THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 43, pp. 31206–31216, October 25, 2013
© 2013 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

Hepatocyte Nuclear Factor 6 Suppresses the Migration and Invasive Growth of Lung Cancer Cells through p53 and the Inhibition of Epithelial-Mesenchymal Transition*

Received for publication, April 23, 2013, and in revised form, September 5, 2013 Published, JBC Papers in Press, September 10, 2013, DOI 10.1074/jbc.M113.480285

Xin-Wang Yuan, Dong-Mei Wang, Ying Hu, Yun-Neng Tang, Wei-Wei Shi, Xiao-Jie Guo, and Jian-Guo Song[1]
From the Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Background: The role of HNF6 in lung cancer growth and progression remains to be characterized.
Results: HNF6 up-regulates p53 and inhibits EMT, cell migration, and invasive growth in lung cancer cells.
Conclusion: HNF6 suppresses EMT and invasive growth of lung adenocarcinoma cells through p53.
Significance: HNF6 is potentially a molecular marker and target for diagnostic and therapeutic purpose for lung adenocarcinoma cancers.

Epithelial-mesenchymal transition plays an important role in many patho-physiological processes, including cancer invasion and metastatic progression. Hepatocyte nuclear factor 6 (HNF6) has been known to be an important factor for both physiological and pathological functions in liver and pancreas. However, its role in EMT and lung cancer progression remains unidentified. We observed that HNF6 level can be down-regulated by TGF-β1 in human lung cancer cells. Knockdown of HNF6 induced EMT and increased cell migration. In contrast, ectopically expression of HNF6 inhibited cell migration and attenuated TGF-β1-induced EMT. The data suggest that HNF6 plays a role in maintaining epithelial phenotype, which suppresses EMT. HNF6 also inhibits both colony formation and proliferation of lung cancer cells. It pronouncedly reduced the formation of tumor xenografts in nude mice. In addition, HNF6 can activate the promoter activity of p53 by directly binding to a specific region of its promoter and therefore increase the protein level of tumor suppressor p53. p53 knockdown induced EMT and increased cell migration, whereas the opposite effect was generated by p53 overexpression. p53 knockdown also inhibited the effect of HNF6 on EMT and cell migration, indicating that p53 is required for the functions of HNF6 herein. Moreover, there is a high positive correlation among the expression levels of HNF6, p53, and E-cadherin in human lung cancer cells and tissues. The data suggest that HNF6 inhibits EMT, cell migration, and invasive growth through a mechanism involving the transcriptional activation of p53.

Epithelial-mesenchymal transition (EMT)[2] is a precisely regulated process through which epithelial cells lose polarity and cell to cell junction and gain a fibroblast-like morphology. During EMT, the epithelial protein level, such as E-cadherin and γ-catenin (1), are down-regulated, whereas mesenchymal protein such as N-cadherin, fibronectin, and vimentin are up-regulated, and the cell skeleton undergoes rearrangement. Higher invasive growth and metastasis of cancers is often correlated with higher grade malignancy and poorer prognosis. More and more evidence show that EMT is a critical step for cancer invasion and metastatic progression (2, 3).

EMT was initially observed in mammalian embryonic development. Several liver and pancreas development and functional related genes controlling EMT have been identified. For instance, FoxA1/2 (4, 5) and HNF4α (6), which are important in liver and pancreas development and function, were shown to maintain the epithelial phenotype and inhibit EMT and metastasis. HNF6 is a liver-enriched transcription factor, which is involved in complex biological processes best known in liver and pancreas such as cell differentiation and organogenesis, glucose metabolism, biliary duct repair, and cell proliferation (7). Some studies have shown that HNF6 is required for normal pancreas and biliary duct development (8, 9). HNF6 expression was found to be decreased during biliary duct obstruction and injury, suggesting that its down-regulation in liver is necessary for the repair of biliary duct (10), a process in which cells may need to undergo EMT, suggesting a possible relationship of HNF6 with EMT. HNF6 stimulates the proliferation of normal hepatocytes during mouse liver regeneration (11). It has been reported that HNF6 inhibits cell proliferation in some colon and liver cancer cell lines (12). The different effects of HNF6 on cell proliferation suggest a complex regulatory mechanism of its function, which is important to be further explored. It has been shown that HNF6 may activate or cooperate with FoxA2 to regulate the signaling pathway important in liver cells (13, 14). FoxA2 has also been shown to be implicated in the control of EMT and tumor metastasis, which suggests a potential connection between HNF6 and EMT. HNF6 has also been shown to promote hepatic precursor cells to differentiate into mature hepatocytes by inhibiting the expression of TGF-β and activin (15), implying a potential inhibitory effect of HNF6 on TGF-β

* This work was supported by grants from the Chinese Ministry of Science and Technology (2011CB966200) and Natural Science Foundation of China (30730023).
[1] To whom correspondence should be addressed: Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China. Tel.: 86-21-54921167; Fax: 86-21-54921167; E-mail: jgsong@sibcb.ac.cn.
[2] The abbreviations used are: EMT, epithelial-to-mesenchymal transition; HNF6, hepatocyte nuclear factor 6; ZEB1/2, zinc finger E-box-binding homeobox 1/2; HNF4α, hepatocyte nuclear factor 4α; FoxA1/2, forkhead box protein A2; qPCR, quantitative PCR.

signaling. Both HNF6 and FoxA2 have been implicated in colorectal liver metastases (12). The expression level of HNF6 was found to be much lower in both pancreas cancer tissues and cell lines compared with normal pancreas tissues and cell lines, implying a possible tumor suppressor role of HNF6 in pancreas (17).

p53 is a very important tumor suppressor gene, which is mainly involved in cell cycle arrest, apoptosis, senescence, DNA repair, and the inhibition of angiogenesis (18–20). Recent studies indicate that p53 is involved in the inhibition of EMT through regulation of micro-RNAs, such as miR-34, miR-200, and miR-130b by targeting ZEB1/ZEB2. Mutation or loss of p53 expression may induce EMT and metastasis in cancer cells (21–23). However, the regulation of p53 by HNFs and its biological significance in the control of EMT and relevant events have not been identified. In exploring the role of HNF6 in tumor growth and progression, we observed that HNF6 is implicated in maintaining the epithelial phenotype of lung cancer cells and is a positive regulator for p53 expression. Further studies showed that HNF6 plays suppressive role in the control of EMT, migration and invasive growth of lung cancer cells. These roles of HNF6 have been closely related with its regulatory effect on p53 expression.

EXPERIMENTAL PROCEDURES

Materials

293T, A549, NCI-H446, NCI-H358, and NCI-H1650 cells were originally purchased from the American Type Culture Collection (Manassas, VA). DMEM and RPMI 1640 were bought from HyClone (Thermo Fisher Scientific). FBS and transfection reagents were purchased from Invitrogen. TGF-β1 was from Chemicon (Millipore, Billerica, MA). Rabbit anti-HNF6 antibody (ab83564) were purchased from Abcam (Cambridge, MA), Rabbit anti-p53 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Mouse anti- E-cadherin, N-cadherin, γ-catenin, vimentin, β-actin, and FLAG were all from BD Biosciences. Lentivirus plasmids pCDH-CMV-MCS-EF1-Puro, Δ8.9, VSV-G, and pLKO.1-TRC were purchased from Addgene (Cambridge, MA).

Methods

Cell Culture and Transfection

A549 and 293T cells were cultured in DMEM containing 10% FBS supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml). H446, H358, and H1650 cells were grown in RPMI 1640 medium containing 10% FBS supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Transfection was carried out using Lipofectamine 2000 as transfection reagent according to the manufacturer's instructions.

Western Blotting and Immunofluorescence Staining

Western blotting and immunofluorescence staining was performed as described previously (24).

Plasmids and shRNAs

For knockdown of HNF6 and p53, two independent shRNA sequences targeting human HNF6 and p53 were used respectively, with one scramble shRNA sequence as a control. shRNAs were inserted into pLKO.1-TRC plasmid following the Addgene instructions. All shRNAs were from Generay Biotech Co. (Shanghai, China). shRNA sequences targeting human HNF6 are as follows: 5′-GCCTCCATGAATAACCTCTAT-3′ and 5′-TGGAAGTAATTCAGGGCAGAT-3′, and the latter sequence was used for most experiments. shRNAs for human p53 were as follows: 5′-GTCCAGATGAAGCTCCCAGAA-3′, and 5′-CACCATCCACTACAACTACAT-3′, and the latter sequence was used for most experiments. The scramble shRNA sequence is 5′-CCTAAGGTTAAGTCGCCCTCG-3′. For overexpression of HNF6 and p53, N-terminal FLAG-tagged full-length human wildtype HNF6 and p53 gene sequences were inserted into the pCDH-CMV-MCS-EF1-Puro plasmid, respectively.

