression was undetected (Fig. 2*A*). A similar result was also observed in ZR-75-1 cells (data not shown). The differential expression (light or dark brown) of p53 mutants in MCF-7 cells may be the result of variable transfections of mutant *p53* gene. We selected R-175H or R-273H missense mutants for these studies because these two mutants are very common in breast cancer and exhibit a gain of function or dominant negative phenotype or both under certain conditions (33, 34). Subsequently, we determined the status of CCN5/WISP-2 mRNA level in the transiently transfected cells. As shown in Fig. 2*B*, the expression of CCN5/WISP-2 was markedly reduced in both MCF-7 and ZR-75-1 after the transfection of p53 missense mutants, whereas this effect is undetected in Lipofectin controls. The p53 mutants mediated down-regulation of CCN5/WISP-2 was massive in MCF-7 cells compared with ZR-75-1 cells. This could be due to the differential efficacy of transfection of the vectors in these two cell lines.

Mutant p53-induced morphologic alterations and migration is mediated by suppressing CCN5/WISP-2 in MCF-7 cells. Recently, we found that CCN5/WISP-2 recombinant protein is able to reverse the epithelial-to-mesenchymal transition (EMT), a hallmark of invasive carcinoma (8). Therefore, we sought to determine whether exogenous overexpression of mutant p53 is able to alter the morphology of MCF-7 cells from round-shaped epithelial to spindle-shaped mesenchymal types. To do so, MCF-7 cells were transiently transfected with mutant p53 (R-273H). After transfection, cells were treated with CCN5/WISP-2 recombinant protein for 48 hours or left untreated; and the morphologic shift was evaluated by labeling these cells with a fluorescent dye (CFDA-SE). As shown in Fig. 3*A*, the cobble stone–like appearance of MCF-7 cells was changed to a spindle-like, mesenchymal type after the transfection of mutant *p53* genes. This phenotypic conversion from EMT can be blocked after the addition of 100 ng/mL of CCN5/WISP-2 recombinant protein for 48 hours. The unique phenotypic changes observed in MCF-7/R-273H cells with or without CCN5/WISP-2 recombinant protein led us to investigate the status of molecular markers for EMT signature under different microenvironments using immuno-Western blot analysis. These include E-cadherin, β-catenin, and vimentin. We found a complete loss of an epithelial marker, the E-cadherin protein, and significant reduction of β-catenin protein expression in both MCF-7/R-273H and ZR-75-1/R-273H cells (Fig. 3*B* and *C*). On the other hand, the expression of a mesenchymal marker, Vimentin, increased markedly in mutant p53–transfected cells. Addition of CCN5/WISP-2 recombinant protein restores β-catenin expression and simultaneously abolishes Vimentin expression in MCF-7/R-273H cells (Fig. 3*C*). Hence, both the morphologic and molecular changes in MCF-7/R-273H and ZR-75-1/R-273H cells showed that gain of oncogenic function of mutant p53 induces EMT and CCN5/WISP-2 recombinant protein is required for reversion of this phenomenon.

EMT with respect to motility is essential for tumor invasion (35). To understand this phenotypic trait of EMT, we explored the

migratory behavior of MCF-7/R-273H cells. To do so, MCF-7/R-273H cells or parent cells were seeded on the upper chamber of a Boyden chamber, and after 24 h, the migration of these cells was determined. We observed ~3.6-fold increase of motile MCF-7/R-273H cells compared with MCF-7/Lipofectin cells (Fig. 4). These results show that the overexpression of mutant p53 leads to the acquisition of EMT-like properties, which results in escalating cell migration. A similar phenomenon was observed in MCF-7 cell after the silencing of endogenous CCN5 by shRNA. Moreover, in the presence of CCN5/WISP-2 recombinant protein, migration of MCF-7/R-273H cells decreased by ~7 fold compared with untreated cells (Fig. 4). The gain of oncogenic function of p53 mutant indicates that the loss of *CCN5/WISP-2* in MCF-7 cells induces an EMT, thus facilitating motility and invasiveness.

