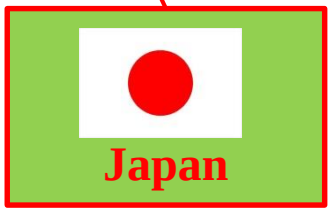
The 8th Meet the Experts Transporter Conference

Mikihisa Takano, PhD, Hiroshima University, Japan

Hungary, Japan, and Hiroshima

Distance: approx. 9,000 km



Hiroshima University (Medical Campus) @Hiroshima City

Department of Pharmaceutics and Therapeutics

Graduate School of Biomedical & Health Sciences
Hiroshima University

Major research interests

- **Membrane transport of small molecules**
- **Endocytosis of large molecules**
- **Drug-induced cellular toxicity and its prevention**

Especially in recent years, we are focusing our research on the drug transport and drug/xenobiotic toxicity in the lung alveolar epithelial cells.

"The 8th Meet the Experts Transporter Conference"
April 26-27, 2018, Budapest, Hungary

Effect of cigarette smoke extract on the function and expression of membrane transporters in alveolar epithelial cells

Mikihisa Takano

Graduate School of Biomedical & Health Sciences,
Hiroshima University, Japan

Outline of today's talk

1) General characteristics of alveolar type II and type I cells
2) Functional expression of P-glycoprotein and PEPT2 in alveolar type II and type I-like cells in primary culture
3) Effect of cigarette smoke extract on P-glycoprotein function in alveolar type I-like cells in primary culture
4) Development of a new alveolar epithelial cell line expressing functional P-glycoprotein
5) Effect of cigarette smoke extract on the function and expression of P-glycoprotein in A549/P-gp cells and PEPT2 in NCI-H441 cells

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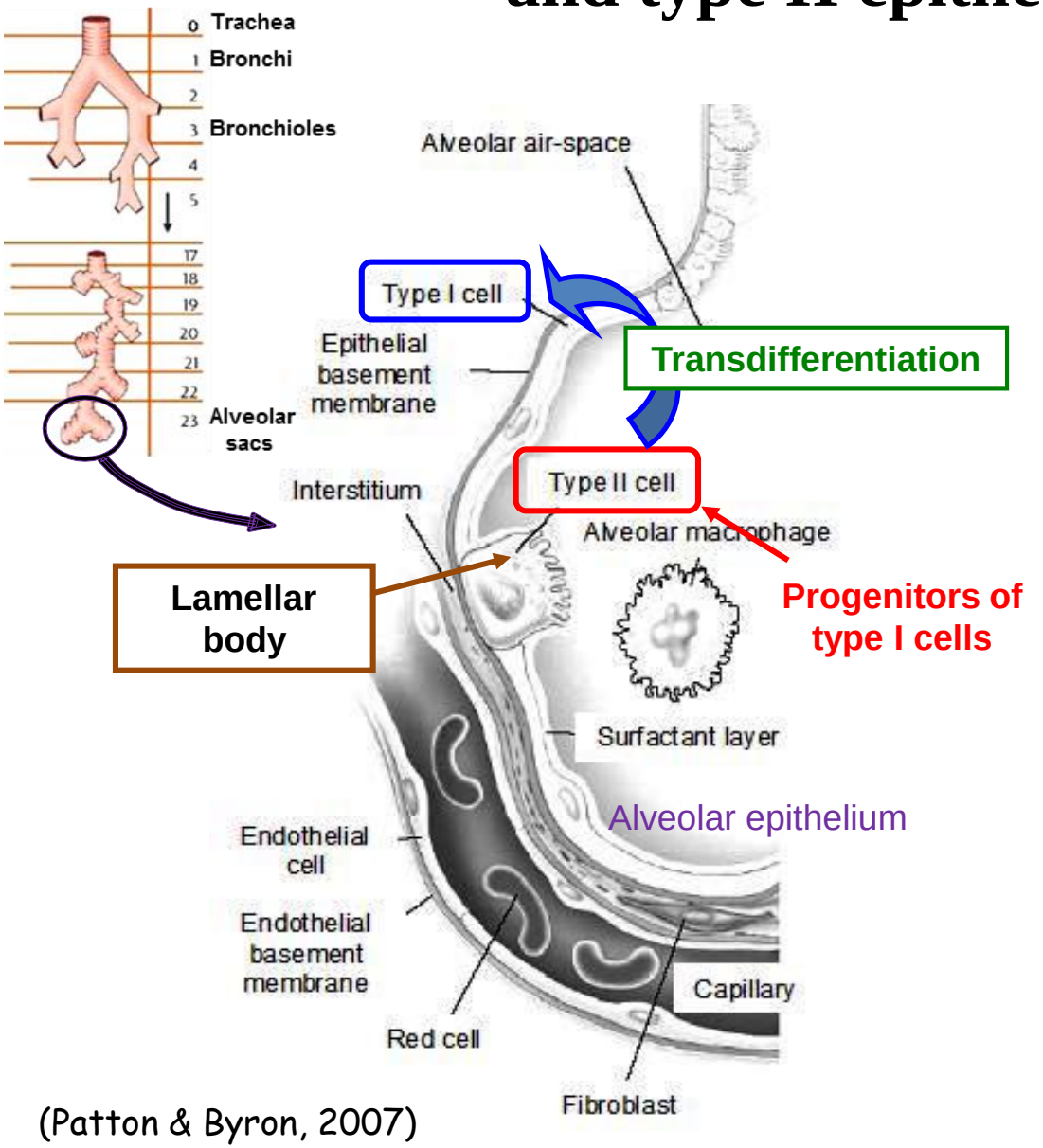
Structure and function of lung alveolus and type I and type II epithelial cells



(Patton & Byron, 2007)

In the human distal lungs, there are 400-500 million alveoli, and the total surface area is > 100 m^2. The alveolar epithelium is comprised of ---

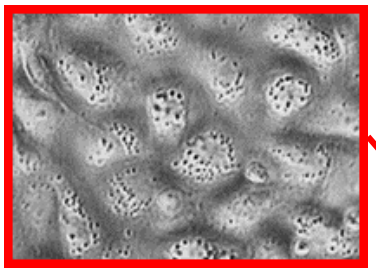
Type I cells:

- squamous and thin epithelial cells
- 90-95% of surface area
- gas exchange

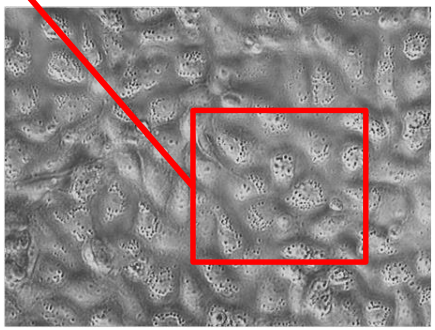
Type II cells:

- cuboidal epithelial cells
- 5-10% of surface area
- surfactant production

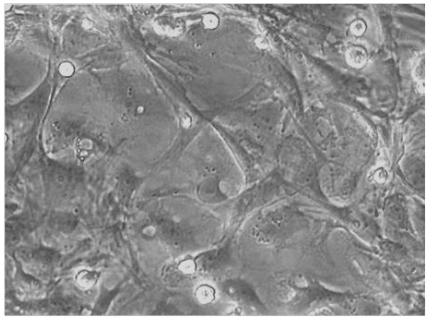
Morphological feature of rat primary cultured alveolar epithelial cells (type II and type I-like cells)



Phase-contrast micrographs

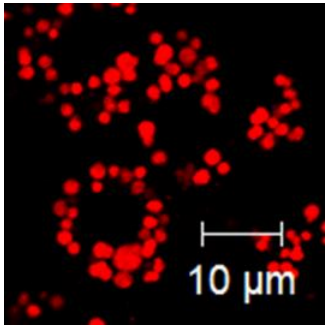


Type II cells
(5 x 10^6 cells/35 mm dish/2 days)

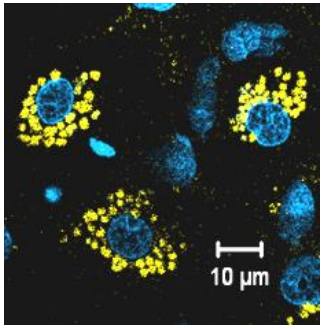


Type I-like cells
(2 x 10^6 cells/35 mm dish/6 days)

Lamellar bodies in type II cells
Confocal laser scanning microscopy (CLSM)



LysoTracker Red



Nile Red

Type II cells were isolated from rat lungs, and were cultured for two days (type II cells) or six days (type I-like cells) at the seeding density described above.

Change in mRNA expression profile during transdifferentiation of type II cells into type I-like cells

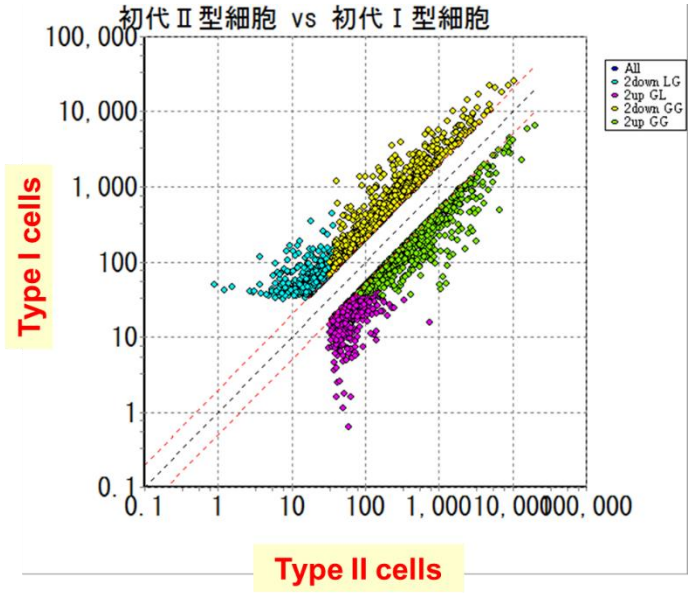
Real-time PCR and Microarray analyses

Table II. Change in the Expression Level of Marker Gene mRNA in Rat Alveolar Epithelial Cells During Culture

Gene Name	Type II Day 2	Type I-like Day 6	Ratio (day 6/day 2)
Type I cell marker			
RTI40	0.12±0.07	1.55±0.19**	12.6
IGFBP6	0.37±0.07	2.00±0.46*	5.5
mdr1a	0.15±0.10	2.17±0.65*	14.2
caveolin-1	0.21±0.15	11.26±1.67**	52.5
Type II cell marker			
SP-B	1.26±0.12	0.19±0.02**	0.15
CINC-1	1.17±0.12	0.03±0.01**	0.03

Total RNA was extracted from the cells cultured for 2 or 6 days after seeding 5 or 2×10^6 cells/35-mm dish, respectively. Each value represents the mean±SE of relative expression level normalized by *GAPDH* (n=3).
*p<0.05
**p<0.01 vs. day2.

CINC-1: chemokine-induced neutrophilic chemoattractant–1



The expression of about 1200 mRNAs increased more than 2-fold and decreased to less than 50%, respectively, along with transdifferentiation (total probes 35000).

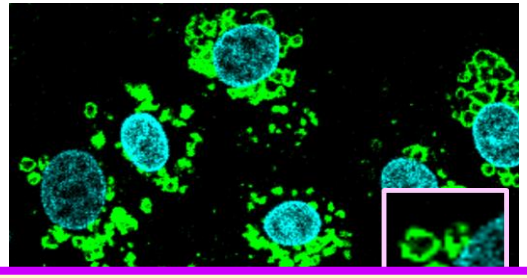
In both methods, we observed that the expression level of many mRNAs changed drastically before and after transdifferentiation. For example, in Real-time PCR analysis, caveolin-1, a type I cell marker mRNA, increased markedly, to about 50-fold, while CINC-1, a type II cell marker mRNA, decreased markedly, to about 3%, after transdifferentiation.

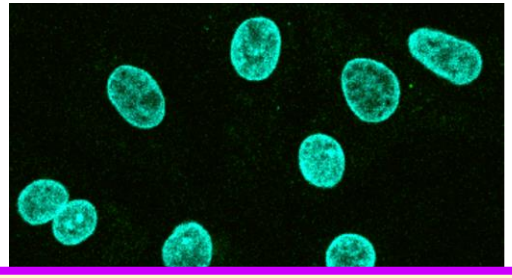
Immunostaining of ABCA3 and caveolin-1 protein in primary cultured rat alveolar epithelial cells

Type II cell

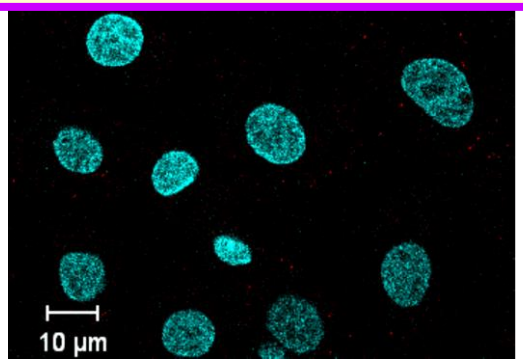
Type I-like cell

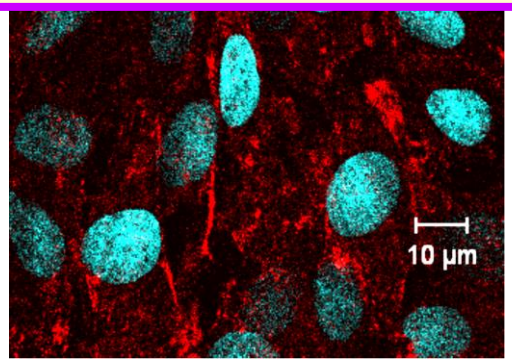
ABCA3 (type II cell marker)





Primary cultured alveolar epithelial cells we have been using have type II and type I cell phenotype, respectively.





