Mechanical stretch activates piezo1 in caveolae of alveolar type I cells to trigger ATP release and paracrine stimulation of surfactant secretion from alveolar type II cells

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Abstract
Secretion of pulmonary surfactant in the alveoli of the lungs is essential to maintain lung function. Stretching of alveoli during lung inflation is the main trigger for surfactant secretion. Yet, the molecular mechanisms how mechanical distension of alveoli results in surfactant secretion are still elusive. The alveolar epithelium consists of alveolar epithelial type I (ATI) and surfactant secreting type II (ATII) cells. ATI, but not ATII cells, express caveolae, small plasma membrane invaginations that can respond to plasma membrane stresses and serve mechanotransductive roles. Within this study, we investigated the role of caveolae as mechanosensors in the alveolus. We generated a human caveolin-1 knockout ATI cell (hAELVi<sup>cav−/−</sup>) using CRISPR/Cas9. Wildtype (hAELVi<sup>wt</sup>) and hAELVi<sup>cav−/−</sup> cells grown on flexible membranes responded to increasing stretch amplitudes with rises in intracellular Ca<sup>2+</sup>. The response was less frequent and started at higher stretch amplitudes in hAELVi<sup>cav−/−</sup> cells. Stretch-induced Ca<sup>2+</sup>-signals depended on Ca<sup>2+</sup>-entry via piezo1 channels, localized within caveolae in hAELVi<sup>wt</sup> and primary ATI cells. Ca<sup>2+</sup>-entry via piezo1 activated pannexin-1 hemichannels resulting in ATP release from ATI cells. ATP release was reduced in hAELVi<sup>cav−/−</sup> cells. In co-cultures resembling the alveolar epithelium, released ATP stimulated Ca<sup>2+</sup> signals and surfactant secretion from neighboring ATII cells when co-cultured with hAELVi<sup>wt</sup> but not hAELVi<sup>cav−/−</sup> cells. In summary, we propose that caveolae in ATI cells are mechanosensors within alveoli regulating stretch-induced surfactant secretion from ATII cells.

KEYWORDS
lung, mechanobiology, piezo-1, purinergic signaling

Abbreviations: ATI, alveolar type I epithelial cell; ATII, alveolar type II epithelial cell; hAELVi, human alveolar epithelial lentivirus immortalized cells; LB, lamellar body; PDMS, Polydimethylsiloxane.
1 | INTRODUCTION

Secretion of pulmonary surfactant in the alveoli of the lungs is essential to maintain lung function. The alveolar epithelium consists of alveolar type I (ATI) and type II (ATII) cells and surfactant is secreted from ATII cells via Ca\(^{2+}\)-dependent exocytosis of lamellar bodies (LBs). The strongest stimulus for surfactant secretion is the mechanical deformation/expansion of alveoli. It has been demonstrated that the stretch of ATII cells triggers strain-induced Ca\(^{2+}\)-entry and the resulting rise in intracellular Ca\(^{2+}\) levels stimulates LB exocytosis and surfactant secretion. However, ATI cells, which cover >95% of the alveolar surface, are distended almost two times as much as ATII cells during inflation. Accordingly, ATI cells can act as mechanosensors in the alveoli and stimulate surfactant secretion from ATII cells in a paracrine way. Hyperinflation of the lungs resulted in an increase in Ca\(^{2+}\) in ATI cells that was transmitted to ATII cells via gap junctions and stimulated LB exocytosis. Signals can also be transmitted by extracellular ATP, acting on purinergic receptors on ATII cells. Mechanical deformation results in Ca\(^{2+}\)-dependent ATP release from ATII and ATI cells that stimulates surfactant secretion in ATII cells. In line, inflation of rat lungs ex vivo induces ATP release in alveoli. ATP can be released from ATI cells following the activation of purinergic P2X7 receptors, probably via pannexin hemichannels.

Despite the importance of mechanical stimuli for surfactant secretion and lung function, it is still unclear how mechanical deformation is sensed at the molecular level within intact alveoli and in alveolar epithelial cells.

Recently, caveolae have emerged as vital plasma membrane sensors that can respond to plasma membrane stresses and serve mechanotransductive and mechanoprotective roles. Caveolae are 50-100 nm, bulb-shaped invaginations of the plasma membrane present in most mammalian cell types. The formation of caveolae depends on an 80S caveolar coat complex of caveolin and cavin proteins, being essential for the formation of caveolae. Caveolae are particularly abundant in endothelial cells, adipocytes, muscle cells, and ATII cells—suggesting an important role of these invaginations in the physiology of the alveolus. Indeed, lungs from cavin-1−/− mice have constricted alveolar spaces, associated with a thickening of the alveolar wall and resembling a fibrotic phenotype.

Interestingly, caveolae are absent from ATI cells. Next to their role in mechanosensation and mechanoprotection, caveolae have also been described to function in endocytosis and transcytosis, in maintaining membrane lipid composition, as well as acting as signaling platforms. Increased membrane tension and stretching of the plasma membrane causes disassembly and flattening of caveolae to provide extra membrane and protect cells from mechanical damage. Mechanical cues also elicit intracellular signals via caveolae to trigger transcriptional or adaptive responses. This includes ATP release and intracellular Ca\(^{2+}\) signals with caveolae having been found to constitute microdomains for stretch-dependent Ca\(^{2+}\) signaling.

Within this study, we, therefore, employed the co-culture of primary ATII cells and a recently established ATI cell line on an elastic PDMS substrate to investigate the effects of mechanical stretch on the alveolar epithelium and the role for caveolae as mechanosensors at the single-cell level. To decipher the role of caveolae, we used CRISPR/Cas9 gene-editing to generate ATI cells lacking caveolin-1 expression (hAELVi\(^{cav−/−}\)). Stretch of wild-type hAELVi (hAELVi\(^{wt}\)) and hAELVi\(^{cav−/−}\) cells with increasing amplitudes triggered rises in intracellular Ca\(^{2+}\) with increasing stretch amplitudes. The response was less frequent and started at higher stretch amplitudes in hAELVi\(^{cav−/−}\) cells. We further identified that mechanotransductive ion channel piezo1 is expressed in hAELVi cells and primary ATII cells and co-localized with caveolin-1 in hAELVi\(^{wt}\) cells. Stretch-induced Ca\(^{2+}\)-signals depended on Ca\(^{2+}\)-entry via piezo1 channels, activated pannexin-1 hemichannels, and resulted in ATP release from ATI cells. ATP measurements with microbiosensors confirmed that ATP release was reduced in hAELVi\(^{cav−/−}\) cells. Released ATP stimulated Ca\(^{2+}\) signals and surfactant secretion from neighboring ATII cells in co-cultures of hAELVi cells with primary ATII cells resembling the alveolar epithelium. In summary, our findings suggest that caveolae within ATI cells serve as mechanosensors within the alveolus regulating stretch-induced surfactant secretion from ATII cells.