Lentivirus Transduction

Lentiviruses for knockdown and overexpression were produced as described (25).

Real Time qPCR

mRNA were extracted from cells with TRIzol reagent (Invitrogen), and 1 μg for each were use for reverse transcription by the ReverTra Ace (Toyobo, Japan) following the product instructions. Real time qPCR was carried out in the ABI-7500 systems by following the instructions from ABI. In brief, cycles were as follows: 95 °C for 10 min, then 95 °C for 15 s, 60 °C for 1 min, 40 cycles, and adding the annealing curve. For real time qPCR, primer sequences were as follows: p53, 5′-GCG-AGCACTGCCCAACAACA-3′ (forward) and 5′-GGATCTG-AAGGGTGAAATATTCT-3′ (reverse); GAPDH-F, 5′-GCGCG-TGCCTTCATCAC-3′ (forward) and 5′-TCTGCGCCATAAG-GTGGTAG-3′ (reverse).

Cell Migration Assays

Scratch Assay—Cells were scratched by a pipette tip when cell confluence reached ~90% and further incubated with fresh medium; media were changed every day. Photographs were taken just after scratching (0 h) and at time points of 24 h and 48 h, at ×40 magnifications.

Transwell Assay—Cell migration assays were performed using Transwell migration chambers (8-μm pore size, Costar) according to the manufacturer's instructions. For all cells, 2×10^4 cells were plated into the insert of the well, and representative photos were taken at ×100 magnifications.

Colony Formation in Soft Agar

Base agar was made by mixing of volume 1:1 of melted 1.2% agar (DNA grade) and 2× DMEM, 1 ml for each 35-mm dish. Top Agar final concentration in the DMEM-agar mixture was 0.6%, and 5×10^3 cells were seeded in top agar in each dish. After the top agar melted, 1 ml of DMEM with 10% of FBS was added to each dish. Cells were scored after 2 weeks.

Animal Experiments

All animal experiments were performed under the ethical guidelines of the Institute of Biochemistry and Cell Biology (Shanghai Institutes for Biological Sciences, Chinese Academy

of Sciences). For each mouse, 1×10^7 cells in 200 μl of DMEM were injected subcutaneously. And the mice were narcotized and photographed after 6 weeks. To obtain the blood of the mice injected subcutaneously with lung cancer cells, mice were narcotized, and blood was gathered by cutting the neck. Then tumors were finally weighed and statistically analyzed. Then, RNA for whole blood was extracted and reverse-transcribed into cDNA. Human cells in circulating blood were examined by real time qPCR by detecting the ratio of human tubulin expression to mouse GAPDH expression. The specific primers for human tubulin were as follows: 5′-CAGATGCCCAGTGACAAGACC-3′ (forward) and 5′-CAATGACCGTAGGCTCCAGAT-3′ (reverse); and for mouse GAPDH, the specific primers were as follows: 5′-AGGTCGGTGTGAACGGATTTG-3′ (forward) and 5′-GGCCTCACCCCATTTGATGT-3′ (reverse).

Luciferase Assay

The 2.8 kb (-2810 bp ~ 0 bp), 300 bp (-2810 bp ~ -2446 bp), and 2.5 kb (-2446 bp ~ 0 bp) of p53 promoters was cloned into pGL3-basic plasmid. The primers used for PCR were as follows: -2810 bp ~ 0 bp, 5′-GACTGGTACCGTCTGTCTCCCACACAGAAAGCAA-3′ (forward) 5′-GTCACTCGAGCCCAATCCCATCAACCCCT-3′ (reverse); -2810 bp ~ -2446 bp, 5′-GACTGGTACCCCACACAGAAAGCAAACCAGCCCTT-3′ (forward) and 5′-GTCACTCGAGGTCACCCAGGCTGGAGTGCTGT-3′ (reverse); -2446 bp ~ 0 bp, 5′-GACTGGTACCGCCTGGGTGACAGAGCAAGACCTT-3′ (forward) and 5′-GTCACTCGAGCGCCATGACAAGTAAGGGCAAGTAA-3′ (reverse). Luciferase assays were performed according to the manufacturer's instructions by Promega (Madison, WI).

ChIP

ChIP assays were performed according to the manufacturer's instructions (Millipore). HNF6 antibody was used to precipitate DNAs cross-linked with HNF6 in the nuclear extracts of A549 cells, and normal rabbit IgG (Santa Cruz Biotechnology) performed in samples in parallel as controls. Enriched DNAs were then used as templates to assess the binding intensity of HNF6 to putative binding sites in p53 promoters. Binding intensity was detected by real time qPCR, and relative binding intensity was calculated by the ratio of ChIP-enriched DNA over control IgG input DNA and normalized. Primers used in this assay are as follows: primer-1, 5′-GTCCTGATGCTTGCTGGTTT-3′ (forward) and 5′-TGGGCAGGCTGATATCAAAT-3′ (reverse); primer-2, 5′-CGTCTTGAAAAACAGTCCCGTTT-3′ (forward) and 5′-GGAGCAGG TGGAATATGCAGAAA-3′ (reverse); primer-NC, 5′-CGGGACGTGAAAGGTTAGAA-3′ (forward) and 5′-GACAGGTCTGAAGCCTGGAG-3′ (reverse).

Tissue Microarray Immunohistochemistry

Human lung adenocarcinoma tissue microarrays were from National Engineering Center For Biochip at Shanghai, China, and the product ID of the tissue microarray is Hlug-Ade060PG-01. The concentrations of antibodies were as follows: HNF6, 1/150; E-cadherin, 1/500; and p53, 1/75. The immunohistochemistry was done as described (26).

Statistical Analysis

Quantitative data are reported as means ± S.D. Statistical significance was determined by the Student's t test. A p value of < 0.05 was considered statistically significant. *, $p < 0.05$; **, $p < 0.01$.

RESULTS

Knockdown of HNF6 Induces EMT and Cell Migration—Our previous work showed that TGF-β1 can induce EMT in human lung cancer cell A549 cells (24, 27). To investigate the potential role of HNF6 in EMT and other relevant cell functions, we examined whether HNF6 can be regulated by TGF-β1 during EMT induction. As shown in Fig. 1*A*, HNF6 levels were decreased in a time-dependent manner by TGF-β1 in A549 cells, suggesting a role of HNF6 in controlling the epithelial-mesenchymal plasticity. Interestingly, the HNF6 levels are positively correlated with epithelial marker E-cadherin and γ-catenin and reversely correlated with mesenchymal marker N-cadherin, as detected in three human lung cancer cell lines (Fig. 1*B*), implying a role of HNF6 as an suppressor of EMT. To determine this possibility, we knocked down HNF6 in A549 cells and then examined the effect generated. Surprisingly, HNF6 knockdown caused a morphological change of cells from flat to elongated shape (Fig. 1*C*, *left*). A corresponding decrease in the levels of E-cadherin and γ-catenin and an increase of N-cadherin can be observed (Fig. 1*C*, *right*). HNF6 knockdown also apparently decreased plasmic membrane level of E-cadherin and increased the F-actin rearrangement as shown by immunofluorescence assay (Fig. 1*D*). These results indicate that HNF6 plays a role in maintaining the cell epithelial phenotype, which is important in the control of EMT. In accompaniment with EMT, there is usually an increase in cell mobility. Consistent with this phenomenon, HNF6 knockdown pronouncedly increased the cell migration of A549 cells as examined by scratch and Transwell assays (Fig. 1*E*).

HNF6 Overexpression Increases Epithelial Cell Phenotype and Attenuates TGF-β1-induced EMT and Cell Migration—To further investigate the function of HNF6 in EMT, we ectopically expressed HNF6 in several lung cancer cell lines. In NCI-H446 cells, which have relatively strong mesenchymal-like properties, overexpression of HNF6 increased E-cadherin and γ-catenin levels but decreased N-cadherin levels (Fig. 2*A*, *right*), and the cells changed from a scattered and less adherent morphology to a relatively adherent and more epithelial-like shape (Fig. 2*A*, *left*), suggesting a MET-like event was induced. In another lung cancer cell line, NCI-H1650, overexpression of HNF6 also increased E-cadherin level and decreased N-cadherin and fibronectin levels (Fig. 2*B*). Overexpression of HNF6 in A549 cells also caused an increase in the levels of epithelial marker E-cadherin and γ-catenin and a decrease in the levels of mesenchymal marker protein N-cadherin and reduced TGF-β1-induced EMT (Fig. 2*C*). Because HNF6 knockdown increases the migration of A549 cells, we also determined whether HNF6 overexpression has the opposite effect. As expected, the migration ability of H446, H1650, and A549 cells were decreased as examined by Transwell (Fig. 2*D*) and scratch (Fig. 2*E*) assays.

