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p53 Attenuates the oncogenic Ras-induced epithelial–mesenchymal transition in human mammary epithelial cells

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ABSTRACT

Inactivation of the tumor suppressor p53 and activation of the oncogene Ras are the two most pivotal events in tumor development. However, potential intersection between p53 and Ras activity during an EMT process, which plays a crucial role during malignant tumor progression, remains elusive. Here, we report that increased expression of wild type p53 suppressed H-Ras^{V12}-induced EMT phenotypes and restrained stem cell properties, through downregulation of MEK-ERK signaling pathways. *In vivo* experiments showed that p53 was able to inhibit H-Ras^{V12}-induced tumor growth of human mammary epithelial cells. This study elucidates a novel correlation between the tumor suppressor gene p53 and the oncogene Ras in regulating EMT program, and expands the knowledge about the function of p53 in EMT process.

1. Introduction

Epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) are involved in cell morphologic changes between epithelial and mesenchymal states as key steps during embryonic morphogenesis [1]. Increasing evidence suggests that EMT is now implicated in tumorigenesis and tumor metastasis [2]. In the process of EMT, epithelial cells shed their differentiated characteristics through disruption of cell–cell adhesion, apical-basal polarity, and acquisition of mesenchymal features, such as migratory and invasive properties [3]. Recent findings further illustrate that the EMT can produce cancer cells that exhibit stem cell characteristics [4].

Ras protein family members belong to the protein superfamily of small GTPases, which are activated in response to a variety of extracellular stimuli [5,6]. Ras signal transduction pathway controls a diverse range of cellular activities such as cell proliferation, adhesion, motility, survival and differentiation [7,8]. Ras is one of the most frequently mutated oncogenes in human cancers. Oncogenic Ras mutants constitutively activate numerous pathways including the RAF-MEK-ERK and PI3K-Akt signaling pathways, both of which play significant roles in cancer progression [6,9]. Recent studies have shown that EMT inducers Twist1 and Twist2, in combination with the activated oncogenic Ras (H-Ras^{V12}) promote even more dramatic characteristics of EMT, indicating that oncogenic Ras plays an important part during an EMT program [10].

The tumor suppressor p53 plays a vital role in regulation of many fundamental cellular processes such as cell-cycle delay, DNA damage repair, apoptosis and senescence [11,12]. Transgenic mice with mutant p53 expression or p53 knockout mice are prone to the spontaneous development of tumors at a high frequency [13]. Significantly, p53 gene mutations or deletion occur in most human cancers, indicating that loss of p53 function is a frequent and important event in tumorigenesis. In addition, recent work demonstrates that p53 is able to suppress cancer progression and metastasis through regulating EMT and EMT associated tumor stem cell properties [14].

Many studies demonstrate that the synergistic action of inactivation of p53 gene and activation of the oncogene Ras is the most common event in the progression of many human cancers [15]. In this study, we intended to investigate the function of p53 in EMT induced by oncogenic Ras. We showed that H-Ras^{V12} was able to trigger an EMT program in mammary epithelial cells. Meanwhile, the tumor suppressor p53 suppressed H-Ras^{V12} -induced EMT phenotype, restrained stem cell properties, and inhibited *in vivo* tumorigenesis through inactivating MEK-ERK signaling pathway. This study elucidates the novel relationship between tumor suppressor gene p53 and oncogene Ras in regulating EMT program.

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2. Materials and methods

2.1. Antibodies and reagents

Antibodies against E-cadherin, N-cadherin, β-catenin, Vimentin (BD Biosciences), ERK, p-ERK, Akt, p-Akt (Cell Signaling Technology), β-actin, p53 (Sigma) and Ras (Millipore) were used. Recombinant human hEGF and hFGF proteins were purchased from R&D Systems. Insulin, B27 and horse serum were obtained from Gibico. Cholera toxin, hydrocortisone, MEK/ERK inhibitor U0126 and PI3K/Akt inhibitor Wortmannin were purchased from Sigma.

2.2. Cell culture

MCF10A and 293T cell lines were obtained from the American Type Culture Collection (Manassas, VA). MCF10A cells were cultured as described previously [16] in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 μg/ml Hydrocortisone, 100 ng/ml Cholera toxin, 10 μg/ml insulin and pen/strep. 293T cells were cultured in DMEM containing 10% FBS.

