ARTICLE
Metastasis

Pharmacological blockage of transforming growth factor-β signalling by a Traf2- and Nck-interacting kinase inhibitor, NCB-0846

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BACKGROUND: Metastasis is the primary cause of death in cancer patients, and its management is still a major challenge. Epithelial to mesenchymal transition (EMT) has been implicated in the process of cancer metastasis, and its pharmacological interference holds therapeutic promise.

METHODS: Traf2- and Nck-interacting kinase (TNIK) functions as a transcriptional coregulator of Wnt target genes. Given the convergence of Wnt and transforming growth factor-β (TGFβ) signalling, we examined the effects of a small-molecule TNIK inhibitor (named NCB-0846) on the TGFβ1-induced EMT of lung cancer cells.

RESULTS: NCB-0846 inhibited the TGFβ1-induced EMT of A549 cells. This inhibition was associated with inhibition of Smad and Mad-Related Protein-2/3 (SMAD2/3) phosphorylation and nuclear translocation. NCB-0846 abolished the lung metastasis of TGFβ1-treated A549 cells injected into the tail veins of immunodeficient mice. The inhibition of EMT was mediated by suppression of the TGFβ receptor type-I (TGFBR1) gene, at least partly through the induction of microRNAs targeting the TGFBR1 transcript [miR-320 (a, b and d) and miR-186].

CONCLUSIONS: NCB-0846 pharmacologically blocks the TGFβ/SMAD signalling and EMT induction of lung cancer cells by transcriptionally downregulating TGFBR1 expression, representing a potentially promising approach for prevention of metastasis in lung cancer patients.

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resulting in reduction of EMT marker (N-cadherin and vimentin) expression.\textsuperscript{14} TNIK phosphorylates the conserved serine 154 residue of TCF4.\textsuperscript{12} TGFβ1 induces the phosphorylation of TCF4, and the induction is inhibited by NCB-0005.\textsuperscript{14} Kaneko et al. identified TNIK as one of the kinases responsible for α-helix 1 phosphorylation of SMADs, which reduces SMAD interaction with TGFβ/bone morphogenetic protein (BMP) receptor kinase.\textsuperscript{15} NCB-0005 inhibits TGFβ1-induced migration and invasion of lung cancer cells,\textsuperscript{14} but the degree of the EMT inhibition was modest and the underlying molecular mechanism has remained unexplored.

NCB-0846 is a newly identified small-molecule TNIK-inhibitory compound with a backbone structure different from NCB-0005.\textsuperscript{16} NCB-0846 inhibits the transcriptional coactivator function of TNIK by modulating its conformational structure. We have previously revealed that this compound abrogates colorectal cancer stemness through suppression of Wnt target gene expression. NCB-0846 also suppresses the expression of SMAD2, vimentin and EMT-activating transcription factors (TFs) including Wnt targets, Snail and Twist, in colorectal cancer cells.\textsuperscript{16} Given the convergence of TGFβ and Wnt signalling, we assumed that NCB-0846 might also inhibit EMT. Here we report that NCB-0846 blocks the TGFβ/SMAD signalling and EMT induction of lung cancer cells through novel miRNA-mediated silencing of the TGFBR1 gene.

\section*{METHODS}

\subsection*{Ethical issues}

All the animal experimental protocols in this study were reviewed and approved by the institutional ethics and recombination safety committees of the National Cancer Center Research Institute (Tokyo, Japan). The number of animals necessary for obtaining reliable results was minimised, with strict attention to animal rights and welfare protection.

\subsection*{Antibodies and reagents}

All antibodies used in this study and their suppliers are listed in Supplementary Table S1. Recombinant human TGFβ1 was from R&D Systems. NCB-0846 and its diastereomer, NCB-0970, have been described previously.\textsuperscript{16}

\subsection*{Cell lines}

The human non-small-cell lung cancer (NSCLC) cell lines, A549 and NCI-H2228, were obtained from the Cell Bank of the American Type Culture Collection (ATCC). Cells were maintained in Eagle’s minimum essential medium (Nacalai Tesque) or RPMI1640 medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% nonessential amino acid (Thermo Fisher Scientific) in a humidified incubator supplied with 5% CO2. Absence of mycoplasma contamination was monitored routinely using the e-Myco VALiD Mycoplasma PCR Detection Kit (iNtRon Biotechnology). None of these cell lines is listed in the International Cell Line Authentication Committee database of cross-contaminated or misidentified cell lines, and all the cell lines were authenticated by short tandem repeat (STR) DNA profiling by the JCRB Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan).

