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Original Research Article

MicroRNA-541-3p/Rac2 signaling bridges radiation-induced lung injury and repair

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ABSTRACT

Background: While radiation-induced lung injury decreases quality of life and suppresses efficacy of radiotherapy, to date, the relationship between radiation-induced lung injury and repair remains unclear. Our previous studies revealed that TNFRSF10B-RIPK1/RIPK3-MLKL signaling induces necroptosis of alveolar epithelial cells and potentiates radiation-induced lung injury. We also found that microRNA-541-3p is differentially expressed in radiation-damaged lungs. The connection between microRNA-541-3p, TNFRSF10B signaling, and TGFβ1 signaling is also unclear.
Objective: This study was performed to explore the regulatory effects of microRNA-541-3p on TNFRSF10B and TGFβ1 signaling.
Methods: Mouse alveolar epithelial cells were transfected with a vector expressing microRNA-541-3p to regulate expression of target genes. Flow cytometry, polymerase chain reaction, and western blotting were used to analyze cell necroptosis, target gene expression, and target protein expression, respectively.
Results: Overexpression of microRNA-541-3p positively regulated TNFRSF10B-RIPK1/RIPK3-MLKL signaling through Rac2 to induce cell necroptosis. MicroRNA-541-3p negatively regulates Rac2. MicroRNA-541-3p and Rac2 regulate the expression of Tgf-beta1 and its encoded proteins.
Conclusions: The Rac2 gene synchronously regulates TNFRSF10B-RIPK1/RIPK3-MLKL and TGFβ1 signaling. MicroRNA-541-3P/Rac2 act as mediators of radiation damage and repair signaling.

1. Introduction

Radiation-induced lung injury affects patients quality of life during progression from inflammation to fibrosis and limits the efficacy of radiotherapy for patients with thoracic tumors. Release of cytokines induced by radiation triggers cascading reactions that underlie the molecular mechanisms of radiation-induced lung injury. Type I alveolar epithelial cells undergo programmed cell death following radiation exposure. Cytokine release results in the accumulation of inflammatory cells. A sustained inflammatory response leads to increased differentiation of macrophages into M2 type. M2 macrophages secrete many growth factors, including TGFβ, which promotes 1) the transformation of fibroblasts into myofibroblasts that originate from various cell types, possess contractility, and promote wound healing [1]; 2) collagen deposition in the extracellular matrix; and 3) induction of fibrosis. Pro-inflammatory and pro-fibrotic signaling molecules are crucial in radiation-induced lung injury [2–13].

In previous transcriptomics studies to elucidate mechanisms that protect the lungs from radiation damage, we found that Tnfrsb10b and microRNA-541-3p were differentially expressed in radiation-induced lung injury. We demonstrated that necroptosis (a form of programmed cell death that differs from apoptosis in that after cell death, the cell

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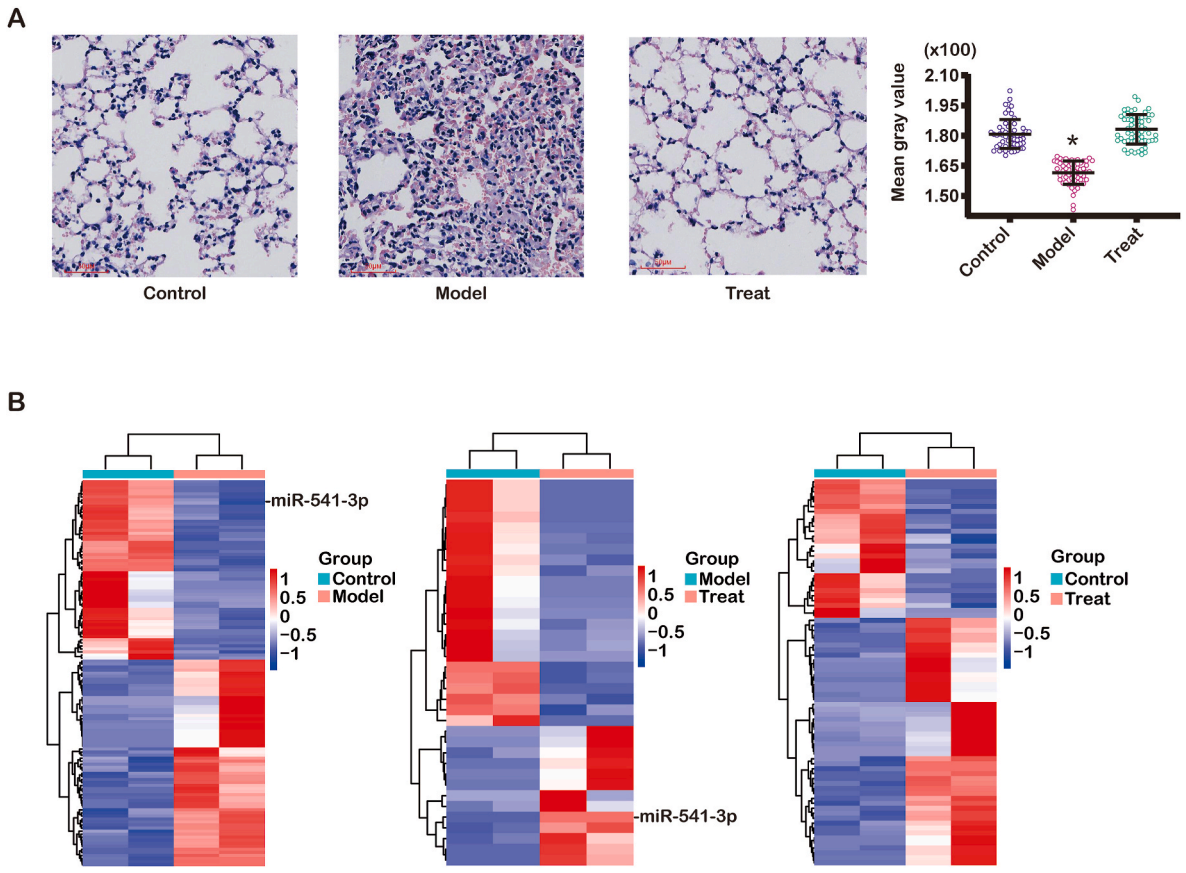

Fig. 1. Construction of radiation-induced lung injury mouse model and transcriptomic analysis (A) HE staining and quantitative analysis of lung tissue in mice. The mean gray value of HE-stained images quantitatively reflected the degree of lung damage. * represented $p < 0.05$ when compared with the control group. (B) Heatmaps of microRNA expression in mouse lungs. The left and right panels were heatmaps comparing the model and treatment groups with the control groups. The panel in the middle compares the model group and the treatment group. Both the model group and the treat group mice received a single dose of 15Gy to the chest.

membrane is compromised and intracellular components leak out [14]) induced by TNFRSF10B-RIPK1/RIPK3-MLKL signaling potentiates radiation-induced lung injury [15]. Programmed cell death induced by TNFRSF10B signaling initiates damage, and TGFβ-induced fibrosis is the result of repair efforts. Whether these two signaling systems are linked remains unclear.