2 | MATERIALS AND METHODS

2.1 | Materials

Fluorescently labeled secondary antibodies and fluorescent dyes were purchased from Molecular Probes (Thermo Fisher Scientific, Darmstadt, Germany), α-caveolin-1 (cat#. ab2910 for immunofluorescence and co-IP, cat#. ab36152 for Western blotting) and α-P180 lamellar body protein (ABCa3, cat#. ab24751) antibodies were purchased from Abcam (Cambridge, UK). α-Piezo1 antibody (28511-1-AP) was purchased from Proteintech (Manchester, UK). Edelfosine, GsMTx4, Yoda1, and PanX were purchased from Tocris Bioscience (Bio-Technne GmbH, Wiesbaden, Germany), YM-254890 was purchased from tebu-bio GmbH (Offenbach am Main, Germany). Silencer Select siRNAs were purchased from Thermo Fisher Scientific. All other chemicals were purchased from Sigma (Steinheim, Germany) unless stated otherwise.
2.2 | Cell isolation and cell culture

ATII cells were isolated from lung tissue according to the procedure of Dobbs et al for isolation of ATII cells from rat lung\textsuperscript{39} with minor modifications as recently described.\textsuperscript{40} Lungs were harvested from male Sprague-Dawley rats in accordance with a protocol reviewed and approved by the Regierungspräsidium Tübingen. Following the isolation, ATII cells were collected in MucilAir Medium (Epithelix Sàrl, Geneva, Switzerland) with 25.6 µg/mL of Gentamicin (Thermo Fisher Scientific), counted, and seeded in appropriate densities.

hAELVi cells are a recently established human alveolar epithelial cell line, with type I-like characteristics, functional tight junctions, and maintaining a stable barrier at air-liquid culture conditions.\textsuperscript{38} hAELVi\textsuperscript{wt} and hAELVi\textsubscript{cav−/−} cells were maintained at 37°C/5% CO\textsubscript{2} in huAEC-Medium (human Alveolar Epithelial Cell-Medium, InSCREENeX, Braunschweig, Germany) supplemented with 50 U/mL of penicillin and 50 µg/mL of streptomycin (Thermo Fisher Scientific) in cell culture flasks coated with huAEC (human Alveolar Epithelial Cell) coating solution (InSCREENeX).

For mechanical strain experiments, cells were seeded on PDMS membranes (USP class VI silicone, thickness: 0.005″, Specialty Manufacturing, Inc, Saginaw, MI, USA) coated with PDMS membranes (USP class VI silicone, thickness: 0.005″, Specialty Manufacturing, Inc, Saginaw, MI, USA) coated with huAEC coating solution. Cells were seeded 24-48 hours prior to the experiments at a density of 1.5 × 10\textsuperscript{5} hAELVi (hAELVi monaculture), 4 × 10\textsuperscript{5} ATII (ATII monaculture) or 1 × 10\textsuperscript{5} hAELVi + 2 × 10\textsuperscript{5} ATII (co-culture) cells, respectively.

2.3 | Genome editing

Deletion of endogenous CAV1 to generate hAELVi\textsubscript{cav−/−} cells was achieved using the CRISPR/Cas9 system as described in 41. Two guide RNA (gRNA) sequences (gRNA 1:5′ CACCGTGGGGGCAAAATTACGTAGACT; gRNA 2:5′ CACCGTGGGGCAAAATTACGTAGACT) were designed in the beginning and the end of the CDS in exon 1 and 2 of human CAV1, respectively (Figure 1A), were designed using the R&D Benching software (https://www.benchling.com) and cloned into pSpCas9(99)-2A-GFP (PX458), pSpCas9(99)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138; http://n2t.net/adding;48138; RRID:Addgene_48138). hAELVi cells were transfected with Lipofectamine LTX with PLUS Reagent (Thermo Fisher Scientific). About 96 hours after transfection, cells were FACs-sorted for GFP expression and positive cells seeded as single clones in 96 well plates. After 3 weeks of growth, monoclonal colonies were expanded in 6-well plates and evaluated by RT-PCR, Western blot, immunofluorescence, and Sanger sequencing to identify double positively transfected clones.

2.4 | siRNA

hAELVi\textsuperscript{wt} cells grown in 6-well plates were transfected with 0.1 µM Silencer Select siRNA (Thermo Fisher Scientific) using Lipofectamine RNAi Max transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s recommendations. After 48 hours of cell culture, cells were re-transfected with siRNA again and maintained in standard culture conditions for further 24 hours before seeding on flexible PDMS membranes at a density of 1.5 × 10\textsuperscript{5} (monoculture) or 1 × 10\textsuperscript{5} hAELVi\textsuperscript{wt} cells plus 2 × 10\textsuperscript{5} ATII cells (co-culture). Experiments and validation of knockdown efficiency by Western blotting were performed 24 hours thereafter. The following siRNAs were used for the knockdown of piezo1, pannexin-1 or as non-targeting (nt) negative control, respectively: Silencer Select siRNA Piezo1 hs (# s18891), Silencer Select siRNA Panx1 hs (# s24426), and Silencer Select nt siRNA (# AM4621) (all from Thermo Fisher Scientific).

2.5 | Fura-2 measurements and cell strain

Prior to stretch cells were loaded with 7.5 µM Fura-2 AM (Thermo Fisher Scientific) at 37°C/5% CO\textsubscript{2} for 30 minutes. Cells were washed twice with bath solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 5 mM glucose, 10 mM HEPES, 2 mM CaCl\textsubscript{2}, pH 7.4: 310 mOsm/l), incubated with respective inhibitors or agonists for 10 minutes before the start of the experiment and maintained in the solution (except the control cells) for the entire duration of the experiment. In Ca\textsuperscript{2+}-free bath solution, CaCl\textsubscript{2} was replaced by 10 mM EGTA.

For stretching assays, cells were stretched using a Cell Stretcher DCCS10 straining device (Innerbichler GmbH, Kundl, Austria) mounted on an inverted Zeiss Axiovert 200M imaging system equipped with a 20x lens (EC plan-NEOFLUAR 20x/0.5). The stretching device was recently described in detail.\textsuperscript{42} Cells were stretched intermittently with increasing amplitudes to a maximum of 75% longitudinal distension of the cell substrate (ie, PDMS membrane) (Figure 2B). About 75% longitudinal extension resulted in 18% lateral compression of the membrane, perpendicular to the longitudinal distension. The resulting increase in the cell-substrate area was therefore approx. 40%. The table below indicates the changes in the length of the membrane (increases due to longitudinal extension), width of the membrane (decreases due to lateral compression), and substrate area at different strain amplitudes.
**FIGURE 1** Generation of hAELVicav−/− cells using CRISPR/Cas9. A, Strategy for cav1 deletion by CRISPR/Cas technology. Top: schematic scheme of the cav1 genomic locus, CDS coding sequences are indicated by the blue lines. Middle: wt CDS sequence illustrating the targeting sequences for gRNA1 and 2. Bottom: Resulting sequence after gene editing. The resulting changes in the aa sequence are highlighted in red. B, Western blot of caveolin-1 and caveolin-2 in hAELViwt cells and 3 independent KO clones. Vinculin was used as a loading control. C, Real-time RT-PCR analysis of caveolin transcripts in hAELViwt cells and clone c5 (hAELVicav−/−) from (B) confirms the complete deletion of caveolin-1 expression in clone c5. Caveolin-2 expression is unaffected by caveolin-1 deletion. Caveolin-3 is not expressed in hAELViwt cells. Data are expressed as fold expression of housekeeping gene hmbs. Values are means from 5 individual cell isolations. D, Immunofluorescence for caveolin-1 (green) in hAELViwt cells and clone c5 from (B) and (C). Scale bar: 50 μm.
**FIGURE 2** Knock-out of caveolin-1 reduces stretch induced Ca\(^{2+}\) signaling in an ATI cell line. A, Schematic illustration of the experimental straining setup. Cells grown on silastic membranes were mounted in a cell stretching device fixed to the stage of an inverted microscope. The straining device allows precise control of mechanical distension whilst simultaneously monitoring individual cells. Changes in intracellular Ca\(^{2+}\) levels were followed analyzing fura-2 ratios (top insert). Scale bar: 20 μm. B, Representative fura-2 traces and stretch amplitudes for single cells depicted in (A). 