\subsection*{Immunoblot analysis}

Whole-cell extracts and nuclear protein fractions were prepared using RIPA buffer (Cell Signaling Technology) and Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific), respectively. Protein concentration was determined using Direct Detect (Merck). Equal amounts of proteins (10 or 15 µg) were fractionated on 4–12% NuPAGE Bis-Tris gels (Thermo Fisher Scientific) and transferred to Immobilon-P membranes (Merck) as described previously.\textsuperscript{7} Following incubation with primary antibodies (listed in Supplementary Table S1) at 4 °C overnight, the blots were reacted with relevant horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG antibody (Cell Signaling Technology). Blot signals were detected with the Western Lighting ECL Pro (PerkinElmer) and the LAS 4010 system (GE Healthcare), and signal intensity was quantified using the ImageQuant TL software package (GE Healthcare).

\subsection*{Immunofluorescence microscopy}

Cells were seeded at 2.5 or 5.0 × 10^4 per well onto collagen-coated 8-well culture slides (BioCoat). Following 24-h serum starvation, the cells were cultured with dimethyl sulfoxide (DMSO) alone (control), TGFβ1 (5 ng/ml) and DMSO, TGFβ1 and NCB-0846 (1 µM) or TGFβ1 and NCB-970 (1 µM) for 48 h and fixed in 4% paraformaldehyde. The fixed cells were immunostained with primary antibodies (listed in Supplementary Table S1) as described previously.\textsuperscript{18} Following overnight incubation at 4 °C, the cells were incubated with relevant secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 568 (Thermo Fisher Scientific) and co-stained with TOTO3 (Thermo Fisher Scientific) or phalloidin (Thermo Fisher Scientific) for visualisation of nuclei or filamentous actin, respectively. Cells were examined with the LSM 5 PASCAL laser confocal microscopy system (Carl Zeiss).

\subsection*{Wound-closure assay}

A549 cells were seeded at 5.0 × 10^5 per well in a 6-well plate and serum-starved for 24 h prior to creation of a scratch on the confluent cell monolayer. The wounded monolayers were then treated with DMSO alone (control), TGFβ1 (5 ng/ml) and DMSO, TGFβ1 and NCB-0846 (3 µM), or TGFβ1 and NCB-970 (3 µM) for 48 h. The degree of migration was determined by measuring the width of cell-free areas in triplicate.

\subsection*{Automated real-time cell migration assay}

Cells were plated (1.0 × 10^4 cells/well) in triplicate onto a microelectrodes-embedded xCelligence E-culture plate (ACEA Biosciences). When cells reached confluence (indicated by steady-state impedance), scratches were created with sterile P200 micropipette tips and the cells were treated with DMSO alone (control), TGFβ1 (5 ng/ml) and DMSO, TGFβ1 and NCB-0846 (3 µM), or TGFβ1 and NCB-970 (3 µM) for 40 h. The impedance of cell monolayers before and after scratching as well as the recovery of full impedance were monitored in real time with the xCelligence instrument (ACEA Biosciences).

\subsection*{Cell invasion assay}

Cell invasion was assessed using 16-well transwell plates with 8-µm pores (CIM plate 16; ACEA Biosciences) precoated with 20 µl of Matrigel (BD Biosciences) diluted 1:40 in MEM medium. Microelectrodes were located on the underside of membranes in the upper chambers. Medium containing 10% FCS was added to the lower chamber, and cells were seeded into the upper chamber at 4 × 10^4 per well in serum-free medium. A549 cells were treated with DMSO alone (control), TGFβ1 (5 ng/ml) and DMSO, TGFβ1 and NCB-0846 (3 µM), or TGFβ1 and NCB-970 (3 µM). Impedance of migrated cells was monitored in real time with the xCelligence instrument. Data analysis was carried out using RTCA software 2.0 supplied with the instrument (ACEA Biosciences).

