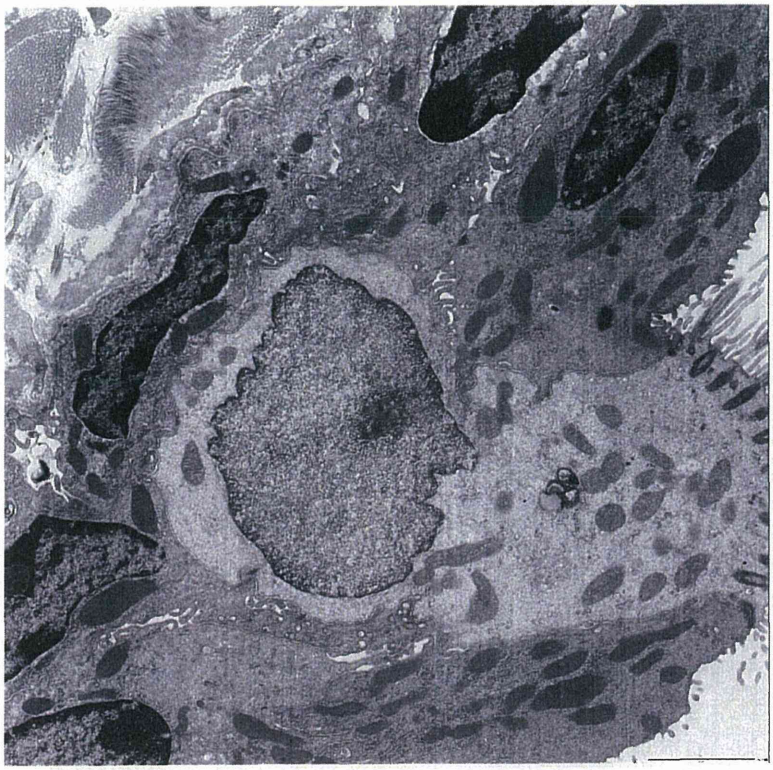

図 9 極低濃度ホルムアルデヒドに 28 日間暴露したマウス肺の超微細形態学的（TEM）検索
検索ポイント① 気管支上皮（線毛上皮細胞、クララ細胞）、1.0 ppm 暴露群

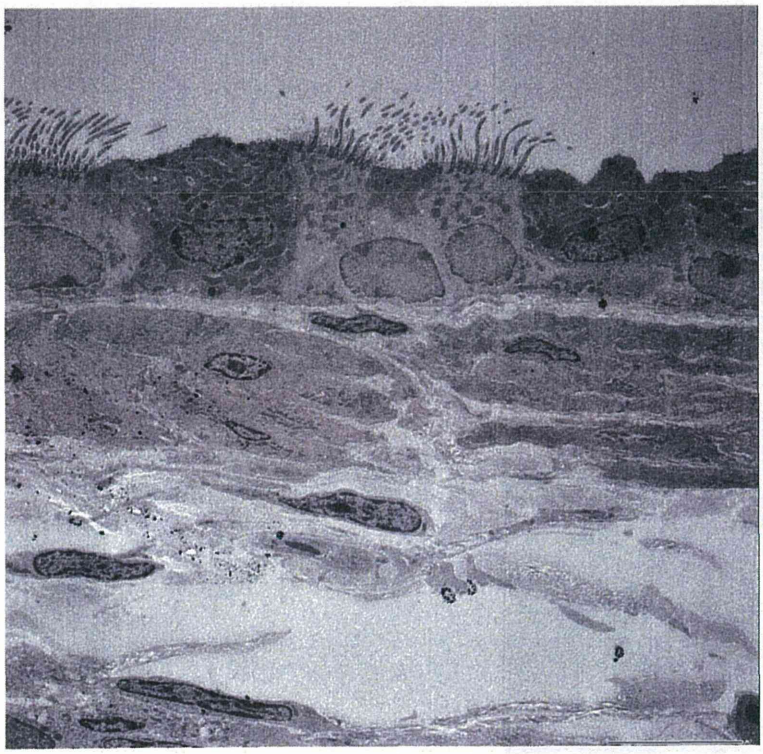

図 10 極低濃度ホルムアルデヒドに 28 日間暴露したマウス肺の超微細形態学的（TEM）検索
検索ポイント② 細気管支上皮（線毛上皮細胞、クララ細胞）、1.0 ppm 暴露群

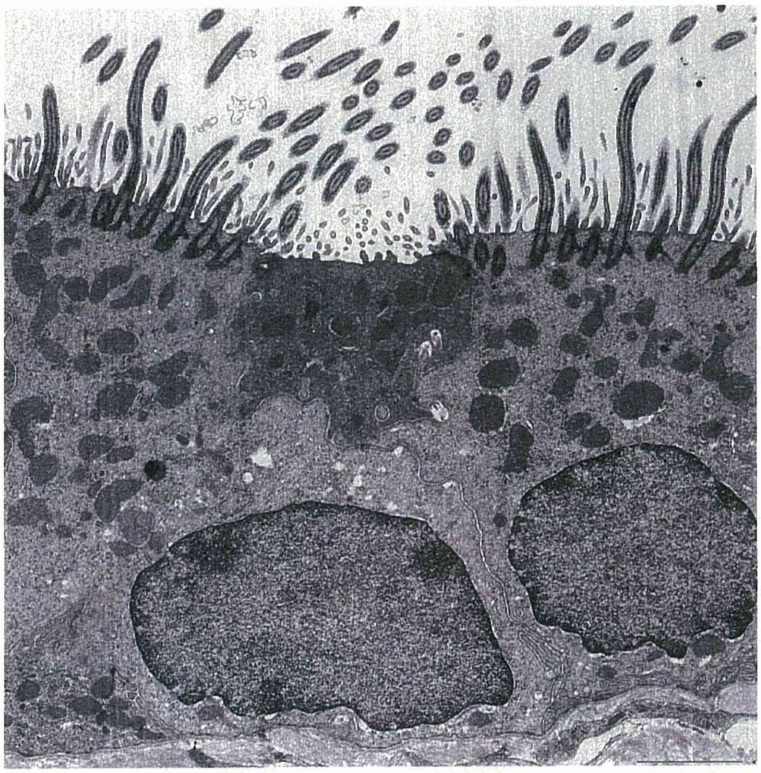

図 11 極低濃度ホルムアルデヒドに 28 日間暴露したマウス肺の超微細形態学的（TEM）検索
検索ポイント② 細気管支上皮（線毛上皮細胞）、1.0 ppm 暴露群