2.3. Plasmid construction, viral production and infection of target cells

The H-Ras^{V12} ORF was amplified by PCR using following primers (forward primer 5′-CGCGGATCCATGACCGAATACAAGCTTGT-3′ and reverse primer 5′-CCGGAATTCTCAGGAGAGCACACACTTGC-3′) from the pMSCV-H-Ras^{V12}-IRES-GFP construct (a generous gift from Dr. Patrick Humbert), and the amplified fragment was subcloned into the *Bam*HI/*Eco*RI sites of lentivrial vector pWPXLd. The lentiviral expression plasmid of wild-type p53 (pLenti6/V5-p53_wt p53) was a generous gift from Dr. Bernard W. Futscher. Lentiviruses were produced by co-transfecting 293T cells with one of the expression plasmids and packaging plasmids (psPAX2 and pMD2.G). Viral supernatants were collected 48 h later, centrifuged to remove cell debris, filtered through 0.45-μm filters (Millipore), and concentrated using Amicon Ultra centrifugal filters (Millipore). MCF10A cells were transduced with the lentivirus for 12 h in the presence of polybrene (8 μg/ml).

2.4. Immunoblot

Cells were lysed in RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100 and 0.1% SDS) containing a protease inhibitor cocktail tablet (Roche). Protein lysates were resolved by SDS–PAGE, transferred to PVDF membranes, detected with primary antibody overnight at 4 °C, and then incubated with HRP-conjugated secondary antibodies. Western blots were visualized with ECL reagent (GE Healthcare).

2.5. Immunofluorescence

Cells were seeded onto glass coverslips in 24-well plates, washed with PBS, fixed in 4% formaldehyde solution and permeabilized with 0.2% Triton X-100/PBS. Cells were blocked with 2% BSA in PBS for 30 min. Coverslips were incubated with primary antibodies for 1 h, followed by incubation with FITC-conjugated secondary antibodies for 1 h, and then stained with DAPI. Finally, coverslips were examined under a fluorescence microscope.

2.6. Mammosphere formation assay

Mammosphere assay was performed as described elsewhere [17] with minor modifications. Single cells were plated at 10,000 cells/ml on 6-well ultra-low attachment plates (Corning) in serum-free DMEM/F12 medium supplemented with 20 ng/ml bFGF, 20 ng/ml EGF, 4 μg/ml insulin, 4 μg/ml heparin, 1 μg/ml hydrocortisone, 0.4% BSA and B27. Fresh medium was supplemented every three days. After 7 d of incubation, the mammospheres were counted.

2.7. Flow cytometry

1×10^6 Cells were resuspended in 100 μl PBS containing 2% FBS (FACS buffer), and then incubated on ice for 10 min. CD44-APC and CD24-PE (BD Biosciences) were added to the cell suspension and incubated on ice for 30 min. Cells were washed and resuspended in 500 μl FACS buffer and analyzed using a FACS Calibur Flow Cytometer (BD Biosciences).

2.8. Xenograft mouse experiments

1×10^7 Cells in 100 μl of PBS were injected subcutaneously into 3-week-old female BALB/c nude mice. Five mice per group were used in each experiment. Tumor volume was measured weekly using a vernier caliper and calculated according to the formula: $\pi/6 \times \text{length} \times \text{width}^2$. Three months later, the mice were sacrificed, and tumors were collected and photographed. All animal experiments were approved by the Animal Care Committee of the Northeast Normal University, China.

2.9. Statistical analysis

Data are presented as mean ± SD. The Student's *t* test (two-tailed) was used to determine statistically the significance of differences between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Activated oncogenic Ras induced EMT in human mammary epithelial cells

We first overexpressed H-Ras^{V12} in immortalized normal human mammary epithelial cell line MCF10A by using lentiviral infection (Supplementary Fig. SA). We observed that MCF10A cells transfected with vector maintained their cobblestone-like morphology with tight cell–cell adhesion, whereas cells expressing exogenous H-Ras^{V12} displayed elongated fibroblast-like morphology typically associated with mesenchymal phenotype (Supplementary Fig. SB). This morphologic change in MCF10A-H-Ras^{V12} cells was accompanied by a significant reduction in expression of epithelial markers E-cadherin and β-catenin, together with a simultaneous increase in expression of mesenchymal markers N-cadherin and Vimentin, as shown by immunoblotting (Supplementary Fig. SC). Meanwhile, the immunofluorescence also revealed that the E-cadherin protein disappeared from cell membrane in MCF10A-H-Ras^{V12} cells, whereas the Vimentin protein was markedly induced in MCF10A-H-Ras^{V12} cells (Supplementary Fig. SD). These results suggested that H-Ras^{V12} triggered the morphological changes in human mammary epithelial cells that represent typical EMT characteristics.