\subsection*{Quantitative PCR}

Total RNA was isolated with a RNeasy Mini Kit or miRNasy Mini Kit (Qiagen) in accordance with the manufacturer’s instructions. cDNA was prepared using a High-Capacity cDNA reverse transcription kit (Applied Biosystems) or MicroRNA Reverse Transcription Kit (Applied Biosystems). The relative expression of mRNA and miRNA was measured using the TaqMan Gene Expression Assay (Applied Biosystems) or TaqMan MicroRNA Assay (Applied Biosystems),
We tested the effects of a small-molecule TNK modulator, NCB-0846, on TGFβ1-induced EMT in two NSCLC cell lines (A549 and H2228). NCB-0846, but not its inactive diastereomer NCB-0970, inhibited the TGFβ1-induced morphological conversion from the cobblestone-like epithelial phenotype to the spindle cell-like mesenchymal phenotype in A549 cells and modestly in H2228 cells (Fig. 1a). This inhibitory effect of NCB-0846 on the TGFβ1-triggered EMT of A549 cells was supported by restoration of E-cadherin expression and suppression of the mesenchymal cell markers, vimentin and N-cadherin, whereas NCB-970 had no such effect on the expression of these EMT markers. The marker switching was not obvious in H2228 cells (Fig. 1b). Furthermore, NCB-0846, but not NCB-0970, upregulated the gene expression of E-cadherin (CDH1) and downregulated that of N-cadherin (CDH2) and vimentin (VIM) in A549 cells (Supplementary Fig. S1). Immunofluorescence microscopy supported these observations. NCB-0846 restored the cell membrane localisation of E-cadherin and a tight junction protein, zonula occludens-1 (ZO-1), and suppressed the expression of mesenchymal cell markers (Fig. 1c). We were unable to detect any cells that showed dual positivity for E-cadherin and vimentin. The proper distribution of ZO-1 in NCB-0846-treated A549 cells indicated the restoration of epithelial cell polarity. NCB-0846, but not NCB-0970, decreased the TGFβ1-induced motility of A549 cells in a conventional wound-closure assay (Fig. 1d) and also as demonstrated by automated real-time cell tracking (Supplementary Fig. S2). Real-time trans-Matrigel cell monitoring also demonstrated complete inhibition of TGFβ1-induced cell invasion by NCB-0846 (Supplementary Fig. S3). However, the motility and invasion of A549 cells treated with TGFβ1 and NCB-0864 was reduced to below that with the DMSO control, indicating the cytotoxic effect of NCB-0846.

Abrogation of stemness is not involved in EMT inhibition NCB-0846, but not NCB-0970, inhibited the expression of TNFα as well as the expression of Wnt target gene products, including Axin1, Axin2 and c-Myc, in A549 lung cancer cells (Supplementary Fig. S4a). NCB-0846 abrogates the stemness of colorectal cancer by blocking the transcription of Wnt target genes.16,20 We had previously observed that NCB-0846 downregulated the expression of stem cell markers, such as CD133, CD44 and aldehyde dehydrogenase-1 (ALDH1) in colorectal cancer cells,16 whereas such an effect was barely evident in the lung cancer cells (Supplementary Fig. S4b), suggesting that the effects of NCB-0846 are cell context-dependent and that its cancer stemness-inhibitory activity is not necessary for EMT suppression.

Effects on SMAD signalling Canonical TGFβ1 signalling is initiated by binding of TGFβ1 to the TGFβ type-II receptor (TGFβRII). TGFβRII is a serine/threonine protein kinase, which transphosphorylates and activates the TGFβ type-I receptor (TGFβRI). The activated TGFβRII recruits and phosphorylates Smad and Mad-Related Proteins-2 and -3 (SMAD2/3), thus facilitating the formation of a complex with SMAD4, and translocation of the complex into the nucleus,21,22 where the SMAD complex mediates the transcription of EMT-activating transcription factors; including the Snail, Twist and zinc finger E-box-binding homeobox (ZEB) families.23 NCB-0846, but not NCB-0970, completely inhibited TGFβ1-driven phosphorylation of SMAD2 and SMAD3 (Fig. 2a) as well as their subsequent nuclear translocation (Fig. 2b) in A549 cells. Paradoxically, the inhibition of SMAD phosphorylation and nuclear translocation in H2228 cells occurred despite the lack of TGFβ1-driven EMT induction. In addition, a luciferase reporter assay for SMAD-binding element (SBE) confirmed that NCB-0846 dose-dependently inhibited TGFβ1-driven SMAD-mediated transcriptional activity (Supplementary Fig. S5). Consistently, NCB-0846 suppressed the expression of Snail completely and that of ZEB1 modestly in A549 cells (Fig. 2c).

