

## Original Paper

# Microarray-Based Gene Expression Profiling Suggests adaptation of Lung Epithelial Cells Subjected to Chronic Cyclic Strain

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Rolf-Edgar Silber<sup>a</sup> Babett Bartling<sup>a</sup><sup>a</sup>Department of Cardio-thoracic Surgery, Heart Center of the University Hospital Halle, Martin Luther University Halle-Wittenberg, <sup>b</sup>Center of Medical Basic Research, Medical Faculty, Martin Luther University Halle-Wittenberg, Halle /Saale, Germany**Key Words**

Epithelial cells • Cyclic strain • Gene microarray • Lung • mRNA expression • qPCR

**Abstract**

**Background/Aims:** Mechanical strain of the lung tissue is a physiological process that affects the behavior of lung cells. Since recent evidence also suggests alterations in the expression of certain genes as a consequence of mechanotransduction, our study aimed at the analysis of the gene expression profile in lung epithelial cells subjected to chronic cyclic strain. **Methods:** Various human lung epithelial cell lines (A549 as principal adherent cell line and four others) were subjected to cyclic strain (16 % surface distension, 12 min<sup>-1</sup>) in a Strain Cell Culture Device for 24 h. In comparison to static controls, expression analyses were performed by gene microarray and qPCR. **Results:** Microarray analysis revealed many differences in the gene expression but at moderate levels. Altogether 25 genes were moderately down-regulated (0.86-fold ± 0.06) and 26 genes were up-regulated (1.18-fold ± 0.10) in A549 and the others. Strain-regulated genes often code for transcription factors, such as E2F4 and SRF. qPCR analyses confirmed the up-regulation of both transcription factors and further genes, such as PLAU (urokinase-type plasminogen activator) and S100A4 (S100 protein A4). Moreover, we showed the down-regulations of AGR2 (anterior gradient 2) and LCN2 (lipocalin 2). **Conclusions:** We identified many genes of which the expression was moderately altered in lung epithelial cells subjected to chronic cyclic strain. Although many moderate changes in the gene expression profile might affect cellular behavior, it also suggests an effective adaptation of cells to mechanical forces in long-term conditions.

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## Introduction

Mechanical forces influence biological processes in cells and tissues. This is particularly the case in the alveolar tissue of the lung, which is continuously subjected to mechanical strain due to breathing. Mechanical strain plays a central role in lung development and maturation as shown by studies of the effect of mechanical forces on fetal lung cells. Chronic mechanical distension of these cells enhances cell proliferation and increases expression of elastin and surfactant proteins [1, 2]. In addition, mechanical strain supports the transition of alveolar type (AT) II cells to AT I cells [3], a process important to maintenance and repair of the alveolar epithelium [4]. However, non-physiological strain of the lung tissue can contribute to pulmonary diseases. While over-distension by mechanical ventilation can cause acute lung injury (ALI) [5], under-distension in stiffer fibrotic areas has been suggested to support the development of lung fibrosis [6].

During normal tidal breathing, alveoli undergo periodical changes in the volume; however, the magnitude of mechanical strain experienced by alveolar cells *in vivo* is difficult to determine. Morphometric analyses indicate that inflation from the functional residual capacity to the normal tidal volume causes minimal cell surface area distension only (0 to 10 %), whereas inflation from the residual capacity to the total lung capacity (TCL) causes a higher distension of approximately 15 to 45 % [7]. These values might vary in old or injured lungs because the distension of cells depends on many factors, including the functional residual capacity [8]. Since it is currently not possible to determine exactly the cellular distension *in vivo*, most research groups studying the effect of mechanical strain on lung cells *in vitro* perform their experiments with a cell surface elongation between 10 and 20 % to mimic physiological conditions [1, 2, 9-12]. Depending on the cell type higher magnitudes result in a pro-inflammatory cell phenotype and cell death [13, 14]. In these studies, self-constructed or commercially available devices were used to apply static or cyclic strain to lung cells attached to flexible membranes *in vitro*. We also designed and constructed a cell culture device, in which adherent cells can be subjected to multi-axial strain at variable magnitudes and frequencies [15].

Mechanical strain modifies the phenotype of cells following strain-induced conformational changes of transmembrane proteins, such as cell adhesion molecules like integrins, surface receptors and ion channels, and then reorganization of the cytoskeleton [16]. In lung epithelial monolayers, mechanotransduction by cyclic distension leads to reorganization of F-actin into a perijunctional actin ring [15, 17, 18]. The mechanotransduction might lastly alter the expression of various genes, as already demonstrated for SFTPC (coding for surfactant protein C) in fetal AT II cells [1, 19], IL8 (interleukin 8) in bronchial and alveolar epithelial cells [20, 21], and further genes in the same cell types or in other types of lung cells.

Gene microarray studies have been performed to identify genes, which are up- or down-regulated in alveolar epithelial cells subjected to cyclic mechanical strain *in vitro* [11, 22-24]. However, a comparison of these studies revealed only few parallels probably because of differences in the cell type used and strain conditions applied. Moreover, most experimental studies applied strain conditions rather related to the respiratory frequencies in newborns or infants than to frequencies in adults ( $\geq 30 \text{ min}^{-1}$ ) except for the study of Yerrapureddy *et al.* ( $15 \text{ min}^{-1}$ ) [24]. Also, the time of analysis varied strongly (1 to 24 h), and only one study analyzed long-term effects of cyclic strain on the gene expression [11]. Therefore, our study aimed at the identification of genes, which are differentially expressed in various lung epithelial cells each subjected to long-term cyclic distension at adult frequency conditions.

## Material and Methods

### *Adherent lung cells and culturing conditions*

Various lung alveolar epithelial cells (AT II cell-like A549 [25], serous glandular epithelial cell-like Calu-3 [26], Clara cell-like NCI-H322 and NCI-H358 [27]) and immortalized bronchial epithelial BEAS-

2B cells [28] were studied (all cells from the ATCC cell bank, Manassas, VA). Alveolar epithelial cells were cultured on plastic cell culture dishes in DMEM (for A549, NCI-H322, and NCI-H358) or MEM (for Calu-3) each supplemented with 10 % fetal calf serum (FCS; Perbio, Bonn, Germany) and a mixture of 50 units/mL penicillin and 50 µg/mL streptomycin (Life Technologies, Darmstadt, Germany). BEAS-2B cells were cultured on pre-coated plastic dishes in AECG medium (Promocell, Heidelberg, Germany). Pre-coating was performed overnight with 8.6 µg/cm<sup>2</sup> collagen A (Biochrom), 2.8 µg/cm<sup>2</sup> fibronectin (Biochrom, Hamburg, Germany), and 2.8 µg/cm<sup>2</sup> bovine serum albumin (BSA; Sigma, Steinheim, Germany). Cells were cultured at 37 °C in an incubator (95 % humidity, 5 % CO<sub>2</sub> atmosphere). Potential mycoplasma contaminations were examined by PCR [29].

