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Loss of p53 in Stromal Fibroblasts Promotes Epithelial Cell Invasion through Redox-Mediated ICAM1 Signal

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Abstract

Tumor microenvironment plays a major role in cancer development. Understanding how the stroma affects epithelial transformation will provide a basis for new preventive strategies. Recent evidences suggest that oxidative stress in stroma may play a role in cancer progression and loss of *p53* function in the stromal cells were associated with poor prognosis and high tumor recurrence. However, the underlying mechanisms remain poorly understood. Here, we investigated the role of *p53* loss in fibroblasts on epithelial transformation and the mechanistic involvement of reactive species. Using 3D-organotypic culture and other assays, we report that the stroma containing *p53*-deficient fibroblasts could transform the non-tumorigenic epithelial cells of oral and ovarian tissues origins to become invasive through reactive nitrogen species (RNS)-mediated release of cytokine ICAM1. The *p53*-deficient fibroblasts have increased RNS production and accumulation of oxidative DNA damage products associated with specific up-regulation of endothelial nitric oxide synthase (eNOS). Suppression of RNS production by siRNA of eNOS or antioxidant NAC reduced ICAM1 expression and prevented the stroma-mediated epithelial invasion. Our study uncovers the novel mechanism by which redox alteration associated with loss of *p53* in stromal fibroblasts function as a key inducer of epithelial transformation and invasion via RNS-mediated-ICAM1 signaling. Thus, modulation of the redox signaling in microenvironment may serve as a new approach to prevent epithelial transformation and suppress cancer invasion.

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Keywords

fibroblast; ICAM1; Invasion; RNS; p53; eNOS

Introduction

Cancer cells have increased level of reactive oxygen/nitrogen species (ROS and RNS) and are under oxidative stress [1–2]. Oxidative stress in cancer cells was known to play role in the hallmarks of cancer including cell transformation, cell proliferation, cell survival, angiogenesis, invasion and metastasis [3–4]. The “inflammatory microenvironment” is considered as a new cancer hallmark [5]. Growing evidences suggested that cancer-supporting cells such as stromal fibroblasts are also under oxidative stress [6–9]. Lisanti and his colleagues showed that breast cancer cells release ROS to induce stress in the stromal fibroblasts. The stromal oxidative stress in turn further mutagenizes the cancer cells and drive them be highly aggressive [6–7]. On the other hand, our recent study showed that bone marrow stromal cells could modulate redox status of leukemia cells and promote their survival and drug resistance [10]. These observations underscore the important roles of stroma in promoting the cancer development and progression [11]. However, how the stromal cells exert such profound effect on cancer cells remain largely unknown, and the intrinsic genetic factors of the stromal cells that affect their interactions with cancer cells remain to be investigated.

One of the common genetic abnormalities in cancer-associated fibroblasts is the mutation or deletion of *p53* gene [12–14]. Clinical studies showed a strong correlation between stromal defects in *p53* gene and cancer progression [14]. A study in mice inoculated with *pRb*-deficient fibroblasts indicated loss of *p53* as a factor in tumor-promoting effect of the fibroblasts [13]. Importantly, p53 seems to function as antioxidant molecule in cancer cells, and the *p53* genetic defects may lead to redox imbalance and oxidative stress [15]. The stress in cancer cells associated with *p53* loss was known to induce DNA damage and genetic instability, promoting cancer progression [16–17]. Nevertheless, it is unknown whether redox alteration associated with loss of *p53* in fibroblasts could play any role in epithelial transformation.

Significant progress has been made in recent years in the area of preventive and therapeutic strategies targeting cancer cells. However, recent studies also suggest that the ability of anti-cancer agents to eliminate cancer cells *in vivo* can be severely compromised by tumor microenvironment, which promotes cancer cell survival, drug resistance, and disease recurrence [18]. In order to overcome these challenges, it is important to target both the cancer cells and their microenvironment [18]. Using organotypic culture and other assays, we studied the role of redox alteration associated with loss of p53 in fibroblasts on epithelial transformation and invasion. The insights from this work will provide an important basis for developing new strategies to suppress cancer development and progression by modulating the redox state in tissue microenvironment.

Materials and methods

Cell culture

The *p53*-deficient fibroblasts (NOF/hTERT shRNA p53) were established by stable transfection of immortalized non-tumorigenic fibroblasts (NOF/hTERT) with short-hairpin siRNA for p53 as described [19]. NOF/hTERT and NOF/hTERT shRNA p53 were both cultured in medium consisting of a 1:1 mix of MCDB105 medium and M199 medium (Sigma-Aldrich, St. Louis, MO) with 15% fetal bovine serum and 10 ng/ml epidermal

growth factor. The immortalized non-tumorigenic oral epithelial cells (OKF6/TERT2) were established by Dr. Rheinwald and colleague as described [20]. T72 cells are immortalized human normal ovarian epithelial cell line harboring SV-40 T/t and hTERT as described previously [21]. These cells are non-tumorigenic and unable to form colonies in anchorage-independent assay [22]. OKF6/TERT2 cells were grown in KSFM media (Invitrogen (Gibco), Carlsbad, CA) with bovine pituitary extract, CaCl_2 , Glutamine and epidermal growth factor. T72 cells were maintained in the media mixture similar to that of NOF/hTERT.