C, **Left:** Percentage of cells that respond to stretch with an intracellular Ca\(^{2+}\) signal at different stretch amplitudes. **Right:** Quantitative analysis of the percentage of cells that responded at a stretch amplitude of 75%. Generation of Ca\(^{2+}\) signals was strictly dependent on extracellular Ca\(^{2+}\) and significantly reduced in hAELVi\(^{wt}\) cells, when compared to hAELVi\(^{wt}\) cells. Data represent 5-17 independent experiments.

| Strain amplitude (% distension) | Lenght of membrane (mm) | Width of membrane (mm) | Substrate area (mm\(^2\)) | Increase in cell-substrate area (%) |
|---------------------------------|-------------------------|------------------------|---------------------------|-----------------------------------|
| 0                               | 40.0                    | 20.0                   | 800.0                     |                                   |
| 10                              | 44.0                    | 19.2                   | 844.8                     | 5.6                               |
| 20                              | 48.0                    | 18.4                   | 883.2                     | 10.4                              |
| 30                              | 52.0                    | 17.8                   | 925.6                     | 15.7                              |
| 40                              | 56.0                    | 17.2                   | 963.2                     | 20.4                              |
| 50                              | 60.0                    | 16.8                   | 1008.0                    | 26.0                              |
| 60                              | 64.0                    | 16.6                   | 1062.4                    | 32.8                              |
| 70                              | 68.0                    | 16.4                   | 1115.2                    | 39.4                              |
Images were acquired at an acquisition rate of 0.2 Hz and an exposure time of 100 ms at 340 and 380 nm, respectively, using VisiView (Visitron Systems GmbH, Puchheim, Germany). Image acquisition was started 1 minutes before the first and finished 1 minutes after the last stretch. Individual stretches were intermitted by a relaxed period of 1 minutes. Images were analyzed using FIJI as described previously.44

2.6 | Plasmids & adenoviruses

To investigate the potential colocalization of caveolin-1 with pannexin-1 and piezo1 in hAELVi cells, split GFP assays were performed as described in 45. CDS encoding caveolin-1, pannexin-1, and piezo1 was amplified from cDNA isolated from hAELVi<sup>wt</sup> cells and cloned into pmGFP10C-Tau and pmGFP11C-Tau to replace Tau, respectively, using In-Fusion HD cloning kit (Takara Bio Inc, Kusatsu, Japan). pmGFP10C-Tau and pmGFP11C-Tau were a gift from Henri Huttunen (Addgene plasmid #71433; RRID:Addgene_71433, Addgene plasmid # 71434; http://n2t.net/addgene:71434; RRID:Addgene_71434, Addgene plasmid #71434; http://n2t.net/addgene:71434; RRID:Addgene_71434).46 hAELVi<sup>wt</sup> and hAELVi<sup>−/−</sup> cells were seeded 24 hours before transfection, co-transfection of plasmid combinations was performed using Lipofectamine LTX with PLUS Reagent (Invitrogen, Thermo Fisher). 24 hours post-transfection, cells were analyzed for GFP signals.

pAD-CMV-Caveolin-1-CMV-GFP was a gift from Andrew Brooks (Addgene plasmid # 83272; http://n2t.net/addgene:83272; RRID:Addgene_83272). Adenoviruses were produced according to the manufacturer’s protocol and purified using the ViraBind™ Adenovirus Purification Kit (Cell Biolabs, San Diego, USA). Viral transduction efficiency was >95% after 24 hours. About 1 × 10<sup>5</sup> virus-infected hAELVi<sup>−/−</sup> cells and 2 × 10<sup>5</sup> freshly isolated ATII cells were seeded on flexible PDMS membranes 48 hours before stretch experiments.

To generate cav1-GFP for pull-down assays, caveolin-1 was amplified from cDNA isolated from hAELVi<sup>wt</sup> cells and cloned into pmGFP11C-Tau to replace Tau using In-Fusion HD cloning kit (Takara Bio Inc, Kusatsu, Japan). Accordingly, GFP was amplified from pEGFP-N1 (Clontech, USA) and cloned into pmGFP11-C-Cav-1 to replace GFP11C.

2.7 | Pull-down assays

hAELVi<sup>wt</sup> cells were transfected with cav1-GFP and pEGFP-N1, respectively, 48 hours prior to cell collection. Co-immunoprecipitation was performed using μMacs Epitope Tag Protein Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as described by Hansen et al. In brief, cells were lysed in lysis buffer supplemented with Pierce Protease and Phosphatase Inhibitors (Thermo Fisher Scientific) and 1% octyl β-D-glucopyranoside (OG), solubilized for 2 hours at 4°C and 100 rpm horizontal shaking. Lysate was then centrifuged at 14 000 rpm (10 minutes, 4°C) and 20% of the supernatant was separated as lysate fraction sample. The residual sample was supplemented with magnetic anti-GFP coated beads and incubated for 1 hour (4°C, 100 rpm horizontal shaking). The samples were applied on μMACS columns and extensively washed with wash buffer containing 0.2% OG. Bead-bound cav1-GFP and cav1 co-immunoprecipitates were eluted by pH shift (0.1M Triethyl Amine, 0.1% Triton X100, pH11). Immunodetection was performed via SDS-PAGE and Western Blot.

For the pull-down of endogenous panx-1, 100 µg BcMag Protein G magnetic beads (Bioclone Inc, San Diego, CA, USA) was coated with 6 µg anti-pannexin-1-antibody (Santa Cruz, sc-515941) overnight on a horizontal shaker at 4°C (100 rpm). Antibody-coated beads were applied for 1 hour at 4°C on lysates from hAELVi<sup>wt</sup> cells, collected, and solubilized as described above. Sample collection and immunode-
tection were performed as described above.

2.8 | FM 1-43 fusion assay

Cells were loaded with 10 nM LysoTracker Red (LTR) DND-99 (Thermo Fisher Scientific) for 20 minutes at 37°C/5% CO<sub>2</sub>. LTR accumulates in acidic LBs<sup>49</sup> and thus was used to identify ATII cells. Cells were then washed twice with bath solution and maintained in bath solution supplemented with 100 nM FM 1-43 (Thermo Fisher Scientific) for the duration of the experiment. The cells were stretched once for either 30% or 60% and afterward imaged for 5 minutes with an acquisition rate of 3 seconds and an exposure time of 100 ms at an excitation wavelength of 480 nm for FM 1-43. Exocytosis of individual LBs was indicated by a bright FM 1-43 signal. Determination of LB fusion by FM 1-43 fluorescence was recently described in detail.<sup>4,49,50</sup> This method is based on the cell-impermeant, surfactant-staining properties of FM 1-43, resulting in a very bright, localized fluorescence signal emanating from single LBs after fusion when FM 1-43 enters lamellar bodies from the bath solution through the fusion pore. Importantly, FM 1-43 is non-fluorescent in aqueous solutions, permitting fusion to be monitored in the continuous presence of the dye in the bath. At the end of each experiment an image at 568 nm (LTR excitation) was acquired to identify single ATII cells and to assign individual LB fusions to single ATII cells for calculating the percentage of ATII cells with at least one LB fusion event.