All cells were strained in a self-constructed Strain Cell Culture Device, which applies cyclic multi-axial strain to adherent cells in four separate strain chambers of 30 mm internal diameter [15]. Cells are stretched in radial and circumferential direction, and the strain amplitude depends on the position of the cells in the strain chamber [30]. For strain experiments, the adherent cells were reseeded from plastic dishes to the portable strain chambers containing a pre-coated flexible StageFlexer® silicone membrane (Flexcell International Corp, Hillsborough, NC). In some cases, the silicone membrane had to be pre-coated overnight. We used FCS for Calu-3, a mixture of 8.6 µg/cm<sup>2</sup> collagen A (Biochrom), 2.8 µg/cm<sup>2</sup> fibronectin (Biochrom), and 2.8 µg/cm<sup>2</sup> BSA (Sigma) for BEAS-2B, or 1.4 µg/cm<sup>2</sup> RGD peptide (Bachem AG, Bubendorf, Switzerland) photochemically immobilized with 2.8 µg/cm<sup>2</sup> Sulfo-SANPAH (Thermo Scientific, Dreieich, Germany) [31] for NCI-H358.

After two to four days of cell growth until a confluent monolayer was formed, the portable strain chambers were placed into the Strain Cell Culture Device at 37 °C in an incubator. For cyclic strain and static controls, we only used chambers showing comparable confluent monolayers. Experiments were performed as duplicates and samples were finally mixed. Cells were subjected to cyclic strain at a frequency of 12 min<sup>-1</sup> and surface elongation ( $A_m$ ) of maximum 16 % (equivalent to a linear distension of 10 %) with an equal duration of stretch and relaxation. The required maximal deflection of the silicone membrane ( $h$ ) was calculated according to the formula of a spherical segment ( $A_m = \pi \times (\text{radius of the cell chamber}^2 + h^2)$ ). The chosen  $A_m$  corresponds to the change in the epithelial basement membrane  $A_m$  between residual volume and approximately 75 % total lung capacity (TLC) according to the formula  $\% \Delta A_m = 0.0057 \times (\% \text{TLC})^2 - 0.2608 \times (\% \text{TLC}) + 4.8021$  [32], which is derived from morphometric studies on rat lungs [8]. Control cells were also cultured in the portable strain chambers but without application of strain. After finishing the strain experiment the integrity of the cell monolayer was checked by inverted light microscopy.

#### Gene microarray analysis

Total RNA was isolated from cells by use of the TRIzol® reagent (Life Technologies) in combination with the InviTrap Spin Cell RNA kit (Stratek, Berlin, Germany). RNA concentration was determined with the NanoDrop spectrophotometer (Thermo Scientific), and RNA quality was checked with the RNA Nano Chips in the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The mRNA of the total RNA was specially reverse transcribed to double-stranded cDNA, transcribed to antisense cRNA, and then reverse transcribed to single-stranded cDNA by simultaneous incorporation of deoxyuridine (Ambion® WT Expression kit; Applied Biosystems, Darmstadt, Germany). After fragmentation and labeling with biotin (WT terminal Labeling kit; Affymetrix, Santa Clara, CA), cDNA was subjected to hybridization procedure in the GeneChip Fluidics station 450 using GeneChip Human Gene 1.0 ST Arrays (analysis of 28,869 genes) and the Hybridization Wash and Stain kit (all compounds from Affymetrix). After scanning the arrays in the GeneChip Scanner 7G with GeneChip Command Console 3.1 software, quality assessment, background adjustment by the GC Robust Multi-array Average (GCRMA) algorithm, and data calculation were performed with the Affymetrix® Expression Console 1.3.1 software (all compounds from Affymetrix). GCRMA is an optimized Robust Multi-array Average (RMA) method, which additionally uses sequence information and GC content of the hybridization probe to estimate non-specific bindings for background correction [33]. Gene Ontology (GO) for *Homo sapiens* was used to annotate all genes.

#### Relative quantitative real-time polymerase chain reaction (qPCR)

cDNA was synthesized from total RNA by use of ABgene® anchored oligo-dT primers (Thermo Scientific), random hexamer primers, dNTPs, and RNase H<sup>-</sup> M-MLV Reverse Transcriptase (all compounds from Promega, Mannheim, Germany). cDNA was then amplified by gene-specific primer pairs (Table 1)

**Table 1.** Human-specific primer pairs used for qPCR

Gene name	Reference number	5' - 3' sequence		Fragment size (bp)	Annealing (°C)
		sense	antisense		
18S rRNA	M_10098	GTTGGTGGAGCGATTGTCTGG	AGGGCAGGGACTTAATCAACGC	345	58
ACTB	NM_001101	GAAGTGTGACGTGGACATCCG	AGCATTTCGGGTGGACGAT	290	58
AGR2	AF007791	GCCAAAAAGGACACAAAGGA	GGACAACTGCTCTGCCAAT	232	58
CALM1	AK289937	CCCAAGTTGACTTGACTTTG	CACATCTCCAGCCTTCAT	231	58
E2F4	AB451292	CCCTAGCTGTACGCCAGAAG	GTTCTGCACGCTCTGTGA	257	58
ECE1	AK304167	CAATGAATCCGAGCCTATTGTG	CCGCACCAGGTTCAGATC	117	54
FLII	AK297836	TCTGAACCTGTCCCGAAATC	GATCTCCGTACGGAAATGGA	277	60
GAPDH	AB062273	GACCACAGTCCATGCCATCAC	TCCACCACCTGTTGCTGTAG	453	58
LCN2	AK316217	TGTGGTAGGCCTGGCAGGGAA	ACCTGTGCACTCAGCCGTCG	452	60
PGK1	AK291081	GCCACTTGCTGTGCCAAATG	CCCAGGAAGGACTTTACCTT	101	58
PLAU	AK297940	TCACCACCAAAATGCTGTGT	AGGCCATTCTCTTCCTTGGT	223	55
PLAT	AK289387	ACATGCTGTGTGCTGGAGAC	GTCGCATGTTGTCACGAATC	219	55
PRKCD	NM_006254	GAAGAAGACCGGAGTTGCTG	GTCGATCAGGACCACATCCT	214	58
S100A4	AK292083	CTGACTGCTCATATGGCGTGCC	CCAACCGGATCCGAGGAGTTTT	341	52
SERPINE1	AK293248	CTCTGCCACCTGCAGCACCC	GGCACCTGCTTCCACCCGTC	279	60
SRF	AK300467	ACGACCTTCAGCAAGAGGAA	AAGCCAGTGGCACTCATTCT	258	58
TSPN8	BC005246	CCAGAGCATATTGCAGGACA	TGTCCACAGCAACGTAGGAG	247	55

