**A missing piece of the puzzle in pulmonary fibrosis: anoikis resistance promotes fibroblast activation**

**Running title:** Anoikis resistance promotes fibroblast activation

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**Material and methods**

***1. Western blot analysis***

Western blot analysis was performed as described in a previous study [14]. Primary antibodies against ZC3H4 (1:1000), TrkB (1:800), Caspase-3 (1:500), Cleaved Caspase-3 (1:500), JNK (1:1000), p-JNK (1:1000), Akt (1:1000), p-Akt (1:1000), P38 (1:1000), p-P38 (1:1000), Bax (1:1000), Npnt (1:1000) and Bcl-xL (1:1000) were used. All western blots are representative of three or more independent experiments. The protein band intensities were quantiﬁed using ImageJ v1.48 software.

***2. Immunofluorescence staining***

The mouse lung tissue was fixed with OCT gel. When the OCT gel solidified, 5- to 10-μm-thick tissue slices were obtained by frozen sectioning and set on slides. The edge of the slides was surrounded with hydrogel, and the sections were fixed with 4% paraformaldehyde. The OCT glue was removed with PBS before the slides were blocked with 10% normal goat serum in 0.3% Triton X-100 for 2 h at room temperature followed by incubation with primary antibodies targeting ZC3H4, TrkB or Npnt at 4°C overnight. The next day, the tissue was incubated with the appropriate fluorescent secondary antibodies (Alexa Fluor, Thermo Fisher Scientific). A fluorescence microscope (Olympus IX70, Olympus America, Inc., Center Valley, PA, USA) was used to observe the tissue.

***3. Cell viability***

Cells were seeded in 96-well plates at 5000 per well and stimulated with macrophage-conditioned medium (CM). After the reaction, CCK8 reagent (APExBIO, USA) was added at a ratio of 10:1 and incubated with the cells for 30 min in the dark. Finally, the absorbance at 450 nm was measured with a microplate reader (BioTek, USA)

***4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)***

Real-time quantitative PCR (qRT-PCR) was performed to determine the relative expression of circ-ZC3H4 circRNA and Zc3h4 mRNA. Total RNA was extracted from HPF-a cells with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. After the extraction of total RNA, the concentration was measured on a NanoDrop One (Thermo Fisher Scientific). The volumes of the samples were adjusted, and 300 ng or 400 ng of RNA was reverse-transcribed into cDNA, which were used as templates for real-time qRT-PCR. The cycle threshold (Ct) and ΔCT values were analyzed. The ΔΔCT quantification method was applied using Opticon Monitor software (Bio-Rad). The relative quantitative expression of RNA was normalized to that of GAPDH (endogenous reference gene).

***5. Apoptosis cell assessment by ﬂow cytometry***

FCM was performed according to the manufacturer’s instructions (Keygentec, KGA108, China). The stained cells were incubated in the dark for at least 15 min at 4°C, and the percentages of apoptotic cells were quantiﬁed using a FACSCalibur ﬂow cytometer within 1 h of staining.

***6. Nested matrix model and cell migration assay***

As mentioned earlier, we used a three-dimensional (3D) migration model with some modifications [16]. For the nested attachment matrix, standard fibroblasts were transplanted into ECM and incubated for 48 h in the attachment state in DMEM containing 5% FBS. The ECM was then removed from the culture well and placed into 60 mL of fresh acellular collagen matrix solution centered around the scratch area (12 mm diameter). The newly metastatic fibroblast-populated collagen matrix (FPCM) was then covered with 140 mL of acellular collagen matrix solution. The matrix system was polymerized at 37°C and 5% CO2 for 1 h. Then, 1 mL of DMEM containing 10% FBS was added to the well. In these experiments, we used CM derived from THP-1 macrophages. Fresh medium was mixed with the prepared CM at a 1:1 ratio. The migration of cells from the ECM to the acellular matrix was observed. Cell migration observed at 6, 12, and 24 h by fluorescence microscopy was compared with that observed at 0 h. Digital images of the interface between the ECM and the acellular matrix were captured using an EVOS FL cell imaging microscope (Thermo Fisher Scientific). Migration of lung fibroblasts (PFB) from the ECM was quantified by counting the number of cells that significantly migrated from the nested matrix to the acellular matrix. The maximum migration distance was quantified by determining the cell with the maximum distance from the nested matrix to the element-free matrix and measuring that distance. The average number of cells in each field that migrated from the nested matrix and the maximum migration distance of each field were recorded.



**Supplementary Figure S1.** (A). Immunofluorescence staining of the expression of the epithelial marker Cdh1 and the anoikis marker TrkB. Scale bar=100 μm. (B). Immunofluorescence staining of the expression of the endothelial cell marker Cdh5 and the anoikis marker TrkB. Scale bar=100 μm.

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**Supplementary Figure S2.** (A). BEAS-2B cells were suspended for 48 hours, cocultured with CM for 24 h, and double stained with Annexin V and PI. The sum of the counts in Q2-1, Q2-2 and Q2-4 was defined as the number of apoptotic cells. (B). HPMECs were treated as described in (A). The sum of the counts in Q2-1, Q2-2 and Q2-4 was defined as the number of apoptotic cells. (C). Statistical analysis of three independent experiments of BEAS-2B cell apoptosis by flow cytometry (n=3); \*p < 0.05 vs. the attached group. (D). Statistical analysis of three independent experiments of HPMEC apoptosis by flow cytometry (n=3); \*p < 0.05 vs. the attached group.



**Supplementary Figure S3.** Representative western blot showing the transfection efficiency of ZC3H4-NIC in HPF-a cells.



**Supplementary Figure S4.** Spatial transcriptomics showed the co-expression of *Itgb1* and *Vimentin*.