MicroRNAs regulate target gene expression by interacting with their 3′ untranslated regions. miRNAs are biomarkers for disease diagnosis, and miRNA-targeted therapies are in development [16,17]. MicroRNA-541-3p (gene sequence: UGGCGAACACAGAAUCCAUACU) confers radiation resistance and promotes distant metastasis of tumor cells. It also regulates lipoproteins involved in atherosclerosis. MicroRNA-541-3p reportedly exerts a regulatory effect on ferroptosis, a type of programmed cell death [18–25]. Studies of microRNA-541-3p are limited, and its relationship with radiation-induced lung injury signaling remains unclear. Differential expression of microRNA-541-3p and Tnfrsf10b co-occurs, but whether the two molecules interact remains unclear.

We explored the relationships between microRNA-541-3p, TNFRSF10B signaling, radiation damage, and repair to facilitate a more comprehensive understanding of radiation-induced lung injury. MicroRNA-541-3p may be a new target for prevention and treatment of radiation-induced lung injury. We thoroughly explored the interaction between microRNA-541-3p and Tnfrsf10b and its regulation of radiation damage and repair signals.

2. Materials and methods

2.1. Experimental animals and cells

In our previous experiments [15], we used C57BL/6 mice to construct a radiation-induced lung injury model (Supplementary Material 1). Mouse alveolar epithelial cells (MLE-12) were purchased from Wuhan Sunnyvale Biotechnology Co., Ltd. (SNL-414). Cells were cultured in DMEM/F12 (SNLM-414, Sunncell) + 10 % FBS + 1 % P/S at 37 °C in 5 % CO_2. We divided MLE-12 cells into a control group (labeled Control), an overexpressed gene group (labeled OE_gene), and a silenced gene group (labeled siR_gene).

2.2. Cell transfection

2.2.1. Lipofectamine DNA transfection

One day before transfection, we seeded 1×10^5 MLE-12 cells per well into 24-well plates and added 500 μL of medium for routine culture. We diluted DNA and Lipofectamine 2000 (11668019, Thermo) each in 50 μL of OPTI-MEM (31985062, Gibco) and labeled them solutions A and B. Solutions A and B were mixed (100 μL total volume per well) and kept at room temperature for 20 min. Cells were washed twice with OPTI-MEM, resuspended in 400 μL of OPTI-MEM, and 100 μL of mixed transfection solution was added to each well. Cells were then cultured for 48 h before testing.

2.2.2. Lipofectamine siRNA transfection

One day before transfection, we seeded 1×10^5 MLE-12 cells per well into 24-well plates and added 500 μL of culture medium for routine

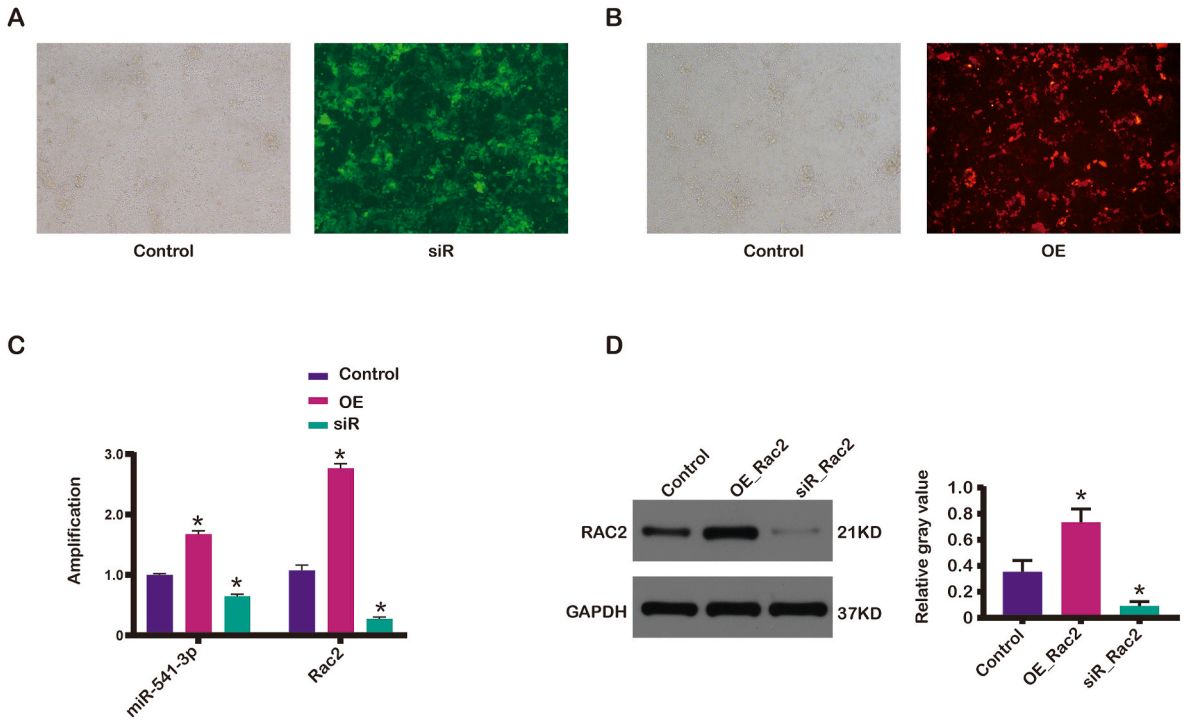

Fig. 2. Effect of gene transfection in MLE-12 cells (A) Images of MLE-12 cells before and after silencing transfection. The left panel showed images of normal cells before transfection. The right panel showed green fluorescence images of cells after silencing transfection. (B) Images of MLE-12 cells before and after overexpression transfection. The left panel showed images of normal cells before transfection. The right panel showed red fluorescence images of cells after overexpression transfection. (C) PCR analysis of gene expression in MLE-12 cells transfected with target genes. * represented $p < 0.05$ when compared with the control group. (D) Western blotting analysis of protein expression in MLE-12 cells transfected with target genes. The left panel showed the band images after protein electrophoresis. The right panel showed the quantitative analysis of the relative gray value. * represented $p < 0.05$ when compared with the control group. The above results were obtained 48 h after transfection of cells. Three independent experiments were performed.

culture. We diluted siRNA in 30 μL Opti-MEM, added 3 μL Lipofectamine 2000 to prepare the mixture, and incubated at room temperature for 15 min. This transfection mixture was added to 465.75 μL of culture medium and mixed evenly. Cells were tested after 48 h of post-transfection culture.

