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ARTICLES

DNA Microarray Reveals Novel Genes Induced by Mechanical Forces in Fetal Lung Type II Epithelial Cells

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ABSTRACT: Mechanical forces are essential for normal fetal lung development. However, the cellular and molecular mechanisms regulating this process are still poorly defined. In this study, we used oligonucleotide microarrays to investigate gene expression in cultured embryonic d 19 rat fetal lung type II epithelial cells exposed to a level of mechanical strain similar to the developing lung. Significance Analysis of Microarrays (SAM) identified 92 genes differentially expressed by strain. Interestingly, several members of the solute carrier family of amino acid transporter (Slc7a1, Slc7a3, Slc6a9, and tumor-associated protein 1) genes involved in amino acid synthesis (Phgdh, Psat1, Psph, Cars, and Asns), as well as the amiloride-sensitive epithelial sodium channel gene (Scnn1a) were up-regulated by the application of force. These results were confirmed by quantitative real-time PCR (qRT-PCR). Thus, this study identifies genes induced by strain that may be important for amino acid signaling pathways and protein synthesis in fetal type II cells. In addition, these data suggest that mechanical forces may contribute to facilitate lung fluid reabsorption in preparation for birth. Taken together, the present investigation provides further insights into how mechanical forces may modulate fetal lung development. **(*Pediatr Res* 60: 118–124, 2006)**

Normal lung growth and development during fetal life are critical for extrauterine survival. Premature infants are often born before sufficient lung maturation has occurred, and, as a result, they experience a high rate of long-term pulmonary complications such as bronchopulmonary dysplasia. Understanding the mechanisms of prenatal lung growth and development is a crucial step for the design of preventive and therapeutic strategies.

Mechanical forces generated *in utero* by repetitive breathing movements and by fluid distension are essential to mammalian lung development (1–3). Previous *in vitro* experiments have demonstrated that application of force to cultured type II epithelial cells induces cell proliferation (4) and differentiation (5). Other studies have begun to identify mechanoreceptors and cell signaling pathways mediating fetal lung growth and maturation (4–8). However, the precise molecular and cellular mechanisms by which lung cells sense mechanical stimuli to influence lung development are still poorly defined.

DNA microarray technology provides a powerful tool for rapid, comprehensive, and quantitative analysis of gene expression profile. This technique has been previously used to assess changes in gene expression in endothelial cells (9), bladder smooth muscle cells (10), and human pulmonary A549 (11) and H441 (12) epithelial cells exposed to mechanical strain.

Therefore, the goal of the present study was to identify genes differentially expressed by mechanical stress of fetal type II epithelial cells. We used an *in vitro* model system in which embryonic d (E) 19 type II cells are exposed to mechanical forces similar to that observed in the developing lung. These studies revealed that mechanical forces induce genes related to amino acid transporter, amino acid synthesis, and sodium transport across the cell membrane.

METHODS

Cell isolation and mechanical distention protocol. Fetal rat lungs were obtained from timed-pregnant Sprague-Dawley rats (Charles Rivers, Wilmington, MA) and E19 type II cells were isolated as previously described (6). Epithelial cells were then seeded onto Bioflex plates precoated with collagen-1 (Flexcell Corporation, Hillsborough, NC) and maintained in serum-free Dulbecco's modified Eagle's medium (DMEM) for 24 h. Plates containing adherent cells were mounted in a Flexercell FX-4000 Strain Unit (Flexcell Corp.). Equibiaxial elongation of 5% was applied at intervals of 60 cycles per minute for 15 min plus 2.5% continuous distention for the remaining 45 min of each hour, for 16 h. This regimen was chosen to simulate mechanical forces experienced by type II epithelial cells during lung development (13), and it is

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Abbreviations: ENaC, amiloride-sensitive epithelial sodium channel; **qRT-PCR,** quantitative real-time PCR; **SAM,** Significance Analysis of Microarrays

based on previous studies from our laboratory (5,8). Cells grown on non-strained substrates were treated in an identical manner and served as controls.

