

**Involvement of vitamin D receptor in the intestinal induction of human *ABCB1***

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## Running title page

Running title: Intestine-selective role of VDR in human ABCB1 induction

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The number of text pages: 34

The number of tables: 0

The number of figures: 7

The number of references: 39

The number of words in Abstract: 248

The number of words in Introduction: 648

The number of words in Discussion: 1313

### List of nonstandard abbreviations:

PXR pregnane X receptor, RXR; retinoid X receptor, CAR constitutive androstane receptor, DR4; direct repeat separated by 4 bases, VDR; vitamin D receptor, 1,25-VD<sub>3</sub>; 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, LCA; lithocholic acid, VDRE; vitamin D receptor responsive element, ER6; everted repeat separated by 6 bases, DR3; direct repeat separated by 3 bases, DMSO; dimethyl sulfoxide, GAPDH; glyceraldehyde-3-phosphate dehydrogenase, TK; thymidine kinase, SEAP; secreted alkaline phosphatase, siRNA; small interfering RNA, NURREM; nuclear receptor responsive module.

## Abstract

ABCB1 (P-glycoprotein) is an efflux transporter that limits the cellular uptake levels of various drugs in intestine, brain and other tissues. The expression of human *ABCB1* has recently been reported to be under control of nuclear receptor NR1I subfamily members, pregnane X receptor (PXR; NR1I2) and constitutive androstane receptor (CAR; NR1I3). Here we have investigated the involvement of another NR1I member, vitamin D receptor (VDR; NR1I1), in the *ABCB1* expression. In human colorectal adenocarcinoma cell line LS174T, which abundantly expresses VDR, both  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> (1,25-VD<sub>3</sub>) and lithocholic acid (LCA) increased *ABCB1* mRNA levels. Reporter gene assays in LS174T cells with constructs containing various length of the *ABCB1* regulatory region revealed that the region containing multiple nuclear receptor binding motifs located at -7.8 kb (termed NURREM), to which PXR and CAR also bind, is essential for the VDR-mediated *ABCB1* transactivation. Further reporter assays with constructs containing truncated NURREM and gel shift assays suggested simultaneous binding of multiple VDR/retinoid X receptor  $\alpha$  heterodimers to NURREM. Furthermore, knockdown of VDR expression in LS174T cells blocked the LCA- as well as 1,25-VD<sub>3</sub>-induced transcription of *ABCB1* reporter genes. In human hepatoma HepG2 cells, in contrast to LS174T cells, 1,25-VD<sub>3</sub> activated the *ABCB1* transcription only in the presence of ectopically expressed VDR. These results suggest that the NR1I subfamily members regulate the *ABCB1* expression sharing the binding sites within NURREM and that the physiologically produced LCA and 1,25-VD<sub>3</sub> may modulate the *ABCB1* expression in human intestines, possibly associated with interindividual variations of *ABCB1* expression.

## Introduction

ABCB1 (P-glycoprotein) plays an important physiological role as an efflux transporter. It is highly expressed in the epithelial cells of gastrointestinal tract and renal tubules, the canalicular membrane of hepatocytes, the capillary endothelial cells of the brain, testes, and ovaries. In these tissues, ABCB1 functions as a biological barrier to the systemic exposure of chemical substances by effluxing a broad range of hydrophobic compounds from the intracellular to the extracellular compartment (Fromm, 2004). In the intestine, ABCB1 limits the oral bioavailability of drugs and mediates excretion of the drugs into the intestinal lumen (Hsing et al., 1992; Leu and Huang, 1995; Sparreboom et al., 1997). Intestinal level of ABCB1 expression shows wide inter-individual variability, which may contribute to the variation in pharmacokinetics of drugs (Lown et al., 1997; Lindell et al., 2003; Urquhart et al., 2007). However, the mechanism underlying the interindividual variations in the basal expression level of ABCB1 remains unclear.

In addition, some drugs and steroid hormones increase intestinal ABCB1 levels, which may result in drug-drug interactions (Schuetz et al., 1996; Kim and Benet, 2004). For example, oral bioavailability of  $\beta$ -blocker talinolol, which is a substrate for ABCB1 but not oxidized in humans, was decreased after co-administration of rifampicin or St John's wort (Westphal et al., 2000; Schwarz et al., 2007). This induction of ABCB1 in the intestine resulted mainly from the activation of human *ABCB1* gene expression. The rifampicin-mediated activation of the *ABCB1* expression has been shown to be through the nuclear receptor, pregnane X receptor (PXR; NR1I2). PXR binds as a heterodimer with

retinoid X receptor  $\alpha$  (RXR $\alpha$ ; NR2B1) to the PXR-responsive element located at -7.8 kb upstream regions of *ABCB1* (Geick et al., 2001). Recently, constitutive androstane receptor (CAR; NR113) has also been shown to bind the same motif, the direct repeat 4 (DR4) element of *ABCB1* (Burk et al., 2005).

PXR and CAR also regulate the gene expression of drug metabolizing enzymes such as CYP3A4 (Lehmann et al., 1998; Goodwin et al., 2002). CYP3A4 and *ABCB1* show similar profiles of tissue distribution and substrate specificity, and are often co-induced by PXR ligands. They are thus considered to cooperate the defense against the potentially harmful chemical substances (Zhang and Benet, 2001). In addition to PXR and CAR, the *CYP3A4* expression is also enhanced by vitamin D receptor (VDR; NR111), which belongs to the same nuclear receptor NR11 subfamily as PXR and CAR (Thummel et al., 2001). VDR, forming a heterodimer with RXR, is activated by  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> (1,25-VD<sub>3</sub>) and a secondary bile acid lithocholic acid (LCA), and enhances the transcription of target genes through binding to VDR responsive elements (VDREs) in their promoter sequences. In *CYP3A4*, the proximal everted repeat separated by 6 bases (ER6) and distal direct repeat separated by 3 bases (DR3) motifs bind to VDR as well as PXR and CAR (Drocourt et al., 2002; Thompson et al., 2002). These facts raise the possibility that VDR is also involved in the expression of *ABCB1*. In fact, the mRNA level of *ABCB1* was increased by treatment with 1,25-VD<sub>3</sub> in human colorectal adenocarcinoma Caco-2 and LS180 cell lines (Schmiedlin-Ren et al., 1997; Thummel et al., 2001). However, little is known about the VDR regulation of the *ABCB1* expression.

In the present study, we have tested our hypothesis of the VDR-mediated *ABCB1* expression. To this end, we have investigated the molecular mechanism of the transcriptional activation of human *ABCB1* in response to 1,25-VD<sub>3</sub> and LCA, known VDR ligands (Makishima et al., 2002; Jurutka et al., 2005; McCarthy et al., 2005), using reporter gene assays in human colon carcinoma LS174T cells and gel shift assays. Our results show that multiple VDR/RXR $\alpha$  heterodimers simultaneously bind to the several VDR-responsive motifs within the regulatory cluster region located in the 5'-upstream of the *ABCB1* gene, which is identical with that for PXR and CAR, suggesting the complex regulation of the human *ABCB1* expression via NR11 subfamily members.