2.3. Flow cytometry

After washing cells with PBS, 0.25 % trypsin (0.5 mL) was added, and cells were incubated until they readily detached. Detached cells were centrifuged (300× *g* for 5 min), resuspended in PBS, and recentrifuged. Supernatants were discarded, and cells were resuspended in 300 μL of binding buffer. We added 5 μL of Annexin V-FITC (AO2001-02P-H, Sungene) to the resuspended cells and incubated them in the dark for 10 min, then added 5 μL of PI and incubated them in the dark for 5 min. Flow cytometry was performed within 1 h.

Methods to assess necroptosis: We added Nec-1 (Necrostain-1 [26], a specific inhibitor of necroptosis, 20 μmol/L, Cas: 4311-88-0, TargetMol) to the culture medium and analyzed the effect of Nec-1 on the proportion of PE-positive cells. PE positivity indicates cell necrosis. Nec-1 treatment abrogated the increased numbers of PE-positive cells, indicating that necroptosis was the dominant form of cell death [27,28].

2.4. PCR analysis

We removed culture medium from adherent cells, washed the cells with 1 mL of 4 °C PBS, resuspended the cells in 1 mL of TRIpure (EP013, ELK Biotechnology) solution, added 250 μL of chloroform, mixed evenly, and placed on ice for 5 min. We centrifuged the mixtures at low temperature and high speed (4 °C, 10,000× *g*, 10 min), kept the supernatants, added equal volumes of 4 °C isopropanol, mixed, and incubated at −20 °C for 15 min. We centrifuged the mixtures, retained the precipitates, added 1 mL of 75 % ethanol at 4 °C to wash the RNA precipitates, centrifuged again, allowed the ethanol to evaporate, and added 10 μL RNase-Free Water to dissolve the RNA. An M-MLV Reverse Transcriptase Kit (EQ (002); ELK Biotechnology) was used for first-strand cDNA synthesis. We used the QuFast SYBR Green PCR Master Mix Kit (EQ (001), ELK Biotechnology) and performed reactions using a real-time PCR instrument (StepOne™, Life Technologies). Primer information is shown in Supplementary Material (Supplementary Material 3, Tables 1 and 2).

Reaction Procedure:

Pre-denaturation, 95 °C, 1 min;
Cycle 40 times: 95 °C, 15 s, 58 °C, 20 s, 72 °C, 45 s.
Melting curve: 60°C–95 °C, increasing by 1 °C every 20 s.

Data analysis method: $\Delta\Delta CT$.
$A = CT_{(Target\ gene\_test)} - CT_{(Reference\ gene\_test)}$.
$B = CT_{(Target\ gene\_control)} - CT_{(Reference\ gene\_control)}$.
$K = A - B$.
Amplification $= 2^{-k}$

2.5. Western blotting

Adherent cells were washed three times with PBS, and then lysed for 5 min in RIPA total protein lysis buffer (AS1004, ASPEN). Lysates were placed in an ice bath for 30 min, centrifuged at low temperature and high speed for 5 min, and supernatants containing total protein were collected. A BCA protein concentration assay kit (AS1086, ASPEN) was used to determine each lysate's protein concentration. We added 5× protein loading buffer (AS1011, ASPEN) to each protein sample and