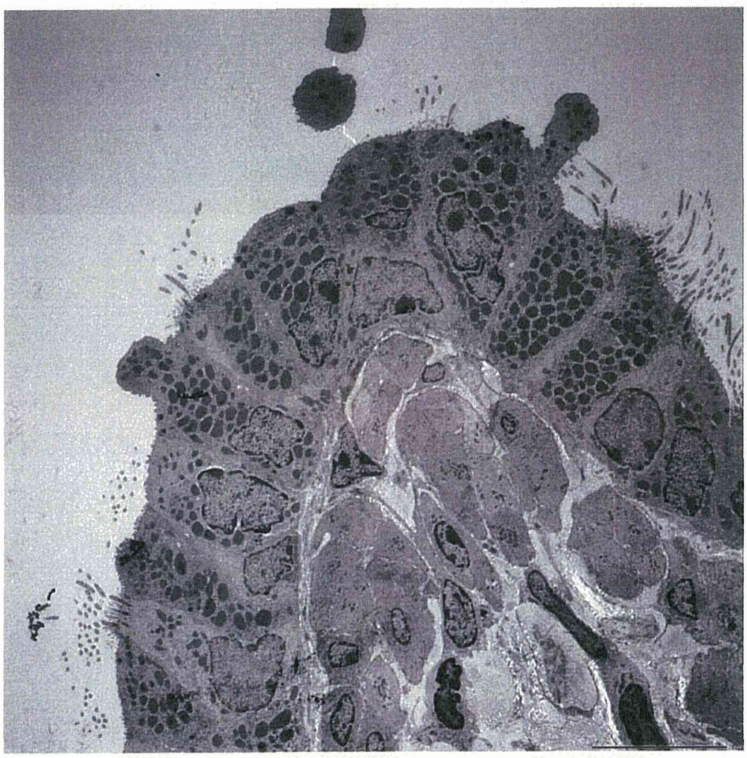

図 12 極低濃度ホルムアルデヒドに 28 日間暴露したマウス肺の超微細形態学的（TEM）検索
検索ポイント③ 細気管支上皮(線毛細胞、クララ細胞）、1.0 ppm 暴露群

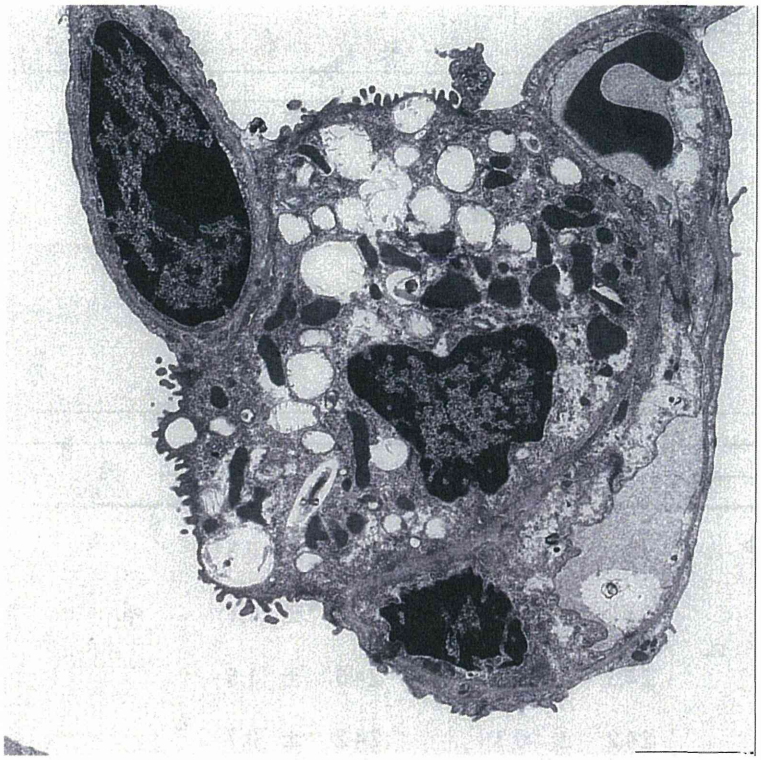

図 13 極低濃度ホルムアルデヒドに 28 日間暴露したマウス肺の超微細形態学的（TEM）検索
検索ポイント④ 終末細気管支、細葉中心領域の肺胞上皮（Ⅱ型細胞、Ⅰ型細胞）と
毛細血管、1.0 ppm 暴露群

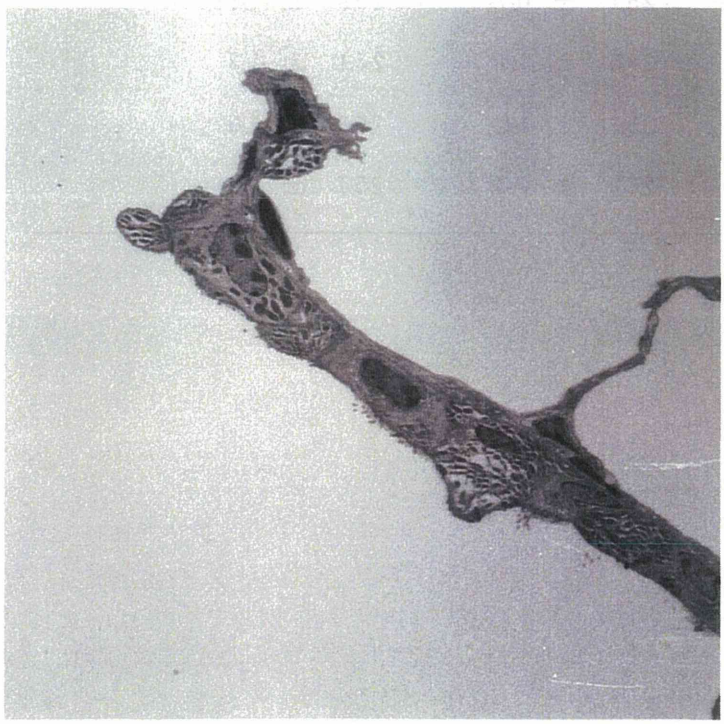

図 14 極低濃度ホルムアルデヒドに 28 日間暴露したマウス肺の超微細形態学的（TEM）検索
検索ポイント④ 終末細気管支上皮遠位端（クララ細胞、線毛細胞）、1.0 ppm 暴露群

表1　体重推移　（キシレン：22時間/日、28日間）

Exp. Group	Nomber of animals	Exposure 0	4	7	(day)
Control	6	23.7 ± 0.4	23.8 ± 0.5	23.5 ± 0.4	
0.2 ppm	6	24.2 ± 0.8	24.1 ± 1.0	23.9 ± 0.8	
0.7 ppm	6	24.1 ± 1.0	23.9 ± 1.0	23.8 ± 1.3	
2.0 ppm	6	23.7 ± 0.5	23.8 ± 1.0	23.4 ± 0.6	

Exp. Group	Nomber of animals	Exposure 11	14	18	(day)
Control	6	23.8 ± 0.4	23.9 ± 0.4	24.4 ± 0.5	
0.2 ppm	6	24.0 ± 1.1	24.3 ± 1.0	24.6 ± 1.1	
0.7 ppm	6	24.0 ± 1.3	24.3 ± 1.3	24.6 ± 1.5	
2.0 ppm	6	23.9 ± 0.6	24.2 ± 0.7	24.2 ± 0.7	

Exp. Group	Nomber of animals	Exposure 21	25	28	(day)
Control	6	24.5 ± 0.4	25.1 ± 0.7	25.9 ± 1.0	
0.2 ppm	6	24.7 ± 1.0	24.5 ± 1.1	25.1 ± 1.3	
0.7 ppm	6	25.1 ± 1.3	25.2 ± 1.2	25.8 ± 1.0	
2.0 ppm	6	24.3 ± 0.5	25.0 ± 0.7	25.7 ± 0.8	

平均値±標準偏差（*P<0.05,**P<0.01）

表2　摂餌量推移　（キシレン: 22時間/日、28日間）

Exp. Group	Nomber of animals	Exposure 4	7	11	(day)
Control	6	4.4 ± 0.4	3.7 ± 0.2	4.0 ± 0.2	
0.2 ppm	6	4.4 ± 0.2	3.8 ± 0.2	4.0 ± 0.4	
0.7 ppm	6	4.2 ± 0.2	3.8 ± 0.5	4.1 ± 0.2	
2.0 ppm	6	4.3 ± 0.4	3.7 ± 0.2	4.1 ± 0.2	