Mutant p53-mediated suppression of CCN5/WISP-2 expression is nullified by estrogen in MCF-7 cells. The objective of this study was to determine the effect of ectopic expression of mutant p53 on the ER-α protein expression and also determined whether estrogen signaling, which up-regulates CCN5 expression and inhibits invasive and reverses EMT in ER-positive noninvasive breast cancer cells (5, 36, 37), is able to nullify the suppressive effect of p53 mutants on CCN5 in MCF-7 cells. Consistent with the previous work of Angeloni and colleagues (32), we found that p53 mutant enhances the ER-α level significantly in MCF-7 cells (Supplementary Fig. S1). Moreover, when these transfected cells were exposed to 17β-estradiol (10 nmol/L) for 24 hours, the inhibitory effect of mutant p53 on CCN5 expression was blocked (Fig. 5*A*). Finally, CAT promoter assays in MCF-7–transfected cells (pCAT-3-*CCN5* promoter and with or without R-273H-p53 mutant vectors) exhibit that estrogen is able to induce *CCN5* promoter-mediated CAT activity in the absence or presence of mutant p53 protein (Fig. 5*B* and *C*). As expected, unstimulated MCF-7/R-273H/*CCN5* promoter cells show background level CAT activity. Collectively, these studies suggest that the up-regulation of ER-α and suppression of CCN5 expression by mutant p53 are two independent events. Under the mutant p53 microenvironment, ER-α stayed functionally active, and thus, in conjunction with its ligand (estrogen), they nullify the suppression of CCN5 by mutant p53.

Discussion

CCN5/WISP-2 has been implicated as having an important role in carcinogenesis with a particular relevance to human breast and pancreatic disease (1, 5, 7, 8). *CCN5/WISP-2* is a two-faced gene, and its functional multiplicities are depending on the cellular microenvironment. Our recent studies suggested *CCN5* plays a