Summary 1

- Alveolar type II epithelial cells in primary culture rapidly transdifferentiated into type I-like cells along with the culture days (about 6 days in our culture condition).
- Cell morphology including intracellular lamellar body formation was quite different between type II and type I-like cells.
- The expression pattern of mRNA and protein changed drastically along with the transdifferentiation.

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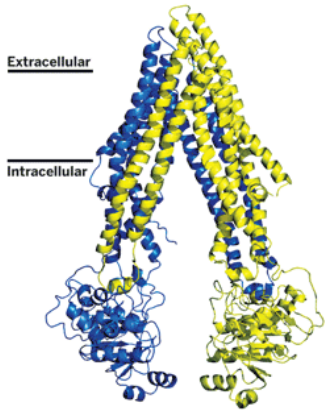
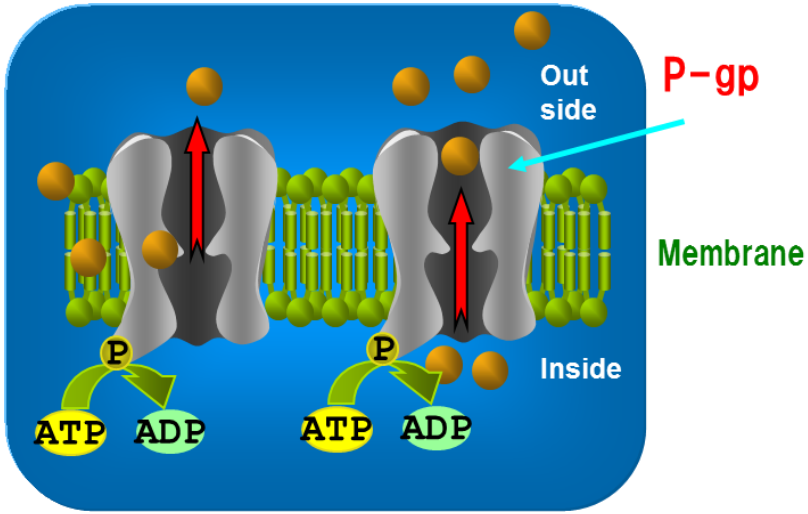
Structure and function of an ABC efflux transporter P-glycoprotein (P-gp; ABCB1)



Science 323, 1718 (2009)



: P-gp substrate

P-Glycoprotein is a member of ABC transporter family (ABCB1), and transports various drugs such as anticancer drugs and immunosuppressants.

It is a primary active efflux transporter, and actively pumps out its substrates from the cells.

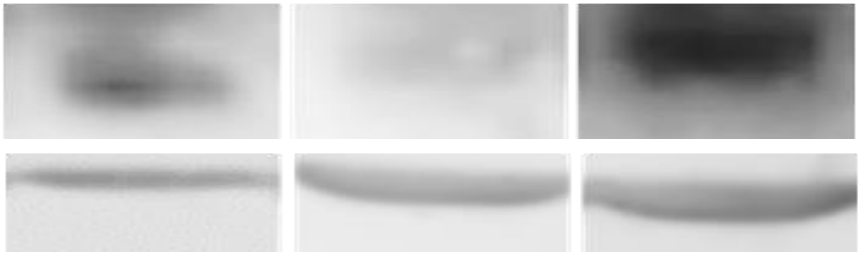
It is well known that P-gp confers multidrug resistance in cancer cells.

In the lungs, the expression of P-gp within the whole lungs and within the alveolar epithelium has been reported. However, the relationship between P-gp expression and transdifferentiation was not clear, and was controversial. So, we examined this point using primary cultured alveolar epithelial cells.

Expression of P-gp protein in primary cultured alveolar type II and type I-like cells

Western blotting

rBBM Type II Type I-like

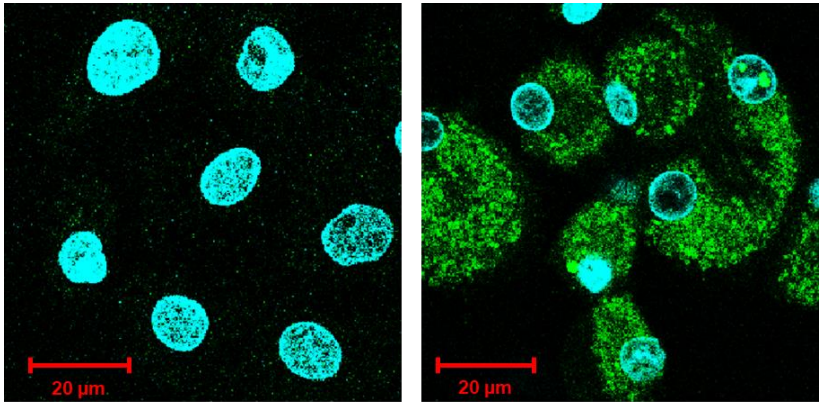


P-gp

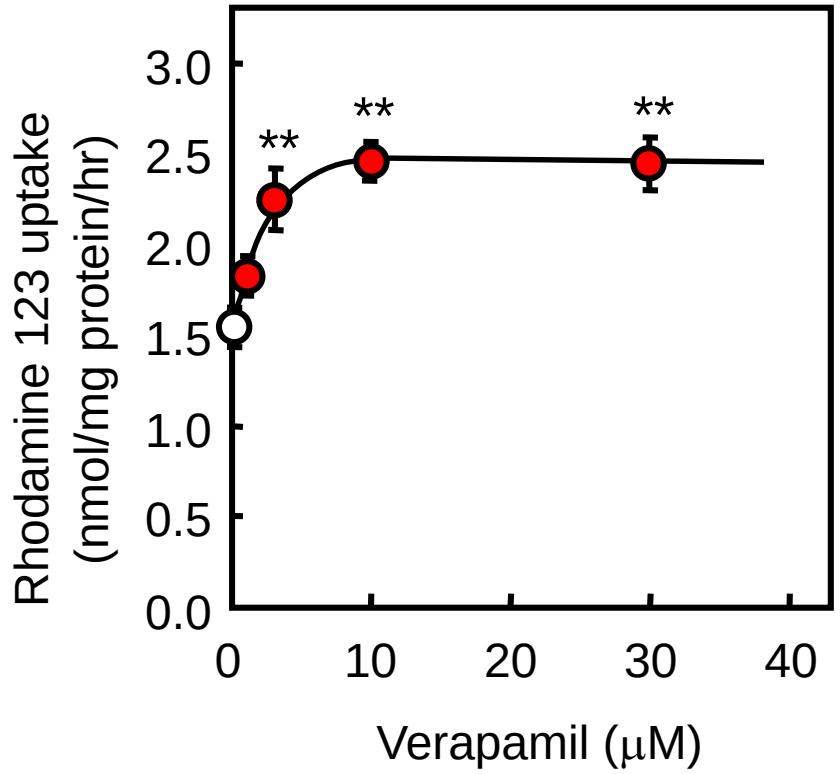
β-Actin

Immunostaining

Type II Type I-like

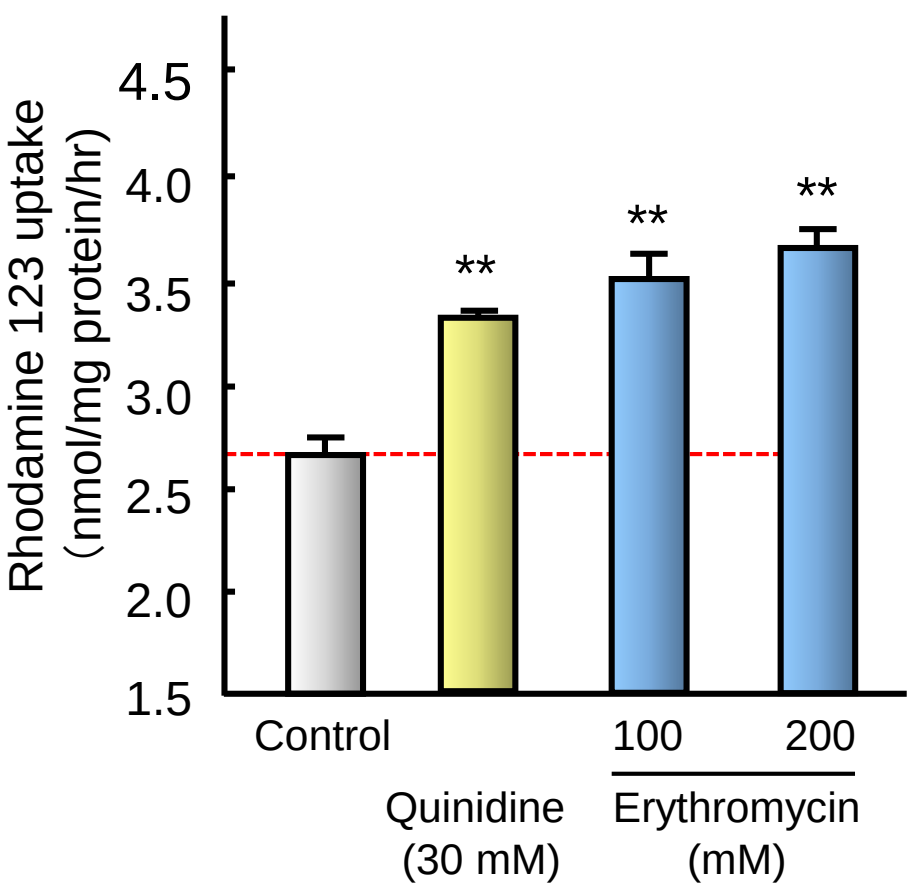


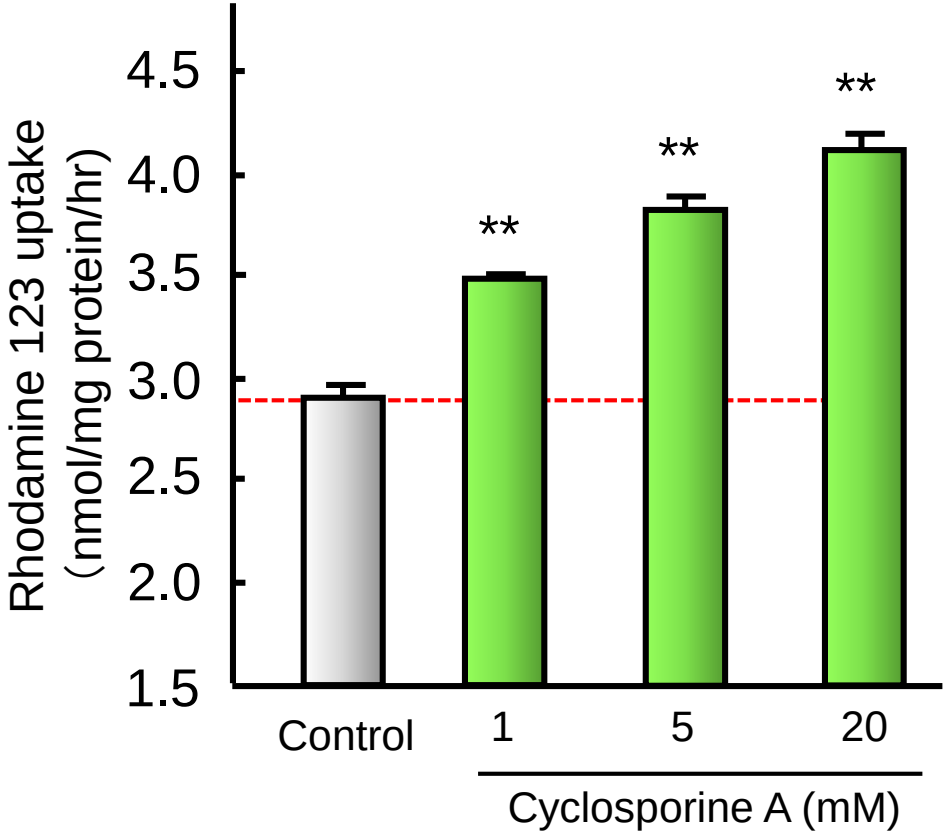
Effect of verapamil, a P-gp inhibitor, on the uptake of rhodamine 123 in alveolar type I-like cells