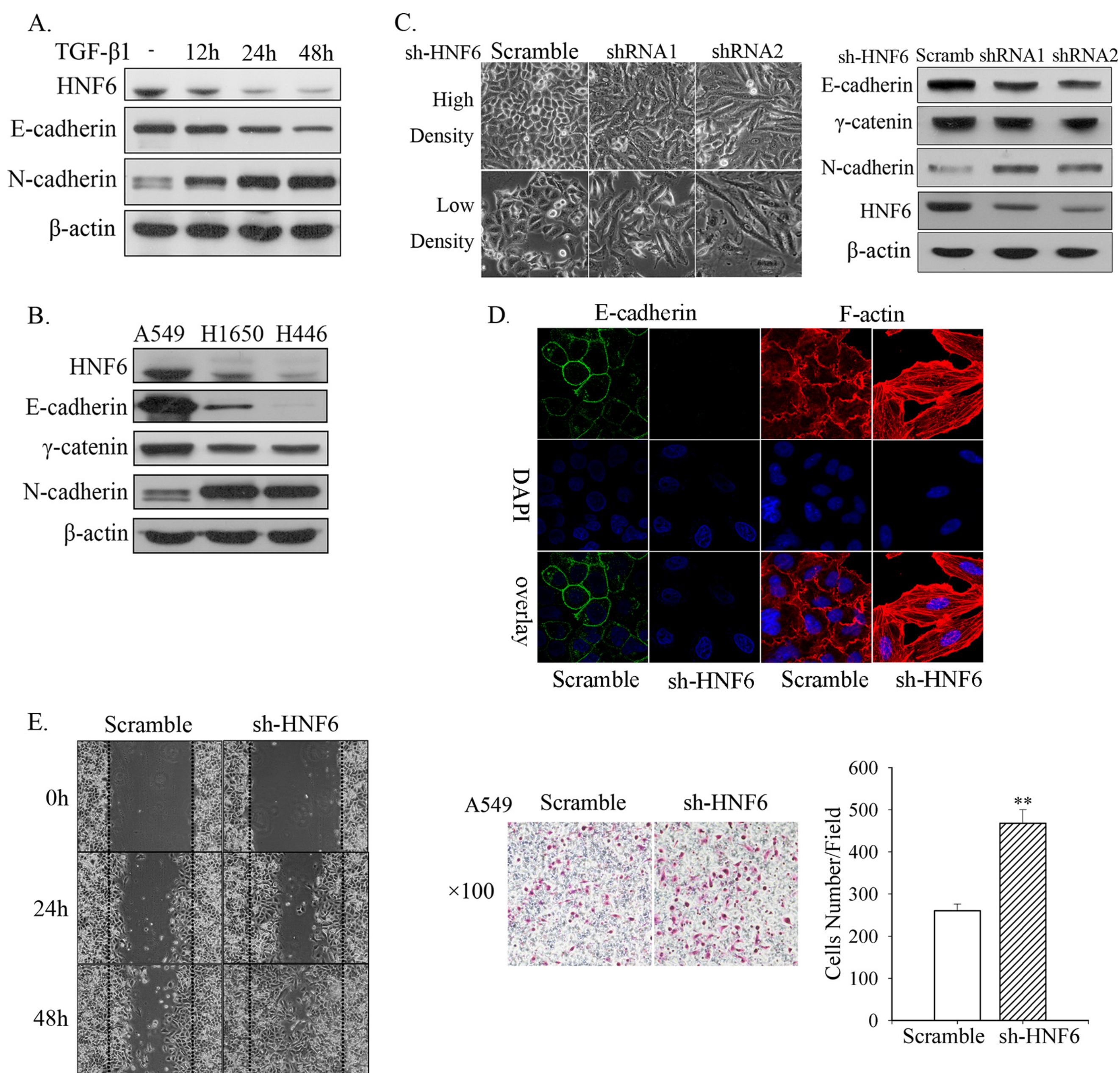

FIGURE 1. **Knockdown of HNF6 induces spontaneously EMT and cell migration.** *A*, A549 cells were treated with TGF-β1 (5 ng/ml) for the time indicated, the protein level of HNF6, E-cadherin, and N-cadherin were detected by Western blotting. *B*, HNF6, E-cadherin, and N-cadherin expression level in human lung cancer cells, A549, H1650, and H446 were detected by Western blotting. *C*, the effects of HNF6 knockdown on EMT were detected by observing the morphology and protein marker levels. Two RNAi sequences loaded by pLKO.1 lentivirus system were used to knockdown HNF6. *D*, the distribution and expression of E-cadherin and the arrangement of cytoskeleton protein F-actin were detected by immunofluorescence. *E*, the effect of HNF6 knockdown on cell migration was detected by scratch assay at the indicated time point (*left*) and also by Transwell assay (*right*). A representative result of three independent experiments and statistic data were presented.

Overexpression of HNF6 Represses Anchorage-independent Growth, Cell Proliferation, and Tumor Xenograft Growth and Invasion—Anchorage-independent growth is an important indicator of cancer malignancy, and EMT increases the anoikis resistance ability of cells thus increases the anchorage-independent growth of cancer cells. Our investigations showed that HNF6 can significantly decrease the colony formation of cells in soft agar (Fig. 3*A*). In contrast, HNF6 knockdown increased an anchorage-independent growth of cells (Fig. 3*B*). Tumor formation ability is an important indicator of the malignancy of cancer cells, so we next detected the effect of HNF6 on tumor formation by subcutaneously A549 cell injection of mice. Surprisingly, overexpression of HNF6 strongly inhibited tumors growth (Fig. 3*C*, *left*). The invasion ability of lung cancer cells from primary tumor to circulating blood was also examined by detecting the human tubulin mRNA levels compared with mouse GAPDH mRNA levels in the whole blood of mice by real time qPCR. The results showed that HNF6 can significantly decrease the cancer cells invasion to blood vessels (Fig. 3*C*, *right*). We also detected the effect of HNF6 overexpression on cell proliferation rate of A549 cells. As shown in Fig. 3*D*, overexpression of HNF6 significantly inhibited cell proliferation in A549 cells,

