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ERK/GSK3 β /Snail signaling mediates radiation-induced alveolar epithelial-to-mesenchymal transition

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Abstract

Radiotherapy is one of the major treatment regimes for thoracic malignancies, but can lead to severe lung complications including pneumonitis and fibrosis. Recent studies suggest that epithelial to mesenchymal transition (EMT) plays an important role in tissue injury leading to organ fibrosis. To investigate whether radiation can induce EMT in lung epithelial cells and also understand the potential mechanism(s) associated with this change, rat alveolar type II lung epithelial RLE-6TN cells were irradiated with 8 Gy of ¹³⁷Cs γ -rays. Western blot and immunofluorescence analyses revealed a time-dependent decrease in E-cadherin with a concomitant increase in α -SMA and vimentin after radiation, suggesting that the epithelial cells acquired mesenchymal-like morphology. Protein levels and nuclear translocation of Snail, the key inducer of EMT, were significantly elevated in the irradiated cells. Radiation also induced a time-dependent inactivation of glycogen synthase kinase-3 β (GSK3 β), an endogenous inhibitor of Snail. A marked increase in phosphorylation of ERK1/2, but not JNKs or p38, was observed in irradiated RLE-6TN cells. Silencing ERK1/2 using siRNAs and the MEK/ERK inhibitor U0126 attenuated the radiation-induced phosphorylation of GSK3 β and altered the protein levels of Snail, α -SMA and E-cadherin in RLE-6TN cells. Pre-incubating RLE-6TN cells with N-acetyl cysteine, an antioxidant, abolished the radiation-induced phosphorylation of ERK and altered protein levels of Snail, E-cadherin and α -SMA. These findings reveal, for the first time, that radiation-induced EMT in alveolar type II epithelial cells is mediated by the ERK/GSK3 β /Snail pathway.

Keywords

Ionizing radiation; Alveolar type II epithelial cells; Epithelial-to-mesenchymal transition; ROS; ERK; Snail; GSK3 β ; E-cadherin; α -SMA

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INTRODUCTION

Lung cancer is a major public health problem and is one of the most fatal cancers in the US, accounting for 29% and 26% of all estimated cancer deaths in both men and women, respectively [1]. Radiotherapy is a mainstay in the treatment of locally advanced lung cancer, but can result in normal tissue complications including pneumonitis and fibrosis. Pulmonary fibrosis is characterized by alveolar epithelial cell injury, accumulation of fibroblasts, myofibroblasts, collagen and other extracellular matrix (ECM) proteins with subsequent scar formation resulting in impaired lung function [2]. Myofibroblasts play a central role in the pathogenesis of pulmonary fibrosis; their origin has become the subject of intense investigation [3]. Myofibroblasts are believed to derive mainly from resident fibroblasts but more recently, evidence suggests that injured epithelial cells may directly serve as a source of myofibroblasts by the process of “epithelial-mesenchymal transition” (EMT) [3]. Under inflammatory stress, 30% of myofibroblasts can arise via EMT whereas resident fibroblast contribute only 23% in the kidney [4]. Zeisberg *et al.* reported that 45% fibroblasts are derived from hepatocytes via EMT and suggested that EMT is a promising therapeutic target for the attenuation of liver fibrosis [5]. EMT is a highly regulated process by which fully differentiated epithelial cells can undergo transition to a mesenchymal phenotype that can give rise to matrix producing myofibroblasts. This transition is characterized by loss of epithelial proteins such as E-cadherin and acquisition of new mesenchymal markers including vimentin and α -smooth muscle actin (α -SMA). At the molecular level, down-regulation of E-cadherin expression can be achieved by transcriptional suppression mediated by members of the basic helix-loop-helix family, including Snail [6]. Glycogen synthase kinase 3 β (GSK 3 β) is a constitutively active serine/threonine kinase contributing to a variety of biological events, including embryonic development, cell differentiation, apoptosis, and insulin response [7]. GSK3 β is also essential to maintain the epithelial architecture of the cells; inhibiting GSK3 β causes the acquisition of mesenchymal morphology [8]. Accumulating evidence indicates that GSK3 β can maintain epithelial morphology by binding to Snail and facilitating its proteasomal degradation [9]. In addition, GSK3 β can be inactivated by MAPK upon phosphorylation [10]. Ding *et al.* have reported that ERK primed GSK3 β for its inactivation in cells infected with hepatitis B virus [11]. Studies have demonstrated that the MAPK pathway regulates EMT induced by TGF- β in various epithelial cell lines including mammary [12], liver [13] and lung [14].

In the lung, the alveolar epithelium is composed of type I and type II cells that are morphologically and functionally distinct. Following lung injury, alveolar type II epithelial cells (AE2) are able to self-renew and can give rise to alveolar type I epithelial cells to re-establish a functional alveolar epithelium [15]. Alveolar epithelial injury followed by abnormal epithelial repair appears to be a key pathological feature of lung fibrosis [15]. Increased proliferation/hyperplasia of AE2 cells has been frequently observed in injured lungs, including following irradiation [16, 17]. Injury in alveolar type II epithelial cells has been linked with the development of lung fibrosis [18]. AE2 cells from patients with idiopathic pulmonary fibrosis (IPF) expressed high level of EMT associated protein markers [19, 20]. Furthermore, co-expression of AE2 and mesenchymal markers was detected in IPF patients in two independent studies [19, 21], suggesting that AE2 cells acquired a mesenchymal phenotype [20]. By using genetically modified mice in which AE2 cell fate can be tracked, Kim *et al.* found that AE2 cells were progenitors for mesenchymal cells and contributed significantly to the pool of expanded fibroblasts after lung injury [19]. Tanjore *et al.* reported that approximately one third of lung fibroblasts were derived from the lung epithelium in a bleomycin-induced lung fibrosis model [22]. Although hyperproliferation of AE2 is frequently observed in irradiated lungs, transdifferentiation of AE2 cells into mesenchymal-like cells has not been previously reported. We have observed the expression

of mesenchymal protein α -SMA in the AE2 cells of FVB/N mice following thoracic radiation with a single dose of 12 Gy (Zhao *et al.* unpublished results). In the present study, we used an *in vitro* model to: i] investigate radiation-induced EMT in AE2 cells and ii] understand the possible signaling mechanisms associated with radiation-induced EMT. Our data reveal, for the first time, that radiation-induced transdifferentiation of AE2 to mesenchymal phenotypic cells is mediated, at least in part, by the ERK/GSK3 β /Snail signaling pathway.