Organotypic culture and analysis

Procedure—Organotypic cultures of oral and ovarian mucosa were established following a new protocol modified from published works [23–24]. Major steps include stroma construction, epithelial cells seeding, air-lifting and tissue harvesting. We utilized deep-well plate (BD, Biocoat) and 3 μm -polycarbonate inserts (BD Falcon) which prevents contamination during the air-lift stage. First, we constructed 3D collagen matrices by mixing on ice rat tail collagen solution (BD Biosciences), polymerization premix, FBS (final 2%) and 4.5×10^5 fibroblasts/3 ml of gel, then the mixture was poured to culture insert and polymerized in 37°C incubator. The matrices were fed with fresh fibroblast media every three days. (Polymerization premix was made by mixing on ice 10X reconstitution buffer (22mg/ml sodium bicarbonate in 20 mM HEPES in 0.062N NaOH,) 5x DMEM (from 10x DMEM powder, Fisher), Glutamine 200 mM and 100x Penicillin/Streptomycin. All solutions were passed through 0.2 μm filter for sterilization. The final concentrations of collagen matrices were 1.5% for oral model and 2% for ovarian model. If the fibroblasts grew well in the matrices, within 7 days, the top of the matrices would shrink to 1.5 cm diameter due to fibroblasts-mediated contraction of collagen. At this point, 1×10^6 epithelial cells were seeded on top of the matrices. To ensure optimum proliferation, epithelial media were added inside the insert and fibroblast media were added outside the insert, feeding the culture from the bottom. After 5–7 days, the culture would be air-lifted. We can simply lift the culture exposing it to air by completely removing media on the top of culture and feed only the outside of insert with air-lift media. Epithelial cells would no longer receive nutrient from the top. Instead, the epithelial-stroma interaction would be the key of epithelial survival and tissue organization. This mimics the *in vivo* situation, which epithelia are spared from blood supply and induced to differentiate by atmospheric oxygen [25–26]. Fresh air-lift media consisting of 3:1 v/v of DMEM:Ham F-12 media, 0.4ug/ml Hydrocortisone, 2×10^{-11} M of thyroid hormone T3, 1.8×10^{-4} M of Adenine, 5 ug/ml Transferrin, 10^{-10} M Cholera toxin, 2mM L-Glutamine, 5% FBS, 100 IU/ml Pen/100 ug/ml Streptomycin and 5ug/ml Insulin, would be replaced every three days. If the epithelial layer formed well, within 12–15 days we would observed the granular white disc appeared on top of the culture [23–24]. At this point, we harvested the tissue by fixing the whole piece of culture and insert with 10% formalin. Then, the tissue on membrane would be cut from the insert frame and the 5 mm- strip of middle part of culture would be sectioned for histologic analysis.

Histologic analysis and quantitation—The fixed organotypic tissue was processed with gradients of alcohol and embedded in paraffin-wax (*embedded on edge*). The tissue blocks were sectioned at 6 μm thickness. Morphologic phenotype of tissue was analyzed by hematoxylin-eosin (H&E) staining similar to standard pathologic staining procedure. The organotypic mucosa was quantitated as previously described [27]. Invasion index was calculated as $N \times A \times \text{MCY}$, where N was the number of particle below the imaginary basement membrane, A was the sum of area of the remaining particles, and MCY was the mean invasion distance.

ICAM1 and NAC treatment on organotypic culture—For addition of ICAM1 into microenvironment of the culture, 10 ng/ml of human recombinant ICAM1 (cat#150-05, Peprotech, NJ, USA) was added in the process of collagen matrix fabrication and in air-lift media throughout the procedure. Fresh media containing 10 ng/ml of ICAM1 were replaced every three days to maintain the concentration of cytokine. Mixing the ICAM1 to be part of collagen matrix was to mimic the cell-cell contact signaling, whereas adding the cytokine to culture media was to mimic the paracrine signaling of soluble ICAM1.

For addition of NAC into microenvironment of the culture, air-lift media containing 1mM of N-Acetylcysteine (NAC) (Sigma-Aldrich (St. Louis, MO) were applied and replaced every three days throughout the procedure.

Measurement of cytokine level

Cytokine screening assay—Alteration of cytokine profile after knocking down *p53* was investigated by cytokine array. The Human Cytokine Array Panel A (cat. # ARY005) was purchased from R&D Systems, Inc. (MN, USA). To compare the expression profile of the *p53*-intact fibroblasts and *p53*-deficient fibroblasts in single culture, 2×10^5 cells of each were grown in separate 25 ml culture flask. For comparison of co-culture with epithelial cells, 1×10^5 of each type of fibroblasts and 1×10^5 of epithelial cells were mixed well and seeded together in the same 25 ml flask. Single culture of epithelial cells was also used as control. When the cells grown to 90% confluent, 3 ml of fresh media were replaced and incubated for two days. The conditioned media were collected for analysis of cytokine expression following the standard protocol from the company. All samples were analyzed in the same experiment. Briefly, nitrocellulose membrane coated with antibody against 36 different cytokines (double spots for each cytokine) was blocked with array buffer for one hour. The conditioned medium (800 μ l) was mixed with detection antibody cocktail for one hour. Then, the blocked membrane was incubated with the conditioned media/detection antibody cocktail mixture at 4 °C overnight on a rocking platform. After washing the membrane for 3×10 min with wash buffer, the antibody-cytokine complexes were detected by streptavidin-HRP and exposed to x-ray film for 10 min. The data from each sample was compared to identify the different spots. Then, identification of each spot can be revealed by overlaying the transparency template on the array data and tracing back the types of cytokine corresponding with the co-ordinates of each spots. Quantitative analysis was performed according to the protocol suggested by the manufacturer. Briefly, the array image had been scanned and the pixel density of each spot had been quantified. The average signal (pixel density) from the pair of duplicate spots was calculated. The corresponding signals on different arrays were then compared and analyzed.

Detection of ICAM1 protein level—The protein levels of soluble form of ICAM1 (sICAM1) and cellular ICAM1 were detected by western blot analysis. The conditioned media were collected in a similar fashion as the cytokine screening assay and used to analyze sICAM1 level. 4x non-reducing Laemmli buffer (containing SDS, Urea, glycerol and bromophenol blue, without β -mercaptoethanol) was added to the conditioned media. The cells which were the source of conditioned media were lysed in non-reducing Laemmli buffer and cooked at 95 °C for 10 min. The cell lysates were used to analyze cellular ICAM1 level. Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Primary antibody against active form of ICAM1 which can detect both soluble and cellular forms of ICAM1 (cat# BBA3), was purchased from R&D Systems, Inc. (MN, USA). After blocking with 5% milk, each blot was incubated with 1 μ g/ml ICAM1 antibody at 4 °C overnight on a rocking platform. The signals were detected by secondary antibody conjugated with HRP, followed by ECL and x-ray film exposed. The blot was stripped and reprobed for actin at concentration of 1:20,000 for 1 hour at room temperature. For

quantitation, the radiographic image had been scanned and the band density of each spot had been quantified. The density was compared with actin of the same sample and calculated as “fold of actin”. The corresponding signals of different cells/treatment were compared and analyzed.