and GoTaq qPCR mix (Promega). Real-time PCR was performed by use of the iCycler iQ™ system (Bio-Rad; Hercules, CA). cDNA concentration-dependent standard curves with sub-sequent linear regression analysis revealed the efficiencies for the qPCR amplification of each gene (1.6 to 2.0). The mRNA expression levels were then calculated according to the efficiency-corrected  $\Delta\Delta CT$  mathematical method for the relative quantification in real-time PCR per internal reference gene [34]. We used the mean-corrected CT value of two reference genes for data normalization.

#### Immunoblot analysis

Total protein was isolated from cells by use of the TRIzol® reagent (Life Technologies). Protein precipitates were resolved in 2 % SDS solution containing 62.5 mM sodium lauroyl sarcosinate (pH 8.0). Protein concentration was determined by the bicinchoninic acid protein assay (Thermo Scientific). Protein samples were subjected to standard immunoblot procedure. For immunostaining rabbit anti-lipocalin 2 (Abcam, Cambridge, UK) or rabbit anti-GAPDH (used as reference antigen; Santa Cruz Biotech, Dallas, TX) were applied. Bound antibodies were detected by a horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany) and the Western Blotting Luminol reagent (Santa Cruz). All visualized signals were densitometrically estimated by use of the LAS 3000 computer-based imaging system (FUJIFilm; Tokyo, Japan).

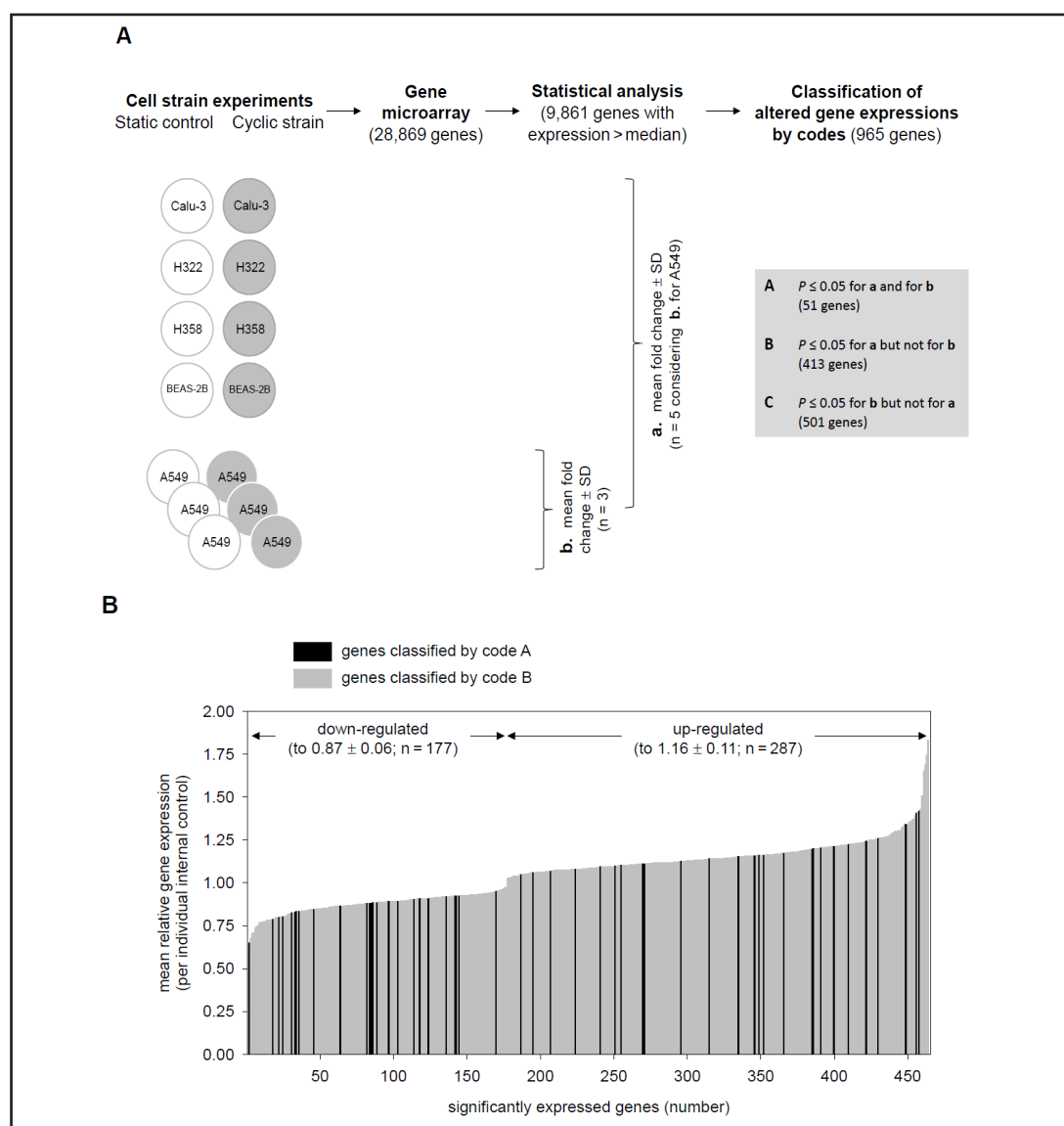
#### Statistical analysis

All data are reported as mean with standard deviation (SD) and number of time-independent experiments (n). The effect of strain on the expression level was always calculated as fold change per static control. *P* values  $\leq 0.05$  (paired Student's *t*-test) indicate a statistical significant difference per control. Statistical calculations and data presentations were performed with the SigmaPlot 10 software (Systat Software Inc., San Jose, CA).