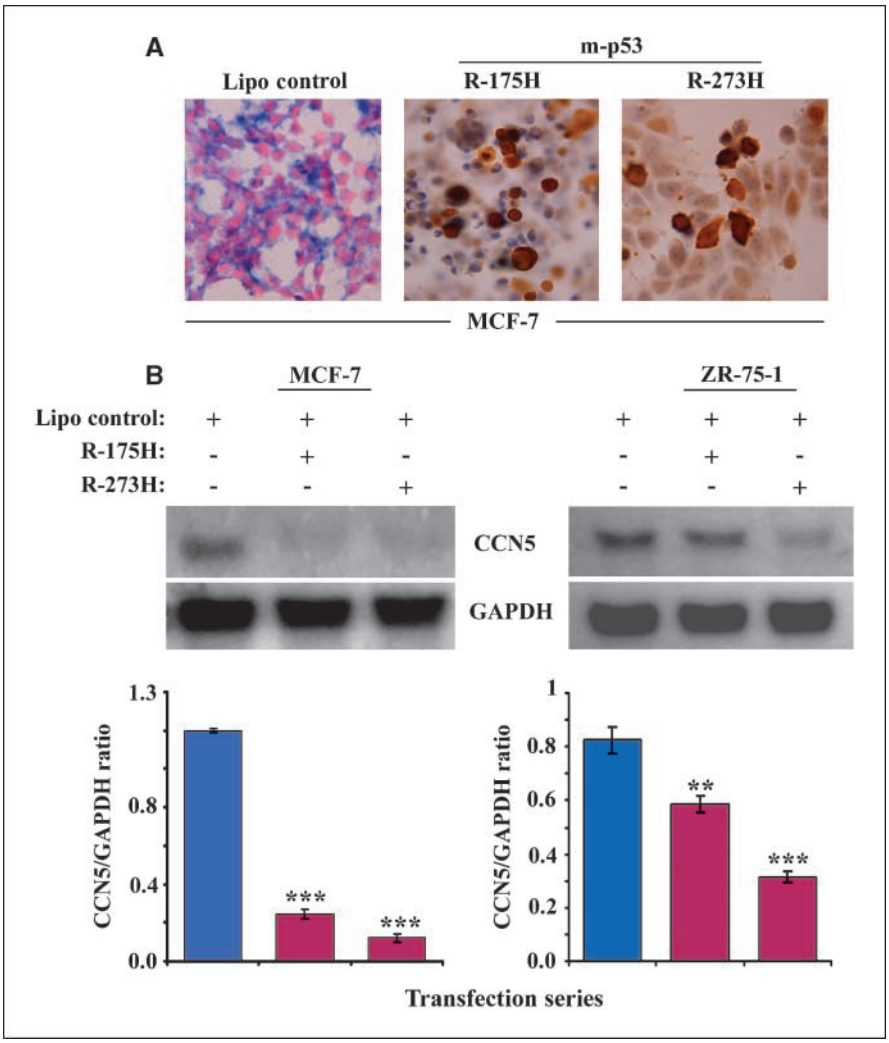


Figure 2. Ectopic expressions of p53 mutants suppress CCN5 expression in noninvasive human breast cancer cells. *A,* MCF-7 and ZR-75-1 breast cancer cells were transfected with either Lipofectin vehicle alone or two p53 missense mutants (R-175H or R-273H), and enforced mutant p53 expression efficiency was evaluated by combined *in situ* hybridization and immunohistochemistry. Note that mutant p53 is expressed differentially in MCF-7 cells. *B,* CCN5 mRNA expression was measured by Northern blot analysis in MCF-7 and ZR-75-1 cells after transfection of R-175H or R-273H. The mRNA levels of GAPDH were determined as loading control. *Columns,* data from three independent experiments; *bar,* SD. *P* values were generated by Student's *t* test. *, $P < 0.001$ versus Lipofectin-transfected controls; **, $P < 0.01$ versus Lipofectin-transfected controls.

preventive role in the progression of cancer from noninvasive to an invasive phenotype, as it participates in the morphologic alterations from MET of pancreatic adenocarcinoma cells (8). This proposition was further supported by recent work (9). Although *CCN5/WISP-2* has been considered as an antiinvasive gene, the mechanism of silencing of this molecule during the progression of the disease from noninvasive to invasive has been elusive. Because mutational oncogenic activations of *p53* are a prerequisite genetical event for the progression of >50% of breast cancers (38), the aim of this study was to develop a detailed understanding as to whether mutant p53 is able to silence the CCN5/WISP-2 pathway in noninvasive breast cancer cells and, if so, then what is the pathobiological relevance of silencing of CCN5/WISP-2 by mutant p53. The major conclusions of the present study, as depicted in Fig. 6, are that the hotspot p53 mutants (i.e., 175H and 273H) are able to silence the *CCN5/WISP-2* gene expression in MCF-7 and ZR-75-1 cells (noninvasive microenvironment) after transient transfections with varied efficiencies. The suppressive effect of p53 mutant can be abolished by estrogen signaling in these cells through the transcriptional activation of the *CCN5* gene. Moreover, the studies also showed that the inactivation of CCN5/WISP-2 by mutant p53 is required to exert mutant p53-mediated induction of the invasive phenotypes (Fig. 6). This phenomenon may be applicable in human breast tumor development under the influence of p53 mutants. To the best of our knowledge, this is the first study showing that p53 mutant protein-induced invasive phenotypes in noninvasive breast cancer cells are mediated by inactivating *CCN5/WISP-2*. This pathobiological event could be repealed in these cells by estrogen treatment.