Determination of cellular RNS, superoxide, oxidative DNA damage products and thiols

Reactive nitrogen species (RNS) content were analyzed as described [22]. Briefly, 4×10^5 cells were incubated with $3 \mu\text{M}$ of 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) for 60 min, and fluorescent intensity was analyzed by flow cytometer (a FACSCalibur equipped with CellQuest Pro software). Reactive oxygen species such as hydrogen peroxide and superoxide were analyzed using CMH2-DCF-DA and dihydroethidium (Het) as described [22]. The cells were stained and processed similar to DAF-FM except 100 ng/ml of Het or $3 \mu\text{M}$ of DCF-DA were used instead. DAF-FM, CMH2-DCF-DA and Het were purchased from Invitrogen (Molecular Probes), Carlsbad, CA. For detection of major products of DNA oxidation, we utilized the Oxidative DNA damage kit from Kamiya biomedical company (Seattle, WA), as previously described [16]. First, 4×10^5 cells were fixed and permeabilized. Then, the cell pellets were incubated with FITC-labeled protein conjugate, which would bind to 8-oxoguanine moiety presented in 8-oxo-guanosine of oxidized DNA. The presence and quantitation of oxidized DNA were analyzed by flow cytometer (a FACSCalibur equipped with CellQuest Pro software). For detection of total free intracellular thiols (both protein-bound SH groups and free non-protein thiols (mainly GSH), we used 5-chloromethylfluorescein diacetate (CMF-DA) as previously described [28]. Comparative study using NOF/ht and NOF/ht/p53-shRNA was performed on the same day using same analytical conditions and equipment voltage set-up. For repeated experiments, we used freshly-prepared dye solutions to minimize potential auto-oxidation of the dye in solution. To ensure the consistency of the assay conditions for samples at different days, we used a reference cell line (SKOV3 cells) stained under identical conditions as a control for comparable parameter settings.

Determination of gene expression by Real-Time reverse transcription PCR (qRT-PCR)

The relative mRNA expression levels of *ICAM1*, *eNOS*, *iNOS*, *Nrf-2*, *GCLC* and *GCLM* genes were determined. Total RNA were extracted using the RNeasy kit (QIAGEN, CA) followed by DNAase (Ambion, TX) treatment according to the manufacturer's instructions. Then, first strand cDNA were prepared using TaqMan Reverse Transcription Reagents (Invitrogen, CA) from total RNA. Specific primers for *ICAM1*, *eNOS*, *iNOS*, *Nrf-2*, *GCLC* and *GCLM* were designed using invitrogen OligoPerfect™ Designer, and Quantitative real-time PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR System. Relative mRNA quantization was analyzed using the PFAFFL method [29]. β -actin was selected as the internal control gene.

Suppression of eNOS by siRNA

Three pairs of eNOS siRNA and scramble siRNA control were purchased from Invitrogen (Carlsbad, CA). The nucleotide sequences of the siRNA are pair 1: sense - UUCGAGGGAC ACCACGUCAUACUCA, antisense - UGAGUAUGACGUGGUGUCCUCGAA; pair 2: sense - UGUGUUACUGGACUCC UUCCUCUUC, antisense - GAAGAGGAAGGAGUCCAGUAACA CA; pair 3: sense - UUUCCACUGAGCGGAUUGUAGCCUG, antisense - CAGGCUACAAU CCGCUCAGUGGAAA. Transfection of siRNA was performed using Lipofectamine 2000 Transfection Reagent, following the manufacture's instruction. Briefly, the cells were grown to 50% confluent and the media were replaced with Opti-MEM Reduced Serum Medium. Then, 100 pmol/well of siRNA or control siRNA were transfected to NOF/hTERT shRNA p53 cells in 6-well plate and incubated for 6 hours. After that, the media were

replaced with whole serum culture medium and the cells were harvested at 48 hours for further analysis.

Matrigel Invasion Assay

We used BD BioCoat™ Matrigel™ Invasion Chamber to perform the assay, following the protocol from the company (BD Biosciences Discovery Labware, Bedford, MA). Briefly, suspension of 4×10^5 fibroblasts was placed in each well of lower chamber 6-well plate. Then, the upper chamber (Matrigel culture insert), containing 2×10^5 OKF6/TERT2 cells was placed on top of the lower chamber. The Invasion chambers were then incubated for 22 h in a humidified tissue culture incubator, at 37 °C, 5% CO₂. After the incubation, non-invading cells were removed by scrubbing the upper surface of the Matrigel membrane using cotton swab. The invading cells appeared on the lower surface of the membrane are stained with Diff-Quik™ stain (containing fixative and two stained solutions similar to Wright-Giemsa staining). For quantitation, the stained membrane was photographed and the number of invading cells was counted under microscope. For analysis, invasion index was calculated by dividing % of invasion in the tested cells with % of invasion in the control cells. In this study, we would like to compare the sample of NOF/shRNA p53 between with and without siRNA of eNOS pair #3. Therefore, we used NOF/ht as control cells.

Statistical analyses

The statistical significance of the difference in invasion index (either from organotypic culture or from invasion assay), expression of ICAM1 and eNOS and the level of nitric oxide between the *p53*-intact and *p53*-deficient fibroblasts was evaluated using the unpaired t-test (two-tailed). Comparison of the cytokines' levels between the *p53*-intact and *p53*-deficient fibroblasts was analyzed by two-way ANOVA with Bonferrini post-hoc test. The statistical significance of the difference in ICAM1 expression and nitric oxide level between the NAC-treated and the control groups was analyzed using the paired test (two-tailed). These data analyses were performed using the Prism software (GraphPad, San Diego, CA). Analysis of flow cytometric histogram overlay was performed using Cell Quest Pro software. K-S statistic was used to test the significance of difference between those histograms. The *P* value of less than 0.05 was considered statistically significant, and the *P* value of less than 0.001 was considered highly significant.