MATERIALS AND METHODS

Cell Culture

RLE-6TN cells, a rat alveolar type II epithelial cell line, and A549, a human lung carcinoma epithelial cell line, were obtained from ATCC (Manassas, VA) and routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin and 100 μ g/mL streptomycin (purchased from Invitrogen, Carlsbad, CA) at 37° C with 5% CO₂ in air.

Irradiation

Once cells reached >80% confluence, the DMEM medium was replaced with serum-free medium for 24 h prior to irradiation. Cells were then irradiated with a single dose of 8 Gy γ rays using a ¹³⁷Cs irradiator at a dose rate of 3.64 Gy/min. All irradiations were performed at room temperature.

Western Blot analysis

Cells were lysed using RIPA buffer containing 1 mM PMSF, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 mM Na₃VO₄ and 1 mM NaF, and stored in aliquots at –80°C until assayed. The lysate (20 μ g) was mixed with an equal volume of sample buffer, denatured by boiling, and then separated on 10–15% polyacrylamide mini-gel. The proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL), blocked with 5% milk and incubated overnight with E-cadherin (Abcam, Cambridge, MA), Snail (Abcam), p-GSK3 β (s9) (Cell Signaling Technology, Danvers, MA), GSK3 β (Cell Signaling Technology), p-GSK3 β (T216) (Santa Cruz Biotechnology, Santa Cruz, CA), α -SMA (Abcam), phosphorylated ERK (Santa Cruz Biotechnology), ERK (Santa Cruz Biotechnology), p-p38, p-JNK (Cell Signaling Technology) and β -actin antibodies (Sigma, St. Louis, MO). The blots were then incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase conjugated antibodies (GE Healthcare, Piscataway, NJ) for 1 h at room temperature. Finally, the signal was detected using Amersham ECL plus (GE Healthcare).

RT-PCR

Total RNA was isolated from cells using an RNeasy Mini kit according to the manufacturer's recommendations (QIAGEN, Valencia, CA) with addition of DNase digestion with RNase-free DNase set (QIAGEN). One μ g of Total mRNA was used as template for cDNA synthesis with the First-Strand cDNA Synthesis kit (Invitrogen) according to the recommendations of the manufacturer. The PCR amplifications were carried out in 50 μ L reaction solution containing 2 μ L of RT product, 5 μ L of 10 $\times$ PCR buffer, 0.15 mM MgCl₂, 1 μ L of 10 mM dNTP, 15 pmol of sense and antisense primers, and 2.5 U Taq polymerase (Promega). The PCR reaction solution was denatured initially at 94°C for 2 min, followed by 25–30 cycles through a 1-min denaturing step at 94°C, a 40 sec annealing step at 50–55°C, and a 40 sec elongation step at 72°C. Mouse E-cadherin and GAPDH primers were designed and synthesized by Integrated DNA Technologies (Iowa City, IA). Forward and reverse primers were 5'- ATG TTC ACT GTC AAT AGG GAC

ACT -3' and 5'-TCA TCA GTC ACC TTG AGT GTG GCA -3' for E-cadherin and 5'-TGA CTC TAC CCA CGG CAA GTT CAA -3' and 5'-TCT CGT GGT TCA CAC CCA TCA CAA' for GAPDH.

Immunoprecipitation

Five hundred μg of cell lysate were incubated with 10 μL of the GSK3 β antibody overnight at 4°C in a rotating wheel. After overnight incubation, 50 μL of protein A/G agarose were added and the solution incubated for 4 h at 4°C with rotation. The complexes were harvested by centrifugation, washed three times with RIPA buffer and then dissociated from the beads by addition of 50 μL of SDS sample loading buffer and heated for 5 min at 95 °C. The protein level of Snail was analyzed using Western blot.

siRNA transfection

RLE-6TN cells were cultured in 6-well plates in DMEM medium supplemented with 10 % fetal bovine serum for 24 h. Fifty to 70% confluent cells were transfected with 100 pmol of siRNA using 5 μL of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Six hours after transfection, antibiotic and serum free medium was replaced with full culture medium. Cells were then irradiated with a single dose of 8 Gy of ^{137}Cs γ rays at 48 h post-transfection, and cell lysates were harvested at the required time point for protein analysis.

Reactive oxygen species (ROS) generation

ROS generation was measured using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA , Invitrogen, CA) as described previously [23]. Cells were incubated with 10 μM H_2DCFDA (in PBS) for 30 min, washed and then irradiated with 8 Gy of ^{137}Cs γ rays; the cells were then returned to the incubator for 1 h. The fluorescence intensity was then measured at excitation wavelength 485 nm and emission wavelength 530 nm using a microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT).

Immunofluorescence staining

Cells grown on 8-well chamber slides were irradiated with 8 Gy of ^{137}Cs γ rays and then fixed with 4% neutral formalin for 30 minutes. After washing three times with $1 \times \text{PBS}$, the cells were blocked with 3% BSA for 1h. The cells were then incubated with $\alpha\text{-SMA}$ (Abcam), Snail (Abcam), vimentin (Epitomics, Burlingame, CA), $p\text{-GSK3}\beta$ (Cell Signaling Technology) antibodies at 4 C overnight. After washing with PBS, the sections were incubated with Alexa Fluor® 488 dye (Invitrogen) anti-mouse and Texas Red conjugated anti-rabbit (Vector Laboratories, Burlingame, CA) antibodies at room temperature for 30 min. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI), and the slides were analyzed using a fluorescence microscope.

Statistical Analysis

Statistical analysis was performed using one-sample Student's t test to compare the differences between the irradiated and unirradiated groups. A p value of 0.05 was considered significant.