Affymetrix GeneChip Analysis. Total RNA was extracted from E19 type II cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and purified further using the Rneasy Mini Kit (Invitrogen). Five micrograms of total RNA was used for the synthesis of labeled target in the Center for Genomic and Proteomics, Brown Medical School. Briefly, RNA was reverse-transcribed into cDNA using the Superscript Double Stranded cDNA Synthesis Kit (Invitrogen) with a T7-(dT)24 oligomer to prime the first-strand synthesis. DNA polymerase and DNA ligase were included in the synthesis of the second strand. After phase lock gel phenol/chloroform cDNA extraction and ethanol precipitation, conversion of double-stranded cDNA into biotin-labeled cRNA was accomplished using the BioArray High Yield RNA Transcript Labeling kit (T7) (Enzo Diagnostics, Farmingdale, NY) according to the manufacturer's instructions. Following *in vitro* transcription reactions purification and RNA cleanup, fragmentation of the labeled cRNA for target preparation was done according to the Affymetrix GeneChip Expression Analysis Protocol. After hybridization cocktails preparation, the Affymetrix rat E230A GeneChip array was hybridized with the fragmented labeled cRNA for 16 h at 45°C as described in the Affymetrix Technical Analysis Manual. GeneChip arrays were then loaded into the Affymetrix GeneChip Fluidics Station 400 for washing and staining, following the appropriate Affymetrix fluidics protocol. GeneChips were then scanned using the Agilent Technologies G2500A GeneArray Scanner (Agilent Technologies, Palo Alto, CA).

Microarray data analysis. Prior to analysis, data generated from the six hybridizations (three pairs from control and strain samples, using different litters for each pair) were processed using Microarray Suite software, version 5.0, to yield signal values. These expression values were calculated from the combined, background-adjusted "perfect match" and "mismatch" for each probe set, using a statistical algorithm described in Affymetrix Statistical Manual. Hybridization data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (GEO) (http://www.ncbi.nlm.nih.gov/geo) with GEO accession number GSE3541. To allow for comparison of signal intensity across arrays, the data were first normalized by adjusting expression values according to average percentiles (quantile normalization). In addition, to reduce noise, certain genes were eliminated from subsequent analysis, including those with low expression levels (<200 on all chips), genes with invariant changes [(control − strain) in absolute value <50 of all three pairs] and genes with contradictory changes of direction during the repeated experiments. In total, around 75% of genes were filtered, leaving 2030 genes for further analysis.

To identify fetal type II cell genes that were differentially expressed in response to mechanical strain, the SAM statistical method (14) was used. In SAM analysis, each gene is assigned a numerical score (d) that is derived from the change in gene expression relative to the SD of repeated measurements across data sets. For genes with score greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, the False Discovery Rate. SAM analysis was performed on paired, unlogged data with eight blocked permutations. As an additional control, a fold change cutoff value of 1.5 was selected.

Validation of gene expression changes by real-time PCR. Real-time PCR (qRT-PCR) was performed on 12 genes to verify microarray results. Predesigned TaqMan primers were purchased from Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA). Standard curves were generated for each primer set and housekeeping gene 18S ribosomal RNA. Linear regression revealed efficiencies between 96 and 99%. Therefore, fold expressions of strained samples relative to controls were calculated using the $\Delta\Delta C_T$ method for relative quantification (RQ). Samples were normalized to the 18S rRNA. No differences in RQ values for 18S were found between control and strain samples. Three micrograms of total RNA extracted from strained and control E19 type II cells were reverse-transcribed into cDNA using the ¡Script cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. To amplify the cDNA by qRT-PCR, 5 μL of the resulting cDNA were added to a mixture of 25 μL of TaqMan Universal PCR Master Mix (Applied Biosystems) and 2.5 μL of 20× Assays-on-Demand Gene Expression Assay Mix containing forward and reverse primers and TaqMan-labeled probe (Applied Biosystems). The reactions were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). All assays were performed in triplicate.

Statistical analysis. Paired *t* test was used to compare RQ values obtained from qRT-PCR from E19 type II epithelial cells exposed or not to mechanical strain for 16 h. A level of $p < 0.05$ was considered significant.