## **Materials and Methods**

### *Materials*

Rifampicin, LCA, and 1,25-VD<sub>3</sub> were purchased from Sigma-Aldrich (St Louis, MO). Dimethyl sulfoxide (DMSO) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Bio (Ohtsu, Japan).

Oligonucleotides were prepared by Nisshinbo Industries (Tokyo, Japan) and Fasmac Co., Ltd. (Atsugi, Japan). pSV- $\beta$ -galactosidase was from Promega (Madison, WI).

### *Cell culture*

LS174T cells were obtained from Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and cultured in Minimum Essential Medium containing 10% fetal bovine serum (Sigma-Aldrich or BioWest, Nuaille, France), minimal essential medium nonessential amino acid (Invitrogen, Carlsbad, CA), and penicillin-streptomycin (Invitrogen). HepG2 cells were obtained from Riken Bioresource Center and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, minimal essential medium nonessential amino acid, and penicillin-streptomycin.

### *RNA isolation and Quantitative Real-time PCR analysis*

Total RNA was extracted from LS174T and HepG2 cells using acid guanidine thiocyanate-phenol-chloroform method. Reverse transcription reactions were performed using

Ready-To-Go Your-Prime First-Strand Beads (GE Healthcare, Little Chalfont, UK) with oligo(dT) primer.

Quantitative real-time PCR was performed with TaqMan<sup>®</sup> Universal PCR Master Mix using ABI PRISM

7000 (Applied Biosystems, Foster City, CA). All samples were quantified using the comparative Ct

method for relative quantification of gene expression, normalized to glyceraldehyde-3-phosphate

dehydrogenase (GAPDH). The following primers and probe sets were used in this study: Human

*ABCB1* (5'-CTCAGACAGGATGTGAGTTGGTTT-3', 5'-ATTACAGCAAGCCTGGAACCTATAG-3' and

5'-FAM-CACCACTGGAGCATTGACTACCAGGCTC-3'); human *GAPDH*

(5'-AACAGCCTCAAGATCATCAGCAA-3', 5'-GACTGTGGTCATGAGTCCTTCCA-3' and

5'-VIC-CCAAGTCATCCATGACAACCTTTGGTATCG-3').

#### *Construction of plasmid DNA*

The human *ABCB1* 5'-flanking fragment including bases from -14556 to +125 was amplified by PCR with human genomic DNA as a template and oligonucleotides,

5'-cgacgcgtCTCAAGATAGCCTACACATCCTGCAGG-3' containing an MluI site and

5'-gcgtcgaCGCTCCTTGG AACGGCCACCAAGACG-3' containing an Sall site, using *TAKARA LA Taq*

(Takara Bio). The PCR fragment was subcloned into pCR-XL-TOPO (Invitrogen). The 15-kb DNA

fragment obtained by digesting the plasmid with MluI and Sall was inserted into the pSEAP2-basic

vector (Clontech, Mountain View, CA) at MluI and XhoI sites to obtain a reporter gene plasmid

(p-14566). Constructs p-8935, p-7472, and p-4179 were prepared by digesting p-14566 with Ball or



SpeI and ligating themselves.

pSEAP2-TK plasmid was constructed by inserting the thymidine kinase (TK) promoter

(Sueyoshi et al., 1999) between BglIII and HindIII sites of pSEAP2-basic plasmid. To construct

p-7880/7810-TK plasmid, oligonucleotides,

5'-ACGTTACCTCATTGAACTAAGTCTGACCTTGCTCCTGGGAGAGAGTTCATTTGAGATTAACAAGT

TCAAAG-3' and 5'-

CTTTGAACTTGTTTAATCTCAAATGAACTCTCTCCCAGGAGCAAGGTCAAGTTAGTTCAATGAGGTA

ACGTG-3' were annealed and the double-strand oligonucleotide was ligated into the MluI and XhoI

sites of pSEAP2-TK. For the construction of p-7880/7847-TK and p-7846/7810-TK, the

oligonucleotides pairs, 5'- GTACCACGTTACCTCATTGAACTAAGTCTGACCTTGCTCCA-3'/5'-

GATCTGGAGCAAGGTCAAGTTAGTTCAATGAGGTAACGTG-3' and 5'-

GTACCTGGGAGAGAGTTCATTTGAGATTAACAAGTTCAAAGA-3'/5'-

GATCTCTTTGAACTTGTTTAATCTCAAATGAACTCTCTCCCAG-3', were annealed, respectively, and

the double-strand oligonucleotides were ligated into the MluI and XhoI sites of pSEAP2-TK. The

sequences of these constructs were confirmed by direct sequencing.

To construct human VDR mammalian expression plasmid (pT-hVDR), human VDR cDNA was

amplified by PCR with primers, 5'- AGGGATGGAGGCAATGGCGGCCAG-3' and 5'-

TAGTCAGGAGATCTCATTGCCAAAC-3', and cDNA from HEK293 cells (Riken Bioresource Center,

Tsukuba, Japan) as the template using KOD-FX (Toyobo, Osaka, Japan), and ligated into pTarget

plasmid (Promega). The cDNA fragment obtained by digesting pT-hVDR with NotI and MluI was inserted into pTNT plasmid (Promega) to obtain pTNT-hVDR for in vitro transcription/translation. pTNT-hRXR $\alpha$  for in vitro transcription/translation of human RXR $\alpha$  was constructed previously (Toriyabe et al., 2009).

*Transient transfection and secreted alkaline phosphatase (SEAP) assay*

One day before transfection, cells were seeded in 12-well or 24-well plates. Reporter plasmid and pSV- $\beta$ -galactosidase were transfected using Cellfect Transfection kit (GE Healthcare) according to the manufacturer's protocol. pSV- $\beta$ -galactosidase was used for the normalization of transfection efficiency. For human VDR expression experiments, pT-hVDR or pTarget (as a control) was also transfected. Six to twelve hours after transfection, the cells were washed with phosphate-buffered saline and then treated with compounds or vehicle (0.1%(v/v) DMSO) for 48 h. Then, an aliquot of the medium was collected and used for the determination of SEAP activity with Great EscAPe™ SEAP Assay System (Clontech) or SEAP Reporter Gene Assay, chemiluminescent (Roche Applied Science, Mannheim, Germany) according to the manufacturers' protocols. The cells were harvested with Reporter Lysis Buffer (Promega) and  $\beta$ -galactosidase activity was measured using  $\beta$ -galactosidase Enzyme Assay System (Promega) or as described previously (Toriyabe et al., 2009). The reporter gene (SEAP) activity was normalized to the corresponding  $\beta$ -galactosidase activity and the results are expressed as normalized values. Fold induction (ratio of activities in compound-treated cells to those in

vehicle-treated cells) was calculated with the normalized values.

