

II. 研究成果の刊行に関する一覧表

1. Nakajima T, Jo T, Meguro K, Oonuma H, Ma J, Kubota N, Imuta H, Takano H, Iida H, Nagase T, Nagata T. Effect of dexamethasone on voltage-gated Na⁺ channel in cultured human bronchial smooth muscle cells. *Life Sci* 82:1210-1215, 2008.
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Effect of dexamethasone on voltage-gated Na⁺ channel in cultured human bronchial smooth muscle cells

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ABSTRACT

Voltage-gated Na⁺ channel (I_{Na}) encoded by SCN9A mRNA is expressed in cultured human bronchial smooth muscle cells. We investigated the effects of dexamethasone on I_{Na} by using whole-cell voltage clamp techniques, reverse transcriptase/polymerase chain reaction (RT-PCR), and quantitative real-time RT-PCR. Acute application of dexamethasone (10^{-6} M) did not affect I_{Na} . However, the percentage of the cells with I_{Na} was significantly less in cells pretreated with dexamethasone for 48 h, and the current-density of I_{Na} adjusted by cell capacitance in cells with I_{Na} was also decreased in cells treated with dexamethasone. RT-PCR analysis showed that α and β subunits mRNA of I_{Na} mainly consisted of SCN9A and SCN1B, respectively. Treatment with dexamethasone for 24–48 h inhibited the expression of SCN9A mRNA. The inhibitory effect of dexamethasone was concentration-dependent, and was observed at a concentration higher than 0.1 nM. The effect of dexamethasone on SCN9A mRNA was not blocked by spironolactone, but inhibited by mifepristone. The inhibitory effects of dexamethasone on SCN9A mRNA could not be explained by the changes of the stabilization of mRNA measured by using actinomycin D. These results suggest that dexamethasone inhibited I_{Na} encoded by SCN9A mRNA in cultured human bronchial smooth muscle cells by inhibiting the transcription via the glucocorticoid receptor.

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Introduction

Glucocorticoids are the most effective therapy currently available for the treatment of asthma. They act primarily as anti-inflammatory agents to inhibit recruitment of inflammatory cells and suppress the release of pro-inflammatory mediators and cytokines from activated inflammatory and airway epithelial cells (Barnes, 1996). They also have other several effects in asthma managements including inhibition of proliferation of smooth muscle (Stewart et al., 1995; Dixon et al., 1999), reducing airway hyperresponsiveness (Kim et al., 2004), and preventing or reversing structural remodeling in airways (Kumar et al., 2003). Thus, airway smooth muscle cells play essential roles in the effects of steroids (Hirst and Lee, 1998), but little is known about the action of glucocorticoids on the function of airway smooth muscle cells.

Ionic channels play essential roles in regulating intracellular Ca²⁺ ions of smooth muscle cells, smooth muscle tone, and then proliferation and hypertrophy of the cells. Glucocorticoids have been known to affect Na⁺-K⁺ ATPase (Chalaka et al., 1999) and several ion

channels such as K_v1.5, a non-inactivating K⁺ current (Takimoto et al., 1993; Takimoto and Levitan, 1994). The voltage-gated Na⁺ channel (I_{Na}) exists in a wide variety of excitable cells including nerves, heart and skeletal muscle. In response to depolarizing stimuli, the channels open and play an essential role in the rising phase of action potential, which is important for impulse generation and conduction. Until now, ten different types of α -subunit Na⁺ channel genes denoted as SCN1A to SCN11A have been identified (Goldin, 1999; Goldin, 2002), and are distributed among a wide variety of mammalian cells. I_{Na} is expressed in several types of freshly isolated phasic smooth muscle cells including vascular, urinary and gastrointestinal muscle (Xiong et al., 1993; Hollywood et al., 1997; Belevych et al., 1999), while it appears to be rarely expressed in tonic smooth muscle cells such as bronchial smooth muscle. However, I_{Na} is identified in cultured human smooth muscle cells such as bronchial, and coronary arterial smooth muscle cells (Snetkov et al., 1996; Quignard et al., 1997; Cox et al., 1998; Boccarda et al., 1999; Choby et al., 2000a; Choby et al., 2000b; Jo et al., 2004). We have previously reported that I_{Na} expressed in cultured human smooth muscle cells is mainly composed of Na_v1.7 encoded by SCN9A mRNA (Jo et al., 2004). The physiological significance of I_{Na} remains unsettled, but may be related to the cultured conditions, where cellular dedifferentiation and proliferation are involved (Snetkov et al., 1996; Quignard et al., 1997; Cox et al., 1998; Boccarda

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et al., 1999; Choby et al., 2000a; Choby et al., 2000b). These changes may occur under various pathophysiological conditions such as asthma (Hirst et al., 2000; Johnson et al., 2001; Vignola et al., 2001). Therefore, it is interesting to examine the effects of dexamethasone, a potent glucocorticoid, on I_{Na} in cultured human bronchial smooth muscle cells.

The purpose of the present study is to investigate the effects of dexamethasone on I_{Na} in cultured human bronchial smooth muscle cells. Here, we provided novel evidence that acute application of dexamethasone did not affect I_{Na} , but dexamethasone inhibited I_{Na} encoded by SCN9A mRNA in cultured human bronchial smooth muscle cells by inhibiting the transcription via the glucocorticoid receptor.

Materials and methods

Cell preparation

Culture cells isolated from normal adult human bronchus ($n=4$) were purchased from the Clonetics Corporation (San Diego, U.S.A.) as previously described (Jo et al., 2004). These cells were cultured in the culture medium supplemented with 5% fetal bovine serum, human epidermal growth factor (0.5 $\mu\text{g/ml}$), insulin (5 mg/ml), human fibroblast growth factor (1 $\mu\text{g/ml}$), gentamycin (50 $\mu\text{g/ml}$), and amphotericin B (0.05 $\mu\text{g/ml}$) [SmGM-2 Buffer-Kit, Clonetics] in an atmosphere of 5% CO_2 and 95% air at 37 °C. When cells became confluent, they were subcultured in the same medium. At confluence, the cells were passaged using 0.05% trypsin in 0.02% EDTA. Medium was replaced twice weekly. Cells before confluence at passages 3–7 were detached from culture dish with 0.25% trypsin in 0.02% EDTA, and used for later experiments. The cells were identified as smooth muscle cells, by which the expression of α -actin was confirmed by immunostaining with biotin-conjugated antibody as shown previously (Oonuma et al., 2002; Jo et al., 2004).

Solutions and drug

The composition of the control extracellular Tyrode solution was as follows (in mM): NaCl 136.5, KCl 5.4, CaCl_2 1.8, MgCl_2 0.53, glucose 5.5 and HEPES–NaOH buffer 5.5 (pH 7.4). To block K^+ currents, the patch pipette contained (in mM): CsCl 140, EGTA 10, MgCl_2 2, Na_2ATP 3, guanosine-5'-triphosphate (GTP, sodium salt, Sigma) 0.1, and HEPES–CsOH buffer 5 (pH 7.2). In addition, 4-aminopyridine (4-AP, 4 mM), tetraethylammonium (2 mM), and Ba^{2+} (1 mM) were added to the control solution to block K^+ currents and record I_{Na} . Nifedipine, 4-AP, TEA, tetrodotoxin (TTX), actinomycin D and dexamethasone were obtained from Sigma Chemicals Co. Spironolactone and mifepristone were also purchased from Sigma.

Recording technique and data analysis

Membrane currents were recorded with tight-seal whole-cell clamp techniques using a patch-clamp amplifier (EPC-7, List Electronics, Darmstadt, Germany) (Hamill et al., 1981; Nakajima et al., 1999). The heat-polished patch electrode had tip resistance of 3–5 M Ω . All data were acquired, stored, and analyzed on Power Macintosh 7100/80 using the PULSE+PULSEFIT software (HEKA Electronic) and Igor PRO (Wave Metrics, Lake Oswego, OR) as previously described (Terasawa et al., 2002).

RNA extraction and quantitative real-time reverse transcriptase/polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using ISOGEN (Nippon Gene, Tokyo). For RT-PCR, complementary DNA (cDNA) was synthesized from 1 μg of total RNA with reverse transcriptase with random primers

(Toyobo, Osaka, Japan) as shown previously (Jo et al., 2004). The reaction mixture was then subjected to PCR amplification with specific forward and reverse oligonucleotide primers consisting of heat denaturation, annealing, and extension. PCR products were size-fractionated on 2% agarose gels, and visualized under UV light. Primers were chosen on the basis of the sequence of human genes as follows: SCN9A (403 bp), sense, GAGGCTGTTTCACAGATGG; antisense, TGGGGCCAAGATCTGAGTAG; GAPDH (906 bp), sense, GAGTCAACG-GATTGGTCCT; antisense, TGACAAAGTGGCTGTGAGG. Total RNA of human fetal brain and adult human left ventricle (Toyobo, Osaka) were used for positive control.

The quantitative real-time RT-PCR was performed with the use of real-time Taq-Man technology and a sequence detector (ABI PRISM $\text{J} 7000$, Applied Biosystems, Foster City, CA) as previously described (Jo et al., 2004). Gene-specific primers and Taq-Man probes were used to analyze transcript abundance. The 18S ribosomal RNA level was analyzed as an internal control and used to normalize the values for transcript abundance of SCN1A, 2A, 3A, 4A, 5A, 6A, 8A, 9A and SCN β 1–4.

Statistical analysis

Data were expressed as the means \pm S.E.M. Student's *t*-test and 2×2 Chi square test were used for statistical analysis and $P < 0.05$ was considered as significant.