3.2. p53 Suppressed the oncogenic Ras-induced EMT in human mammary epithelial cells

To determine whether increased p53 protein expression could reverse the cell phenotypes from the mesenchymal pattern to the epithelial pattern, we exogenously expressed p53 in H-Ras^{V12}-expressing MCF10A cells through lentiviral infection (Fig. 1A). We detected that enhanced p53 expression was able to change

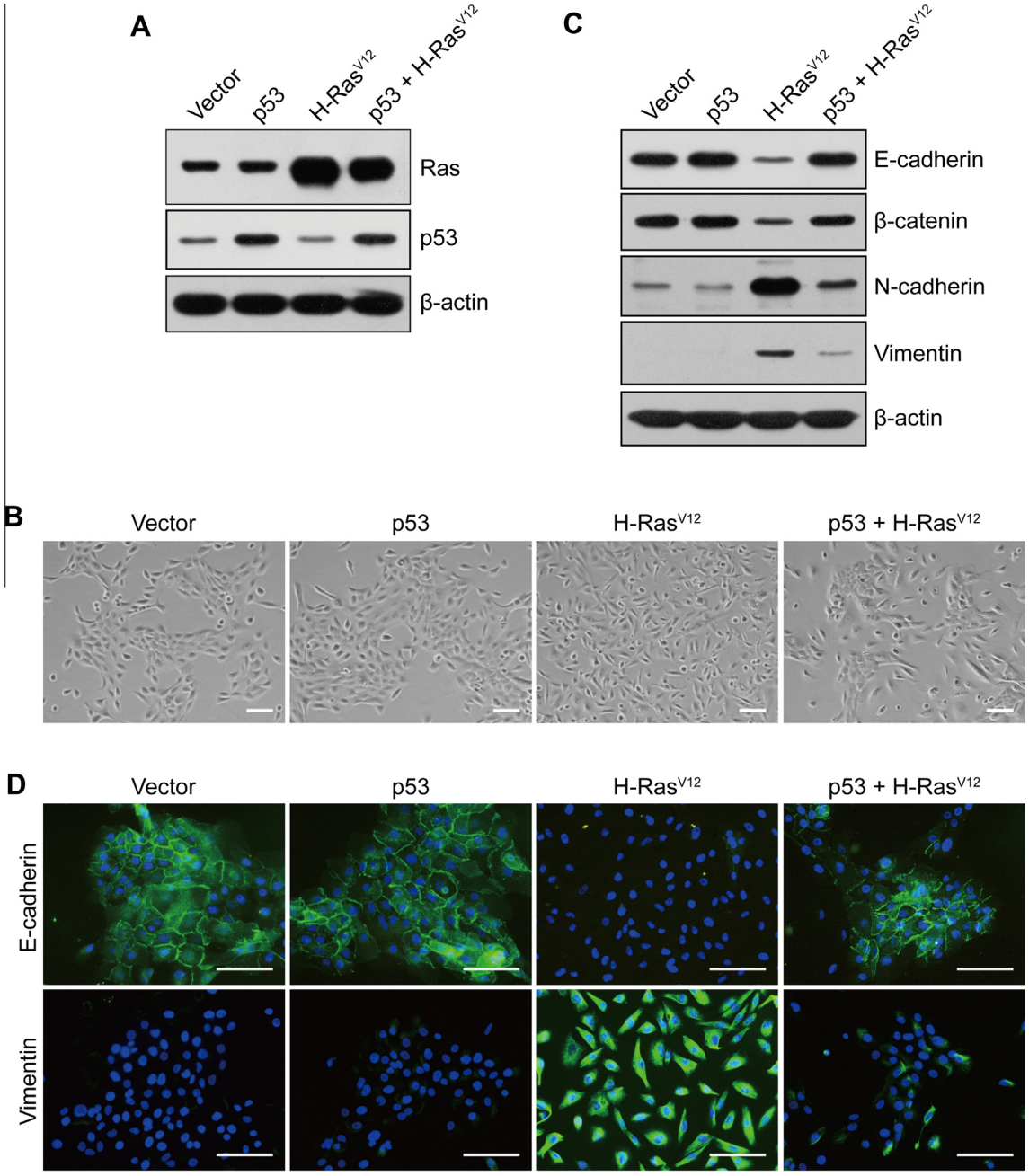

Fig. 1. Increased expression of p53 reversed the EMT phenotype. (A) p53 was introduced into MCF10A and MCF10A-H-Ras^{V12} cells; immunoblotting assessment of ectopic expression of H-Ras^{V12} and p53 after lentiviral infection in MCF10A and MCF10A-H-Ras^{V12} cells. (B) Phase contrast images of the morphology of MCF10A cells expressing vector, p53, H-Ras^{V12} and p53 + H-Ras^{V12}. Scale bar = 100 μm. (C) Expression levels of the epithelial markers E-cadherin, β-catenin, and the mesenchymal markers N-cadherin and Vimentin were examined by immunoblotting in MCF10A cells expressing vector, p53, H-Ras^{V12} and p53 + H-Ras^{V12}, respectively. β-actin was used as a loading control. (D) Immunofluorescence staining for E-cadherin and Vimentin in MCF10A cells expressing vector, p53, H-Ras^{V12} and p53 + H-Ras^{V12}, respectively. Scale bar = 100 μm.