#### *RNA interference*

Two different small interfering RNA (siRNA) duplexes to target human VDR were purchased from Invitrogen. Their sequences were 5'-UUUGGAUGCUGAACUGACCAGGUC-3' (VDR-HSS111273; hVDR-siRNA-1) and 5'-UAGCAUUGAAGUGAAAGCCAGUGGC-3' (VDR-HSS111272; hVDR-siRNA-2). Slicer® negative control siRNA (Applied Biosystems) was used as a control siRNA. LS174T cells were seeded in 24-well plate ( $1 \times 10^5$  cells/well) and transfected with 15 pmol of each siRNA using siPORT™ Neo FX™ Transfection Agent (Applied Biosystems) according to the manufacturer's protocol. Two days after seeding and transfection, the cells were transfected with p-8935 (0.5 µg) and pSV-β-galactosidase (1 µg) as described above. Twelve hours later, the cells were washed with phosphate-buffered saline and treated with vehicle (0.1% DMSO), 100 nM 1,25-VD<sub>3</sub>, 10 µM LCA, or 10 µM rifampicin for 24 h. Reporter activities were determined as described above.

#### *Gel shift assay*

Human VDR and RXR $\alpha$  were synthesized in vitro from pTNT-hVDR and pTNT-hRXR $\alpha$ , respectively, using the TNT SP6 Coupled Reticulocyte Lysate Systems (Promega) following the manufacturer's protocol. Double-strand oligonucleotide probes (shown in Fig. 4A) were labeled with [ $\gamma$ -<sup>32</sup>P]ATP (PerkinElmer, Waltham, MA) using T4 polynucleotide kinase and purified by NICK columns

(GE Healthcare). The binding reaction was carried out with a reaction mixture (15  $\mu$ l) containing 10 mM Tris-HCl (pH 8.0), 5% glycerol, 100 mM KCl, 1 mM dithiothreitol, 1  $\mu$ g of poly(dI-dC) (GE Healthcare), and 0.5  $\mu$ l each of synthesized VDR- and/or RXR $\alpha$ -containing lysate, or control lysate. Reaction mixtures were preincubated on ice for 1 hr before the addition of  $^{32}$ P-labeled probe (105 fmol). The samples were kept on ice for additional 30 min and then separated on 4% polyacrylamide gel in 0.25 x Tris-boric acid-EDTA buffer at 20 mA. The gel was dried and exposed to an imaging plate to detect DNA-protein complexes with Imaging Analyzer FLA-3000 (Fuji Film, Tokyo, Japan). In some reactions, 2  $\mu$ g of anti-human VDR polyclonal IgG (H-81X, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture before preincubation.

## RESULTS

### *Effect of 1,25-VD3 and LCA treatment on ABCB1 mRNA level in LS174T cells*

To investigate the effect of VDR ligands on the expression of human *ABCB1*, the *ABCB1* mRNA levels in LS174T cells were determined after treatment with 1,25-VD3 or LCA (Fig. 1). The levels of *ABCB1* mRNA were increased 22-fold and 2.5-fold in the presence of 250 nM of 1,25-VD3 and 10  $\mu$ M LCA, respectively. Rifampicin, a human PXR ligand, also increased the mRNA levels (2.9-fold) as expected. In contrast, 1,25-VD3 and LCA as well as rifampicin had no effect on the *ABCB1* mRNA levels in HepG2 cells (Fig. 1).

### *Transcriptional activation of ABCB1 reporter genes by 1,25-VD3 and LCA*

To analyze the molecular mechanism of the *ABCB1* transcriptional activation by 1,25-VD3 or LCA, we constructed a reporter gene plasmid containing approximately 15 kb of the upstream region of *ABCB1* (p-14556), which included the previously reported regulatory cluster region located at -7.8 kb. The reporter plasmid was transiently transfected into LS174T cells and SEAP activity was determined after 48-h treatment with 100 nM 1,25-VD3, 10  $\mu$ M LCA or vehicle. As shown in Fig. 2A, treatment with either VDR ligand increased the reporter activity. Rifampicin treatment also enhanced the reporter activity of p-14556 as expected (Fig. 2B). These ligands, however, had no effect on the SEAP activity of pSEAP2-basic vector (data not shown). The results indicated that VDR responsive region was located within the 15 kb upstream of the human *ABCB1* gene.

To identify the region responsible for the VDR-mediated transcriptional activation of human *ABCB1*, a series of deletion constructs were prepared and transfected into LS174T cells. A deletion construct containing about 9 kb of the *ABCB1* promoter (p-8935) also responded to 1,25-VD3 and LCA, whereas shorter constructs lacking the regulatory cluster region at -7.8 kb (p-7472 and p-4179) were no longer transactivated by the VDR ligands (Fig. 2A). As expected, rifampicin response was observed only with the constructs containing the regulatory cluster region (p-7472 and p-4179; Fig. 2B). These results suggest that the regulatory cluster region at around -7.8 kb is necessary for the VDR ligand-induced *ABCB1* transcription as an enhancer element.

Geick et al. (Geick et al., 2001) found that the regulatory cluster at -7.8 kb contains several potential nuclear receptor-responsive elements, including DR3, DR4(I), DR4(II), DR4(III), and ER6 between -7880 and -7810. This 71-bp sequence was essential for the PXR- and CAR-mediated expression of *ABCB1* (Geick et al., 2001; Burk et al., 2005). To investigate a possible role of this region in the VDR-mediated transactivation, we constructed a reporter gene containing the 71-bp sequence fused to the thymidine kinase promoter (p-7880/7810-TK). As shown in Fig. 3A, this construct, but not the construct without the 71-bp sequence (pSEAP2-TK), was transactivated by treatment with either 1,25-VD3 or LCA in LS174T cells. Rifampicin response was also observed with p-7880/7810-TK (Fig. 3B). These results suggested that the 71-bp sequence is sufficient for the transcriptional activation of *ABCB1* via VDR as well as other NR11 subfamily members, PXR and CAR. The 71-bp sequence was thus termed NURREM (nuclear receptor responsive module).

*Multiple VDR/RXR $\alpha$  heterodimers bind to NURREM*

NURREM comprises several candidates of VDRE such as DR3, DR4(I), DR4(II), DR4(III), and ER6 (Fig. 4A). In order to identify a principal VDRE involved in the VDR-dependent transcription of *ABCB1*, we performed electrophoretic mobility shift assays with in vitro transcribed/translated human VDR and human RXR $\alpha$  proteins, and <sup>32</sup>P-labeled double-strand oligonucleotide probes (Fig. 4A). As shown in Fig. 4B, a heterodimer of VDR/RXR $\alpha$  bound to the DR3, DR4(I), and ER6 motifs, and weakly to DR4(II). The interaction of VDR/RXR $\alpha$  with DR4(I) was strongest among the probes tested. In addition, VDR monomer, but not RXR $\alpha$  monomer, bound to DR4(II), although the complex was barely detected in the presence of both VDR and RXR $\alpha$  (Fig. 4B and data not shown). Bands indicated with asterisk in Fig. 4B were nonspecific because they were also observed with control lysate that contained neither VDR nor RXR $\alpha$  (data not shown). As shown in Fig. 4C, at least 2 protein-DNA complexes with different electrophoretic mobility were detected when NURREM probe was incubated with in vitro synthesized VDR and RXR $\alpha$ . Preincubation of VDR and RXR $\alpha$  with anti-VDR antibody before addition of NURREM probe resulted in the formation of multiple supershifted complexes. Addition of normal IgG did not affect the bindings (data not shown). These results suggested simultaneous bindings of VDR/RXR $\alpha$  heterodimers to the several VDRE motifs with in NURREM.