RESULTS

Radiation induces a mesenchymal phenotype in rat type II alveolar RLE-6TN cells

Although lung EMT has been reported in human tissues, animal models and *in vitro* studies, it is not clear whether alveolar epithelial cells can also undergo EMT following radiation treatment. To test that, rat type II alveolar RLE-6TN cells were irradiated with a single dose

of 2, 4, 6, or 8 Gy γ -rays. Cell morphology was observed at 24 h postirradiation. Cells irradiated with 2–8 Gy lost their cuboidal appearance and showed an elongated mesenchymal-like morphology (Fig. S1). To determine if radiation can modulate EMT associated proteins, RLE-6TN cells were irradiated with a single dose of 8 Gy and cells were harvested at various time points ranging from 15 min to 72 h postirradiation, followed by Western blot and immunohistochemical analysis. Western blot analysis of E-cadherin showed a marked reduction in the protein level of E-cadherin at 48h and 72 h postirradiation compared to non-irradiated control levels (Fig. 1A). Densitometric analysis revealed that the protein levels of E-cadherin were decreased 45% and 50%, respectively, at 48 and 72 h postirradiation. Loss of E-cadherin expression is thought to be one of the most important events in EMT. α -SMA and vimentin are often used as markers of mesenchymally-derived cells. Radiation enhanced the protein level of α -SMA in a time-dependent manner (Fig. 1B), which was confirmed by immunofluorescence staining (Fig. 1C). We also observed a dose-dependent increase in the protein level of α -SMA in cells exposed with 2–8 Gy of radiation (Fig. S2). Immunofluorescence analysis showed a mild expression of vimentin in non-irradiated control cells, whereas an increase was seen in the irradiated cells in a fibril-associated pattern (Fig. 1D). These data suggest that radiation induces AE2 transdifferentiation to a mesenchymal-like phenotype.

Radiation increases the protein level and nuclear translocation of Snail—Snail, a zinc finger-type transcription factor, is the most prominent EMT transcriptional regulator that contributes to EMT by mainly acting as an E-cadherin repressor [24]. To analyze the effect of radiation on Snail, RLE-6TN cells were exposed to 8Gy and protein was harvested from 15 min to 7h postirradiation. Western blot analysis showed that radiation caused a time-dependent increase in Snail protein level (Fig. 2A). Densitometric analysis revealed that the mean fold increase in Snail protein levels in the cell lysate collected at 15 min, 30 min, 1 h, 3 h and 7 h postirradiation were 1.6 ± 0.09 , 2.05 ± 0.13 , 2.2 ± 0.1 , 2.86 ± 0.27 and 2.91 ± 0.3 , respectively, compared with levels observed in control cells (Fig. 2B). A dose-dependent change of Snail protein was also seen in the cells exposed with 2–8 Gy of radiation (Fig. S3). Snail is functionally active when it is transported into the nucleus. Thus, we conducted an immunofluorescence analysis to determine the translocation potential of Snail in irradiated RLE-6TN cells. The cells were stained with Snail antibody (red fluorescence), and counterstained with Dapi (blue fluorescence) (Fig. 2D). Overlay of the red fluorescence with the blue nuclear staining resulted in a purple nucleus, an indicative of nuclear localization of active Snail. Most of cells in the irradiated group showed a purple staining in the nucleus; few cells with purple nucleus were also observed in the non-irradiated control. We next examined the effect of radiation on gene expression of E-cadherin. RT-PCR analysis showed a time-dependent reduction in the message level of E-cadherin in the irradiated RLE-6TN cells from 7 h to 48 h post-irradiation (Fig. 2C). Our data showed a concomitant change in the level of Snail protein and E-cadherin mRNA following radiation, suggesting an association between repression of E-cadherin mRNA and activation of Snail during radiation-induced EMT.

Radiation leads to inactivation of GSK3 β by phosphorylation and enhances disassociation of GSK3 β with Snail in RLE-6TN cells

GSK3 β is an active kinase of Snail. The catalytic activity of GSK3 β is negatively regulated through phosphorylation of serine 9 residues (s9) [25] and positively regulated by the phosphorylation of tyrosine 216 (T216) [26]. As shown in Fig. 3A & B, phosphorylation of GSK3 β (s9) was significantly increased in irradiated RLE-6TN cells in a time-dependent manner, whereas the level of total GSK3 β and p-GSK3 β (T216) remained unchanged which indicated that radiation inactivated GSK3 β by phosphorylating specifically at serine 9 residue. We also observed a dose-dependent increase in the p-GSK3 β (s9) in cells exposed

with 2–8 Gy of radiation (Fig. S4). These results showed that GSK3 β is post-translationally modulated by radiation. Increased phosphorylation of GSK3 β (s9) was further confirmed by immunofluorescence analysis (Fig. 3C). Phosphorylation at serine 9 inactivates GSK3 β [25], leading to inhibition of GSK-3 toward its substrates, such as Snail. Thus, we next examined the physical association between GSK3 β and Snail using a co-immunoprecipitation assay (Fig. 3D). Proteins were immunoprecipitated with GSK3 β antibody and the precipitants were subjected to immunoblotting using anti-Snail antibody. Association of Snail and GSK3 β was seen in protein samples collected from non-irradiated controls and at 1 h postirradiation. However, at 3 h postirradiation we observed a reduced association of both proteins, indicating that the interaction of GSK3 β with Snail was reduced following radiation.

Irradiation enhances ERK1/2, but not p38 and JNK, phosphorylation in RLE-6TN cells

MAPK have been reported to play a crucial role during EMT. Thus, we investigated the possible involvement of MAPK signaling pathways (ERK1/2, p38 and JNK) in the radiation-induced EMT in RLE-6TN cells (Fig. 4A). Western blot analysis showed an increased ERK phosphorylation at 15 min to 1 h postirradiation. The maximum increase was seen at 15 min, followed by a gradual return to control levels at 3 h postirradiation; the level of total ERK was not altered (Fig 4A & B). Surprisingly, no significant changes were observed in the protein level of phosphorylated p38 and JNK (Fig. 4A). These data indicate that ERK may play a role in radiation-induced EMT in lung epithelial cells.