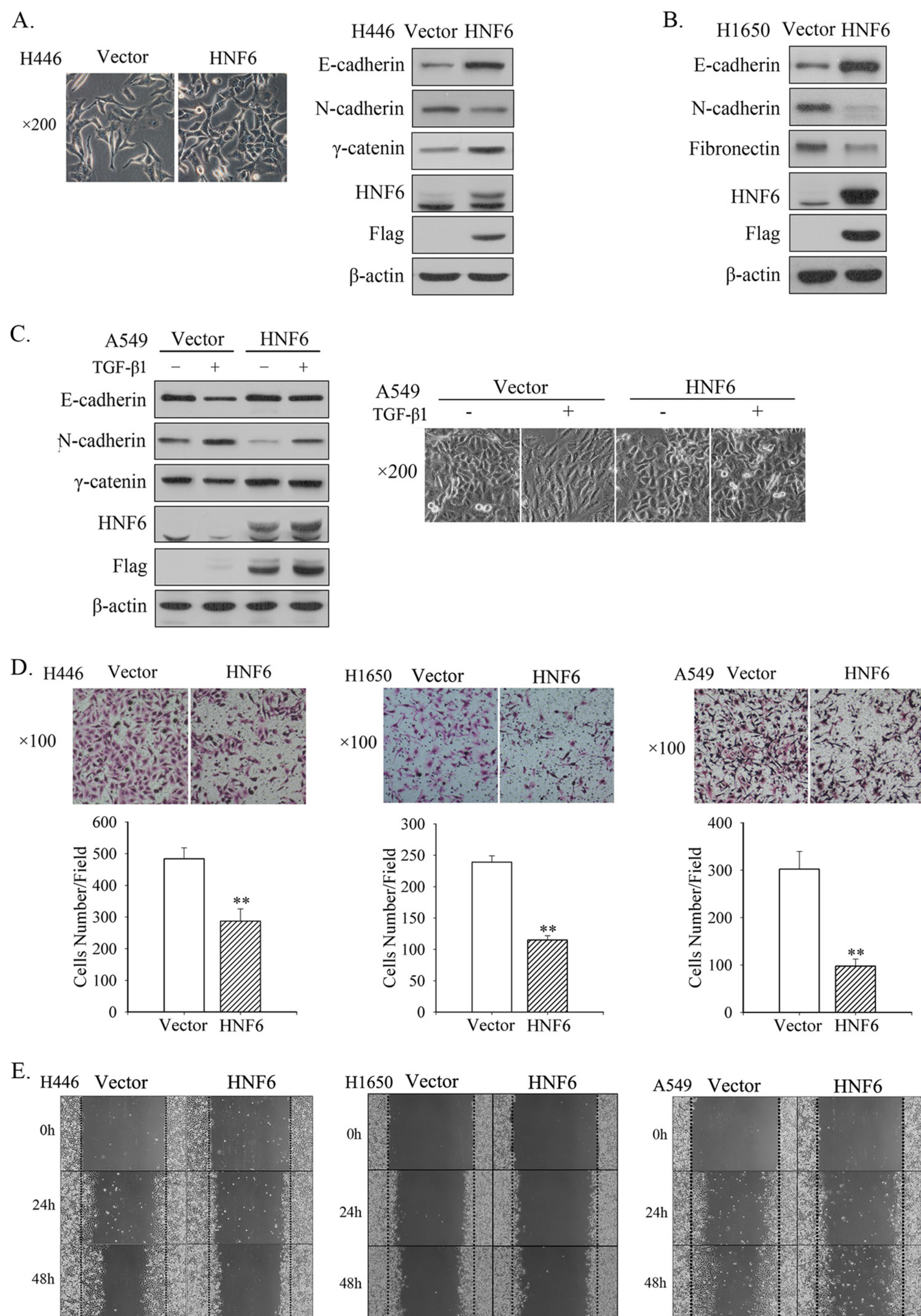

FIGURE 2. **The effects of HNF6 overexpression on EMT and cell migration.** *A*, ectopically expressed FLAG-tagged HNF6 and vector plasmid were transfected into H446 lung cancer cells. The cell morphology and EMT marker protein level were examined. *B*, the effect of HNF6 overexpression in H1650 lung cancer cells were examined by detecting the EMT marker protein level. *C*, the effect of HNF6 overexpression on TGF-β1 induced EMT in A549 cells were determined by cell morphology and relevant protein markers. Cells were treated with or without TGF-β1 (3 ng/ml) for 48 h. *D*, for Transwell assay in H446, H1650, and A549 cells, a representative result of three independent experiments, and the statistic data were presented. *E*, decreased cell migration ability by HNF6 in A549 cells were detected by scratch assay at the time point indicated.

suggesting that tumor suppressive effect of HNF6 may also be linked with its effect on cell proliferation besides on EMT.

Correlation between the Level and Effect of HNF6 and p53—p53 has been in recent years reportedly implicated in the inhibition of EMT. Interestingly, p53 levels are not only positively correlated with that of HNF6 in A549, H1650, and H446 lung cancer cells but also positively correlated with the levels of E-cadherin and reversely correlated with N-cadherin (Fig. 4*A*). The data suggest that p53 functions in maintaining epithelial phenotype. This observation is consistent with the reported work that shows a loss of wild type p53 in A549 cells activated EMT (28). Knockdown of p53 in A549 cells induced a morphological change with the characteristic of EMT (Fig. 4*B*, *left*). Decrease in E-cadherin and increase in N-cadherin level was also detected (Fig. 4*B*, *right*). Correspondingly, a marked increase in cell migration was observed as detected by Tran-

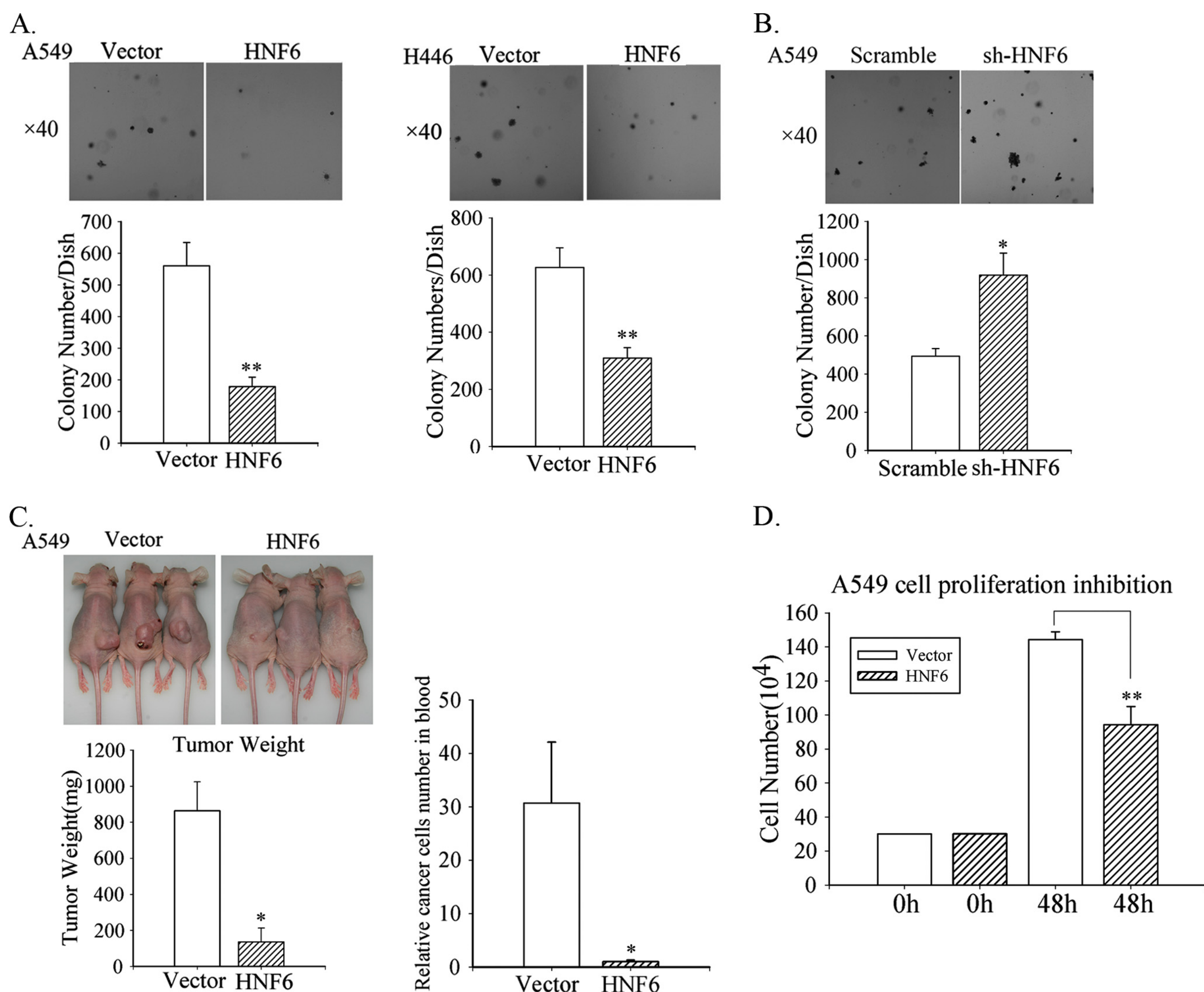

FIGURE 3. **HNF6 overexpression represses anchorage-independent growth, cell proliferation, tumor xenograft growth, and invasion.** Experiments were performed as indicated. A representative result of three independent experiments and the statistic data for each result were presented. *A*, the effect of HNF6 overexpression on anchorage independent growth ability in A549 cells and H446 cells were detected by colony formation assay in soft agar. *B*, the effect of HNF6 knockdown on anchorage-independent growth ability in A549 cells were detected by colony formation assay in soft agar. *C*, the effect of HNF6 on tumor formation ability was determined by subcutaneously injection of HNF6 overexpressed and control A549 cells to nude mice as indicated. Mice were executed after 6 weeks and tumors were weighted. For invasion, the cancer cells invaded to blood vessels were examined through detecting the mRNA level of human tubulin and mouse GAPDH in the whole blood of mice by real time qPCR (*right*). *D*, the effect of HNF6 on cell proliferation in A549 cells were determined by counting the cells number. 3×10^5 cells were seeded in each dish, and the cell numbers were counted after 48 h.

swell and scratch assays (Fig. 4*C*). In contrast, overexpression of wide type p53 in A549 cells increased E-cadherin and γ-catenin levels and decreased N-cadherin level (Fig. 4*D*, *left*). In line with the above effect, p53 overexpression suppressed cell migration (Fig. 4*D*, *right*). Similar effects of p53 overexpression in H446 cells were also observed (Fig. 4*E*).