To verify whether several putative VDREs contribute to the activation of the *ABCB1* gene, two distinct half-deleted constructs of p-7880/7810-TK-SEAP were prepared; the 3'-half-deleted NURREM

construct (p-7880/7847-TK) lacking DR4(II), DR4(III), and ER6, and the 5'-half-deleted NURREM construct (p-7846-7810-TK) lacking DR3 and DR4(I). After transfection of these constructs into LS174T cells, the cells were treated with 1,25-VD3 for 48h. As shown in Fig. 5, the reporter activities of both constructs were decreased by 40-50% compared to that of intact p-7880/7810-TK, suggesting that not only DR4(I)/DR3 in the 5'-half of NURREM, but ER6/DR4(III) in the 3'-half of NURREM are also involved in the VDR-mediated transcriptional of human *ABCB1*.

#### *LCA-induced transcription of ABCB1 reporter genes through VDR*

We demonstrated that LCA and 1,25-VD3 transactivate *ABCB1* through the common regulatory region NURREM. LCA is known to interact with multiple nuclear receptors including PXR and FXR as well as VDR. Thus, we have examined the role of VDR in the LCA-induced *ABCB1* transcription using siRNA technique (Fig. 6). LS174T cells were transfected with two distinct siRNA for human VDR and then with the *ABCB1* reporter gene p-8935. Treatment with LCA as well as 1,25-VD3 increased reporter activities in the cells transfected with control siRNA. On the other hand, the transfection of either human VDR-targeting siRNA completely abolished the response to LCA and drastically reduced the response to 1,25-VD3. In contrast, rifampicin responses were clearly observed even in the presence of the VDR-targeting siRNAs (Fig. 6). These results indicate that the LCA-induced transactivation of *ABCB1* is mediated through VDR.



*VDR-dependent transcription of ABCB1 reporter genes in HepG2 cells*

The VDR-dependent regulation of the *ABCB1* expression was observed in LS174T cells. As mentioned above, 1,25-VD<sub>3</sub> and LCA, however, had no effect on the *ABCB1* mRNA levels in HepG2 cells. To assess the mechanism underlying the difference in responses to VDR ligands between these cell lines, the influence of the ectopic expression of human VDR on the *ABCB1* reporter activities was investigated in HepG2 cells. A reporter plasmid containing the 9-kb upstream region of *ABCB1* fused to pSEAP2-basic vector (p-8935) was transfected into HepG2 cells, with either human VDR expression plasmid (pT-hVDR) or empty plasmid (pTarget). As shown in Fig. 7A, without co-transfection of pT-hVDR, the treatment with 1,25-VD<sub>3</sub> had no significant effect on the reporter activity of p-8935. However, with co-transfection of pT-hVDR, the reporter activity was increased significantly in response to 1,25-VD<sub>3</sub>. Similar results were obtained with the p-7880/7810-TK (Fig. 7B): Treatment of HepG2 cells with 1,25-VD<sub>3</sub> significantly increased the reporter activity in the presence, but not in the absence, of ectopic human VDR.

## Discussion

In the present study, we have demonstrated that endogenous mRNA levels and *ABCB1* reporter activities are inducible in human colon cancer LS174T cells in response to 1,25-VD3 and LCA (Figs. 1, and 2A). In addition, the 71-bp sequence termed NURREM located in the upstream region from -7880 to -7810 of the *ABCB1* gene is essential for the transactivation mediated by VDR in addition to PXR and CAR (Figs. 2A, 2B, 3A, 3B, and 5). Thus, the NR1I subfamily member VDR as well as PXR and CAR enhances the gene expression of human *ABCB1*.

In contrast to the results obtained in LS174T cells, the *ABCB1* transactivation was not observed in human hepatoma HepG2 cells (Fig. 7). Furthermore, we did not observe an increase of the *ABCB1* mRNA level in response to 1,25-VD3 in cryopreserved human hepatocytes, in spite of the significant increase after rifampicin treatment (Tachibana et al., unpublished results). These are probably due to the low or little expression of VDR in HepG2 cells and human hepatocytes. In fact, *VDR* mRNA was barely detected in HepG2 cells, whereas it was detected at much higher level in LS174T cells (Matsubara et al., 2008). In addition, VDR protein was detected in human intestine at much higher levels (approximately 320 fmol/mg protein) than in the liver (Berger et al., 1988; Ebeling et al., 1992; Stumpf, 1995). Interestingly we found that co-transfection of VDR expression plasmid into HepG2 cells conferred the transactivation of the *ABCB1* reporter genes on the cells (Fig. 7). These results suggest that the expression level of VDR is a major determinant for the induction of *ABCB1* in response to 1,25-VD3 and LCA in these cell lines and probably in human tissues. Consistently, previous studies

demonstrated increased *ABCB1* mRNA levels after treatment with 1,25-VD<sub>3</sub> in Caco-2 and LS180 cell lines, in which VDR is abundantly expressed (Thummel et al., 2001).

VDR, in addition to other nuclear receptors including PXR, CAR and FXR, has been recognized as a key regulator of bile acid homeostasis in the intestine. LCA, a secondary bile acid, is thought to activate the expression of VDR target genes in the gastrointestinal tract to protect intestinal cells from bile acid toxicity (Makishima et al., 2002). McCarthy et al. (2005) have suggested that the physiologically relevant site for the VDR-dependent regulation of *Mrp3* expression is the gastrointestinal tract. In this study, we showed that the LCA-mediated activation of *ABCB1* reporter gene was completely blocked with siRNA for human VDR in LS174T cells (Fig. 6), which express both PXR and VDR (Matsubara et al., 2008). Recently, we have also reported that LCA enhances the *CYP3A4* expression in the intestine but not in the liver, which is mediated by VDR rather than PXR and FXR (Matsubara et al., 2008). Thus, VDR probably plays a critical role in response to LCA for the intestinal expression of human *ABCB1* as well as *CYP3A4*.

Similar to *CYP3A4*, the intestinal levels of *ABCB1* expression show large inter-individual variability (Lown et al., 1997; Lindell et al., 2003; Urquhart et al., 2007). Although genetic factors are thought to influence, in part, the expression level (Leschziner et al., 2007), the mechanism underlying the variation is still not fully understood. Based on our results, LCA and 1,25-VD<sub>3</sub> could modulate *ABCB1* expression levels in the intestine. As reported in the literatures, LCA levels in intestines could reach high enough to activate VDR (Batta et al., 1999). One of most important physiological roles of

1,25-VD<sub>3</sub> is to stimulate the absorption of dietary calcium and phosphate in the intestine. Although the formation of 1,25-VD<sub>3</sub> from 25-hydroxyvitamin D mainly occurs in the kidney, CYP27B1, which catalyzes the 1 $\alpha$ -hydroxylation of 25-hydroxyvitamin D to form 1,25-VD<sub>3</sub>, is expressed at various extra-renal sites including the colonic epithelial cells (Zehnder et al., 2001). In these tissues, 1,25-VD<sub>3</sub> is considered to act as a VDR autocrine signal. Therefore, 1,25-VD<sub>3</sub> produced locally in the intestine might affect the VDR-mediated gene expression. These facts support the idea that at least in part LCA and 1,25-VD<sub>3</sub> are possible factors causing the variable expression of ABCB1 in the intestine.