Blocking and silencing of ERK inhibits the radiation-induced changes in α -SMA, E-cadherin, p-GSK3 β and Snail

As noted above, phosphorylation at serine 9 inhibits GSK3 β activity. To determine whether the MEK-ERK axis signaling affects GSK3 β phosphorylation, cells were preincubated with the selective MEK1/2 inhibitor U0126 for 2 h prior to irradiation. As shown in Fig. 5A, U0126 inhibited phosphorylation of ERK1/2 and effectively abolished the radiation-induced phosphorylation of GSK3 β (s9). Furthermore, blocking MEK/ERK signaling also inhibited the radiation-induced increase in protein levels of Snail (Fig. 5A) and α -SMA (Fig. 5B). In contrast, blocking MEK/ERK signaling increased the expression of E-cadherin following irradiation (Fig. 5B). To further confirm the role of ERK in radiation-induced EMT, we knocked down the expression of ERK1/2 using ERK1/2 siRNA. Transient transfection of ERK1 and ERK 2 siRNA resulted in an ~70 and 95% reduction in ERK1 and ERK2, respectively (Fig. 5C). Knocking-down ERK1/2 effectively inhibited the radiation-induced EMT by modulating the protein levels of p-GSK3 β (s9), Snail, α -SMA and E-cadherin (Fig 5D & E). Thus, MEK/ERK signaling appears essential for radiation-induced EMT in RLE-6TN cells.

Pre-incubating RLE-6TN cells with N-acetyl cysteine (NAC) abrogates the radiation-induced changes in α -SMA, E-cadherin, Snail and p-ERK

The biological effects of radiation are mainly mediated by ROS [27]. We initially measured the generation of ROS following radiation using the oxidation sensitive fluorescent probe DCFH₂-DA. Irradiating RLE-6TN cells with 8 Gy of ¹³⁷Cs γ rays led to a 2.8 fold increase in ROS generation at 1 h postirradiation, compared with that observed in non-irradiated controls (Fig. 6A). To determine the role of ROS in radiation-induced EMT in RLE-6TN cells, cells were pre-treated with 5 mM of NAC, an antioxidant, for 3 h and then irradiated with a single dose of 8 Gy. Preincubating cells with NAC decreased ROS production in irradiated RLE-6TN cells (Fig. 6A). Moreover, pre-incubating cells with NAC inhibited the radiation-induced changes in EMT associated proteins including Snail, α -SMA and E-cadherin; and also ERK phosphorylation (Fig. 6B). These data indicate that the radiation-induced EMT in AE2 is mediated, at least in part, via increased ROS production.

Is ERK/GSK3 β /Snail signaling pathway also involved in the radiation-induced EMT in human A549 cells?

To further investigate whether similar mechanisms are involved in radiation-induced EMT in AE2, we examined the effects of radiation using A549 cells, a human lung cancer cell line with characteristics of AE2 cells [28, 29]. Irradiating A549 cells resulted in a time-dependent increase in the protein levels of α -SMA at both 24 and 48 h postirradiation; E-cadherin levels were reduced 48 hours after radiation (Fig. 7A). A time dependent increase in the protein level of Snail and phosphorylated GSK3 β (s9) was seen from 30 min to 7 h postirradiation (Fig. 7A). Moreover, radiation enhanced the phosphorylation of all the three ERK, JNK and p38 in A549 cells (Fig. 7B). ERK phosphorylation was enhanced at 15 min and maintained till 3 h postirradiation, but by 7h, the phosphorylated protein level of ERK was similar to that seen in the non-irradiated controls. On the other hand, a gradual increase in the expression of phosphorylated p38 and JNK was observed from 15 min to 7 h. Blocking MEK/ERK signaling pathway with U0126 partially inhibited radiation-induced phosphorylation of GSK3 β (s9) and increased the protein level of Snail (Fig. 7C). Western blot analysis of α -SMA and E-cadherin showed that pre-incubation with U0126 abrogated the radiation-induced increase in α -SMA protein and decrease in the expression of E-cadherin (Fig. 7D). These data suggest that radiation-induced EMT in A549 cells is mediated, in part, through the ERK/GSK3 β /Snail signaling pathway.

DISCUSSION

Pulmonary fibrosis is characterized by activation, expansion of the fibroblast/myofibroblast population, and ECM remodeling, resulting in an irreversible distortion of the lung architecture [30]. Myofibroblasts have been recognized as the major effectors involving in excessive deposition of ECM under pathological condition. However, emerging evidence suggests that injured epithelial cells undergoing EMT are also an important source of myofibroblasts in fibrosis [31]. Here we demonstrate, for the first time, that radiation induces EMT in normal alveolar type II epithelial cells. MEK/ERK signaling appears to be required for the induction of EMT following radiation. Blockage of ROS generation and MEK/ERK signaling reduced the radiation-induced deactivation of GSK3 β , activation of Snail and induction of EMT.

EMT is defined by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype [32]. A critical molecular feature of EMT is down-regulation of E-cadherin [33]. E-cadherin, present in the plasma membrane of normal epithelial cells, is a calcium-dependent adhesion molecule that plays a major role in maintaining cellular polarity and morphological structure of cells [34]. Loss of E-cadherin disrupts the cell-cell junction and affects the structural integrity of cells [33]. In our study, reduction in protein level of E-cadherin and alteration in cell morphology from cuboidal to an elongated spindle like structure in irradiated RLE-6TN cells indicated that these cells lost their epithelial phenotype. α -SMA and vimentin are often used to define the mesenchymal phenotype [21]. Our data showed that the expressions of vimentin and α -SMA was significantly increased in RLE-6TN cells following radiation, further evidence in support of the hypothesis that radiation induces EMT in these cells. Jung *et al.* reported a reduction of E-cadherin assessed using image analysis in A549 cells following irradiation with 6 and 12 Gy doses [35]. In contrast, Akimoto *et al.* showed increased protein level of E-cadherin in the same cell line following radiation with 10 Gy using Western blot [36]. The lack of normalization using an appropriate loading control in the Western blot analysis may explain this apparent variation in E-cadherin expression. Zhou *et al.* observed phenotypic changes in alveolar epithelial cells consisting of increased levels of α -SMA and vimentin and decreased E-cadherin under hypoxic conditions [37]. Similar changes have been observed in TGF- β -induced EMT in lung and kidney epithelial cells [14, 38]. An early study suggested that irradiation with 2 Gy

enhanced TGF- β 1-induced EMT in human mammary epithelial cells [39]. Recent studies have revealed the occurrence of radiation-induced EMT in malignant cancer cells [35, 40, 41], which was associated with their invasive potential. Consistent with these early findings obtained from cancer cells, our data indicate that radiation can induce a transition of normal lung AE2 RLE-6TN cells to a mesenchymal phenotype.