RESULTS
NCB-0846 blocks TGFβ1-induced EMT
We tested the effects of a small-molecule TNK modulator, NCB-0846, on TGFβ1-induced EMT in two NSCLC cell lines (A549 and
Repression of TGFBR1 gene expression
To clarify the mechanism underlying the inhibitory effect of NCB-0846 on TGFβ1-induced SMAD signalling, we looked at its upstream receptors TGFβRI and TGFβRII. TGFβ1 did not affect the expression of TGFβRII, but strongly induced the expression of TGFβRI (Fig. 3a). NCB-0846 inhibited the TGFβ1-mediated induction of TGFβRI protein (Fig. 3a) and TGFBR1 gene expression (Fig. 3b).

To confirm the involvement of TGFβRI in the EMT inhibition by NCB-0846, we introduced a small interfering RNA (siRNA) recognising TGFβRI(siTβRI) into A549 cells. siTβRI, but not control RNA (siNC), restored the cobblestone-like appearance and cell-to-cell adhesion of TGFβ1-treated cells (Fig. 3c). TGFβRI knockdown restored the cell membrane localisation (Supplementary Fig. S6) of E-cadherin, downregulated the expression of phosphorylated SMAD2/3 (pSMAD2/3) (Fig. 3d), and inhibited the nuclear localisation

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Fig. 1 NCB-0846 inhibits TGFβ1-triggered EMT. a Morphological conversion of A549 (upper panels) and H2228 (lower panels) cells. Following 24-h serum starvation, cells were treated with DMSO alone (control), TGFβ1 (5 ng/mL) and DMSO, TGFβ1 and NCB-0846 (3 µM), or TGFβ1 and NCB-0970 (3 µM) for 48 h and photographed. Scale bar, 100 µm. b Immunoblot analysis of epithelial (E-cadherin) and mesenchymal cell markers (vimentin and N-cadherin) and γ-tubulin (loading control) in A549 (upper panels) and H2228 (lower panels) cells treated as indicated. c Immunofluorescence confocal microscopy of epithelial (E-cadherin and ZO-1) and mesenchymal cell markers (vimentin and fibronectin) in A549 cells treated as indicated. The nuclei were stained with TOTO3 (blue). Scale bar, 20 µm. d Cell migration evaluated by a wound-closure assay. Dotted and solid lines indicate the edges of wounds immediately and 48 h after creation of the wounds, respectively. The width of wounds relative to their baseline (set to one) is shown in the bar graph (right).
of pSMAD2/3, ZEB1 and Snail proteins (Supplementary Fig. S6). These results confirmed that NCB-0846 inhibited EMT through downregulation of TGFBR1 gene expression.

**TGFBR1-targeting miRNAs**

Noncoding microRNAs (miRNAs) have been increasingly recognised as key players in gene silencing. miRNAs bind to specific target sequences present in the 3’ untranslated regions (UTR) of transcripts and post-transcriptionally silence the corresponding gene expression. To better understand the mechanism underlying the reduction of TGFBR1 expression by NCB-0846, we profiled the miRNA expression of A549 cells treated with TGFβ1 and NCB-0846 or NCB-0970 using oligonucleotide miRNA microarrays (Supplementary Table S3). Among 61 miRNAs whose expression was increased > 2-fold by NCB-0846 treatment, we found using two publicly available algorithms (TargetScan; http://www.targetscan.org and TargetSpy; http://webclu.bio.wzw.tum.de/targetspy) that miR-186-5p and miR-320a, 320b, and 320d were predicted to bind to the 3’UTR of TGFBR1 (Supplementary Fig. S7). Sharp induction of miR-186-5p and miR-320 (a, b and d) by NCB-0846 was confirmed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 4a).