Induction of the intestinal ABCB1 may lead to drug-drug interactions, usually resulting in the reduced absorption of drugs and their AUC. Our results implicate in addition to PXR ligands that VDR ligands could cause such drug-drug interactions involving ABCB1. Recently, 1,25-VD<sub>3</sub> and its synthetic analogs have been indicated for their potential applications to the treatment of osteoporosis, inflammation, dermatological indications, cancers and autoimmune diseases through activation of VDR (Takahashi and Morikawa, 2006). Thus, these drugs have potential risks of drug-drug interactions caused by the ABCB1 induction in the intestine.

Several putative VDREs exist within NURREM, overlapping with the PXR- and CAR-responsive elements (Geick et al., 2001; Burk et al., 2005). Among these VDREs, DR4(I) was reported to be a high affinity element for PXR/RXR $\alpha$  heterodimers. As for the CAR-mediated transactivation of human *ABCB1*, DR4(I) and DR4(II) were important for the receptor binding as a heterodimer with RXR $\alpha$  or as a monomer, respectively (Geick et al., 2001; Burk et al., 2005). In the

present study, VDR monomer also bound to DR4(II) as CAR monomer (Fig. 4B). However, its physiological relevance is unclear because the binding was not observed in the presence of both VDR and RXR $\alpha$  (Fig. 4B). We also found the high affinity binding of VDR/RXR $\alpha$  to DR4(I) (Fig. 4B), suggesting that DR4(I) mainly contributes to the VDR-mediated *ABCB1* transactivation. Our findings, however, show that in addition to DR4(I), DR3 and ER6 elements significantly contribute to the bindings of VDR/RXR $\alpha$  to NURREM (Fig. 4B), which is in contrast to the binding preference for DR4 by PXR and CAR as heterodimers with RXR $\alpha$ . Although the well-known VDREs are DR3 or DR4 (Hausler et al., 1998; Quack and Carlberg, 2000), ER6 is also functional in the promoter region of *CYP3A4* (Drocourt et al., 2002). In the present study, gel shift assays demonstrated that multiple VDR/RXR $\alpha$  heterodimers could simultaneously bind to NURREM (Fig. 4C). Consistently, neither 5'- nor 3'-half deletion of NURREM could completely abolish the reporter activity (Fig. 5). Such a VDR-mediated regulation via multiple VDREs has been also described in other VDR target genes, such as rat *CYP24* (Chen and DeLuca, 1995), human *cyclin C* (Sinkkonen et al., 2005), and *CYP3A4* (Drocourt et al., 2002; Thompson et al., 2002). The significance of the multiple functional VDREs within a single gene is still unknown. The functional cooperation between other transcription factors and VDR/RXR heterodimers in one of two VDREs within rat *CYP24* was demonstrated (Dwivedi et al., 2000). Whether other transcription factor(s) could stabilize a VDR/RXR heterodimer on each VDRE is an interesting issue for future studies. Nevertheless, our present results clearly indicate that each VDRE within NURREM of *ABCB1* can serve as a specific binding site of a VDR/RXR $\alpha$  heterodimer, and that the multiple VDREs

may act synergistically.

During the preparation of this manuscript, Saeki et al., (Saeki et al., 2008) have demonstrated the activation of human *ABCB1* expression by 1,25-VD<sub>3</sub> via VDR in Caco-2 cells. They have also reported that multiple VDR/RXR heterodimers bind to several VDREs within NURREM with DR4(I) showing the highest affinity. These are consistent with our present results. In addition to these results, we first demonstrate in this manuscript that LCA, an activator of multiple nuclear receptors, enhances the *ABCB1* expression through VDR in human colon cells (Figs 1, 2A, 3A, and 6).

In conclusion, we have demonstrated the VDR-dependent regulation of human *ABCB1* expression through the previously reported cluster of nuclear receptor binding motifs (termed NURREM in this study). Thus, the nuclear receptors in NR11 subfamily, namely VDR, PXR, and CAR, have overlapping functions in the detoxification and excretion of xenobiotics through regulating the expression of both *ABCB1* and *CYP3A4*. Because the *ABCB1* expression was enhanced by LCA in addition to 1,25-VD<sub>3</sub>, our present results also imply novel mechanisms that may explain the interindividual variability of *ABCB1* levels in the human intestine.

## **Acknowledgements**

We thank Masakiyo Ohya for his technical assistance.

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**Footnote**

This work was presented in part at the 8th ISSX International meeting, Oct 9–12, 2007, Sendai, Japan. This work was supported, in part, by Grant-in-Aid from Ministry of Education, Culture, Sports, Sciences and Technology of Japan, Ministry of Health, Labor, and Welfare of Japan and Comprehensive Research and Education Center for Planning of Drug Development and Clinical Education, Tohoku University 21<sup>st</sup> Century “Center of Excellence” Program.

## Legends for Figures

### Figure 1. Effects of 1,25-VD<sub>3</sub>, LCA, and rifampicin on *ABCB1* mRNA levels in LS174T cells.

LS174T and HepG2 cells were exposed to 250 nM 1,25-VD<sub>3</sub>, 10 μM LCA, 10 μM rifampicin, or vehicle (0.1% DMSO) for 48 h. Then total RNA was extracted from the cells and *ABCB1* mRNA levels were quantified by quantitative reverse transcription PCR as described under Materials and Methods. The *ABCB1* mRNA levels were normalized with those of GAPDH. Data are expressed as fold increase over vehicle-treated cells.

### Figure 2. Identification of VDR-responsive region in human *ABCB1*.

Schematic structures of reporter gene constructs are shown on the left. The closed boxes represent the regulatory cluster region previously reported (Geick et al., 2001), which is termed NURREM in this study. LS174T cells ( $2 \times 10^5$  cell/well in 12-well plate) were transfected with each reporter plasmid (1 μg) with pSV-β-galactosidase (1 μg). Six hours after transfection, the cells were treated with vehicle (0.1% DMSO), 100 nM 1,25-VD<sub>3</sub>, or 10 μM LCA in **A**, or vehicle (0.1% DMSO), 100 nM 1,25-VD<sub>3</sub>, or 10 μM rifampicin in **B** for 48 h, and SEAP and β-galactosidase activities were determined as described under Materials and Methods. Values of SEAP activities were normalized with β-galactosidase activities. Data are expressed as fold activation over vehicle-treated cells for each construct. Data are the mean ± SD (n = 4).