Exp. Group	Nomber of animals	Exposure 14	18	21	(day)
Control	6	4.0 ± 0.2	3.9 ± 0.2	3.6 ± 0.3	
0.2 ppm	6	4.0 ± 0.4	4.1 ± 0.3	3.9 ± 0.3	
0.7 ppm	6	4.2 ± 0.2	4.0 ± 0.5	3.8 ± 0.4	
2.0 ppm	6	4.1 ± 0.3	3.8 ± 0.3	3.7 ± 0.2	

Exp. Group	Nomber of animals	Exposure 25	28	(day)
Control	6	4.1 ± 0.3	4.1 ± 0.2	
0.2 ppm	6	3.9 ± 0.5	4.6 ± 0.5	
0.7 ppm	6	4.1 ± 0.3	4.0 ± 0.3	
2.0 ppm	6	4.0 ± 0.1	4.0 ± 0.3	

平均値±標準偏差（*P<0.05,**P<0.01）

表3　各臓器の絶対・相対重量及び最終体重（キシレン:22時間×28日間）

肝

Exp. Group	Liver (g/100g)	Liver (g)	Body weight (g)
Air control	5.700 ± 0.248	1.478 ± 0.107	25.9 ± 1.0
0.2 ppm	5.446 ± 0.240 *	1.371 ± 0.121	25.1 ± 1.3
0.7 ppm	5.595 ± 0.107	1.444 ± 0.052	25.8 ± 1.0
2.0 ppm	5.678 ± 0.168	1.459 ± 0.066	25.7 ± 0.8

腎

Exp. Group	Kidny (g/100g)	Kidny (g)	Body weight (g)
Air control	1.142 ± 0.062	0.296 ± 0.010	25.9 ± 1.0
0.2 ppm	1.157 ± 0.047	0.291 ± 0.014	25.1 ± 1.3
0.7 ppm	1.099 ± 0.035	0.284 ± 0.017	25.8 ± 1.0
2.0 ppm	1.157 ± 0.025	0.297 ± 0.008	25.7 ± 0.8

脾

Exp. Group	Spleen (g/100g)	Spleen (g)	Body weight (g)
Air control	0.278 ± 0.014	0.072 ± 0.005	25.9 ± 1.0
0.2 ppm	0.274 ± 0.027	0.069 ± 0.009	25.1 ± 1.3
0.7 ppm	0.274 ± 0.010	0.071 ± 0.003	25.8 ± 1.0
2.0 ppm	0.258 ± 0.016	0.066 ± 0.003	25.7 ± 0.8

心

Exp. Group	Heart (g/100g)	Heart (g)	Body weight (g)
Air control	0.515 ± 0.016	0.133 ± 0.006	25.9 ± 1.0
0.2 ppm	0.529 ± 0.022	0.133 ± 0.007	25.1 ± 1.3
0.7 ppm	0.504 ± 0.019	0.130 ± 0.005	25.8 ± 1.0
2.0 ppm	0.505 ± 0.024	0.130 ± 0.004	25.7 ± 0.8

脳

Exp. Group	Brain (g/100g)	Brain (g)	Body weight (g)
Air control	1.726 ± 0.080	0.447 ± 0.015	25.9 ± 1.0
0.2 ppm	1.762 ± 0.086	0.442 ± 0.012	25.1 ± 1.3
0.7 ppm	1.724 ± 0.050	0.445 ± 0.006	25.8 ± 1.0
2.0 ppm	1.723 ± 0.050	0.442 ± 0.010	25.7 ± 0.8

平均値±標準偏差（*P<0.05,**P<0.01）

表 4　病理組織学的検査結果

濃度	0 ppm	0.2 ppm 群	0.7 ppm 群	2.0 ppm 群
検索数	6	6	6	6
肝臓	炎症性細胞集簇巣 1+:2	炎症性細胞集簇巣 1+:1	炎症性細胞集簇巣 1+:2	炎症性細胞集簇巣 1+:2
腎臓	著変なし	著変なし	著変なし	著変なし
脾臓	著変なし	著変なし	著変なし	著変なし
心臓	著変なし	著変なし	著変なし	著変なし
脳	著変なし	著変なし	著変なし	著変なし
胸腺	著変なし	著変なし	著変なし	著変なし

1+:軽度　　肺の病理組織学的検査は切り出し位置を確定次第実施する.

病理組織学的検査で各臓器にキシレンの暴露による影響を認めなかった.

