Supplementary information (SI)

Materials and Methods

Materials

Mouse monoclonal anti-PC1/3, rabbit polyclonal anti-ChrA, mouse monoclonal anti-E-cadherin, mouse monoclonal anti-β-actin, goat polyclonal anti-collagen I, goat polyclonal anti-MMP-2, goat polyclonal anti-Twist, Texas Red-conjugated goat anti-rabbit IgG, and FITC-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-PC1/3 was purchased from Millipore (Billerica, MA). Mouse monoclonal anti-MUC5AC was obtained from Thermo Scientific (Fremont, CA). Mouse monoclonal anti-acetylated tubulin, mouse monoclonal anti-pan cytokeratin clone C-11, FITC-phallloidin, Mitomycin C, protease inhibitors, and colloidal Coomassie Brilliant Blue (CBB) G250 were purchased from Sigma-Aldrich (Saint Louis, MO). Mouse monoclonal anti-Vimentin was obtained from DakoCytomation (Glostrup, Denmark). Rabbit monoclonal anti-N-cadherin, rabbit monoclonal anti-Snail, and rabbit monoclonal anti-Slug were obtained from Cell Signaling (Beverly, MA). Fluorogenic peptide substrate, L-PyroGlu-Arg-Thr-Lys-Arg-7-amido-4-methyl Coumarin (pERTKR-AMC) was purchased from R&D Systems (Minneapolis, MN). RcCMV expression vector containing mouse PC1/3 cDNA and rabbit polyclonal anti-mouse PC1/3 were kindly provided by Dr. Iris Lindberg**.**

Cell Culture, Transfection, and Selection

The Institutional Review Board of Yonsei University College of Medicine provided approval for this study (IRB# 4-2012-0136). Normal human nasal epithelial (NHNE) cells were obtained from patients after surgical turbinectomies. The air-liquid interface (ALI) culture system was used for NHNE cells as previously described (1). Cells were cultured in a 1:1 mixture of BEGM and DMEM containing all supplements. Briefly, passage-1 NHNE cells were seeded on a 24-mm, 0.45-μm-pore Transwell-Clear culture insert (Costar Co, Cambridge, MA, USA) at 2 x 10^5 cells/well. The medium was removed from the apical chamber after 9 days in culture and the cells were maintained at an ALI until 11 days in culture. Then the cells were treated basolaterally with either 10 ng/ml of TGF-β1 (R&D Systems, MN, USA) or cytomix (5 ng/ml of TNF-α and 5 ng/ml of IL-1β, R&D Systems) for 72 h.

NCI-H292 cells were cultured in RPMI 1640 medium containing 10% FBS. To establish PC1/3 stable expressing cells, NCI-H292 cells were transfected with either RcCMV plasmid alone or RcCMV encoding mouse PC1/3 with FUGENE™6 (Roche, Indianapolis, IN, USA). Stable cells were selected with 1 mg/ml 50% active G418 (Invitrogen, Gaithersburg, MD, USA). Two independent high-expressing cells were used for subsequent experiments.

RNA isolation, Real-Time PCR, and Semi-quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's protocol. The cDNA was synthesized and amplified by PCR using specific primers for the indicated genes, as previously described (1). Real-time PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and DyNAmo™HS SYBR®Green qPCR Kit (Finnzymes, Espoo, Finland). Sequences of PCR primer sets used in this study are shown in Tables 1 and S1.

Western blotting

Normal human nasal mucosa (NM) and nasal polyps (NP) were ground to a fine powder in liquid nitrogen with a mortar and pestle and stored at -80℃ until use. Ground tissues and NCI-H292 cells were homogenized in cell lysis buffer (Invitrogen, Camarillo, CA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and the Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) on ice. Samples were subjected to electrophoresis on either 8%/10% SDS-PAGE gels, followed by western blotting using the appropriate antisera. Proteins were transferred from gels to PVDF membranes

and the membranes were preincubated in 5% nonfat milk in Tris-buffered saline (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 0.5% Tween 20 (TTBS) for 1 h at room temperature prior to incubation overnight at 4℃ with antiserum diluted 1:1000 in either 5% nonfat milk in TTBS or 5% BSA in TTBS (for antibodies from Cell signaling). Membranes were washed three times with TTBS followed by incubation at room temperature for 1 h with secondary antibody (1:2000 dilution, anti-mouse/rabbit IgG coupled to alkaline phosphatase). Specific bands were detected by Thermo Scientific Pierce ECL Western Blotting Substrate (Rockford, IL) and visualization was performed by exposure of the membranes to X-ray film.