Results

Effects of dexamethasone on voltage-gated Na^+ channels (I_{Na})

Fig. 1A shows I_{Na} in cultured human bronchial smooth muscle cells. The cells were held at -80 mV, and the command voltage pulses (100 ms in duration) were applied to $+0$ mV. A fast transient inward current was elicited. TTX (1 μM , Fig. 1A) completely abolished it, indicating that the transient inward current is composed of TTX-sensitive Na^+ channels (I_{Na}) as previously reported (Snetkov et al., 1996; Jo et al., 2004). Fig. 1A also shows the effects of dexamethasone on I_{Na} . The acute application of dexamethasone (1 μM) for ~ 5 min did not affect the I_{Na} significantly. Similar results were observed in 5 experiments tested. The effects of dexamethasone (1 μM) on I_{Na} were shown at various command voltage steps in Fig. 1B–D. The current-voltage relationships for I_{Na} obtained from 5 different cells are indicated in Fig. 1E. Dexamethasone (1 μM , Fig. 1C) did not affect the amplitude of I_{Na} peak at any command voltage pulses significantly, compared with the control (Fig. 1B), but TTX completely abolished it (Fig. 1D). These results suggest that acute application of dexamethasone did not affect I_{Na} . Then, we investigated chronic effects of dexamethasone on I_{Na} . The expression of I_{Na} was compared in between cells treated with dexamethasone (1 μM) for 48 h and cells untreated with dexamethasone (control cells), by using the whole-cell voltage clamp techniques. In control cells, I_{Na} was detected in 44% of the total cells tested ($n=50$), while the percentage of the cells with I_{Na} were significantly less in cells treated with dexamethasone (24% of the total cells tested ($n=50$, $P < 0.05$ vs. control cells)). The current-density of I_{Na} adjusted by cell capacitance in cells with I_{Na} was significantly decreased in cells treated with dexamethasone (4.3 ± 2.5 pA/pF in control and 1.3 ± 0.4 pA/pF ($n=20$) in dexamethasone-treated cells, $P < 0.05$) as shown in Fig. 1F.

Effects of dexamethasone on SCN9A and Na^+ channel β subunit mRNA expression

To identify the gene of I_{Na} expressed in these cells, conventional RT-PCR and quantitative real-time RT-PCR analysis were applied (Fig. 2). By using RT-PCR, the expression of SCN9A mRNA was observed (Fig. 2A). The amplitude of SCN9A cDNA fragments was of predicted

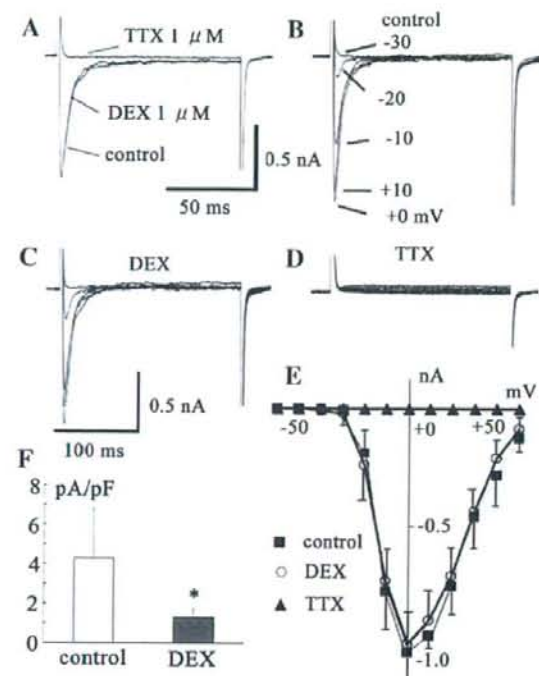


Fig. 1. Voltage-gated Na^+ channels (I_{Na}) and the effects of dexamethasone (DEX) in cultured human bronchial smooth muscle cells (hBSMCs). (A) Acute effects of DEX on I_{Na} . The cell was held at -80 mV, and depolarizing pulses at $+0$ mV were applied at 0.2 Hz. The current traces are shown in control, -5 min after the application of DEX ($1 \mu\text{M}$), and in the presence of TTX ($1 \mu\text{M}$). The data are representative of 5 different experiments. (B–E) Effects of DEX on I_{Na} . The cell was held at -80 mV, and the command voltage pulses to various membrane potentials are indicated in control (B) and in the presence of DEX ($1 \mu\text{M}$, C) for 5 min, and in the presence of TTX ($1 \mu\text{M}$, D). The current-voltage (I - V) relations (control, closed squares; DEX, open circle; TTX, closed triangle) measured at the peak after the leakage currents were subtracted are illustrated in E. The data represent the mean value \pm S.E.M. from four different cells. (F) Effect of chronic treatment of dexamethasone on I_{Na} . The cells were treated with dexamethasone ($1 \mu\text{M}$) or without (control) for 48 h. The current-density of I_{Na} adjusted by cell capacitance in cells with I_{Na} was shown in control cells and cells treated with dexamethasone. * $P < 0.01$ vs. control.

molecular size, identical to cDNA fragments amplified from reversely transcribed mRNA. In addition, expression of SCN channel family members (SCN1A, 2A, 3A, 4A, 5A, 6A, 8A and 9A) was also investigated by quantitative real-time RT-PCR. Expression of SCN10A and 11A was not investigated in this study, because these genes form TTX-insensitive I_{Na} . Transcript levels were normalized to 18S ribosomal housekeeping gene. As shown in Fig. 2B, expression levels of SCN9A mRNA were much higher than those of the other SCN families (SCN2A, 3A, 8A). The expression of SCN1A, 4A, 5A, 6A was not detected. Thus, it is likely that I_{Na} expressed in these cells is mainly composed of $\text{Na}_v1.7$ encoded by SCN9A as previously described (Jo et al., 2004).

Fig. 2C and D shows the effects of dexamethasone on SCN9A mRNA steady-state levels. To study this, we treated cells in the culture medium with or without dexamethasone ($1 \mu\text{M}$) for 24–48 h, and measured steady-state levels of SCN9A mRNA. Fig. 2C shows the effects of chronic treatment with dexamethasone on the expression of SCN9A mRNA by using conventional RT-PCR. The cells were treated with and without dexamethasone ($1 \mu\text{M}$). Treatment with dexamethasone for 24 h inhibited the expression of SCN9A mRNA, compared with the internal control (GAPDH). To confirm it, the expression of SCN9A mRNA was investigated by real-time quantitative

RT-PCR, and transcript levels were normalized to 18S ribosomal housekeeping gene. As shown in Fig. 2D, treatment with dexamethasone ($1 \mu\text{M}$) for 24–48 h significantly decreased SCN9A mRNA expression, compared with that in control samples.

I_{Na} is composed of a pore-forming α subunit encoded by SCN family and auxiliary β subunit, and four kinds of β subunit mRNA (SCN β 1–4) have been identified until now. Expression of SCN β channel family members (SCN β 1–4) was investigated by quantitative real-time RT-PCR in cultured human bronchial smooth muscle cells, and compared with normal human left ventricle (Fig. 3). In human adult heart (Fig. 3A), β subunits were mainly composed of SCN β 1 and SCN β 2, while they are mainly composed of SCN β 1 in cultured human bronchial smooth muscle cells (Fig. 3B). Fig. 3B also shows the effects of dexamethasone on SCN β mRNA. Cultured human bronchial smooth muscle cells were treated with or without dexamethasone ($1 \mu\text{M}$) for 24 h, and the steady-state levels of SCN β mRNA were measured. In contrast to the effects of dexamethasone on SCN9A mRNA, dexamethasone ($1 \mu\text{M}$) did not change the SCN β mRNA levels significantly. Thus, dexamethasone appears to selectively affect the expression of SCN α subunit mRNA, but not auxiliary β subunit mRNA.

The effects of various concentrations of dexamethasone (10^{-10} – 10^{-6} M) were also investigated in Fig. 4A. Dexamethasone decreased the mRNA steady-state levels in a concentration-dependent manner. Significant inhibitory effects were observed at concentrations higher than 0.1 nM. Fig. 4B shows the effects of spironolactone, a mineral corticoid receptor blocker, and mifepristone, a glucocorticoid receptor blocker on the action of dexamethasone. The inhibitory effect of dexamethasone (100 nM) on SCN9A mRNA was not blocked by spironolactone ($1 \mu\text{M}$), but significantly inhibited by mifepristone ($1 \mu\text{M}$). Similarly, mifepristone ($10 \mu\text{M}$), but not spironolactone ($10 \mu\text{M}$), antagonized the effects of dexamethasone ($1 \mu\text{M}$) on SCN9A mRNA. These results suggest that dexamethasone inhibits SCN9A mRNA expression via the glucocorticoid receptor.

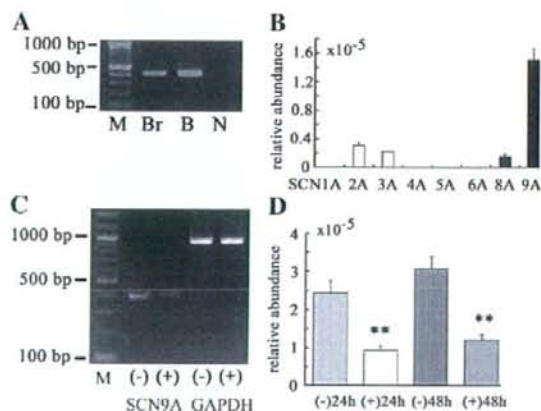


Fig. 2. Effects of chronic treatment of dexamethasone (Dex) on the mRNA steady-state levels of SCN9A in cultured human bronchial smooth muscle cells (hBSMCs). (A) Expression of SCN9A mRNA detected by RT-PCR. PCR cycle is 35. M: Marker, Br (human fetal brain); B (hBSMCs), N (negative). (B) The quantitative real-time RT-PCR. The expression levels of SCN channel family genes (SCN1A, 2A, 3A, 4A, 5A, 6A, 8A and 9A) were normalized to those of the 18S ribosomal RNA levels. Data are means \pm S.E.M. from six independent samples. (C) RT-PCR analysis of SCN9A and GAPDH. Cells were treated with and without Dex ($1 \mu\text{M}$) for 24 h in culture medium. PCR cycle is 28. (D) Effects of Dex on SCN9A mRNA expression by using quantitative real-time RT-PCR. The expression of SCN9A mRNA was evaluated by quantitative real-time RT-PCR, and transcript levels were normalized to 18S ribosomal housekeeping gene. The level of mRNA expression of SCN9A in the presence of Dex for various intervals (24–48 h) was compared with that in the absence of Dex (control). The data represent the mean value \pm S.E.M. from four different cells. ** $P < 0.01$ vs. control.

