Effect of AR gene-specific knockout on the process of radiation-induced pulmonary fibrosis and its mechanism

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Abstract
Numerous studies have proved that epithelial-mesenchymal transition (EMT) of lung epithelial cells is one of the important causes of radiation-induced pulmonary fibrosis (RIPF). Aldose reductase (AR) is a monomer enzyme in the polyglycolic metabolic pathway and belongs to the aldo-keto reductase protein superfamily. Our previous studies have found that AR as one of the most significantly up-regulated genes was associated with the development of bleomycin-induced PF in rats. It is not clear whether aldose reductase is related to the regulation of radiation-induced EMT and mediates RIPF. AR-knockout mice, wild-type mice and lung epithelial cells were induced by radiation to establish a RIPF animal model and EMT system, to explore whether AR is meditated to RIPF through the EMT pathway. In vitro, AR deficiency significantly alleviated radiation-induced histopathological changes, reduced collagen deposition and inhibited collagen I, matrix metalloproteinase 2 (MMP2) and Twist1 expression. In addition, AR knockout up-regulated E-cadherin expression and up-regulated α-SMA and Vimentin expression. Knockdown or inhibition of AR inhibited the expressions of Twist1, MMP2 and collagen I, and reversed cell migration and reversed radiation-induced EMT. These results indicated that aldose reductase may be related to radiation-induced lung epithelial cells EMT, and that inhibition of aldose reductase might be a promising treatment for RIPF.

Introduction
Radiation-induced pulmonary fibrosis (RIPF) is a late complication of radiation-induced lung injury. It is a pathological reaction caused by the radiation dose of the lung tissue in the radiation field exceeding the threshold of the biological effect of the lung tissue when receiving radiation therapy for thoracic malignancy or bone marrow transplantation (1). The main pathological manifestations of RIPF were lung epithelial cell damage, increased accumulation of extracellular matrix (ECM) and fibrous connective tissue, alveolar structure destruction and parenchymal cell reduction (2). Persistent progress can cause the failure of lung function and endanger human life and health. The pathogenesis of RIPF is complex, and its mechanism has not been fully understood. The occurrence and development of RIPF are often related to the damage of lung epithelial cells and macrophages (3). When the damage occurs, it is very easy to cause the activation of fibroblasts, which in turn leads to the release of a series of cytokines, the activation of fibrosis-related signal pathway transduction, the interaction between cytokines, that causes epithelial-mesenchymal transition (EMT) of lung epithelial cells and excessive deposition of ECM, which ultimately leads to RIPF (4,5). Therefore, exploring the influencing factors of EMT in lung epithelial cells is the focus of the treatment of RIPF.

Aldose reductase (AR) belongs to the superfamily protein of aldosterone reductase, which is mainly involved in the metabolism of polyols in vivo by reducing glucose to sorbitol. It is a member of the nicotinamide adenine dinucleotide phosphate (NADPH) dependent aldo-keto reductase family, which is mainly involved in the metabolism of polyols in vivo by reducing glucose to sorbitol (6). The polyol pathway plays an important role in the pathogenesis of diabetes and its complications. Therefore, AR is believed to play a key role in diabetic complications, including diabetic nephropathy, retinopathy, heart disease, neuropathy, etc. (6). However, more and more studies have shown that the optimal substrate for AR is not glucose, but some toxic aldehydes produced in the process of lipid metabolism, such as 4-HNE. The binding ability of AR for these substrates is much higher than that of carbohydrate compounds, and it plays a more important physiological role in the body than the regulation of the polyol pathway and glucose metabolism (7,8). Research shows that AR knockout mice have resistance to ragweed pollen-induced airway inflammation, including significantly reduced proinflammatory cytokines and chemokines (9). Recent studies have found that AR gene knockout has an obvious neuroprotective effect on spinal cord injury in mice (10). It has also been demonstrated that AR mediates the lens
epithelial cells EMT, and inhibition of AR activity by AR inhibitor can significantly block the EMT and alleviate the development of cataracts (11). In addition, AR was also found to be involved in the EMT of glomerular mesangial cells, and inhibition of AR could effectively prevent glomerulonephritis and renal interstitial fibrosis (12). Our previous study found that Epalrestat (AR inhibitor) reduced the proliferation of TGF-β1 induced pulmonary fibroblasts by inhibiting the expression of AR, thereby improving bleomycin-induced pulmonary fibrosis (PF) in rats (13). We also found that Epalrestat could significantly reduce renal interstitial fibrosis after inhibiting AR expression (14). However, the role of AR in RIPF is still unclear, which is worth further study.