Result

p53 - deficient fibroblasts induce transformation and invasion of oral and ovarian mucosa

To investigate the role of stromal stress associated with the loss of p53 in fibroblasts on epithelial transformation, we utilized a pair of immortalized human fibroblasts with *p53* gene knocked-down by short hairpin-RNA (NOF/hTERT/shRNA p53) and their parental *p53*-wild type cells (NOF/hTERT) [19], as an experimental model system to compare their effect on epithelial cell growth in 3-dimensional organotypic culture. As shown in Fig 1A, shRNA was effective in knocking down *p53* expression in fibroblasts. Such shRNA suppression also substantially reduced the ability of p53 to respond to DNA damage caused by doxorubicin (Supplemental Fig S1A). The *p53*-deficient cells also exhibited certain morphological changes such as rounder and less spindle-like shape (Supplemental Fig. S1B), likely reflect their intrinsic stress due to increase in reactive nitrogen species (see below). Interestingly, organotypic co-culture of the fibroblasts and the non-tumorigenic oral keratinocytes (OKF6/TERT2, [20]) showed that the epithelial cells became disorganized and highly invasive when they were cultured on top of collagen gel containing the *p53*-deficient stromal fibroblasts, but not on the collagen gel containing wt-*p53* fibroblasts (Fig 1A). Statistical analysis showed that organotypic culture of oral mucosa with *p53*-deficient fibroblasts had significantly higher level of invasive index than that of *p53*-intact cells

($p < 0.0001$, unpaired t-test). Time course experiments of organotypic culture showed kinetic process of the invasion (Supplemental Fig. S2).

Consistently, immunohistochemistry showed a decrease in expression of the epithelial marker E-cadherin and an up-regulation of mesenchymal marker MMP9 at the invasive border of epithelia on organotypic culture with p53-deficient fibroblasts (Supplemental Fig. S3A–B), suggesting the loss of p53 in the stromal cells might induce the epithelial cells to undergo epithelia-to-mesenchyme transition (EMT), the process associated with epithelial invasion [30–31]. Likewise, organotypic culture of non-tumorigenic ovarian epithelial cells (T72, [21]) also showed that p53-deficient stroma induced an invasive transformation of the ovarian epithelia (Fig 1B). Interestingly, organotypic culture of ovarian cancer cells (SKOV3) showed that the cancer epithelia become more invasive when co-cultured with p53-deficient fibroblast (Fig 1C). Taken together, the data indicated that a loss of p53 in the stromal cells could induce epithelial transformation and promote invasion.

P53-deficient fibroblasts have increased reactive nitrogen species associated with specific up-regulation of eNOS

To investigate the underlying mechanisms, we first monitored the redox status of the fibroblasts since the p53-deficient cells seemed to exhibit stressed morphology and p53 is known to affect mitochondrial function and ROS production [15–16]. P53-deficient fibroblasts exhibited 200% increased level of DAF fluorescent from 17 to 34 units (Fig 2A-left panel). Statistical analysis demonstrated that the increase in DAF fluorescent was highly significant (Fig 2A - right panel, $p < 0.001$), while Het fluorescent signal did not show any significant change (Supplemental Fig. S4A, $p = 0.1743$). Interestingly, we observed the decrease in DCF fluorescent signal (Supplemental Fig. S4B, $p < 0.05$), indicating a decline in overall reactive oxygen species content. These data suggested that suppression of p53 by shRNA resulted in an increase of reactive nitrogen species but not the overall reactive oxygen species in the fibroblasts.

Although diaminofluorescein-dyes (DAFs) are widely used for visualizing nitric oxide and other reactive nitrogen species in biological systems, recent works showed that DAF fluorescent signal can also be originated from DAF-reactive compounds [32]. Therefore, we tested whether the increase of DAF-signal in the fibroblasts was derived from nitric oxide and its derivatives (reactive nitrogen species). As shown in Fig. 2B–C, the DAF-fluorescent signal could be profoundly suppressed by L-NAME, an inhibitor of Nitric oxide synthases (NOSs), and could also be enhanced by SNAP, a nitric oxide donor. These data confirmed that the increased DAF signal was mainly from the increase of reactive nitrogen species (RNS).

Since nitric oxide (NO) is the precursor of most reactive nitrogen species, we speculated that the increase of RNS may be the result of increased NO production. qRT-PCR analysis of NO-producing enzymes, nitric oxide synthase (NOS), demonstrated that knocking down p53 lead to a 5-fold increase in expression of the endothelial NOS (*eNOS*) while the inducible *iNOS* decreased to essentially zero (Fig 2D–E) These suggested that the p53-deficient fibroblasts had increased production of RNS associated with specific up-regulation of *eNOS*.

P53-deficient fibroblasts have accumulation of oxidative products, alteration in glutathione-synthesis enzymes and up-regulation of redox regulated cytokines

The increased reactive nitrogen species suggested that the p53-deficient fibroblasts may have alteration in cellular redox states. Analysis of 8-oxoguanosine (8-oxo-Gua), major products of DNA oxidation showed that the p53-deficient fibroblasts have significant

increased accumulation of 8-oxo-Gua (Fig. 3A), suggesting that these cells might have been under elevated oxidative stress. Interestingly, analysis of cellular thiols and glutathione synthesis enzymes demonstrated that the *p53*-deficient fibroblasts had an increased level of cellular thiols and elevated gene expression of *Nrf-2* transcription factor, *GCLC* and *GCLM*, the catalytic and modulatory subunit of glutamate cysteine ligase that is a rate-limiting enzyme in glutathione synthesis (Fig. 3B–C). These data together suggested that the *p53*-deficient cells had higher intrinsic oxidative stress with adaptive increase in antioxidant molecules to cope with such ROS stress [1].

Alteration in redox states may lead to changes in expression and function of many proteins [4]. Interestingly, our cytokine array analysis revealed a highly significant increase of three cytokines (GM-CSF, sICAM-1 and Serpin E1) released from the *p53*-deficient fibroblasts (NOF/ht shRNA *p53*), compared to the parental cells ($P < 0.0001$, Two-way ANOVA with Bonferrini post test) (Fig 3D). Since expression of these cytokines was primarily regulated by reactive oxygen and nitrogen species [33–35], it is likely that the elevation of those cytokines was a consequence of the elevation in reactive nitrogen species in the *p53*-deficient fibroblasts.