RESULTS

Data normalization and SAM analysis. Before statistical analysis, raw data were normalized. Normalization removes nonbiological systematic variations across chips. Figure 1*A* shows box plots of the six arrays (three pairs of control and strain samples) before and after quantile normalization, side by side. Before normalization, the second and fourth arrays showed slightly longer range of expressions (stretched boxes) than the others. Normalization scaled the data, so the medians, quartiles, and range were now equal for all six arrays. Figure 1*B* shows bivariate plots of control and strain for non-normalized and normalized expressions. The data points cluster around the 45 degree line, indicating that most genes are expressed at similar levels in both groups. After the data were normalized and filtered, SAM statistical software was used to identify genes in which their expression levels differed significantly between control and strain samples. Using a threshold value of 0.735, we identified 92 genes (63 up-regulated and 29 down-regulated) out of 2030 to be differentially expressed by 1.5-fold or greater in E19 type II cells exposed to mechanical strain. The false discovery rate was 11.9% (Fig. 2). These genes are listed in Tables 1 and 2, along with their output scores, fold change, and *q* values, and ordered by their functions.

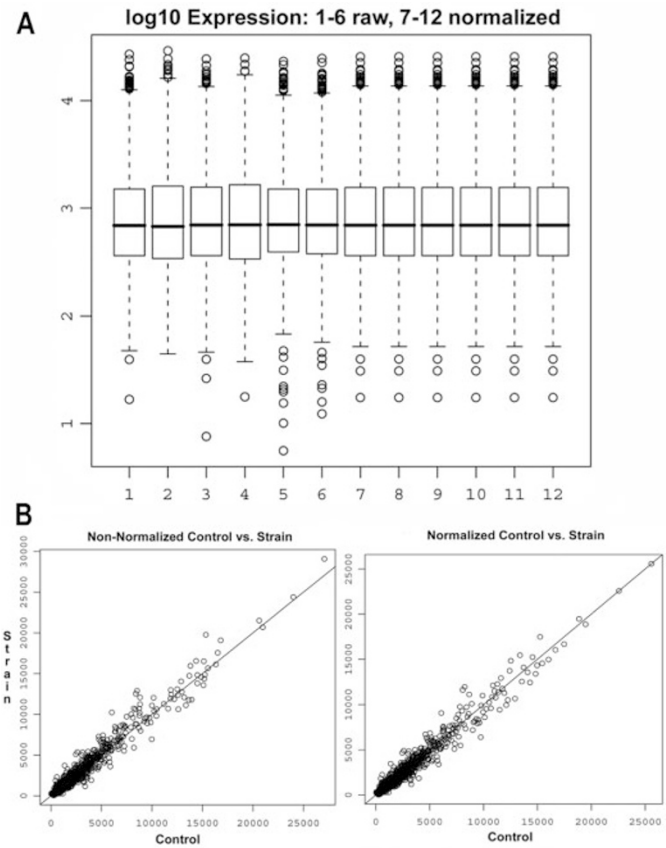

Figure 1. Normalization of the microarray data and SAM analysis. (*A*) Box plots of non-normalized (*arrays 1–6*) and normalized expressions (*7–12*). (*B*) Bivariate plots for control and strain expressions before and after normalization. The expression values depicted are for the first array pair.

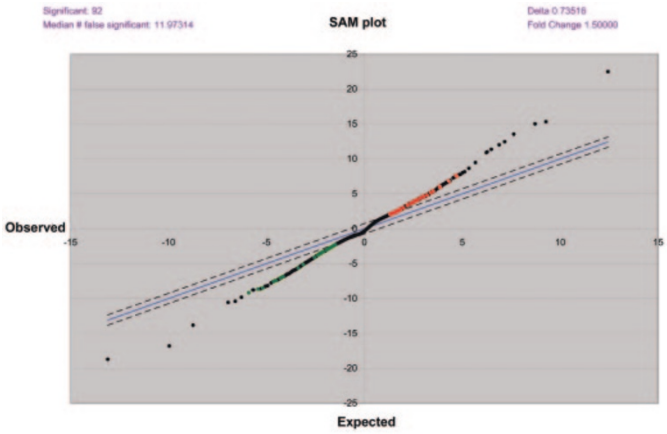

Figure 2. SAM graph plot of expected *vs* observed values of expression. The dotted lines parallel to 45 degree mark the boundary of the chosen threshold value (0.73516). The genes outside this threshold were selected as significantly expressed genes. A total of 92 genes, 43 up-regulated (in red) and 16 down-regulated (in green), out of 2030 were identified to be differentially expressed by 1.5-fold or greater in E19 type II cells exposed to mechanical strain. The false discovery rate was 11.9%.