## Results

### Moderate effect of cyclic strain on the gene expression in lung epithelial cells

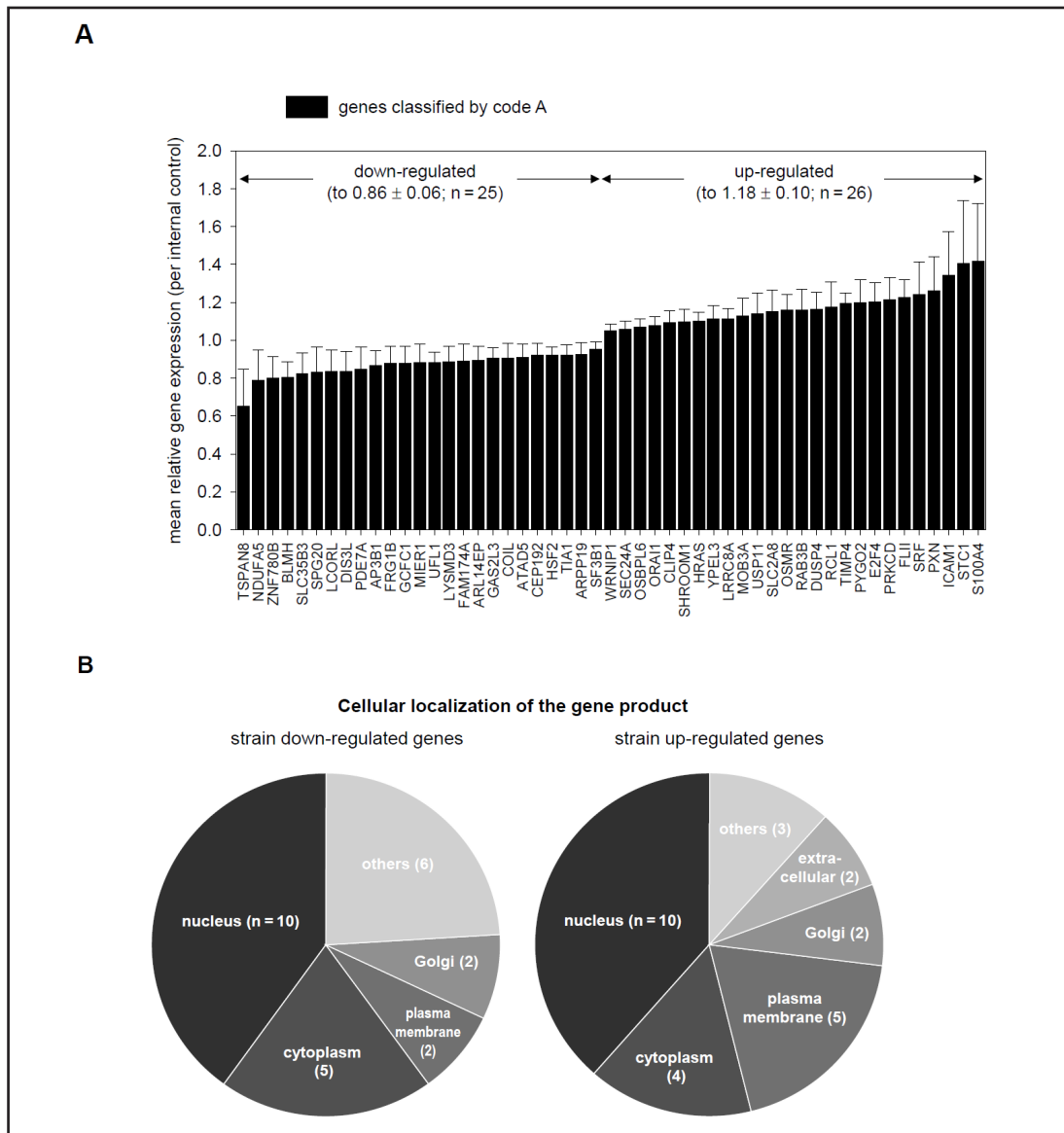
We aimed at the identification of genes of which the expression was commonly altered in lung epithelial cells in response to chronic cyclic strain. Since lung epithelial cell lines have been shown to respond well to cell distension [9, 12, 22, 35, 36], we selected various types of them. All cell lines were subjected to the following cyclic strain conditions: frequency of 12 min<sup>-1</sup>, which corresponds to the normal breathing frequency of human adults; elongation of 16 % in the cell surface area, which is assessed as physiological strain in lung [7]; and duration of 24 h to observe long-term effects.



**Fig. 1.** Experimental procedure and basic analysis of the gene microarray data. (A) Procedure for the gene microarray analysis of human lung epithelial cell lines subjected to chronic cyclic strain. (B) Mean relative changes of the expressions per internal static control are given for genes, which were classified by either code A or code B.

A549 cells form a typical epithelial monolayer on silicone membranes. Therefore, A549 was selected as the principal cell line for our gene microarray study (Fig. 1A). In addition, we analyzed the cell lines Calu-3, NCI-H322, NCI-H358, and BEAS-2B to assess potential findings on A549 cells (Fig. 1A). The statistical analysis of our gene microarray data included only genes, which were expressed above the median expression level of all genes in static cells to exclude minor expressed genes. In total, we identified 965 well-expressed genes of which the expression was altered in A549 cells and/or in the other lung epithelial cell lines due to chronic cyclic strain. As given in figure 1A, we classified the microarray data by codes to make data interpretations easier. According to this classification, cyclic strain altered the expression of 464 genes in all cell lines (Fig. 1B). Of them, 51 genes were differentially expressed when relative expression data of A549 had been averaged and when data of all cell lines had been averaged (code A), and 413 genes were differentially expressed only when





**Fig. 2.** Detailed microarray analysis of strain-regulated genes classified by code A. (A) Level of the differential expression of strain-regulated genes. Mean relative changes  $\pm$  SD per internal static control result from the microarray calculation of all five cell lines (see figure 1). (B) Main cell localization for the gene products of strain-regulated genes according to GO classification.

data of all cell lines had been averaged (code B) (Fig. 1B). Moreover, we identified 501 genes of which the expression was altered exclusively in A549 without similar tendency in the other cell lines (code C) (Fig. 1A).

A more detailed analysis of code A-classified genes showed that 25 of the 51 genes were down-regulated and 26 genes were up-regulated (Fig. 2A). Gene ontology analysis revealed that these genes often code for proteins localized in the nucleus (Fig. 2B). Among the nuclear proteins are some transcription factors, such as E2F4 and SRF (Table 2). However, the levels of expression alterations induced by chronic cyclic strain are rather low (Fig. 2A). In maximum, A549 and the other lung epithelial cells showed a down-regulation to  $0.65\text{-fold} \pm 0.20$  (TSPAN8) and up-regulation to  $1.42\text{-fold} \pm 0.30$  (S100A4) in response to chronic cyclic strain (Fig. 2A; see also Table 2).