*p<0.05, **p<0.01; significantly different from each control

Effect of P-gp inhibitors on rhodamine 123 uptake in alveolar type I-like cells

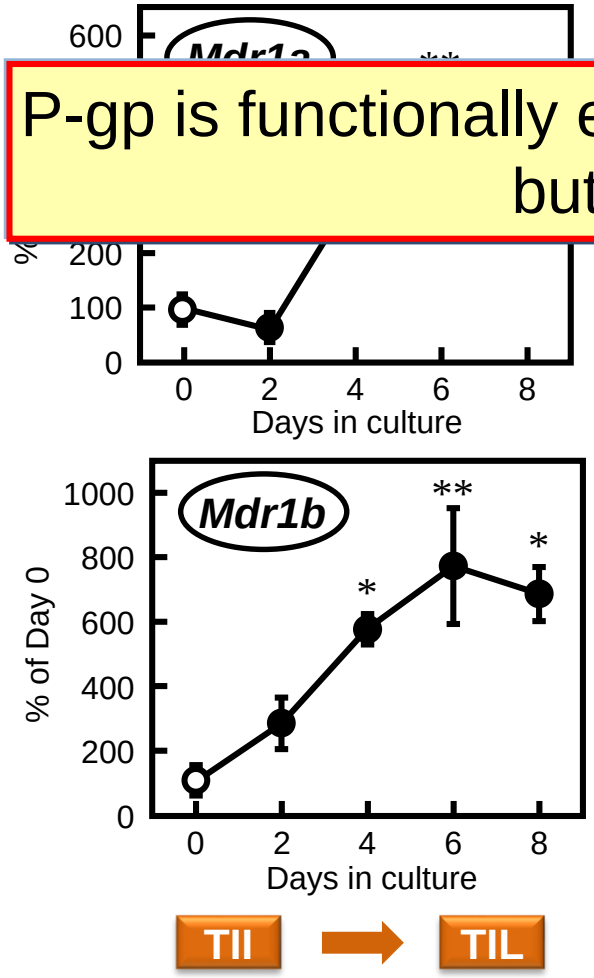




**p<0.01

mRNA expression and function of P-gp in alveolar epithelial cells during transdifferentiation

Mdr1a/b mRNA expression



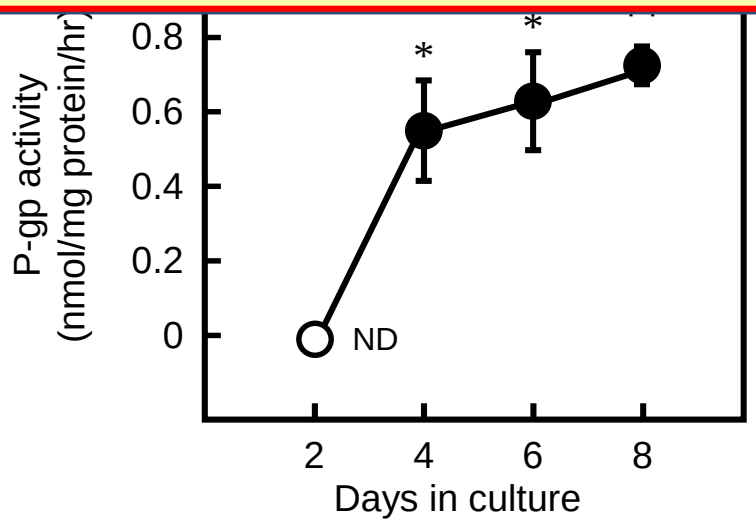
TII → TIL

* $p<0.05$, **$p<0.01$; significantly different from the value of day 0

P-gp activity

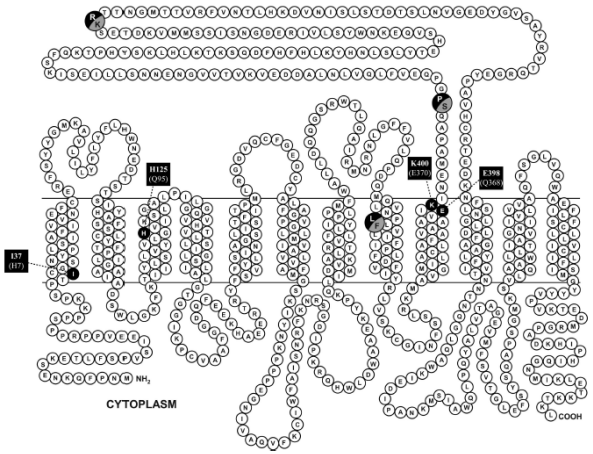
・Substrate : Rho123 (20 μM)

P-gp is functionally expressed in alveolar type I-like cells, but not in type II cells.

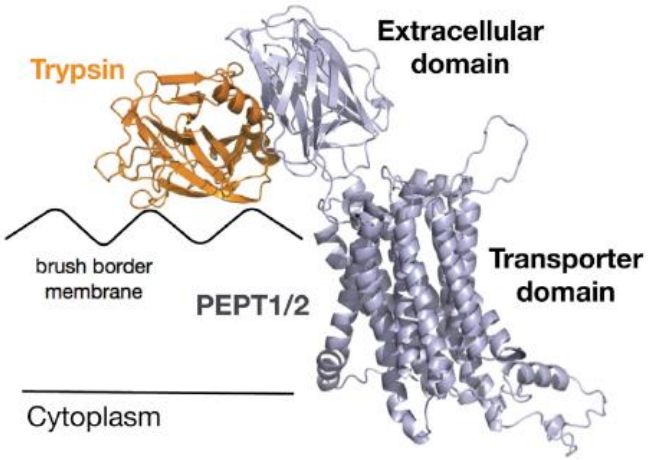


$p<0.05$, **$p<0.01$; significantly different from the value of day 2
ND: not detected

Peptide transporter 2 (PEPT2)



Am J Physiol Renal Physiol 294: F1422–F1432, 2008

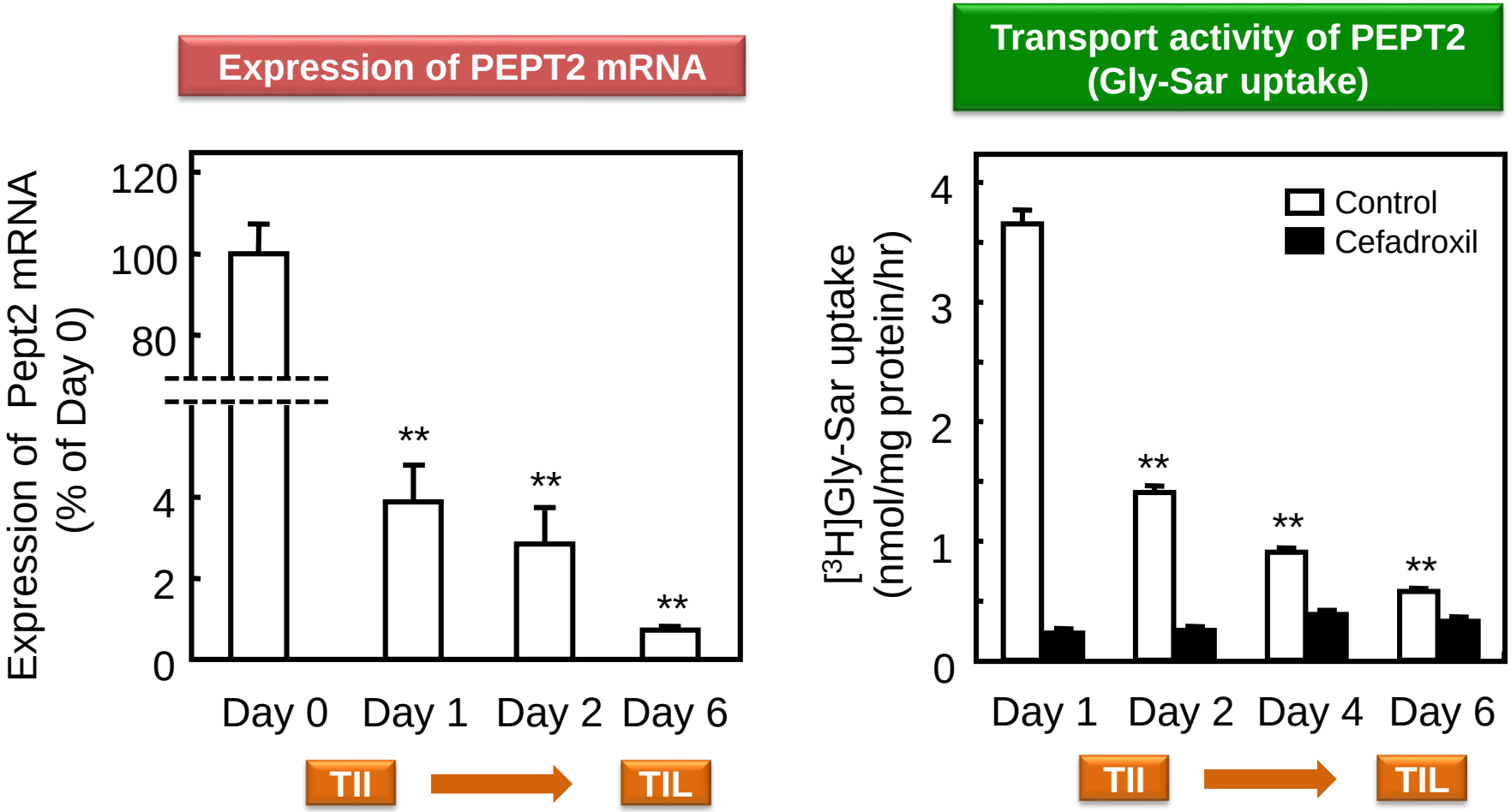


Structure 23, 1889–1899, 2015

PEPT2 is

- H^+/oligopeptide cotransporter.
- a high-affinity/low-capacity system when compared with PEPT1.
- expressed in the kidney, choroid plexus, mammary gland and **lungs**.
- However, the relationship between the transdifferentiation of alveolar epithelial cells and functional expression of PEPT2 was not clear. In addition, there was little information concerning the transport characteristics of PEPT2 at the alveolar cell level. Therefore, we studied and compared the expression and function of PEPT2 in primary cultured alveolar type II and type I-like epithelial cells.

mRNA expression and transport activity of PEPT2 in alveolar epithelial cells during transdifferentiation

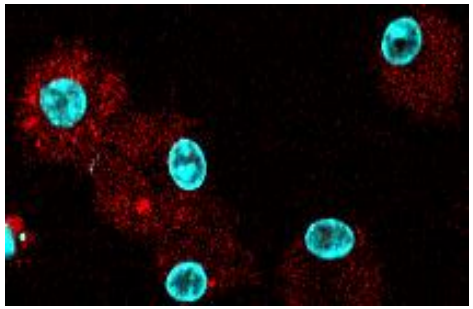


The mRNA expression and PEPT2 activity decreased along with the transdifferentiation of alveolar epithelial cells.

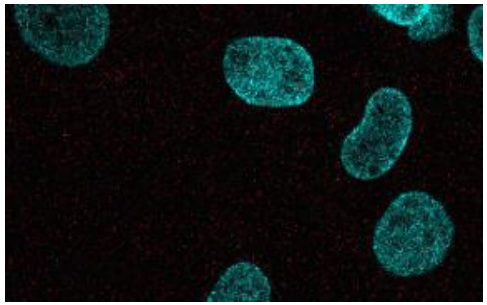
Expression of PEPT2 protein in alveolar type II and type I-like epithelial cells