The induction of epithelial invasion by *p53*-deficient fibroblasts was mediated by ICAM1

Because cytokines are major players in cell-cell communications including epithelial and fibroblast interactions [36–37], we further investigated the key cytokine released from the stressed fibroblasts (*p53*-deficient) that promotes the invasive phenotype of the epithelial cells. Cytokine array analysis revealed a highly significant increase of only one cytokine (sICAM-1) released from the co-culture between oral keratinocyte (OKF6/TERT2) and *p53*-deficient fibroblasts (NOF/ht shRNA *p53*), compared to co-culture with the parental cells ($P < 0.0001$, Two-way ANOVA with Bonferrini post test) (Fig 4A). Further analysis showed that sICAM-1 was found only in the single culture of the fibroblasts (Fig 3D), but not detectable in the epithelial cells. Thus, in the co-culture system, sICAM-1 was mostly released from the *p53*-deficient fibroblasts and might then exert its effect on the epithelial cells. Since ICAM-1 is a membrane-bound polypeptide which can be cleaved to yield soluble ICAM1 (sICAM-1) [38–39], the increase of sICAM-1 could result from the increase of its synthesis and/or an increase in its cleavage. Western blot analysis and qRT-PCR revealed an increase in both ICAM-1 protein and ICAM-1 mRNA (Fig 4B–C). Quantitative analysis showed 6–8 fold increase of cellular ICAM1 protein and gene expression in *p53*-deficient fibroblasts ($P < 0.0001$, unpaired t-test). Thus, the increased secretion of sICAM-1 from the stressed fibroblasts (*p53*-deficient) was likely due to an increase in the overall production of ICAM-1 and its processing to sICAM-1. Since ICAM1 is known to play an important role in promoting tumor invasion [39–40], we postulated that ICAM-1 might be a key cytokine released from the *p53*-deficient fibroblasts to mediate the interaction with epithelial cells and promote their invasive behaviors. To further prove this, we tested the ability of recombinant ICAM-1 to promote epithelial invasion and showed that this cytokine indeed induced OKF6/TERT2 cells to become invasive in organotypic culture with *p53*-wt fibroblasts (NOF/hTERT) (Fig 4D). Statistical analysis showed significantly increased invasion index in organotypic culture with ICAM-1 addition ($P < 0.0001$, unpaired t-test). This confirms the importance of ICAM1 in induction of epithelial invasion.

siRNA of eNOS suppressed ICAM-1 synthesis in *p53*-deficient fibroblasts and prevented their inductive capability of epithelial invasion

Transcription of ICAM-1 is primarily regulated by reactive oxygen and nitrogen species including nitric oxide [41–42], though other factors could also contribute [35]. Based on our observation, reactive nitrogen species (RNS) were increased in *p53*-deficient fibroblasts. Thus, we postulated that the elevated ICAM-1 level might be caused by NO and other RNS.

Since *p53*-deficient fibroblasts exhibited specific *eNOS* up-regulation, we transiently suppressed RNS level by siRNA of *eNOS* and measure ICAM-1 expression. As shown in Fig 5A, the *eNOS* siRNAs (#1–3) were significantly effective in suppressing cellular ICAM1 levels. The *eNOS* siRNA #3 was the most effective. This siRNA also induced a significant decrease in DAF-fluorescent signal (Fig 5B). Consistently, siRNA of *eNOS* #3 suppressed the ability of *p53*-deficient fibroblasts in inducing epithelial invasion of oral non-tumorigenic OKF6/TERT2 cells (Fig 5C). Statistical analysis showed highly significant decrease in their invasion index ($p < 0.001$, unpaired t-test). Taken together, the data suggested that the elevation of ICAM-1 in *p53*-deficient fibroblast was likely the consequence of increased NO and other RNS production due to *eNOS* up-regulation.

Antioxidant NAC inhibited ICAM-1 synthesis and prevented the stroma-induced epithelial invasion

To further confirm that oxidative stress in the stroma was the key inducer of epithelial invasion through activation of ICAM-1 signaling, we utilized a powerful antioxidant N-acetylcysteine (NAC) to test if the decrease of oxidative stress could suppress the ICAM1 release from the *p53*-knocked down cells. As shown in Fig 6A–B, the generation of sICAM1 and the expression of ICAM1 mRNA and protein were profoundly suppressed by NAC. Consistently, DAF fluorescent was also significantly suppressed by NAC (Fig 6C). Consequently, the presence of NAC in the organotypic culture remarkably suppressed the invasive phenotype of oral and ovarian epithelia in the presence of *p53*-deficient fibroblasts (Fig 6D and Supplemental Fig S4, respectively). Quantitative analysis showed highly significant decrease in their invasion index ($p < 0.001$, unpaired t-test). This further confirmed the key role of RNS in promoting invasive behavior of the epithelial cells.

In summary, we showed that knocking down of *p53* in fibroblasts led to up-regulation of *eNOS* and increased production of reactive nitrogen species, which in turn stimulated the expression of ICAM1 and release of its soluble form to the tissue environment to promote epithelial cell transformation and invasion (Fig 7).

Discussion

Recent evidences suggest that oxidative stress in stroma may play a role in cancer progression [6–10] and loss of *p53* function in the stromal cells were associated with poor prognosis and high tumor recurrence [14, 43–44]. However, the underlying mechanisms remain poorly understood. This study discovers the novel mechanism by which intrinsic oxidative stress associated with loss of *p53* in stromal fibroblasts function as a key inducer of epithelial transformation and invasion via RNS-ICAM1 signaling. This work not only uncovers the mechanism of epithelial induction via *eNOS*-RNS-mediated ICAM1 stimulation, but also provides the implication of using redox-based intervention to prevent transformation and invasion of epithelial cancer.

Recently, loss of functional *p53* gene in the tumor stroma was found associated with disease progression and poor prognosis [14]. However, it is still unclear how the *p53* loss may affect epithelial cell behaviors. Using *p53*-knocked down fibroblasts and organotypic culture, we found that the loss of *p53* in fibroblasts affect the interaction between fibroblasts and epithelium, promoting the epithelial invasion. Previous studies had shown that mutation of *p53* in epithelial cells drive themselves to be invasive [13]. In this study, we provide new evidence that functional defect in *p53* of stromal cells could drive the neighbor epithelial cells to become invasive through signaling of cytokines such as ICAM1. Since ICAM1 is membrane-attached protein which can be cleaved yielding soluble factor, the signal of ICAM1 could be transmitted by either binding to its membrane receptor during direct contact or binding of its secretory form to its receptor at distant target [35]. In this study, the

p53-deficient fibroblasts seemed to have up-regulation of both soluble and cellular form of ICAM1. Therefore, the invasion-promoting effect of ICAM1 observed here is likely mediated by both direct contact of epithelium-fibroblasts and paracrine effect of soluble ICAM1.