Snail is a critical transcriptional regulator of E-cadherin for epithelial cells to adopt a fibroblastic phenotype and acquire mesenchymal properties [42]. Snail binds to the E-boxes of the human E-cadherin promoter, and represses E-cadherin expression. Knocking-down Snail using siRNA reduced high glucose-induced EMT in renal proximal tubular epithelial cells [43]. Further studies have shown that Snail is a key mediator of TGF β 1-induced EMT in lung fibrosis [44]. We observed a markedly increase in the protein level of Snail and a concomitant decrease in the message level of E-cadherin following radiation in RLE-6TN cells. Snail activity is regulated by GSK3 β -mediated phosphorylation [10]. In many cells, GSK3 β is constitutively active and can bind to and phosphorylate Snail to facilitate its degradation [10]. GSK3 β can be inactivated by phosphorylation at its N-terminal serine 9 residue through serine/threonine kinases [25]. Inactivation of GSK3 β promotes Snail stabilization, nuclear translocation and subsequent EMT induction [10]. Yoshino *et al.* reported that inhibition of GSK3 β using lithium or thiadiazolidinone-8 induced accumulation of Snail protein in human kidney HKC-8 cells and suggested that the increase in Snail paralleled the inactivation of GSK3 β [45]. In the current study, Western blot and immunofluorescence analyses showed that radiation increased the phosphorylation of GSK3 β (s9) and stimulated the nuclear translocation of Snail in RLE-6TN cells. Co-immunoprecipitation studies using GSK3 β and Snail antibody showed that GSK3 β protein bound with Snail in the control cells. Conversely, a reduced protein level of Snail in the precipitant was observed at 3 h postirradiation, indicating the dissociation of Snail from GSK3 β . Our data suggest that radiation enhanced phosphorylation of GSK3 β at serine 9 active site, which led to its disassociation with Snail, thereby facilitating the nuclear translocation of Snail.

The biological effects of ionizing radiation are largely attributable to energy deposition and subsequent ROS generation in irradiated cells [27]. ROS can cause biological damage by activating multiple cellular signal transduction pathways in the cells. Activation of MAPK pathways have been associated with radiation-induced injuries [46] and EMT in both normal and tumor cells [47–50]. We investigated the response of MAPK to radiation in RLE-6TN cells. Phosphorylation of ERK1/2, but not JNK and p38 was observed following radiation, suggesting that ERK may be involved in the radiation-induced EMT in RLE-6TN cells. Of interest, ERK activation has been linked with inactivation of GSK3 β [51, 52]. Inhibition of ERK suppressed TGF- β 1-induced GSK3 β phosphorylation in lung fibroblasts [52]. There is no direct evidence showing that radiation can inactivate GSK3 β via ERK1/2. Thus, we hypothesize that the radiation can inhibit GSK3 β and activate snail via activation of the MEK/ERK signaling pathway. In support of this hypothesis, pretreatment of cells with the MEK inhibitor U0126, or knocking down ERK1/2 using siRNA abolished the radiation-induced increase in phosphorylated GSK3 β protein, implying that MEK/ERK signaling is required for radiation-induced inactivation of GSK3 β . In addition, inhibition of ERK activity using U0126 or ERK1/2 siRNA prior to radiation prevented the radiation-induced modulation of E-cadherin, α -SMA and Snail protein level in the irradiated RLE-6TN cells. Altogether, our data suggest that MEK/ERK signaling is necessary for radiation-induced EMT in RLE-6TN cells.

ROS have also been shown to play a role in TGF- β 1-induced EMT in renal tubular epithelial cells through activation of MAPK [53] and in matrix metalloproteinase 3-induced EMT by stimulating the expression of Snail [54]. In our study, increased ROS generation

was also observed in irradiated RLE-6TN cells. Therefore, we hypothesized that blocking of ROS would inhibit EMT in irradiated RLE-6TN cells. The role of ROS in mediating EMT was investigated using the thiol antioxidant NAC. NAC can either directly quench intracellular free radicals or raise intracellular glutathione levels [55]. Pre-incubating cells with NAC abrogated the radiation-induced changes in EMT-associated proteins, including E-cadherin and α -SMA. Furthermore, NAC reduced radiation-induced ROS generation, ERK phosphorylation and increased Snail expression, indicating that the radiation-induced EMT in RLE-6TN cells is mediated, in part, via ROS generation and activation of the MEK/ERK signaling.

A549 cells have been used as a model of AE2 cells to study drug metabolism [28, 29] and other biological events, i.e. EMT [52]. Thus, we used A549 cells as an additional *in vitro* model to confirm our findings obtained from RLE-6TN cells. As observed with RLE-6TN cells, A549 also showed a mesenchymal-like phenotype with increased protein level of p-GSK3 β and Snail following radiation treatment. An early activation of ERK was noted at 30 min to 3 h postirradiation. Interestingly, p38 and JNK were also activated following radiation, which was not observed in the irradiated RLE-6TN cells. Although inhibition of MEK/ERK signaling by U0126 abolished radiation-induced increase in p-ERK and Snail, radiation-induced alterations in E-cadherin, α -SMA and p-GSK3 β were only partially recovered, suggesting that MEK/ERK signaling is not the only pathway involved in the radiation-induced EMT in A549 cells.

Based on our findings, we propose a mechanism for radiation-induced EMT in RLE-6TN cells as shown in Fig. 8. Radiation, through the increased generation of ROS, leads to activation of the MEK/ERK signaling pathway. Activated ERK1/2 causes the phosphorylation and resultant inactivation of GSK3 β , resulting in the disassociation of GSK3 β and Snail. Unbound Snail then migrates to the nucleus and functions as repressor of E-cadherin, eventually leading to a mesenchymal-like phenotypic alteration of RLE-6TN cells. The high prevalence of EMT in experimental lung fibrotic models suggests [19–21] that EMT is an important remodeling process. The involvement of EMT in terms of radiation-induced lung fibrosis has not been explored *in vivo*. Our current *in vitro* findings cannot be simply extrapolated to *in vivo* conditions. Therefore, these *in vitro* observations need to be further confirmed using appropriate animal models to support the potential importance of redox regulation, MEK/ERK signaling and GSK3 β inactivation in the radiation-induced EMT of AE2 cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Reference List

- [1]. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60:277–300. [PubMed: 20610543]
- [2]. Sime PJ. The antifibrogenic potential of PPARgamma ligands in pulmonary fibrosis. *J Investig Med.* 2008; 56:534–8.
- [3]. Grgic I, Duffield JS, Humphreys BD. The origin of interstitial myofibroblasts in chronic kidney disease. *Pediatr Nephrol.* 2011