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2.9 | Semi-quantitative RT-PCR

Total RNA was isolated using my-budget RNA Mini Kit (Bio-Budget Technologies, Krefeld, Germany) and the RNase-free DNase Set (Qiagen, Hilden, Germany). Reverse transcription was performed on 0.8 µg total RNA using the SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific). The following validated QuantiTect primer assays (Qiagen) were used to determine transcript expression: hmb5 (QT00014462 (hs), QT00179123 (rm)), cav1 (QT0012607 (hs)), cav2 (QT0031920 (hs)), cav3 (QT00204071 (hs)), piezo1 (QT00088403 (hs), (QT00387604 (rm)), piezo2 (QT00046375 (hs), (QT01294510 (rm)). Amplification was performed on a StepOne-Plus qPCR cycler (Applied Biosystems, Foster City, CA, USA) using EvaGreen QPCR Mix II (Bio-Budget Technologies). Each reaction was carried out on cDNA from ≥5 independent preps. Specificity of PCR reactions was confirmed by melting point analysis of PCR products. StepOne Software 2.3 (Applied Biosystems, Germany) was used for data acquisition and analysis. Correction for PCR performance as well as quantification relative to housekeeping gene HMBS was carried out as described.51

2.10 | Western blotting

Cells were washed twice in PBS, solubilized in NuPAGE LDS Sample Buffer (Thermo Fisher Scientific), separated on an SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary antibody overnight at 4°C, immuno-detection was completed using secondary antibodies conjugated to alkaline phosphatase and subsequent chromogenic substrate application (WesternBreeze Chromogenic Kit, Thermo Fisher Scientific).

2.11 | Immunofluorescence

For immunofluorescence staining, cells were washed twice in DPBS (pH 7.4, Biochrom, Berlin, Germany) fixed for 20 minutes in 4% paraformaldehyde in DPBS and permeabilized with 0.2% saponin, 50 mM HEPES, and 10% FBS (Thermo Fisher Scientific, Bonn, Germany) in DPBS. Immunofluorescence staining for piezo1 was performed without permeabilization.52 For cells grown on PDMS membranes, membranes were cut and mounted on a Superfrost Plus microscope slide (Thermo Fisher Scientific). Cells were subsequently stained with primary (1:200) and secondary (1:300) antibodies in PBS, 0.2% saponin, 50 mM HEPES, and 10% FBS. Images were taken on an inverted confocal microscope (Leica TCS SP5, Leica, Germany) using a 63x lens (Leica HCX PL APO lambda blue 63.0 x 1.40 OIL UV), Images for the blue (Hoechst 33342), green (Alexa Fluor 488), red (Alexa Fluor 568), and far red (Alexa Fluor 647) channels were taken in sequential mode using appropriate excitation and emission settings.

2.12 | Precision-cut lung slices

Precision-cut lung slices were prepared as described previously.53 After pneumonectomy, the lungs were washed, instilled with 10 mL of low melting point agarose (3% w/v, 37°C), and put into PBS (4°C) for gelling. With a razor blade, the tissue was reduced to small pieces and embedded in agarose. Slices of 150 µm were cut using a vibratome, transferred to gelatine-coated microscope slides, and fixed with 4% PFA. Immunofluorescence staining was performed as described.

2.13 | ATP microbiosensors and quantification of ATP release

ATP measurements were performed using an electrochemical biosensor based on surface immobilized glucose oxidase/hexokinase as recently described.54,55 ATP microbiosensors were prepared by immobilizing glucose oxidase (from Aspergillus niger, 1500.000 U/g, Sigma-Aldrich Chemie GmbH) and hexokinase (from yeast, 2500 IU solid, Calbiochem) in a poly(benzoxazine) layer.54 Pt microelectrodes with a radius of 25 µm were modified by electrodeposition using mixtures of BA-TEPA and GOD/HEX in a ratio of 1:2.5 w/w; the activity ratio for the two enzymes was 1:1. The synthesis of the precursor was prepared as previously described.56 Depositions were performed with a potential pulse sequence of 1.7 V/0.05 s; 0.0 V/0.5 s and 14 repetitions of the pulse cycle. Calibrations were performed in tris-buffer at a potential of 0.7 V vs Ag/AgCl (3 M KCl) with the addition of glucose and ATP aliquots, respectively. All electrochemical experiments were performed with a three-electrode configuration with the microelectrode, microelectrode assembly or microbiosensor, respectively, as a working electrode, a Pt as a counter electrode (CE), and Ag/AgCl (3 M KCl) reference electrode unless otherwise stated.

Glucose oxidase catalyzes the oxidation of glucose that is present in the medium. This leads to the formation of hydrogen peroxide as byproduct when oxygen is the electron acceptor. Hydrogen peroxide is then oxidized at the sensor surface leading to an increase in current, which represents the baseline signal. If ATP is present in solution (eg, is released by the cells) hexokinase catalyzes the phosphorylation of glucose resulting in ADP and glucose-6-phosphate, hence consuming glucose and reducing the glucose concentration that is available for the glucose oxidase catalyzed process.
Therefore, the decrease in hydrogen peroxide reflected by a decrease in the faradaic current is proportional to the concentration of ATP.

To quantify stretch-induced ATP release, the sensor was positioned 200 µm above the cells and a constant potential of +0.7 V (vs. AgQRE) was applied. 8 mM glucose was added to the bath solution. After leveling of the current signal, the cells were stretched 20%-75% in longitudinal distension (3 seconds each) interrupted by unstretched periods of 60 seconds. The addition of 6 µM ATP at the end of the experiment was used to calculate ATP concentrations. Control experiments, without glucose in the bath solution, were
performed to exclude significant endogenous production of H₂O₂ in response to stretch. We did not observe a significant change in current in any of these experiments, indicating no change in endogenous H₂O₂ levels upon the stretch.

2.14 | Statistics

PSTrace 5.5 (PalmSens BV, Houten, Netherlands), MS Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 7 (GraphPad, San Diego, CA, USA) were used for statistics, curve fitting, and graph design. Unless otherwise stated all data are presented as mean ± SEM. Statistical significance was determined using analysis of variance (ANOVA with Bonferroni’s correction) for multiple comparisons and an unpaired, two-tailed Student’s t-test for the comparison of two independent samples. Data were considered significant if the P value was < .05, * indicates P < .05, ** indicates P < .01, and *** indicates P < .001.