**Figure 3. Functional evaluation of NURREM as a VDR responsive region.**

**A.** LS174T cells ( $2 \times 10^5$  cell/well in 12-well plate) were transfected with each reporter plasmid (2  $\mu$ g) with pSV- $\beta$ -galactosidase (1  $\mu$ g). Twelve hours after transfection, the cells were treated with vehicle (0.1% DMSO), 10  $\mu$ M LCA, or 100 nM 1,25-VD3 for 48 h. **B.** LS174T cells ( $1 \times 10^5$  cell/well in 24-well plate) were transfected with each reporter plasmid (0.5  $\mu$ g) with pSV- $\beta$ -galactosidase (1  $\mu$ g). Twelve hours after transfection, the cells were treated with vehicle (0.1% DMSO), 100 nM 1,25-VD3, or 10  $\mu$ M rifampicin for 48 h. Reporter activities were determined as described under Materials and Methods. Values of SEAP activities were normalized with  $\beta$ -galactosidase activities. Data are expressed as fold activation over vehicle-treated cells for each construct. Data are the mean  $\pm$  SD (n = 4).

**Figure 4. In vitro binding of VDR and RXR $\alpha$  heterodimer to NURREM.**

**A.** The sequences and positions of probes used are shown as double-headed arrows. **B and C.** Gel shift assays were performed as described under Materials and Methods with radiolabeled probes shown in **A** and *in vitro* synthesized human VDR and/or human RXR $\alpha$ . The complex of VDR/RXR $\alpha$ /probe (shifted complex), and the free probe are marked. An asterisk indicates nonspecific bindings. Polyclonal antibody against VDR was used for supershift assays in **C**.

**Figure 5. Reporter assays with truncated NURREM-reporter gene constructs.**

Schematic structures of reporter gene constructs are shown on the left. LS174T cells ( $1 \times 10^5$  cell/well



in 24-well plate) were transfected with each reporter plasmid (0.5  $\mu$ g) and pSV- $\beta$ -galactosidase (1  $\mu$ g). Twelve hours after transfection, the cells were treated with vehicle (0.1% DMSO) or 100 nM 1,25-VD3 for 48 h, and then reporter activities were determined. Values of SEAP activities were normalized with  $\beta$ -galactosidase activities. Data are expressed as fold activation over vehicle-treated cells for each construct. Data are the mean  $\pm$  SD (n = 4).

**Figure 6. Influence of siRNA targeting VDR on the transcription of *ABCB1* reporter gene.**

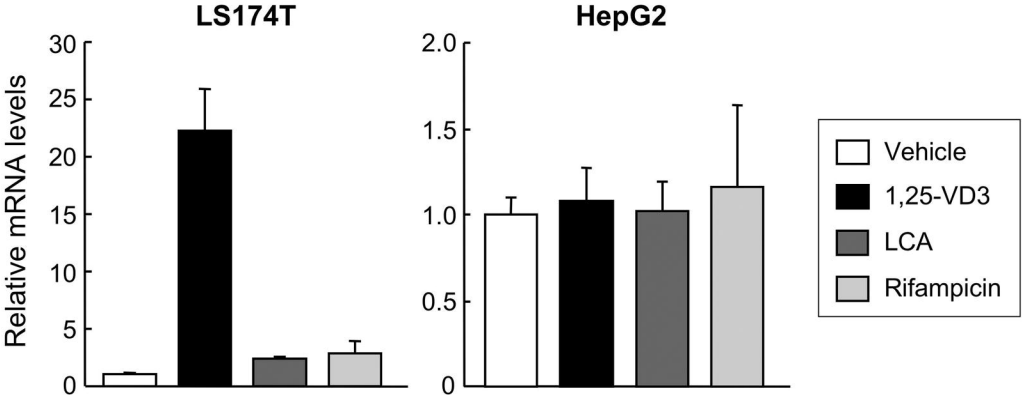
LS174T cells were transfected with 15 pmol of VDR-targeting siRNA or control siRNA when being seeded in 24-well plate ( $1 \times 10^5$  cells/well). Two days after seeding, the cells were transfected with p-8935 (0.5  $\mu$ g) and pSV- $\beta$ -galactosidase (1  $\mu$ g). Twelve hours later, the cells were treated with vehicle (0.1% DMSO), 100 nM 1,25-VD3, 10  $\mu$ M LCA, or 10  $\mu$ M rifampicin for 24 h, and then SEAP and  $\beta$ -galactosidase activities were determined as described under Materials and Methods. Values of SEAP activities were normalized with  $\beta$ -galactosidase activities. Data are expressed as relative activities to those in the vehicle-treated cells transfected with control siRNA. Data are the mean  $\pm$  SD (n = 4).

**Figure 7. Influences of ectopic expression human VDR in HepG2 cells on the activation of *ABCB1* reporter genes.**

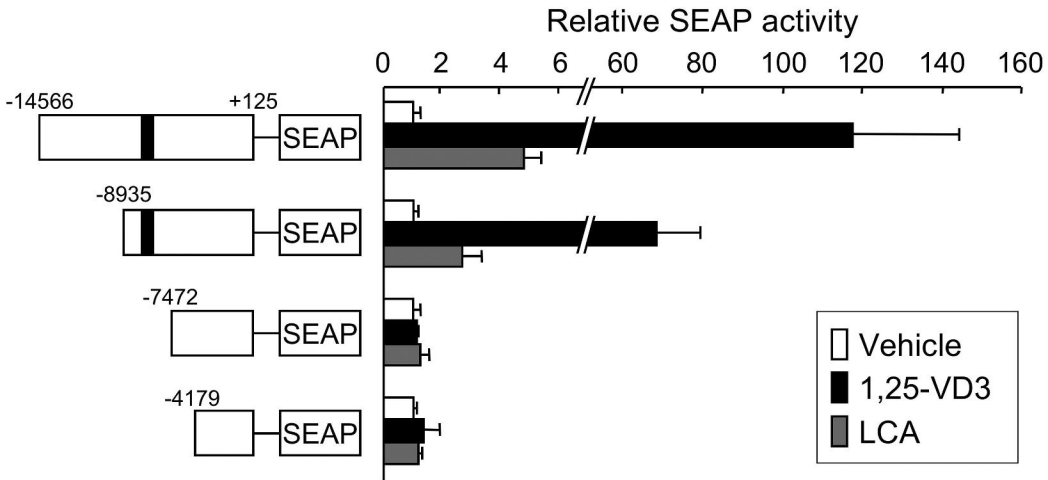
Schematic structures of reporter gene constructs are shown on the left. The closed box in the construct represents NURREM. LS174T cells ( $1 \times 10^5$  cell/well in 24-well plate) were transfected with each

reporter plasmid (0.5  $\mu$ g) and pSV- $\beta$ -galactosidase (1  $\mu$ g) in the absence or presence of hVDR expression plasmid pT-hVDR (+; 0.25  $\mu$ g, ++; 0.5  $\mu$ g). Empty pTarget vector was used as the control for pT-hVDR. Twelve hours after transfection, the cells were treated with vehicle (0.1% DMSO) or 100 nM 1,25-VD<sub>3</sub> for 48 h, and then reporter activities were determined as described under Materials and Methods. Values of SEAP activities were normalized with  $\beta$ -galactosidase activities. Data are expressed as relative activities to those in the vehicle-treated and pSEAP2-basic/pTarget-transfected (**A**) and vehicle-treated and pSEAP2-TK/pTarget-transfected (**B**) cells (the top bar in each figure). Numbers next to the closed bars represent the fold-activation of reporter activities over the corresponding vehicle-treated cells. Data are the mean  $\pm$  SD (n = 4).