Twist1 belongs to the basic helix-loop-helix transcription factor family, which can bind to the E-box region of various gene promoter regions and regulate various biological functions such as embryonic development, EMT and fibrosis (15,16). The expression of twist1 was significantly increased in the lung tissues of patients with idiopathic pulmonary fibrosis (IPF). Overexpression of twist1 could significantly promote the EMT of lung epithelial cells, while twist1 gene knockout could significantly inhibit the EMT of lung epithelial cells (17,18). In addition, it was also found that twist1 expression was significantly increased in the lung tissue of radiation-induced lung fibrosis in mice, and inhibition of twist1 expression can significantly reverse radiation-induced lung epithelial cells EMT and reduced RIPF (19). Our previous research also found that Twist1 was highly expressed in bleomycin-induced PF, and TGF-β1-induced EMT in type II alveolar epithelial cells is related to its activation of the Notch-1/ Twist1 signaling pathway (20). In this study, we took AR gene knockout mice and mouse lung epithelial cells as the research object and took AR/Twist1 signaling pathway as the breakthrough to explore the mechanism of EMT and RIPF using a mouse model of RIPF and radiation-induced EMT system. It is hoped to provide theoretical basis for the pathogenesis of RIPF.

Materials and Methods

Animals

Wild-type (WT, AR+/+) Male C57BL/6 mice (specific pathogen-free, 8 weeks, 22–24 g) were obtained from the SiPeiFu (SPF) Biotechnology Co., Ltd (certificate No: SCXK (jing) 2019-0010; Beijing, China). AR Knockout (KO, AR−/−) mice (C57BL/6 background, homozygous) were purchased from Cyagen (Suzhou, China) Biotechnology Co., Ltd (No: KOAIB220407LJ9). The mice were raised in the feeding room of the SPF Animal Laboratory Center of Wannan Medical College (certificate No: SYXK (wan) 2018-004), with 5 mice in each cage. Feeding conditions: Animals drink and eat freely, temperature (22±2)°C, humidity (30%-40%), light/dark time: 12 hrs / 12hrs, the light intensity: 15-20lux. All the animal experiments were approved by the Medical Science Animal Management Committee of Wannan Medical College.

Radiation-induced pulmonary fibrosis

Twenty WT mice were randomly divided into two groups: the WT control (WT + Con) group and the WT radiation (WT +RT) group. Twenty AR-knockout mice were randomly divided into two groups: KO + Con group and KO + RT group. Mice were fixed in the prone position in a self-made fixing device and covered a 10-mm bolus material on the surface after being anaesthetized with 10% chloral hydrate (i.p., 400 mg/kg; Sigma, St. Louis, MO, USA). A 6 MV X-ray accelerator (Varian 600C/D, VARIAN Medical System, Palo Alto, CA, USA) was used to replicate the mouse model of RIPF with a single whole-chest radiation of 15 Gy, a radiation area of about 3.5 cm × 4.0 cm (from the upper to the two axillary pits and the lower to the xiphoid process of sterna, and the rest was shielded by 10 cm thick lead brick), a source target distance of 100 cm, and a dose rate of 500 cGy/min. Normal control mice were anesthetized without chest radiation. Mice were killed 16 weeks after radiation and lung tissues were collected for follow-up experiments.

Histology and Immunohistochemistry

According to previous research methods (21), the lung tissue was fixed with 4% paraformaldehyde solution and prepared into 4 μm paraffin sections. HE staining and Masson's trichromatic staining (KeyGEN Biotech, Nanjing, China) were performed to observe the lung histopathological changes and collagen deposition under a light microscope. Protein expressions of collagen I and Matrix Metallopeptidase 2 (MMP2) in lung tissues were detected by streptavidin-biotin complex-alkaline phosphatase (SABC-AP) immunohistochemistry, the positive expression of collagen I and MMP2 were yellow to brownish-yellow particles. MMP2 antibody (sc-13595, 1:250) and collagen I antibody (sc-59772, 1:500) were purchased from Santa Cruz.

Cell experiments

Mouse lung epithelial cells (MLE-12) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (ATCC® CRL-2110™, Manassas, VA, USA). DMEM/F12 medium and fetal bovine serum (FBS) were purchased from GIBCO (New York, NY, USA) and penicillin and streptomycin were purchased from Invitrogen (California, Carlsbad, CA, USA). When the cells were fused to more than 80%, 0.25% trypsin digested the cells and was used for the next experiment after passage. To verify the effect of AR inhibitor Tolrestat (SML1573, Sigma, St. Louis, MO, USA) on radiation-induced EMT in lung epithelial cells. The cells were divided into control (Con), radiation (RT), dimethyl sulfoxide (DMSO) and Tolrestat groups. The cells were pre-treated with Tolrestat (25 mM) for 2 h, and then the cells were radiated with 8 Gy X-ray (dose rate 1.0 Gy/min). To research the effect of AR small interference RNA (siRNA) on radiation-induced EMT in lung epithelial cells. The cells were divided into Con, RT, siRNA negative control (NC) and AR siRNA (si AR) group. The cells were pre-treated with si AR for 24 h, and then X-ray radiation was performed. The cells were collected 48 h after radiation for follow-up experiments.