Regulation of p53 by HNF6 and Its Involvement in EMT and Cell Migration—The fact that p53 is abundant in A549 cells, and its levels are positively correlated with that of HNF6 in A549, H446, and H1650 lung cancer cells suggests that HNF6 may act as an upstream regulator of p53 expression in the control of EMT and cell migration and growth. To test this possibility, we examined the effect of HNF6 on p53 level in A549 cells and found that HNF6 knockdown apparently reduced p53 mRNA and protein levels (Fig. 5*A*). While overexpression of HNF6 increased p53 mRNA and protein levels (Fig. 5*B*). The increase of p53 protein levels by overexpression of HNF6 can also be observed in H446 cells and H358 cells (Fig. 5*C*). We also tested the effect of HNF6 on EMT and p53 expression in xenograft tumors. The results showed that HNF6 overexpression increased the protein levels of E-cadherin and γ-catenin. In contrast, a weak decrease in the level of mesenchymal marker protein fibronectin in HNF6 overexpressed tumors was observed. In addition, increased p53 level can be detected in HNF6 overexpressed tumors (Fig. 5*D*). To examine whether p53 is implicated in the function of HNF6, we knocked down p53 in HNF6-overexpressing cells. As shown, p53 knockdown fundamentally abolished MET-like change and the inhibition of cell migration mediated by HNF6 (Fig. 5, *E* and *F*).

HNF6 Regulates p53 through Directly Transcriptional Activation of Its Promoter—Because HNF6 is a transcription factor, it is likely that HNF6 regulates the p53 level through transcrip-

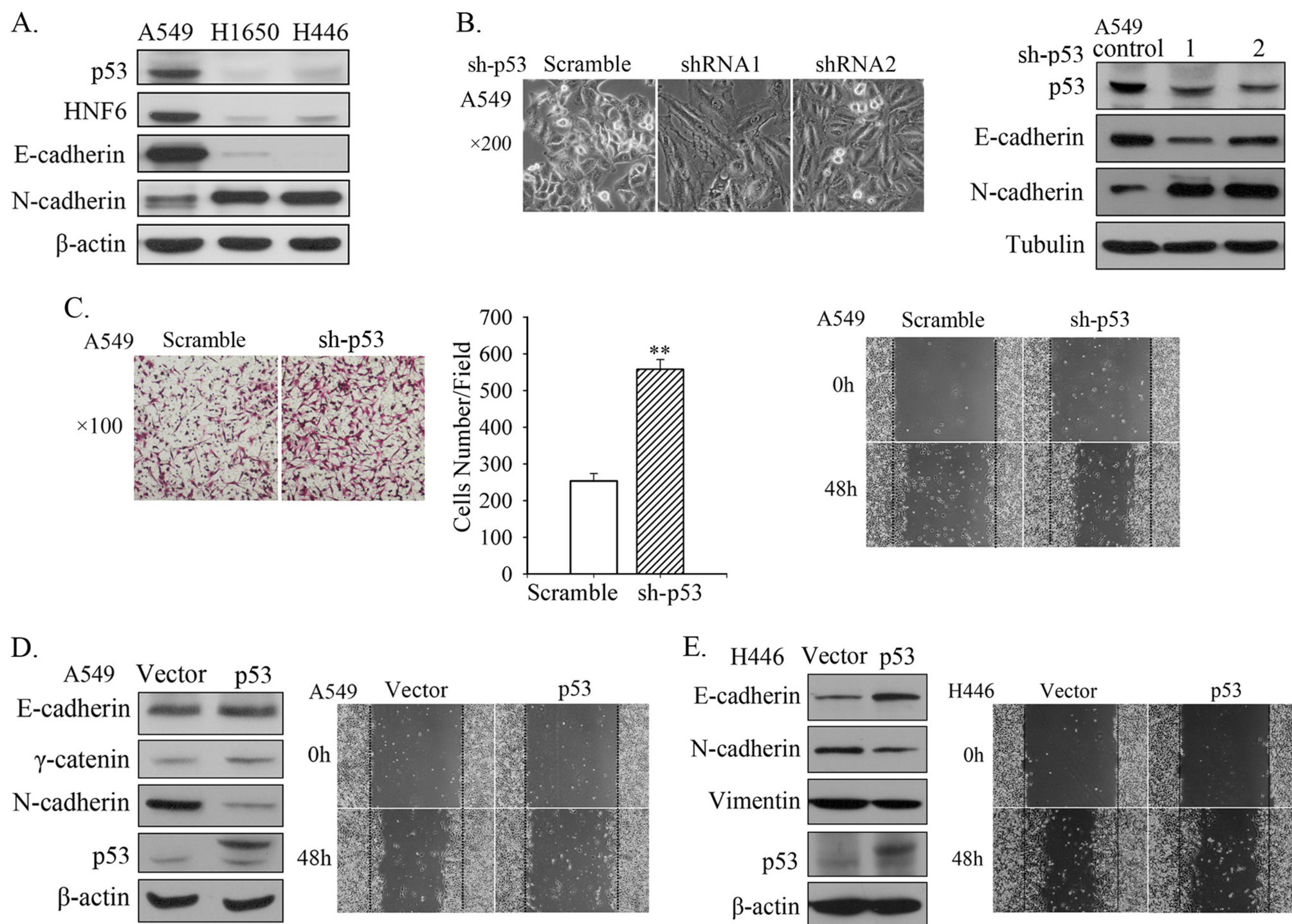

FIGURE 4. **p53 inhibits EMT and cell migration.** *A*, the protein level of p53, HNF6, and several epithelial and mesenchymal marker protein levels in A549, H1650, and H446 cells were examined. *B*, the effect of p53 knockdown on EMT was assessed by cell morphology and E-cadherin and N-cadherin levels. *C*, p53 knockdown increases the cell migration ability as examined by Transwell assay and scratch assay. *D*, the effect of p53 overexpression on the protein level of E-cadherin, γ-catenin, and N-cadherin in A549 cells were examined (*left*). The effect of p53 overexpression on A549 cell migration ability was determined by scratch assay at the time point indicated (*right*). E. The effect of p53 overexpression on the protein level of E-cadherin, N-cadherin and vimentin in H446 cells (*left*). The effect of p53 overexpression on cell migration in H446 cells were examined by scratch assay (*right*).

tional activation p53 gene. By an assay using a Dual-Reporter pGL3 luciferase assay system, we found that HNF6 significantly increased p53 promoter activity (Fig. 6*A*). We also determined whether HNF6 can directly bind to the p53 gene promoter by ChIP assay. There are two putative HNF6 binding sites in the upstream of the p53 cDNA sequence region, which are AAGTCAATCA and ATTGAT (13, 30). HNF6 can directly bind to p53 promoter through the AAGTCAATCA sequence (Fig. 6*B*). In addition, the region of -2.8 kb $\sim$ -2.5 kb of the p53 promoter, which contains the AAGTCAATCA sequence, was identified to be essential for the activation of p53 promoter activity by HNF6 (Fig. 6*C*).

Correlation of the E-cadherin and p53 Levels with HNF6 Levels in Human Lung Cancer Tissues—To confirm the relationship between the expression levels of HNF6 and E-cadherin or p53 protein levels, we also examined and analyzed their expression levels in human lung adenocarcinoma tissue microarrays by immunohistochemistry. As shown in Fig. 7*A*, the HNF6 and E-cadherin levels are highly correlated in human lung adenocarcinoma tissues. Surprisingly, the high expression level of HNF6 always correlated with high levels of E-cadherin in 21 cases examined. Similarly, Fig. 7*B* showed a high correlation between the HNF6 and p53 levels. These data further suggest that HNF6 is a regulator for p53 expression and a suppressor of EMT. Analysis of one microarray data set from NCBI GEO profiles revealed that during colorectal cancer metastasis, HNF6 expression was decreased in lymph node metastasis, as compared with primary tumor (Fig. 7*C*). In addition, during the multistep pancreatic carcinogenesis, HNF6 expression was also obviously decreased in intraductal papillary mucinous adenoma, intraductal papillary mucinous carcinoma, and invasive cancer of intraductal papillary mucinous neoplasm, as compared with normal pancreatic tissues (Fig. 7*D*). These data suggest that HNF6 can function as an important suppressor for tumor progression.