Transient transfection with miR-320 (a, b and d) and miR-186 mimics resulted in reduced expression of TGFβRI (Fig. 4b), whereas transient transfection with inhibitors of miR-320 (a, b and d) and miR-186 increased the expression of TGFβRI protein (Fig. 4c). These data indicated that NCB-0846 repressed the expression of TGFβRI at least partially through the induction of these miRNAs.

![Fig. 2 NCB-0846 blocks TGFβ1-mediated SMAD activation. a Immunoblot analysis of SMAD2 phosphorylated at the 465/467 serine residues (pSMAD2), total SMAD2/3, SMAD3 phosphorylated at the serine 465/467 residues (pSMAD3), and γ-tubulin (loading control) in A549 (left) and H2228 (right) cells treated as indicated. b Immunofluorescence confocal microscopy of pSMAD3, phosphorylated SMAD2 (Ser465/467)/SMAD3 (Ser423/425) (pSMAD2/3), ZEB1 and Snail in A549 cells and pSMAD3 and pSMAD2/3 in H2228 cells treated as indicated. Filamentous actin is co-stained with phalloidin (red). Scale bar, 20 μm. c Immunoblot analysis of EMT-related transcription factors (Twist, Snail and ZEB1) and Lamin-B1 (loading control) in the nuclear protein extracts prepared from A549 cells treated as indicated.](image-url)
Finally, we explored whether the EMT inhibitory activity of NCB-0846 affects metastasis. A549 cells were treated with TGFβ1 in the absence or presence of either NCB-0846 or NCB-0970 for 48 h in vitro, and then injected into immunodeficient mice (eight per group) via the tail vein. Seven weeks after injection, the mice were sacrificed, and their lung metastases were digitally quantified in tissue sections (Fig. 5a). This animal experiment is used mainly to evaluate the trans-endothelial migration/extravasation capability of cancer cells embolised in peripheral lung vessels immediately after systemic injection.

Metastatic lesions occupied 38% of the lung area in mice injected with TGFβ1-treated cells but were significantly (P < 0.001) reduced in the lungs of mice injected with NCB-0846-treated cells (Fig. 5b). We confirmed the complete absence of metastasis by microscopic inspection of tissue sections. In parallel, the average lung weights of mice injected with NCB-0846-treated cells were significantly decreased due to lack of the pulmonary metastatic
burden, compared with those of mice injected with cells that had been treated with DMSO (control) or NCB-0970 (Fig. 5c). These results support the notion that inhibition of EMT by NCB-0846 compromises the TGFβ1-induced metastatic potential of lung cancer cells.

DISCUSSION

TNIK is a member of the STE20 (sterile 20) serine/threonine protein kinase family, which comprises TNIK, misshapen-like kinase 1 (MINK1), and mitogen-activated protein kinase kinase kinase 4 (MAP4K4).25–27 We and others almost simultaneously identified TNIK as a component of the β-catenin and TCF4 transcriptional complex in human and mouse, respectively, through similar large-scale proteome analyses.11,28 TNIK was essential for colorectal cancer growth and the transactivation of Wnt target genes (such as MYC, AXIN2 and CD44) by TCF4.12 Increased expression of TNIK is associated with an unfavourable clinical outcome in patients with pancreatic, colorectal and hepatocellular carcinomas,29–31 and the TNIK gene is amplified in 7% of gastric cancers.32 A recent meta-analysis of cancer genome-sequencing studies identified TNIK as a driver oncogene.33 Based on these experimental and clinical findings, various classes of TNIK inhibitors have been developed.20,34 In this study we found that the activity of TGFβ signalling was dynamically regulated by the expression level of a cell surface receptor of TGFβ1 (Fig. 3). Stimulation of NSCLC cells with TGFβ1 induced the expression of TGFβRI, and a small-molecule TNIK inhibitor, NCB-0846, downregulated the TGFBR1 gene expression and inhibited the TGFβ1-induced EMT of NSCLC cells. NCB-0970 has a similar inhibitory profile over the human kinome except for STE20 member kinases.16 We carefully included the