Small interference RNA Transfection

The AR siRNA and negative control siRNA were designed according to the mouse AR cDNA sequence by GenePharma (Suzhou, China). The si AR sequences: sense, 5'-CCCCUGAAAGUGCUAUGATT-3’ and antisense, 5’-UCAUGCAACUUUCACGGTT-3’. The cell transfection was performed according to siRNA and Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) instructions.
Silencing of AR was confirmed with reverse transcription quantitative PCR (RT-qPCR) and Western blot.

**Transwell assay**

After cells were treated according to the above cell experiment protocol, cells were collected and then re-suspended with serum-free DMEM/F12 medium. 400 µL of the cell suspension was taken and added into the upper compartment of the invading compartment and the lower compartment of the invading compartment plus DMEM/F12 medium containing 10% FPS for 48 h. Take out the chamber, 4% paraformaldehyde solution fixed for 30 minutes, crystal violet staining for 30 minutes. The mean value was calculated to indicate the invasion ability of cells by the number of cells penetrating the membrane. The experiment was repeated three times.

**Immunofluorescence staining**

After cells were treated according to the above cell experiment protocol and fixed in 4% paraformaldehyde. The immunostaining solution (Triton X-100) was incubated for 10 min. QuickBlock™ blocking buffer (P0260, Beyotime, Shanghai, China) was added and closed for 10 min. The blocking buffer was discarded, and rabbit anti-E-Cadherin monoclonal antibody (#14472, CST, Danvers, MA, USA) and mouse anti-ɑ-SMA monoclonal antibody (#19245, CST) were added and incubated at 4°C overnight. After rinsing with PBS for 3 times, fluorescently labeled secondary antibodies (A0460, A0423, Beyotime, Shanghai, China) were added and incubated for 2 h away from light. The anti-fluorescence quenched sealing solution (including DAPI) was added and placed for 5 min, then Confocal Laser Scanning Microscope (Carl Zeiss, CLSM 710, Germany) was performed and photographs were taken.

**RT-qPCR**

Total RNA was extracted from lung tissues and MLE-12 cells in the enzyme-free environment using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). After concentration determination, RNA was reversely transcribed into cDNA. RT-qPCR was amplified by Step-One-Plus™ Real-Time PCR System using 2 µl cDNA as template and 20 µL reaction system. The PCR reaction conditions refer to our previous research results (21). The primary antibody concentrations of related proteins are as follows: AR (1:1000, ab153897, Abcam, Hong Kong, China), GAPDH (1:3000, ab8425, Abcam, Hong Kong, China); Twist1 (1:1000, #69366, CST, Danvers, MA, USA), E-cadherin (1:1000, #14472, CST, Danvers, MA, USA), Vimentin (1:2000, #5741, CST, Danvers, MA, USA), ɑ-SMA (1:2000, #19245, CST, Danvers, MA, USA); MMP2 (1:1000, sc-13595, Santa Cruz, Santa Cruz, CA, USA) and collagen I (1:1000, sc-59772, Santa Cruz, Santa Cruz, CA, USA). The gray values of each protein strip were measured by the Western blot analysis function of Image Studio Lite software.

**Statistical analysis**

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The result is expressed as mean ± standard deviation. Multiple groups were compared by One-Way ANOVA or Two-Way ANOVA. The Dunnet t-test was used to analyze the differences between the two groups. P<0.05 was considered statistically significant.

**Results**

**AR knockout alleviated radiation-induced pulmonary fibrosis**

H&E staining showed that unirradiated WT mouse and AR KO mice had normal lung structure, no thickened alveolar septum, and no obvious inflammatory cell infiltration. The lung structure of irradiated WT mice was severely damaged, and the normal lung tissue structure was lost. There was a large area of fibrosis, a large number of inflammatory cell infiltration, and even "honeycomb lung" changes. However, the lung injury of irradiated AR KO mice was relieved to varying degrees, with normal lung structure, slight thickening of alveolar walls, and occasional fibrous bands or small fibrous masses (Figure 1A).