We discovered ICAM1 as the key cytokine released from the stressed fibroblasts (*p53*-deficient) and signaled to induce epithelial invasion. This is consistent with a recent report showed that fibroblasts adjacent to the invasive border of melanoma expressed increased level of ICAM1, compared to fibroblasts in normal tissue far from the cancerous area [45]. Previous study using inducible expression of *p53* discovered *ICAM1* as a target gene of p53 transcription factor [46]. Theoretically, when we knocked down p53, ICAM1 should be decreased. Paradoxically, we observed an up-regulation of ICAM1 in stably p53 knocked-down cells. This seemingly conflicting result could be explained by another mechanism i.e. RNS-mediated ICAM1 transcription [38, 41–42]. It appears that in fibroblasts with stable knockdown of *p53*, the RNS-mediated up-regulation of ICAM expression may be a dominant event. In fact, a study in squamous cell carcinoma also suggested the important role of NO on ICAM1 transcription [41]. This suggested that under the stressed condition, RNS may play more prominent role in regulation of ICAM1 synthesis, than the p53 itself. This shed the light on the significant role of reactive species in affecting gene transcription and epithelial invasion. Since our data showed that knocking down of *p53* was upstream of elevated nitric oxide generation, thus these two events are closely linked and it may be difficult to draw a conclusion whether the high invasive behavior is solely dependent on increase in nitric oxide or due to p53 deficiency. It is likely that the loss of p53 in fibroblasts was an initial event, and the increase in nitric oxide generation in the fibroblasts may be a key down-stream mechanism that promotes epithelial invasion. The direct role of nitric oxide from fibroblasts on epithelial invasion can be further investigated using *p53*-wt fibroblasts with stably eNOS over-expression or knocking down eNOS to test their effect on epithelial cell migration and invasion.

In *p53*-deficient fibroblasts, reactive nitrogen species seems to be the key player in inducing epithelial invasion. This discovery is in part based on the observation that DAF signal, but not Het and DCF signals, was highly increased. To detect DAF and DCF signal, we used DAF-FM and CM-H2-DCF-DA, the fluorophores with ester parts, to ensure maximum uptake of the dye into the cells. However, the dye DAF-FM and DCF-DA will not fluorescence unless acetyl group is cleavage by esterase enzyme [47]. This would post a challenge if the *p53* deficient and *p53*-intact fibroblasts have different levels of esterase enzyme. If the observed increase of DAF signal had been due to the difference levels of esterase enzyme, we would have seen similar degree of increase in signal of both DAF and DCF signal. In contrast, we observed the increase in DAF signal (Fig. 2A), while DCF signal has decreased in p53-deficient cells. This suggests that the observed increase of DAF was not due to the difference levels of esterase cleavage, but likely reflect the increase in reactive nitrogen species.

Production of nitric oxide is actively catalyzed by nitric oxide synthase (NOS), which has multiple isoforms including neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [48]. Previously, *iNOS* was shown transcriptionally regulated by p53 [49]. Thus, down-regulation of iNOS expression observed in our *p53*-deficient cells was not surprising. However, we observed for the first time that loss of functional *p53* led to specific up-regulation of *eNOS* (Fig 2D), followed by the increase of reactive nitrogen species. These suggested that eNOS might play dominant role in NO production of *p53*-deficient cells. This is consistent with previous report showing that under standard condition with no inflammatory stimuli, eNOS play dominant role in NO production of human fibroblasts [50]. It is likely because NO production by eNOS can utilize both intracellular and

extracellular Arginine as substrate, while iNOS depend solely on the extracellular Arginine [51]. Furthermore, iNOS usually can impair the NO synthesis from eNOS, providing the checks and balances system to prevent excessive production of NO [52]. However, in our *p53*-deficient fibroblasts the iNOS was almost undetectable, rendering the iNOS - eNOS balance to shift in favor of NO production by eNOS, as observed. Interestingly, a recent work reported the expression of eNOS in transformed cells and proposed its tumor-maintaining role [53]. Our study showed that suppression of eNOS by siRNA could prevent the ability of stromal fibroblasts in inducing epithelial invasion. Owing to its potential role in both tumor themselves and the stromal environment, eNOS might be a novel molecular target for cancer, which warrant further investigation.

The levels of reactive oxygen/nitrogen species are determined by the balance between ROS/RNS production by various biological processes and the elimination by cellular antioxidant systems [4]. Therefore, the observed increase in RNS indicated that the redox balance in the *p53*-deficient fibroblasts was shifted toward increased RNS accumulation. As shown in Fig 3B–3C, the cells exhibited up-regulation of GSH synthesis enzymes and increased total free thiols. This was likely a mechanism to counterbalance with the increased RNS. However, it seems that such increase of antioxidants was not sufficient to reduce RNS back to the basal level as in the *p53*-wt cells. Consequently, the increased RNS led to a redox-mediated up-regulation of ICAM1 in *p53*-deficient cells, and may play an important role in promoting invasive phenotype of oral epithelial cells. Thus, redox imbalance in *p53*-deficient cells seems to be an inducer of epithelial invasion, and application of exogenous agents which promote antioxidant capacity may be an effective way to reduce RNS level and suppress the stroma-induced epithelial invasion. Indeed, addition of N-acetylcysteine, a precursor of endogenous GSH synthesis, could decrease cellular RNS and ICAM 1 expression in *p53*-deficient cells, and suppress their invasive phenotype (Fig 6).

Since *p53* has multiple functions in cells, the redox-mediated ICAM1 signaling might not be the only mechanism of epithelial invasion induced by fibroblasts. In fact, the *p53*-deficient fibroblasts have a slight increase in proliferation rate, compared to their parental cells (doubling time of 2.5 days and 3 days, respectively). This difference might potentially contribute to the increased cell invasion associated with elevated RNS and ICAM1 secretion.

Tumor microenvironment was known to drive tumor metastasis, therapeutic resistance and disease recurrence [54–56]. A retrospective study found that SMA-positive myofibroblastic stroma is the strongest death predictor of oral cancer [57]. Recent studies revealed that oxidative stress in stroma was strongly associated with aggressiveness and poor survival rate of breast cancer patients [9, 58–59]. Our study provides new evidence that stromal ROS stress is linked to defects in *p53* and may play a major role in epithelial transformation and invasion. As redox alteration in fibroblasts is the early event, application of antioxidants such as NAC to the microenvironment could suppress ICAM1 production and thus might block epithelial transformation and invasion. Since over 50% of recurrent cancers have inactivation of *p53* in stromal cells adjacent to the tumors [43–44], targeting stromal oxidative stress may be a potential approach to prevent cancer invasion and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Loss of p53 in stromal fibroblasts up-regulates eNOS and increases NO and RNS production.

