Supplementary Material

Osteopontin drives KRAS-mutant lung adenocarcinoma.

Ioanna Giopanou, Nikolaos I. Kanellakis,Anastasios D. Giannou, Ioannis Lilis, Antonia Marazioti, Magda Spella,Vassilios Papaleonidopoulos, Davina C.M. Simoes, Dimitra E. Zazara, Theodora Agalioti, Charalampos Moschos, Sophia Magkouta, Ioannis Kalomenidis, Vily Panoutsakopoulou, Anne-Sophie Lamort, Georgios T. Stathopoulos, and Ioannis Psallidas

**SUPPLEMENTARY METHODS**

**Assessment of lung inflammation:** Bronchoalveolar lavage (BAL) was performed with 3 x1000 μl sterile saline. Fluid was combined and centrifuged (260g, 10 min), cells were resuspended in 1 ml phosphate-buffered saline and 1% bovine serum albumin, total cell counts were determined using a grid hemocytometer1.

**Enzyme-linked immunosorbent assay (ELISA):** SPP1 protein levels of Bronchoalveolar lavage (BAL) was determined using dedicated murine ELISA kits according to the manufacturer’s instructions (Peprotech, London, UK and R&D Systems, Minneapolis, MN).

**qPCR and microarray:** RNA was isolated using Trizol (Invitrogen) followed by RNAeasy (QIAGEN), RNA was reverse transcribed using Superscript III (Invitrogen), and reverse transcriptase or qPCR was performed using specific primers (Supplemental Table 2). For microarray, 5 μg RNA pooled from triplicate samples was tested for quality, labeled, and hybridized to GeneChip Mouse Gene 1.0 or 2.0 ST arrays (Affymetrix). Microarray data are available at the GEO database (https://www.ncbi.nlm.nih.gov/geo/) using accession ID GSE94981.

**Flow cytometry:** Cells were analyzed on a FACS Calibur (BD Biosciences) for endogenous fluorescence. Data were examined using FlowJo (Ashland, OR).

**Immunoblotting:** Whole cell extracts were prepared using RIPA buffer (Sigma Aldrich, (St Louis, MO), separated by 10% SDS-PAGE, and

electroblotted to PVDF membranes (Merck-Millipore, Darmstadt, Germany). Membranes were labeled using the indicated antibodies (Supplemental Table 3) followed by incubation with the appropriate HRP-conjugated secondary antibodies at the manufacturers’ indicated dilutions and were visualized using enhanced chemiluminescence substrate (Merck Millipore, Darmstadt, Germany).

**Histology and Cytology:** Lungs were exsanguinated, inflated at 20 cmH2O with 10% neutral-buffered formalin, and fixed overnight. Lung tumor number and diameter (δ) were measured under a Stemi DV4 stereoscope (Zeiss; Jena, Germany) and tumor volume (V) was calculated as πδ3/6 and averaged/summed. Lung volume was measured by saline immersion, lungs were embedded in paraffin. Lungs were fixed in 10% paraffin or in 4% paraformaldehyde overnight, were embedded in paraffin or in optimal cutting temperature (OCT; Sakura, Tokyo, Japan) and were stored at room temperature or -80o C, respectively, till further analyses. Five-μm paraffin or 10-μm-cryosections were mounted on glass slides. Sections were mounted on glass slides and stained with hematoxylin and eosin (H&E). The proportion (percent of total lung lesions) consisting of each type of distinct lung lesions, including atypical alveolar hyperplasia (AAH), adenoma (AD), and adenocarcinoma (AC), on the sections from each lung were evaluated by two blinded readers (IP and GTS). Lung tumor burden was determined by point counting of the ratio of the area occupied by tumor versus the lung area and by extrapolating the average ratio per mouse to total lung volume, as described elsewhere3. Alternatively, tissue sections were immune-labeled with specific antibodies against SPP1, PCNA, factor VIII-related antigen (fVIIIra) and TUNEL as described previously4. The number of immunoreactive cells in lungs (bronchial and alveolar epithelium) and lung tumors was evaluated by two blinded readers in at least five high-power visual fields of at least five different lung or tumor regions per lung. The results were averaged per mouse. Sections were labeled using the indicated antibodies (Supplemental Table 3) and detected by Envision/diaminobenzidine detection (Dako, Glostrup, Denmark) kit or counterstained with hematoxylin/eosin working solution or with Hoechst 33258 (Sigma-Aldrich) and were mounted with Entellan new (Merck Millipore, Darmstadt, Germany) or with Mowiol 4-88 (Calbiochem, Gibbstown, NJ). A 100-point-grid was superimposed on ≥ 5 random non-overlapping fields of ≥ 10 sections/lung using Fiji and lung tumor burden was determined by extrapolating tumor-to-lung point counts to lung volume3. For isotype control, primary antibody was omitted. Bright-field images were captured with an AxioLab.A1 microscope connected to an AxioCam ERc 5s camera (Zeiss, Jena, Germany). Fluorescent microscopy was carried out on an AxioObserver.D1 inverted (Zeiss) or a TCS SP5 confocal microscope (Leica MS-20 Microsystems, Heidelberg, Germany) and digital images were processed with Fiji software5.

**SUPPLEMENTARY REFERENCES**

1. Kourepini E, Aggelakopoulou M, Alissafi T, Paschalidis N, Simoes DC, Panoutsakopoulou V. Osteopontin expression by CD103- dendritic cells drives intestinal inflammation. Proc NatlAcadSci U S A 2014; 111: E856-865.