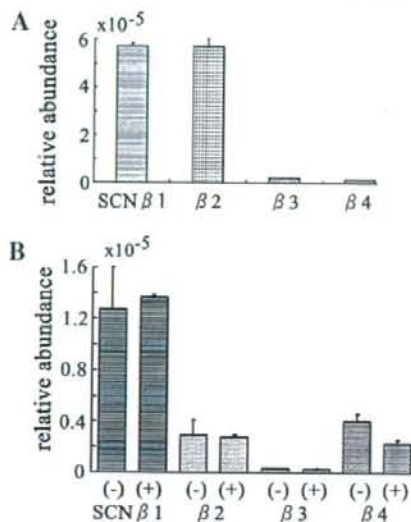


Fig. 3. (A) Expression of SCNβ1–4 in human adult heart. (B) Effects of dexamethasone (Dex) on the mRNA steady-state levels of SCNβ subunit in cultured human bronchial smooth muscle cells. In B, cells were treated with and without Dex (1 μM) for 24 h. The expression of SCNβ1–4 mRNA was evaluated by quantitative real-time RT-PCR, and transcript levels were normalized to 18S ribosomal housekeeping gene. The level of mRNA expression of SCNβ1–4 in the presence of Dex was compared with that in the absence of Dex. Each data represent the mean value ± S.E.M. from four different cells.

Effects of dexamethasone on SCN9A mRNA half-life

We investigated whether the decreased mRNA steady-state levels induced by dexamethasone were related to changes in half-life of SCN9A mRNA. Cells were treated with actinomycin D for various

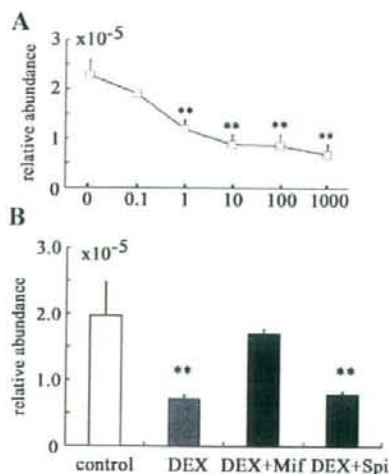


Fig. 4. (A) Concentration-dependent effects of dexamethasone (Dex) on the mRNA steady-state levels of SCN9A. Cells were exposed to the indicated concentrations (0.1–1000 nM) of Dex for 24 h. (B) Effects of spironolactone (Spi, 1 μM) and mifepristone (Mif, 1 μM) on the inhibitory actions of Dex. Cells were treated with Dex (100 nM) for 24 h in the presence or absence of these agents. In A and B, the expression of SCN9A mRNA was evaluated by real-time quantitative RT-PCR, and transcript levels were normalized to 18S ribosomal RNA. The data represent the mean value ± S.E.M. from four different cells. *P < 0.05 vs. control.

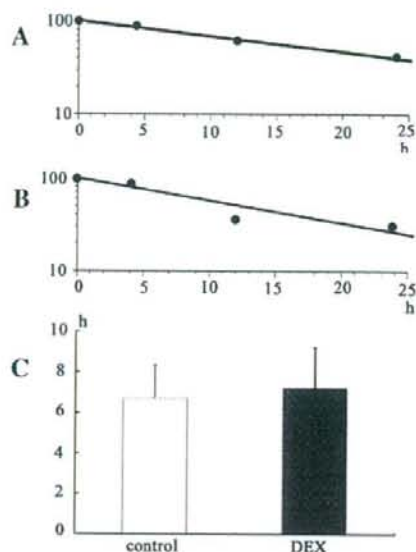


Fig. 5. Effects of dexamethasone on SCN9A mRNA half-life. Cells were exposed to dexamethasone (1 μM) for 24 h and to actinomycin D for various time intervals. All time points were normalized to time 0 for that given condition (with (A) or without dexamethasone (B)); therefore, all time 0 points start as 100%. Typical data are shown in A and B. The data were shown as the normalized mRNA levels for the different time points measured by quantitative RT-PCR analysis. The data were best fit by a linear line. (C) SCN9A mRNA half-life in control and dexamethasone-treated cells. The data represent the mean value ± S.E.M. from four different experiments included in A and B.

intervals to inhibit transcription and mRNA levels for the different time points were measured and plotted on a log scale, and the half-life was calculated. Each time point was normalized to time 0 for that specific condition (with and without dexamethasone). Fig. 5A and B shows typical data of the calculated half-life. It was 7.9 h for dexamethasone-treated cells (Fig. 5A) and 5.3 h for control cells (Fig. 5B). Similarly, the calculated half-life was not statistically different in between control (6.7 ± 1.6 h, n=4) and dexamethasone-treated cells (7.2 ± 2.0 h, n=4) as shown in Fig. 5C. Therefore, the decreased SCN9A mRNA levels by dexamethasone were not due to a decrease in mRNA half-life.

Discussion

The present study provides the first evidence that dexamethasone failed to affect I_{Na} directly, but dexamethasone inhibited I_{Na} encoded by SCN9A mRNA in cultured human bronchial smooth muscle cells by inhibiting the transcription via the glucocorticoid receptor.

Airway smooth muscle plays a principal role in the pathogenesis of airway remodeling and then asthma (Hirst and Lee, 1998; Hirst et al., 2000; Johnson et al., 2001; Vignola et al., 2001), where increased airway smooth muscle content and subsequently airway wall thickening are occurring as a result of proliferative and/or hypertrophic growth of smooth muscle cells. Primary cultures are being used to investigate airway myocyte proliferation and hypertrophy (Hall and Kotlikoff, 1995). In vitro, when airway smooth muscle is stimulated to proliferate, the muscle differentiates from a contractile phenotype to a more synthetic one (Chamley-Campbell et al., 1979). Similarly, chronic stimulation by cytokines or growth factors in the inflamed airway walls of asthmatic subjects results in the proliferation and dedifferentiation of the airway smooth muscle, making it more synthetic (Halayko et al., 1999). I_{Na} is not expressed in freshly isolated tonic smooth muscle cells such as bronchial smooth muscle, while it

has been identified under the cultured conditions (Snetkov et al., 1996; Quignard et al., 1997; Cox et al., 1998; Boccarda et al., 1999; Choby et al., 2000a; Choby et al., 2000b; Jo et al., 2004). The present study also showed the presence of I_{Na} in cultured human bronchial smooth muscle cells. Thus, the expression of I_{Na} may be related to the culture conditions, where cell proliferation or differentiation may be involved as previously described ((Snetkov et al., 1996; Quignard et al., 1997; Cox et al., 1998; Boccarda et al., 1999; Choby et al., 2000a; Choby et al., 2000b). These changes also occur under various pathophysiological conditions such as asthma (Hirst et al., 2000; Johnson et al., 2001; Vignola et al., 2001), proposing that the expression of I_{Na} may play a role in these pathophysiological conditions. However, since it is difficult to get freshly isolated human preparations, the possible expression of I_{Na} under these conditions is worth considering and further studies are necessary to clarify the physiological significance of the channel.

Glucocorticoids are the most effective therapy currently available for the treatment of asthma, and have been reported to affect ionic channels and then the electrical activity of excitable cells by regulating the transcription (Takimoto et al., 1993; Takimoto and Levitan, 1994; Chalaka et al., 1999). Glucocorticoids have been known to affect the expression of Na⁺-K⁺ ATPase (Chalaka et al., 1999) and several ion channels such as K_v1.5, a non-inactivating K⁺ current (Takimoto et al., 1993; Takimoto and Levitan, 1994). Dexamethasone has been also reported to downregulate voltage-gated Na⁺ channel in clonal rat pituitary cells, by using patch-clamp techniques, and saxitoxin binding assay (Avila et al., 2003). In the present study, short-term application of dexamethasone did not affect I_{Na} significantly. On the other hand, chronic treatment with dexamethasone inhibited SCN9A mRNA levels, and also reduced the current-density of the channel. The analysis of the protein level by using Western blotting was lacking, because the suitable antibody for human Na_v1.7 could not be obtained. However, these results suggest that dexamethasone affects the expression of voltage-gated Na⁺ channel, by inhibiting the transcription. The significant inhibitory effects were observed at concentrations higher than 0.1 nM. The effect of dexamethasone on SCN9A mRNA was not blocked by spironolactone, a mineral corticoid receptor blocker, but inhibited by mifepristone, a glucocorticoid receptor blocker, suggesting that dexamethasone inhibits SCN9A mRNA expression via the glucocorticoid receptor. The exact mechanism by which dexamethasone inhibits SCN9A mRNA has not been elucidated. But, our study using the RNA stability results indicates that dexamethasone inhibited transcription of SCN9A gene as a mechanism of inhibiting mRNA steady-state levels. Dexamethasone has been reported to inhibit mitogen-stimulated DNA syntheses and proliferation of smooth muscle including human airways by inducing an arrest in the G1 phase of the cell cycle (Stewart et al., 1995; Dixon et al., 1999). However, in our experimental conditions using fetal bovine serum, human epidermal growth factor, insulin, and human fibroblast growth factor as mitogens, dexamethasone did not inhibit cell proliferation significantly (data not shown). Dixon et al. (1999) also showed that the inhibitory effect of dexamethasone on DNA synthesis was variable depending on the growth factors: significant effect was observed on beta-hexosaminidase A and insulin; no significant effect was observed on epidermal growth factor and fetal bovine serum. Thus, it is unlikely that the antiproliferative effects of glucocorticoids may contribute to underlie the mechanism of the inhibitory effects of dexamethasone on I_{Na} in cultured human bronchial smooth muscle cells.