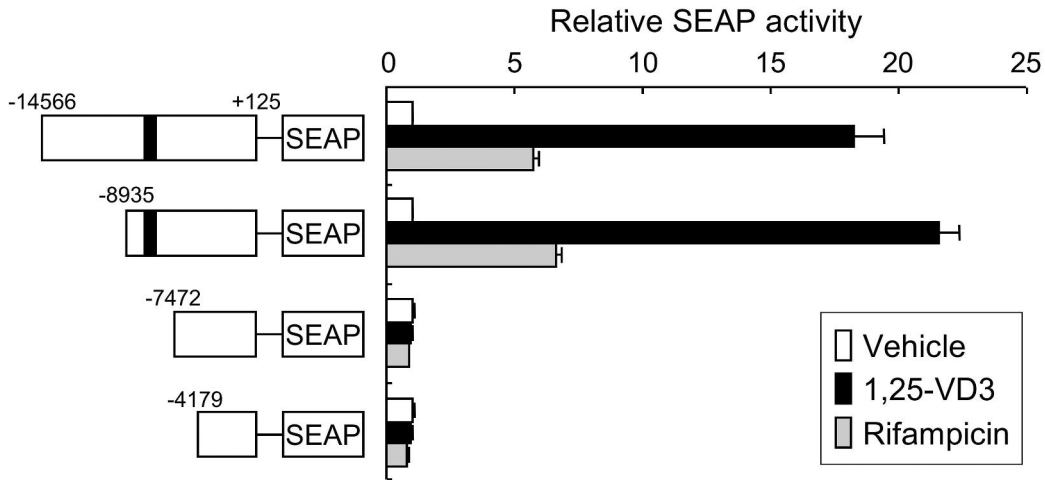
**Fig. 1**

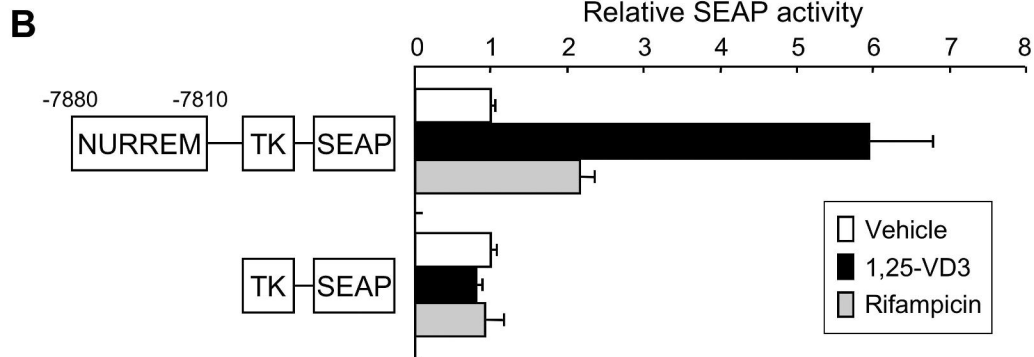
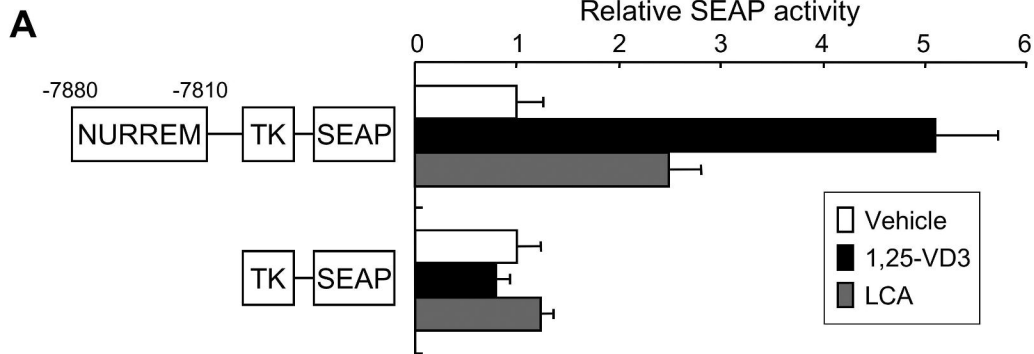