3 | RESULTS

3.1 | Knock-out of caveolin-1 reduces stretch-induced Ca²⁺ signaling in an ATI cell line

We generated a caveolin-1 knock-out (KO) ATI cell line (hAELViwt) by CRISPR/Cas9-based gene-editing (Figure 1A) of a recently established ATI cell line (hAELViwt). hAELViwt cells display ATI-like characteristics, form functional tight and gap junctions and maintain a stable barrier under air-liquid culture conditions. Successful deletion of cav1 and caveolin-1 expression was verified by Sanger sequencing, PCR-based genotyping, Western blot (Figure 1B), gene expression analysis (Figure 1C), and immunofluorescence staining for caveolin-1 (Figure 1D). hAELViwt cells express α (24 kDa) and β (21 kDa) isoforms of caveolin-1. Both isoforms were absent in hAELViwt cells (Figure 1B). In line with previous studies, KO of cav1 reduced expression of cav2 on the protein level (Figure 1B), but not on the mRNA level (Figure 1C). Muscle-specific isoform cav3 is not expressed in hAELVi cells (Figure 1C).

Initially, we investigated the effects of mechanical strain on Ca²⁺ signaling in hAELViwt and hAELViwt cells. ATI cells were grown on flexible PDMS substrates. For mechanical stimulation, cells were mounted in a straining device placed on an inverted microscope (Figure 2A). The straining device compensated the lateral displacement during the stretch and enabled almost continuous observation of individual cells during mechanical stimulation. This allowed the analysis of intracellular Ca²⁺ signals at the single cell level (Figure 2B). Periodic stretching pulses (3s) with increasing amplitudes triggered intracellular Ca²⁺ signals at stretch amplitudes ≥15% in hAELViwt cells. The threshold was increased to ≥25% in hAELVi cells. Quantitative analysis of the percentage of ATI cells that responded to mechanical strain was significantly reduced in hAELVi cells (Figure 2C).

3.2 | Stretch-induced Ca²⁺ signaling in ATI cells depends on the activation of piezo1 localized to caveolin-1

Detailed analysis of stretch-induced Ca²⁺ signals revealed two different types of Ca²⁺ increase. A pronounced Ca²⁺ peak correlating with individual stretches that could be suppressed by extracellular apyrase, indicating ATP-dependent Ca²⁺ signaling via purinergic receptors. In addition, these distinct Ca²⁺ peaks were always preceded by little Ca²⁺ “humps” that were not responsive to apyrase, but depended on extracellular Ca²⁺ (Figure 2C).
and immunofluorescence confirmed the expression of piezo1 (Figure 3B,C). Piezo2 was not expressed in hAELVi cells. Piezo1 is also expressed in endogenous ATI cells as confirmed by immunofluorescence staining of piezo1 in caveolin-1 positive ATI cells in native precision-cut lung slices (Figure 3D).
FIGURE 4 Piezo1 co-localizes with caveolin-1 at the plasma membrane, but does not co-immunoprecipitate with caveolin-1. A, Illustration of split GFP complementation strategy. GFP1-10 (GFP10) and GFP11 are tagged to proteins of interest. When tagged proteins interact with each other to bring GFP10 and GFP11 sufficiently close they interact and generate green fluorescence. B, Left: Immunofluorescence images, Scale bar: 25 μm. Right: live cell images, Scale bar: 20 μm. Expression of caveolin1 tagged to GFP1-10 (cav1-GFP10), GFP11 (cav1-GFP11) or piezo1 tagged to GFP1-10 (piezo1-GFP10) does not result in green fluorescence in transfected cells. Co-expression of caveolin1 tagged to GFP1-10 (cav1-GFP10) with GFP11 (cav1-GFP11) and co-expression of piezo1 tagged to GFP1-10 (piezo1-GFP10) and GFP11 (cav1-GFP11) resulted in a GFP signal at the PM of hAELVi\(^{cav−/−}\) cells when transfected with both constructs. Please note that in the last panel 3 cells express piezo1-GFP10, but a GFP signal is only visible in the cell also expressing cav1-GFP11. C, GFP-Pull down and co-immunoprecipitation of endogenous caveolin1 (left) and piezo1 (right) from non-transfected cells, cells transfected with GFP-N1 or caveolin-1-GFP (cav1-GFP). Caveolin-1, but not piezo1 co-immunoprecipitated with caveolin1-GFP. L: cell lysate; E: bead eluate.

We used pharmacological inhibition as well as the siRNA knockdown of piezo1 to investigate a role for piezo1 channels in the generation of stretch-induced Ca\(^{2+}\) signals. siRNA knockdown reduced piezo1 expression by >80% in hAELVi\(^{wt}\) when compared to non-treated cells or cells transfected with non-targeting (nt) siRNA (Figure 3E). Inhibition (5 μM GsMTx4) and knockdown of piezo1 significantly reduced stretch-induced Ca\(^{2+}\) signals in hAELVi\(^{wt}\) cells to levels observed in hAELVi\(^{cav−/−}\) cells. Inhibition had no effect on stretch-induced Ca\(^{2+}\) signaling in hAELVi\(^{cav−/−}\) cells. A selective activator of piezo1 (10 μM Yoda1), however, rescued the response to mechanical strain in hAELVi\(^{cav−/−}\) cells to hAELVi\(^{wt}\) cell levels (Figure 3F), strongly suggesting a role for caveolae in stretch-dependent piezo1 activation. To further characterize the interplay between piezo1 and caveolae, we used split GFP bimolecular fluorescence complementation (biFC) and co-immunoprecipitation assays to investigate co-localization and direct interaction of piezo1 and caveolin-1, respectively. Expression of single split-GFP constructs did not result in GFP fluorescence. Expression of caveolin1-GFP1-10 (cav1-GFP10) and caveolin1-GFP11 (cav1-GFP11) or piezo1-GFP1-10 (piezo1-GFP10) and cav1-GFP11 resulted in a substantial fluorescent signal, suggesting close co-localization of caveolin-1 and piezo1 in hAELVi cells, respectively (Figure 4A,B). However, piezo1 did not co-immunoprecipitate with caveolin1-GFP (Figure 4C). In summary, these results indicate mechanical-stretch induced activation of Ca\(^{2+}\) entry via piezo1 which is in close proximity to, but does not directly interact with caveolin-1.

3.3 Stretch induced Ca\(^{2+}\) signals trigger ATP release from ATI cells via pannexin hemichannels and amplify Ca\(^{2+}\) signals in ATI cells

Subsequent measurements of stretch-induced ATP release using ATP microbiosensors\(^54\) (Figure 5A) confirmed that ATP release from hAELVi cells is dependent on the expression of caveolin-1 and Ca\(^{2+}\) entry via piezo1. ATP release from hAELVi\(^{wt}\) and hAELVi\(^{cav−/−}\) cells increased with increasing stretch amplitudes, however, release from hAELVi\(^{cav−/−}\) was significantly lower. Inhibition and knockdown of piezo1 significantly reduced ATP release in \(^{wt}\) cells to levels released from \(^{cav−/−}\) cells and release from \(^{cav−/−}\) cells was strongly amplified by Yoda1.

ATP release from hAELVi\(^{wt}\) cells was also almost completely blocked by \(^{10}\)panx, a small peptide inhibitor of pannexin hemichannels, and siRNA knockdown of pannexin-1 (Figure 5B,C). Upon release, ATP stimulated Ca\(^{2+}\) signaling in hAELVi cells via P2 purinergic receptors, likely P2Y\(_2\) receptors. Knockdown of pannexin-1 and inhibitors of P2Y receptors (suramin), G\(_{q}\)-signaling (YM-254890) and PLC activation (edelfosine) significantly and almost completely inhibited pronounced Ca\(^{2+}\) peaks in hAELVi\(^{wt}\) cells. There was little effect on Ca\(^{2+}\) signaling in hAELVi\(^{cav−/−}\) cells, in line with the reduced ATP release in these cells (Figure 5D). Pannexin-1 also co-localized with caveolin-1 (Figure 6A), but did not co-immunoprecipitate with caveolin1-GFP and neither did caveolin1 co-immunoprecipitate with endogenous pannexin-1 (Figure 6B).