The results of some of the genes potentially relevant to fetal lung development are discussed below according to their functional categories.

Mechanical strain of fetal type II cells regulates the expression of genes related to amino acid transport and synthesis. Microarray analysis identified a group of genes related to amino acid transport and amino acid synthesis significantly increased by strain. The cationic amino acid transporters y^+ system, solute carrier family 7, member 1 (Slc7a1) and solute carrier family 7, member 3 (Slc7a3) (L-arginine and L-lysine transport) increased by 1.72- and 2-fold, respectively. Solute carrier family 6, member 9 (Slc6a9), involved in glycine transport, increased by 1.74-fold. Similarly, Slc7a5 (tumor-associated protein 1), another cationic amino acid transporter highly expressed during liver development (15) increased by 2-fold (Table 1). The up-regulation of these genes by strain was confirmed by qRT-PCR (Fig. 3). Slc3a2, a dibasic and neutral amino acid transporter that increased after 16 h of strain by microarray analysis did not change by qRT-PCR (Fig. 3).

Our studies also revealed strain-mediated up-regulation of genes involved in L-serine biosynthesis, including 3-phosphoglycerate dehydrogenase (Phgdh) (1.69-fold), phosphoserine aminotransferase (Psat1) (1.67-fold), and phosphoserine phosphatase (Psph) (1.7-fold). Likewise, cysteinyl-tRNA synthetase (Cars, cysteine biosynthesis) increased by 1.8-fold and asparagine synthetase (Asns, asparagine biosynthesis) increased by 1.85-fold (Table 1). Microarray results were confirmed by qRT-PCR (Fig. 4). Taken together, these studies demonstrate that mechanical strain is a potent stimulus for induction of amino acid transporter and amino acid synthesis genes in fetal lung type II epithelial cells.

Mechanical strain modulates sodium transport across the type II cell membrane. In the current study, E19 fetal type II epithelial cells exposed to mechanical strain for 16 h showed up-regulation of the sodium channel nonvoltage-gated, type I, alpha polypeptide (Scnn1a) gene by 2.1-fold (Table 1). This amiloride-sensitive epithelial sodium channel (ENaC) gene modulates Na^+ transport across the cell membrane and is critical to facilitate lung fluid reabsorption around birth (16). These results were validated by qRT-PCR (Fig. 5). Another ion transport gene, solute carrier family 19, member 1 (Slc19a1), involved in Na^+/H^+ exchanger and folate transport, up-regulated by microarray analysis could not be confirmed by qRT-PCR (Fig. 5).

Other genes differentially expressed by mechanical strain of fetal type II epithelial cells. SAM analysis identified genes induced by strain that participate in the Wnt/β-catenin signaling pathway, including Fos-like antigen 1 (Fosl1), a transcription factor of the fos-related family genes (by 1.65-fold) and the seven in absentia 2 gene (sina), required for eye development in *Drosophila* (by 1.83-fold) (17).

Tissue remodeling by programmed cell death or apoptosis is important for normal lung morphogenesis (18). Our data show that mechanical strain differentially expresses genes involved in apoptosis, including ubiquitin-like domain member 1 (3.1-fold), DNA-damage inducible transcript 3 (1.87-fold), heme oxygenase 1 (1.87-fold), and transglutaminase 2 (0.57-fold) (Tables 1 and 2).

Several other genes participating in metabolism (bilirubin conjugation, nucleotide and nucleic acid metabolism, methyltransferase activity, gluconeogenesis, cholesterol biosynthesis, proteolysis), signaling (MAPK activation, protein phosphorylation and dephosphorylation, calcium signaling, G-protein-coupled receptor, Ras GTPase, zinc ion binding), transcription and translation, electron transport, extracellular matrix/cytoskeleton, and so on, were also modulated by mechanical forces (Tables 1 and 2).