Immunohistochemistry

Formalin-fixed paraffin sections (4 μm) were de-waxed in xylene (Sigma Chemicals, St Louis, MO), rehydrated in successive ethanol baths, and subjected to antigen retrieval by microwave in 0.01 mol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 3% methanolic hydrogen peroxide for 10 min at room temperature. Nonspecific binding was blocked by incubation with 10% normal serum from VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Primary antibody (1:200 dilution) was applied at 4℃ for 24 h. Following washing in TBS, slides were incubated with peroxidase-conjugated goat anti-mouse/rabbit antibodies (1:200 dilution, Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Signal was amplified using the indirect immunoperoxidase technique using the DAKO Envision kit (Dako, Kingsgrove, Australia) Tissue sections were counterstained with Gill's hematoxylin (Sigma Chemicals, St Louis, MO), dehydrated, and mounted with DPX (ProSciTech, Thuringowa, Australia). Staining was visualized using an Olympus U-TV0.63XC microscope with the DP Controller software (Hamburg, Germany). The experiment was performed using four NM and four NP specimens.

Immunofluorescence staining

Cyto-spin slides were prepared from epithelial cells of four NM and of four NP specimens. Cells were fixed, permeabilized, and then blocked with 10% BSA. Cells were incubated with either antisera against mouse anti-human PC1/3 and rabbit anti-human MUC5AC, antisera against PC1/3 and acetylated tubulin , or antisera against PC1/3 and chrA (diluted 1:100 in DakoCytomation antidody diluent, respectively) overnight at 4 °C. For immunostaining of NHNE cells, cells were incubated with antiserum against mouse anti-human E-cadherin (diluted 1:100 in DakoCytomation antidody diluent). Cells were then incubated with either secondary anti-rabbit Texas red-conjugated IgG (1:250, for PC1/3 and E-cadherin) or secondary anti-mouse FITC-conjugated IgG (1:250, for MUC5AC and acetylated tubulin) for 1 h at room temperature. For F-actin staining, cells were incubated with 10 μg/ml FITC–conjugated phalloidin for 40 min. After extensive washing, coverslips were mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and analyzed by confocal laser-scanning microscopy (LSM510, Carl Zeiss MicroImaging GmbH, Jena, Germany).

Enzyme assays

NM and NP were briefly sonicated in 100 mM sodium acetate, pH 5.5, and 1% Triton X-100 in the presence of a protease inhibitor cocktail (1 μM pepstatin, 1 μM E-64, and 1 mM PMSF). After centrifugation for 2 min at 12,000 x g, the supernatants were used for PC enzyme assays. Enzyme assays were conducted in triplicate in 96-well black polystyrene microtiter plates using 100 μg of each sample in a total volume of 100 μl containing 200 μM Pyr-Glu-Arg-Thr-Lys-Arg-methylcoumarinamide (R&D Systems, Minneapolis, MN) as a substrate and 100 mM sodium acetate buffer (pH 5.5) containing 5 mM $CaCl_2$, 0.5% Triton X-100 in the presence of a protease inhibitor cocktail (1 μM pepstatin, 0.28 mM TPCK, 1 μM E-64, and 0.14 mM TLCK). NCI-H292 cells were cultured in a 12-well plate overnight. Medium was changed to Opti-MEM containing 100 μg/ml aprotinin. The conditioned medium was collected after 16 h and briefly centrifuged, and then assayed for PC1/3 activity. The rate of released 7-amino-4-methylcoumarin was measured with a Fluoroskan Ascent fluorometer (LabSystems) using an

excitation wavelength of 380 nm and an emission wavelength of 460 nm for 2 h at 37 °C.

Gelatin zymography
Zymography was performed to detect the activity of secreted MMP-2. NCI-H292 cells were cultured in a 6-well plate overnight. Medium was changed to Opti-MEM and collected after 24 h. Conditioned medium samples were concentrated 40-fold using Amicon Ultracel-30K (Millipore, Cork, Ireland) according to the manufacture. Protein content was normalized and samples were incubated in 2x non-reducing sample buffer (0.5 M Tris–HCL, pH 6.8, 10% SDS, 50% v/v glycerol and 1% bromophenol blue) for 30 min on ice to achieve full denaturation. Samples were electrophoresed in 10% SDS-PAGE containing 0.1% gelatin. Gel was washed for 30 min each in l00 ml of buffer 1 (50 mM Tris, 2.5% Triton X-100, pH 7.5) and buffer 2 (50 mM Tris, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5% Triton X-100, pH 7.5), followed by incubation in buffer 3 (50 mM Tris, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.5) for 16 h at 37°C with gentle shaking. Gels were atained with Coomassise staining solution (25% methanol/0.7% acetic acid/0.1% Coomassie blue G250) for 1 h, followed by destaining (4% methanol/8% acetic acid).