Immunostaining

Type II cells

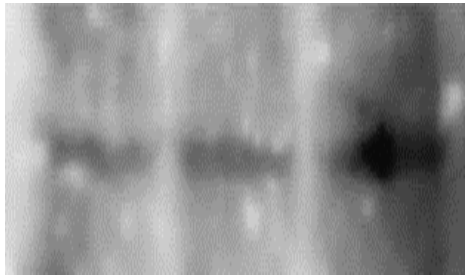


Type I-like cells

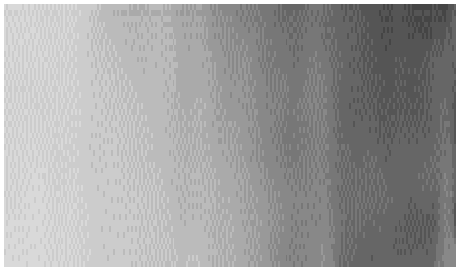


Western blotting

Type II cells

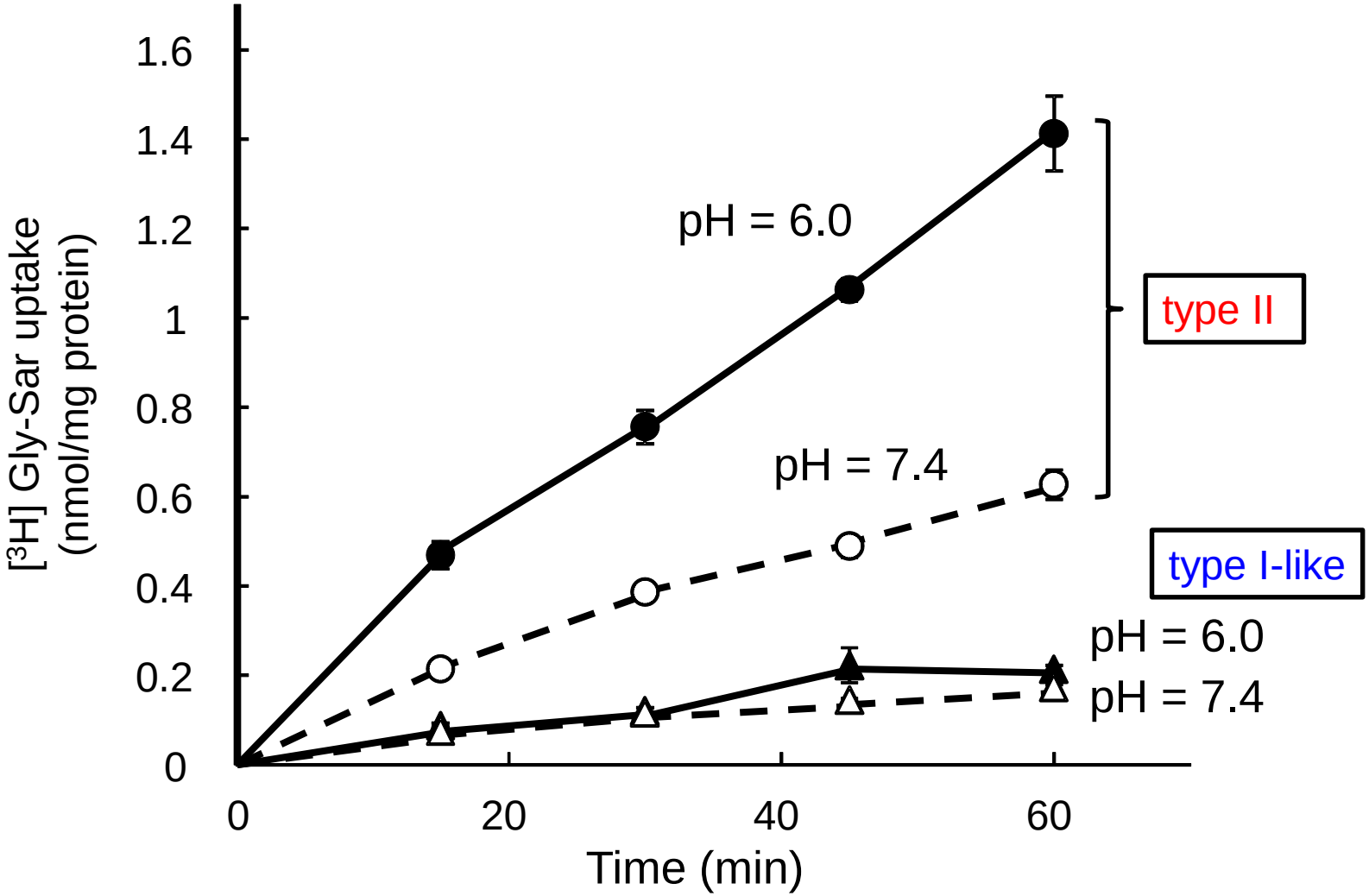


Type I-like cells

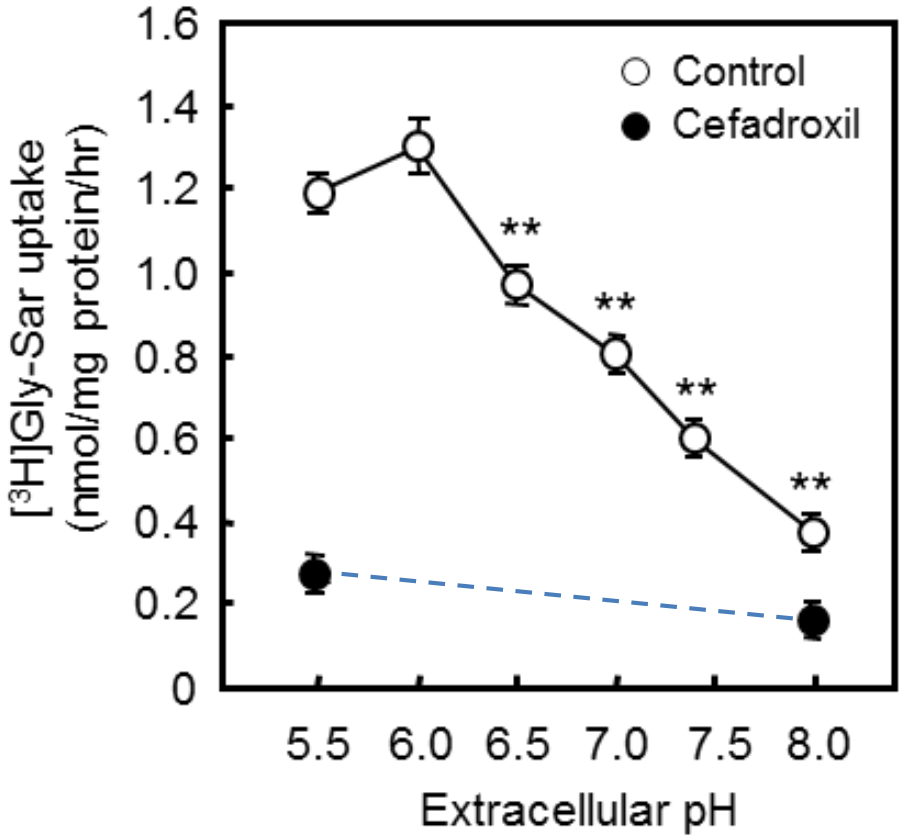


Characteristics of Gly-Sar uptake

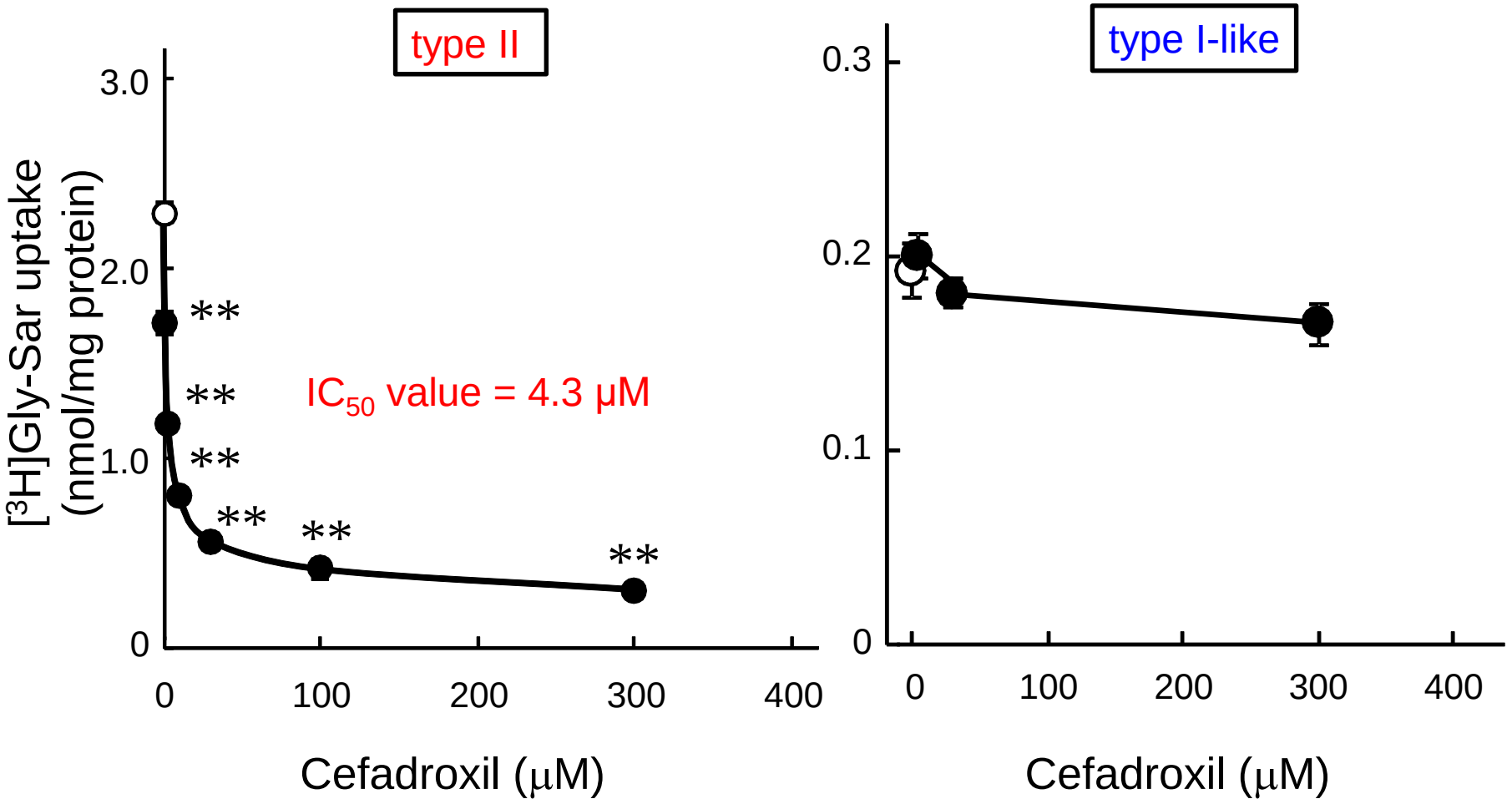
Effect of extracellular pH on Gly-Sar uptake in alveolar epithelial cells



pH-dependence of Gly-Sar uptake in alveolar type II cells (pH 5.5 – 8.0)

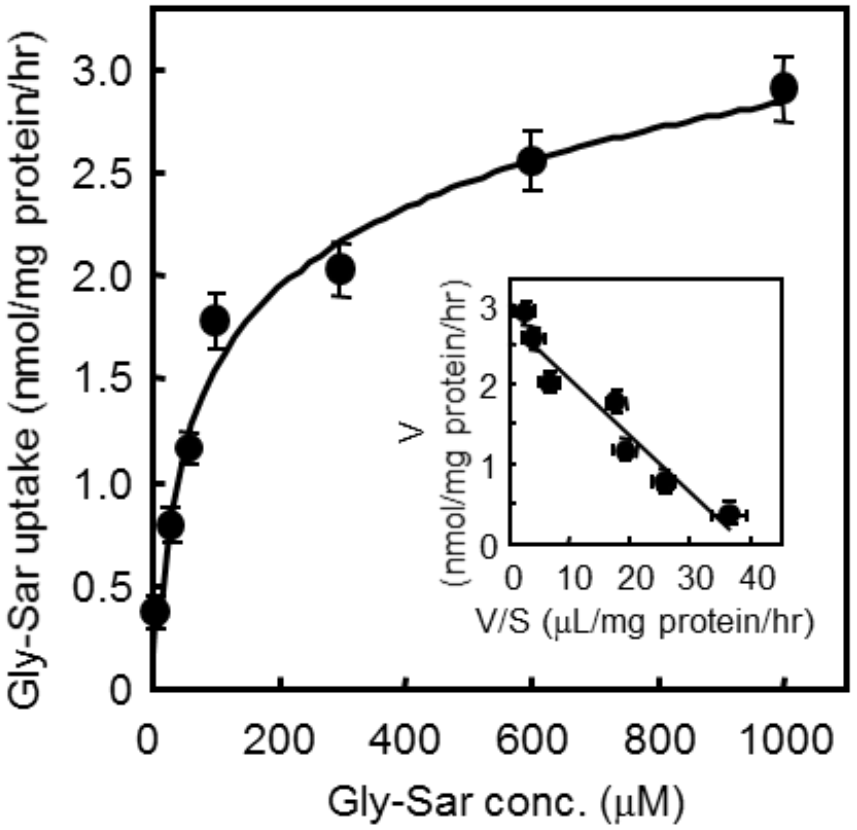


Concentration-dependent effect of cefadroxil on Gly-Sar uptake in alveolar epithelial cells (pH 6.0)



** P < 0.01

Concentration-dependence of Gly-Sar uptake in alveolar type II cells (pH 6.0)



Km = 72.0 μM

These transport characteristics of Gly-Sar in alveolar type II cells were comparable to those of PEPT2 reported in other types of cells.

Summary 2

The relationship between transdifferentiation of alveolar epithelial cells and functional expression of P-gp and PEPT2

- P-gp protein was expressed in type I-like cells, but not in type II cells, and the expression of P-gp mRNAs, mdr1a and mdr1b, as well as P-gp activity increased along with the transdifferentiation.

- In contrast, PEPT2 is functionally expressed in type II cells, but the expression decreased along with the transdifferentiation, and PEPT2 was almost completely lost in type I-like cells.