- [4]. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest.* 2009; 119:1420–8. [PubMed: 19487818]
- [5]. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, et al. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J Biol Chem.* 2007; 282:23337–47. [PubMed: 17562716]
- [6]. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer.* 2007; 7:415–28. [PubMed: 17508028]
- [7]. Kim JW, Lee JE, Kim MJ, Cho EG, Cho SG, Choi EJ. Glycogen synthase kinase 3 beta is a natural activator of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1). *J Biol Chem.* 2003; 278:13995–4001. [PubMed: 12584189]
- [8]. Mishra P, Senthivinayagam S, Rana A, Rana B. Glycogen Synthase Kinase-3beta regulates Snail and beta-catenin during gastrin-induced migration of gastric cancer cells. *J Mol Signal.* 2010; 5:9. [PubMed: 20637111]
- [9]. Doble BW, Woodgett JR. Role of glycogen synthase kinase-3 in cell fate and epithelial-mesenchymal transitions. *Cells Tissues Organs.* 2007; 185:73–84. [PubMed: 17587811]
- [10]. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, et al. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol.* 2004; 6:931–40. [PubMed: 15448698]
- [11]. Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, et al. Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. *Mol Cell.* 2005; 19:159–70. [PubMed: 16039586]
- [12]. Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration. *J Cell Sci.* 2002; 115:3193–206. [PubMed: 12118074]
- [13]. Nitta T, Kim JS, Mohuczy D, Behrns KE. Murine cirrhosis induces hepatocyte epithelial mesenchymal transition and alterations in survival signaling pathways. *Hepatology.* 2008; 48:909–19. [PubMed: 18712785]
- [14]. Chen XF, Zhang HJ, Wang HB, Zhu J, Zhou WY, Zhang H, et al. Transforming growth factor-beta1 induces epithelial-to-mesenchymal transition in human lung cancer cells via PI3K/Akt and MEK/Erk1/2 signaling pathways. *Mol Biol Rep.* 2011
- [15]. Adamson IY, Young L, Bowden DH. Relationship of alveolar epithelial injury and repair to the induction of pulmonary fibrosis. *Am J Pathol.* 1988; 130:377–83. [PubMed: 3341452]
- [16]. Coggle JE. Proliferation of type II pneumonocytes after X-irradiation. *Int J Radiat Biol Relat Stud Phys Chem Med.* 1987; 51:393–9. [PubMed: 3494694]
- [17]. McCormack FX, King TE Jr, Bucher BL, Nielsen L, Mason RJ. Surfactant protein A predicts survival in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med.* 1995; 152:751–9. [PubMed: 7633738]
- [18]. Sisson TH, Mendez M, Choi K, Subbotina N, Courey A, Cunningham A, et al. Targeted injury of type II alveolar epithelial cells induces pulmonary fibrosis. *Am J Respir Crit Care Med.* 2010; 181:254–63. [PubMed: 19850947]
- [19]. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, et al. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci U S A.* 2006; 103:13180–5. [PubMed: 16924102]
- [20]. Marmai C, Sutherland RE, Kim KK, Dolganov GM, Fang X, Kim SS, et al. Alveolar epithelial cells express mesenchymal proteins in patients with idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol.* 2011; 301:L71–L78. [PubMed: 21498628]
- [21]. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, et al. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol.* 2005; 166:1321–32. [PubMed: 15855634]
- [22]. Tanjore H, Xu XC, Polosukhin VV, Degryse AL, Li B, Han W, et al. Contribution of epithelial-derived fibroblasts to bleomycin-induced lung fibrosis. *Am J Respir Crit Care Med.* 2009; 180:657–65. [PubMed: 19556518]

- [23]. Zhao W, Spitz DR, Oberley LW, Robbins ME. Redox modulation of the pro-fibrogenic mediator plasminogen activator inhibitor-1 following ionizing radiation. *Cancer Res.* 2001; 61:5537–43. [PubMed: 11454704]
- [24]. Battle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol.* 2000; 2:84–9. [PubMed: 10655587]
- [25]. Bauer K, Dowejko A, Bosserhoff AK, Reichert TE, Bauer RJ. P-cadherin induces an epithelial-like phenotype in oral squamous cell carcinoma by GSK-3 β -mediated Snail phosphorylation. *Carcinogenesis.* 2009; 30:1781–8. [PubMed: 19654099]
- [26]. Cai G, Wang J, Xin X, Ke Z, Luo J. Phosphorylation of glycogen synthase kinase-3 β at serine 9 confers cisplatin resistance in ovarian cancer cells. *Int J Oncol.* 2007; 31:657–62. [PubMed: 17671694]
- [27]. Riley PA. Free radicals in biology: oxidative stress and the effects of ionizing radiation. *Int J Radiat Biol.* 1994; 65:27–33. [PubMed: 7905906]
- [28]. Foster KA, Oster CG, Mayer MM, Avery ML, Audus KL. Characterization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism. *Exp Cell Res.* 1998; 243:359–66. [PubMed: 9743595]
- [29]. Nardone LL, Andrews SB. Cell line A549 as a model of the type II pneumocyte. Phospholipid biosynthesis from native and organometallic precursors. *Biochim Biophys Acta.* 1979; 573:276–95. [PubMed: 444551]
- [30]. Ramos C, Becerril C, Montano M, Garcia-De-Alba C, Ramirez R, Checa M, et al. FGF-1 reverts epithelial-mesenchymal transition induced by TGF- β 1 through MAPK/ERK kinase pathway. *Am J Physiol Lung Cell Mol Physiol.* 2010; 299:L222–L231. [PubMed: 20495078]
- [31]. Selman M, Pardo A. Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. *Proc Am Thorac Soc.* 2006; 3:364–72. [PubMed: 16738202]
- [32]. Flier SN, Tanjore H, Kokkotou EG, Sugimoto H, Zeisberg M, Kalluri R. Identification of epithelial to mesenchymal transition as a novel source of fibroblasts in intestinal fibrosis. *J Biol Chem.* 2010; 285:20202–12. [PubMed: 20363741]
- [33]. Mohamet L, Hawkins K, Ward CM. Loss of function of e-cadherin in embryonic stem cells and the relevance to models of tumorigenesis. *J Oncol.* 2011; 2011:352616. [PubMed: 21197469]
- [34]. Saha B, Chaiwun B, Imam SS, Tsao-Wei DD, Groshen S, Naritoku WY, et al. Overexpression of E-cadherin protein in metastatic breast cancer cells in bone. *Anticancer Res.* 2007; 27:3903–8. [PubMed: 18225549]
- [35]. Jung JW, Hwang SY, Hwang JS, Oh ES, Park S, Han IO. Ionising radiation induces changes associated with epithelial-mesenchymal transdifferentiation and increased cell motility of A549 lung epithelial cells. *Eur J Cancer.* 2007; 43:1214–24. [PubMed: 17379505]
- [36]. Akimoto T, Mitsuhashi N, Saito Y, Ebara T, Niibe H. Effect of radiation on the expression of E-cadherin and alpha-catenin and invasive capacity in human lung cancer cell line in vitro. *Int J Radiat Oncol Biol Phys.* 1998; 41:1171–6. [PubMed: 9719129]
- [37]. Zhou G, Dada LA, Wu M, Kelly A, Trejo H, Zhou Q, et al. Hypoxia-induced alveolar epithelial-mesenchymal transition requires mitochondrial ROS and hypoxia-inducible factor 1. *Am J Physiol Lung Cell Mol Physiol.* 2009; 297:L1120–L1130. [PubMed: 19801454]
- [38]. Aoyagi-Ikeda K, Maeno T, Matsui H, Ueno M, Hara K, Aoki Y, et al. Notch Induces Myofibroblast Differentiation of Alveolar Epithelial Cells via TGF- β /Smad3 Pathway. *Am J Respir Cell Mol Biol.* 2010
- [39]. Andarawewa KL, Erickson AC, Chou WS, Costes SV, Gascard P, Mott JD, et al. Ionizing radiation predisposes nonmalignant human mammary epithelial cells to undergo transforming growth factor β induced epithelial to mesenchymal transition. *Cancer Res.* 2007; 67:8662–70. [PubMed: 17875706]
- [40]. Chen C, Wei Y, Hummel M, Hoffmann TK, Gross M, Kaufmann AM, et al. Evidence for epithelial-mesenchymal transition in cancer stem cells of head and neck squamous cell carcinoma. *PLoS One.* 2011; 6:e16466. [PubMed: 21304586]