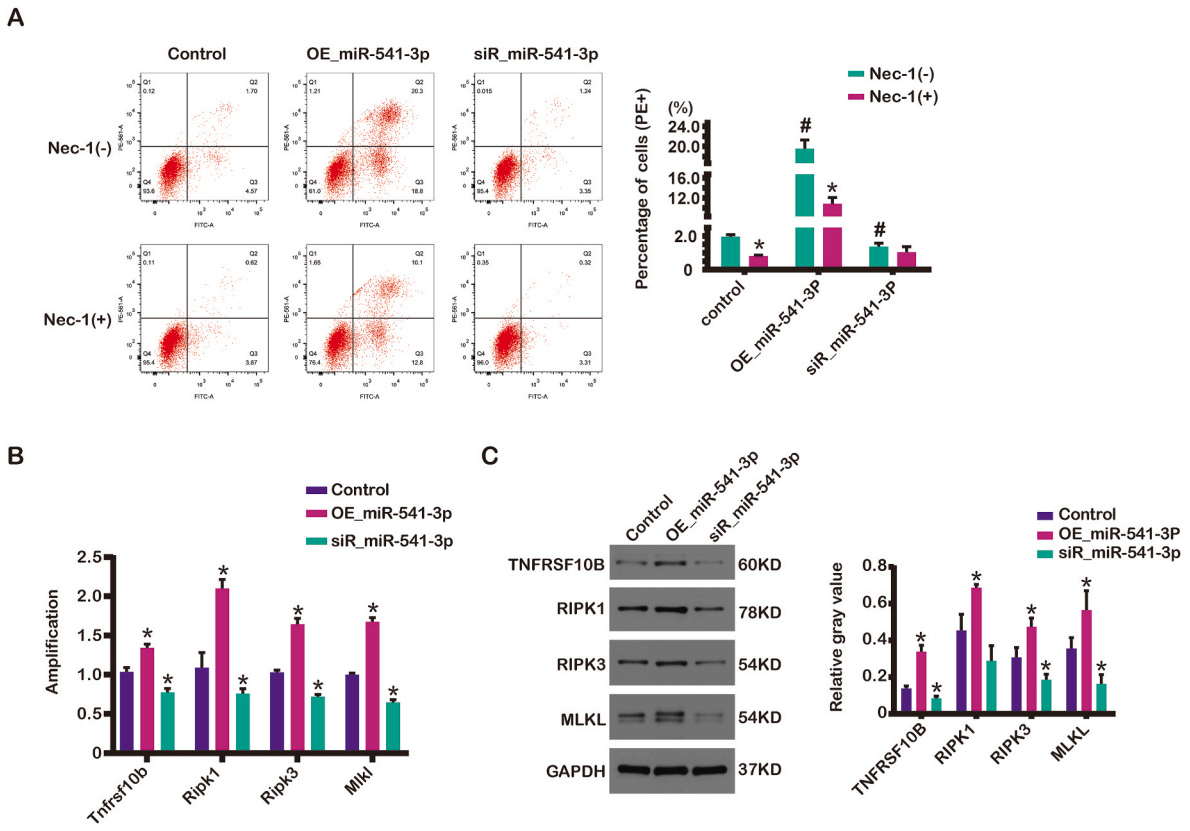

Fig. 3. Regulation of TNFRSF10B-RIPK1/RIPK3-MLKL signaling and necroptosis by microRNA-541-3p (A) Necroptosis in MLE-12 cells transfected with microRNA-541-3p. The left panel showed a flow cytometric analysis of MLE-12 cells transfected with microRNA-541-3p. The right panel showed the quantitative analysis of PE-positive cells. * represented $p < 0.05$ when compared with the Nec- (intra-group). # represented $p < 0.05$ when compared with the control group. (B) Tnfrsf10b, Ripk1, Ripk3, and Mlkl genes were expressed in MLE-12 cells transfected with microRNA-541-3p. * represented $p < 0.05$ when compared with the control group. (C) TNFRSF10B, RIPK1, RIPK3, and MLKL proteins were expressed in MLE-12 cells transfected with microRNA-541-3p. The left panel showed the band images after protein electrophoresis. The right panel showed the quantitative analysis of the relative gray value. * represented $p < 0.05$ when compared with the control group. Three independent experiments were performed.

boiled them in a 95–100 °C boiling water bath for 5 min. We performed electrophoresis with 40 μg of protein from each group (separation gel, 120 V; concentration gel, 80 V; constant voltage). We activated a 0.45 μm PVDF membrane (IPVH00010, Millipore) in methanol for 3 min, then transferred proteins to the membrane at a constant current of 300 mA. The PVDF membrane was then placed in blocking solution at room temperature for 1 h. After removing the blocking solution, the primary antibody was added, and the membrane was incubated at 4 °C overnight. After washing the PVDF membrane to remove excess primary antibody, secondary antibody was added, and the membrane was incubated at room temperature for 30 min. The membrane was then washed four times, and freshly prepared ECL solution (AS1059, ASPEN) was added. Film was exposed and developed in a dark room to obtain the image. Antibody information is shown in Supplementary Material (Supplementary Material 3, Table 3).

Protein expression analysis was performed using ImageJ software (freely available at https://imagej.net/ij/) to calculate gray-scale intensities of the protein band images. We calculated gray-scale intensity ratios between target and internal reference protein bands. The gray-scale intensity ratios were used as the basis for quantification of target protein expression.

2.6. Statistical analyses

Statistical analyses and graph processing were performed using GraphPad Prism version 9.0. Statistical results are presented as means ± standard deviations (SD). Statistical methods used two-way *t*-tests and univariate ANOVA. Statistical significance was set at $p < 0.05$. Three independent experiments were performed.

3. Results

3.1. MicroRNA-541-3p is differentially expressed in radiation lung injury

In a previous study, we constructed a mouse model of radiation-induced lung injury. HE staining of lung tissues revealed that the alveolar walls of radiation-damaged mouse lungs were significantly thickened, alveolar volume was reduced, and some alveoli had fused to form bullae (Fig. 1A). Alveolar damage was significantly alleviated in mice that received protective treatment (Fig. 1A). Quantitative analysis of mean gray values revealed a significantly lower mean gray value in the model group than in the control group. No other difference was observed between treatment and control groups (Fig. 1A). These results indicate that we have successfully constructed a radiation-induced lung injury model and can effectively protect the lungs from radiation damage.

Our study of the mechanism underlying lung protection from radiation injury revealed that TNFRSF10B-RIPK1/RIPK3-MLKL signaling is involved. Through transcriptomic testing, we found that microRNA-541-3p showed reduced expression in radiation-damaged mice (Fig. 1B, left) and elevated expression in the lungs of mice that received protective treatment (Fig. 1B, middle). Expression of microRNA-541-3p in the lungs of protective-treated mice was comparable to that in normal mice (Fig. 1B, right). These results indicate that microRNA-541-3p expression is altered by radiation injury in the lungs.

Table 1
Possible target gene information of microRNA-541-3p.

Gene	Full title	Summary
*Tcf7l2	transcription factor 7 like 2	This gene encodes a high mobility group (HMG) box-containing transcription factor that plays a key role in the Wnt signaling pathway. The protein has been implicated in blood glucose homeostasis. Genetic variants of this gene are associated with increased risk of type 2 diabetes. Several transcript variants encoding multiple different isoforms have been found for this gene. [provided by RefSeq, Oct 2010]
Trim3	tripartite motif-containing 3	Enables ubiquitin protein ligase activity. Involved in protein ubiquitination. Located in cytoplasm. Is expressed in several structures, including adrenal gland; cardiovascular system; genitourinary system; gut gland; and nervous system. Orthologous to human TRIM3 (tripartite motif containing 3). [provided by Alliance of Genome Resources, Dec 2024]
Tada2a	transcriptional adaptor 2A	Predicted to enable chromatin binding activity and transcription coactivator activity. Predicted to contribute to histone acetyltransferase activity. Involved in regulation of cell cycle; regulation of cell division; and regulation of embryonic development. Acts upstream of or within several processes, including chromatin organization; regulation of protein modification process; and regulation of protein stability. Located in mitotic spindle and nucleus. Part of ATAC complex. Orthologous to human TADA2A (transcriptional adaptor 2A). [provided by Alliance of Genome Resources, Dec 2024]
Rac2	**Rac family small GTPase 2**	**Predicted to enable GTP binding activity; GTPase activity; and protein kinase binding activity. Acts upstream of or within several processes, including defense response to other organism; leukocyte chemotaxis; and regulation of neutrophil migration. Located in cytoplasm. Is expressed in blood island; caudal hematopoietic tissue; neutrophil; rostral blood island; and thymus. Human ortholog(s) of this gene implicated in immunodeficiency 73a with defective neutrophil chemotaxis and leukocytosis; immunodeficiency 73b with defective neutrophil chemotaxis and lymphopenia; and immunodeficiency 73c with defective neutrophil chemotaxis and hypogammaglobulinemia. Orthologous to human RAC2 (Rac family small GTPase 2). [provided by Alliance of Genome Resources, Dec 2024]**
Gskip	GSK3B interacting protein	Predicted to enable protein kinase A regulatory subunit binding activity; protein kinase binding activity; and protein kinase inhibitor activity. Predicted to be involved in intrinsic apoptotic signaling pathway in response to oxidative stress; negative regulation of protein kinase activity; and positive regulation of canonical Wnt signaling pathway. Predicted to be located in nucleus and perinuclear region of cytoplasm. Predicted to be active in cytoplasm. Is expressed in several structures, including gut; liver; metanephros; spleen; and testis. Orthologous to human GSKIP (GSK3B interacting protein). [provided by Alliance of Genome Resources, Dec 2024]
Tmem191c	transmembrane protein 191	Predicted to be located in membrane. Orthologous to several human genes including TMEM191B (transmembrane protein 191B). [provided by Alliance of Genome Resources, Dec 2024]
Smyd5	SET and MYND domain containing 5	Enables histone H3K36 trimethyltransferase activity and histone H4K20 methyltransferase activity. Involved in regulation of stem cell differentiation; regulation of stem cell division; and transposable element silencing by heterochromatin formation. Predicted to be active in cytoplasm. Is expressed in several structures, including brain; heart; hemolymphoid system; incisor; and skeletal musculature. Orthologous to human SMYD5 (SMYD family member 5). [provided by Alliance of Genome Resources, Dec 2024]
Chm	CHM Rab escort protein	Predicted to enable enzyme-substrate adaptor activity and small GTPase binding activity. Acts upstream of or within blood vessel development. Predicted to be part of Rab-protein geranylgeranyltransferase complex. Predicted to be active in cytosol and nucleus. Is expressed in urethra. Used to study choroideremia. Human ortholog(s) of this gene implicated in choroideremia. Orthologous to human CHM (CHM Rab escort protein). [provided by Alliance of Genome Resources, Dec 2024]
Fshb	follicle stimulating hormone beta	The pituitary glycoprotein hormone family includes follicle-stimulating hormone, luteinizing hormone, chorionic gonadotropin, and thyroid-stimulating hormone. All of these glycoproteins consist of an identical alpha subunit and a hormone-specific beta subunit. This gene encodes the beta subunit of follicle-stimulating hormone. In conjunction with luteinizing hormone, follicle-stimulating hormone induces egg and sperm production. [provided by RefSeq, Aug 2015]
Ccdc148	coiled-coil domain containing 148	Is expressed in cranial ganglion; dorsal root ganglion; neural retina; and pons mantle layer. Orthologous to human CCDC148 (coiled-coil domain containing 148). [provided by Alliance of Genome Resources, Dec 2024]