the mesenchymal phenotypes induced by H-Ras^{V12}, such as the elongated fibroblast-like morphology and scattered distribution, into the epithelial phenotypes with increased cell–cell adhesion (Fig. 1B). Meanwhile, ectopic expression of p53 alone in MCF10A cells retained their cobblestone-like morphology (Fig. 1B). Consistent with the morphologic changes, re-expression of p53 abolished the suppression of epithelial markers E-cadherin and β-catenin, and the upregulation of mesenchymal markers N-cadherin and Vimentin, induced by H-Ras^{V12} overexpression in MCF10A cells as determined by immunoblotting (Fig. 1C). Moreover, immunofluorescence studies also demonstrated that the E-cadherin protein was increased and the Vimentin protein was reduced in MCF10A-H-Ras^{V12} cells that re-expressed p53 (Fig. 1D). These data indicated that p53 inhibited H-Ras^{V12}-triggered EMT in human mammary epithelial cells.

3.3. p53 Suppressed the oncogenic Ras-mediated mammary stem-like cell generation

Mammary epithelial cells undergoing EMT have been implicated in stem cell-like phenotypes such as the increased CD44^{high}/CD24^{low} cell population and the mammosphere-forming ability [4]. To determine whether p53 could also influence the oncogenic Ras-mediated stem cell properties, we performed FACS analysis to

assess the CD44^{high}/CD24^{low} populations. We found that the H-Ras^{V12}-expressing MCF10A cells exhibited a significant increase in the CD44^{high}/CD24^{low} stem cell population, compared with vector-infected cells, whereas re-expression of p53 significantly decreased the CD44^{high}/CD24^{low} populations induced by H-Ras^{V12} in MCF10A cells (Fig. 2A). Meanwhile, as shown in Fig. 2B and C, re-expression of p53 in H-Ras^{V12}-expressing MCF10A cells also reduced both the size and the number of mammospheres compared with the MCF10A cells expressing H-Ras^{V12} alone. These results indicated that p53 suppressed the EMT-associated properties of stem cells.

3.4. p53 Suppressed the oncogenic Ras-induced tumor growth of human mammary epithelial cells

It was reported that the CD44^{high}/CD24^{low} breast cancer cell subpopulations, which possessed the cancer stem cell properties, enhanced the tumorigenicity in a xenograft model [18]. Our results