3.4 Deletion of cav1 in ATI-like cells reduces stretch-induced paracrine stimulation of Ca\(^{2+}\) signals and LB exocytosis in ATI cells

To investigate the effects of caveolin-1 and piezo1-dependent mecano-signaling and ATP release from ATI cells on surfactant secretion in the alveolar epithelium, we reconstituted the alveolar epithelium in vitro, co-culturing hAELVi cells, and primary ATI cells. Co-culture resulted in a mixed monolayer of ATI and hAELVi cells at a ratio of approx. 40:60. In line with previous reports,\(^27,57\) only ATI-like cells but not primary ATI cells expressed caveolin-1 (Figure 7A). Connexin-43, a constituent of connexin hemichannels, was also expressed at ATI-ATII and ATII-hAELVi cell junctions (Figure 7B).

Ca\(^{2+}\) signaling and LB exocytosis in ATI cells were significantly increased in co-culture with hAELVi\(^{wt}\) cells compared to ATI cells maintained in monoculture. The effect was significantly reduced when ATI cells were...
co-cultured with hAELVi\textsuperscript{cav−/−} cells (Figure 7C,D). In particular, the percentage of primary ATII cells with at least one LB fusion event within 5 minutes of a short 60% stretch stimulus (% responding ATII cells) was significantly increased in ATII co-cultured with hAELVi\textsuperscript{wt} cells when compared to ATII monoculture or co-culture with hAELVi\textsuperscript{cav−/−} cells (Figure 7D). These findings suggest that the stretch-dependent secretion of pulmonary surfactant
FIGURE 5 Stretch induced Ca\textsuperscript{2+} signals trigger ATP release from ATI cells via pannexin hemichannels and amplify Ca\textsuperscript{2+} signals in ATI cells. A, Left: Schematic illustration of the biosensor experimental straining setup. The ATP biosensor is positioned 200 μm above the cells. Right: Time-current graph recorded with an ATP biosensor (black) and respective stretch amplitudes (grey). 8 mM Glucose was added at the start of the experiment as a substrate for the enzymatic glucose oxidase/hexokinase reaction. Release of ATP or addition of ATP (6 μM, t = 590s) resulted in a decrease in current. B, Knockdown of panxin-1 in untreated hAELVi\textsuperscript{wt} cells (wt), hAELVi\textsuperscript{wt} cells transfected with non-targeting (nt), and panx-1 siRNA, respectively. C, Left: ATP concentrations under different stretch amplitudes for hAELVi\textsuperscript{wt} (wt) cells under control conditions, following knockdown/pharmacological inhibition of piezo1 (piezo1 siRNA, GsMTx4) or pannexin-1 (panx-1 siRNA, 10\textsuperscript{panx}). Also depicted, hAELVi\textsuperscript{cav−/−} cells under control conditions (cav\textsuperscript{−/−}) or in the presence of activator of piezo1 (Yoda1). Right: Quantitative analysis of ATP concentrations at a stretch amplitude of 75%. Knockdown of piezo1 (piezo1 siRNA) or panxin-1 (panx-1 siRNA) and 10\textsuperscript{panx} significantly inhibited ATP release from hAELVi\textsuperscript{wt} cells, whereas Yoda1 increased ATP release from hAELVi\textsuperscript{cav−/−} cells. Transfection with non-targeting siRNA (nt siRNA) had no effect on Ca\textsuperscript{2+} signals in hAELVi\textsuperscript{wt} cells. Data represent 5-6 independent experiments. D, Left: Percentage of hAELVi\textsuperscript{wt} (wt) cells that responded to stretch with an intracellular Ca\textsuperscript{2+} signal at different stretch amplitudes following knockdown of panxin-1 (panx-1 siRNA) or under inhibition of purinergic receptors (suramin, YM-254890) or PLC (edelfosine). Right: Quantitative analysis of the percentage of hAELVi\textsuperscript{wt} (wt) and hAELVi\textsuperscript{cav−/−} (cav\textsuperscript{−/−}) cells that responded at a stretch amplitude of 75%. Knockdown of panxin-1 (panx-1 siRNA) and inhibition of P2Y receptor signaling (suramin, YM-254890, edelfosine) significantly inhibited the generation of Ca\textsuperscript{2+} signals in hAELVi\textsuperscript{wt} cells. A similar, but weaker effect was observed in hAELVi\textsuperscript{cav−/−} cells. Data represent 5-16 independent experiments.

from ATII cells can be stimulated by ATI cells in a caveolin-1-dependent manner. 

Co-culture-dependent Ca\textsuperscript{2+} signaling in ATII cells was, again, strictly dependent on extracellular Ca\textsuperscript{2+}. Moreover, amplification of Ca\textsuperscript{2+}-signaling in ATII cells required close proximity to ATI-like (hAELVi) cells. The response was significantly reduced in ATII cells without a hAELVi\textsuperscript{wt} cell within a 30 μm perimeter. Previous reports suggested a role for direct cell-cell communication and Ca\textsuperscript{2+} signaling via gap junctions in ATII-dependent stimulation of Ca\textsuperscript{2+} signals in ATII cells. However, we could neither detect direct gap junctional communication by fluorescent dye (Calcein AM) transfer nor did heptanol inhibit ATII-dependent, stretch-activated Ca\textsuperscript{2+} signals in ATII cells. Inhibition and/or knockdown of purinergic signaling, pannexin hemichannels or piezo1, however, significantly reduced ATII-dependent Ca\textsuperscript{2+} signaling in ATII cells co-cultured with hAELVi\textsuperscript{wt} cells. The percentage of responding cells at 75% strain was no longer significantly higher than signals observed in ATII mono-culture or ATII cells co-cultured with hAELVi\textsuperscript{wt} cells. In ATII/ hAELVi\textsuperscript{cav−/−} co-cultures, inhibition of purinergic signaling, pannexin hemichannels or piezo1 had no significant effect on Ca\textsuperscript{2+} signals in ATII cells. Yet, Yoda1 treatment and reconstitution of caveolin-1 expression almost restored the response observed in the co-culture of ATII and hAELVi\textsuperscript{wt} cells (Figure 7E).

In summary, our data suggest a model in which stretch of the alveolar epithelium leads to caveolin-1 dependent activation of piezo1 in ATI cells. Ca\textsuperscript{2+}-entry via piezo1 activates pannexin hemichannels, by a yet unknown mechanism, and results in ATP release from ATI cells. Extracellular ATP then amplifies Ca\textsuperscript{2+}-signaling in ATII cells and stimulates Ca\textsuperscript{2+} signals and LB exocytosis in neighboring ATII cells via the activation of P2Y\textsubscript{2} receptors (Figure 8).