The descriptions of the genes in the list come from the website https://www.ncbi.nlm.nih.gov/gene/. The genes marked with * originate from human tissues, while the other genes originate from mice.

3.2. *Overexpressed microRNA-541-3p positively modulates TNFRSF10B-RIPK1/RIPK3-MLKL signaling*

Target gene expression in MLE-12 cells was modulated by transient transfection. We confirmed that transfections were successful by observing intracellular fluorescence of co-transfected fluorescent proteins (Fig. 2A & B). We further examined the expression of our target genes, namely microRNA-541-3p and Rac2, and the expression of RAC2 protein, and found that in cells treated with silencing constructs, microRNA-541-3p and Rac2 gene expression decreased, and RAC2 protein expression was reduced. Increased expression was uniformly observed in cells treated to achieve overexpression (Fig. 2C & D).

To explore the regulation of TNFRSF10B-RIPK1/RIPK3-MLKL signaling by microRNA-541-3p, we transfected microRNA-541-3p into MLE-12 cells and analyzed necroptosis using flow cytometry. We found that PE-positive cell proportions increased in microRNA-541-3p-overexpressing cells (19.57 % OE_miR-541-3p vs. 1.96 % Control). In cells treated with Nec-1, the proportion of PE-positive cells decreased (19.57 % Nec- vs. 10.85 % Nec+). In microRNA-541-3p-silenced cells, the percentage of PE-positive cells decreased (1.37 % siR_miR-541-3p vs. 1.96 % Control) and was not further altered by Nec-1 (Fig. 3A). We further analyzed the expression of proteins and genes involved in