**Fig. 2A**

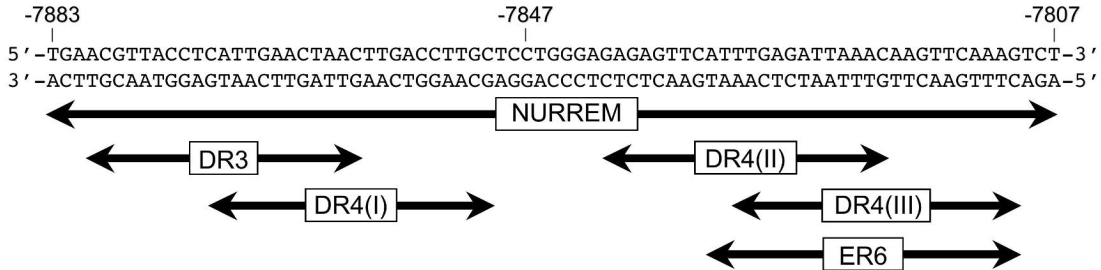


**Fig. 2B**



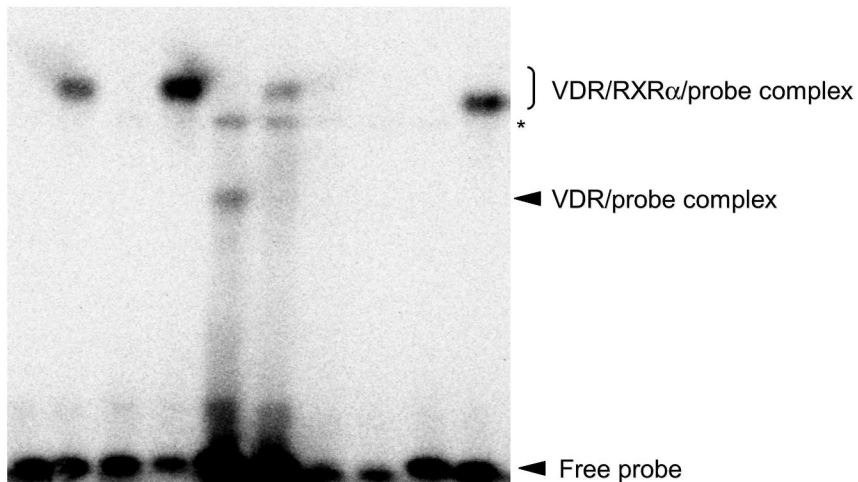
**Fig. 3**

**Fig. 4A**



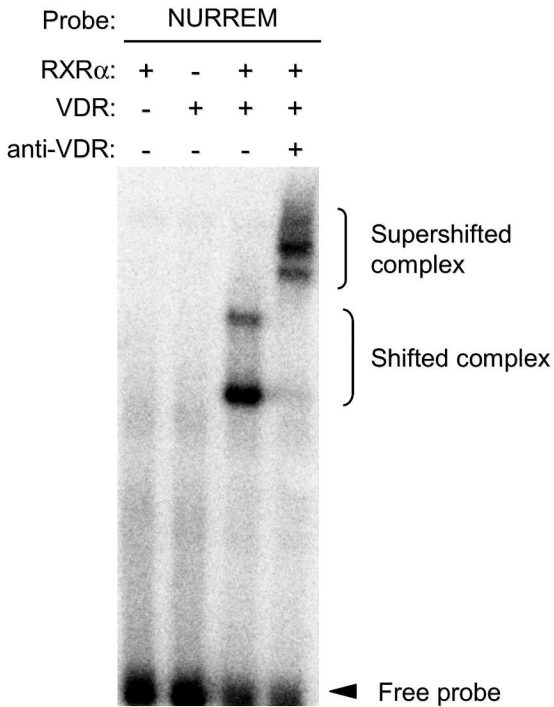
**Fig. 4B**

Probe:	DR3		DR4(I)		DR4(II)		DR4(III)		ER6	
VDR:	+	+	+	+	+	+	+	+	+	+
RXR $\alpha$ :	-	+	-	+	-	+	-	+	-	+

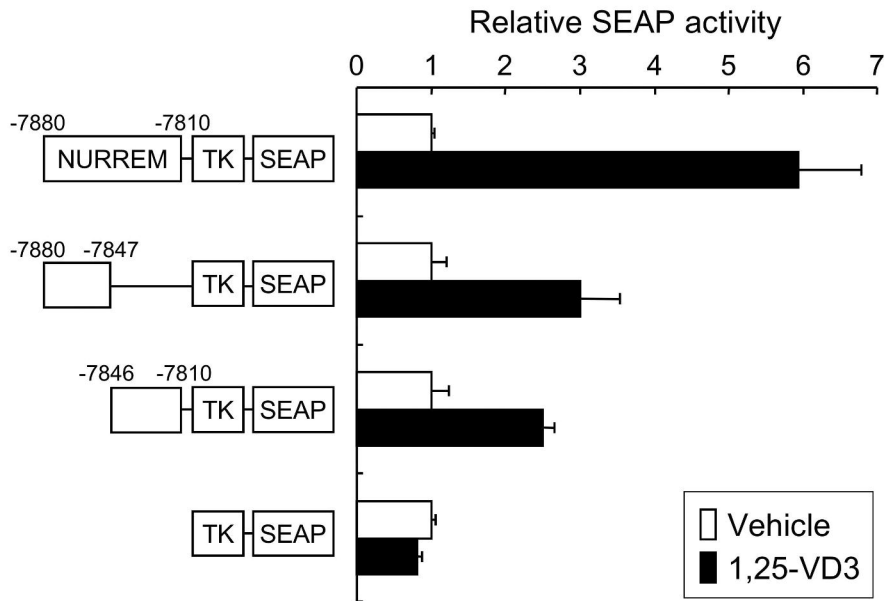




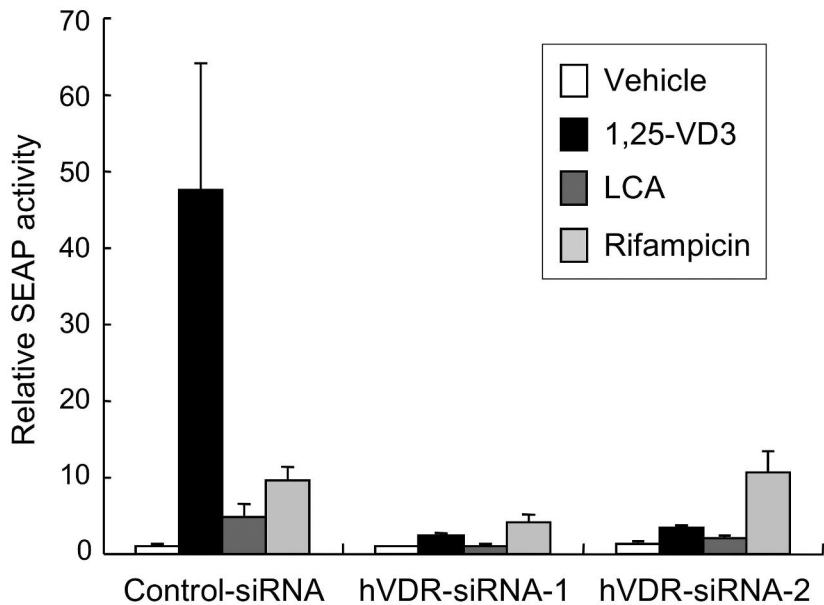
**Fig. 4C**



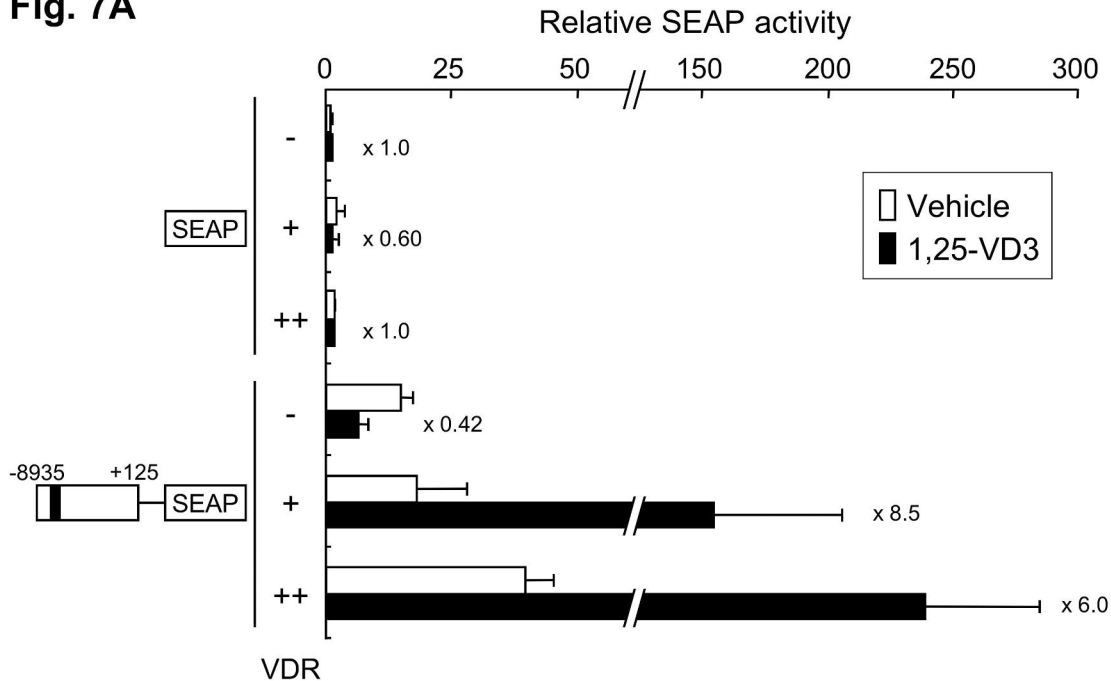
**Fig. 5**



**Fig. 6**



**Fig. 7A**



**Fig. 7B**