Elevated NO and RNS in the fibroblasts up-regulates ICAM-1, promoting epithelial cell invasion.

Knocking down *eNOS* in the fibroblasts suppresses ICAM-1 synthesis and prevents epithelial invasion.

Suppressing NO and RNS by antioxidant blocks ICAM-1 synthesis and prevents epithelial invasion.

Modulation of redox signaling in stroma may be a novel strategy to prevent epithelial invasion.

Figure 1. *p53*-deficient fibroblasts transformed organotypic oral and ovarian epithelia Organotypic cultures were fixed, paraffin-embedded, sectioned, hematoxylin & eosin stained and seen under light microscope.

A: Transformation of oral epithelium. The *p53*-deficient fibroblasts or *p53*-intact fibroblasts were organotypically co-cultured with non-tumorigenic oral (OKF6/TERT2) epithelial cells. Scale bars, 100 μ m. Bar graph represents the invasion index (in arbitrary unit (AU) and error bar indicates S.D. from 5 repeatable experiments for *p53*-deficient culture and 3 repeatable experiments for *p53*-intact culture. The data indicates significant increases of invasion index in the organotypic mucosa containing *p53*-deficient fibroblasts ($P < 0.0001$, unpaired t-test). Western blots of cellular p53 in each fibroblast. Actin was used as loading control.

B: Transformation of ovarian epithelium. The *p53*-deficient fibroblasts or *p53*-intact fibroblasts were organotypically co-cultured with non-tumorigenic ovarian (T72) epithelial cells. Scale bars, 200 μ m. Bar graph represents the invasion index (in arbitrary unit (AU) and error bar indicates S.D. from 2 repeatable experiments. The data indicates significant increases of invasion index in the organotypic mucosa containing *p53*-deficient fibroblasts ($P = 0.0069$, unpaired t-test).

C: Induction of ovarian cancer invasion. The *p53*-deficient fibroblasts or *p53*-intact fibroblasts were organotypically co-cultured with ovarian cancer cell line (SKOV3). Scale bars, 100 μ m. Bar graph represents the invasion index (in arbitrary unit (AU) and error bar indicates S.D. from two repeatable experiments. The data indicates significant increases of invasion index in the organotypic mucosa containing *p53*-deficient fibroblasts ($P = 0.0082$, unpaired t-test).

Figure 2. Knocking down *p53* gene resulted in increase of reactive nitrogen species associated with upregulation of NOS

A: Increased DAF-fluorescent in *p53*-deficient fibroblasts. Reactive nitrogen species levels of *p53*-intact and *p53*-deficient fibroblasts were measured by flow cytometric analysis of DAF-FM. Overlaying Histograms were representatives of triplicate experiments. Bar graph represents the mean fluorescent intensity and error bar indicates S.D. from triplicate experiments. The data indicates significant increases of DAF-fluorescent in *p53*-deficient fibroblasts ($P < 0.001$, unpaired t-test).

B–C: Suppression of DAF-fluorescent by NOS inhibitor. The *p53*-intact and *p53*-deficient fibroblasts were treated with 30 μ M of L-NAME, an inhibitor of nitric oxide synthase, for 30 min or 100 μ M of SNAP, a nitric oxide donor, for 1 h and the DAF-fluorescents were measured by flow cytometric analysis of DAF-FM. The data indicates that

DAF-signal in both fibroblasts could be suppressed by L-NAME but could be enhanced by SNAP ($P < 0.001$, K-S statistical test).

D–E: Increased expression of *eNOS* and decreased expression of *iNOS* in *p53*-deficient fibroblasts. The gene expression of *eNOS* and *iNOS* in *p53*-deficient and *p53*-intact fibroblasts was analyzed by qRT-PCR. Bar graph represents the mean of relative expression level and error bar indicates S.D. from triplicate experiments. The data indicates 5-fold increase of *eNOS* gene synthesized in the *p53*-deficient fibroblasts ($P < 0.001$, unpaired T-test). The data indicates undetectable level of *iNOS* gene in the *p53*-deficient fibroblasts

Figure 3. *p53* – deficient fibroblasts have accumulated oxidative products, alteration in GSH-synthesis enzymes and up-regulation of redox regulated cytokines

A: Increased oxidative DNA damage product, 8-oxo-Gua in *p53*-deficient fibroblasts.

Oxidative DNA damage products in *p53*-intact and *p53*-deficient fibroblasts were measured by flow cytometric analysis of FITC- protein conjugate binding to 8-oxoguanine moiety in 8-oxoguanosine of damaged DNA. Overlaying Histograms represents duplicate experiments. Bar graph represents the mean fluorescent intensity and error bar indicates S.D. from duplicate experiments. The data indicates significant increases of 8-oxo-Gua in *p53*-deficient fibroblasts ($P < 0.001$, unpaired t-test).

B: Increased cellular thiols in *p53*-deficient fibroblasts. Total cellular thiols in *p53*-intact and *p53*-deficient fibroblasts were measured by flow cytometric analysis of CMF-DA.

Overlaying Histograms were representatives of duplicate experiments. The data indicates significant increases of total thiols in *p53*-deficient fibroblasts ($P < 0.001$, K-S stat test).

C: Upregulation of glutathione synthesis enzymes. The gene expression of *Nrf-2* transcription factor, GCLC and GCLM, the glutathione synthesis enzymes, in *p53*-intact and *p53*-deficient fibroblasts were measured by Real time PCR. Bar graph represents the relative level of gene expression (normalized with actin loading control) and error bar indicates S.D. from triplicate experiments. The data indicates significant increases of Nrf2, GCLC and GCLM in *p53*-deficient fibroblasts ($P < 0.001$, unpaired t-test).