2. Giopanou I, Lilis I, Papaleonidopoulos V, Agalioti T, Kanellakis NI, Spiropoulou N, Spella M, Stathopoulos GT. Tumor-derived osteopontin isoforms cooperate with TRP53 and CCL2 to promote lung metastasis. Oncoimmunology 2017; 6: e1256528.

3. Hsia CC, Hyde DM, Ochs M, Weibel ER. An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. Am J RespirCrit Care Med 2010; 181: 394-418.

4. Darzynkiewicz Z, Galkowski D, Zhao H. Analysis of apoptosis by cytometry using TUNEL assay. Methods 2008; 44: 250-254.

5. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012; 9: 676-682.

**SUPPLEMENTARY TABLES**

**Supplementary Table 1.** Number of experimental mice (*n*) used for these studies.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Strain designation** | **Jackson Laboratory Stock #** | **Short strain designation** | ***n*** |
| **Parental strains** | *C57BL/6J* | 000664 | *C57BL/6* | 67 |
| B6.129S6-*Spp1tm1Blh/J* | 004936 | *Spp1-/-* | 59 |
| *FVB/NJ* | 001800 | *FVB* | 30 |
| B6.129S4-*Krastm4Tyj/J* | 008179 | *LSL.KRAS*G12D | 16 |
| FVB-Tg*(CAG-luc,-GFP)L2G85Chco/J* | 008450 | *CAG-luc-eGFP* | 8 |
| NOD.CB17-*Prkdc<scid>/J* | 001303 | *NOD/SCID* | 14 |
| *Spp1-stopf/f/CreERT1* | - | *LSL.Spp1*Tg | 9 |
| **Inter-crosses** | - | - | *SPP1+/-* | 40 |
| - | - | *LSL.KRAS*G12D*;Spp1*+/- | 4 |
| - | - | *LSL.KRAS*G12D*;Spp1*-/- | 8 |
| - | - | *LSL.KRAS*G12D*;LSL.Spp1*Tg | 9 |
| **Total** | | | | **264** |

**Supplementary Table 2.** PCR primers used for these studies.

|  |  |  |
| --- | --- | --- |
| **Methoda** | **Primer** | **Sequence** |
| qPCR | mSpp1F | CCCTTTCCGTTGTTGTCCTG |
|  | mSpp1R | GATGAACAGTATCCTGATGCCAC |
|  | CD44F | CGTCCAACACCTCCCACTAT |
|  | CD44R | TGGTAACCGGTCCATCGAAG |
|  | GusbvF | TTACTTTAAGACGCTGATCACC |
|  | GusbR | ACCTCCAAATGCCCATAG |
|  | ItgavF | TGGCTATTCAATGAAGGGAG |
|  | ItgavR | AGGGTACACTTCAAGGCCAG |
|  | Itgb1F | TCCAGCTAATCATCGATGCCT |
|  | Itgb1R | TTCTCCTGTCCCATTCACC |
|  | Itgb3F | CTGGCAAGTACTGTGAGTGC |
|  | Itgb3R | AGTAGTAGCCAGTCCAGTCC |
|  | Itgb5F | ACCGAGATACCAGACCAATCC |
|  | Itgb5R | CCTCTGCTTCCTCACTTCCT |
| PCR | MycoplasmaF | GGGAGCAAACAGGATTAGATACCCT |
|  | MycoplasmaR | TGCACCATCTGTCACTCTGTTAACCTC |
|  | mSpp1F (*LSL.Spp1*Tg) | CTGCTAGTACACAAGCAGAC |
|  | mSpp1R (*LSL.Spp1*Tg) | GCCTCTTCTTTAGTTGACCTC |
|  | mSpp1F1 | CCATACAGGAAAGAGAGACC |
|  | mSpp1F2 | CGTCCTGTAAGTCTGCAGAA |
|  | mSpp1R | AACTGTTTTGCTTGCATGCG |

a Application: qPCR, quantitative real-time polymerase chain reaction; PCR, polymerase chain reaction.

**Supplementary Table 3.** Antibodies used in these studies.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Methoda** | **Target** | **Providerb** | **Catalog#** | **Dilution** | **Conjugatec** |
| WB | KRAS2A | Santa Cruz | sc-522 | 1:200 | - |
|  | α-Tubulin | Sigma | T5168 | 1:4000 | - |
|  | Goat anti-mouse IgG | Southern Biotech | 1030-05 | 1:8000 | HRP |
|  | Goat anti-rabbit IgG | Southern Biotech | 4030-05 | 1:8000 | HRP |
| IHC/IF | SPP1 | Abcam | ab91655 | 1:1000 | - |
|  | PCNA | Abcam | ab2426 | 1:2000 | - |
|  | F8A | Invitrogen | PA5-61382 | 1:1000 | **-** |
|  | ITGB3 | Sigma-Aldrich | SAB4501586 | 1:1000 | **-** |
|  | donkey anti-rabbit & anti-mouse IgG | Invitrogen | A21206 -A21202 | 1:1000 | Alexa 488 |

aApplication: WIB, Western immunoblotting; IF, immunofluorescence; IHC, immunochistochemistry; bProviders: Santa Cruz Biotechnology, San Diego, CA; Sigma Aldrich, Taufkirchen, Germany; Abcam, Cambridge, UK; Southern Biotech, Birmingham, AL; Invitrogen, Carlsbad, CA. cConjugates: HRP, horse radish peroxidase.

**Supplementary Figure 1.** Uncropped gells from the electrophoresis shown in Figure 6A of the manuscript.

**Supplementary Figure 2:** Validation of SPP1 expression in *LSL.Spp1*Tg, *LSL.KRAS*G12D, and *LSL.KRAS*G12D; *LSLSpp1*Tg mice upon Ad-*Cre* administration as shown in Figure 6J.