Fig. 4 miR-320s and miR-186 target TGFβRI. a The induction of miR-320s and miR-186 by NCB-0846. A549 cells were treated with DMSO alone (control), TGFβ1 (5 ng/mL) and DMSO, TGFβ1 and NCB-0846 (3 µM), or TGFβ1 and NCB-0970 (3 µM), and the expression of indicated miRNAs was quantified in triplicate by qRT-PCR. The values for cells treated with DMSO alone are set to one. Error bars represent SD. b Reduced expression of TGFβRI protein by introduction of miR-320s and miR-186 mimics. A549 cells were transfected with the indicated miRNA mimics, and 48 h later TGFβRI protein was detected by immunoblotting (upper panels, γ-tubulin as a loading control). The bottom panel indicates signal intensity relative to the control mimic (miR-Ctr). c Increased expression of TGFβRI protein by introduction of miR-320s and miR-186 inhibitors. A549 cells were transfected with the indicated miRNA inhibitors, and 48 h later TGFβRI protein was detected by immunoblotting (upper panels, γ-tubulin as a loading control). The bottom panel indicates signal intensity relative to the control inhibitor (miR-Ctr).
As phosphorylation of SMAD proteins by TGF/β has been considered a good target for drug development. Galunisertib (LY2157299) is an orally available small-molecule kinase inhibitor of TGF/RI, and its anti-tumour activity has been confirmed in animal models of various cancers. Galunisertib in combination with gemcitabine significantly improved the overall survival of patients with unresectable pancreatic cancer over gemcitabine monotherapy. NCB-0846 blocked TGF/β/SMAD signalling by downregulating TGF/BR1 gene expression. This novel mode of action (MOA) distinguishes the compound from the preceding TGF/BR1/ALK5 kinase inhibitors.

Several lines of evidence indicate the involvement of Wnt signalling in TGF/β-induced EMT. TNIK is an essential regulator of Wnt signalling, and two small-molecule TNIK inhibitors with different chemical structures (NCB-0005 and NCB-0846) reproducibly blocked the TGF/β/SMAD signalling and EMT of NSCLC cells, confirming the feasibility of targeting TNIK. Pharmacological TNIK inhibition would be potentially applicable to the management of various EMT-associated disorders; not only metastasis, but also cancer stemness and chemotherapy resistance. NCB-0846 is now under preclinical development aimed at investigational new drug (IND) application.

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AUTHOR CONTRIBUTIONS

T.S., M.M. and T.Y. conceived the study concept. N.M., S.K., Y.U., H.M. and M.Sa. provided materials. T.S., M.M., F.T., T.H., N.G. and S.K. performed the experiments. All authors analysed and interpreted the data. T.S., M.M. and T.Y. drafted the paper. All authors revised the manuscript, approved the final version of the paper and provided their consent for publication.

ADDITIONAL INFORMATION

Ethics approval and consent to participate All the animal experimental protocols in this study were reviewed and approved by the institutional ethics and recombination safety committees of the National Cancer Center Research Institute (Tokyo, Japan). Two human NSCLC cell lines, A549 and NCI-H2228, were obtained from the ATCC (Manassas, VA, USA).

Data availability The datasets generated and/or analysed during the present study are not publicly available but may be available from the corresponding author on reasonable request. The miRNA microarray data have been deposited in the NCBI’s Gene Expression Omnibus database under the accession number GSE95766.

Competing interests The authors declare no competing interests. S.K., Y.U., H.M. and M.S. are employees of Carna Biosciences, Inc. (Kobe, Japan). T.Y. and M.M. have received a research grant from the company. The remaining authors declare no conflict of interest.

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Fig. 5 NCB-0846 inhibits metastatic potential. a Representative images of lungs (from top to bottom: unfixed, formalin-fixed, and HE-stained tissue sections) resected from mice 7 weeks after injection of A549 cells treated with TGF-β1 (5 ng/mL) and DMSO, TGF-β1 and NCB-0846 (3 µM) or TGF-β1 and NCB-0970 (3 µM) for 48 h. Scale bar represents 5 mm. b Summed areas of metastases relative to the total lung areas of mice injected with A549 cells treated as described in the legend to Fig. 5a. Error bars represent SD. ***P < 0.001. c Wet lung weights of mice injected with A549 cells treated as described in the legend to Fig. 5a. Error bars represent SD. ***P < 0.001; **P < 0.01.
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