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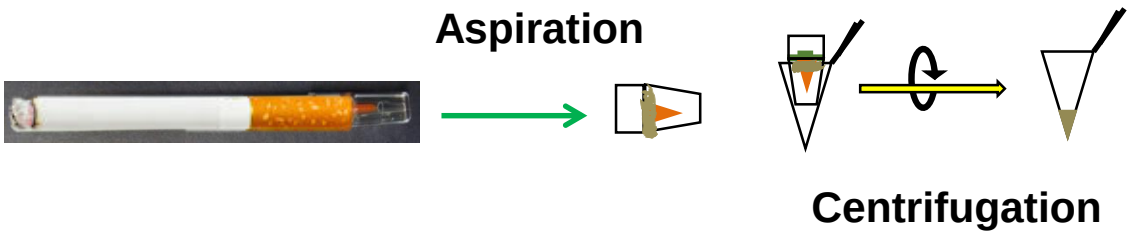
Preparation of cigarette smoke extract (CSE)

- Cigarette: MEVIUS SUPER LIGHTS; tar 6 mg, nicotine 0.5 mg (JAPAN TOBACCO INC, JAPAN)

- Filter for CSE collection: SUPER25 filter (commercially available filters for smoking; KAMAYA, JAPAN)

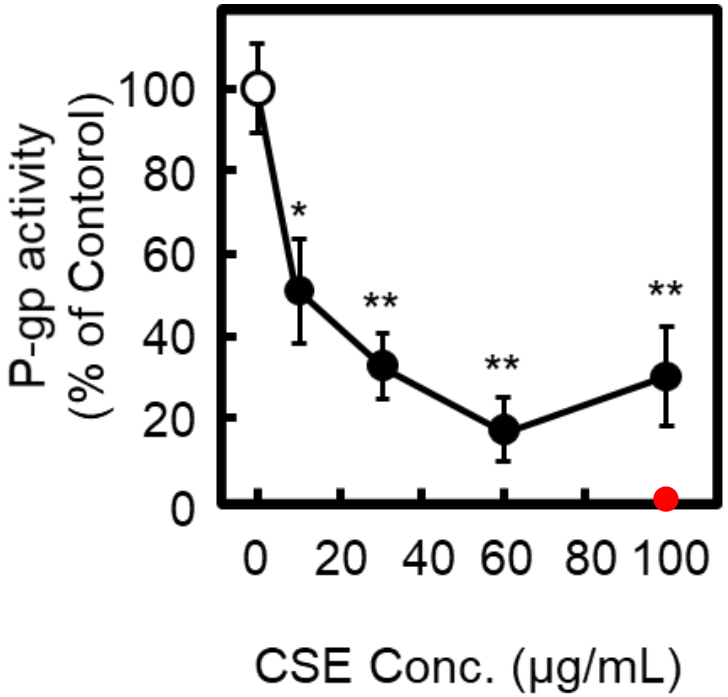


The amount of CSE obtained from one cigarette was about 4 mg.

Effect of CSE on P-gp activity in type I-like cells (co-incubation; uptake study)

P-gp activity (rhodamine 123 uptake study)

Alveolar type I-like cells were preincubated with CSE (0-100 μg/mL) for 30 min, and incubated with Rho123 w/ or w/o CSE and a P-gp inhibitor erythromycin for 60 min.



Assuming that alveolar fluid volume in human lungs is 40 mL and CSE from one cigarette (4 mg) is rapidly dissolved in alveolar lining fluid, the concentration of CSE is 100 μg/mL.

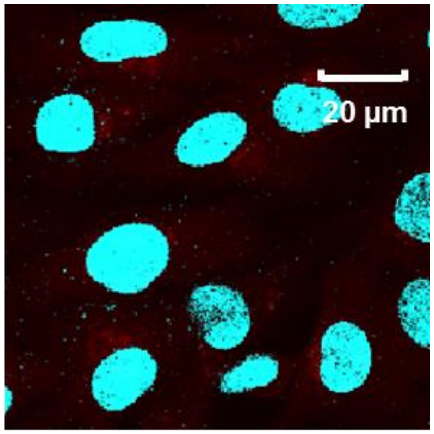
Effect of CSE on P-gp activity in type I-like cells
(co-incubation; confocal laser scanning microscopy (CLSM))

Rhodamine 123 accumulation (CLSM)

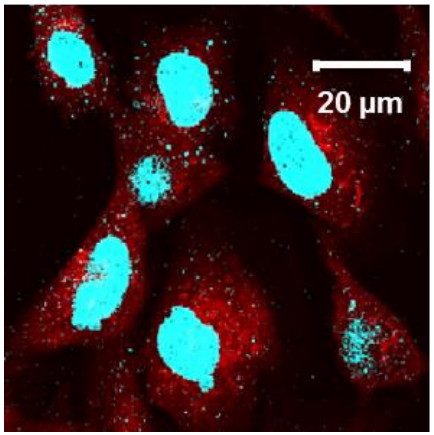
Alveolar type I-like cells were incubated with Rho123 w/ or w/o CSE, and were observed by CLSM.

Rho123 (red) and nucleus (blue, Hoechst 33342).

Control



CSE 60 μg/mL



Summary 3

- P-gp activity estimated from Rho123 accumulation was inhibited by co-incubation with CSE in a concentration-dependent manner in type I-like cells, which was also confirmed by CLSM.

We next attempted to examine the effect of long term treatment with CSE on P-gp. However, the phenotype of primary cultured alveolar type I-like cells is not necessarily stable. In addition, we could not detect substantial P-gp activity in widely used alveolar epithelial cell lines such as A549 and NCI-H441 (derived from human lungs), and RLE-6TN (derived from rat lungs).

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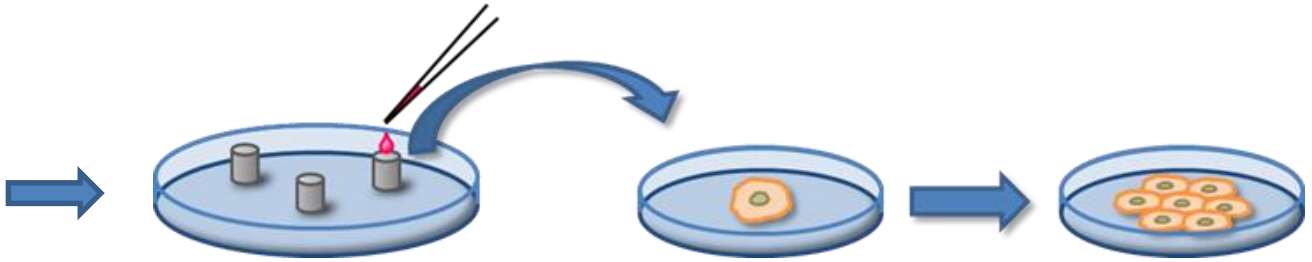
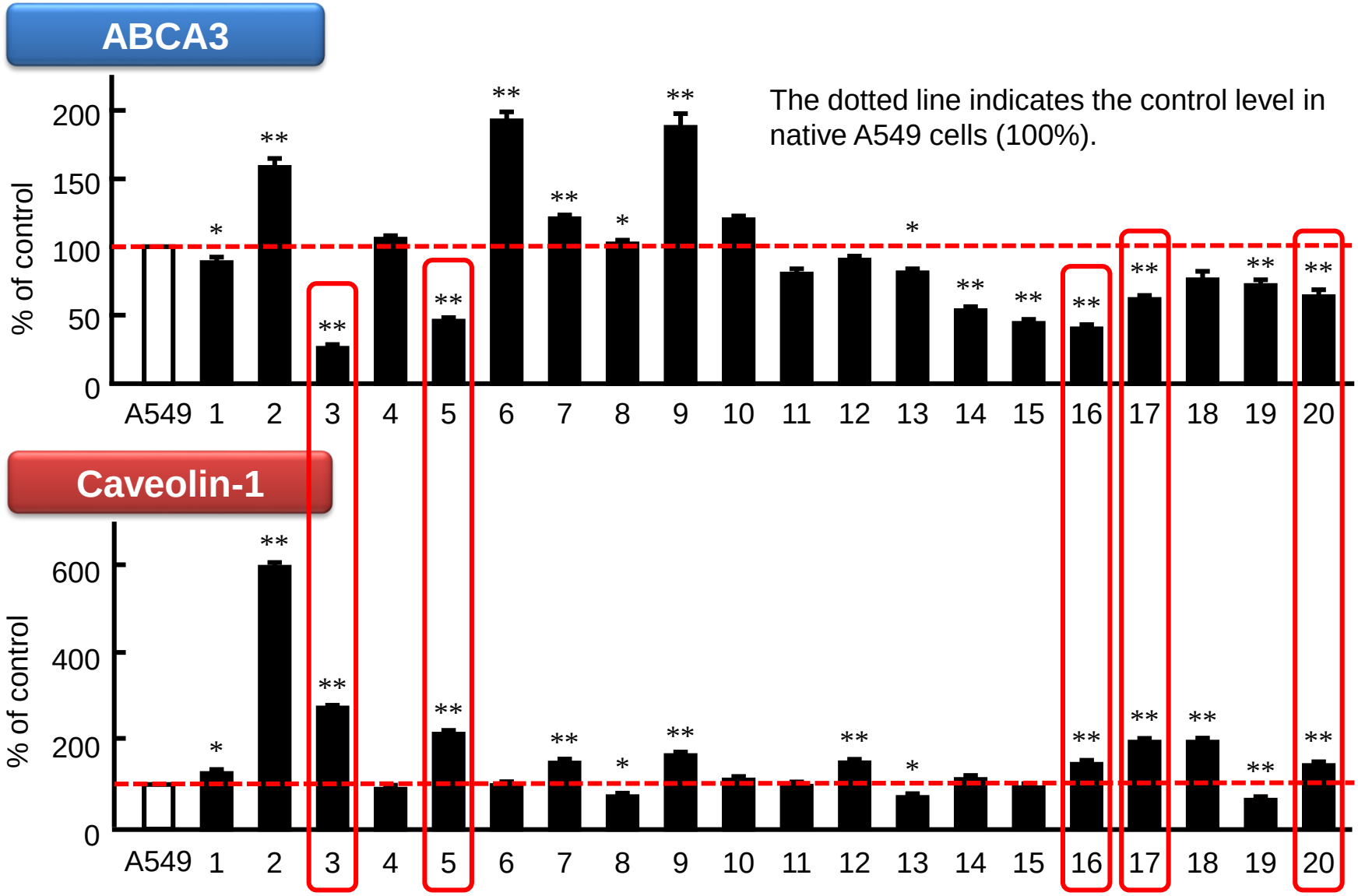
Isolation of an A549 clone expressing functional P-glycoprotein

A549 cells were seeded at a density of 20 cells/100-mm dish



Schematic diagram of cell cloning
(limiting dilution method)

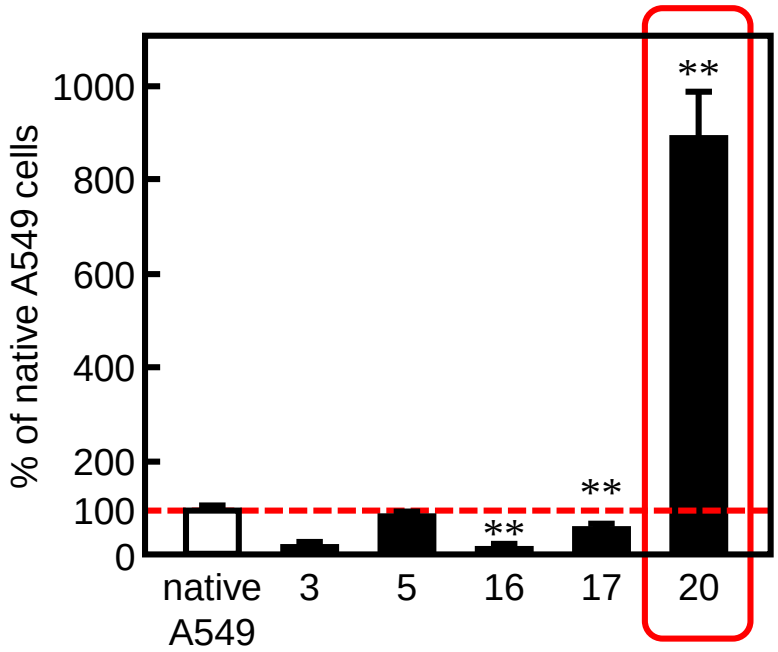
Expression of mRNAs of ABCA3 (a TII cell marker) and caveolin-1 (a TI cell marker) in twenty A549 clones



* $p<0.05$, **$p<0.01$; significantly different from the value of native A549 cells

Expression of MDR1 mRNA in selected five clones

Real-time PCR (MDR1 mRNA)

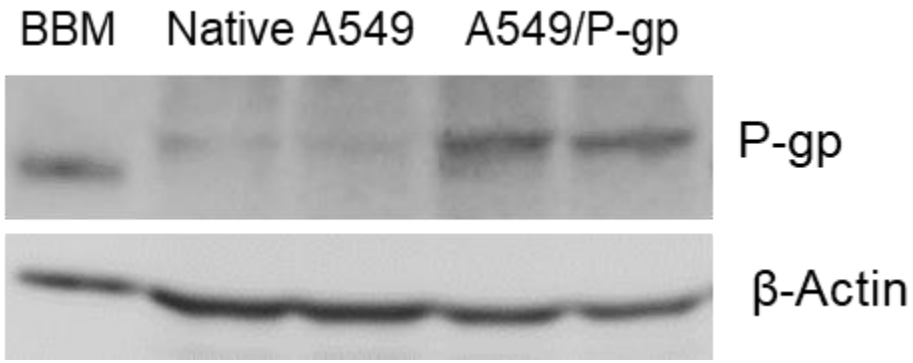


A549/P-gp

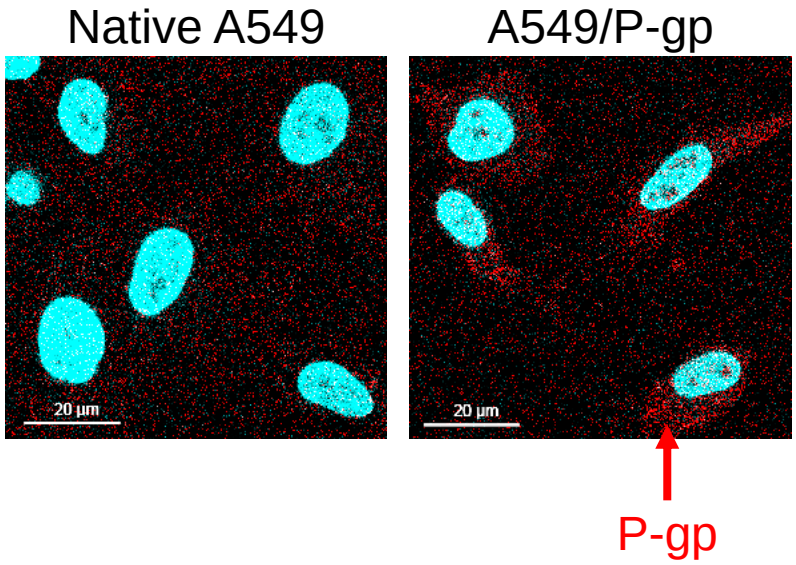
**p<0.01；significantly different from the value of native A549 cells