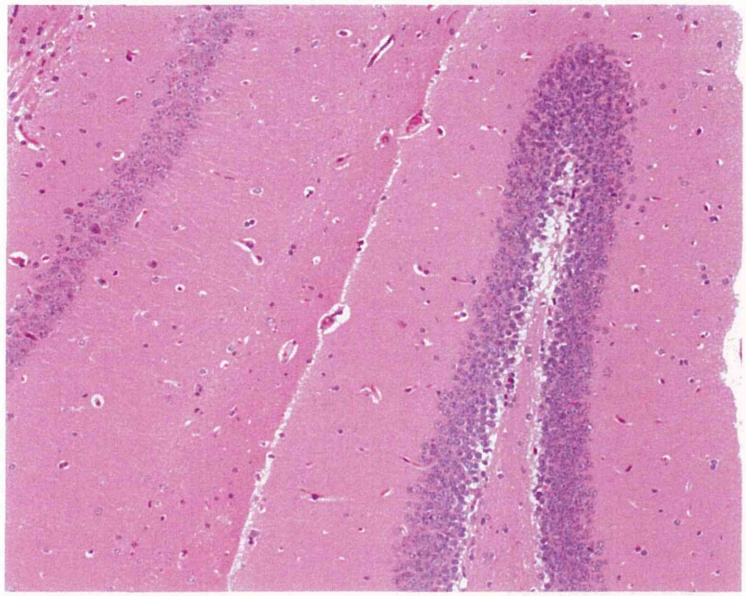

図 15. 極低濃度キシレンを 28 日間反復吸入暴露したマウスの病理組織学的検査
脳（海馬）、ヘマトキシリン・エオジン染色、キシレン 2.0ppm 暴露群

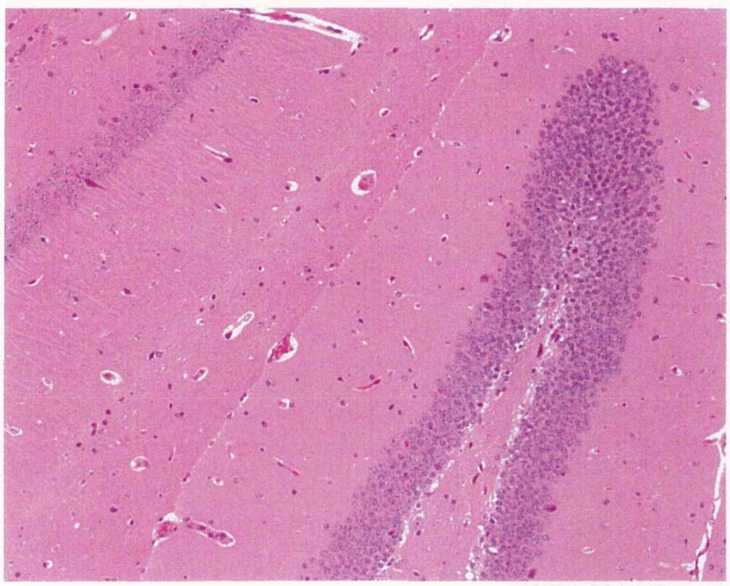

図 16. 極低濃度キシレンを 28 日間反復吸入暴露したマウスの病理組織学的検査
脳（海馬）、ヘマトキシリン・エオジン染色、対照群

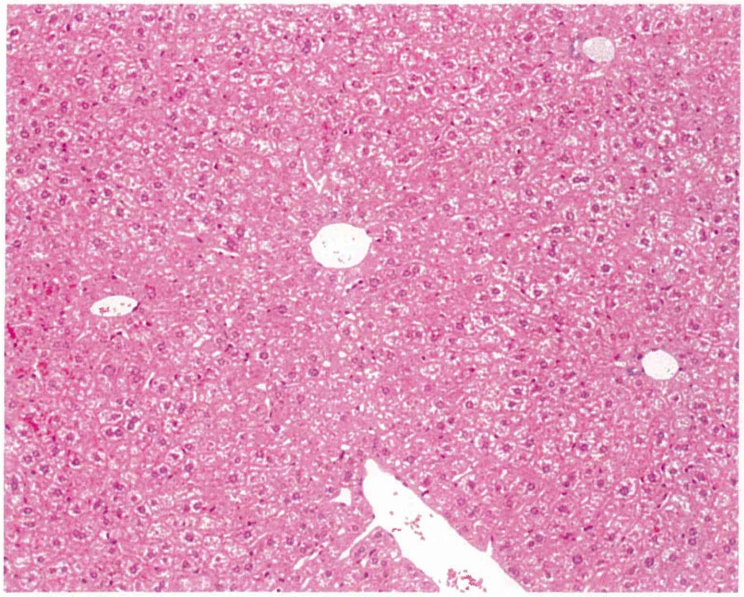

図 17. 極低濃度キシレンを 28 日間反復吸入暴露したマウスの病理組織学的検査
肝臓、ヘマトキシリン・エオジン染色、キシレン 2.0ppm 暴露群

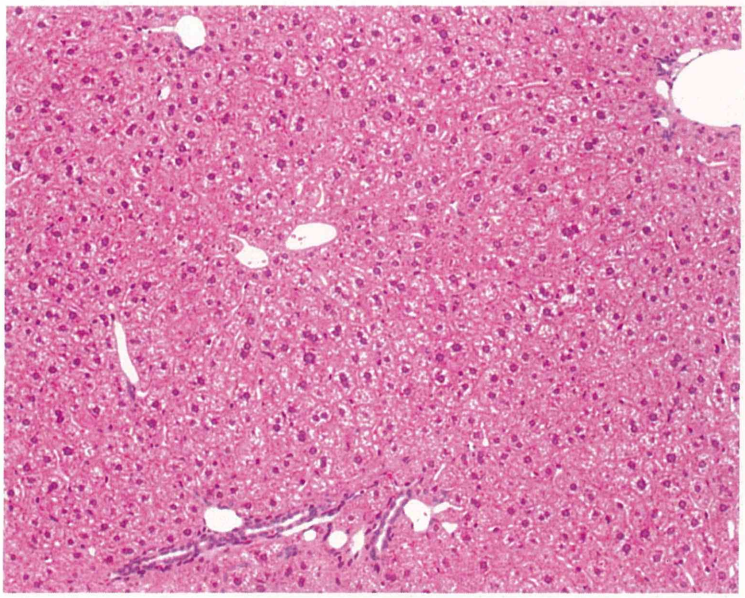

図 18. 極低濃度キシレンを 28 日間反復吸入暴露したマウスの病理組織学的検査
肝臓、ヘマトキシリン・エオジン染色、対照群

Ⅲ. 研究成果の刊行に関する一覧表

別添５

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Igarashi K, Kitajima S, Aisaki K, Tanemura K, Taquahashi Y, Moriyama N, Ikeno E, Matsuda N, Saga Y, Blumberg B, Kanno J.	Development of humanized steroid and xenobiotic receptor mouse by homologous knock-in of the human steroid and xenobiotic receptor ligand binding domain sequence.	J Toxicol Sci.	37 (2)	373 - 380	2012
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Noguchi S, Hamano E, Matsushita I, Hijikata M, Ito H, Nagase T, Keicho N.	Differential effects of a common splice site polymorphism on the generation of OAS1 variants in human bronchial epithelial cells.	Hum Immunol	74 (3)	395 - 401	2013
Noguchi S, Hijikata M, Hamano E, Matsushita I, Ito H, Ohashi J, Nagase T, Keicho N.	MxA transcripts with distinct first exons and modulation of gene expression levels by single-nucleotide polymorphisms in human bronchial epithelial cells.	Immunogenetics	65 (2)	107 - 114	2013

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Abe S, Kurata M, Suzuki S, Yamamoto K, Aisaki K, Kanno J, Kitagawa M.	Minichromosome maintenance 2 bound with retroviral Gp70 is localized to cytoplasm and enhances DNA-damage-induced apoptosis.	PLoS One	7 (6)	e40129	2012
Swedenborg E, Kotka M, Seifert M, Kanno J, Pongratz I, Rüegg J.	The aryl hydrocarbon receptor ligands 2,3,7,8-tetrachlorodibenzo-p-dioxin and 3-methylcholanthrene regulate distinct genetic networks.	Mol Cell Endocrinol.	362 (1-2)	39 - 47	2012
Fujimoto H, Woo GH, Inoue K, Igarashi K, Kanno J, Hirose M, Nishikawa A, Shibutani M.	Increased cellular distribution of vimentin and Ret in the cingulum induced by developmental hypothyroidism in rat offspring maternally exposed to anti-thyroid agents.	Reprod Toxicol.	34 (1)	93 - 100	2012
Nagano, K., Gotoh, K., Kasai, T., Aiso, S., Nishizawa, T., Ohnishi, M., Ikawa, N., Eitaki, Y., Yamada, K., Arito, H. and Fukushima, S.	Two- and 13-week Inhalation Toxicities of Indium-Tin Oxide and Indium Oxide in Rats	Journal of Occupational Health	53	51 - 63	2011
Nagano, K., Nishizawa, T., Eitaki, Y., Ohnishi, M., Noguchi, T., Arito, H. and Fukushima, S.	Pulmonary Toxicity in Mice by 2- and 13-week Inhalation Exposures to Indium-tin Oxide and Indium Oxide Aerosols	Journal of Occupational Health	53	234 - 239	2011

Ⅳ. 研究成果の刊行物・別刷

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Vol.37, No.2, 373-380, 2012

Original Article

Development of humanized steroid and xenobiotic receptor mouse by homologous knock-in of the human steroid and xenobiotic receptor ligand binding domain sequence