- [41]. Tsukamoto H, Shibata K, Kajiyama H, Terauchi M, Nawa A, Kikkawa F. Irradiation-induced epithelial-mesenchymal transition (EMT) related to invasive potential in endometrial carcinoma cells. *Gynecol Oncol.* 2007; 107:500–4. [PubMed: 17905419]
- [42]. Yang Z, Rayala S, Nguyen D, Vadlamudi RK, Chen S, Kumar R. Pak1 phosphorylation of snail, a master regulator of epithelial-to-mesenchyme transition, modulates snail's subcellular localization and functions. *Cancer Res.* 2005; 65:3179–84. [PubMed: 15833848]
- [43]. Lee YJ, Han HJ. Troglitazone ameliorates high glucose-induced EMT and dysfunction of SGLTs through PI3K/Akt, GSK-3{beta}, Snail1, and {beta}-catenin in renal proximal tubule cells. *Am J Physiol Renal Physiol.* 2009
- [44]. Jayachandran A, Konigshoff M, Yu H, Rupniewska E, Hecker M, Klepetko W, et al. SNAI transcription factors mediate epithelial-mesenchymal transition in lung fibrosis. *Thorax.* 2009; 64:1053–61. [PubMed: 19850962]
- [45]. Yoshino J, Monkawa T, Tsuji M, Inukai M, Itoh H, Hayashi M. Snail1 is involved in the renal epithelial-mesenchymal transition. *Biochem Biophys Res Commun.* 2007; 362:63–8. [PubMed: 17692821]
- [46]. Dent P, Yacoub A, Fisher PB, Hagan MP, Grant S. MAPK pathways in radiation responses. *Oncogene.* 2003; 22:5885–96. [PubMed: 12947395]
- [47]. Brown KA, Aakre ME, Gorska AE, Price JO, Eltom SE, Pietenpol JA, et al. Induction by transforming growth factor-beta1 of epithelial to mesenchymal transition is a rare event in vitro. *Breast Cancer Res.* 2004; 6:R215–R231. [PubMed: 15084245]
- [48]. Xie L, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro. *Neoplasia.* 2004; 6:603–10. [PubMed: 15548370]
- [49]. Javle MM, Gibbs JF, Iwata KK, Pak Y, Rutledge P, Yu J, et al. Epithelial mesenchymal transition (EMT) and activated extracellular signal-regulated kinase (p-Erk) in surgically resected pancreatic cancer. *Ann Surg Oncol.* 2007; 14:3527–33. [PubMed: 17879119]
- [50]. Santibanez JF. JNK mediates TGF-beta1-induced epithelial mesenchymal transdifferentiation of mouse transformed keratinocytes. *FEBS Lett.* 2006; 580:5385–91. [PubMed: 16989819]
- [51]. Cho KB, Cho MK, Lee WY, Kang KW. Overexpression of c-myc induces epithelial mesenchymal transition in mammary epithelial cells. *Cancer Lett.* 2010; 293:230–9. [PubMed: 20144848]
- [52]. Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z. TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res.* 2005; 6:56. [PubMed: 15946381]
- [53]. Rhyu DY, Yang Y, Ha H, Lee GT, Song JS, Uh ST, et al. Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol.* 2005; 16:667–75. [PubMed: 15677311]
- [54]. Radisky DC, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, et al. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature.* 2005; 436:123–7. [PubMed: 16001073]
- [55]. Galis ZS, Asanuma K, Godin D, Meng X. N-acetyl-cysteine decreases the matrix-degrading capacity of macrophage-derived foam cells: new target for antioxidant therapy? *Circulation.* 1998; 97:2445–53. [PubMed: 9641697]

Highlights

We investigated potential mechanisms involved in radiation-induced EMT in lung type II epithelial cells.

Radiation elevated the expression and nuclear translocation of Snail and suppressed GSK-3 β activity by phosphorylation of GSK3 β at serine 9

Silencing ERK1/2 attenuated the radiation-induced changes in the protein levels of phosphorylated GSK3 β (s9), Snail, α -SMA and E-cadherin.