Expression of P-gp protein in native A549 and A549/P-gp cells

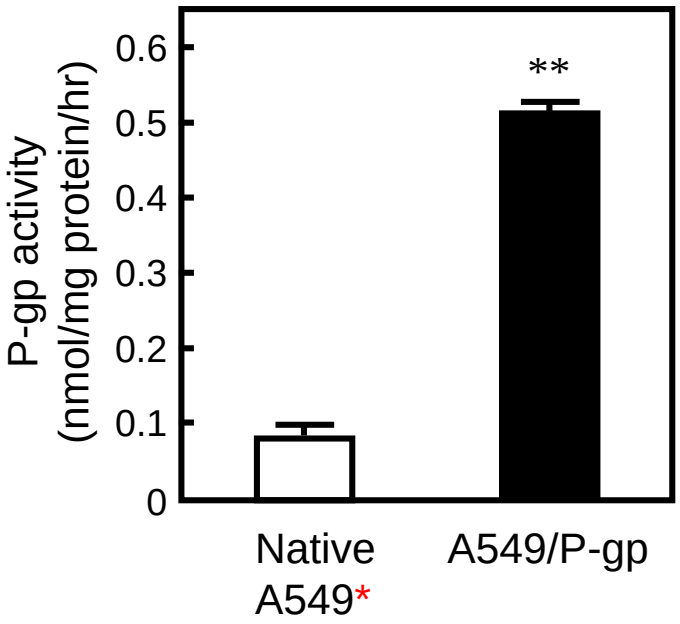
P-gp (Western blotting)



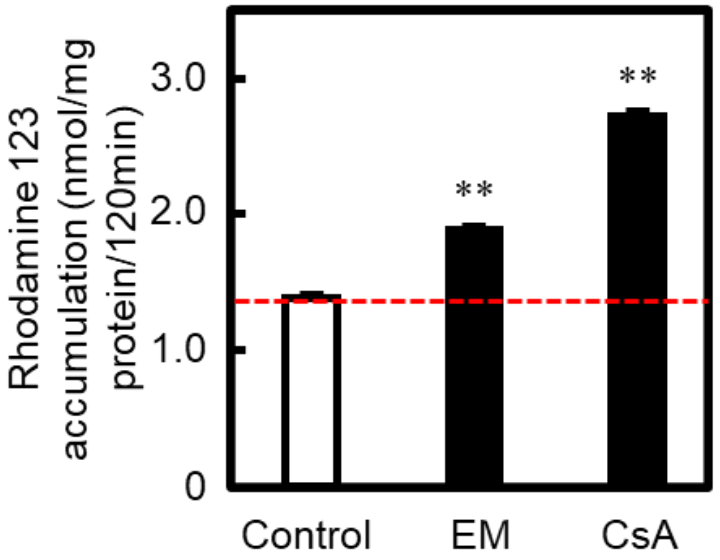
P-gp (immunostaining)



P-gp activity in native A549 and A549/P-gp cells



Effect of P-gp inhibitors on Rho123 uptake in A549/P-gp cells



* In some experiments, P-gp activity could not be detected in native A549 cells.

Substrate: Rho123 (10 μM)
Inhibitor: Verapamil, erythromycin (EM), cyclosporine A (CsA)
***P<0.01*; vs native A549 cells or control A549/P-gp cells

Summary 4

- Using limiting dilution method, an A549 clone (A549/P-gp) expressing MDR1 (P-gp) mRNA and protein was successfully obtained.
- Transport activity of P-gp was confirmed in A549/P-gp cells.

A549/P-gp cells may be a good in-vitro model to study the regulation of P-gp expression and function in alveolar type I cells.

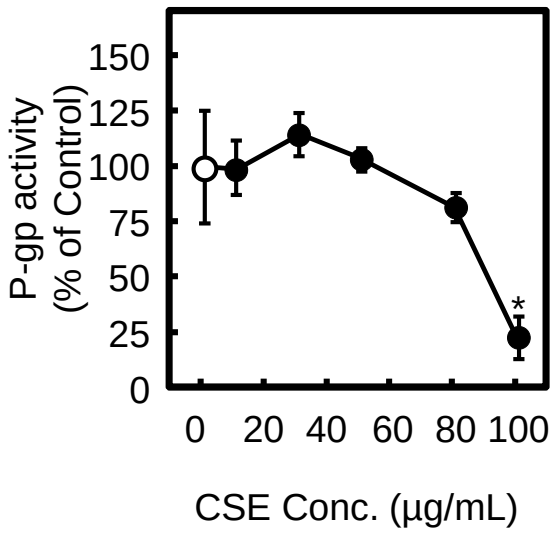
Outline of today's talk

1) General characteristics of alveolar type II and type I cells
2) Functional expression of P-glycoprotein and PEPT2 in alveolar type II and type I-like cells in primary culture
3) Effect of cigarette smoke extract on P-glycoprotein function in alveolar type I-like cells in primary culture
4) Development of a new alveolar epithelial cell line expressing functional P-glycoprotein
5) Effect of cigarette smoke extract on the function and expression of P-glycoprotein in A549/P-gp cells and PEPT2 in NCI-H441 cells

P-gp study

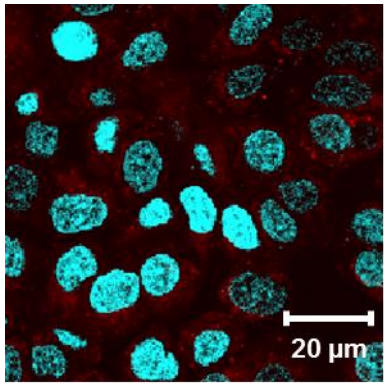
Effect of co-incubation with CSE on P-gp activity in A549/P-gp cells

P-gp activity (rhodamine 123 uptake study)

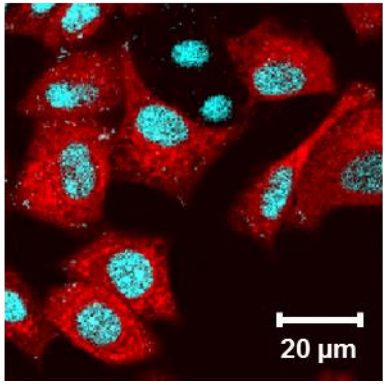


Rhodamine 123 accumulation (CLSM)

Control



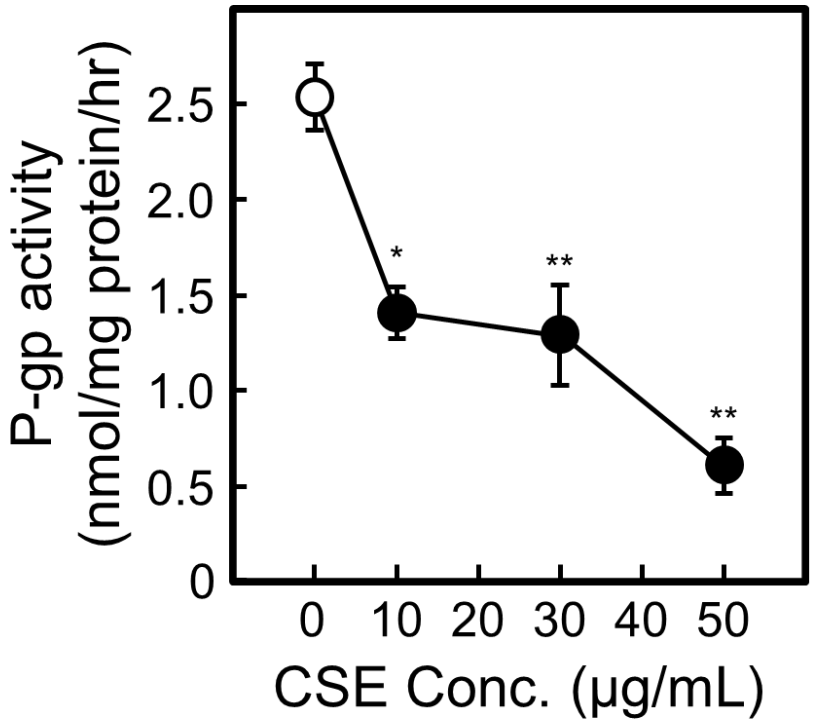
CSE 100 µg/mL



Long term treatment

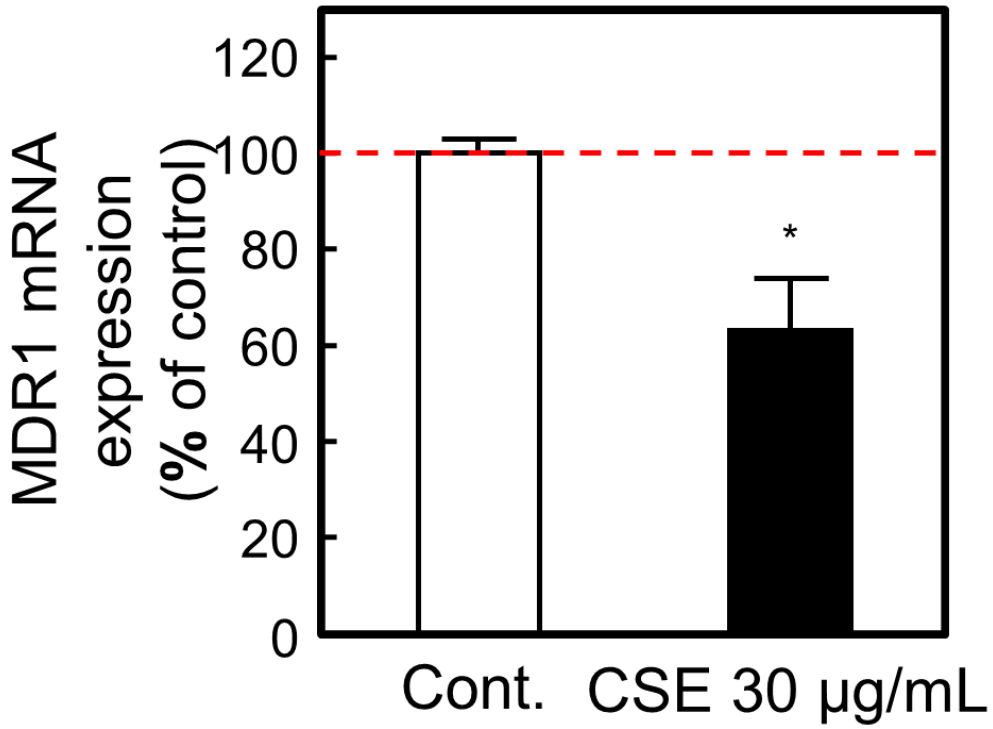
Effect of long-term CSE treatment on P-gp activity in A549/P-gp cells

(pretreatment for 96 hrs)



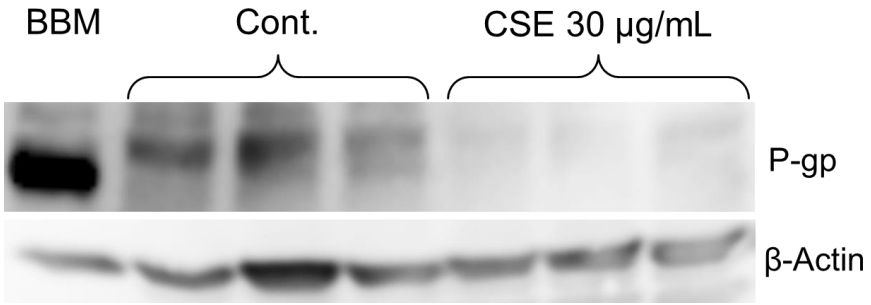
Effect of long-term CSE treatment on MDR1 mRNA expression in A549/P-gp cells

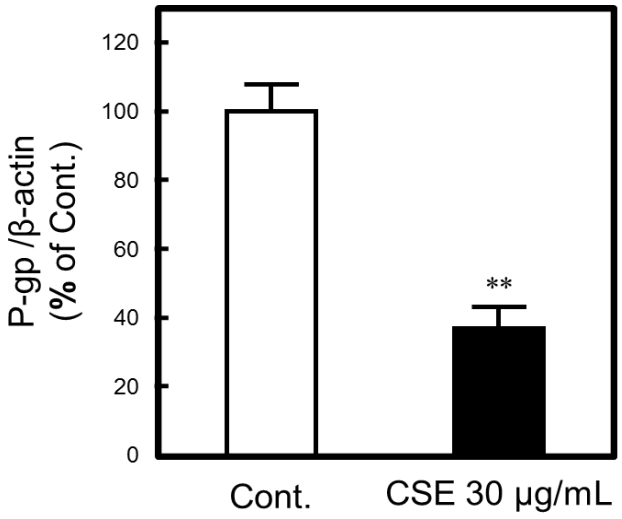
(pretreatment for 96 hrs)