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ABSTRACT — The human steroid and xenobiotic receptor (SXR), (also known as pregnane X receptor PXR, and NR1I2) is a low affinity sensor that responds to a variety of endobiotic, nutritional and xenobiotic ligands. SXR activates transcription of Cytochrome P450, family 3, subfamily A (CYP3A) and other important metabolic enzymes to up-regulate catabolic pathways mediating xenobiotic elimination. One key feature that demarcates SXR from other nuclear receptors is that the human and rodent orthologues exhibit different ligand preference for a subset of toxicologically important chemicals. This difference leads to a profound problem for rodent studies to predict toxicity in humans. The objective of this study is to generate a new humanized mouse line, which responds systemically to human-specific ligands in order to better predict systemic toxicity in humans. For this purpose, the ligand binding domain (LBD) of the human SXR was homologously knocked-in to the murine gene replacing the endogenous LBD. The LBD-humanized chimeric gene was expressed in all ten organs examined, including liver, small intestine, stomach, kidney and lung in a pattern similar to the endogenous gene expressed in the wild-type (WT) mouse. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the human-selective ligand, rifampicin induced Cyp3a11 and Carboxylesterase 6 (Ces6) mRNA expression in liver and intestine, whereas the murine-selective ligand, pregnenolone-16-carbonitrile did not. This new humanized mouse line should provide a useful tool for assessing whole body toxicity, whether acute, chronic or developmental, induced by human selective ligands themselves and subsequently generated metabolites that can trigger further toxic responses mediated secondarily by other receptors distributed body-wide.

Key words: Steroid and xenobiotic receptor, Pregnane X receptor, Humanized mouse, Ligand binding domain, Knock-in mouse

INTRODUCTION

Most orally administered xenobiotics are metabolized first by the intestine and then by the liver after portal transport. The expression levels of enzymes involved in xenobiotic metabolism are regulated at the transcriptional level by key xenobiotic sensors including the steroid and xenobiotic receptor (SXR), also known as the pregnane X receptor (PXR), pregnane activated receptor (PAR) and NR1I2 (Bertilsson *et al.*, 1998; Lehmann *et al.*, 1998; Blumberg *et al.*, 1998). SXR is important in the field of toxicology for at least two reasons. Firstly, this receptor system induces the expression of CYP3A and CYP2B enzymes, the major metabolizers of pharmaceu-

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ticals and xenobiotics. Therefore, SXR is a key mediator of drug- and chemical-induced toxicity as well as drug-drug and drug-nutrient interactions (Zhou *et al.*, 2004). Secondly, the orthologous rodent and human receptors exhibit differential sensitivity for a subset of chemical ligands important in the field of toxicology. For example, rifampicin (RIF) is a specific and selective activator of human SXR, whereas pregnenolone 16a-carbonitrile (PCN) is selective for the rodent orthologue.

Rodent-human differences in CYP3A and CYP2B-mediated responses to xenobiotics can be a profound problem in toxicologic studies where rodents are used to predict the toxicity of a compound in humans (Ma *et al.*, 2007). Therefore, development of a murine model that reconstructs the SXR-mediated systemic response of humans is of a great significance in toxicology.

Human and rodent SXRs share ~95% amino acid sequence identity in the DNA-binding domain (DBD) but only about 77% identity in the LBD. Tirona *et al.* (2004) analyzed the ligand selectivity of a human-rat chimeric protein and showed that the species differences are primarily defined by sequence differences in the LBD. Watkins and colleagues showed that the key residues responsible for the majority of the ligand selectivity were Leu 308 (human) and Phe305 (rat and mouse). Crystallographic analysis located these amino acids within or neighboring the flexible loop that forms a part of the pore to the ligand-binding cavity. Swapping the rodent and human-specific residues was shown to modulate the activation by the human-selective activator RIF *in vitro* (Watkins *et al.*, 2001). According to those findings, a simple replacement of the mouse LBD with the human sequence should be sufficient to "humanize" the ligand binding properties as well as activation of the downstream target genes.

Three kinds of humanized mice have already been generated. One is the SXR-null/Alb-SXR mouse (Alb-SXR mouse) made by crossing the SXR knockout mice with a transgenic mouse line that expresses human SXR in liver under the control of the albumin promoter (Xie *et al.*, 2000). Gonzalez and colleagues generated a transgenic mouse expressing a human BAC containing the entire hSXR gene in a SXR null background, thus controlled under human SXR promoter (SXR BAC mouse) (Ma *et al.*, 2007). Another mouse is the human SXR genome knock-in mice (hSXR genome mouse) (Scheer *et al.*, 2008). The human SXR genomic region from exon 2 to exon 9 was knocked-in to mouse SXR exon 2. This mouse expresses the human full length SXR mRNA under the control of mouse SXR promoter regulation. Although useful for toxicology studies, these mice have disadvantages in that the human SXR is expressed only in the liver (Alb-SXR mouse), hSXR mRNA is not expressed in all of the tissues where SXR is known to be expressed (SXR BAC mouse), and there might be potential differences in the binding affinities of hSXR DNA-binding domain (DBD) to *cis*-acting elements in mouse SXR target genes (hSXR genome mouse).

As noted above, it is known that the critical differences between human and rodent ligand-selectivity reside in the LBD. Therefore, when our project to generate a humanized SXR mouse was initiated, we reasoned that altering the LBD would be sufficient to generate a humanized ligand selectivity. We decided to retain the mouse DBD to avoid any potential differences between the binding affinities of the chimeric receptor for cis-acting elements in the mouse genome. To maintain the tissue-specific expression pattern of the endogenous gene, we inserted the human cDNA encoding the region carboxyl-terminal to the DBD into the mouse gene. This retains all of the 5' and 3' regulatory elements in the mouse gene, as well as introns 1 and 2, which contain important elements for regulating SXR expression (Jung *et al.*, 2006).

Here we report a new line of mouse (hSXRki mouse) in which a cDNA encoding the human LBD is homologously recombined into the mouse gene after exon 3. The tissue distribution of the resulting chimeric mouse DBD-human LBD mRNA is comparable to that of the WT mouse. The hSXRki mouse showed a fully humanized response to the human-selective activator RIF in that the Cyp3a11 mRNA was induced in liver and mucosa of small intestine in response to RIF, but not the rodent-selective compound PCN. This new mouse line should provide a useful tool for assessing the whole body toxicity induced by a human selective SXR ligand itself and its subsequently generated metabolite(s) that can trigger further toxic responses through other pathways body-wide.

MATERIALS AND METHODS

Generation of hSXRki knock-in mice

A DNA fragment of mouse SXR intron 2 to exon 3 was PCR amplified using mouse BAC DNA (BAC clone No. RP23-351P21) as a template. Primers used were BAC39486FW and mSXR462RV (for sequences of the primers see Table1). This fragment was connected to the LBD of human SXR cDNA from amino acid 105 through the carboxyl terminus amplified by the PCR primers: hSXR904FW and hSXR1887RVEcoRI (template; human SXR cDNA). The 3'UTR of bovine growth hormone (BGH) was added to 3' to the terminal codon. This concatenated fragment was introduced to a vector, which