DISCUSSION

Although mechanical forces are critical for normal lung development, the mechanisms regulating this process remain to be defined. In the current study, we used DNA microarray technology to assess changes in gene expression of E19 fetal type II epithelial cells exposed to mechanical strain. Our results demonstrate 92 genes that were differentially expressed in response to mechanical forces. Among them, we identified a group of genes not previously described to be modulated by force, including genes related to amino acid transporter, amino acid synthesis and sodium transport across the membrane.

Amino acids are involved in biosynthetic pathways and are essential for metabolic processes (19). They do not permeate cell membranes without specialized transport system carriers. The present study identifies two members (Slc7a1 and Slc7a3) of the arginine transporters activated by strain. L-arginine is a necessary precursor for protein and creatinine biosynthesis and plays a critical role in regulating nitrogen balance. L-arginine is metabolized to nitric oxide (NO) and L-citrulline (20). Cyclic strain, inflammatory mediators, growth factors and lysophosphatidylcholine stimulate L-arginine transport in vascular smooth muscle cells (21,22). The significance of our findings in fetal lung development needs to be determined. Strain-induced up-regulation of genes modulating L-arginine

Table 1. *Genes up-regulated by mechanical strain*

Probe set ID	Accession ID	Gene title	GO Biological process description	Score (*d*)	Fold change (%)	*Q* value
		Amino acid transport				
1368391_at	NM_013111	Solute carrier family 7 (cationic amino acid transporter, y^+ system), member 1 (Slc7a1)	Basic amino acid transport	7.56	1.72	11.36
1368582_at	NM_017217	Solute carrier family 7 (cationic amino acid transporter, y^+ system), member 3 (Slc7a3)	L-arginine transport L-lysine transport	3.63	2.05	13.17
1369772_at	NM_053818	Solute carrier family 6, member 9 (Slc6a9)	Glycine transport	2.37	1.74	13.17
1387280_at	NM_017353	Tumor-associated protein 1 (Slc7a5)	Amino acid transport	5.16	2.07	11.95
1398771_at	NM_019283	Solute carrier family 3, member 2 (Slc3a2)	Dibasic and neutral amino acid transport	6.02	1.82	11.95
		Amino acid synthesis				
1367811_at	NM_031620	3-phosphoglycerate dehydrogenase (Phgdh)	L-serine biosynthesis	4.81	1.69	11.95
1372665_at	NM_198738	Phosphoserine aminotransferase 1 (Psat1)	L-serine biosynthesis	6.8	1.67	11.95
1375964_at	NM_001009679	Phosphoserine phosphatase (Psph)	L-serine biosynthesis	3.64	1.7	13.17
1374034_at	XM_215134	Cysteinyl-tRNA synthetase (Cars)	Cysteine biosynthesis	4.01	1.79	13.17
1387925_at	NM_013079	Asparagine synthetase (Asns)	Asparagine biosynthesis	7.57	1.85	11.36
		Ion transport				
1368203_at	NM_031548	Sodium channel, nonvoltage-gated, type I, alpha polypeptide (Scnn1a)	Amiloride-sensitive sodium Channel activity	2.58	2.1	13.17
1369412_at	NM_017299	Solute carrier family 19, member 1 (Slc19a1)	Folic acid transport	2.21	1.53	13.17