Effect of long-term CSE treatment on P-gp protein expression in A549/P-gp cells

(pretreatment for 96 hrs)





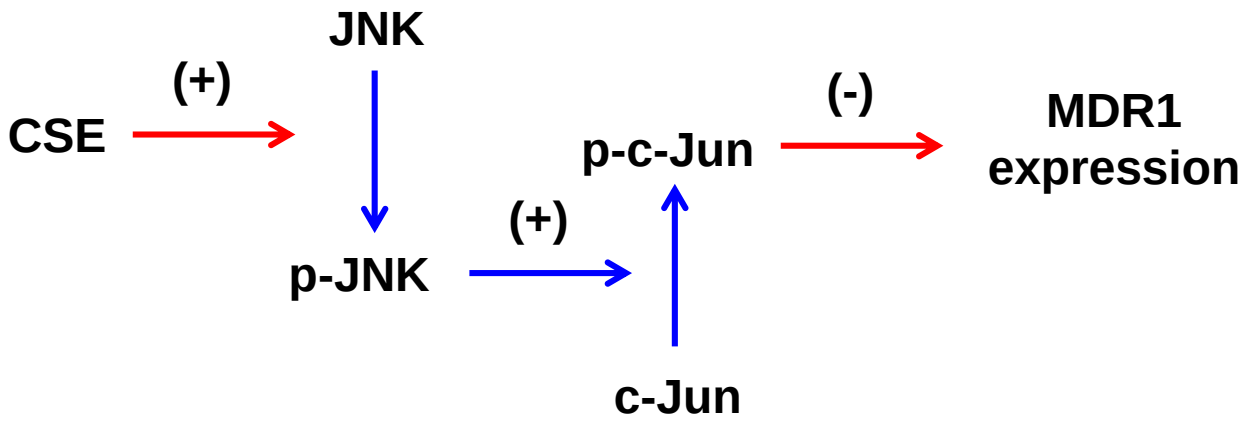
What is the molecular mechanism of P-gp suppression by long-term CSE treatment?

Cancer Research (2003) 63: 1131-1136
Transcription Factor c-Jun Activation Represses mdr-1 Gene Expression (doxorubicin-resistant human leukemia K562 cell line)
Ze-Hong Miao and Jian Ding

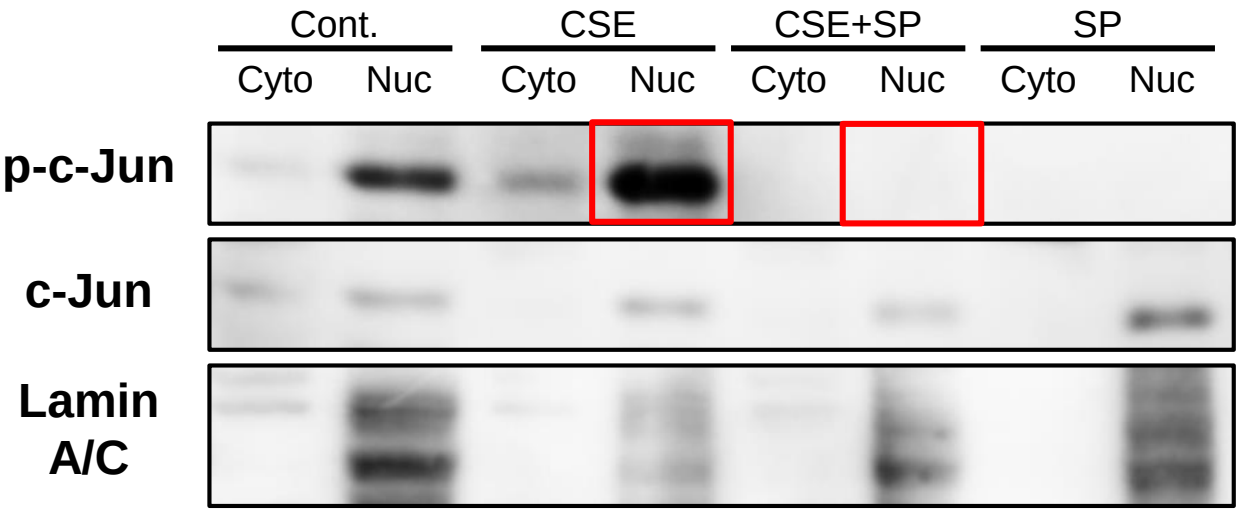
Cancer Chemother Pharmacol (2010) 65:1131–1136
PSC833, cyclosporine analogue, downregulates MDR1 expression by activating JNK/c-Jun/AP-1 and suppressing NF-kB (doxorubicin-resistant lung cancer cell subline SK-MES-1/DX1000)
H. Bark and Cheol-Hee Choi

Some reports suggest that the activation of JNK/c-Jun pathway downregulates MDR1 expression.

CSE may downregulate MDR1 expression by activating JNK/c-Jun pathway in alveolar epithelial cells



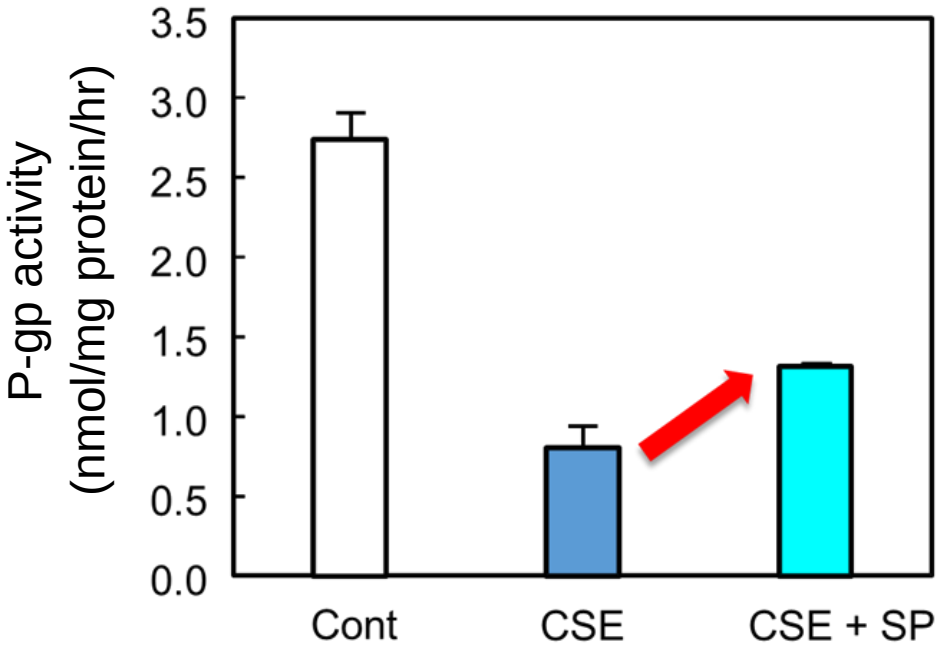
Effect of long-term CSE treatment on phospho-c-Jun level in A549/P-gp cells



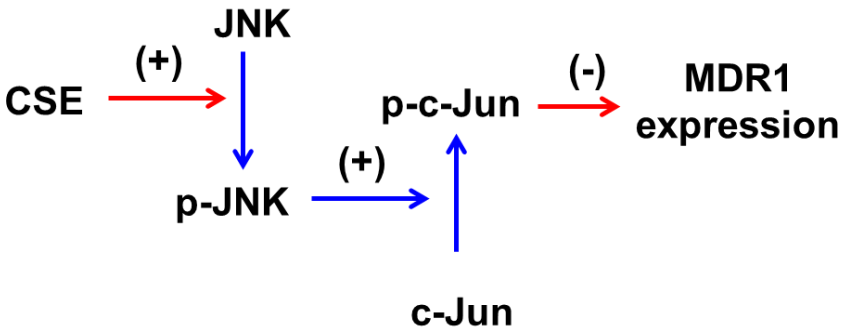
CSE: 50 μg/mL
SP: SP600125 (JNK inhibitor) 5 μM
Cyto: cytosol
Nuc: nucleus

Possible molecular mechanism of P-gp suppression by long-term CSE treatment

Effect of SP600125 on CSE-induced suppression of P-gp activity in A549/P-gp cells (preliminary study)



CSE may downregulate MDR1 expression by activating JNK/c-Jun pathway in A549/P-gp cells



PEPT2 study

Effect of cigarette smoke extract on the function and expression of PEPT2

Studies with primary cultured alveolar type II cells

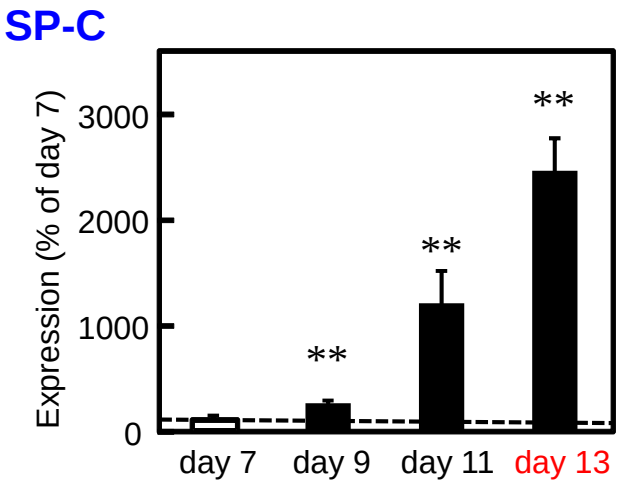
- Co-incubation with CSE inhibited PEPT2 activity (Gly-Sar uptake) in a concentration-dependent manner.
- The Km value of Gly-Sar uptake was increased, whereas Vmax value was not altered, by co-incubation with CSE (competitive?).

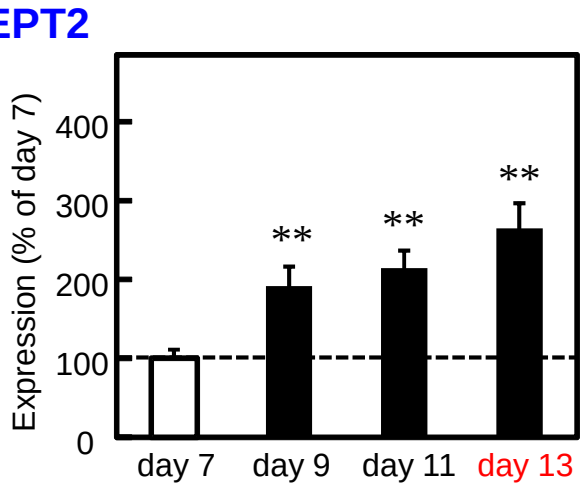
Effect of cigarette smoke extract on the function and expression of PEPT2

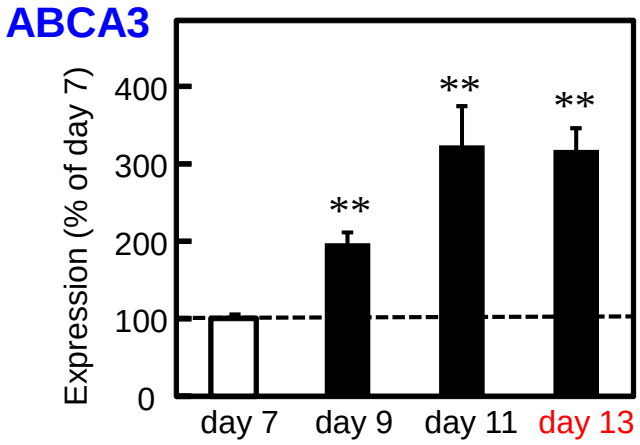
Because of the instability of PEPT2 expression, primary cultured type II cells were not suitable model to study the effect of long-term CSE treatment.

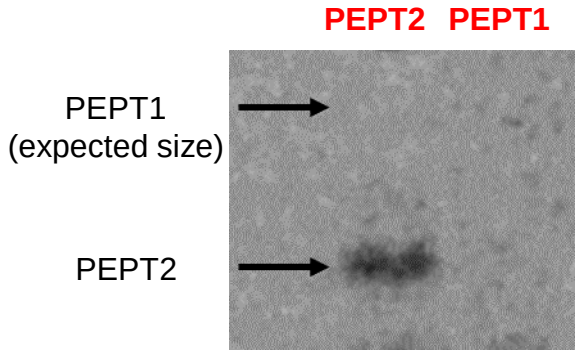
We examined functional expression of PEPT2 in several commonly used type II cell models. A549 and RLE-6TN cells did not have PEPT2 activity, but we found that PEPT2 was functionally expressed in human lung-derived NCI-H441 (H441) cells.