![Figure 1](image-url)
Meanwhile, Masson staining showed that more collagenous fiber deposition (blue) was observed in WT mice after 15Gy local irradiation for 16 weeks, but in the AR KO mice, the collagen fiber deposition is significantly reduced (Figure 1B).

**AR knockout reduced radiation-induced collagen I and MMP2 expression in lung tissues**

In previous studies, it was found that the collagen I and MMP2 expressions in lung tissues were significantly increased in mice with radiation which were an important pathological feature of RIPF (22). In this study, the results of immunohistochemistry, WB and RT-qPCR showed that the expression of collagen I and MMP2 in lung tissue of mice in the WT + RT group was significantly increased compared with the WT + Con group (P<0.001). Compared to the WT + RT group, the expression of collagen I and MMP2 in lung tissue of the AR KO + RT group was significantly reduced (P<0.01) (Figure 2).

**AR knockout decreased EMT in the radiated mouse model**

It has been reported that the levels of interstitial cell markers (α-SMA and Vimentin) were obviously increased and epithelial cell marker (E-cadherin) was progressively decreased in the radiated mouse model (23). In keeping with previous research, the expressions of E-cadherin were down-regulated and the expressions of α-SMA and Vimentin were significantly up-regulated in lung tissues of the WT + RT group compared with WT + Con group (P<0.001). But, compared to the WT + RT group, the expression of E-cadherin was significantly up-regulated, and the expression of α-SMA and Vimentin was significantly down-regulated in AR KO + RT group (P<0.01) (Figure 3).

**AR knockout reduced radiation-induced twist1 expression in lung tissues**

Our research group’s previous study found that the expression of AR was significantly increased in the lung tissues of bleomycin-induced PF in rats (13). In this research, we found for the first time that AR expression was significantly up-regulated in the WT + RT group compared to the WT + Con group (P<0.001) (Figure 4). A previous study has shown that the expression levels of transcription factor Twist1 and markers of interstitial cells in lung tissue of IPF increased significantly in lung epithelial cells (17). In our study, the expression of Twist1 in lung tissue of the WT + RT group was significantly increased compared to WT + Con group (P<0.001). However, compared to the WT + RT group, Twist1 expression was significantly down-regulated in AR KO + RT group (P<0.01) (Figure 4).

**Inhibition or knockdown of AR could alleviate the EMT process of MLE-12 cells induced by radiation**

Previous studies have found that radiation can induce EMT in lung epithelial cells (24,25). In keeping with the previous study, Western blot and RT-qPCR results showed that 48 h after X-ray radiation of MLE-12 cells, the content of E-cadherin obviously decreased, while the content of α-SMA and Vimentin significantly increased, which together with the immunofluorescence results indicated that radiation could induce EMT in lung epithelial cells. We further found that the inhibition or knockdown of AR significantly reversed the radiation-induced EMT in MLE-12 cells compared with the RT group (Figures 5 and 6).
Inhibition or knockdown of AR suppressed cell invasion and collagen I and MMP2 expression in radiation-induced MLE-12 cells

In accordance with the previous study (26,27), X-ray radiation can significantly promote the expression of collagen I and MMP2 and obviously enhance cell migration ability in MLE-12 cells. But when re-treatment of AR inhibitor Tolrestat or AR siRNA can inhibit collagen I and MMP2 expressions, and reduce cell migration ability (Figures 7 and 8).

Radiation-induced twist1 expression of MLE-12 cells was alleviated by inhibition or knockdown of AR

Previous research found that radiation-induced EMT in lung epithelial cells is regulated by the expression of the upstream nuclear transcription factor Twist1 (17). In this study, it was found that the AR and Twist1 expressions were significantly increased in the RT group compared with the Con group. However, the expression of radiation-induced Twist1 was significantly inhibited after the inhibition or knockdown of AR (Figure 9). These results suggest that AR may mediate radiation-induced EMT through Twist1.

Discussion

RIPF is the late change of radiation lung injury and is one of the common and serious complications of radiotherapy for thoracic tumors (28). The pathogenesis of RIPF includes the early inflammatory reaction stage and the late fibrosis stage. It is believed that pulmonary fibrosis is caused by the loss of repair regulation and abnormal reconstruction of damaged lung tissue and is the result of the co-regulation of a series of cytokines and growth factors through multiple signal transduction pathways (29). However, previous studies believed that the main mechanism of RIPF is the continuous inflammatory response leading to lung injury and fibrosis formation, while the cur-
rent study more believes that the abnormal repair of alveolar epithelial cells after an injury is the main mechanism of RIPF (2). Therefore, clarifying the molecular mechanism of RIPF and searching for new therapeutic targets is one of the current researches focuses. In our research, the pathological findings showed that the alveolar fusion and integrity were reduced, the alveolar septum was thickened, and the number of interstitial cells and the significant collagen hyperplasia suggested the successful construction of the RIPF model.