DISCUSSION

HNF6 was initially been identified as an important factor in liver and pancreas development and organogenesis, and it is also essential in supporting the normal functions of these organs. In recent years, a few reports showed that decreased expression levels of HNF6 are associated with the tumor metastasis and increased cell proliferation, which suggest a possibility that HNF6 may be implicated in the control of tumor growth

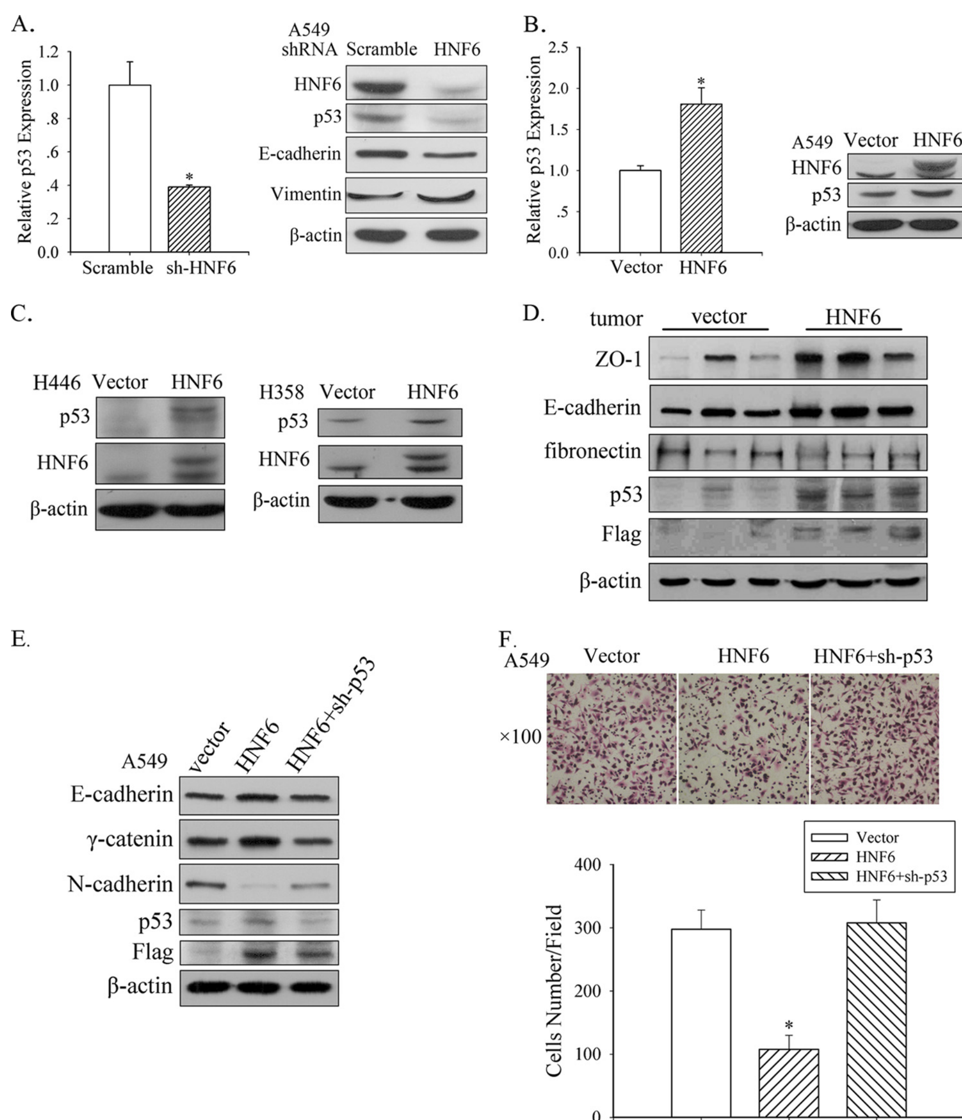

FIGURE 5. **Regulation of p53 by HNF6 and its involvement in EMT and cell migration.** *A*, the effect of HNF6 knockdown on p53 mRNA level and protein level in A549 cells were detected by real time qPCR and Western blotting. *B*, the effect of HNF6 overexpression on p53 mRNA and protein levels in A549 cells were detected by real time qPCR and Western blotting. *C*, overexpression of HNF6 in H446 cells and H358 cells increased p53 protein levels. *D*, the effect of HNF6 overexpression on E-cadherin, γ-catenin, fibronectin, and p53 in A549 xenograft tumors were detected by Western blotting. *E* and *F*, depletion of the effect of p53 on epithelial and mesenchymal marker proteins level and cell migration in HNF6 overexpressed A549 cells were detected by Western blotting and Transwell assays.

and metastasis. However, no experimental evidence and further investigation have been reported. The functions of HNF6 in EMT, migration, cancer cell growth, tumor formation, and invasion remain unclear. In this study, we determined the role and potential mechanism of HNF6 in EMT, migratory ability, and invasive growth of A549 lung adenocarcinoma cells. The disappearance of epithelial phenotype and acquisition of mesenchymal phenotype constitute a basic molecular and morphological manifestation of EMT. This process increases cell mobility and constitutes a critical step in cell migration, which is associated with various biological processes, including cancer invasion and metastasis. Thus, the maintenance of epithelial phenotype and suppression of EMT have been increasingly recognized to be important for preventing cancer progression. Loss of HNF6 in A549 cells causes the lost of epithelial phenotype and the gain of mesenchymal phenotype and also the increase of cell migration. Overexpression of HNF6 can shift the epithelial-mesenchymal balance toward the side of epithelial phenotype and decrease the intensity of EMT induced by TGF-β. These data indicate that HNF6 is essential for the maintenance of epithelial phenotype and changes in its expression level will disrupt the balance between the epithelial-mesenchymal phenotypes. Similar to HNF6, some other molecules have been shown to be critical for maintaining the epithelial phenotype of cells, such as IRS1, FoxA2, and HNF4α, suggesting that a signaling network is orchestrated to regulate the balance between the epithelial-mesenchymal properties. Down- and up-regulation of molecule(s) required for the maintenance of the epithelial phenotype causes a loss or gain of the epithelial phenotype. Likewise, down- and up-regulation of molecule(s) important for the mesenchymal phenotype such as Snail, Slug, Twist, ZEB1, and ZEB2 (31) will cause a converse shift between the balance of epithelial-mesenchymal phenotype. In most epithelial cells, the expression levels or activity of these two groups