The mRNA expression of type II cell markers (SP-C and ABCA3) and PEPT2 in H441 cells



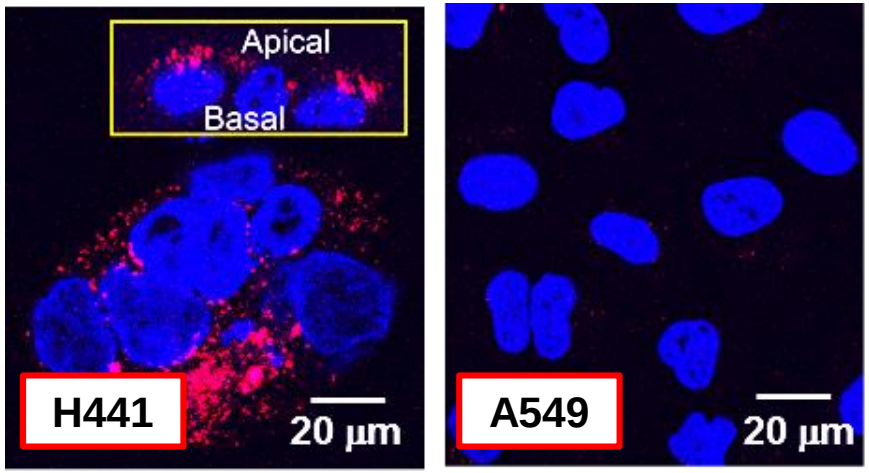




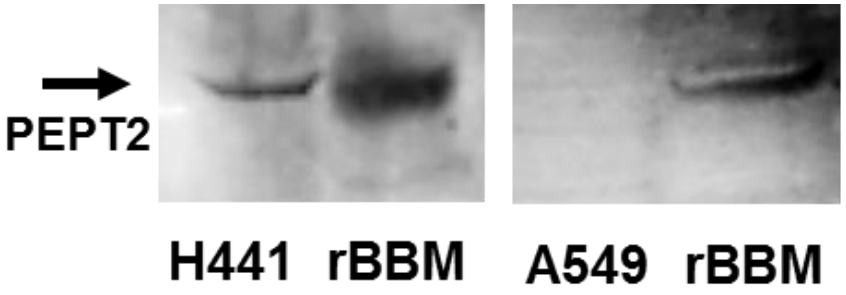


The expression of PEPT2 protein in H441 and A549 cells

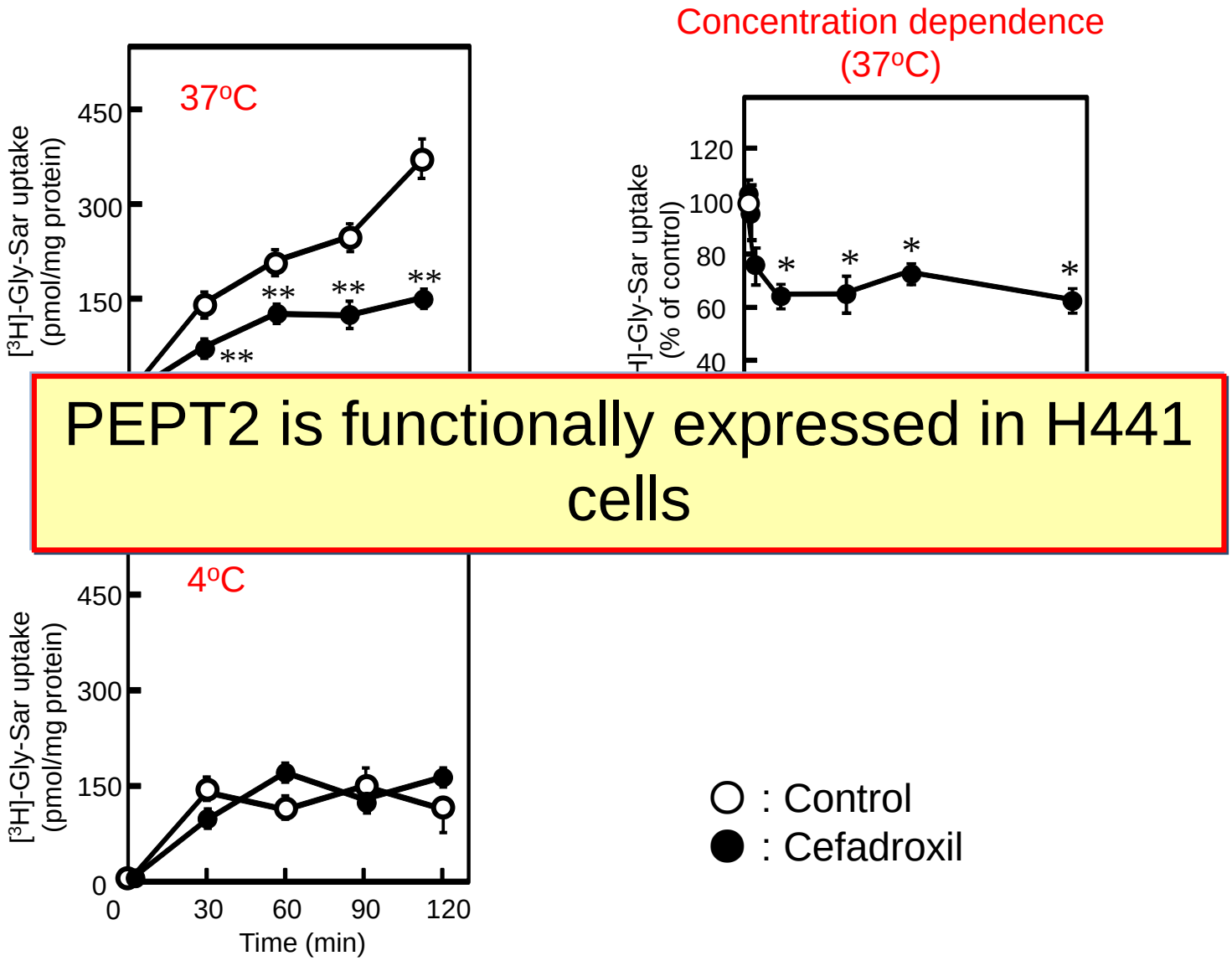
Immunostaining



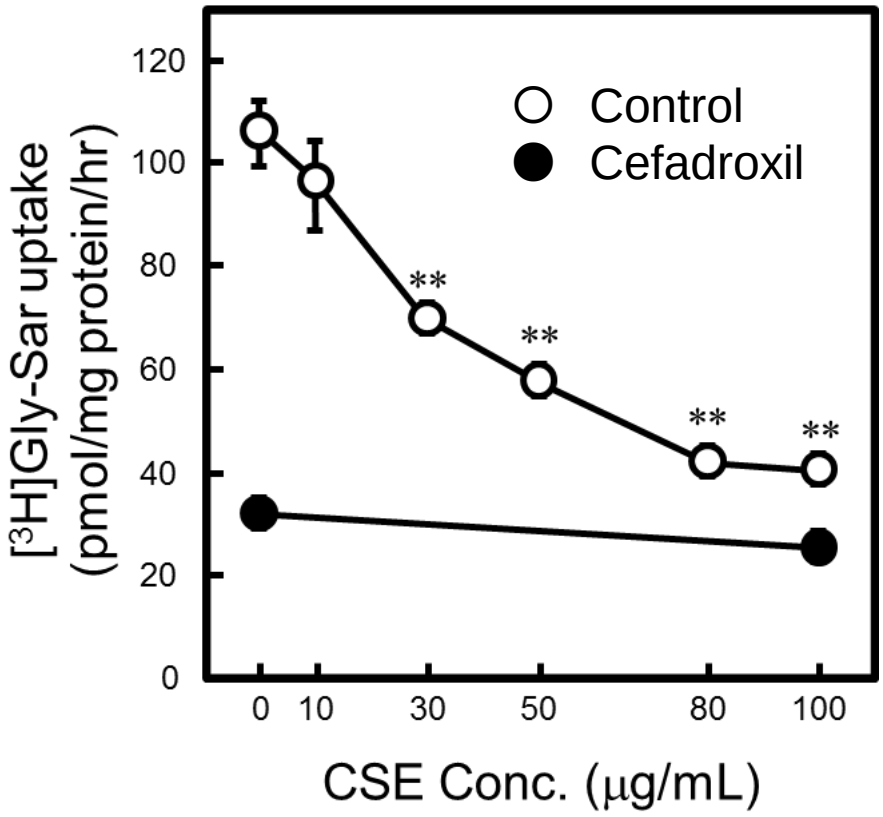
Western Blotting



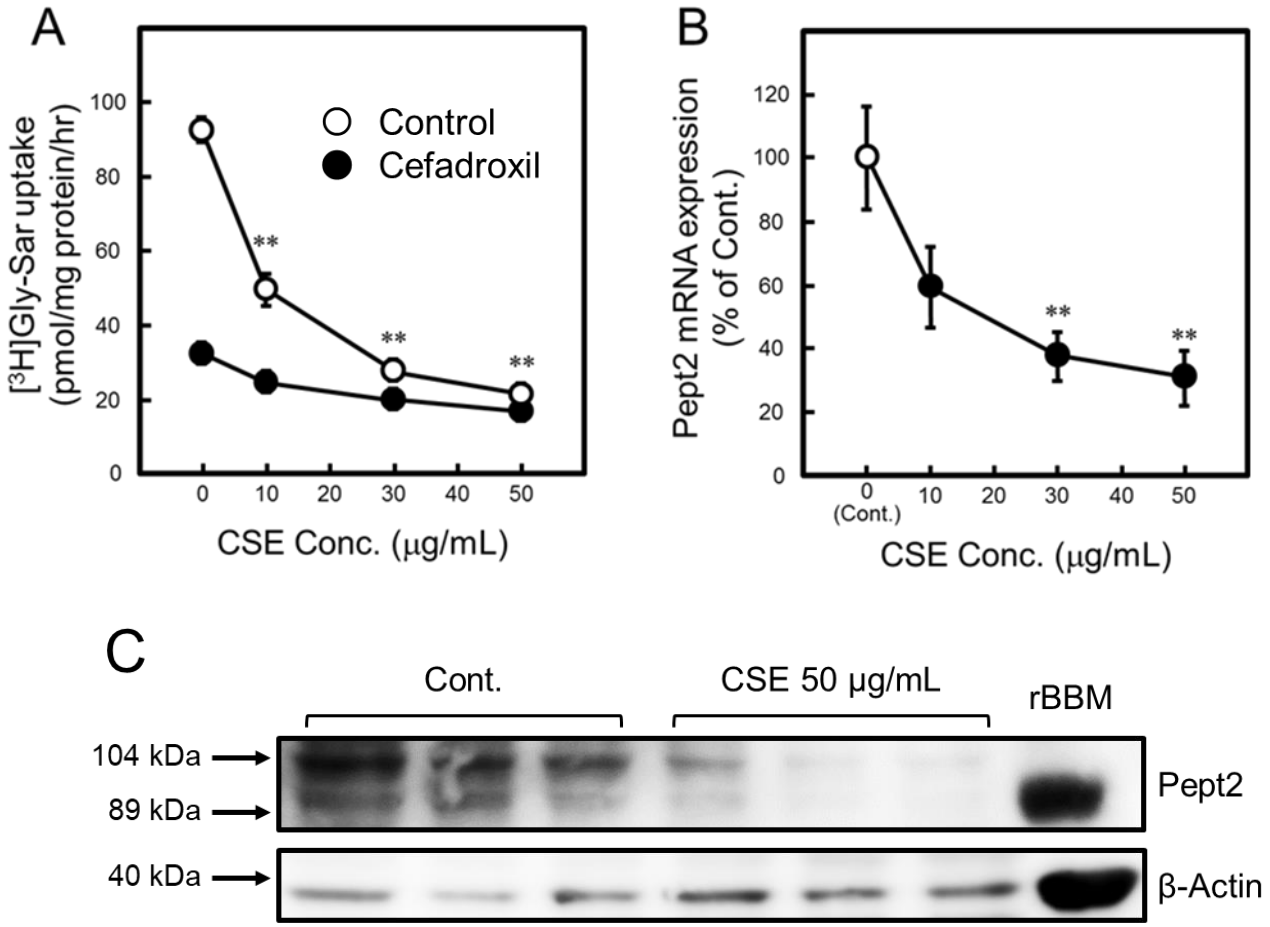
Effect of cefadroxil on Gly-Sar uptake in H441 cells



Effect of co-incubation with CSE on Gly-Sar uptake in H441 cells



Effect of long-term treatment with CSE on Gly-Sar uptake (A), PEPT2 mRNA expression (B) and PEPT2 protein expression (C) in H441 cells



Summary 5-1
(P-gp study)

- Co-incubation with CSE inhibited P-gp activity in A549/P-gp cells, as observed in type I-like cells.
- Long-term treatment (96 hrs) with CSE suppressed P-gp activity, MDR1 mRNA expression, and P-gp protein level in A549/P-gp cells.
- JNK/c-jun pathway may be activated by CSE, which may be involved in P-gp suppression. Further studies are needed to clarify the molecular mechanisms underlying P-gp suppression by CSE.

Summary 5-2
(PEPT2 study)

- PEPT2 was functionally expressed in H441 cells, probably on the apical membrane of the cells.
- As observed in primary cultured type II cells, co-incubation with CSE inhibited PEPT2 activity in H441 cells.
- Long-term treatment (96 hrs) with CSE suppressed PEPT2 activity, mRNA expression, and protein level in H441 cells.
- The molecular mechanisms underlying PEPT2 suppression by CSE is now under study.

Thank you very much for your kind attention!