**Table 2.** Fold changes of the gene expression in lung epithelial cell lines exposed to chronic cyclic strain. The ratio per internal static control was calculated for each cell line and each experiment; hence, data of the individual static controls are 1. For definition of code A and B see figure 1A. Data are given as mean  $\pm$  SD with \*  $P \leq 0.05$  and \*\*  $P \leq 0.01$  per static control

Gene name	Gene product	Code	Gene microarray analysis		qPCR verification (n $\geq$ 8 each)						
			Relative expression per control								
			all cell lines (n = 5)	A549 (n = 3)	A549	Calu-3	NCI-H322	NCI-H358	BEAS-2B	see figure	
TSPN8	tetraspanin 8	A	0.65 $\pm$ 0.20 *	0.51 $\pm$ 0.32 *	×	×	–	–	–	3C	
LCN2	lipocalin 2	B	0.68 $\pm$ 0.26 *	0.82 $\pm$ 0.29	×	–	×	–	×	3A	
AGR2	anterior gradient 2	B	0.74 $\pm$ 0.23 *	0.90 $\pm$ 0.22	×	–	–	–	×	3B	
E2F4	E2F transcription factor 4	A	1.20 $\pm$ 0.10 **	1.06 $\pm$ 0.02 *	×	–	×	–	×	3D	
PRKD	protein kinase C $\delta$	A	1.21 $\pm$ 0.12 *	1.23 $\pm$ 0.07 *	–	–	–	×	–		
PLAT	tissue-type plasminogen activator	B	1.23 $\pm$ 0.11 **	1.43 $\pm$ 0.48	×	–	–	–	–		
FLII	flightless-1 homolog	A	1.23 $\pm$ 0.10 **	1.11 $\pm$ 0.04 *	×	–	–	–	×	3E	
SRF	c-fos serum response element-binding transcription factor	A	1.24 $\pm$ 0.17 *	1.17 $\pm$ 0.04 *	×	–	–	–	×	3F	
ECE-1	endothelin-converting enzyme 1	B	1.24 $\pm$ 0.12 *	1.25 $\pm$ 0.38	×	–	–	–	–		
S100A4	S100 calcium-binding protein A4	A	1.42 $\pm$ 0.30 *	1.51 $\pm$ 0.19 *	×	–	–	–	×	3I	
PLAU	urokinase-type plasminogen activator	B	1.65 $\pm$ 0.40 *	2.01 $\pm$ 0.96	×	×	×	–	–	3H	

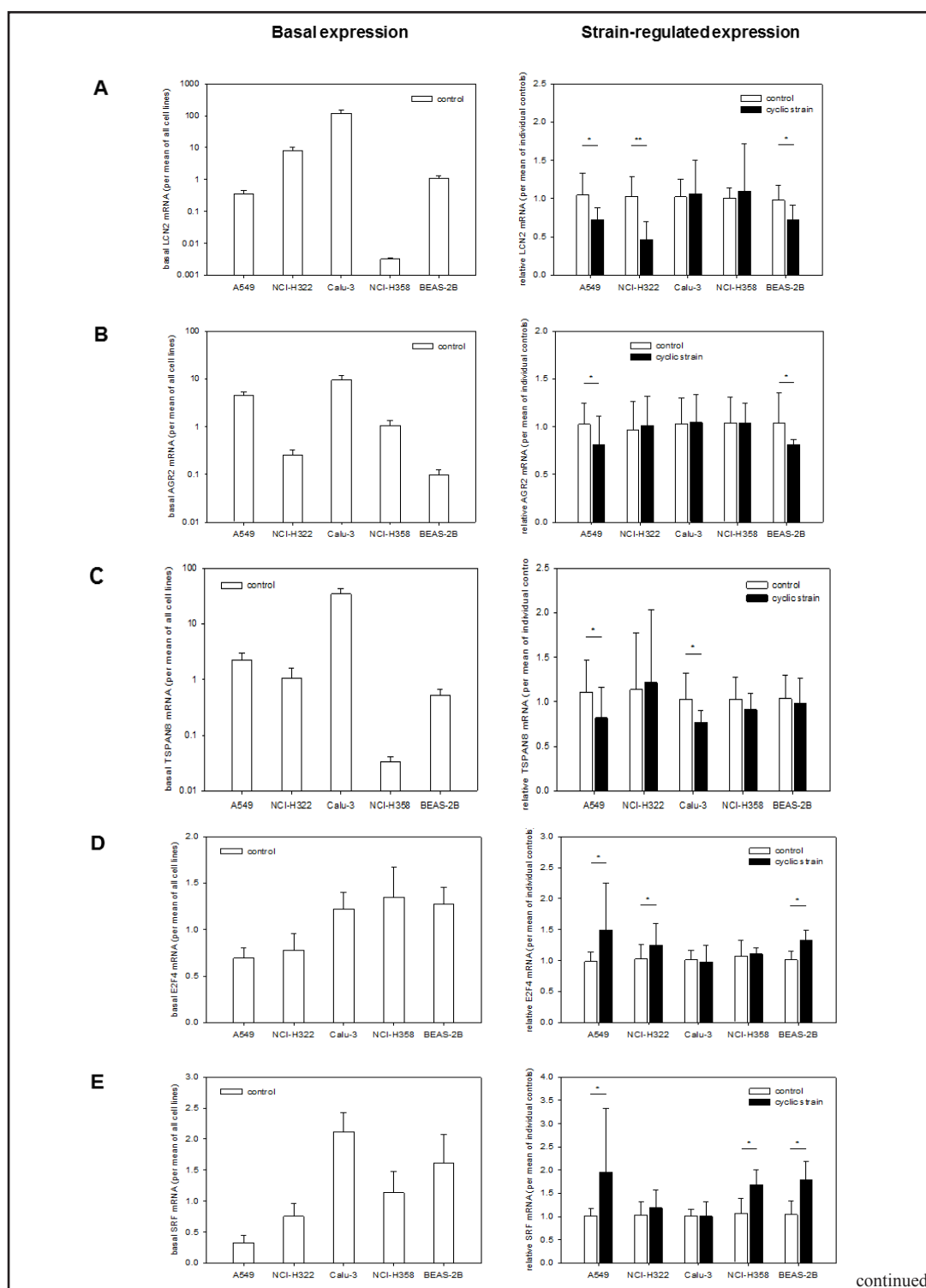
#### Comparison with strain-regulated genes in lung cells identified before