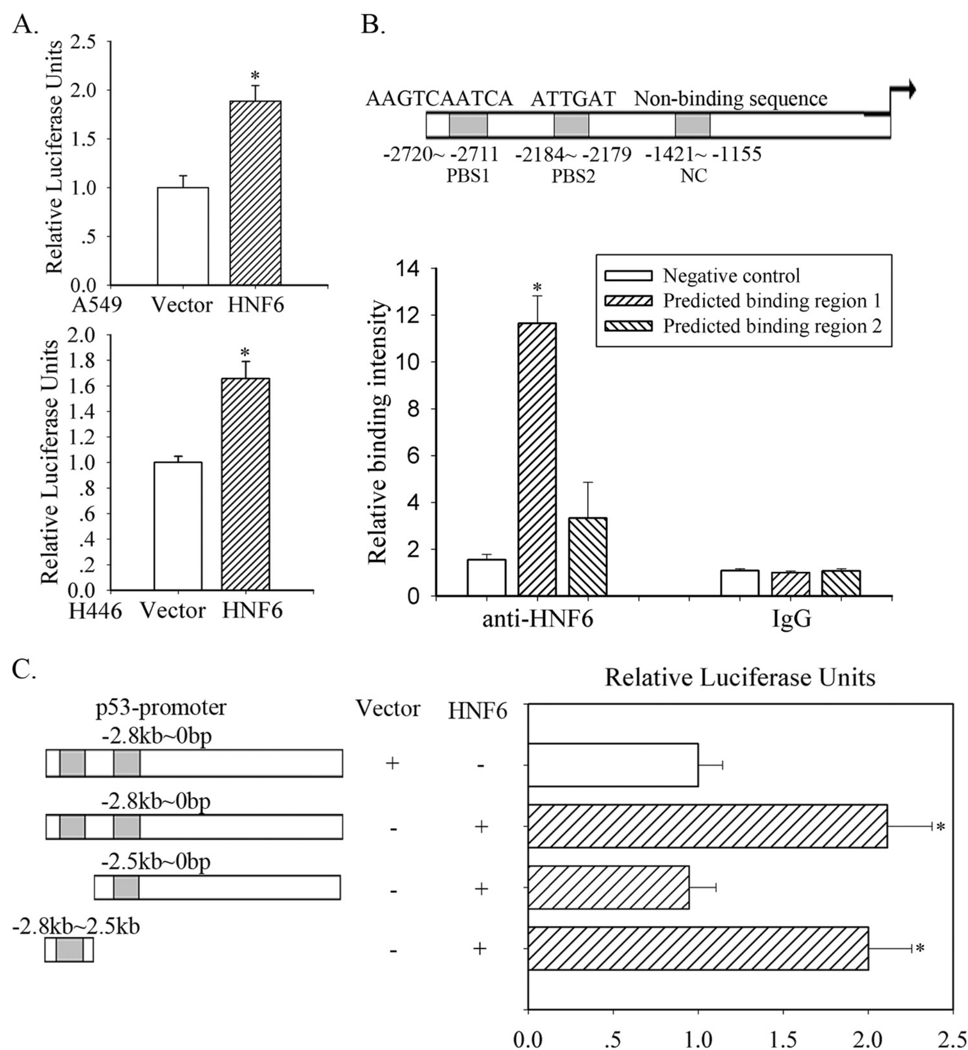

FIGURE 6. **HNF6 regulates p53 through directly transcriptional activation of its promoter.** *A*, the effect of HNF6 on p53 promoter activity in A549 cells and H446 cells were detected by the Dual-Reporter pGL3 luciferase system. Luciferase activities were detected at 24 h after transfection. *B*, binding of HNF6 to predicted regions in the p53 promoter were detected by ChIP assay. *C*, the effects of HNF6 on the luciferase activity of truncations of p53 promoters were detected by luciferase assay.

of molecules are stably balanced, with the epithelial gatekeeper molecules activated and the mesenchymal inducers repressed at a relative low level, keeping the tissue and organ in a state of homeostasis. In this report, we show for the first time that, HNF6 is a gatekeeper molecule for epithelial phenotype, adding a new member to this group.

Overexpression of HNF6 increased the epithelial phenotype and inhibited the EMT and the invasive growth of lung tumor cells. Because anchorage-independent growth is an important indicator of cancer malignancy, and it could often be increased after the EMT of cells, HNF6-mediated suppression of anchorage independent growth in soft agar and the tumor formation and invasion *in vivo* is therefore more likely due to its inhibitory effect on EMT and cell proliferation.

p53 is an important tumor suppressor gene. It plays important roles in apoptosis, DNA repair, and cell proliferation inhibition, and it has been emerged in recent years a critical inhibitor of EMT. A large number of molecules have been reported to be regulated by p53 (32), and many molecules are shown to control the stability and activity of p53 (33). While much less molecules have been reported to regulate p53 expression through transcriptional regulation of its mRNA level. In this report, we found that HNF6 can positively regulate p53 expression by directly activate its promoter activity, suggesting the roles of HNF6 on EMT, cell migration, cell proliferation, and tumor growth may at least partially through its up-regulation of p53. Besides the roles of p53 mentioned above, stemness inhibition is also an important function of p53 reported in recent years (34, 35). The inhibitory effect of p53 on cell stemness may also be related to its inhibitory effect on EMT because EMT was considered to increase stemness in some circumstances (22, 36). However, as an upstream molecule of p53, whether HNF6 is involved in the regulation of cell stemness remains to be investigated.

E-cadherin is one of the most important indicators of epithelial phenotype. In clinical diagnosis, E-cadherin could be used as a prognostic factor in some types of cancers (16, 29). High E-cadherin expression level correlated with less metastatic ability of tumors. HNF6 expression level was highly correlated with E-cadherin not only in lung cancer cell lines but also in human

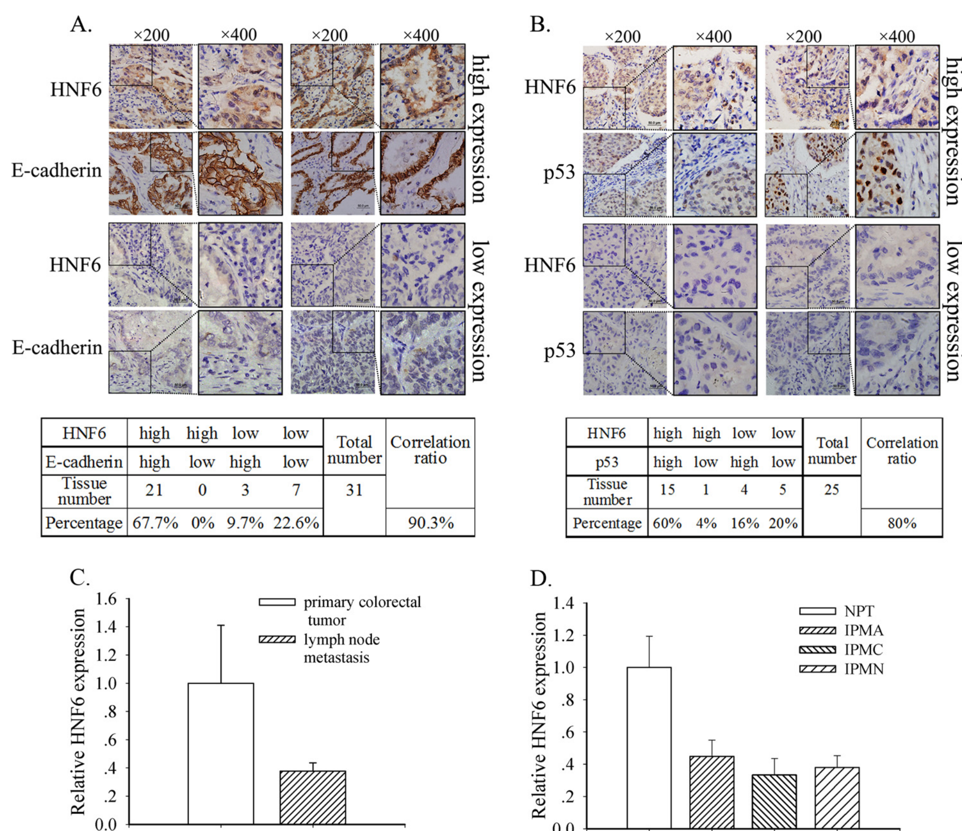

FIGURE 7. **The correlation between the protein levels of HNF6 and E-cadherin or p53 in human lung cancer tissues.** Protein expression in lung cancer tissues were detected by immunohistochemistry. *A*, two representative lung cancer tissue samples with high (*upper*) and low (*lower*) expression levels of HNF6 and E-cadherin, respectively, were shown. The *dark brown* color staining represents HNF6 and E-cadherin. The statistical data were provided in the table *below*. *B*, two representative lung cancer tissue samples with high (*upper*) and low (*lower*) expression levels of HNF6 and p53 were shown. The *dark brown* shade stands for the staining of HNF6 and p53. The statistical data were shown in the table *below*. *C*, HNF6 expression levels in primary colorectal tumor and lymph node metastasis were from NCBI GEO profiles GDS1780, and data were standardized and analyzed. *D*, HNF6 expression levels in normal pancreatic tissues (*NPT*), intraductal papillary mucinous adenoma (*IPMA*), intraductal papillary mucinous carcinoma (*IPMC*), and invasive cancer of intraductal papillary mucinous neoplasm (*IPMN*) were from NCBI GEO profile GDS3836, and data were standardized and analyzed.

lung cancer tissues, and HNF6 can up-regulate E-cadherin in several lung cancer cell lines. High expression of HNF6 correlated with more epithelial phenotype and less metastatic ability and decreased proliferation. These observations suggest a potential diagnostic value of HNF6 in early clinical cancer diagnosis. In addition, factors that are able to restore or up-regulate the expression of HNF6 may be considered as potential therapeutic candidate molecules in the treatment of some cancers.

Acknowledgments—We thank Dr. Dang-Sheng Li for helpful and critical comments of this work and Wei-Qiao Ding for certain technical assistance. We also thank other members of the laboratory for many helpful discussions.