I_{Na} is composed of a pore-forming α subunit encoded by SCN family and auxiliary β subunit (Catterall, 1993; Goldin, 1999; Goldin, 2002). Until now, four kinds of β subunit mRNA (SCN β 1–4) have been identified, and the abnormality of β subunits has been reported. The increase of SCN1 β mRNA has been reported in lung tissues from patients with idiopathic and familial pulmonary arterial hypertension (Geraci et al., 2001). In the present study, β subunits were mainly composed of SCN β 1 in cultured human bronchial smooth muscle cells.

Thus, it is most likely that I_{Na} expressed in cultured human bronchial smooth muscle cells is encoded by SCN9A and SCN β 1. Dexamethasone did not affect SCN β 1 mRNA in comparison with SCN9A mRNA, suggesting that dexamethasone may affect I_{Na} by selectively inhibiting the transcription of SCN9A.

Physiological significance of I_{Na} expressed in cultured human bronchial smooth muscle cells remains unclear. But, it seems likely that the activation of I_{Na} induces an increase of Na influx and intracellular Na⁺ concentration ([Na⁺]_i). The increase in [Na⁺]_i may activate the reverse mode Na⁺/Ca²⁺ exchanger, which may subsequently raise intracellular Ca²⁺ concentration (Boccarda et al., 1999; Saleh et al., 2005). Thus, I_{Na} may alter cell excitability, contractility, and remodeling of human bronchial smooth muscle cells. The present study showed that chronic treatment of dexamethasone inhibited I_{Na} expression, proposing that dexamethasone may reduce the excitation-contraction coupling, and then smooth muscle tone via regulation of I_{Na} expression. Alternatively, Na⁺ influx through I_{Na} may regulate cell volume (Bortner and Cidlowski, 2003), induce changes in ion flux, and affect the cytoskeleton, protein kinase activity and gene expression. These effects of I_{Na} may enhance cellular behaviours linked to secretion and motilities in cultured human bronchial smooth muscle cells as reported in cancer cells (Mycielska et al., 2003; Fraser et al., 2003; Onganer and Djamgoz, 2005). Further studies are needed to clarify the physiological significance of I_{Na} in cultured human bronchial smooth muscle cells.

Conclusion

The present study provides the evidence that dexamethasone failed to affect I_{Na} directly, but dexamethasone inhibited I_{Na} encoded by SCN9A mRNA in cultured human bronchial smooth muscle cells by inhibiting the transcription via the glucocorticoid receptor.

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Sequential and Quantitative Analysis of a Murine Model of Elastase-Induced Emphysema

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Emphysema, one of chronic obstructive pulmonary disease (COPD), is characterized as destruction of airway wall and small airway inflammation. To assess the kinetics of disease progression in murine model of elastase-induced emphysema, we used micro-computed tomography (CT) compared with morphological changes. Two week after elastase administration, a significant increase in the volume of low-density areas, recognized as -800—-600 Hounsfield units by micro-CT, was observed. Coefficient of correlation between mean linear intercept (Lm) and low-density area examined by CT, was 0.79 ($p < 0.01$). Micro-CT can quantitatively and sequentially detect murine emphysematous changes, offering a practical method to sequentially analyze the therapeutic effects of treatments in a murine model of emphysema.

Key words emphysema; computed tomography; elastase

Chronic obstructive pulmonary disease (COPD) is one of the serious causative diseases in death of aging people.¹⁾ Emphysema, one of COPD, is characterized as destruction of airway wall and small airway inflammation.¹⁾ An imbalance between elastolytic proteases and their natural inhibitors is thought to play a role in the pathogenesis of emphysema. One animal model of emphysema is made by administering of elastase into the lungs.^{2–4)} Cigarette smoke-induced emphysema is another common model of emphysema. A study of elastase-deficient mice has revealed that elastase is also critical in smoke-induced emphysema.⁵⁾ As several months are required to establish cigarette smoke-induced emphysema, the elastase-induced model is more convenient for assessing drug effects.

To quantitatively assess the murine model of emphysema, historical assessment using mean linear intercept (Lm) has been used.⁶⁾ Because the elastase-induced destruction of airway wall is not homogenous, random several views are taken to estimate the destruction.

The non-homogenous region of the disease is also applicable for human. In human, noninvasive computed tomography (CT) is commonly used for diagnosis and disease staging by assessing areas of low attenuation.^{1,7)} The primary benefits of CT monitoring are the ability to assess the lungs in their entirety and the ability to follow the same mice over time.

Micro-computed tomography has been developed, resulting in low-noise CT images in a small animal. CT images are used to follow-up tumor growth in the lung.⁸⁾ High respiration rates of mice cause difficulty for high quality of images of the lung. Although invasive methods using tracheostomy enable high quality images,^{9,10)} we employed free-breathing method to take the images serially in a deeply-anesthetized condition, in which respiration rates were reduced. The purpose of present study is to clarify whether the kinetics of a murine elastase-induced emphysema model can be assessed using CT and to compare the results with morphological analysis.

MATERIALS AND METHODS

Administration of Elastase C57BL/6N mice were obtained from Charles River Japan (Kanagawa, Japan) and bred in the animal facilities of Musashino University School of Medicine under specific pathogen-free (SPF) conditions. Care and use of animals followed the guidelines of the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research.

Four cycles of sequential CT analysis were performed using 26 mice, six of which were also under histological examination at the end points. For histological analysis only, 12 mice were used for each time points, and in 8 different time points were examined. Another six mice were employed under CT and histological analysis once at the same day.

Mice were anesthetized with ether or intraperitoneal injection of ketamine (90 mg/kg) and xylazine (1 mg/kg) and given intranasal administration of 0.3 or 1.2 units of porcine pancreatic elastase (Sigma-Aldrich, St. Louis, MO, U.S.A.) in 30 μ l saline solution. Control mice were intranasally administered saline alone.

CT Assessment After anesthesia was induced with ketamine (90 mg/kg) and xylazine (1 mg/kg), mice were placed in the chamber of the CT scanner for small animal (LaTheta; Aloka, Tokyo, Japan).⁹⁾ Anesthesia using ketamine and xylazine induces weak and reduced respiration. The calibration of the CT is conducted using the standard phantom followed by manufacturer's instruction. The X-ray intensity and X-ray attenuation are adjusted each time to the level measured at the factory. The X-ray intensity of air is -1000 Hounsfield units (HU) and water is 0 HU. Both X-ray and detector rotated around the mice. The scanning was employed continuously. X-ray was 50 kV with 1 mAs. Scanning time was 4.5 s for one slice. Slice thickness was 0.25 mm. The size of image matrix was 480 \times 480 and the field of view was 48 mm in resolution of 0.10 mm per pixel. Because previous reports have revealed that area of lower than -620 HU are significantly increased in parallel to the degree of emphysematous

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lung and set the threshold level as "around -600 HU",^{10,11} areas between -800 and -600 HU were recognized as areas of low density for lung field analysis. Total volume of low-density areas in the whole lung was calculated by attached software for measuring area of indicted HU in LaThea.⁹ The area of each slice was reconstructed using slice thickness (0.25 mm). Three dimensional analyses were undertaken using software Vgstudio Max (Nihon Visual Science Inc.), using reconstructing images possessing 0.25 mm thickness.

The absorbed radiation dose is 8 mGy per scanning one slice. All mice recovered from anesthesia after several hours. Anesthesia and CT scanning did not influence body weights.

BALF Cell and Histological Examination BALF was obtained from select mice by incubating and washing the lung with 1 ml of saline each time. We repeated BAL until the recovered volume was 5 ml. BALF was centrifuged at 1500 rpm for 10 min at 4°C . Pellets were resuspended in 1 ml PBS and the number of cells was counted. Cytospin specimens were obtained by centrifugation at 640 rpm for 2 min. Cytospin slides were stained with Diff Quik (International Reagents Corporation) and the cell fractions were examined by microscope.

For histological analysis, the lungs were inflated and fixed by intratracheal administration of 10% formalin at a constant pressure of 25 cmH₂O. The lung was serially divided into transverse section as CT scan and embedded by paraffin. Specimens stained with hematoxylin and eosin (HE) were assessed by analyzing LM, as determined on random 20 photomicroscopic images per animal.^{6,12} Photomicroscopic images were taken using Digital sight (Nikon, Kanagawa, Japan).

To measure intercept, horizontal and vertical lines were drawn in the images. Intercepts of alveolar walls with these lines were counted and length of mean linear intercept was estimated.

RNA Extraction and Quantification of mRNAs Lungs were frozen in liquid nitrogen immediately after isolation and were used for RNA extraction. Lung tissue was homogenized at 4°C and total RNA was extracted using ISOGEN, which is a modified acid guanidium-phenol-chloroform method (Nippon Gene Co., Ltd., Tokyo, Japan). RNA was treated with

10 u DNase (Qiagen, Hiden, Germany) following manufacturer's instruction. The purity of the RNA was established by spectrophotometer using a DNA purity calculating program (Hitachi Seisakusho, Tokyo, Japan). The absorbance OD260/280 ratio was 1.8 . After the amount of total RNA was measured, cDNA synthesis was performed with 0.125 μM oligo-dT (Takara Biochemicals, Tokyo, Japan) as previously described.¹³ The levels of mRNA were examined by real-time PCR using the Light Cycler-Fast Start DNA Master Syber Green I kit (Roche Diagnostics, Mannheim, Germany). In this system, double-stranded DNA is labelled with Syber Green I and then detected. Quantification was performed on the basis of the standard curve obtained using serial dilution of specific PCR products. To produce specific PCR products, cDNA were amplified by 35 cycles (30 s at 95°C , 30 s at 58°C , 30 s at 72°C), using specific primer and *tag* polymerase. Initial denaturation step was 2 min at 95°C and final extension period was 5 min at 72°C . PCR products were then electrophoresed using 2% agarose gel with ethidium bromide, followed by purification using QIAquick Gel Extraction kit (Qiagen).