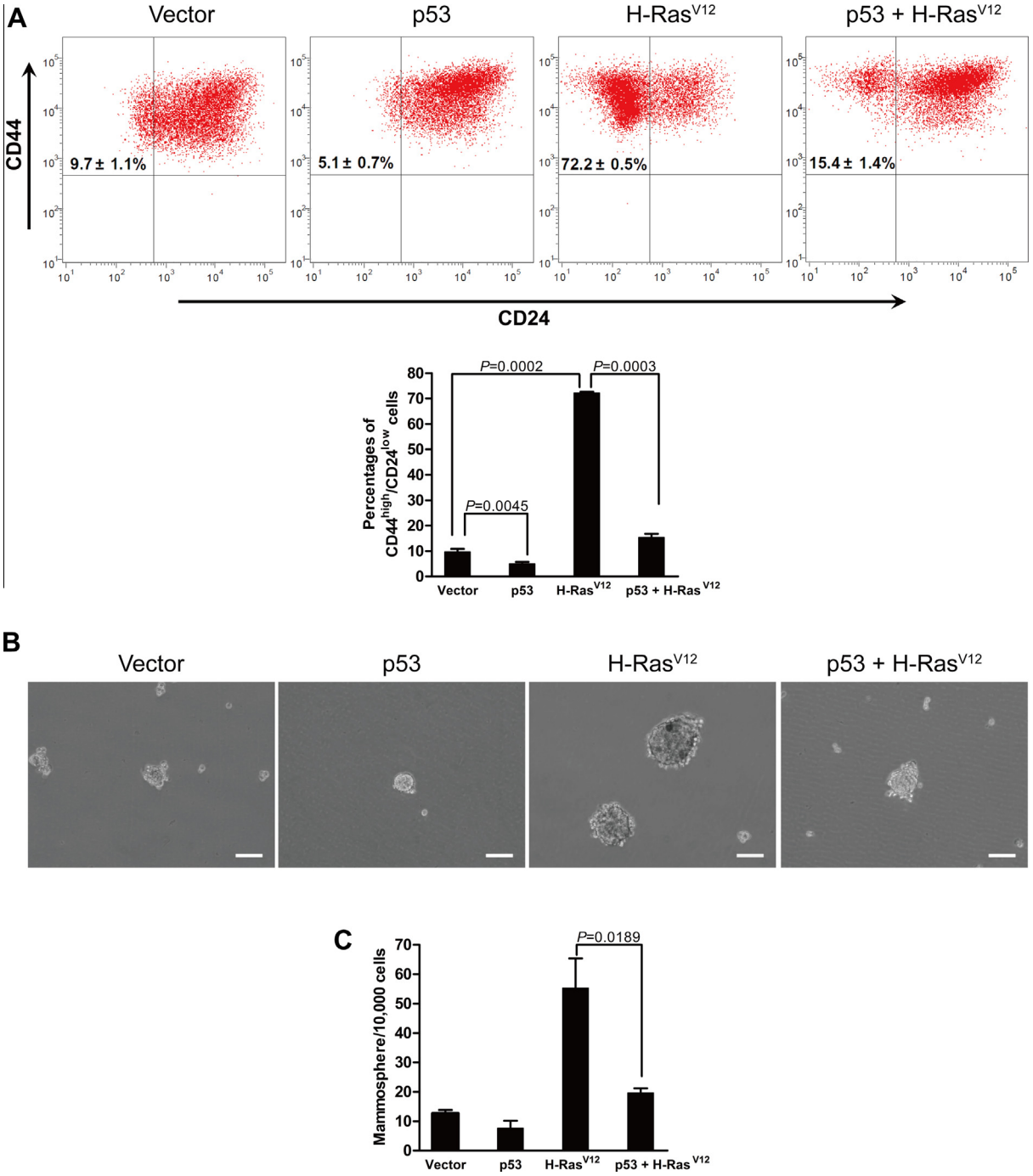

Fig. 2. Restoration of p53 expression suppressed EMT-mediated stem cell phenotype. (A) FACS analysis of cell-surface markers CD44 and CD24 in MCF-10A cells expressing vector, p53, H-Ras^{V12} and p53 + H-Ras^{V12}, respectively. Percentages of mean CD44^{high}/CD24^{low} subpopulation ± SD based on triplicate experiments are indicated in flow charts (Upper) and bar graph (Lower). (B) Phase contrast images of mammospheres formation by MCF-10A cells expressing vector, p53, H-Ras^{V12} and p53 + H-Ras^{V12}, respectively. Scale bar = 100 μm. (C) Quantification of mammosphere numbers formed by MCF10A cells expressing vector, p53, H-Ras^{V12} and p53 + H-Ras^{V12}, respectively. Data are presented as mean ± SD.

above showed that p53 was able to suppress the oncogenic Ras-induced mammary stem cell properties. We next wanted to determine the role of p53 in oncogenic Ras-induced tumorigenesis by using the tumor formation assay in nude mice. As shown in Fig. 3A and B, nude mice subcutaneously injected with MCF10A cells overexpressing H-Ras^{V12} alone gave rise to tumors, whereas re-expression of p53 attenuated the tumorigenic potential of oncogenic Ras-transformed MCF10A cells. As expected, we detected no primary tumor growth in nude mice injected with MCF10A cells either stably expressing vector alone, or expressing p53 alone (Fig. 3A and B). Thus, our results established that enhanced p53 expression reduced the oncogenic Ras-mediated tumor growth *in vivo*.

3.5. Activation of the ERK pathway was critical for the oncogenic Ras-induced EMT

It has been well documented that activated oncogene Ras can activate the RAF/MEK/ERK and the PI3K/Akt pathways [6,7]. As expected, we detected that the level of phosphorylated ERK1/2 and phosphorylated Akt were significantly increased in H-Ras^{V12}-expressing MCF10A cells (Fig. 4A). To further investigate whether