Recently, our studies have shown an inverse correlation between the expression of mutant p53 protein and CCN5/WISP-2 mRNA expression in pancreatic cancer tissue samples or cell lines (8). Here, we showed similar results in breast tumor cells and different breast tumor cell lines. We found CCN5/WISP-2 is always overexpressed in noninvasive breast tumor cells and cell lines where *p53* gene is not mutated, whereas its expression is reduced or undetected in majority of the cells expressing mutant p53 (Fig. 1). This study, therefore, suggests that the gain of oncogenic function of p53 mutants may suppress the CCN5/WISP-2 function in breast tumor cells. Occasionally, CCN5 expression was found to be silenced in the presence of wild-type p53 or in the absence of gain of oncogenic function of mutant p53 in invasive carcinoma samples (depending on their stages and grades; data not included). Therefore, we can anticipate that more than one mechanism may be involved in this process and it is realistically possible because

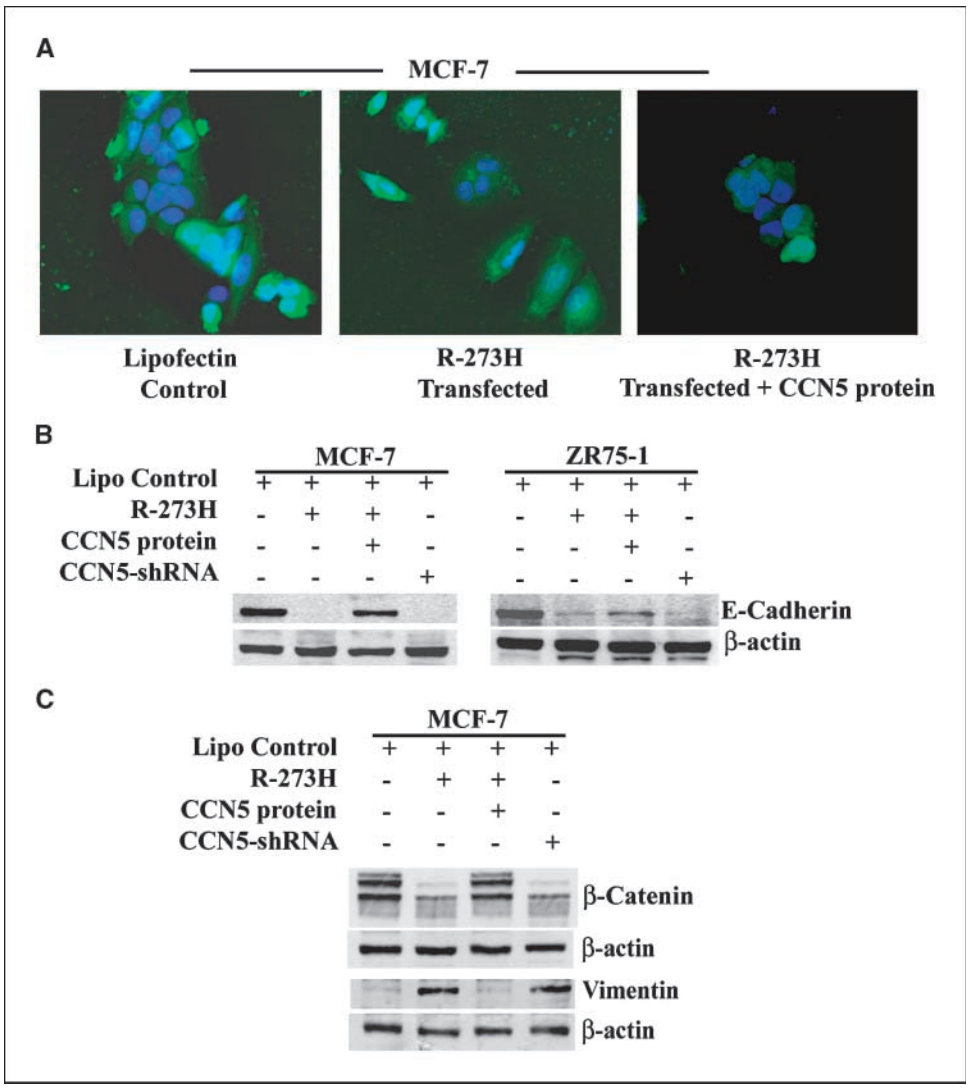


Figure 3. Mutant p53 induced morphologic and molecular marker changes from EMT-like phenotype in MCF-7 cells are restored by CCN5 recombinant protein. *A,* after transfection with either Lipofectin vehicle or R-273H, cells were stained with CFDA SE followed by 4′,6-diamidino-2-phenylindole as described in Materials and Methods. Transfected cells were treated with CCN5 recombinant protein (100 ng/mL) or left untreated for 48 h before staining (*bottom*). The results were confirmed by three independent experiments. *B,* MCF-7 and ZR-75-1 cells were transiently transfected with R-273H or R-273H or CCN5-shRNA followed by the treatment of CCN5 recombinant protein or left untreated as indicated in the figure and Materials and Methods. Total proteins were extracted from these cells for the detection of E-cadherin using immuno-Western blot analysis. *C,* immuno-Western blot analyses were performed with MCF-7/R-275H and MCF-7/R-273H/CCN5 protein and MCF-7/sh-CCN5 transiently transfected cell extracts for the detection of β-catenin and Vimentin levels. β-Actin was measured by Western blotting and included as loading control in each experiment. The experiments were repeated thrice to confirm the results.