4 | DISCUSSION

It is well established that the mechanical deformation/expansion of alveoli constitutes the strongest stimulus for surfactant secretion. However, it has been a long-standing puzzle, how mechanical deformation is sensed at the molecular level within the alveolus and how these mechanical cues are translated into biochemical signals, like an increase in intracellular Ca\textsuperscript{2+}, that trigger LB exocytosis and surfactant secretion. Our results suggest that caveolae within ATI cells may serve as mechanosensors in the alveolus. Caveolae have been found to contribute to mechanosensing in response to various mechanical stimuli, such as membrane stretching, shear stress, hypoosmotic shock, and cell detachment in various cells. It has been proposed that caveolar invitations in the plasma membrane flatten when mechanical tension is imposed upon the membrane, buffering tension forces, and triggering eliciting signals to induce adaptive responses. Such a change in membrane curvature could be the trigger to activate piezo1 channels residing within caveolae. Piezo1 gating can be directly mediated by changes in membrane tension in membrane blebs. We believe that the resulting Ca\textsuperscript{2+} influx leads to the opening of panxin-1 hemichannels and the resulting ATP release stimulates surfactant secretion from ATII cells in a paracrine manner. This is in line with previous findings that a rise in intracellular Ca\textsuperscript{2+} activates panxin-1 hemichannels and leads to ATP release. Whether Ca\textsuperscript{2+} activates panxin-1 directly or indirectly is yet a matter of debate. However, it is tempting to speculate that caveolae serve as signaling hubs for molecular scaffolds, maintaining piezo1, and panx-1 in close proximity within confined domains to ensure efficient signaling and activation of panx-1 by the small Ca\textsuperscript{2+} signals (”humps”) following piezo1 activation. Localization of panx-1 to caveolin-1 and caveolae has been reported in various cell types, and also that caveolin-1 and panx-1 functionally couple with each
other in vascular smooth muscle cells upon adrenergic stimulation. Results from our co-immunoprecipitation experiments do not suggest that panx-1 and caveolin-1 do directly interact; however, we cannot exclude, that panx-1 transiently interacts with caveolin-1 immediately after stretch-induced Ca²⁺-entry. There is also ample evidence that the activation of P2Y₂ receptor signaling and elevation of intracellular Ca²⁺ result in panx-1 opening. All of these reports are in line with our finding that Ca²⁺ entry via piezo1 activates panx-1 and this is further amplified by an autocrine stimulation of P2Y₂ receptors on ATI cells.

In the in vivo situation, ATP is likely secreted to the alveolar lumen. It has been reported that ATP is present in the pulmonary hypophase; however, we cannot exclude, that panx-1 transiently interacts with caveolin-1 immediately after stretch-induced Ca²⁺-entry. There is also ample evidence that the activation of P2Y₂ receptor signaling and elevation of intracellular Ca²⁺ result in panx-1 opening. All of these reports are in line with our finding that Ca²⁺ entry via piezo1 activates panx-1 and this is further amplified by an autocrine stimulation of P2Y₂ receptors on ATI cells.

ATP can also be released from ATII cells and a recent report has found that ATII cells are the primary source of ATP release in mechanically stretched lungs. Although we did not assess ATP release from ATII cells directly, we previously reported that ATP is released from ATII cells upon exocytosis of LBs. These findings are not mutually exclusive. ATP can be released from ATI and ATII cells. However, our data suggest that ATII cells are more sensitive to mechanical strain. This is dependent on the expression of caveolin-1. The study from Tan et al assessed ATP release from primary ATII cells and from ATII-like cells derived from trans-differentiated ATII cells. It is not known whether ATII-derived ATII cells express caveolin-1. ATII cells do not express caveolin-1. Hence, it is possible that both ATI and ATII cells release ATP in response to mechanical stimulation, but with different sensitivity to deformation. This would constitute an integrated mechanism to adjust surfactant secretion to the magnitude of mechanical stretch and hence the extend of lung inflation. In fact, adjusting surfactant secretion to demand. It has been postulated that ATII cells, which are usually located in the alveolar corners, could exhibit lower stretching during alveolar distension than ATI cells located on the alveolar

**FIGURE 6**  Pannexin-1 co-localizes with caveolin-1 at the plasma membrane, but does not co-immunoprecipitate with caveolin-1. A, Co-expression of caveolin-1 tagged to GFP1-10 (cav1-GFP10) and pannexin-1 tagged to GFP11 (panx1-GFP11) resulted in GFP signal at the PM (arrows) of hAELViwt cells. Scale bar: 20 μm. B, Left: GFP-Pull down and co-immunoprecipitation of endogenous pannexin-1 from non-transfected cells, cells transfected with GFP-N1 or caveolin-1-GFP. Caveolin-1, but not panx-1 co-immunoprecipitated with caveolin-1-GFP Right: Pull-down of endogenous pannexin-1 using control (−) or α-pannexin-1 antibody-coated (+) beads and detection of endogenous caveolin-1. L: cell lysate; E: bead eluate.
Hence, at lower levels of alveolar distension, large ATI cells which cover >95% of the alveolar surface, might be sufficiently stretched to trigger caveolin-1 and piezo1-dependent Ca\(^{2+}\)-entry, ATP release and paracrine stimulation of ATII cells. At higher stress levels direct activation of ATII cells then triggers strain-induced Ca\(^{2+}\)-entry via a yet unknown mechanism and exocytic release of ATP from ATII cells. This can amplify surfactant secretion and/
or facilitate surfactant release.\textsuperscript{14,50} Our model does not fully recapitulate this heterogeneous stretching pattern, and hence the contribution of stretch-induced Ca\textsuperscript{2+}-entry in ATII cells could be even overestimated and paracrine stimulation from ATII cells might play the predominant role under physiological stretch conditions. Moreover, we do observe a heterogeneous response of alveolar epithelial cells to mechanical deformation as has been described before.\textsuperscript{9,10,74-76} This likely reflects individual cellular properties such as cell-matrix and cell-cell attachments, cytoskeletal structure, cell morphology, and orientation within the strain field that will affect stretch-induced force generation within single cells in vivo.\textsuperscript{74}

There is also ample evidence that ATP and Ca\textsuperscript{2+} can be delivered directly between cells, most notably via gap junctions. This has also been proposed for the communication between ATI and ATII cells,\textsuperscript{13} as highlighted in the introduction. However, we do not have any indication that this is the case in our model. We find the expression of gap junctional proteins (connexin-43) between hAELVi and ATII cells. However, we could neither detect direct gap junctional communication by fluorescent dye (Calcine AM) transfer nor did the inhibition of gap junctions (heptanol) had any effect on the paracrine stimulation of ATII cells following stretch. Moreover, apyrase and the inhibition of P2Y\textsubscript{2} receptors significantly reduced Ca\textsuperscript{2+} signals in ATII cells to levels elicited in ATII cell monoculture upon stretch. We can also not fully exclude the possibility that either ATI or ATII cells release ATP as a result of temporary membrane breaks that occur and reseal during mechanical stretch. However, we do believe that this could only account for a minor fraction of the released ATP for the following reasons. (1) PM breaks result in an immediate loss of Fura-2 AM from the cytosol.\textsuperscript{10} Loss of Fura-2 from single cells was only observed in very rare cases (<2% at stretch amplitudes >60%). This is in line with previous reports that primary ATII cells tolerate increases to 40–50% in CSA for brief stretching periods\textsuperscript{10,42,74,75} and that ATI-like cells tolerate mechanical deformation even better than ATII cells.\textsuperscript{74} (2) ATP release from ATI cells was very efficiently (almost completely) impaired by the inhibition/KD of panx-1, suggesting release via panx-1 activation as the major ATP source from these cells. Together, our data suggest the dependence on a regulated, extracellular, paracrine and ATP-dependent signaling pathway to transmit mechano-responses from ATI to ATII cells.