D: Increased secretion of three cytokines from *p53*-deficient fibroblast. Cytokine array blots of conditioned media from single culture of each fibroblast (as labeled). Bar graph represents the mean pixel intensity and error bar indicates S.D. from duplicates. (**) indicates highly significant increase of three cytokines (1) GM-CSF, (2) sICAM-1 and (3) Serpin E1, as labeled) released from *p53*-deficient fibroblasts, NOF/ht shRNA p53, compared to that of parental cells ($P < 0.0001$, Two-way ANOVA with Bonferrini post test). (*) indicates moderately significant increases of some other cytokines ($P < 0.05$).

Figure 4. The induction of epithelial invasion by *p53* - deficient fibroblasts was mediated by ICAM1

A: Increased secretion of sICAM1 from *p53* - deficient fibroblast co-culture with oral epithelial cells. Cytokine array blots of conditioned media from co-culture between oral epithelial cells (OKF6/TERT2) and each fibroblast. Bar graph represents mean pixel intensity. Error bar was S.D. from duplicates. (**) indicates highly significant increase of sICAM1 ($P < 0.0001$, Two-way ANOVA with Bonferroni post test). (*) indicates moderately significant changes of other cytokines ($P < 0.05$).

B: Increased protein level of cellular ICAM1 in *p53* - deficient fibroblast. Non-reducing western blots of cellular ICAM1 in each fibroblast from co-culture as Fig. 4A. Actin was used as loading control. Bar graph represents the fold of band intensity of ICAM1 relative to actin. Error bar was S.D. from duplicates. The data indicates 10-fold increase of cellular ICAM1 level in NOF/ht shRNA p53 ($P < 0.001$, unpaired t-test).

C: Increased expression of ICAM1 gene in *p53* - deficient fibroblast. The gene expression of ICAM1 in *p53* - deficient and *p53* - intact fibroblasts was analyzed by RT-PCR. Bar graph represents the mean of relative expression level and error bar indicates S.D. from triplicate experiments. The data indicates at least 6-fold increase of ICAM1 gene synthesized in the *p53*-deficient fibroblasts ($P < 0.001$, unpaired T-test).

D: Induction of invasion by ICAM1. The *p53* - intact fibroblasts were organotypically co-cultured with non-tumorigenic oral (OKF6/TERT2) epithelial cells, in the presence or absence of 10 ng/ml recombinant ICAM1. The tissue sections were H&E stained and investigated through light microscope. Scale bars, 500 μ m. Bar graph represents the invasion index (in arbitrary unit (AU) and error bar indicates S.D. from duplicate experiments. The data indicates significant increases of invasion index in the organotypic mucosa containing recombinant ICAM1 ($P < 0.001$, unpaired t-test).

Figure 5. siRNA of eNOS suppressed ICAM1 production and epithelial invasion

A: Suppression of ICAM1 protein expression by siRNA (s) of eNOS. The p53-deficient fibroblasts were transfected with siRNA of eNOS pair 1, 2, 3 or with scramble siRNA. The ICAM1 protein levels in those cells were analyzed after 48 hours. Bar graph represents the band density. Error bar was S.D. from duplicate experiments. The data indicates significant decrease of ICAM1 protein levels in the fibroblasts containing siRNA of eNOS pair #1, 2 and 3 ($P < 0.001$, unpaired t-test). SiRNA #3 was the most effective.

B: Suppression of reactive nitrogen species by siRNA of eNOS pair # 3. The p53-deficient fibroblasts were transfected with or without siRNA of eNOS pair # 3 or with scramble siRNA. RNS levels in those cells were analyzed after 48 hours. The figure is representative of triplicate experiments. The dash line is at the peak of histogram in the control sample. The histogram of the siRNA pair 3-transfected cells (bottom panel) shows the shift of peak indicating decrease of RNS level ($P < 0.001$, K-S statistic test).

C: Suppression of the fibroblast-induced epithelial invasion by siRNA of eNOS # 3. The p53-deficient fibroblasts (NOF/hTERT shRNA p53) were transfected with or without siRNA of eNOS pair #3 for 2 days. The inductive capability of fibroblasts for epithelial invasion was tested by transwell - matrigel invasion assay. The non-tumorigenic oral (OKF6/TERT2) epithelial cells were placed on the top chamber, while each type of fibroblasts was placed in the bottom chamber. The invasive cells were stained with Diff-Quik™ stain and counted under light microscope. Scale bars, 100 μ m. Bar graph represents the invasion index and error bar indicates S.D. from duplicate experiments. The data indicates significant decrease of invasion index induced by the p53-deficient fibroblasts containing siRNA of eNOS pair # 3 ($P < 0.001$, unpaired T-test).

Figure 6. Antioxidant NAC suppressed RNS - mediated ICAM1 production and epithelial invasion

A–B: Suppression of ICAM1 protein and gene expression by antioxidant NAC. NOF/hTERT shRNA p53 cells were treated with or without 1mM NAC for 2 days. A: Blot of ICAM1 from cells and conditioned media, triplicate experiments B: Bar graph represents the mean of expression of *ICAM1* gene from cells, triplicates. Error bar indicates S.D. from triplicate experiments ($P < 0.001$, paired t-test).

C: Suppression of RNS levels in p53-deficient fibroblasts by NAC NOF/hTERT shRNA p53 cells were treated with or without 1mM NAC for 5h. The cells were collected and RNS levels were analyzed by flow cytometric analysis of DAF-FM. Bar graph represents the mean fluorescent intensity of triplicate experiments and error bar was S.D. from triplicate experiments ($P < 0.001$, paired t-test).

D: Suppression of invasive oral epithelium induced by p53-deficient fibroblasts NOF/hTERT shRNA p53 were organotypically co-cultured with non-tumorigenic oral (OKF6/TERT2) or ovarian (T72) epithelial cells, with or without 1mM NAC. The tissue sections were H&E stained and investigated through light microscope. Scale bars, 100 μ m. Bar graph represents the invasion index (in arbitrary unit (AU) and error bar was S.D. from triplicate experiments. The data indicates significant decrease of invasion index in the organotypic mucosa with the presence of NAC ($P < 0.001$, unpaired t-test).

Figure 7. Conceptual summary

Increase of reactive nitrogen species via eNOS up-regulation augmented expression of cytokine ICAM1. The release of ICAM1 from the p53-deficient fibroblasts provided paracrine signal to epithelium and induced the invasive phenotype. The histologic section of organotypic culture between OKF6/TERT2 and NOF/shRNA p53 was stained with hematoxylin & eosin. Scale bars, 200 μm .