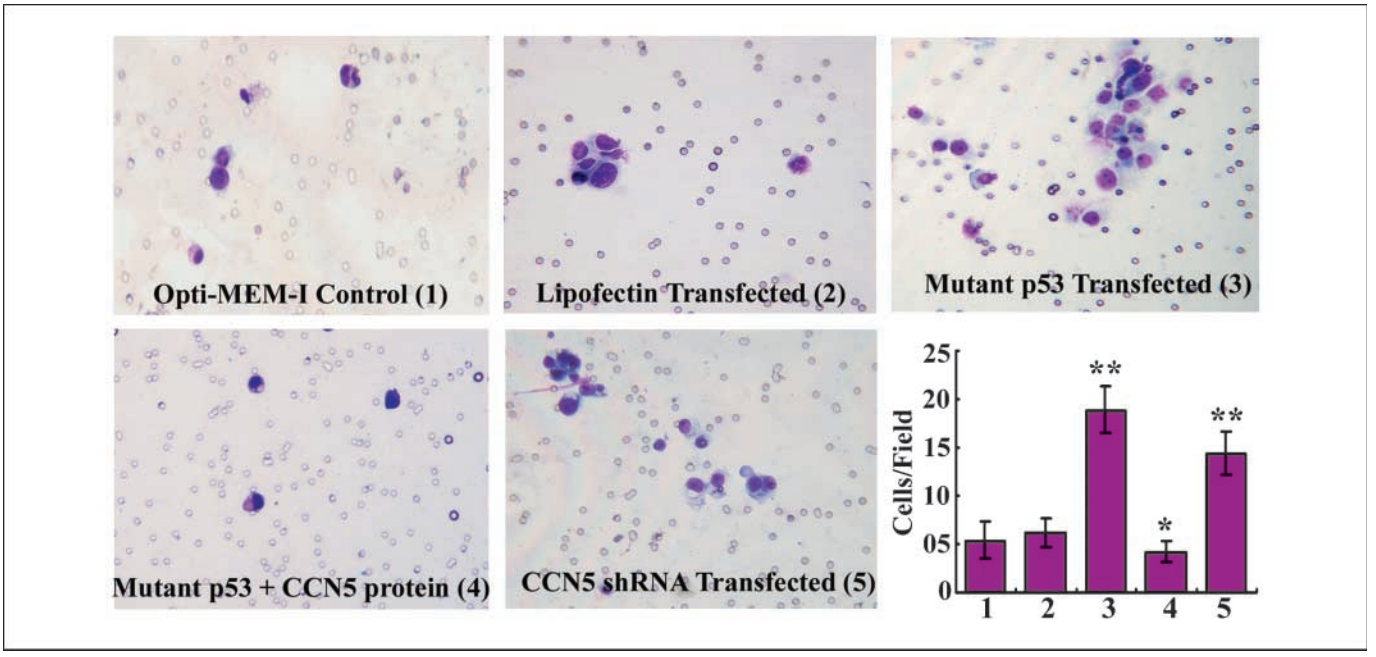


Figure 4. Mutant p53-induced migration of noninvasive breast tumor cells is blocked by CCN5. Mutant p53 (R-273H) or CCN5-shRNA transfected MCF-7 cells were seeded on the transwell filter insert of the modified Boyden chambers. One set of MCF-7/R-273H transient cells was grown in the presence of CCN5 recombinant protein (100 ng/mL) for 48 h, before seeding in the upper chamber of the Boyden chamber. The migrated cells were fixed on the filter after 24 h, stained with Giemsa, and counted under the microscope. *1,* Opti-MEM-I control; *2,* Lipofectin-transfected cells; *3,* R-273H transfected; *4,* R-273H + CCN5 protein; *5,* CCN5 shRNA-transfected cells. *Error bar,* SD from 10 independent fields. *Columns,* mean for three independent experiments; *bars,* SD. **, $P < 0.001$ versus Opti-MEM-I control; *, $P < 0.05$ versus Opti-MEM-I control.

development of genetic heterogeneity is a common phenomenon in the progression of breast cancer (39). Therefore, further studies are warranted. The results of the present study are deviated from our previous qualitative studies on breast cancer samples, which indicated a lack of correlation between p53 overexpression and CCN5 expression (5). This deviation is due to the distinctly different data collection method. In our previous study, we compared the expression profiles of these genes in samples containing heterogeneous lesions with or without *p53* mutations, whereas in the present study we evaluated gene expression at the cellular level, providing direct correlation of p53 mutations and CCN5 expression within individual cells.

The ectotopic transfection of p53 hotspot mutants in different cancer cells have been performed by various laboratories to identify the downstream targeted genes of p53 mutants and their functional relevance (12, 21, 40). These studies provided evidence for the dominant negative and gain-of-oncogenic function effect of different hotspot p53 mutants (12). In the present study, to test our above hypothesis, we transiently transfected two *p53* hotspot mutants ($p53^{R175H}$ and $p53^{R273H}$) in MCF-7 and ZR-75-1 noninvasive wild-type p53 containing breast tumor cells, and CCN5/WISP-2 expression was determined. These two mutations are located in the conserved regions of *p53 gene* (40) and are frequently found human breast tumors. Consistent with this