2D-PAGE electrophoresis/MALDI-TOF/TOF mass spectrometry analysis
Cells were homogenized in cell lysis buffer containing 1 mM PMSF and protease/phosphatase inhibitor cocktail on ice. After centrifugation at 12,000 x *g* for 30 min at 4 °C, 1 mg of protein was run using commercial IPG strips for IEF (pH 3-10 NL, 18 cm) for the first dimension and SDS-polyacrylamide gels with a linear concentration gradient of 9-17% (Bio-Rad, Hercules, CA) for the second dimension. Selected gel pieces were vacuum-dried, rehydrated with an in-gel digestion reagent (Promega, Madison, WI, USA), and desalted using GELoader tips (Eppendorf, Hauppauge, NY, USA) followed by elution with Matrix solution (10 mg/ml α-ciano-hydroxycynamic acid in 70% acetonitrile and 2% formic acid). Eluted samples were analyzed using a 4700 MALDI-TOF/TOF MS (Applied Biosystems, Foster City, CA, USA) and further processed using Data Explorer 4.4 (PerSeptive Biosystems, Framingham, MA, USA). The monoisotopic peptide masses were analyzed using the Matrix Science search engine (http://www.matrixscience,com) with the NCBInr and SWISS-PROT databases.

Supplement Figure Legends

Figure S1: Differential protein production in mock and PC1/3 cells. Whole cell extracts from mock and PC1/3 cells were subjected to 2D-PAGE electrophoresis. 3-10 NL IPG strips were used for the IEF and standard SDS-PAGE for the second dimension. Protein spots indicated with arrows were selected for further characterization by MALDI-TOF/TOF MS. *A*, 2D gel images for proteins up-regulated by PC1/3 expression. *B*, Histogram showing average ratio of protein expression between mock and PC1/3 cells. *C*, RT-PCR study differential gene expression between mock and PC1/3 cells. 18S rRNA expression was used as a control.

Results and Discussion

Proteomic analysis of differentially produced proteins in NCI-H292 cells during PC1/3-induced EMT
To identify the proteins mediating EMT in PC1/3 cells, we employed 2D-PAGE followed by MALDI-TOF/TOF MS. A paired Student's *t*-test was used to select 10 spots that showed a significant increase in PC1/3 cells compared to mock cells (Fig. S1, $P<0.05$). We also selected 5 non-pairing spots found only in PC1/3 cells. We identified GAPDH, annexin A2 (ANXA2), proliferating cell nuclear antigen (PCNA), tropomyosins (TPM, TPM3, TPM4), F-actin-capping protein subunit alpha-1 (CAPZA1), replication protein A 32 kDa subunit (RPA p32), chloride intracellular channel protein 1 (CLIC1), phosphoglycerate mutase 1 (PGAM1), heat shock protein beta-1 (HSPB1), ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1), triosephosphate isomerase 1 (TPI1), galectin-1 (Gal-1), and methylthioadenosine phosphorylase (MTAP) (Table S2). We validated these proteins by RT-PCR analysis and found that

PC1/3 cells exhibited a significant increased expression of the respective RNA compared to mock cells (Fig. S1); however, the expression of *MUC5AC*, a major component of airway mucus, was completely abolished in PC1/3 cells, speculating that PC1/3 cells highly express PC1/3 and turn over the protein in the aggressive way, which could greatly influence the processing state of EMT-related substrates, leading to the induction of full EMT and loss of the epithelial phenotype.

According to biological function, these proteins are implicated in carbohydrate metabolism, cell proliferation, cytoskeletal reorganization, DNA metabolism, and signal transduction (Table S2).

Our proteomic analysis shows that PC1/3 overexpression up-regulates the production level of proteins involved in cell proliferation (PCNA, RPA p32, CLIC1) (2-4) and enzymes in the glycolytic pathway (GAPDH, PGAM1, TPI1) (5), which are essential for cancer cell growth and proliferation. CLIC1 is known to play a role in endothelial cell growth and migration via the regulation of integrin expression (4). RPA p32 is capable of complex formation with the tumor-suppressor protein p53, preventing the transcriptional activation of genes involved in DNA repair and allowing cells with damaged DNA to proceed through the cell cycle (3).