Table 1. List of primer pairs

Purpose	Primer name	Sequence (5' to 3')
Targeting vector construction	BAC39486FW	CCATGGGTACCACGAATAACAA
	mSXR462RV	CATGCCACTCTCCAGGCA
	hSXR904FW	AAGAAGGAGATGATCATGTCCG
	hSXR1887RVEcoRI	CCGAATTCTCATCATCAGCTACCTGTGATACCGAACA
Genotyping	NeoAL2	GGGGATGCGGTGGGCTCTATGGCTT
	SXR RC RV5	TGAGAGTGCACAAGTTCAAGCT
	WTInt5	AGTGATGGGAACCACTCCTG
	WTEx6RV	TGGTCCTCAATAGGCAGGTC
	mhSXRE4	GTGAACGGACAGGGACTCAG
	mhSXRSARV	CTCTCCTGGCTCATCCTCAC
Percellome quantitative RT-PCR	Cyp3a11 FW	CAGCTTGGTGCTCCTCTACC
	Cyp3a11 RV	TCAAACAACCCCCATGTTTT
	Ces6 FW	GGAGCCTGAGTTCAGGACAGAC
	Ces6 RV	ACCCTCACTGTTGGGGTTC
	mouse SXR FW	AATCATGAAAGACAGGGTTC
	mouse SXR RV	AAGAGCACAGATCTTTCCG
	human SXR FW	ATCACCCGGAAGACACGAC
	human SXR RV	AAGAGCACAGATCTTTCCG
	mouse-human SXR FW	CCCATCAACGTAGAGGAGGA

has the neomycin resistance gene with loxP sequence at both ends, removable with Cre recombinase (Saga *et al.*, 1999). A 7kb KpnI fragment containing intron 2 was used as a long arm and 1.3kb PstI-EcoRI fragment containing from exon 8 to intron 8 was used as a short arm for homologous recombination (Fig. 1). The resulting targeting vector was linearized with SacII and introduced by electroporation to TT2 ES cell line (Yagi *et al.*, 1993) and neomycin resistant clones were selected, PCR genotyped, and confirmed by the Southern blotting. For generation of chimeric mice, these ES clones were aggregated with ICR 8-cell embryos and transferred to pseudopregnant female recipients. The chimeric mice born were bred with ICR females. Germ line transmission of the targeted allele was confirmed by PCR. A mouse was crossed with a CAG-Cre transgenic mouse (Sakai and Miyazaki, 1997) to evict the neomycin resistance gene, and back crossed to C57BL/6 CrSlc (SLC, Inc., Shizuoka, Japan) at least 6 generations and used for the analysis.

PCR Genotyping (See Table 1 for primer sequences)

Primers for identification of homologously recombined ES clones were NeoAL2 and SXR RC RV5. DNA purified from the tail of each mouse was used for PCR genotying. Primers for WT detection were WTInt5 and WTEx6RV amplifying a product of 755 bp. Primers for confirmation of removal of the neomycin resistance gene were mhSXRE4 and mhSXRSARV amplifying a product of 1,223 bp.

Southern blot analysis

To confirm homologous recombination, DNA from ES cell cultures was purified and digested with BamHI and XhoI, then electrophoresed and analyzed by Southern hybridization (Saga *et al.*, 1997). Mouse SXR exon 9 region which remains after homologous recombination was used for the probe. The restriction fragments from the WT allele and targeted allele are 2,305 bp and 1,925 bp, respectively.

Chemicals

RIF (molecular weight 822.95) and PCN (molecular weight 341.49) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Corn oil was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Quantitative RT-PCR (Percellome PCR) (See Table 1 for primer sequences)

The method for Percellome quantitative RT-PCR was described previously (Kanno *et al.*, 2006). Briefly, tissue pieces stored in RNAlater (Ambion, Austin, TX, USA) were homogenized and lysed in RLT buffer (Qiagen GmbH., Germany) and 10 μl aliquots were used

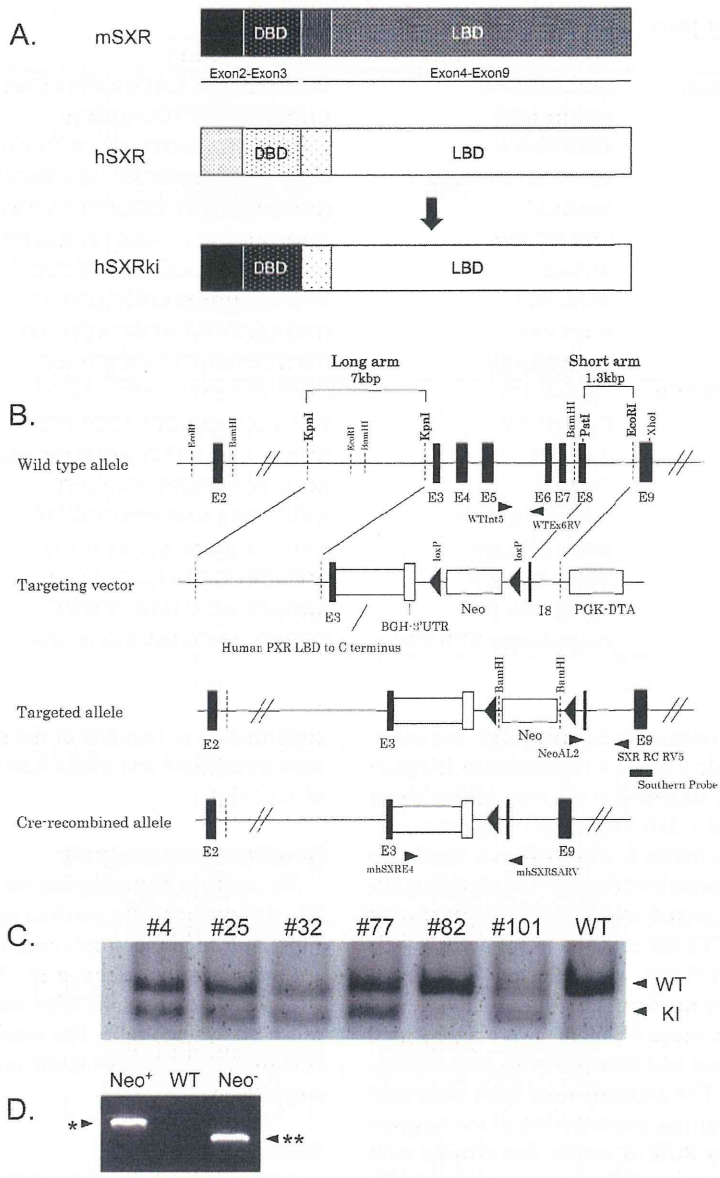

Fig. 1. Targeting strategy used to generate the hSXRki mouse. A) Diagram of hSXRki chimeric protein. Hinge region and ligand binding domain (LBD) of human SXR are knocked-in to mouse SXR, resulting in chimeric protein having murine N-ternial domain and DNA binding domain (DBD). B) Targeting strategy used to generate the hSXRki mouse. The chimeric mouse DBD and human LBD fragment, followed by the BGH 3' UTR were knocked-in to the mouse SXR gene. The genomic region spanning from exon 3 to exon 8 was substituted by the inserted fragment with the remainder of the gene remaining intact. C) Confirmation of homologous recombination by southern blot analysis. Six ES clones positive for recombination by PCR genotyping were further analyzed by southern blot (clones #4 ~ #101). Lower bands (1925 bp) indicate successful homologous recombination; upper bands (2305 bp) correspond to WT allele. Clones #4, #25, #32, #77 and #101 were confirmed as homologous recombinants; clones #4 and #25 were used for the generation of chimeric mice. D) Confirmation of Cre-mediated removal of the neomycin resistance gene. Mouse tail genome DNA was PCR amplified with the primer set, mhSXRE4 and mhSXRSARV. *: 2,858 bp (for the mice having the neomycin resistance gene), **: 1,223 bp (for the mice without the neomycin resistance gene).

for genomic DNA quantification with PicoGreen fluorescent dye (Invitrogen, Carlsbad, CA, USA). A prepared spike mRNA cocktail solution containing known quantity of five mRNAs of bacillus subtilis was added to the tissue lysate in proportion to the DNA quantity. Total RNA was purified from the lysate using the RNeasy kit (Qiagen). One microgram of total RNA was reverse-transcribed with SuperScript II (Invitrogen). Quantitative real time PCR was performed with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems), with initial denaturation at 95°C for 10 min followed by 40 cycles of 30 sec at 95°C and 30 sec at 60°C and 30 sec at 72°C, and Ct values were obtained. Primers for Cyp3a11 were Cyp3a11 FW and Cyp3a11 RV. Primers for Ces6 were Ces6 FW and Ces6 RV. Primers for mouse SXR selective quantification were mouse SXR FW and mouse SXR RV. Primers for hSXRki selective quantification were human SXR FW and human SXR RV. Primers for both mouse SXR and hSXRki quantification were mouse-human SXR FW and mouse-human SXR RV that amplify the DBD region of the chimera.