Results of real time PCR are shown as ratios of the level of mRNAs standardized by the level of β -actin mRNA.

The primers used were as follows; β -actin $5'$ -CCTGTAT-GCCTCTGGTCGTA- $3'$ $5'$ -CCATCTCCTGCTCGAAGTCT- $3'$ 260 bp, TNF- α $5'$ -CAGACCCTCACACTCAGATCA- $3'$ $5'$ -GTCCCTTGAAGAGAACCCTGG- $3'$.

Statistics For comparisons of multiple parameters, we used Wilxon's rank-sum tests or Mann-Whitney's *U* test. Data are expressed as mean \pm standard deviation of the mean (S.D.). The coefficient of correlation was evaluated using Spearman's correlation coefficient by rank. The level of statistical significance was set at $p < 0.05$.

RESULTS

Time Course Assessment of Inflammation Induced by Elastase After elastase administration, significant neutrophil accumulation was observed on day 1 ($p < 0.05$) (Fig. 1A). This inflammation was resolved by 1 week. The cytokine mRNA expression was also examined at the same

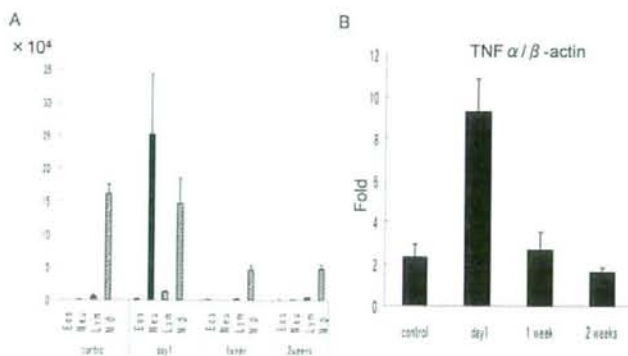


Fig. 1. BALF Cell Analysis (A) and TNF- α mRNA Expression (B)

(A) BALF cells were placed on glass slides using Cytospin. Slides were then stained with Diff Quik, and cell differentiation was assessed microscopically. The ordinate exhibited the number of cells in total BALF (5 ml). Each bar indicates means \pm S.D. of five different mice. (B) TNF- α mRNA expression was determined by real-time PCR. RNA was extracted from whole lung. The ordinate indicated the mRNA expression compared the standardized level of β -actin mRNA. Data are mean \pm S.D. of four different mice.

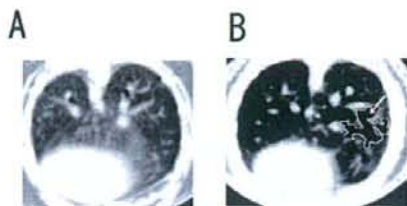


Fig. 2. CT Images for Saline-Treated Mice (A) and Elastase-Treated Mice (B)

Example of low density area -800 and -600 HU is shown by arrow in (B).

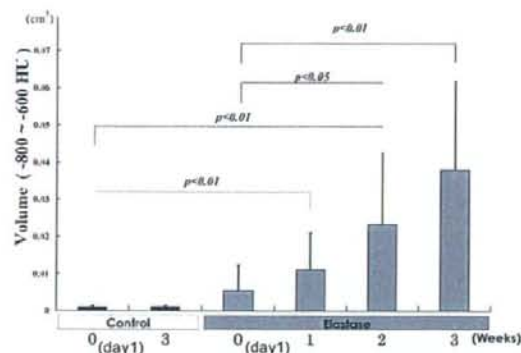


Fig. 3. Sequential Quantification by CT

Areas of lung between -800 and -600 HU were recognized as low-density areas. Total volume of low-density areas was calculated. The trachea and artifacts were removed from calculations. Each bar represents mean \pm S.D. of values obtained from 6–11 mice.

time. TNF- α , which is ascribed one of the causative cytokines of emphysema, was significantly increased on day 1 and returned to baseline by 1 week ($p < 0.01$) (Fig. 1B).

Time Course Assessment of Air Wall Destruction Using CT and Morphometry Elastase-treated mice (Fig. 2B) exhibited a low-density area compared to controls (Fig. 2A). Next, we sequentially analyzed the same mice once a week after administration of elastase (0.3 units), using CT (Fig. 3). For quantitative assessments, areas from -800 to -600 HU were accumulated and showed as volume of low density lung. Volume of low density area for elastase treated mice was $0.01118 \pm 0.00968 \text{ cm}^3$, significantly greater than that of control mice ($0.00096 \pm 0.00034 \text{ cm}^3$) ($n=11$ each group, $p < 0.01$), but not statistically significant compared with elastase treated mice Day 1. At 2 weeks, a significant increase in total volume of low-density areas was observed compared with control mice and elastase-treated mice Day 1 ($p < 0.01$ and $p < 0.05$, respectively), and a further increase was observed at 3 weeks (Fig. 3).

To obtain whole lung images by CT scanning, 3-dimensional reconstruction was performed using software Vgstudio Max (Fig. 4). This revealed an expanding pattern of lung destruction from 2 to 3 weeks (Fig. 4). This analysis also showed that low density lesions were not homogenous and that differences existed in areas of lung destruction (Fig. 4).

To compare time-course analysis of CT with previous methods, histological examination was performed. For histo-

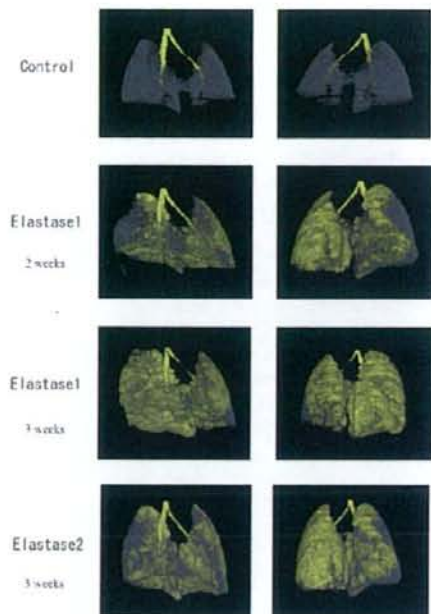


Fig. 4. Three-Dimensional Reconstructions of CT

Yellow areas indicate -800 to -600 HU. Although the trachea and bronchi are shown as yellow areas in this figure, the trachea and bronchi were removed from calculations. The density of trachea and bronchi was about -900 , which was lower than that of the lung. If these areas were included, low density areas were overestimated. Elastase 1 at 2 and 3 weeks are from the same mice. Elastase 1 and Elastase 2 are for different animals treated with elastase in exactly the same manner. Left panel is shown from the front and right panel is from the back of the same mice.

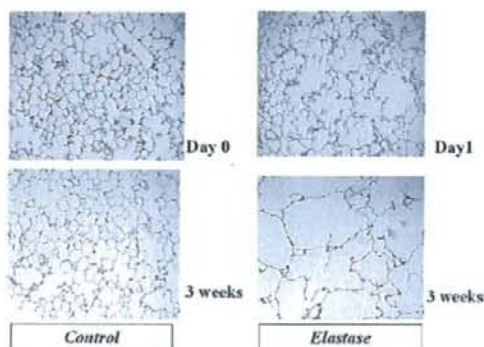


Fig. 5. Histological Analyses by HE Staining

Mice were sacrificed at the day indicated after administration of elastase or saline. Lungs were slowly inflated using 25 cmH₂O pressures to avoid further damage to the lungs. Original magnification $\times 200$.

logical time-course analysis, mice had to be sacrificed at each time point ($n=6$ in each time point). All mice were treated in the same manner as mice in Fig. 2. Histological analysis revealed destruction of the lung from day 1 (Fig. 5). Lung destruction continued to 3 weeks (Fig. 4). Lm was calculated to estimate alveolar wall destruction, as this is the most commonly used method to assess emphysematous lungs. Significant increases in Lm were observed at 1 week

($p < 0.05$; Fig. 6), earlier than the CT evaluation. Control mice did not exhibit any changes during experiments.

After 6 months, 3 elastase-treated mice (same mice in Fig. 3) were examined by CT to obtain follow-up images. Mean volume of accumulated low-density area on CT at 6 months was $0.08 \pm 0.0127 \text{ cm}^3$, significantly greater than that at 3 weeks ($p < 0.01$), suggesting that destruction of alveolar walls had not recovered. Mean Lm for these mice was $52.0 \pm 5.2 \mu\text{m}$, significantly greater than that of control mice ($28.1 \pm 0.5 \mu\text{m}$).

Direct Comparison of CT and Morphometry To clarify correlations between CT and Lm, we analyzed CT results and morphometry using the same mice at the same time. To obtain severe destruction, we used 1.2 units of elastase and shook the mice after elastase administration. This method allowed us to obtain the ratio of destroyed lung to whole lung, reaching $55.6 \pm 5.2\%$ at 3 weeks. Both the percentage of the low-density area (A) and the mean CT level (B) significantly correlated with Lm (Fig. 7). Coefficients of correlation were 0.80 and 0.85, respectively ($p < 0.01$ each).