We are aware that our experiments cannot fully explain the in vivo scenario nor can we exclude that other, maybe even synergistic, mechano-sensitive mechanisms stimulate surfactant secretion in vivo, as surfactant is fundamental for lung function. It has been demonstrated that stretch results in leptin release from lipofibroblasts adjacent to ATII cells in the rodent lung triggering surfactant release in a paracrine manner.\textsuperscript{77} Equally, changes in air-liquid surface tension might impact on surfactant secretion.\textsuperscript{78} However, it was the purpose of this study to selectively focus on the molecular mechanism underlying stretch (mechanical deformation) induced Ca\textsuperscript{2+} signaling, LB exocytosis, and surfactant secretion in the alveolar epithelium with single cell resolution.

We have aimed at replicating the in vivo stretch impact. Stretch amplitudes used in the current study were based on previous works from others\textsuperscript{7,9,74-76,79-83} and ourselves\textsuperscript{10,42} It is yet debatable how inflation (% total lung capacity, TLC) correlates to the % increase in alveolar surface area.\textsuperscript{84} It was estimated that the change in the alveolar epithelial basement membrane surface area is approx. 35%-40% or even higher at total lung capacity (TLC).\textsuperscript{84,85} The maximum of 75% increase in longitudinal extension in our device correlates to
an increase in cell-substrate area (CSA) of approx. 35% as a result from longitudinal extension (75%) and perpendicular compression (18%). The translations from in vivo to in vitro are rough estimations and stretch of individual cells is likely heterogeneous in vivo and depends on many variables. As outlined above, in vivo distension was found to be greater in wall segments associated with ATI cells than those associated with ATII cells. Also, little is known about the impact of compression on alveolar epithelial cells and surfactant secretion in particular. It has been suggested that lung inflation leads to a thinning of the alveolar lining fluid and a concomitant increase in surface tension. Either fluid thinning or increase in surface tension, but most likely both in concert, impose a compressive pressure onto the apical side of ATII cells inducing Ca\(^{2+}\) signals in ATII cells and stimulating surfactant secretion.

The application of cyclic compressive stress-induced sustained liquid secretion that appeared dependent on nucleotide release in the monolayers of primary ATII cells. These reports suggest that compressive forces can induce ATP release and stimulate Ca\(^{2+}\) signals and LB exocytosis in ATII cells. Hence, we cannot exclude that compressive stresses originating from the application of uniaxial, longitudinal stretch contribute to the signals observed in our study. Even in an intact alveolus, distending and compressive forces might act simultaneously during lung inflation and be heterogeneously distributed. The intermittent stretch regimen used had to compromise between being physiologic (intermittent stretch, 3-4s duration of a single distension for a single inflation at a respiratory rate of 15-20 breath/minutes) and technical prerequisites of having a sufficiently long relaxed, inter-stretch period to analyze individual intracellular Ca\(^{2+}\) signals. On average, Ca\(^{2+}\) signals lasted for 15-60s. To prevent super-positioning of Ca\(^{2+}\) signals, we choose an inter-stretch period of 60 seconds to allow for Ca\(^{2+}\) signals to abate and prevent the summation of consecutive stretch-induced Ca\(^{2+}\) signals. Although deformation frequency, magnitude, seeding density, and culture duration and other factors impact cellular responses, we believe that the current study design and protocol allows to specifically investigate the cellular and molecular response of ATI-like cells and ATII cells to a near physiological stretch stimulus without major impact of other biochemical/cellular factors found in an intact alveolus. Of course, one could think of a multitude of different stretch regimens to mimic the in vivo situation even closer. A period of small intermittent distensions followed by a rapid and high stretch to mimic a sigh or yawn, previously reported to stimulate surfactant secretion. Similarly, intermittent stretch with high amplitudes to mimic exercise. Compression to mimic deflation to residual volume. However, this was not the aim of this study. The aims of this study were to decipher molecular mechanisms (1) how alveolar epithelial cells “detect” stretch (ie, identify potential mechanosensors), (2) how stretch is translated into intracellular (Ca\(^{2+}\)) signals, and 3) focus on differences and the cellular interplay between ATI and ATII cells at the single-cell level.

It will certainly be interesting to validate the physiological impact of the mechanism proposed by our results in an in vivo setting. However, deletion of caveolin-1, piezo1 or panx-1 to selectively investigate the effects thereof might induce significant changes in lung mechanics, making it difficult to compare the impact of lung inflation (stretch) on surfactant secretion in wt and knock-out animals.

Lungs from caveolin-1 deficient mice have constricted alveolar spaces, associated with a thickening of the alveolar

**FIGURE 8** Proposed model of mechanosensing and stretch-induced surfactant secretion in the alveolus. *Left:* Proposed localization of piezo1 and pannexin-1 in the caveolae of ATI cells. P2Y\(_{2}\) receptors are expressed in ATI and ATII cells. *Right:* Stretch of the alveolar epithelium leads to caveolin-1 dependent activation of piezo1 in ATI cells. Ca\(^{2+}\)-entry via piezo1 activates pannexin hemichannels by a yet unknown mechanism and results in ATP release from ATII cells. Extracellular ATP then amplifies Ca\(^{2+}\)-signaling in ATII cells and stimulates Ca\(^{2+}\) signals and LB exocytosis in neighboring ATII cells via activation of P2Y\(_{2}\) receptors.
wall and resemble a fibrotic phenotype with reduced lung compliance.29 Hence, the mechanical properties of the distal lung are severely.24,25 In part, this has been attributed to increased ECM production by mesenchymal cells, inflammation, and cell proliferation.29 However, our data suggest another important role of caveolin-1 and caveolae for maintaining alveolar and lung homeostasis via regulating surfactant secretion from ATII cells. Impaired surfactant secretion results in surfactant dysfunction which fails to stabilize alveoli at low lung volumes during ventilation.86 Surfactant dysfunction has been linked with diseases resulting from repetitive or ongoing injury of the alveolar epithelium such as idiopathic pulmonary fibrosis (IPF).87 Although the impact of high surface tension for disease progression in IPF is currently not clear, animal models suggest a relevant causal relationship between ATII cell dysfunction, surfactant dysfunction, high surface tension with mechanical stress, and profibrotic remodeling.88 Restoring surfactant homeostasis via surfactant replacement therapy reduced lung remodeling in a bleomycin model of pulmonary fibrosis.89 Therefore, it is tempting to speculate that impaired stimulation of surfactant secretion due to the loss of caveolin-1 might contribute to the surfactant dysfunction and development of the fibrotic phenotype observed in cavelin-1 deficient mice and rare human patients that lack caveolae due to mutations in cav1.90

Overall, our findings support the notion that the high density of caveolae in ATII cells suggests an important role of these invaginations in the physiology of the lungs. Caveolin-1 expression might not only be vital for regulating surfactant secretion under physiological conditions, but also to protect alveoli from repetitive stresses promoting the development of fibrotic remodeling.

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CONFLICT OF INTEREST
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
M. Frick designed the research. All authors performed the experiments and analyzed the data. K. Diem, C. Kranz, and M. Frick wrote the manuscript.

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