Pre-incubating with NAC abolished the radiation-induced phosphorylation of ERK and alteration of downstream proteins.

These findings reveal that radiation-induced EMT in lung type II epithelial cells is mediated by the ROS/ERK/GSK3 β /Snail pathway.

Fig.1.

Irradiating rat alveolar type II RLE-6TN epithelial cells differentially modulates the expression of E-cadherin, α-SMA and Vimentin. RLE-6TN cells were irradiated with 8 Gy of ^{137}Cs γ -rays and were harvested at 48 and 72 h postirradiation. Panels A and B show representative Western blots and densitometric quantification of E-cadherin and α-SMA protein levels, respectively, in RLE-6TN cells. Data are mean $\pm$ SEM; $n = 3$; * $p < 0.05$ vs. non-irradiated control. Panel C shows immunofluorescence staining of α-SMA (green) and Dapi (blue) in control (upper panel) and irradiated (lower panel) cells at 72 h postirradiation. Panel D shows immunofluorescence staining of vimentin (red) and Dapi (blue) in control (upper panel) and irradiated (lower panel) cells at 72 h postirradiation.

Fig. 2.

Irradiating RLE-6TN cells leads to a significant increase in protein level and nuclear translocation of Snail. RLE-6TN cells were irradiated with 8 Gy of ^{137}Cs γ rays, total RNA and protein samples were collected at various time points after irradiation. A shows representative blot of Snail protein levels determined using Western blot. B shows densitometric quantification of Snail; data are mean $\pm$ SEM; n = 3; * $p < 0.05$ vs. non-irradiated control. C shows RT-PCR analysis of E-cadherin mRNA in the cells collected from 1 h to 48 h post irradiation. D shows immunofluorescence staining of Snail (red) and Dapi (blue) in control (upper panel) and irradiated (lower panel) cells at 3 h postirradiation (400 $\times$).

Fig. 3.

Radiation leads to inactivation of GSK3β and disassociation of GSK3β with Snail in RLE-6TN cells. RLE-6TN cells were irradiated with 8 Gy of ^{137}Cs γ rays and protein lysates collected at 15 min to 7 h postirradiation. Panel A shows representative blots of p-GSK3β (s9), p-GSK3β (T216) and GSK3β protein levels determined using Western blot. β-actin was used as a loading control. Panel B shows densitometric quantification of p-GSK3β (s9), p-GSK3β (T216) and GSK3β; data are mean ± SEM; n = 3; * p < 0.05 vs. non-irradiated control. Panel C shows immunofluorescence staining of p-GSK3β (s9) (red) and Dapi (blue) in control (upper panel) and irradiated (lower panel) cells at 3 h postirradiation (400 ×). Cell lysates were immunoprecipitated with GSK3β antibody and the protein level of Snail in the precipitated protein determined using Western blot (panel D).

Fig. 4. Irradiation leads to increased ERK 1/2 phosphorylation, but not p38 and JNK in RLE-6TN cells. RLE-6TN cells were irradiated with 8 Gy of ^{137}Cs γ rays and cell lysates collected at 15 min to 7 h postirradiation. Panel A shows representative blots of *p*-ERK, ERK, *p*-p38 and *p*-JNK protein determined using Western blot. Panel B shows the densitometric quantification of *p*-ERK; data are mean $\pm$ SEM; $n = 3$; * $p < 0.05$ vs. non-irradiated control.

Fig. 5. Inhibition of ERK abolishes the radiation-induced changes in α -SMA, E-cadherin, p -GSK3 β and Snail. RLE-6TN cells were incubated with/without U0126 for 2 h prior to irradiation with 8 Gy of ^{137}Cs γ rays. Cell lysates were collected and protein levels of p -ERK at 15 min, p -GSK3 β (s9) and Snail at 3 h, and α -SMA and E-cadherin at 72 h postirradiation were measured using Western blot (A & B). RLE-6TN cells were transiently transfected with either siRNA of ERK1/2 or control siRNA prior to irradiation with 8 Gy. Panel C shows significant reduction of ERK in the cells transfected with ERK1/2 siRNA. Panel D shows p -GSK3 β (s9) and Snail protein levels in cells with/without ERK siRNA at 3 h postirradiation. Panel E shows α -SMA and E-cadherin protein levels in cells transfected with/without ERK siRNA at 72 h postirradiation. Data are representative of 3 independent experiments.

Fig. 6.

Pre-incubating RLE-6TN cells with NAC abrogates the radiation-induced changes in *p*-ERK, Snail α -SMA and E-cadherin. RLE-6TN cells were preincubated with 5 mM NAC for 3 h prior to radiation with 8Gy. Intracellular ROS generation was measured using DCFH assay (A). Representative blots show the protein analysis of *p*-ERK (15 min postirradiation [PI]), Snail (3 h PI), α -SMA and E-cadherin (72 h PI) following 8 Gy irradiation with/without NAC (B). Data are representative of 3 independent experiments.

Fig. 7.

MEK/ERK signaling pathway is partially involved in the radiation-induced EMT in human A549 cells. A549 cells were irradiated with 8 Gy γ -ray and were harvested at 15 min to 48 h postirradiation. A: Upper panel shows representative blots of E-cadherin and α -SMA protein levels at 24 and 48 h postirradiation; lower panel shows protein level of *p*-GSK3 β (s9) and Snail at indicated times postirradiation using Western blot. Panel B shows representative blots of *p*-ERK1/2, *p*-p38 and *p*-JNK protein levels determined at indicated time point of postirradiation using Western blot. A549 cells were incubated with/without 2 μ M U0126 for 2h prior to irradiation with 8 Gy. The protein level of *p*-ERK was determined at 15 min postirradiation and at 3 h for *p*-GSK3 β (s9) and Snail (C). α -SMA and E-cadherin were measured at 48 h postirradiation using Western blot (D). Data are representative of 3 independent experiments

Fig. 8.

Schematic representation of the proposed mechanism for radiation-induced EMT in alveolar type II epithelial cells. Radiation-induced generation of ROS leads to activation of the MEK/ERK signaling pathway. Activated ERK1/2 causes the inactivation of GSK3β by phosphorylation of the serine 9 active site, which leads to the disassociation of Snail from GSK3β. Snail then translocates to the nucleus where it functions as a repressor of E-cadherin and initiate EMT in AE2 cells.