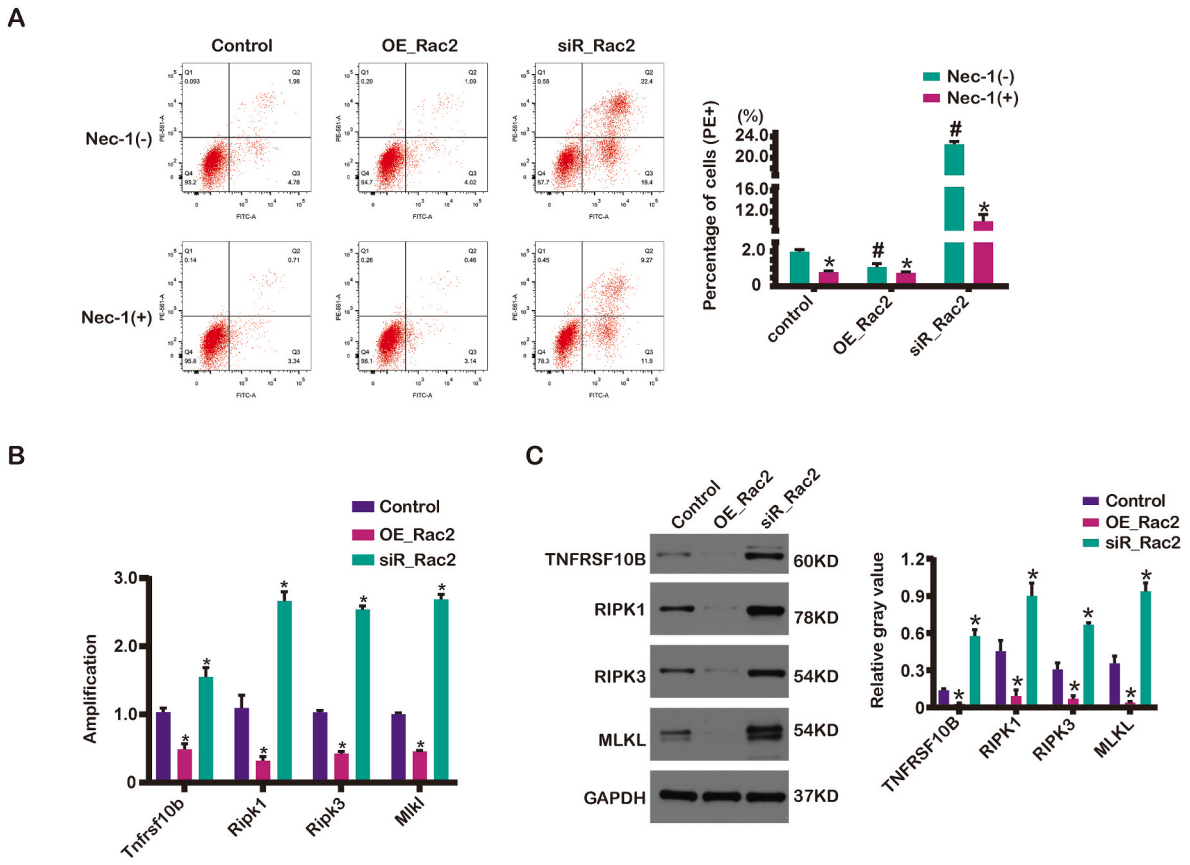

Fig. 4. The regulation of TNFRSF10B-RIPK1/RIPK3-MLKL signaling and necroptosis by Rac2 (A) Necroptosis in MLE-12 cells transfected with Rac2. The left panel showed a flow cytometric analysis of MLE-12 cells transfected with Rac2. The right panel showed the quantitative analysis of PE-positive cells. * represented $p < 0.05$ when compared with the Nec- (intra-group). # represented $p < 0.05$ when compared with the control group. (B) Tnfrsf10b, Ripk1, Ripk3, and Mlkl genes were expressed in MLE-12 cells transfected with Rac2. * represented $p < 0.05$ when compared with the control group. (C) TNFRSF10B, RIPK1, RIPK3, and MLKL proteins were expressed in MLE-12 cells transfected with Rac2. The left panel showed the band images after protein electrophoresis. The right panel showed the quantitative analysis of the relative gray value. * represented $p < 0.05$ when compared with the control group. Three independent experiments were performed.

TNFRSF10B-RIPK1/RIPK3-MLKL signaling. We found that TNFRSF10B, RIPK1, RIPK3, and MLKL proteins and genes encoding these proteins were highly expressed in microRNA-541-3p-overexpressing cells. In microRNA-541-3p-silenced cells, the expression of these genes and proteins was decreased, except for RIPK1. RIPK1 protein expression was not significantly changed in microRNA-541-3p-silenced cells (Fig. 3B & C). These results suggest that overexpressing microRNA-541-3p induces necroptosis of alveolar epithelial cells by positively regulating TNFRSF10B-RIPK1/RIPK3-MLKL signaling.

3.3. The Rac2 gene bridges microRNA-541-3p and TNFRSF10B-RIPK1/RIPK3-MLKL signaling

As an upstream TNFRSF10B-RIPK1/RIPK3-MLKL signaling molecule, the TNFRSF10B protein is regulated by miRNAs [29,30]. Although we demonstrated that microRNA-541-3p regulates Tnfrsf10b expression, it was not a known target gene of microRNA-541-3p in available databases (https://mirdb.org/and https://www.targetscan.org/mmu_72/). We used TargetScan (https://www.targetscan.org/mmu_72/) to identify candidate microRNA-541-3p target genes (Supplementary Material 2). The top ten highest-scoring genes based on cumulative weighted context++ score are listed in Table 1. After analyzing relationships between these ten genes and radiation, we found that Rac2 is closely related to radiation damage [31,32]. RAC2 protein, encoded by the Rac2 gene, is a member of the GTPase subfamily and is involved in biological processes including cytoskeleton remodeling, cell growth and transformation, and gene transcription. Activation of RAC2 can induce inflammatory responses and apoptosis [33–39]. We speculated that microRNA-541-3p regulates TNFRSF10B-RIPK1/RIPK3-MLKL signaling through Rac2.

To confirm our hypothesis, we examined the regulation of Rac2 and TNFRSF10B-RIPK1/RIPK3-MLKL signaling. Flow cytometry results (Fig. 4A) showed that the proportion of PE-positive cells increases in Rac2-silenced cells (22.57 % siR_Rac2 vs. 1.96 % Control). This increase in PE-positive cells is abrogated by Nec-1 treatment (22.57 % Nec- vs. 10.16 % Nec+). In Rac2-overexpressing cells, the percentage of PE-positive cells decreases (1.11 % OE_Rac2 vs. 1.96 % Control), and Nec-1 exerts negligible impact on the proportion of PE-positive cells (1.11 % Nec- vs. 0.77 % Nec+ $p = 0.041$). We next found that TNFRSF10B, RIPK1, RIPK3, and MLKL proteins, and the genes encoding these proteins, are highly expressed in Rac2-silenced cells. However, expression of these proteins and genes decreases in Rac2-overexpressing cells (Fig. 4B & C). These results suggest that Rac2 induces necroptosis in alveolar epithelial cells by negatively regulating TNFRSF10B-RIPK1/RIPK3-MLKL signaling.

To verify the regulatory effect of microRNA-541-3p on Rac2, we analyzed mRNA and protein-level Rac2 expression in transfected cells. We found that Rac2 was weakly expressed in microRNA-541-3p-overexpressing cells and was expressed at levels seen in normal cells in microRNA-541-3p-silenced cells (Fig. 5A & B). Combined with existing data, our results suggest that overexpressing microRNA-541-3p regulates TNFRSF10B-RIPK1/RIPK3-MLKL signaling by negatively regulating Rac2 (Fig. 5C & D).