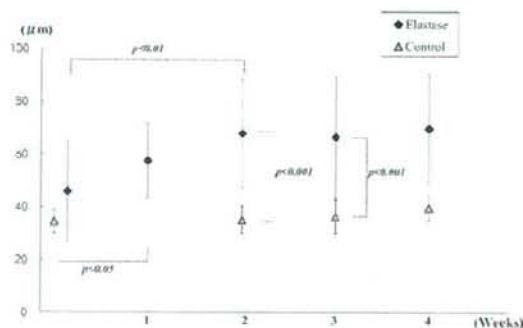


Fig. 6. Quantification by Lm

Mice was sacrificed at the day indicated after administration of elastase or saline. Lm was determined on 20–30 photomicroscopic images per HE-stained specimen. The value of Lm was showed in ordinate. Each bar represents mean \pm S.D. of values obtained from 4 different mice.

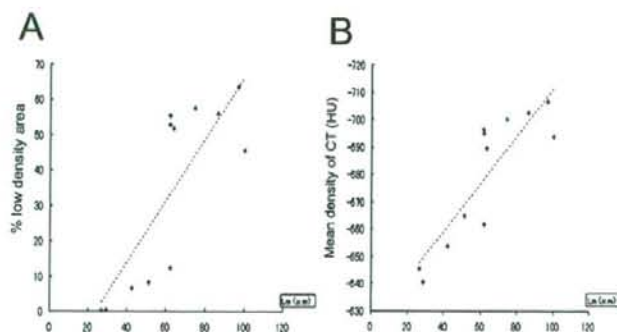


Fig. 7. Comparison of Results from CT and Morphometric Analysis

Correlations between CT and Lm were analyzed using mice with several levels of lung destruction ($n = 12$). (A) Percentage of low-density area compared to total lung volume as determined by CT (y-axis) and Lm (x-axis). The coefficient of correlation is 0.79 ($p < 0.01$). (B) Mean density of CT (HU) (y-axis) and Lm (x-axis). The coefficient of correlation is 0.85 ($p < 0.001$).

DISCUSSION

In the present study, we quantified the kinetics of murine elastase-induced emphysema model by computed tomography, and compared the results with morphological analysis. The primary benefits of CT monitoring are the ability to assess the lungs in their entirety and the ability to follow the same mice over time. Our data demonstrate that CT can detect emphysematous changes in the elastase treated lung, comparable to morphological analysis by measuring Lm. CT images obtained from anesthetized mice could not detect alveolar destruction earlier than morphometric analysis, but had the advantage of allowing the same mouse to be measured sequentially and quantitatively. As intranasal administration of elastase does not damage the lung homogeneously, sequential whole-lung analyses provide more accurate quantification.

Elastase activates protease-activated receptor-2, resulting inflammatory mediator synthesis and neutrophil influx.^{14–16} The both neutrophil and macrophages are proposed to play a critical role in the pathogenesis of emphysema in human and animal models.^{1,3,17} Among inflammatory cytokines, TNF- α is known as a most causative one, which is produced by macrophages and epithelial cells.¹⁷ In elastase-induced model in our experiments, both influx of neutrophil and up-regulation of TNF- α mRNA were observed and returned to normal level by 1 week. Further destruction of airway wall is partly attributable to mechanical stress.^{2,11}

In humans, noninvasive CT follow-up is a common procedure to assess emphysema.^{7,18} In the murine model, quantitative analysis is commonly undertaken by measuring Lm on formalin-fixed specimens. This method of quantifying Lm is authorized,⁶ but cannot estimate the whole lung. Micro-CT has become available to assess various animal models. Micro-CT has also been applied to assess pulmonary fibrosis and metastatic lung cancer.^{8,19,20} The high respiration rate of mice forces using fixed lung or tracheostomized and breath fold lung for precise analysis, resulting in visualization of alveoli and estimation pulmonary function even in small animals.^{10,11,21,22} Recently respiratory-gating methods is developed and provide clearer vision to estimate even airway diameter.²³ The micro-CT, which we used in this manuscript,

has been used for quantifying fat volume and bone mineral density.^{24–26} In order to assess mice sequentially, we used deep anesthetized mice, which have been previously reported to deserve for evaluation.^{7,19,20} Although artifacts due to respiratory movement could not be eliminated in our study and areas of low density may have been slightly underestimated, we found that it is applicable to the sequential estimation of detecting murine emphysematous lung. By using the software contained in CT apparatus, which has been validated for quantifying fat volume and bone mineral density,^{24–26} low density area was calculated and the value exhibited good correlation to the value estimated by Lm. The absorbed radiation dose is 8 mGy per scanning one slice, which is relatively lower compared to other micro-CT and not lethal level.^{27,28}

For human low attenuation area was determined as lower –900 HU.^{9,18} However, studies reveal lowest value is –800 HU and never reached –950 HU in murine emphysematous lung.^{29,30} In addition, mean density of murine normal lung is around –500 to –600 HU by ours and Plathow *et al.*²⁰ and around –400 HU,^{20,29,30} which is quite different from that of human (–700 HU).^{7,18} Therefore, we used the level between –800 and –600 HU for estimating low density area in a murine model.

The estimation of low density area of our study was comparable to previous reports, although CT apparatus and method of anesthesia are different.^{29,30} Froese *et al.* used knockout mice which develop emphysema spontaneously, resulting that correlation of morphology to CT densities was r^2 of 0.53. Pastnov *et al.* have shown possibility of assessment of elastase-induced emphysema in murine model, but the lung was examined once on day 14.²⁹ In this report, we showed usefulness of sequential quantitative analysis. We were able to detect sequential increases in tissue destruction in the same area of the lung using CT images. In addition, these data correlated well with morphometric analyses using Lm (r^2 of 0.72, Fig. 7B). As alveolar wall destruction was not heterogeneous, evaluation of the whole lung is worthwhile in this emphysema model. Micro-CT is expected to provide a practical method for sequential analysis of therapeutic effects in a murine model of emphysema.

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Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking TAZ

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Departments of ¹Physiological Chemistry and Metabolism, ²Developmental Medical Technology (Sankyo), ³Urology, ⁴Respiratory Medicine, and ⁵Laboratory Medicine, Graduate School of Medicine and ⁶Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo; ¹⁰Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto; ¹¹Department of Biomedical Chemistry, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan; and ⁷Division of Cardiology, Departments of Internal Medicine, ⁸Molecular Biology, and ⁹Internal Medicine and Pediatrics, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas

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Makita R, Uchijima Y, Nishiyama K, Amano T, Chen Q, Takeuchi T, Mitani A, Nagase T, Yatomi Y, Aburatani H, Nakagawa O, Small EV, Cobo-Stark P, Igarashi P, Murakami M, Tominaga J, Sato T, Asano T, Kurihara Y, Kurihara H. Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking TAZ. *Am J Physiol Renal Physiol* 294: F542–F553, 2008. First published January 2, 2008; doi:10.1152/ajprenal.00201.2007.—TAZ (transcriptional coactivator with PDZ-binding motif), also called WWTR1 (WW domain containing transcription regulator 1), is a 14-3-3-binding molecule homologous to Yes-associated protein. TAZ acts as a coactivator for several transcription factors as well as a modulator of membrane-associated PDZ domain-containing proteins, but its (patho)physiological roles remain unknown. Here we show that gene inactivation of TAZ in mice resulted in pathological changes in the kidney and lung that resemble the common human diseases polycystic kidney disease and pulmonary emphysema. *Taz*-null/*lacZ* knockin mutant homozygotes demonstrated renal cyst formation as early as embryonic day 15.5 with dilatation of Bowman's capsules and proximal tubules, followed by pelvic dilatation and hydronephrosis. After birth, only one-fifth of TAZ-deficient homozygotes grew to adulthood and demonstrated multicystic kidneys with severe urinary concentrating defects and polyuria. Furthermore, adult TAZ-deficient homozygotes exhibited diffuse emphysematous changes in the lung. Thus TAZ is essential for developmental mechanisms involved in kidney and lung organogenesis, whose disturbance may lead to the pathogenesis of common human diseases.

renal disease; knockout mice; transcription factor

TAZ (TRANSCRIPTIONAL COACTIVATOR with PDZ-binding motif), also called WWTR1 (WW domain containing transcription regulator 1), is a 14-3-3-binding molecule homologous to Yes-associated protein (YAP) (14, 18). TAZ, as well as YAP, possess a WW domain that can bind to the PPXY motif present in some transcription factors. Through this interaction and additional unknown mechanisms, TAZ can act as a coactivator for several transcription factors, including Runx2/Cbfa1, TTF-1/Nkx2.1, Tbx5, and Pax3 (5, 24, 25, 30). The COOH-terminal region of TAZ contains a PSD-95, Dlg, and ZO-1 homology

(PDZ)-binding motif that localizes TAZ to discrete foci in the nucleus and is essential for its activity as a transcriptional coactivator (18). TAZ and YAP also bind to membrane-associated PDZ domain-containing proteins through this motif. In particular, TAZ and YAP bind to sodium/hydrogen exchanger regulatory factor (NHERF)-2 and NHERF, respectively, which may link TAZ and YAP to transmembrane receptors and actin-binding proteins (14, 18). Furthermore, a recent report demonstrated that TAZ may modulate mesenchymal stem cell differentiation into osteogenic and adipogenic lineages by coactivating Runx2/Cbfa1 and repressing peroxisome proliferator-activated receptor- γ -dependent gene transcription (13). Thus TAZ and YAP are now regarded as context-dependent transcriptional modulators in various cell types that may link events at the plasma membrane and cytoskeleton to nuclear transcription, possibly in a 14-3-3-dependent manner (14, 18).

In midgestation mouse embryos, TAZ is mainly expressed in the paraxial mesoderm, limb buds, and the neural tube (25). Later, TAZ is distributed more broadly in various tissues and organs (18 and UniGene's EST ProfileViewer). The broad distribution of TAZ and its interaction with different transcription factors essential for embryonic development led us to investigate the physiological role of TAZ by a gene targeting strategy.