observation is that the protein level of wild-type p53 was undetected in MCF-7 or ZR-75-1 cells expressing CCN5/WISP-2 mRNA (Fig. 2), whereas the basal levels of p53 mutants accumulate differentially, but significantly, in the transfected breast tumor cells parallel with the reduction of CCN5/WISP-2 expression (with different efficiencies) in these cells (Fig. 2). Collectively, these studies indicate that $p53^{R175H}$ and $p53^{R273H}$ hotspot mutations silenced the CCN5/WISP-2 expression in these cells. However, the mechanism of suppression of CCN5/WISP-2 by mutant p53 remains to be elucidated. Several potential mechanisms can be anticipated to be involved in the suppression of the CCN5 gene. The mutant p53 protein may directly bind to an unidentified sequence of the *CCN5* promoter to suppress the transcription of CCN5. Because estrogen abrogates the suppressive action of p53 mutant proteins on CCN5 expression through the transcriptional activation of the *CCN5* gene (Fig. 5), one could anticipate that the mutant p53 protein and estrogen axis may exploit an overlapped regulatory element to either suppress or activate the CCN5 function. Thus, under an estrogenic environment, mutant p53 protein may be unable to interact with this sequence. However, to justify this proposition, further studies are warranted. Additionally, an epigenetic pathway, such as hypermethylation of the *CCN5* promoter by mutant p53, can also be anticipated. This postulation is drawn from the recent studies, which indicate mutant p53 protein enhances cytosine methylation in the promoters of certain genes in cancer cells to silence them, resulting in metastatic growth (41), and it indicates that this could be done through the regulation of the DNA methytransferase gene (42). If this is the case, then we could anticipate that estrogen axis may work differently to repeal the action of p53 mutants. Noninvasive breast cancer cells, such as MCF-7 or ZR-75-1, harbor wild-type *p53*, have higher levels of CCN5, and display a generally less invasive phenotype, implying that wild-type *p53* may positively regulate CCN5 expression and the consequent reduced invasive phenotype. However, this phenomenon is highly unlikely, as previous studies have shown that under an ER-α–noninvasive microenvironment, wild-type *p53* is either functionally inactive (43) or down-regulates ER-responsive genes (44).