Other research groups reported about changes in the expression of certain genes in lung cells, which had been subjected to cyclic strain. Although the experimental protocols differ from our protocol, in particular regarding cycle frequency ( $> 12 \text{ min}^{-1}$  in most cases) and cell types, we identified some similarities. For example, we also identified an up-regulation of ICAM1 (intercellular adhesion molecule 1) for A549 and the other cell lines (Fig. 2A third column from the right) as described before for A549 cells [9, 10, 37]. Also, we identified down-regulation of OCLN (occludin) for A549 cells (0.73-fold  $\pm$  0.08 per static control,  $n = 3$ ,  $P = 0.03$ ) as described before for primary rat AT I-like cells [10, 17]. Several other genes reported could not be confirmed either because of no changes or an expression level below our internal threshold value. Detailed comparisons with another gene microarray study, which revealed a highly altered expression of 18 genes in primary rat AT I-like cells [24], did not identify any similar changes. However, comparisons with the gene microarray study of Wang *et al.*, which revealed 57 differentially expressed genes in primary fetal AT II cells [11], confirmed similar changes for at least four up-regulated genes (SLC7A3, SLC6A9 and SLC29A3 coding for solute carrier (SLC) group of membrane transport proteins, and TRIB3 (Tribbles homolog 3)) in A549 cells (data not shown).

#### Moderate effect of strain on the mRNA expression of selected genes in lung epithelial cells

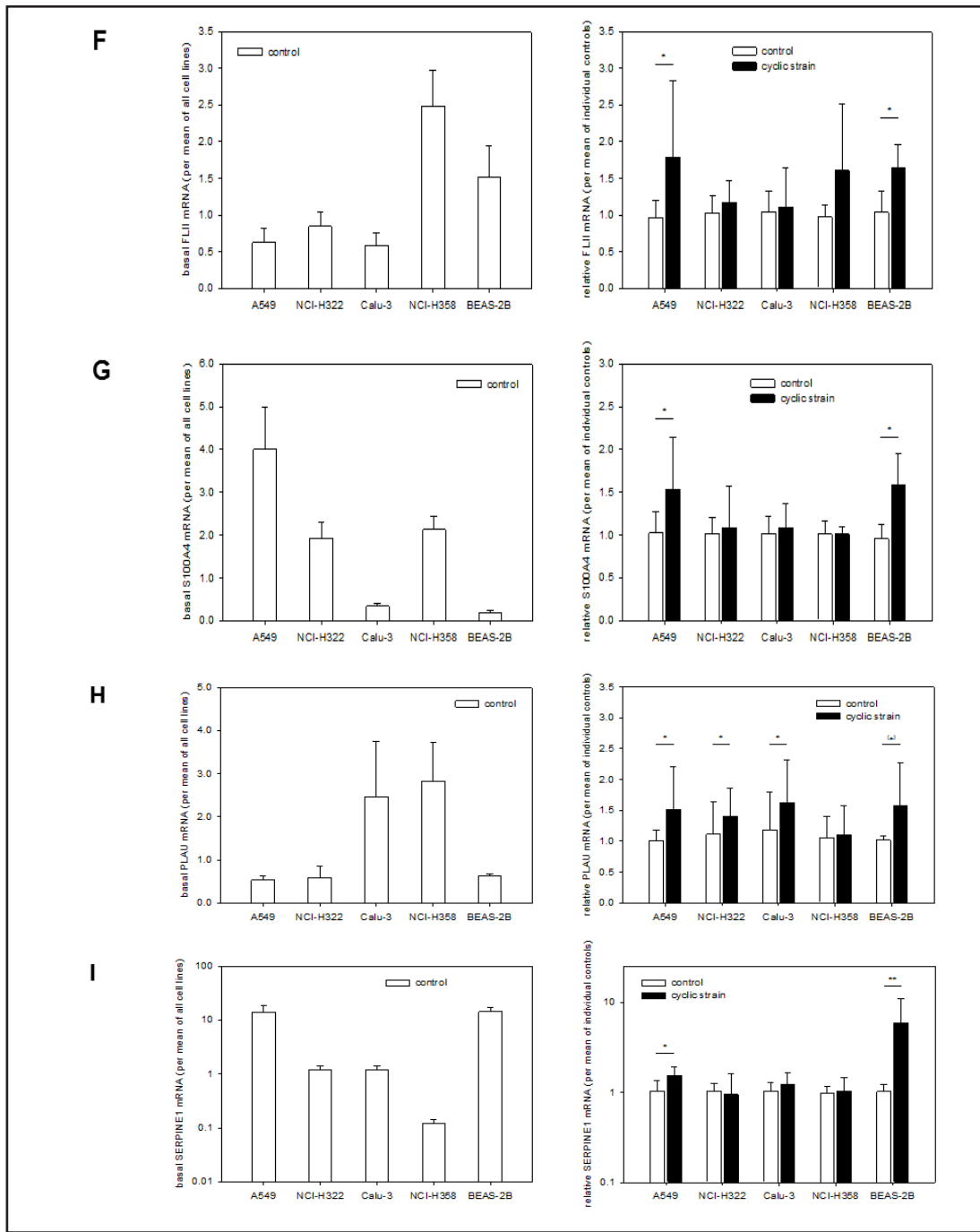
We selected genes classified by code A or code B for a more detailed expression analysis of a higher number of strain experiments by qPCR technology (Table 2). Gene selection based upon extent and significance of the differential expression determined by gene microarray, and upon biological function of the gene product, which might be interesting for the field of pulmonary research (Table 2). The expression analysis by qPCR was, unlike gene microarray analysis, internally corrected to a reference gene without regulation. Since classical reference genes (i.e., 18S rRNA, ACTB, GAPDH) proved to be less suitable in our study, we selected PGK1 (phospho-glycerate kinase 1) and CALM1 (calmodulin 1) as references. qPCR analyses confirmed the low variance of the PGK1 and CALM1 mRNA levels between the time-independent experiments and in response to chronic cyclic strain, and between the five cell lines studied (data not shown). Finally, we used both PGK1 and CALM1 for optimal data normalization of all qPCR analyses. These analyses displayed high differences in the basal expression of each gene selected (Fig. 3, left). Therefore, we again calculated the relative changes of the gene expression per static control for each cell lines to demonstrate the simultaneous effect of chronic cyclic strain (Fig. 3, right).

qPCR analyses verified moderate differences in the mRNA expression for most genes selected in at least one lung epithelial cell line studied (Table 2 for overview; Fig. 3 for genes differentially expressed at the minimum in two cell lines). A strain-regulated expression