We also observed that proteins involved in cytoskeletal reorganization (TPM, TPM4, TPM3, CAPZA1, HSPB1) are up-regulated in PC1/3 cells. Cell motility requires polymerization/depolymerization cycles of actin filaments (6, 7). TPMs are actin-binding proteins and protect actin filaments from Arp2/3 complex-nucleated branching at the base of lamellipodia (8). CAPZA1 (9) and HSPB1 (10) display actin-capping activity and play an important role in the spatial organization of lamellipodia by promoting Arp2/3 complex-mediated branch formation at the leading edge (10). Proteins involved in signal transduction (ANXA2, Gal-1) and MATP were also increased in PC1/3 cells. ANXA2 has been reported to regulate membrane-cytoskeleton contacts by binding to F-actin in a Ca^{2+}-dependent manner (11). Gal-1, a family of beta-galactoside-binding proteins, is associated with cell migration and invasion of many types of cancer through binding to laminin, fibronectin, and vitronectin in the extracellular matrix (12). MTAP is a key enzyme in the salvage pathway of both adenine and methionine (13). Although a MTAP deficiency has been reported in human non-small cell lung cancers (NSCLCs) (14), MTAP production is strongly up-regulated in colon carcinoma by TCF1/β-catenin, resulting in enhanced cell migration and invasion during EMT (13). In line with these reports, our data suggest that PC1/3 overexpression induces a migratory phenotype of airway epithelial cells. Further study will be needed to understand the molecular mechanisms by which PC1/3 functions in this behavior. Interestingly, we found that UCH-L1 expression is up-regulated in PC1/3 cells compared with mock cells. As previously stated, UCH-L1 is a neuroendocrine cell-specific protein used as a marker in assessing lung cancer (15). UCH-L1 belongs to the family of deubiquitinating enzymes and plays a key role in the proteolytic degradation of misfolded and damaged proteins (16). A recent report has shown that UCH-L1 plays an important role in the invasion and metastasis of prostate cancer cells by facilitating the expression of genes including *Snail*, *Slug*, *Twist* and *MMPs* via Akt signaling during EMT (16). Similar effects of UCH-L1 has also been reported in NSCLCs (17). It would be interesting to determine a role of UCH-L1 in epithelial cell functions.

Table S1. RT PCR primer sequences for validated targets.

Gene name	**Forward primer 5' - 3'**	**Reverse primer 5' - 3'**
MUC5AC	CAGCCACGTCCCCTTCAATA	ACCGCATTTGGGCATCC
GAPDH	CCCCTTCATTGACCTCAACTAC	GAGTCCTTCCACGATACCAAAG
ANXA2	CAGAACCAACCAGGAGCT	TTCACTGCGGGAGACCAT
PCNA	GCCGAGATCTCAGCCATATT	ATGTACTTAGAGGTACAAAT
CLIC1	CTCAGCTCTGAACCCTGAGTCC	TTGCTCAAGTACCGATGCAC
PGAM1	GCACCCACTCCCTTCATACAAT	ACGCAGGTTACATTCGTCTTCC
UCH-L1	TGCTGAACAAAGTGCTGTCC	CAGGAATTCCCAATGGTCTG
TPI1	AGGGCGCCTCGGCTCCAG	CTCATTGTTTGGCATTGATGATGTC
Gal-1	AACCTGGAGAGTGCCTTCGA	GTAGTTGATGGCCTCCAGGT

Table S2. List of proteins up-regulated by PC1/3 expression.

Spot No.	Protein description	Short name	NCBI ID	Theoretical MW/p*I*	Protein coverage (%)	Summary score	Function
529	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	gi | 31645	36.20/8.26	50	80	Metabolism
545	Annexin A2 isoform 2	ANXA2	gi | 4757756	38.81/7.57	57	195	Signal transduction
546	Proliferating cell nuclear antigen	PCNA	gi | 2914385	29.07/4.53	56	102	Cell cycle proliferation
574	Tropomyosin isoform	TPM	gi | 854189	28.52/4.89	43	49	Cytoskeleton /mobility
594	F-actin-capping protein subunit alpha-1	CAPZA 1	gi | 5453597	33.07/5.45	52	82	Cytoskeleton /mobility
609	Tropomyosin alpha-4 chain isoform 1	TPM4	gi | 223555975	32,70/4.69	38	55	Cytoskeleton /mobility
610	Replication protein A 32 kDa subunit	RPA p32	gi | 4506585	29.34/5.75	30	65	DNA replication/ metabolism
615	Tropomyosin alpha-3 isoform 3	TPM3	gi | 114155146	29.11/4.79	27	45	Cytoskeleton /mobility
634	Chloride intracellular channel protein 1	CLIC1	gi | 14251209	27.25/5.09	33	60	Cell cycle proliferation
666	Phosphoglycerate mutase 1	PGAM1	gi | 4505753	28.90/6.67	43	137	Metabolism
694	Heat shock protein beta-1	HSPB1	gi | 4504517	22.83/5.98	66	138	Chaperone/ stress protein
703	Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCH-L1	gi | 21361091	25.15/5.33	47	77	Ptotein synthesis/ degradation
704	Triosephosphate isomerase 1 variant	TPI1	gi | 62896835	26.98/6.90	36	80	Metabolism
873	Human galectin-1, chain B	Gal-1	gi | 42542978	14.58/5.34	52	94	Signal transduction
976	Methylthioadenosine phosphorylase	MTAP	gi | 116283224	26.77/6.38	57	83	Metabolism

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 64 are significant ($p<0.05$).
Database : NCBInr 20110527 (14235747 sequences; 4877395943 residues)
Taxonomy : Homo sapiens (human) (237331 sequences)

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