In Situ Hybridization analysis

Digoxigein-labeled cRNA probe for Cyp3a11 was synthesized according to Suzuki *et al.* (2005) by RT-PCR using mouse liver cDNA as a template. The primers used were as follows: forward 5'-GATTGGTTTTGATGCCTGGT-3' and reverse 5'-CAAGAGCTCACATTTTTCATCA-3'. The amplified product was sequence confirmed and ligated with Block-iT T7-TOPO (Invitrogen) Linker, which contains the T7 promoter site. A secondary PCR was performed to generate the sense and antisense DNA templates. For antisense template, Block-iT T7 Primer and Cyp3a11 forward primer (or reverse primer for generation of sense DNA template), the same primer as for the first PCR amplification, were used. With these DNA templates, both sense and antisense digoxigenin-labeled riboprobes were synthesized using a DIG RNA labeling kit (Roche Diagnostics, Germany) according to the manufacturer's protocol.

ISH on paraffin sections was carried out according to Suzuki *et al.* with a modification; permeabilization condition 98°C for 15 min in HistoVT One (Nacalai tesque, Japan).

Animals experiments

Male hSXRki and WT mice were maintained under a 12 hr light/12 hr dark cycle with water and chow (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) provided *ad libitum*. The animal studies were conducted in accordance with the Guidance for Animal Studies of the National Institute of Health Sciences under Institutional approval. The expression level of the hSXRki and WT SXR mRNA of ten organs (brain, thymus, heart, lung, liver, stomach, spleen, kidney, small intestine and testis) were analyzed on 15 weeks old male mice (n = 2) by the Percellome quantitative RT-PCR.

For the demonstration of selective gene induction by RIF and PCN in hSXRki and WT male mice on 13 weeks

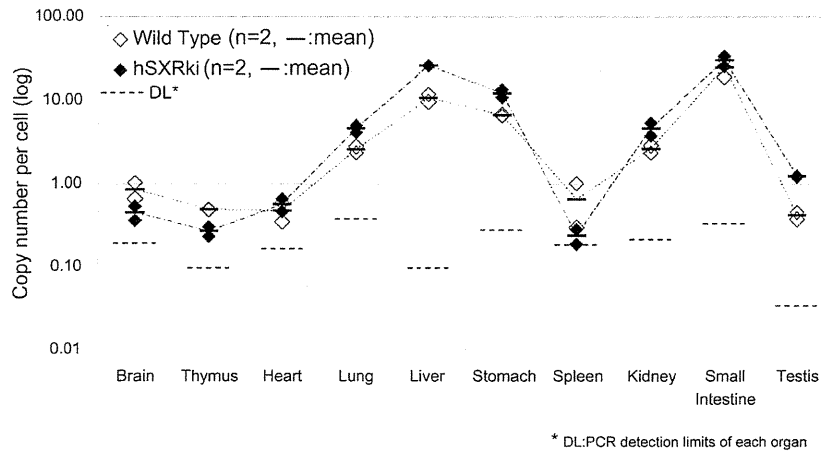

Fig. 2. Conservation of tissue expression patterns of hSXRki mRNA in the knock-in mouse. Percellome quantitative RT-PCR analysis was performed to measure the absolute expression levels of WT SXR mRNA and hSXRki mRNA in ten organs of WT and hSXRki mice. The expression levels of hSXRki mRNA among organs were comparable to WT.

old, three mice per group were singly dosed orally with vehicle (corn oil+0.1% DMSO), 10, 30, or 100 mg/kg of RIF, or 20, 70, or 200 mg/kg PCN (approximately equivalent in molar dose). Eight hours later, mice were sacrificed by exsanguination under ether anesthesia and the liver and the small intestine mucosa were sampled. Liver samples in small pieces were stored in RNA later (Applied Biosystems, Foster City, CA, USA) for further analysis. The small intestine under ice-cooled condition was longitudinally opened, gently rinsed with RNase-free saline and the epithelium was scraped with a glass slide and immersed in RNAlater. For *in situ* hybridization (ISH) of Cyp3a11 in the liver, 15 weeks old male hSXRki and WT mice were dosed orally with vehicle (corn oil), RIF (10 mg/kg), or PCN (40 mg/kg) daily for 3 days and liver sampled 24 hr later. All mice were sacrificed by exsanguination under ether anesthesia.

Statistical analysis

All values are expressed as the means ± S.D. and group differences analyzed by unpaired Student's *t* test or one-way ANOVA followed by Dunnett's post hoc comparison. Level of significance was set at $p < 0.05$.

RESULTS

Generation of hSXRki knock-In mice

Among 144 neomycin resistant TT2 ES clones, six PCR positive clones were further submitted to Southern blotting for the confirmation of homologous recombination. As shown in Fig. 1C, five clones were confirmed, and two (#4 and #25) were used to generate chimeric mice. The resulting mice were backcrossed to ICR strain to confirm germline transmission. One clone (#4) was crossed to a mouse constitutively expressing Cre recombinase to remove the neomycin resistance gene (Fig. 1D) and backcrossed to C57BL/6 CrSlc for at least 6 generations before further analysis.

Tissue distribution of hSXRki mRNA

Ten tissues, i.e., brain, thymus, heart, lung, liver, stomach, spleen, kidney, small intestine and testis from both hSXRki and WT mice were measured for hSXRki or WT SXR mRNA expression by the Percellome quantitative RT-PCR. As shown in Fig. 2, the levels of hSXRki mRNA are comparable to that of SXR in WT mouse and expressed in all tissues analyzed.