		Development				
1387408_at	NM_134457	Seven in absentia 2 (RGD:620778)	Ubiquitin-dependent protein catabolism, apoptosis	2.31	1.83	13.17
1367795_at	NM_019242	Interferon-related developmental regul. 1	Muscle development	2.34	1.53	13.17
1371900_at	NM_001025421	LOC499834	Embryonic development	2.49	1.55	13.17
		Apoptosis				
1367741_at	NM_053523	Homocysteine-inducible, ER stress-inducible, ubiquitin-like domain memb. 1	Response to stress and prevention apoptosis	4.12	3.10	13.17
1369590_at	NM_024134	DNA-damage inducible transcript 3	Regulation of apoptosis	2.37	1.87	13.17
1370080_at	NM_012580	Heme oxygenase 1	Induction of apoptosis	4.14	1.87	13.17
		Metabolism				
1370613_at	NM_012683	UDP glycosyltransferase 1 family	Bilirubin conjugation	3.95	1.6	13.17
1373412_at	XM_231803	5′-nucleotidase, cytosolic III	Nucleotide catabolism	3.35	1.62	13.17
1374713_at	XM_227607	HMT1 hnRNP methyltransferase-like 6	Methyltransferase activity	2.50	2	13.17
1375213_at	XM_341319	Phosphoenolpyruvate carboxykinase 2	Gluconeogenesis	5.96	1.69	11.95
1377112_at	XM_342955	Cytidine deaminase (predicted)	Nucleic acid metabolism	3	2.02	13.17
1389725_at	NM_001013071	Transmembrane 7 superfamily member 2	Cholesterol biosynthesis	3.40	1.55	13.17
		Signaling				
1370695_at	NM_144755	Tribbles homolog 3	(Drosophila) activation of MAPK	2.24	3.99	13.17
1374324_at	NM_017175	Protein kinase N1	Protein phosphorylation	2.81	1.51	13.17
1377064_at	NM_053883	Dual specificity phosphatase 6	Protein dephosphorylation	2.39	2.11	13.17
1375025_at	NM_031338	Calcium/calmodulin-dependent protein kinase kinase 2, beta	Calcium-mediated protein phosphorylation	2.20	1.81	13.17
1387088_at	NM_033237	Galanin	G-protein-coupled receptor binding, neurogenesis	2.50	1.54	13.17
		Transcription and translation				
1368489_at	NM_012953	fos-like antigen 1	Wnt signaling. Regulation of transcription, mitotic.	2.12	1.65	13.17
1372601_at	NM_172336	Activating transcription factor 5	Neurogenesis	2.11	1.99	13.17
1376134_at	NM_001010947	Similar to hypothetical protein MGC3207 (RGD1307789)	Translational initiation	2.76	1.51	13.17
1386888_at	NM_053857	Eukaryotic translation initiation factor 4E binding protein 1	Negative regulation of translational initiation	2.45	1.71	13.17
		Antioxidants				
1368990_at	NM_012940	Cytochrome P450, family 1, subfamily b	Oxidoreductase activity	2.05	1.54	13.17
1386908_at	NM_022278	Glutaredoxin 1 (thioltransferase)	Oxidoreductase activity	2.37	1.75	13.17
1369868_at	NM_053946	Implantation-associated protein	Arsenate reductase activity	2.76	1.58	13.17
		Unclassified				
1368789_at	NM_020072	Acid phosphatase, prostate	Regulation of cell cycle	2.31	1.66	13.17
1370690_at	XM_214583	Heat shock protein, A	Response to heat	3.82	1.69	13.17
1372042_at	XM_226200	Chemokine-like factor super family 3	Chemotaxis	4.07	2.08	13.17
1374221_at	NM_181639	Solute carrier family 29, member 3	Nucleoside transport	2.13	1.56	13.17