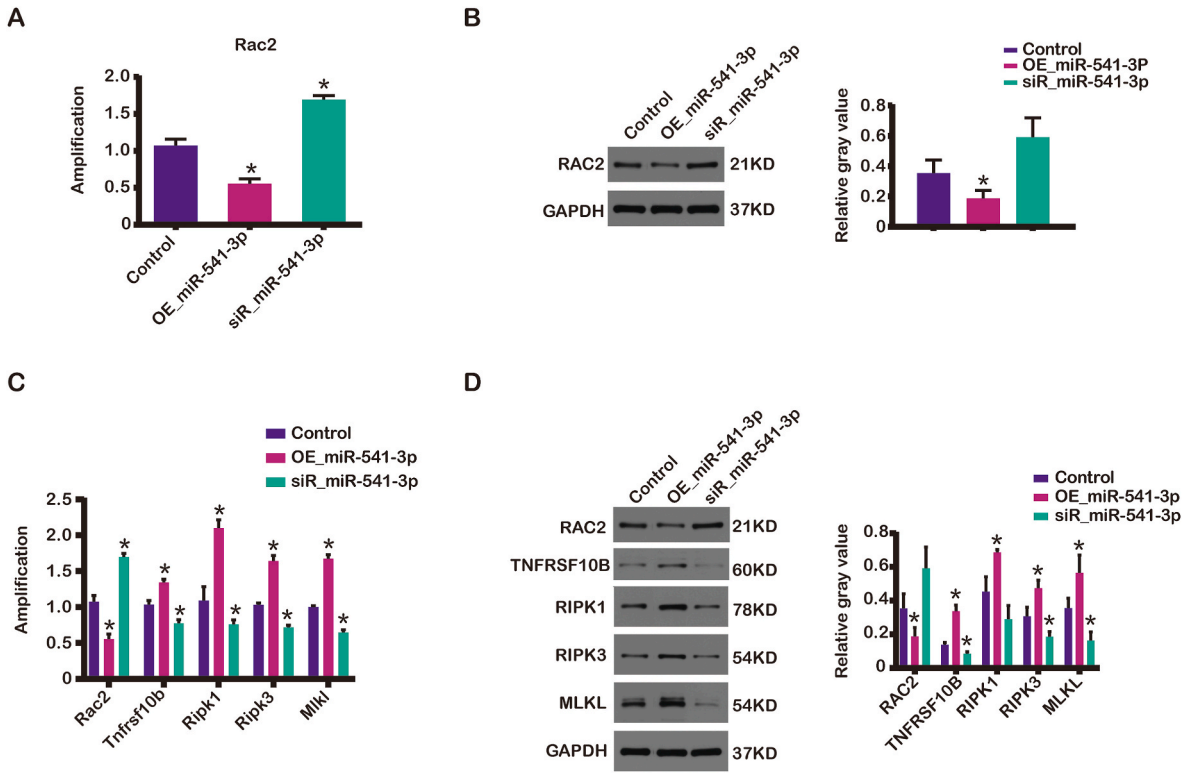

Fig. 5. The regulation of the Rac2 gene and its encoded protein between microRNA-541-3p and TNFRSF10B signaling (A) Expression of Rac2 gene in MLE-12 cells transfected with microRNA-541-3p. * represented $p < 0.05$ when compared with the control group. (B) Expression of RAC2 protein in MLE-12 cells transfected with microRNA-541-3p. The left panel showed the band images after protein electrophoresis. The right panel showed the quantitative analysis of the relative gray value. * represented $p < 0.05$ when compared with the control group. (C) Rac2, Tnfrsf10b, Ripk1, Ripk3, and Mlkl genes were expressed in MLE-12 cells transfected with microRNA-541-3p. * represented $p < 0.05$ when compared with the control group. (D) RAC2, TNFRSF10B, RIPK1, RIPK3, and MLKL proteins were expressed in MLE-12 cells transfected with microRNA-541-3p. The left panel showed the band images after protein electrophoresis. The right panel showed the quantitative analysis of the relative gray value. * represented $p < 0.05$ when compared with the control group. Three independent experiments were performed.

3.4. MicroRNA-541-3p and Rac2 regulate mRNA and protein-level Tgf-beta1 expression

We also explored the regulation of TGFβ1 expression by microRNA-541-3p. We first analyzed microRNA-541-3p, Rac2, and Tgf-beta1 genes and related proteins expression in our radiation-induced lung injury model. Expression of microRNA-541-3p was low in radiation-damaged mouse lungs (model group), whereas Rac2 and Tgf-beta1 genes and the proteins they encode were highly expressed in radiation-damaged lungs (Fig. 6A & B).

To explore regulatory interactions among microRNA-541-3p, Rac2, and Tgf-beta1, we independently transfected cells with Rac2 and microRNA-541-3p. We found that Tgf-beta1 mRNA and protein expression were increased in Rac2-overexpressing cells and decreased in Rac2-silenced cells (Fig. 6C & D). Rac2 and Tgf-beta1 gene expression was reduced in microRNA-541-3p-overexpressing cells and increased in microRNA-541-3p-silenced cells. RAC2 protein was weakly expressed in microRNA-541-3p-overexpressing cells, while TGFβ1 protein expression was elevated in microRNA-541-3p-silenced cells (Fig. 6E & F). These results suggest that microRNA-541-3p and Rac2 regulate Tgf-beta1 gene and its protein expression.

4. Discussion

Previous studies have shown that Tnfrsf10b and microRNA-541-3p are differentially expressed in radiation-induced lung injury, and we have also demonstrated that TNFRSF10B, a TNF receptor, induces alveolar epithelial cell necroptosis by regulating RIPK1/RIPK-MLKL signaling [15]. TNF and TGFβ1 (the first protein among the TGFβ family to recognize trauma and attract other molecules for early wound repair [40]) play essential roles in tissue damage and repair [41–45]. The relationship between TNFRSF10B-mediated tissue damage and TGFβ1-mediated tissue fibrosis in radiation-induced lung injury remains unclear. Some microRNAs can modulate necroptosis [46–50] and TGFβ1 signaling [51–55]. To date, the regulation of these signals by microRNA-541-3p is poorly understood. This study was performed to investigate microRNA-541-3p-mediated regulation of TNFRSF10B-RIPK1/RIPK3-MLKL signaling and its role in repair signaling in response to radiation damage.

Unique to these studies [56–59], we explored the regulation of TNFRSF10B signaling in non-tumor tissues and demonstrated that microRNA-541-3p positively regulates the genes encoding TNFRSF10B-RIPK1/RIPK3-MLKL signaling proteins and negatively regulates the gene encoding TGFβ1 protein. However, RIPK1 protein was unaffected by microRNA-541-3p silencing, and TGFβ1 protein was unaffected by microRNA-541-3p overexpression. We inferred that post-transcriptional regulation of Ripk1 and Tgf-beta1 occurred in MLE-12 cells transfected with microRNA-541-3p. Although RIPK1 protein and gene expression were inconsistent in microRNA-541-3p-silenced cells, they did not affect the microRNA-541-3p-mediated upregulation of alveolar cell necroptosis.

By searching online databases, we found that Tnfrsf10b and Tgf-beta1 are not target genes of microRNA-541-3p. We are the first to confirm that Rac2 is a target gene for microRNA-541-3p, although multiple microRNAs can regulate Rac2 [60–65]. This study demonstrated that microRNA-541-3p negatively regulates Rac2, and Rac2 negatively regulates TNFRSF10B-RIPK1/RIPK3-MLKL signaling proteins and positively regulates TGFβ1 protein. However, RAC2 protein expression was unaltered in microRNA-541-3p-silenced cells, suggesting the presence of post-transcriptional regulation.