Humanized responses in hSXRki mouse

Humanized response of hSXRki was demonstrated by administration of the mouse-specific ligand PCN and the

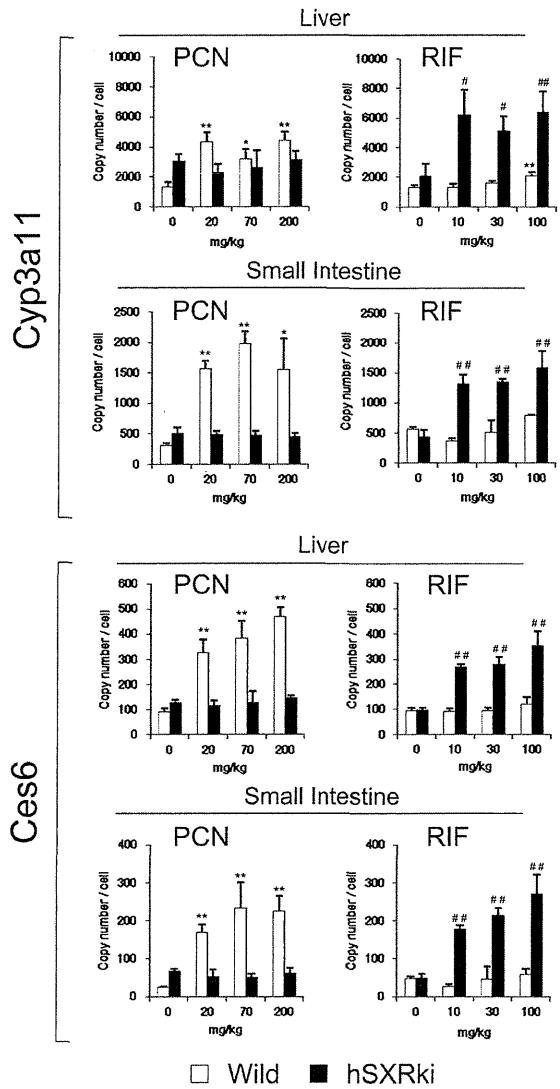

Fig. 3. Humanized response of hSXRki mice to RIF and PCN; Percellome quantitative RT-PCR. WT mice and hSXRki mice (n = 3 each) were singly dosed orally with vehicle (corn oil+0.1% DMSO), 20, 70, or 200 mg/kg PCN, or 10, 30, or 100 mg/kg of RIF (approximately equivalent in molar dose each other). Percellome quantitative RT-PCR data of Cyp3a11 and Ces6, both known as SXR target genes, in liver and small intestinal mucosa showed humanized responses in hSXRki. Bars = S.D., *; $p < 0.05$, **; $p < 0.01$ compared with vehicle group of WT, #; $p < 0.05$, ##; $p < 0.01$ compared with vehicle group of hSXRki. Analyzed by one-way ANOVA followed by Dunnett's post hoc comparison. Level of significance was set at $p < 0.05$.

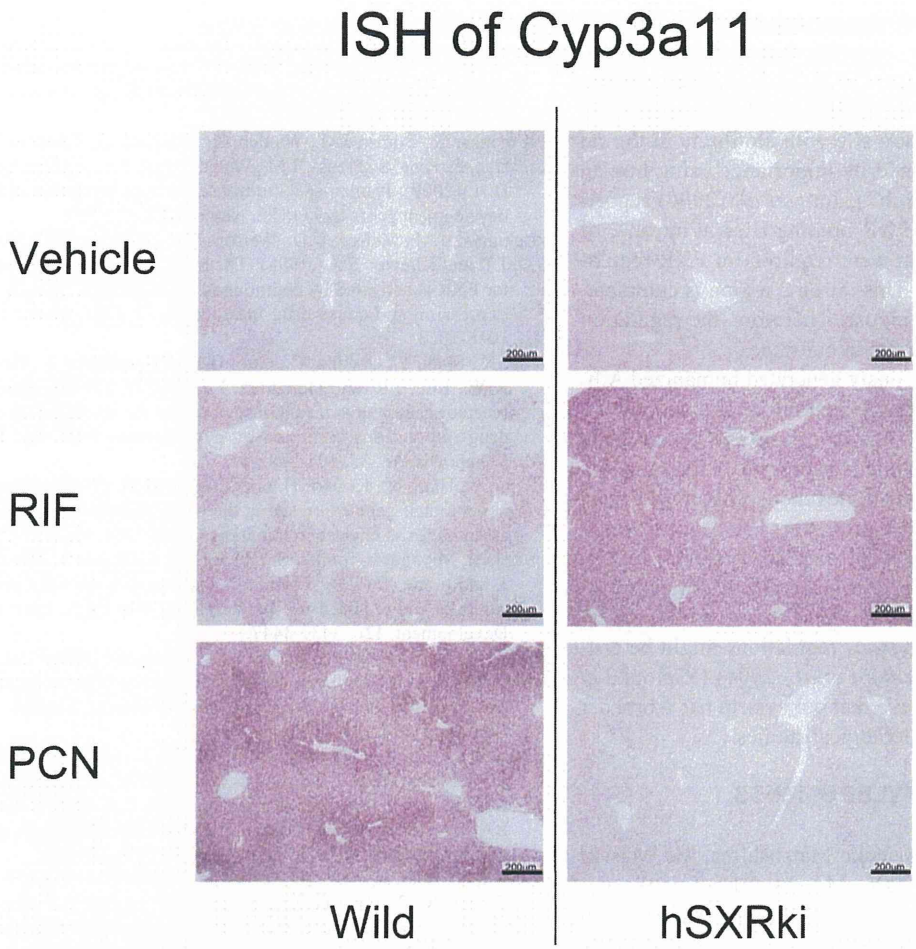

Fig. 4. Humanized response of hSXRki mice to RIF and PCN; *In situ* hybridization for Cyp3a11 mRNA in liver. A DIG-labeled cRNA probe for Cyp3a11 was hybridized and developed for purplish blue chromogenic reaction. Histologically, Cyp3a11 induction was localized around the central veins in both mice with species-specific ligands, respectively.

human-specific ligand RIF to the mice. Induction of the well-known SXR-regulated genes, Cyp3a11 and Ces6 was monitored by Percellome quantitative RT-PCR. As shown in Fig. 3, in the liver and small intestinal mucosa, RIF, but not PCN, induced Cyp3a11 and Ces6 in hSXRki mice (closed column), whereas PCN exclusively induced these genes in WT mice (open column). ISH of Cyp3a11 of the liver also showed humanized responses in hSXRki mice (Fig. 4).

DISCUSSION

We generated a new humanized mouse model in which the ligand binding domain (LBD) of human SXR was homologously knocked-into the murine SXR gene so that systemic response induced by human-selective SXR ligands can be monitored in mice. Firstly, we showed that mRNA from this chimeric gene was expressed at appropriate levels in the same tissues as the endogenous mouse SXR gene in WT mice. Then the humanized response of the mouse was confirmed by monitoring its response to the human-selective activator RIF, and the lack of response to the rodent-selective activator PCN.

There are relatively few reports about the regulation of SXR expression to date. Aouabdi *et al.* (2006) reported the presence of a PPAR alpha binding site 2.2 kb upstream of the transcription start site in human SXR. This site corresponded to the induction site with clofibrate in the rat and they further confirmed its importance using human liver cancer cell line (Huh7). Jung *et al.* (2006) reported the presence of four FXR binding sites in intron 2 of the mouse SXR gene that were required for FXR regulation of SXR expression. This intron 2 region is completely intact in our hSXRki mouse. Therefore, the regulation by FXR should be preserved in our mice.

Compared to the previously generated humanized Alb-SXR, SXR BAC, and hSXR genome mice, we contend that our hSXRki mouse has an advantage because the human-mouse chimeric gene is expressed in the same tissues and at similar levels to endogenous SXR in WT mice under control of the mouse promoter. This feature would make this model suitable not only for systemic toxicity but also toxicity at various stages of development of the embryo and fetus, maturation of infant, and of senescence, where the *cis* and *trans* regulations might be critical in its regulation (Sarsero *et al.*, 2004) (Konopka *et al.*, 2009). Thus, we believe that our system has a broader application range for toxicological studies.

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