**Fig. 3.** qPCR verification of selected genes classified by either code A or code B. Basal and strain-induced differential gene expression in lung epithelial cells for LCN2 (A), AGR2 (B), and TSPAN8 (C), which were frequently down-regulated in response to chronic cyclic strain, as well as for E2F4 (D), SRF (E), FLII (F), S100A4 (G), PLAU (H), and the non-classified gene SERPINE1 (I), which were frequently up-regulated. Basal gene expressions in static controls are shown left for each cell line. Strain-induced relative changes of the gene expressions are shown right. Data are given as mean  $\pm$  SD ( $n \geq 8$  each cell line) with \*  $P \leq 0.05$  and (\*)  $P < 0.1$  per individual static control.





of these genes was observed most frequently for A549 cells, even for genes showing no significant differences in the previous gene microarray analysis (i.e., code B-classified genes), but not that frequent for BEAS-2B cells and even less for the other cell lines (Table 2). In the case of potentially down-regulated genes, we confirmed the altered mRNA expression of LCN2 for A549, BEAS-2B, and especially for NCI-H322 cells (Fig. 3A). Although LCN2 is highly reduced in NCI-H322 cells subjected to cyclic strain for 24 h, this had minor effect on the protein level at the same time-point ( $0.90\text{-fold} \pm 0.14$  per static control;  $n = 8$ ;  $P = 0.09$ ). We also confirmed the reduced mRNA expression of AGR2 and TSPAN8 in some cell lines, but both genes were highly different expressed at basal level (Fig. 3B-C). In the case of potentially up-regulated genes, the altered mRNA levels of the transcription factors E2F4 and

SRF were confirmed for the cell lines A549 and BEAS-2B where E2F4 was also up-regulated in NCI-H322 and SRF in NCI-H358 (Fig. 3D–E). Moreover, moderate up-regulation of FLII as well as S100A4 was determined for A549 and BEAS-2B (Fig. 3F–G). The increased mRNA expression of PLAU has been confirmed in most cell lines subjected to cyclic strain (Fig. 3H), whereas the up-regulation of PLAT was identified for A549 cells only (data not shown). Since the gene products of PLAU and PLAT (urokinase-type and tissue-type plasminogen activator, respectively) are inhibited by the serpine plasminogen activator inhibitor-1 (PAI-1, encoded by SERPINE1 gene), we additionally studied the mRNA expression of SERPINE1. Even though our gene microarray analysis did not reveal strain-induced differences in the SERPINE1 expression, qPCR analysis of a higher number of experiments displayed a significant increase in SERPINE1 mRNA for A549 and BEAS-2B (Fig. 3I).

## Discussion

In this study, we demonstrated that lung epithelial cells display only moderate changes in the gene expression profile when subjected to cyclic strain in long-term conditions. Although our data suggest a well-pronounced adaptation of lung epithelial cells to cyclic strain, the sum of such moderate changes in the gene expression might still be sufficient to affect cellular behavior. In this regard, we identified some strain-regulated genes of which the altered expression could play a significant role *in vivo* depending on the particular epithelial cell type in lung.

We and others already demonstrated that cyclic strain of lung epithelial cells rapidly activates cell signaling molecules, such as protein kinases p42/p44, p38, JNK, or Akt/PKB, but after less than one hour their activation has turned to basal levels [10, 15, 20, 38–40]. This tendency is also shown for transcription factors, such as nuclear factor  $\kappa$  B (NF- $\kappa$  B) [21, 41] and signal transducer and activator of transcription 3 (STAT3) [42]. Moreover, many other studies displayed short-term but not long-term alterations in the mRNA expression of defined genes in lung epithelial cells, such as IL-8 [13, 20, 21], SFTPC [43] or VEGF (vascular endothelial growth factor) [44, 45]. None of them were found to be altered in our long-term study, too.

In contrast to expression studies investigating individual strain-regulated genes in lung epithelial cells, our and other gene microarray studies investigating a large panel of genes were not performed in a time-dependent manner [11, 22, 23]. Merely one study analyzed the entire gene expression profile at two time points, but the maximum time was already at 6 hours of cyclic distension [24]. Nevertheless, we identified an altered expression of at least some genes reported before in short-term studies. This includes the down-regulation of OCLN, which codes for the tight junction protein occludin [10, 17]. Its reduced expression causes an increased permeability of the alveolar epithelial cell layer at high magnitudes of cell distension [10, 17]. Moreover, we identified an increased gene expression of ICAM1, which codes for the intercellular adhesion molecule 1 [9, 37]. ICAM1 is mainly located in AT I cells of the lung tissue [46], and its pulmonary expression is often associated with patho-physiological processes, including the infection of lung cells with rhinoviruses [47, 48]. Although expression alterations of about 25 % for both genes identified in our study were rather moderate, it suggests that chronic cyclic strain contributes to an increased susceptibility of the alveolar epithelial layer to virus infections and integrity loss.

Since others already reported about expression changes of OCLN and ICAM1 in lung epithelial cells subjected to chronic cyclic strain, we focused on the detailed investigation of further genes identified to be differentially expressed in our microarray analysis. This includes genes coding for transcription factors, namely E2F4 and SRF, of which the expression was frequently up-regulated in response to chronic cyclic strain. In particular, the AT II cell-like cell line A549 [25] and immortalized BEAS-2B cells, which have kept many features of primary bronchial epithelial cells [28], responded to cyclic strain with an increased gene expression of E2F4 and SRF.

E2F4, a member of the E2F family of transcription factors, acts via binding to members of the pocket protein family, i.e., retinoblastoma protein (pRb) tumor suppressor, p107 and p130, and plays a crucial role in cell cycle control by repressing E2F-responsive genes [49]. E2F4 is required for normal development of the entire airway epithelium, in particular ciliated cells as well as Clara cells in the proximal lung [50]. The biological role of E2F4 in Clara cells is also indicated by our observation that the gene expression of E2F4 is not only increased in A549 and BEAS-2B subjected to cyclic strain but also in the Clara cell-like NCI-H322 [27]. Via interaction with p130 the transcription factor E2F4 promotes a G0-like state of cells with damaged DNA, thereby preventing their further contribution to growth and development of organs and tissues [51]. Therefore, an increased level of E2F4 in lung epithelial cells subjected to chronic cyclic strain provides additional evidence for the importance of mechanical forces for normal lung development [1, 2, 52]. In contrast to E2F4, the biological function of SRF in lung epithelium is still unknown because SRF, which codes for the c-fos serum response element-binding transcription factor, is mainly examined in smooth muscles cells where it contributes to myogenesis by activating muscle cell-specific gene expression [53]. Smooth muscle cells subjected to sustained distension show an increased expression of full-length SRF by suppressing the expression of the shorter splice variant SRF $\Delta$  5 [54]. This process seems to be defective in lung hypoplasia [54]. Although we did not investigate alternative splicing of SRF, the strain-induced expression of SRF in A549 and BEAS-2B cells proposes similar mechanotransduction in lung epithelial cell lines.