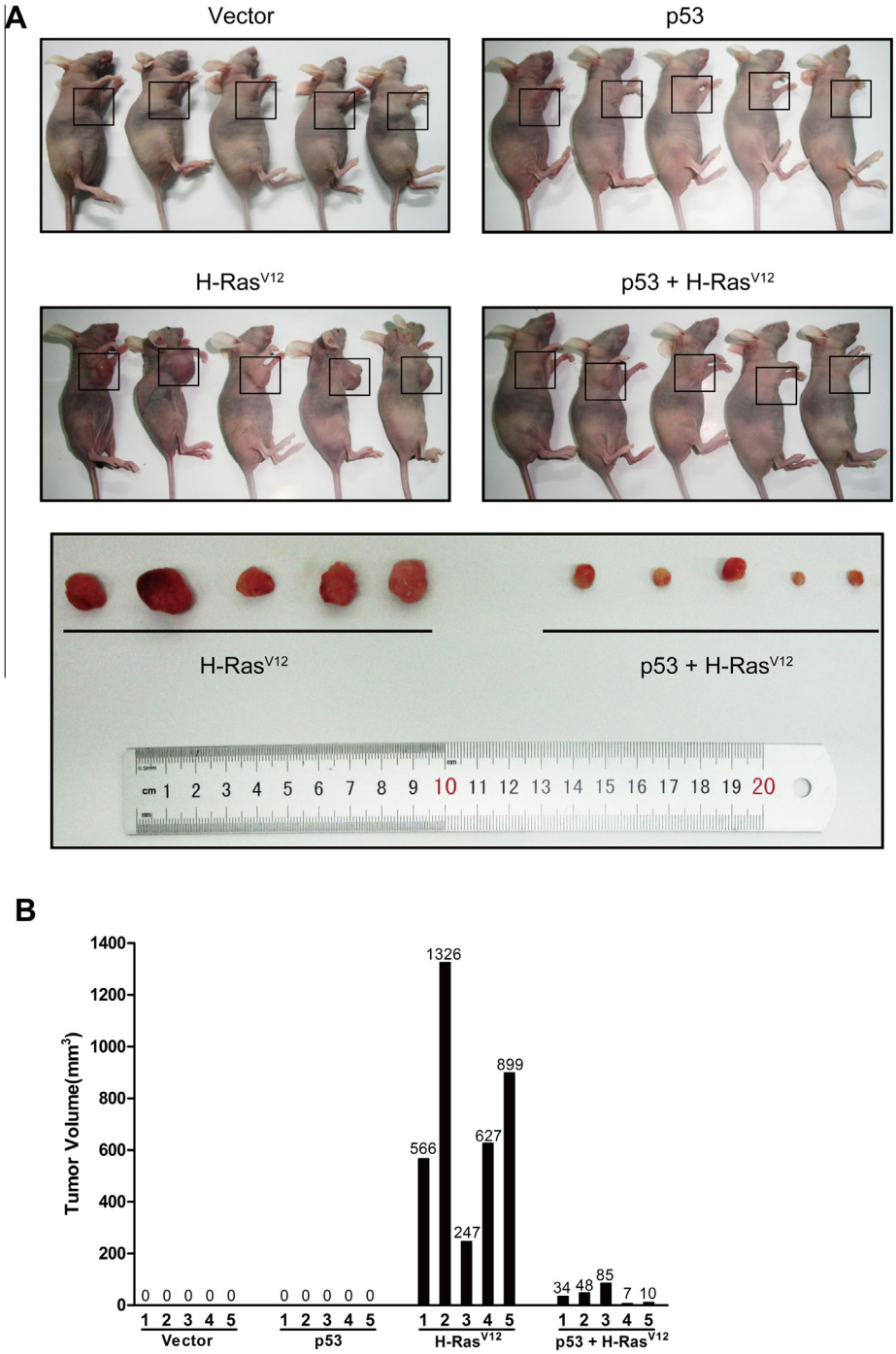

Fig. 3. Enhanced expression of p53 reduced the oncogenic Ras-induced tumor growth of human mammary epithelial cells. (A) MCF10A cells expressing vector, p53, H-Ras^{V12} and p53 + H-Ras^{V12} (1×10^7) were subcutaneously injected into BALB/c female nude mice ($n = 5$ for each experimental group). (B) Individual tumor volume was measured according to the formula: $\pi/6 \times \text{length} \times \text{width}^2$ three months after subcutaneous injection.

