Supplementary Materials

mTOR inhibition with rapamycin mitigates radiation-induced pulmonary fibrosis in a murine model.

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Supplemental Methods and Materials

In Vitro Studies

Isolation of primary pneumocytes: Primary murine pneumocytes were isolated using a previously described method (3) with modification. Mice were anesthetized 10 minutes after intraperitoneal injection of Heparin Sodium (12.5 u/g body weight). Lungs were perfused with 10 ml of HBSS (containing 30 mM HEPES), filled with 1mL enzyme cocktail (Elastase 3 u/ml, 0.01% DNase I and 0.2 % Collagenase in HBSS containing 30 mM HEPES, Sigma Aldrich), and incubated in 5 ml of enzyme cocktail at 37^{o}C for 30 minutes. The digested tissue was carefully teased from the airways and gently swirled for 5 to 10 min. The resulting suspension was successively filtered through 100 μm and 40 μm Falcon cell strainers, then centrifuged at 130 x *g* for 8 min at 4°C and resuspended in HBSS. The crude single cell suspension was applied to Ficoll density gradient isolation solution. Pneumocytes were collected from the layer of density 1.077 ~ 1.080, washed with HBSS, and then resuspended with DMEM media containing 10 % FBS and 1 % antibiotics.

Immunocytochemistry: Isolated primary pneumocytes were treated with vehicle or rapamycin (50 ng/ml) for one hour prior IR (0, 17.5 Gy), and cultured 3 days after IR in chamber slides (Nunc

Lab-Tak, Sigma-Aldrich). X-Gal staining was performed first with these cells using a Senescence detection kit (Abcam). To identify AECII in primary pneumocyte cultures, sequential immunohistochemical staining for pro-SPC was visualized by incubation with anti-pro-SPC primary antibody (Abcam) followed with compatible secondary antibodies conjugated with a fluorophore (Life Technologies, Grand Island, NY). Slides were mounted with Prolong antifade reagent (Life Technologies, Grand Island, NY).

Aniline Blue staining

To assess collagen deposition in irradiated lungs, sections of lung from the 16 week time point were deparaffinized in xylene and rehydrated through a graded alcohol series to water. Sections were incubated in Bouin's solution for 1 overnight and stained using Aniline Blue solution (Sigma-Aldrich) for 15 minutes. Digital micrographs were captured at 10x and 40x magnifications and imported into QCapture (Quantitative Imaging Corporation, Surrey, BC, Canada).

Antibodies

Western blotting: Rabbit antibodies against p70 S6K, S6, 4EBP1, phospho-p70 S6 Kinase (Thr389), phospho-S6 (Ser240/244), and phospho-4EBP1 (Thr37/46) were purchased from Cell Signaling (Danvers, MA). Monoclonal antibody to actin was purchased from EMD Millipore (Billerica, MA). Peroxidase-labeled secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunochemistry: Antibodies against F4/80 (macrophage marker), and CD3 (T-lymphocyte marker) were purchased from Abcam (Cambridge, MA). An antibody for neutrophil elastase

(neutrophil marker) was purchased from EMD Millipore (Billerica, MA). An antibody for S6 was purchased from Cell Signaling (Danver, MA). Secondary antibodies conjugated with HRP were obtained from Vector Laboratories (Burlingame, CA), and antibody conjugated to Alexa Fluor 594 was obtained from Life technologies (Grand Island, NY).

Supplemental Figure Legends

Figure 1. Rapamycin inhibits mTOR signaling in irradiated lung. Lung tissue from C57BL/6NCr mice treated with 5x6 Gy of thoracic IR and control or rapamycin formulated diet was collected at 2 weeks after IR (n=3 per condition) and evaluated by western blotting for phosphorylated/total S6K, 4EBP1, and S6, with actin used as a loading control. The density of each band relative to the indicated loading control is noted below each band. (B) Lung tissue from C57BL/6NCr mice treated with 5x6 Gy of thoracic IR and control or rapamycin formulated diet was collected at 2 weeks after IR (n=3 per condition) and analyzed for hydroxyproline content. (C) Lung tissue sections from C57BL/6NCr mice treated with 5x6 Gy of thoracic IR and control or rapamycin formulated diet were evaluated by immunohistochemistry to determine the number of macrophages (F4/80+ cells) per high power field (HPF, 63x). Mean: columns, bars: SD.

Supplemental Figure 2. Effects of rapamycin on collagen accumulation after radiation. C57BL/6NCr Mice were exposed to 5x6 Gy of thoracic IR and treated with rapamycin or control diet. Lung tissue section from 16 weeks after irradiation were stained with analine blue (collagen: blue). Low and high power views are included for each treatment.