Like other studies investigating the effect of mechanical strain on lung epithelial cells *in vitro*, the findings of our study are subject to certain limitations. Firstly, the magnitude of cell distension applied in our study corresponds to relatively high tidal volumes *in vivo* [32]. However, high cell viabilities of the lung cells studied and moderate changes in the gene expression profile did not indicate patho-physiological magnitudes. In this regard, we identified few genes only, which have been described in association with ventilator-induced ALI [55]. Among these genes are ARPC4 (actin-related protein 2/3, subunit 4) and PLAUR (urokinase-type plasminogen activator receptor) of which the expression was moderately increased in all cell lines studied in our microarray analysis (i.e., code B-classified genes), but they were not analyzed in detail by qPCR. Another gene is SERPINE1, which codes for the plasminogen activator inhibitor-1 (PAI-1). PAI-1 functions as the principal inhibitor of both urokinase-type plasminogen activators (uPA) and tissue-type plasminogen activator (tPA). Especially uPA (encoded by PLAU) is frequently up-regulated in lung epithelial cells subjected to chronic cyclic strain. Since cyclic strain can induce the gene expressions of uPA, its receptor (PLAUR) and its inhibitor (SERPINE1/PAI-1), it suggests a general modification of the plasminogen activation machinery due to mechanical forces.

Despite a relatively high magnitude of cell strain applied for 24 h, the gene expression profile determined by gene microarray showed moderate changes only, and it might still contain some false positive data. Since we might again have missed some significant changes due to the exclusion of genes marginally expressed in lung epithelial cell lines, our observation certainly indicates an effective adaptation of lung epithelial cells to such strain conditions, thereby confirming recent findings of Wilson *et al.* who observed no signs of lung injury in healthy mice ventilated at high tidal volumes [56]. Most likely other factors, such as inflammation, will be necessary to enhance the sensitivity of lung epithelial cells to mechanical forces. Nonetheless, the moderate influence of chronic cyclic strain on the gene expression profile in lung epithelial cells causes another limitation of our study, that is, the verification of our microarray findings by qPCR. Even though PCR is highly sensitive and specific, it is certainly not the best technology to detect very small changes in mRNA expression levels. However, it allowed us to investigate many genes in various lung epithelial cell lines and a high number of independent experiments. Moreover, we could verify the expression of most genes in A549 cells and to lower extent in BEAS-2B cells. One important reason for a more significant influence of mechanical forces and, therefore, higher verification rate by qPCR in A549 and BEAS-2B than in the other cell lines studied might be due to the morphology and organization of the cytoskeleton in both cell types. Since A549 and BEAS-

2B cells spread well on membrane surfaces, mechanical extension of the membrane might more efficiently distend the cell surfaces of A549 and BEAS-2B than of the other cell lines, which grow more clonogenic on membranes. This hypothesis is indirectly supported by the strain-mediated up-regulation of FLII identified in both cell lines. FLII codes for the gelsolin family member of actin-binding proteins flightless-1 homologue [57] and could be essential for the reorganization of the actin cytoskeleton observed for primary alveolar epithelial cells and A549 cells in response to mechanical forces [15, 17, 18].

Chronic under-distension of the lung in stiffer fibrotic areas is suggested to support the development of lung fibrosis due to an altered expression of genes in lung fibroblasts [6]. Chronic under-distension could also play a critical role in the development of solid lung tumors, which are less subjected to mechanical forces than the normal lung tissue. Indeed, we identified the strain-mediated down-regulations of two genes, namely LCN2 and AGR2, which are lower expressed in normal lung tissue than in lung carcinomas [58, 59]. LCN2 codes for lipocalin 2, also called neutrophil gelatinase-associated lipocalin (NGAL), and AGR2 codes for the protein disulfide isomerase anterior gradient 2. In normal lung, both genes are dominantly expressed in mucin-producing cells of the airway epithelium [58, 60]. While the biological function of lipocalin 2 in lung is unclear, recent findings on AGR2 suggest its physiological role in lung development, i.e., differentiation of secretory cells into the goblet cells [61]. However, when expressed at too high level in asthma it promotes allergen-induced overproduction of mucin [60]. Hence, mechanical forces could regulate the mucous secretion in lung via AGR2, but this hypothesis still needs to be proved.

In the case of strain-regulated genes of which the expression was correspondingly up-regulated in normal lung tissue compared to lung carcinomas, we identified SRF and ICAM1, which have been discussed already above. Moreover, we identified S100A4, one of the S100 family members of calcium-binding proteins. Differential expression of S100A4 in lung carcinomas was also determined before by qPCR analysis [62]. Although S100A4 is lower expressed in lung carcinomas than in normal lung tissues [62], many studies demonstrated its growth-promoting and pro-metastatic action in tumor cells [63]. Since S100 proteins mainly modulate the action of other proteins in cells, their effect on the cellular behavior is highly dependent on the cell type [64]. Therefore, the strain-mediated up-regulation of S100A4 in lung carcinoma cells might induce other effects than in normal lung epithelium. It is conceivable that the strain-induced expression of S100A4 promotes proliferation and early migration of lung carcinoma cells placed in the periphery of tumors, an area that is still influenced by mechanical forces in lung. On the other hand, the strain-induced increase in the transcription factor E2F4, which mediates the action of the tumor suppressors pRB, p107, and p130 [49, 51], argues for an anti-tumorigenic effect of mechanical forces.

Here we investigated and discussed a small selection of genes of which the expression might be altered in lung epithelial cells subjected to long-term cyclic distension. According to the function of the gene products described above, our study indicates both beneficial and adverse effects of chronic cyclic strain on the behavior of lung epithelial cells. Moreover, very moderate changes in the gene expression profile indicate an effective adaptation of lung epithelial cells to mechanical forces in long-term conditions. For that reason, it will be of high interest to examine in the future if and to what extent known intrinsic factors, such as growth and aging, or extrinsic factors, such as virus infections and air pollutants, do influence cellular adaptation and, therefore, gene expressions in response to chronic mechanical forces.

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## Disclosure Statement

No financial interest by any author.

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