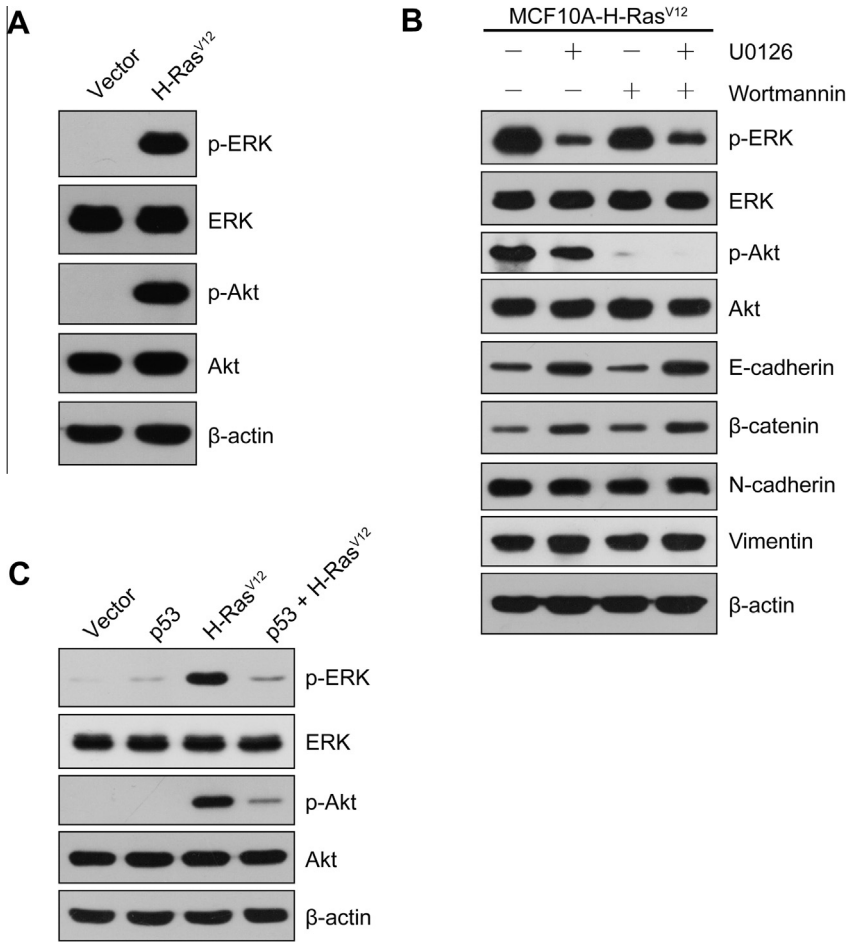

Fig. 4. p53 Downregulated the oncogenic Ras-induced signaling pathways. (A) immunoblotting analysis of phosphorylated ERK (p-ERK), total ERK, phosphorylated Akt (p-Akt) and total Akt protein in MCF10A cells expressing either H-Ras^{V12} or empty vector. (B) MCF10A-H-Ras^{V12} cells were treated with U0126 (20 μM), Wortmannin (0.1 μM), or in combination for 48 h. The expression of the activation of ERK and Akt and epithelial markers E-cadherin, β-catenin and the mesenchymal markers N-cadherin and Vimentin were examined by immunoblotting. (C) The expression of the activation of ERK and Akt were examined by immunoblotting in MCF10A cells expressing vector, p53, H-Ras^{V12} and p53 + H-Ras^{V12}, respectively. β-Actin was used as a loading control.

activation of ERK and Akt pathways was required for H-Ras^{V12}-mediated EMT, we used two pharmacological inhibitors, U0126 and Wortmannin, to specifically block the ERK and Akt pathway, respectively, in H-Ras^{V12}-expressing MCF10A cells. We found that expression of E-cadherin and β-catenin was restored in H-Ras^{V12}-expressing MCF10A cells treated with the ERK pathway inhibitor U0126, whereas treatment with the Akt pathway inhibitor Wortmannin had no effect (Fig. 4B). However, neither U0126 nor Wortmannin had any effect on N-cadherin and Vimentin protein levels (Fig. 4B). Thus, our results implicated that activated ERK pathway, but not PI3K/Akt pathway, was critical for H-Ras^{V12}-induced EMT.

We next determined the role of p53 on the oncogenic Ras-induced ERK activation. As shown in Fig. 4C, re-expression of p53 dramatically inhibited the Ras-induced phosphorylation of ERK. Interestingly, we also observed that the level of phosphorylated Akt was decreased in response to enhanced p53 expression in H-Ras^{V12}-expressing MCF10A cells (Fig. 4C). Together, our data demonstrated that p53 was able to suppress the oncogenic Ras-induced EMT through blocking the activation of ERK pathway.

4. Discussion

The tumor suppressor gene p53 implements a pivotal role in cell's response to DNA damage, which results in the induction of cell cycle arrest, cellular senescence or apoptosis [11,12]. Inactivation or mutation of p53 gene frequently occurs in human cancers. Recent studies suggest that p53 is able to regulate EMT and its associated effects [14]. EMT is a crucial embryonic developmental program implicated in cancer progression and generation of cancer stem cells (CSC), which promotes the tumor-initiating capacity and facilitates execution of the invasion-metastasis cascade [19]. Thus, inhibition of EMT or restoration of MET might efficiently prevent cancer initiation and impair tumor cell dissemination [20]. In this study, we establish that ectopic expression of p53 in H-Ras^{V12}-expressing MCF10A cells can convert the mesenchymal phenotype to the epithelial phenotype, and suppress the H-Ras^{V12}-induced mammary stem cell properties and tumor growth *in vivo*.

A recent study by Chun-Ju Chang and colleagues showed that loss of p53 function was associated with the activation of EMT program in mammary epithelial cells [14]. It was also reported that p53 prevented EMT and EMT associated stemness properties by transcriptional activating miR-200c, which inhibits ZEB1 and ZEB2 expression, the two transcriptional repressors of E-cadherin [14,21]. Moreover, some EMT activators such as Twist1, Twist2 and TGF-β have been shown to cooperate with the activated oncogenic Ras to promote even more dramatic characteristics of EMT [10,22]. We discovered in this study that ectopic expression of H-Ras^{V12} alone in MCF10A cells was sufficient to trigger an EMT program. Nevertheless, potential intersections between tumor