Previous studies have shown that the mutational activation of p53 protein promotes the progression of cancer from noninvasive to metastasis. For example, over production of mutant p53 protein in null *p53* cells enhances the plating efficiencies on agar (33). The

transgenic mice with mutant p53 show accelerated tumor progression in wild-type and Trp53$^{+/-}$ background compared with *p53*-deficient mice (45). Two independent studies have shown that the endogenous *p53* mutant bearing mice exhibit a high frequency of tumor progression and metastasis compared with *p53*-deficient mice (20, 23). Moreover, very recent studies indicate that the mutant p53 is capable of enhancing the expression GEF-H1 oncogene and

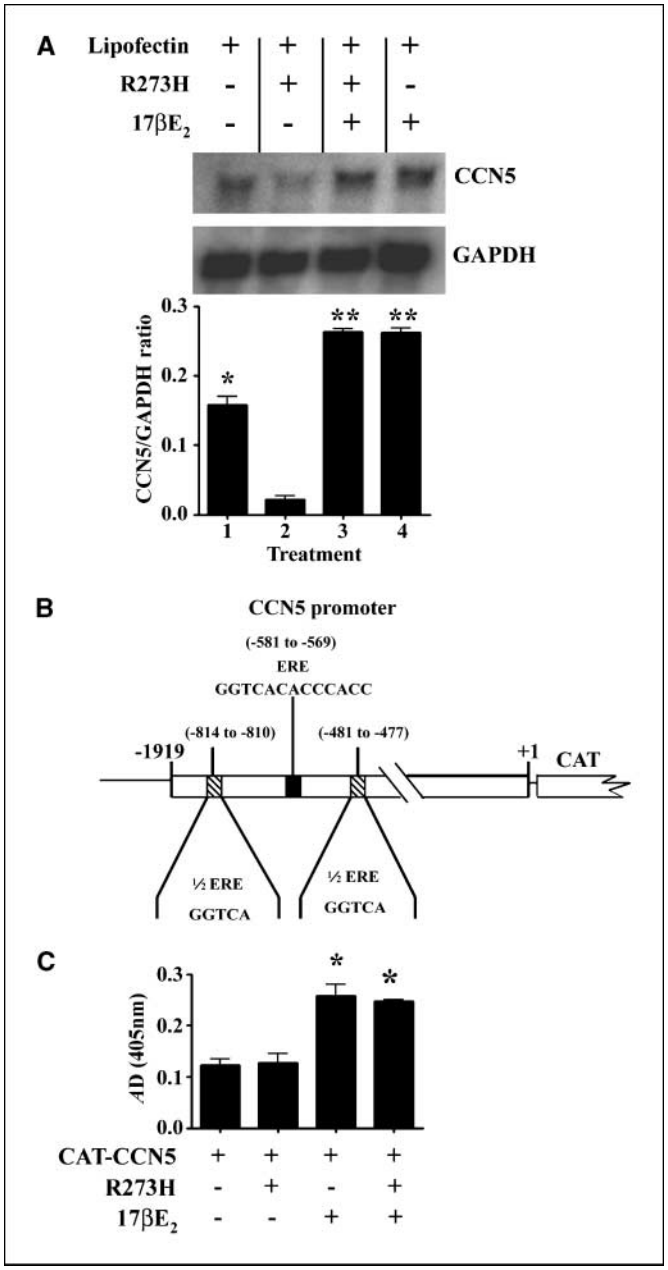


Figure 5. Transcriptional repression of CCN5 expression by mutant p53 can be blocked by estrogen signaling. *A,* MCF-7 cells were transiently transfected with either Lipofectin vehicle or R-273H p53 mutant, and cells were treated with 10 nmol/L 17β-estradiol for 24 h. CCN5 mRNA expression was measured by Northern blot analysis. The mRNA levels of GAPDH were determined as loading control. The experiment was repeated thrice to validate the result. *, $P < 0.01$ versus controls. *B,* schematic representation of the minimal hCCN5 promoter that was recently cloned and characterized (49). Estrogen response element is shown as ERE motif here. *C,* MCF-7 parent cells or MCF-7/R-273H-p53 mutant cells were transiently transfected with CCN5/WISP-2 promoter. After 24 h, transfected cells were exposed to 10 nmol/L 17β-estradiol for 24 h and assayed for CAT activity. Representative of three independent experiments.

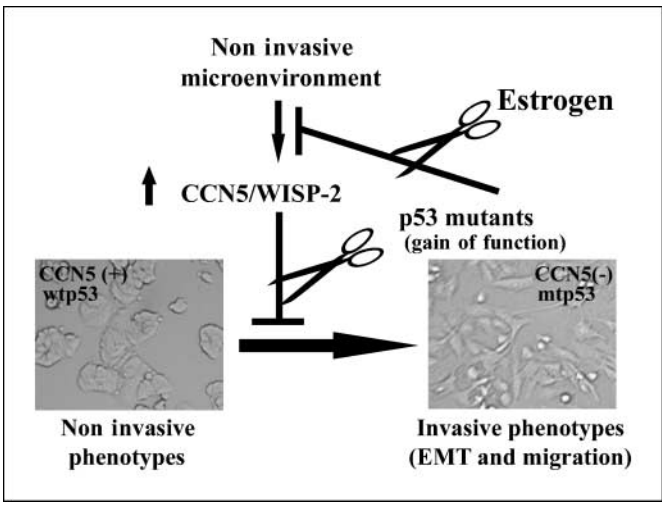


Figure 6. Summary of the study: molecular pathway of CCN5/WISP-2 silencing for tumor cell progression. The diagram showed that the inactivation of CCN5/WISP-2 by mutant p53 is a critical event for mutant p53-mediated induction of invasive phenotypes.