REFERENCES

1. Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R. A. (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117,** 927–939
2. Thiery, J. P. (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer* **2,** 442–454
3. Hugo, H., Ackland, M. L., Blick, T., Lawrence, M. G., Clements, J. A., Williams, E. D., and Thompson, E. W. (2007) Epithelial–mesenchymal and mesenchymal–epithelial transitions in carcinoma progression. *J. Cell. Physiol.* **213,** 374–383
4. Tang, Y., Shu, G., Yuan, X., Jing, N., and Song, J. (2011) FOXA2 functions as a suppressor of tumor metastasis by inhibition of epithelial-to-mesenchymal transition in human lung cancers. *Cell Res.* **21,** 316–326
5. Song, Y., Washington, M. K., and Crawford, H. C. (2010) Loss of FOXA1/2 is essential for the epithelial-to-mesenchymal transition in pancreatic cancer. *Cancer Res.* **70,** 2115–2125
6. Yue, H. Y., Yin, C., Hou, J. L., Zeng, X., Chen, Y. X., Zhong, W., Hu, P. F., Deng, X., Tan, Y. X., Zhang, J. P., Ning, B. F., Shi, J., Zhang, X., Wang, H. Y., Lin, Y., and Xie, W. F. (2010) Hepatocyte nuclear factor 4α attenuates hepatic fibrosis in rats. *Gut* **59,** 236–246
7. Wang, K., and Holterman, A. X. (2012) Pathophysiologic role of hepatocyte nuclear factor 6. *Cell. Signal.* **24,** 9–16
8. Vanhorenbeeck, V., Jenny, M., Cornut, J. F., Gradwohl, G., Lemaigre, F. P., Rousseau, G. G., and Jacquemin, P. (2007) Role of the Onecut transcription factors in pancreas morphogenesis and in pancreatic and enteric endocrine differentiation. *Dev. Biol.* **305,** 685–694
9. Clotman, F., Lannoy, V. J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G. G., and Lemaigre, F. P. (2002) The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* **129,** 1819–1828
10. Holterman, A. X., Tan, Y., Kim, W., Yoo, K. W., and Costa, R. H. (2002) Diminished hepatic expression of the HNF-6 transcription factor during bile duct obstruction. *Hepatology* **35,** 1392–1399
11. Tan, Y., Yoshida, Y., Hughes, D. E., and Costa, R. H. (2006) Increased expression of hepatocyte nuclear factor 6 stimulates hepatocyte proliferation during mouse liver regeneration. *Gastroenterology* **130,** 1283–1300
12. Lehner, F., Kulik, U., Klempnauer, J., and Borlak, J. (2007) The hepatocyte nuclear factor 6 (HNF6) and FOXA2 are key regulators in colorectal liver

metastases. *FASEB J.* **21,** 1445–1462

13. Samadani, U., and Costa, R. H. (1996) The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. *Mol. Cell Biol.* **16,** 6273–6284
14. Rausa, F. M., Tan, Y., and Costa, R. H. (2003) Association between hepatocyte nuclear factor 6 (HNF-6) and FoxA2 DNA binding domains stimulates FOXA2 transcriptional activity but inhibits HNF-6 DNA binding. *Mol. Cell Biol.* **23,** 437–449
15. Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C. E., Van der Smissen, P., Dietz, H. C., Courtoy, P. J., Rousseau, G. G., and Lemaigre, F. P. (2005) Control of liver cell fate decision by a gradient of TGF β signaling modulated by Onecut transcription factors. *Genes Dev.* **19,** 1849–1854
16. Gould Rothberg, B. E., and Bracken, M. B. (2006) E-cadherin immunohistochemical expression as a prognostic factor in infiltrating ductal carcinoma of the breast: a systematic review and meta-analysis. *Breast Cancer Res. Treat.* **100,** 139–148
17. Jiang, X., Zhang, W., Kayed, H., Zheng, P., Giese, N. A., Friess, H., and Kleeff, J. (2008) Loss of ONECUT1 expression in human pancreatic cancer cells. *Oncol. Rep.* **19,** 157–163
18. Levine, A. J. (1997) p53, the cellular gatekeeper for growth and division. *Cell* **88,** 323–331
19. Wade, M., Li, Y. C., and Wahl, G. M. (2013) MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nat. Rev. Cancer* **13,** 83–96
20. Teodoro, J. G., Parker, A. E., Zhu, X., and Green, M. R. (2006) p53-mediated inhibition of angiogenesis through up-regulation of a collagen prolyl hydroxylase. *Science* **313,** 968–971
21. Kim, T., Veronese, A., Pichiorri, F., Lee, T. J., Jeon, Y. J., Volinia, S., Pineau, P., Marchio, A., Palatini, J., Suh, S. S., Alder, H., Liu, C. G., Dejean, A., and Croce, C. M. (2011) p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2. *J. Exp. Med.* **208,** 875–883
22. Chang, C. J., Chao, C. H., Xia, W., Yang, J. Y., Xiong, Y., Li, C. W., Yu, W. H., Rehman, S. K., Hsu, J. L., Lee, H. H., Liu, M., Chen, C. T., Yu, D., and Hung, M. C. (2011) p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. *Nat. Cell Biol.* **13,** 317–323
23. Dong, P., Karaayvaz, M., Jia, N., Kaneuchi, M., Hamada, J., Watari, H., Sudo, S., Ju, J., and Sakuragi, N. (2013) Mutant p53 gain-of-function induces epithelial-mesenchymal transition through modulation of the miR-130b-ZEB1 axis. *Oncogene* **32,** 3286–3295
24. Zhang, K. H., Tian, H. Y., Gao, X., Lei, W. W., Hu, Y., Wang, D. M., Pan, X. C., Yu, M. L., Xu, G. J., Zhao, F. K., and Song, J. G. (2009) Ferritin heavy chain-mediated iron homeostasis and subsequent increased reactive oxygen species production are essential for epithelial-mesenchymal transition. *Cancer Res.* **69,** 5340–5348
25. Tiscornia, G., Singer, O., and Verma, I. M. (2006) Production and purification of lentiviral vectors. *Nat. Protoc.* **1,** 241–245
26. Prudkin, L., Liu, D. D., Ozburn, N. C., Sun, M., Behrens, C., Tang, X., Brown, K. C., Bekele, B. N., Moran, C., and Wistuba, I. I. (2009) Epithelial-to-mesenchymal transition in the development and progression of adenocarcinoma and squamous cell carcinoma of the lung. *Mod. Pathol.* **22,** 668–678
27. Shi, J., Wang, D. M., Wang, C. M., Hu, Y., Liu, A. H., Zhang, Y. L., Sun, B., and Song, J. G. (2009) Insulin receptor substrate-1 suppresses transforming growth factor-beta1-mediated epithelial-mesenchymal transition. *Cancer Res.* **69,** 7180–7187
28. Kim, N. H., Kim, H. S., Li, X. Y., Lee, I., Choi, H. S., Kang, S. E., Cha, S. Y., Ryu, J. K., Yoon, D., Fearon, E. R., Rowe, R. G., Lee, S., Maher, C. A., Weiss, S. J., and Yook, J. I. (2011) A p53/miRNA-34 axis regulates Snail1-dependent cancer cell epithelial-mesenchymal transition. *J. Cell Biol.* **195,** 417–433
29. Rosenau, J., Bahr, M. J., von Wasielewski, R., Mengel, M., Schmidt, H. H., Nashan, B., Lang, H., Klempnauer, J., Manns, M. P., and Boeker, K. H. (2002) Ki67, E-cadherin, and p53 as prognostic indicators of long-term outcome after liver transplantation for metastatic neuroendocrine tumors. *Transplantation* **73,** 386–394
30. Lannoy, V. J., Bürglin, T. R., Rousseau, G. G., and Lemaigre, F. P. (1998) Isoforms of hepatocyte nuclear factor-6 differ in DNA-binding properties, contain a bifunctional homeodomain, and define the new ONECUT class of homeodomain proteins. *J. Biol. Chem.* **273,** 13552–13562
31. Peinado, H., Olmeda, D., and Cano, A. (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat. Rev. Cancer* **7,** 415–428
32. Harris, S. L., and Levine, A. J. (2005) The p53 pathway: positive and negative feedback loops. *Oncogene* **24,** 2899–2908
33. Vousden, K. H., and Lane, D. P. (2007) p53 in health and disease. *Nat. Rev. Mol. Cell Biol.* **8,** 275–283
34. Marión, R. M., Strati, K., Li, H., Murga, M., Blanco, R., Ortega, S., Fernandez-Capetillo, O., Serrano, M., and Blasco, M. A. (2009) A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. *Nature* **460,** 1149–1153
35. Takenaka, C., Nishishita, N., Takada, N., Jakt, L. M., and Kawamata, S. (2010) Effective generation of iPS cells from $CD34^+$ cord blood cells by inhibition of p53. *Exp. Hematol.* **38,** 154–162
36. Schubert, J., and Brabletz, T. (2011) p53 Spreads out further: suppression of EMT and stemness by activating miR-200c expression. *Cell Res.* **21,** 705–707