Probe set ID numbers refer to rat E230A GeneChips from Affymetrix. The score *d* is the relative difference in expression for a particular gene between the control and strain conditions. The *q*value for each gene is the lowest False Discovery Rate at which that gene is called significant. (Note: transcribed loci with unknown functions are not included.)

Table 2. *Genes down-regulated by mechanical strain*

Probe set ID	Accession ID	Gene title	GO Biological process description	Score (*d*)	Fold change (%)	*Q* value
		Development				
1368522_at	NM_031340	Timeless homolog (Drosophila)	Branching morphogenesis	−2.41	0.62	13.17
1368829_at	NM_031825	Fibrillin 1	Skeletal and visual development	−8.65	0.58	8.76
1370026_at	NM_012935	Crystallin, alpha B	Eye development	−4.09	0.63	13.17
1375713_at	NM_024383	Hairy and enhancer of split 5 (Drosophila)	Negative regulation of neuron differentiation	−2.34	0.31	13.17
		Transport				
1370239_at	NM_013096	Hemoglobin alpha, adult chain 1	Oxygen transport	−3.77	0.30	13.17
1372190_at	NM_012825	Aquaporin 4	Water transport	−3.02	0.57	13.17
1373734_at	NM_177481	Solute carrier organic anion transporter family, member 3a1	Ion, prostaglandin transport	−2.35	0.56	13.17
		Apoptosis				
1369943_at	NM_019386	Transglutaminase 2, C polypeptide	Induction of apoptosis	−7.04	0.57	11.36
		Metabolism				
1373329_at	NM_130424	Transmembrane protease, serine2	Proteolysis and peptidolysis	−5.13	0.62	11.95
1389470_at	NM_212466	B-factor, properdin	Proteolysis and peptidolysis complement activation	−3.28	0.56	13.17
		Signaling				
1374902_at	XM_227396	IQ motif containing GTPase activating protein 3	Ras GTPase activator	−2.83	0.63	13.17
1373748_at	XM_232226	PDZ domain containing RING finger 3	Zinc ion binding, signaling	−7.57	0.65	8.76
		ECM/Cytoskeleton				
1367594_at	NM_017087	Biglycan	ECM structural constituent cytoskeleton organization	−2.42	0.65	13.17
1369928_at	NM_019212	Actin, alpha 1, skeletal muscle	and biogenesis	−2.39	0.57	13.17
1370857_at	M22757	Smooth muscle (RGD:621676)	Alpha-actin muscle contraction	−8.48	0.64	8.76
		Unclassified				
1367664_at	NM_013220	Ankyrin repeat domain 1 (cardiac muscle)		−9.16	0.64	8.76
1367776_at	NM_019296	Cell division cycle 2	Homolog A cell cycle, mitosis	−3.61	0.62	13.17

Probe set ID numbers refer to rat E230A GeneChips from Affymetrix. The score *d* is the relative difference in expression for a particular gene between the control and strain conditions. The *q* value for each gene is the lowest False Discovery Rate at which that gene is called significant.

(Note: transcribed loci with unknown functions are not included.)

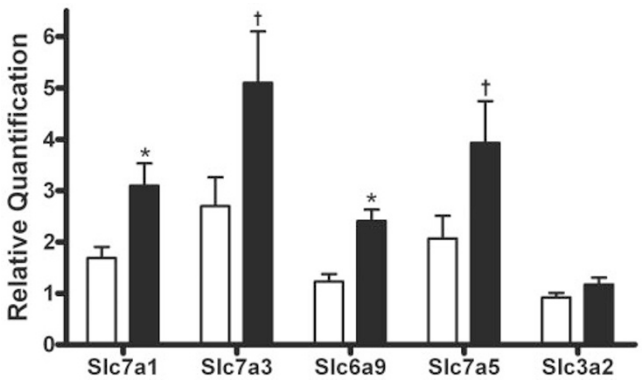

Figure 3. Confirmation of amino acid transporters microarray data by qRT-PCR. Type II cells isolated from E19 rat fetal lung were cultured on flexible silastic membranes coated with collagen 1 and then exposed to strain for 16 h as described in "Methods." Total RNA was reverse-transcribed and the cDNA product was analyzed by qRT-PCR using the $\Delta\Delta C_T$ method for relative quantification (RQ). Results are the mean ± SEM from seven different experiments performed in triplicate (*$p < 0.002$; †$p < 0.007$). *White bars* = control; *black bars* = strain.

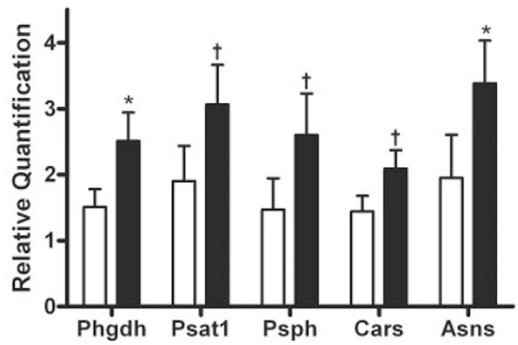

Figure 4. Mechanical strain induces genes involved in amino acid synthesis. qRT-PCR analysis by the $\Delta\Delta C_T$ method validating the microarray findings. Results are the mean ± SEM from seven different experiments performed in triplicate (*$p < 0.004$; †$p < 0.01$). *White bars* = control; *black bars* = strain.

transport may be important for fetal type II cell protein synthesis and/or for NO production. NO regulates not only pulmonary vascular tone during the perinatal transition but also promotes branching morphogenesis (23), angiogenesis (24), and alveolarization (25). NO is a downstream effector of vascular endothelial growth factor (VEGF). Recent studies indicate that VEGF is essential for normal pulmonary parenchymal development (26). In addition, VEGF is expressed in distal lung epithelial cells and its mRNA and protein levels are increased by mechanical forces (27). These studies support a

