Supplementary data

Hypoxia Inducible Factor Regulates Surfactant Protein Expression in Alveolar Type II Cells *in vitro*

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Materials and Methods

Rat ATII cell isolation and culture

Freshly isolated or frozen ATII cells (2.5 million) were plated on 4.2 cm^2 millicell inserts (Millipore Corp, Bellerica, MA) that had been previously coated with a mixture of 20% Matrigel (BD Biosciences, Bedford, MA) and 80% rat-tail collagen in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% rat serum (RS) (Pel-Freez Biologicals, Rogers, AR), 2 mM glutamine, 2.5 μg/ml amphotericin B, 100 μg/ml streptomycin, 100 units/ml penicillin G (all from Mediatech, Inc.), and 10 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO).

Immunoblotting and real-time PCR (RT-PCR)

Polyacrylamide gradient gels (8–16%; Invitrogen Corporation) run in tris glycine buffer were used to separate proteins. Proteins were run in the reduced state except for mature SP-B, which was run unreduced. For western blotting, protein loading was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or beta-actin (β-actin). The primary antibodies were rabbit anti-rat SP-A and SP-D (in-house product), rabbit anti-sheep mature SP-B (Chemicon International, Temecula, CA), rabbit anti-human proSP-C, rabbit anti-human mature SP-C (Seven Hills Bioreagents, Cincinnati, OH**),** rabbit anti-human Pyruvate dehydrogenase kinase (PDK) 1 (Stressgen, Ann Arbor, MI), mouse anti-human HIF1α (BD Biosciences), rabbit anti-mouse/human HIF2α (Novus Biologicals, Littleton CO), mouse anti-rabbit GAPDH (abcam, Cambridge, MA), and mouse anti-β-actin (Sigma-Aldrich). The intensities of the bands were calculated using NIH Image software (version 1.62). For real-time RT-PCR, the expression levels of genes were expressed as a ratio to the expression of the constitutive probe Cyclophilin B (CyB). The specific primers and probes of surfactant proteins and CyB were listed in supplemental Table 1, and those of vascular endothelial growth factor (VEGF) and glucose Glucose transporter 1 (GLUT1) were purchased from Applied Biosystems (Foster City, CA).

Morphology

The cells were fixed in 4% paraformaldehyde, and then the filters were embedded in paraffin as described (26). The primary antibodies included rabbit anti-rat SP-A (in-house product) and rabbit anti-human proSP-C (Seven Hills Bioreagents). The secondary antibody was Alexa Fluor 488 donkey anti-rabbit IgG (H+L) from Invitrogen Corporation (Eugene, OR).

Supplemental Figure 1. Immunocytochemistry of SP-A and proSP-C and electron microscopy (EM) images from rat ATII cells cultured for 7 days

(A) Rat ATII cells were adhered for 1 day and cultured for 6 days in 5% RS and 10 ng/ml KGF. Rat ATII cells were fixed on day 7 were stained by SP-A. Upper panel shows the immunocytochemistry picture from the cells cultured in the A/L interface condition. Lower panel shows the picture from cells cultured in the submerged condition. (B) Rat ATII cells fixed on day 7 were stained by proSP-C. Upper panel shows the immunocytochemistry picture from cells cultured in the A/L interface condition. Lower panel shows the picture from cells cultured in the submerged condition. green: surfactant proteins, blue: DAPI (C) Rat ATII cells were fixed on day 7. Left panel shows an EM image of the cells cultured in the A/L interface condition. Right panel shows an EM image of the cells cultured in the submerged condition.

Supplemental Figure 2. Decreased mRNA levels of surfactant proteins and increased mRNA levels of GLUT1 in rat ATII cells by DMOG

Rat ATII cells were adhered for 1 day and cultured for 6 days in 5% RS, 10 ng/ml KGF, 0.58% ethanol and PBS for 6 days either in A/L interface, in submerged, or in A/L interface conditions with 1mM DMOG dissolved in 0.58% ethanol and PBS. The mRNA levels of surfactant proteins, VEGF and GLUT1 were measured by real-time PCR from 4 independent experiments. These levels were normalized to the constitutive probe cyclophilin B. Values are means ± SEM for 4 independent experiments. *: $p<0.05$.

Supplemental Table 1. Sequence of primer and probes used in this study

Gene Name	Forward Primer	Probe	Reverse Primer
SP-A	CACCAATGGGCAGTCAGTCA	CTCCTGCTCTGGTACACATCTCTTTAATGGTATCAA	CCTCGGGACAGCAATGTTG
SP-B	CCATCCCTCTGCCCTTCTG	ACCCGCTTGATCAGAGTCCTGCAAAG	CACCCTTGGGAATCACAGCTT
SP-C	GTCCCAGGAGCCAGTTTCG	TTCCCTGCTGCCCCGTGCA	CACGATGAGAAGGCGTTTGAG
SP-D	AGAGGAATCAAAGGCGAAAGTG	CTTCCAGACAGTGCTGCTCTGAGGCA	TCCATTCAAAGCCTCCATCTG
CyB	TCGGAGCGCAATATGAAGGT	TCTTCGCCGCCGCCCTCAT	CAGGCAGCAAAAGGAAGACAA

Definition of abbreviation; SP: surfactant protein, CyB: Cyclophilin B