Here, we demonstrate that inactivation of the mouse *Taz* gene in mice results in the formation of multiple cysts in the kidney and greatly enlarged air spaces in the lung. These phenotypes resemble human polycystic kidney diseases (PKD) and pulmonary emphysema, respectively. PKD is a human inherited renal disorder that is the most common genetic cause of renal failure in children and adults (3, 15). Autosomal dominant polycystic kidney disease (ADPKD) is the most prevalent form, with an incidence of 1–2 per 1,000 individuals and is characterized by bilateral formation of multiple cysts arising from any segment of the nephrons and collecting ducts. ADPKD is caused by mutations in either of two genes, *PKD1*

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and *PKD2*, that encode membrane-associated proteins polycystin-1 and polycystin-2, respectively (23, 32, 33). Autosomal recessive polycystic kidney disease (ARPKD) is much less frequent (1/20,000 live births) and is caused by mutations of *PKHD1* (29, 37), which encodes polyductin/fibrocystin, a large transmembrane protein. Although the causative genes have been identified, the molecular mechanism underlying cystic formation remains largely unknown.

TAZ-deficient mice also exhibited urinary concentration defects, polyuria, and hydronephrosis. Although urinary concentrating defects have been observed in human ADPKD and ARPKD, massive polyuria and hydronephrosis are uncommon. These findings suggest that the pathophysiological processes in TAZ-deficient kidneys and human PKD are different in some respects. The similarities and dissimilarities between TAZ-deficient mice and PKD may provide important clues to understanding the pathogenesis of a common human disease.

In addition, the dilatation of air spaces of the lung in TAZ-deficient mice is morphologically reminiscent of human pulmonary emphysema, which is a common disease that causes death and disability, especially in the aged (1, 12). As a manifestation of chronic obstructive pulmonary disease (COPD), pulmonary em-

physema is characterized by enlargement of air spaces and destruction of the alveolar wall. Although α -antitrypsin deficiency is known to cause a congenital form of pulmonary emphysema, little remains known concerning the molecular mechanisms underlying the pathogenesis of this disease. TAZ-deficient mice may also serve as a novel animal model for COPD as well as kidney diseases.

MATERIALS AND METHODS

Generation and genotyping of mutant mice. A C57BL/6J-derived BAC clone containing the mouse *Taz* gene was obtained from BACPAC Resource Center (Oakland, CA). The *nls-lacZ/PGKneo* cassette was made by placing the *lacZ* gene with a nuclear localization signal (*nls-lacZ*) adjacent to the *PGKneo* gene and flanking it with *lox71* at the 5'-end and *lox2272* at the 3'-end to allow recombinase-mediated cassette exchange. A pKO Scrambler NTKV-1904 plasmid (Stratagene) was used as a backbone vector. For the targeting construct, a PCR-amplified 1.2-kb fragment extending from the promoter region to the 5'-untranslated region in exon 2 and an 8.2-kb *NgoMIV-BamHI* fragment from intron 2 were inserted on each side of the *nls-lacZ/PGKneo* cassette (Fig. 1A). The targeting vector was linearized and electroporated into the B6129F1-derived embryonic stem (ES) cell line ATOMI (Amano T et al., unpublished observations).

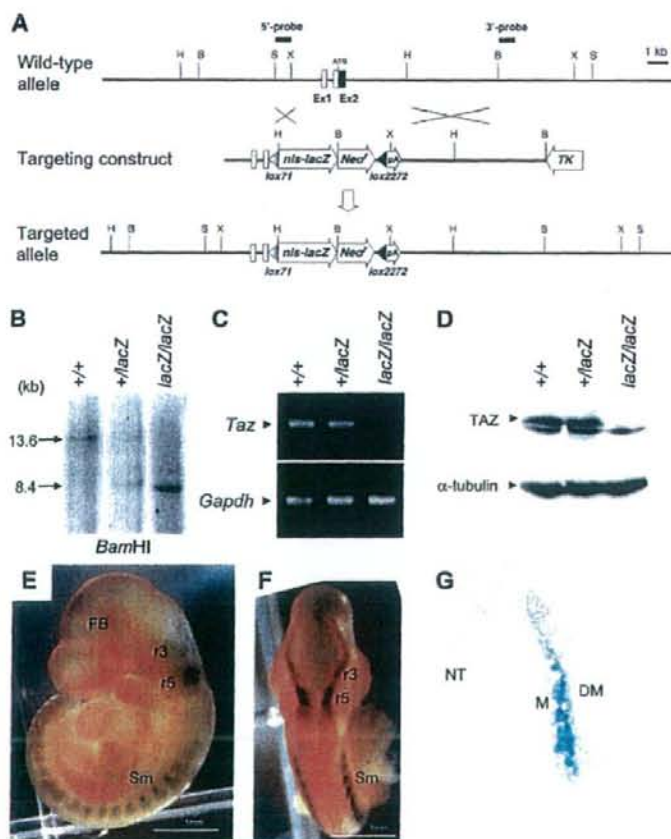


Fig. 1. Targeting of the mouse *Taz* (transcriptional coactivator with PDZ-binding motif) gene. **A**: schematic representation of the targeting strategy employed to knock in a *nls-lacZ* cassette into the *Taz* locus. Two different probes for genotyping are indicated as 5'- and 3'-probes. **B**, *Bam*HI; **H**, *Hind*III; **S**, *S*fuI; **X**, *Xba*I. **B**: representative genotyping of the offspring from an F₁ intercross by Southern blot analysis. Genomic DNA samples were digested with *Bam*HI and probed with the 5'-probe. Bands of 13.6 and 8.4 kb represent wild-type and mutant alleles, respectively. **C** and **D**: RT-PCR (**C**) and Western blot (**D**) confirming the absence of TAZ expression in *Taz*^{lacZ/lacZ} embryos. Total RNA and protein extracts were obtained from embryonic day 15.5 whole embryos. Lower band in **D**, *wp*, represents nonspecific binding. **E-G**: lateral (**E**) and dorsal (**F**) views of an embryonic day 9.5 *Taz*^{lacZ} embryo and a section at the level of the somites (**G**) stained with X-gal. **FB**, forebrain; **DM**, dermomyotome; **M**, myotome; **NT**, neural tube; **r3** and **r5**, rhombomeres 3 and 5 of the hindbrain; **Sm**, somites.

Clones that survived positive-negative selection with neomycin and 1-(2'-deoxy-2'-fluoro-1- β -D-arabinofuranosyl)-5-iodouracil were screened for homologous recombination with diagnostic PCR primers. Targeted clones were injected in ICR blastocysts to generate germline chimeras. Mice homozygous for the *Taz^{lacZ}* allele were obtained by intercrossing F₁ heterozygotes. The genotypes of the offspring were determined by PCR or Southern blot analysis on tail-tip or amnion DNA. All animal experiments were reviewed and approved by the University of Tokyo Animal Care and Use Committee.

RT-PCR. Total RNA was extracted using ISOGEN (Nippon Gene). The reverse transcription reaction was carried out using SuperScriptIII (Invitrogen). PCR was then performed on the resulting cDNA using the primers 5'-GAAATCACCACATGGCAAGACCC-3' and 5'-TTACAGCCAGGTTAGAAAGGGCTC-3' for *TAZ* (product size, 748 bp; annealing, 64°C), 5'-GGCTATGTGCAGTGCATGTC-3' and 5'-CTGTGATATGCCAGTGGTCAG-3' for *Aqp1* (product size, 462 bp; annealing, 58°C), 5'-TGGATTCATGGAGCAGCCCGGT-3' and 5'-TCCTTCCTCGAGCTGCCTTC-3' for *Aqp2* (product size, 312 bp; annealing, 58°C), 5'-ATCAAGCTGCCATCTACAC-3' and 5'-GGCCAGCTTCACATCTC-3' for *Aqp3* (product size, 559 bp; annealing, 56°C), and 5'-GGTGTGAACCCAGAGAAATAT-3' and 5'-AGATCCACGACGGACACATT-3' for *Gapdh* (product size, 335 bp; annealing, 56°C). For comparison of *Pkd1*, *Pkd2*, and *Pkhd1* expression levels, quantitative real-time RT-PCR was performed using the LightCycler system (Roche diagnostics) according to the manufacturer's protocol. Sequences of the primers were as follows: 5'-GGATGGTGATCAGACACCGCTCAA-3' and 5'-TTGGTGCTTCTTCCTCCGACCT-3' for *Pkd1*, 5'-TCTCCTCAGGTTATGGCGGAGTT-3' and 5'-GACATACCGGATCAGTTTACAGG-3' for *Pkd2*, 5'-GGCCACAGACAAATTA-3' and 5'-GCCCTG-CAGTCTAGCTTGGT-3' for *Pkhd1*, and 5'-GGTGGCCCTGGGAGTACCAAGAA-3' and 5'-AATGGGAAGCCCAAGTGCCTC-TGT-3' for *Avpr2*.

Western blotting. Anti-TAZ rabbit polyclonal antibody was previously described (25). Western blotting was performed on lysates from embryonic day 13.5 whole embryos or 12-wk-old kidneys using a standard protocol.

Histology. For histological analysis, samples were fixed in formalin, embedded in paraffin, cut into 2- μ m-thick sections, and stained with hematoxylin and eosin. Photomicrographs were obtained using a computer-assisted microscope (Nikon ECLIPSE 80i).

Urinary analysis. Urine volume was measured using the 48-h frequency/volume analysis system as previously described (4). Briefly, each mouse was placed in a metabolic cage connected to a digital scale and personal computer. Each mouse was provided with free access to food and water. After mice were acclimatized for 2 days in the cage, water intake, urine voiding frequency, and volume per void were recorded for 48 h.