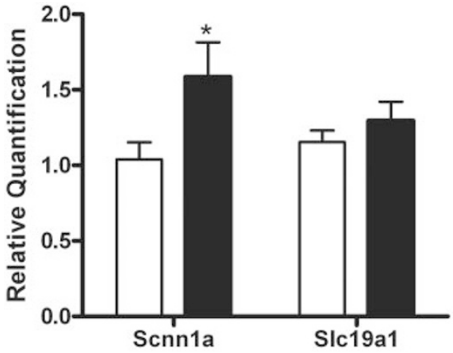

Figure 5. Validation of ion transport microarray findings by qRT-PCR. Graphical depiction of results from seven separate experiments showing that mechanical strain up-regulates the amiloride-sensitive sodium channel gene Scnn1a (*$p < 0.002$). *White bars* = control; *black bars* = strain.

role for VEGF-NO as an autocrine/paracrine regulator of lung development. In this context, mechanical forces may stimulate fetal lung development by promoting the uptake of L-arginine necessary for NO synthesis.

The biologic role of amino acid transporters during lung development is currently unknown. It has been postulated that apical transepithelial amino acid transporters may play important roles in protein removal from the alveolar space to prevent airway obstruction and bacterial overgrowth in adult lungs (28). However, based on the low protein concentration present in the lumen of the fetal lung, it is unlikely that these transporters are localized on the apical membrane of the fetal type II cells. We hypothesize instead they are present on the basolateral surface to uptake amino acids from bloodstream or surrounding interstitial space required for signaling pathways and protein synthesis.

Although maintenance of a constant transpulmonary pressure in the lumen of the fetal lung by epithelial fluid secretion is essential for normal lung development, reabsorption of these fluids during labor and soon after birth is also critical to survive in an air-breathing environment (29). The critical role of ENaC in alveolar fluid homeostasis has been highlighted by the fact that newborn α-ENaC knockout mice died shortly after birth, primarily from failure to clear their lungs of fluid (16). ENaC subunits are differentially regulated during lung development. The α-ENaC subunit is initially detected in E19 fetal rat type II cells whereas β- and γ-subunits are predominantly expressed after birth (30). Dexamethasone induces α-subunit mRNA expression only during the canalicular stage of lung development (E19). This gestational age correlates with an increase in endogenous fetal corticosteroids levels, suggesting that perinatal expression of α-ENaC is, at least in part, regulated by corticosteroids (30). Our data provide evidence that mechanical strain may also modulate α-ENaC expression to prepare the fetal lung for fluid reabsorption during labor.

Our study has the limitations inherent to an *in vitro* experimental system in which E19 type II cells are isolated from their environment. Furthermore, cultured type II epithelial cells have been shown to express different phenotype than freshly isolated type II cells (31). Therefore, our results should be interpreted cautiously. Another potential caveat is that experiments were performed at only one time-point following mechanical strain (16 h). Previous microarray studies (9) have shown that induction of gene expression by mechanical forces changes overtime. Thus, we may have missed other genes differentially modulated by strain. Nevertheless, this investigation has identified genes not previously recognized to be induced by force in fetal type II cells.

The present investigations are consistent with previous genome-wide experiments (11,12,32) demonstrating that mechanical strain is a potent stimulus to modulate genes related to apoptosis, cell signaling, transcription, oxidation, extracellular matrix remodeling, and so on. However, our distinct results may be explained by differences in the experimental model system used, type of cells and distension pressure protocol.

In summary, using microarray analysis we have found genes whose expression levels are influenced by mechanical strain. In particular, we have detected genes related to amino acid transporter, amino acid synthesis, and epithelial sodium channel up-regulated by strain. Based on the critical role played by mechanical forces in lung development, the information derived from these experiments may provide further knowledge in understanding the complex interactions between mechanical forces and fetal type II epithelial cells. Characterization of the biologic function of these genes will be an important focus of future studies, which may have an impact on clinical conditions where lung development is impaired, such as extreme prematurity and pulmonary hypoplasia.

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