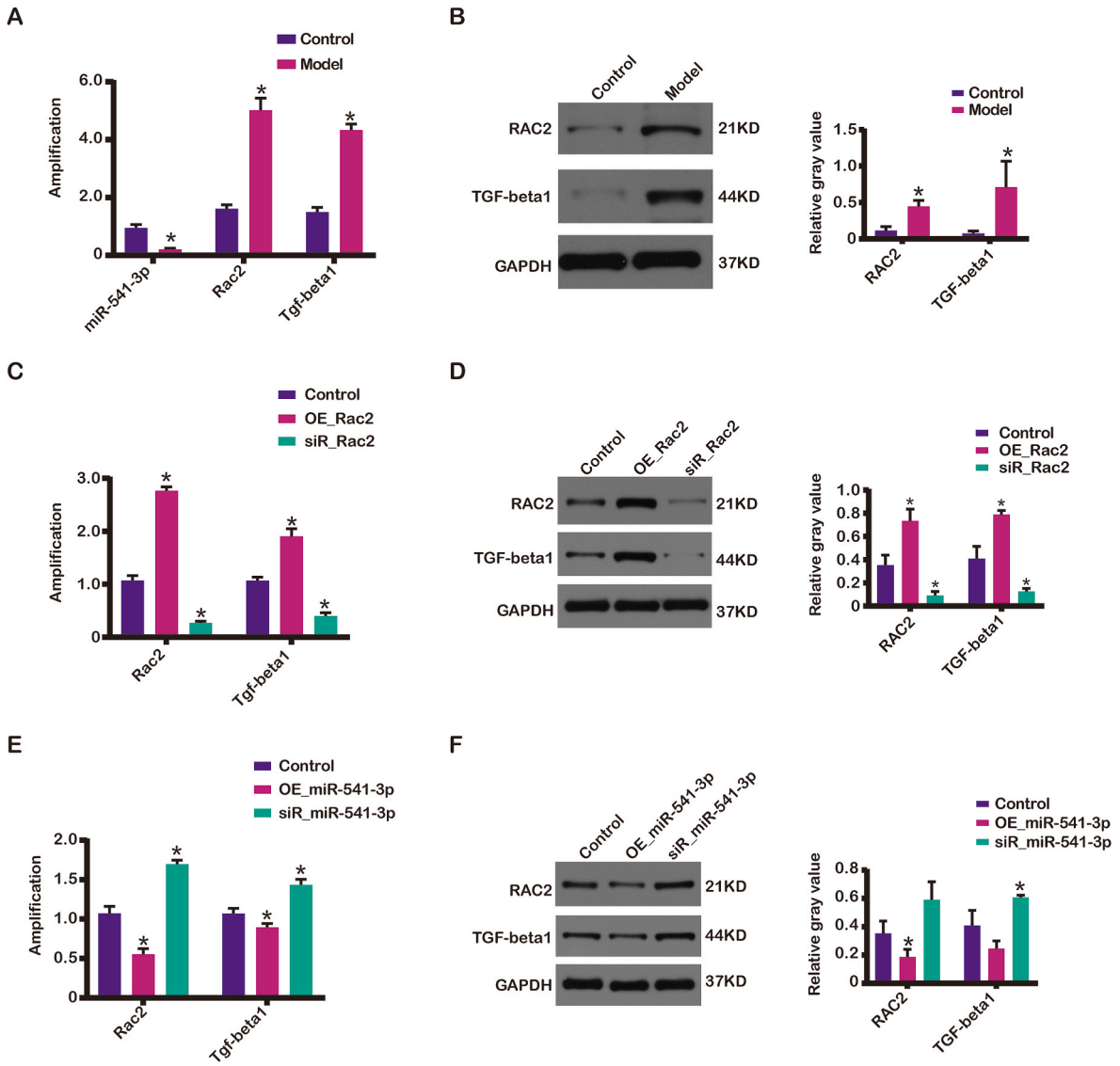

Fig. 6. The regulation of TGFβ1 by microRNA-541-3p and Rac2 (A) MicroRNA-541-3p, Rac2, and Tgf-beta1 genes were expressed in the radiation-induced lung injury mouse model. * represented $p < 0.05$ when compared with the control group. (B) Expression of RAC2 and TGFβ1 proteins in radiation-induced lung injury mouse model. The left panel showed the band images after protein electrophoresis. The right panel showed the quantitative analysis of the relative gray value. * represented $p < 0.05$ when compared with the control group. (C) Expression of Rac2 and Tgf-beta1 genes in MLE-12 cells transfected with Rac2. * represented $p < 0.05$ when compared with the control group. (D) Expression of RAC2 and TGFβ1 proteins in MLE-12 cells transfected with Rac2. The left panel showed the band images after protein electrophoresis. The right panel showed the quantitative analysis of the relative gray value. * represented $p < 0.05$ when compared with the control group. (E) Expression of Rac2 and Tgf-beta1 genes in MLE-12 cells transfected with microRNA-541-3p. * represented $p < 0.05$ when compared with the control group. (F) Expression of RAC2 and TGFβ1 proteins in MLE-12 cells transfected with microRNA-541-3p. The left panel showed the band images after protein electrophoresis. The right panel showed the quantitative analysis of the relative gray value. * represented $p < 0.05$ when compared with the control group. Three independent experiments were performed.

In lungs with significant radiation damage, we found depressed microRNA-541-3p expression and high RAC2 and TGFβ1 protein levels. However, in microRNA-541-3p-silenced cells, RAC2 protein levels were not significantly elevated. Two possible explanations can account for this discrepancy. First, in our cell model, simply using transfection to regulate microRNA-541-3p does not entirely recapitulate the tissue cell damage caused by radiation. Tissue damage caused by radiation is much more complex. In radiation-damaged lungs, RAC2 protein may be regulated by several factors. Second, although we repeated all experiments thrice, we cannot rule out statistical biases caused by small sample size.

This study has some limitations. Radiation damage simultaneously affects the expression of multiple genes and proteins. This study only used transfection to manipulate the expression of specific genes and did not verify these results in our radiation-damaged cell model. Alveolar epithelial cells are classified into types I and II. Although type II alveolar epithelial cells account for only a small proportion of these cells, they play an essential role in radiation damage. Separate experiments were not performed using these two cell types. This study was conducted on mouse alveolar epithelial cells and has not been verified in human cells.

In summary, microRNA-541-3p regulates TNFRSF10B-RIPK1/RIPK3-MLKL signaling by negatively regulating Rac2. MicroRNA-541-3p negatively regulates TGFβ1 via a post-transcriptional non-Rac2 pathway. This is the first study to demonstrate that microRNA-541-3p negatively regulates Rac2 expression. The coordination of microRNA-541-3p/Rac2 effects on TNFRSF10B and TGFβ1 signaling provides a theoretical basis for exploring the relationship between radiation-related injury and repair. MicroRNA-541-3p/rac2 may be a target for

the prevention and treatment of radiation-induced lung injuries.

5. Conclusions

MicroRNA-541-3p negatively regulates Rac2. RAC2 acts as a switch protein, synchronously regulating TNFRSF10B and TGFβ1 signaling in radiation-induced lung injury. MicroRNA-541-3p/Rac2 signaling plays a mediating role in lung radiation injury and repair.

CRediT authorship contribution statement

Jiandong Zhang: Writing – original draft, Validation, Investigation. **Lei Ma:** Validation, Formal analysis. **Limin He:** Validation, Methodology. **Quanxiao Xu:** Writing – original draft, Investigation. **Yan Ding:** Writing – original draft, Visualization, Funding acquisition, Formal analysis. **Lidong Wang:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2025.01.010.

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