The main reason for the formation of RIPF is the reconstruction and deposition of ECM caused by the abnormal repair of lung tissue injury, and collagen I and MMP2 are important ECM related to pulmonary fibrosis (26,27). Therefore, collagen I and MMP2 were selected as factors involved in the occurrence and progression of fibrosis in this study, to reflect the degree of fibrosis. AR is a rate-limiting enzyme in the polyol pathway of glucose metabolism, which is responsible for converting glucose into sorbitol (6). However, recent studies have found that the role of AR is not only limited to the process of glucose metabolism, it is also regarded as a target molecule mediating a variety of inflammatory diseases (30). Recent research demonstrated that AR mediates the EMT of lens epithelial cells, and inhibition of AR expression by AR inhibitors can significantly block the EMT of lens epithelial cells and alleviate the development of cataracts (10). In addition, AR was also found to be involved in the EMT of glomerular mesangial cells, and inhibition of AR could effectively prevent renal interstitial fibrosis (12). Our previous study found that AR inhibitors reduced the proliferation of TGF-β1 induced pulmonary fibroblasts by inhibiting the expression of AR (13). Our results showed that the collagen deposition and collagen I and MMP2 expressions were increased significantly after radiation, and were positively correlated with the increased expression level of AR. It is suggested that the increased expression of AR may be closely related to the progression of RIPF in mice.

It is known that EMT is a dynamic and reversible process regulated by relevant signaling pathways, involving embryonic development, wound healing, cancer metastasis, fibrosis and other links, and is associated with the enhancement of cell migration and invasion ability (31). Epithelial cells with EMT lose contact adhesion and apical-basal polarity, and their shape changes. At the same time, its cytoskeleton changes significantly, acquire the ability of migration and ECM invasion, develop some interstitial cell features and induce fibrosis (32). Previous research found that the levels of α-SMA and Vimentin were obviously increased and E-cadherin expression was progressively decreased in the radiated mouse model (23). Previous studies also have shown that radiation-induced EMT was found in vitro (33,34). In this research, we also found that radiation can significantly induce EMT (the expression of E-Cadherin decreases while the expression of α-SMA and Vimentin increases) in vitro and in vivo. However, we also found that both in vivo specific knockout of the AR gene and in vitro interference or inhibition of AR expression significantly inhibited radiation-induced EMT and cell migration ability. These results indicate that the knockout of the AR gene can inhibit the occurrence of EMT in lung epithelial cells during RIPF, and thus inhibit the RIPF process.

The bHLH transcription factor Twist1 controls mouse embryonic development and contributes to the EMT that occurs in lung cancer (35,36) and pulmonary fibrosis (17). A preliminary study found that he expression of the expression of Twist1 was significantly increased in the lung tissues of patients with idiopathic pulmonary fibrosis (37). Further studies showed that when Twist1 was activated, the expression of E-cadherin was down-regulated while the expression of α-SMA and vimentin was up-regulated, and inducing the occurrence of EMT in lung epithelial cells (38). Recent research demonstrated that Twist1 was up-regulated after radiation, and inhibition of Twist 1 expression can significantly reverse radiation-induced EMT in mouse lung epithelial cells (17). In this research, we have found that the expression of Twist1 was significantly up-regulated in lung tissue after radiation in mice. However, the knockout of AR can significantly inhibit the expression of Twist1 in vivo. In vitro, we also found that Twist1 expression was up-regulated after radiation in MLE-12 cells. Moreover, inhibition or knockdown of AR reduced Twist1 expression induced by radiation. Therefore, these results reveal that AR deficiency alleviated radiation-induced EMT and PF via inhibiting Twist1 expression.

In summary, the present study suggests that AR deficiency effectively alleviated RIPF by decreasing Twist1 expression and inhibiting EMT transformation and MMP2 and collagen I expression of lung epithelial cells epithelial cells, which ultimately affects the occurrence and development of RIPF. Targeting AR may be another effective way to treat RIPF.

Authors’ contributions
ZZ and XL designed the study and performed the experiments, ZL and XC collected the data, ZL, XC and YW analyzed the data, ZZ and XL prepared the manuscript. All authors read and approved the final manuscript.

Data availability
The data used to support the findings of this study are included within the article.

Conflict of interest
The authors declare that they have no conflicts of interest for this work.

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