Lectin and immunofluorescent staining. Lectin staining was performed on cryosectioned kidneys. Embryonic day 18.5 kidneys were dissected, embedded in optimum-cutting temperature (OCT) compound (Miles), and cut into 10- μ m-thick sections. After being blocked with blocking buffer, the sections were incubated for 1 h at 37°C with 20 μ g/ml biotin-conjugated *Dolichos biflorus* agglutinin (DBA) or *Lotus tetragonolobus* agglutinin (LTA) followed by extensive washing with blocking buffer. Lectin staining was visualized by reacting with fluorescein isothiocyanate-conjugated streptavidin, and nuclei were counterstained with propidium iodide. Photomicrographs were obtained using a computer-assisted confocal microscope (Nikon D-ECLIPSE C1).

For immunofluorescent staining, paraformaldehyde-fixed cryosections were stained with antibodies to aquaporin-2 (gift from M. Knepper, National Heart, Lung, and Blood Institute, Bethesda, MD) or aquaporin-3 (Chemicon), as described previously (31). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

In situ hybridization. Isotopic in situ hybridization was performed on paraffin-embedded sections of embryonic day 14.5 and 16.5

embryos using ³⁵S-radiolabeled RNA probes for *Taz/Wnt1* as previously described (27).

β -Galactosidase staining. *LacZ* expression was detected by staining with X-gal (5-bromo-4-chloro-3-indolyl β -D-galactoside). Staining was performed as described by Nagy et al. (26) with minor modifications. Whole embryos and kidneys were washed in ice-cold PBS containing 2 mM MgCl₂ and fixed in 0.1 M phosphate buffer (pH 7.3) containing 0.2% glutaraldehyde, 5 mM EGTA, and 2 mM MgCl₂. Following rinsing three times with 0.1 M phosphate buffer containing 2 mM MgCl₂, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, and 5 mM EGTA (washing buffer), samples were embedded in OCT compound and cryosectioned. Embryos or sections were incubated overnight at 37°C in X-gal-staining buffer (10 mM potassium ferrocyanide, 10 mM potassium ferricyanide and 2 mg/ml X-gal in washing buffer). For costaining with platelet/endothelial cell adhesion molecule, X-gal-stained sections were rinsed with PBS, incubated in the blocking buffer (see *Lectin and immunofluorescent staining*), and reacted with anti-CD31 antibody (BD Bioscience) (1:100) at 4°C overnight. Immunoreactivity was visualized with the VECTASTAIN ABC kit (Vector laboratories). Sections were counterstained with 1% Orange G (Sigma).

Morphometric analysis. The mean linear intercept, as a measure of interalveolar wall distance, was determined as described by Thurlbeck (34). Briefly, lines were drawn across light microscopic images of the lung section stained with hematoxylin and eosin. The mean linear intercept was calculated by dividing the total length of a line by the total number of intercepts encountered in 72 lines/lung.

Statistical analysis. Mann-Whitney nonparametric test was used to compare values between two groups for morphometric analysis. Student's *t*-test was used for the comparison of values in other experiments. Data were represented as means \pm SD. Values of *P* < 0.05 were considered significant.

RESULTS

Generation of *Taz-lacZ* knockin mice. We disrupted the mouse *Taz* locus by replacing the coding region in exon 2 with an *nls-lacZ/PGKneo* cassette (Fig. 1A). Of 683 ES cell clones screened, three clones were positive for the mutant *Taz^{lacZ}* allele. All three clones were injected in ICR blastocysts and gave rise to male germline chimeras, which were subsequently bred with ICR females to produce *Taz^{+/lacZ}* heterozygous mice. The heterozygous mice appeared normal and were fertile. Offspring from *Taz^{+/lacZ}* intercrosses were genotyped by Southern analysis of tail genomic DNA (Fig. 1B). The absence of *Taz* transcripts and protein in *Taz^{lacZ/lacZ}* homozygous mice was confirmed by RT-PCR and Western blotting, respectively (Fig. 1, C and D).

To verify that *Taz*-directed *lacZ* expression reflected the pattern of authentic *Taz* expression, we stained for *lacZ* in

Table 1. Genotypic distribution of offspring from heterozygous matings

Age	Wild-type	<i>Taz^{+/lacZ}</i>	<i>Taz^{lacZ/lacZ}</i>	Total
E8.5	8 (29)	12 (43)	8 (29)	28
E13.5	25 (19)	77 (58)	31 (23)	133
E15.5	52 (23)	114 (50)	61 (27)	227
E18.5	76 (27)	152 (53)	61 (21)	289
P0	31 (35)	45 (51)	12 (14)	88
P21	133 (33)	234 (59)	30 (8)	397

Nos. in parenthesis indicate percentage of the total numbers. E, embryonic day; P, day postpartum.

embryonic day 9.5 $Taz^{+/lacZ}$ embryos. At embryonic day 9.5, $lacZ$ staining was observed in the somitic mesoderm and as in the neuroectoderm within the forebrain and hindbrain (Fig. 1, E and F). Within the somites, $lacZ$ expression was detected in the myotome (Fig. 1G). These expression patterns coincide with that of authentic Taz expression revealed by in situ hybridization (25).

Early mortality in $Taz^{lacZ/lacZ}$ mice. When 309 littermates derived from $Taz^{+/lacZ}$ intercrosses were followed, 24 pups were found dead before 3 wk of age; 9 of 10 dead pups that could be genotyped were homozygous null. Genotyping of the remaining 285 littermates at 3 wk of age identified 90 wild-

type (32%), 177 heterozygous (62%), and 18 homozygous (6%) mice, indicating that only one-fifth of the expected Mendelian ratio of $Taz^{lacZ/lacZ}$ mice were alive at weaning (Table 1).

To determine the stage at which homozygous null mice started to die, offspring from $Taz^{+/lacZ}$ intercrosses were genotyped at different embryonic stages. From embryonic day 8.5 to 15.5, the distribution of genotypes was close to the 1:2:1 Mendelian ratio (Table 1). At embryonic day 18.5 and day 0 postpartum, the numbers of $Taz^{lacZ/lacZ}$ homozygotes were lower than the expected ratio (Table 1), indicating that partial lethality started at the perinatal stage.

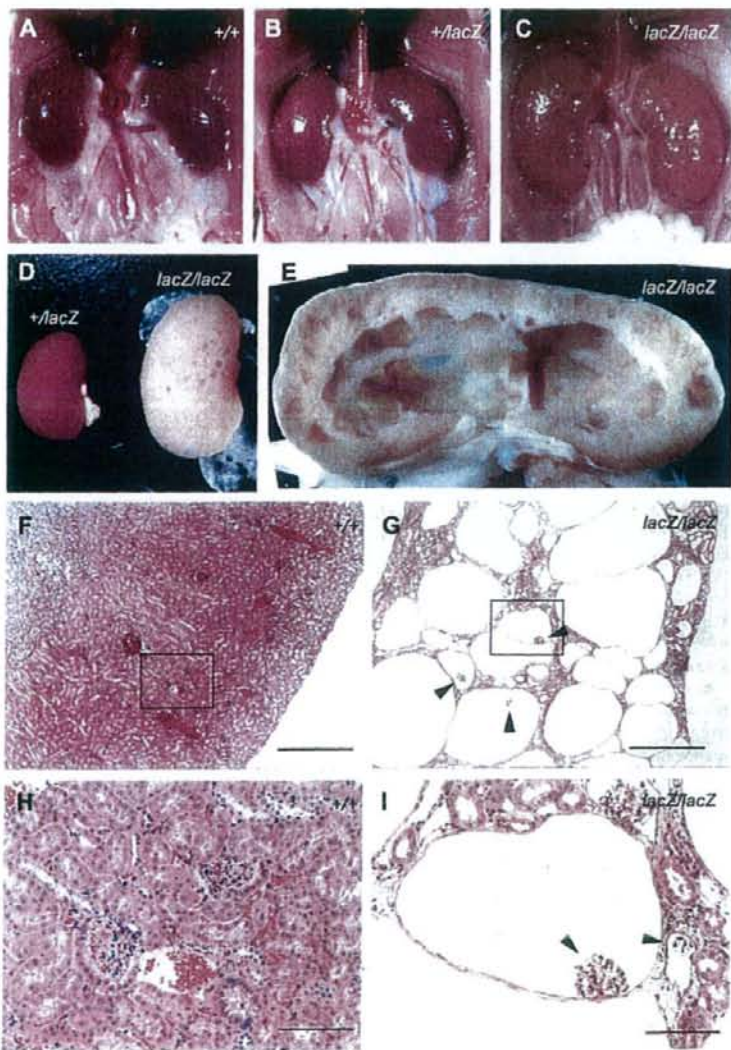


Fig. 2. Enlarged kidneys containing multiple cysts in $Taz^{lacZ/lacZ}$ mice. A-C: gross appearance of kidneys of $Taz^{+/+}$ (A), $Taz^{+/-}$ (B), and $Taz^{lacZ/lacZ}$ (C) mice aged 10 wk. $Taz^{lacZ/lacZ}$ kidneys are enlarged, pale, and filled with numerous cysts. D: comparison of $Taz^{+/+}$ and $Taz^{lacZ/lacZ}$ kidneys. E: section of a $Taz^{lacZ/lacZ}$ kidney showing dilated calyces and thinned parenchyma containing multiple cysts. F-I: histology of $Taz^{+/+}$ (F and H) and $Taz^{lacZ/lacZ}$ (G and I) kidneys at low (F and G) and high (H and I) magnification. Boxed areas in F and G are magnified in H and I, respectively. $Taz^{lacZ/lacZ}$ kidneys exhibit numerous cysts of various sizes that are lined by a flattened epithelial monolayer. Cysts containing a glomerular tuft (arrowheads) are often observed. Scale bars indicate 500 μ m (F and G) or 100 μ m (H and I).