suppressor p53 and oncogenic Ras activity during an EMT process have not been defined previously. Significantly, our data implicate that p53 plays a role in regulating the oncogenic Ras-induced EMT, as well as in reducing the stemness and tumorigenesis of the cells through inhibiting the activation of Ras-mediated signaling pathways. Previous study showed that p53 reduced H-Ras^{V12} activity by transactivating BTG2, which interacted with H-Ras^{V12} and facilitated the change from the Ras-GTP-bound active conformations to Ras-GDP-bound inactive conformations, resulting in inactivation of Ras-mediated signaling pathways [23,24]. Similarly, our results demonstrated that re-expression of p53 resulted in inactivation of MEK/ERK pathway and the PI3K/Akt pathway during H-Ras^{V12}-induced EMT. It has been reported that MEK/ERK and PI3K/Akt pathways are necessary for Ras-mediated transformation and tumorigenesis [6,7]. However, we found that inhibition of the ERK pathway, but not PI3K/Akt pathway, in H-Ras^{V12}-expressing MCF10A cells upregulated epithelia markers E-cadherin and β-catenin expression. This result is consistent with previous reports by Quanwen Li and Raymond R. Mattingly that inhibition of Ras-ERK pathway, but not PI3K/Akt pathway, is able to restore E-cadherin cell–cell contacts and epithelial morphology in cell lines with activated H-Ras^{V12} expression [25]. Moreover, it has been reported that ectopic expression of ERK2 alone is sufficient to induce an EMT program, and inactivation of ERK2 signaling by knocking down endogenous ERK2 expression abolishes the H-Ras^{V12}-induced EMT, whereas inhibition of PI3K/Akt pathway by pharmacological inhibitors does not [26]. It is also documented that TGF-β treatment can induce activation of ERK kinase and the activation of ERK signaling pathway is essential for TGF-β-mediated EMT [27]. Recent findings implicate that inhibition of MEK/ERK signaling effectively attenuates radiation-induced EMT by regulating GSK3β activity and reducing the protein levels of Snail [28]. All of these data point to an important function of the activated Ras-ERK pathway in regulating EMT program. In contrast to these data, it has also been reported that constitutively active Akt is able to trigger an EMT program in the human squamous cell carcinoma lines [29]. A previous study by Binhua P. Zhou and colleagues showed that not only the activation of MAPK/ERK pathway but also the activation of PI3K/Akt pathway suppressed the activity of GSK-3β, which in turn stabilized the Snail level, an EMT inducer, ultimately resulting in EMT process [30]. In addition, other studies come up with the conclusion that activated AKT pathway modulates EMT by regulating EMT inducers such as Snail, Twist and Slug [29,31,32]. Activated Akt pathway has been implicated to be involved in hypoxia signaling and NF-κB signaling-mediated EMT [33,34]. Thus, these studies emphasize the crucial role of Akt pathway in the EMT process. Our data suggested that both ERK and Akt pathways were blocked by enhanced expression of p53 in H-Ras^{V12}-induced EMT. Thus, results from both this study and from other studies suggest that the activated Ras-ERK signaling may be more important to the oncogenic Ras-mediated EMT, whereas the active Akt pathway may partially contribute to H-Ras^{V12}-induced EMT by regulating EMT-associated transcription factors and signaling pathways.

It is well-established that p53 also acts as a master regulator in maintaining differentiated epithelial cell phenotype [35]. A recent study demonstrates that knockdown of endogenous p53 expression induces stem-like cell generation [14]. In the current study, our results demonstrate that enhanced p53 expression inhibited the oncogenic Ras-mediated EMT and the stemness property. Interestingly, we also discovered that overexpression of wild type p53 alone in MCF10A cells diminished the percentage of CD44^{high}/CD24^{low} cell subpopulations compared with MCF10A cells infected with vector (Fig 2A). Moreover, it has been shown that increased p53 expression results in reduction of stem cell subpopulation induced by Snail and TGF-β treatment [14]. These studies point to the importance of p53 in regulating EMT-associated stemness. In fact, there have been indications that p53 modulates EMT by regulating EMT-inducing transcription factors; for instance, p53 has been reported to repress ZEB1 and ZEB2 expression through transactivating miR-200c [14,21]. In addition, p53 has been shown to decrease Snail expression by inducing miR-34 family members [36]. Similarly, p53 can also reduce the stability of Snail protein via MDM2-mediated ubiquitination and proteasomal degradation in hepatocellular carcinoma [37]. Besides, evidence shows that the wild-type p53 is able to inhibit tumor cell invasion and metastasis through inducing MDM2-mediated Slug degradation in non-small cell lung cancer [38]. In the present study, we show, for the first time, that increased p53 expression prevents oncogenic Ras-induced EMT and restrains the stem cell properties mainly by inactivating ERK signaling pathway. Taken together, data arisen from this study expand our understanding towards the role of p53 in EMT process independent of its conventional function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.124.

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