nuclear factor-κB that eventually may contribute to the tumor progression phenotype (46, 47). The goal of this study was to determine whether the silencing of CCN5/WISP-2 by gain of oncogenic function of p53 mutants is necessary to promote the aggressive phenotype of breast cancer cells. We showed that ectopic expression of mutant p53 or nullifying the expression of CCN5 by shRNA in MCF-7 cells induces morphologic alteration from the EMT phenotype (Fig. 3). Consistent with morphologic alterations, some hallmark proteins of epithelial or mesenchymal cells were either decreased or increased during this transition (Fig. 3). These morphologic and molecular changes finally reflect on cellular migration and invasive capability (48). Therefore, we extended our studies and investigated the migration of these cells. We found that the migratory behavior was increased significantly in both mutant p53 overexpressed cells and CCN5 silenced cells (Fig. 4). Interestingly, it is noted that the effect of mutant p53 on the EMT transition of phenotype or migration can be nullified by the addition of recombinant CCN5 protein in the culture. Collectively, these studies suggest that the gain of oncogenic function of p53 mutants induce EMT followed by migration through the inactivating CCN5/WISP-2 signals in noninvasive breast tumor cells with normal p53 background. Further studies are warranted to identify mechanisms regulating CCN5/WISP-2 expression by p53 mutants and CCN5/WISP-2 downstream signaling molecules for a better understanding of the pathobiological roles of *CCN5/WISP-2* in cancer progression and in therapeutic application.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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