

## Response of the ABCG2 promoter in T47D cells and BeWo cells to sex hormone treatment

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**Abstract** The aim of this study was to elucidate the effects of sex hormones on activity of the ABCG2 promoter in different cell lines. T47D cells and BeWo cells were used as models for ABCG2-expressing cell lines, and luciferase assays using ABCG2 promoter–luciferase constructs were performed. It was shown that progesterone increased the response of the ABCG2 promoter in T47D cells but not in BeWo cells. On the other hand, estradiol had no effect on response of the ABCG2 promoter in either cell line. However, response of the ABCG2 promoter was enhanced by overexpression of ER $\alpha$  in both T47D cells and BeWo cells. T47D cells had higher sensitivity to ER $\alpha$  than did BeWo cells. Furthermore, it was shown that the inductive effect of progesterone on the ABCG2 promoter was inhibited by addition of RU486 or mithramycin A. Therefore, it was thought that the ABCG2 promoter responded to stimulation of the progesterone receptor (PR)-Sp1 pathway in T47D cells. Furthermore, progesterone suppressed the response of the ABCG2 promoter by changing the expression levels of PR-A and PR-B in BeWo cells. These findings suggested that there are differences between cell lines in the regulation mechanism of ABCG2 expression by sex hormone treatment.

**Keywords** ABCG2 · Promoter · Progesterone · Estradiol · T47D · BeWo

### Introduction

ABCG2 is one of the transporters in the ATP-binding cassette superfamily [1]. It is known that ABCG2 is expressed in various tissues and contributes to the efflux of various endogenous substances, such as folic acid and protoporphyrin [2, 3]. Moreover, since the blood–brain barrier and the blood–placental barrier express ABCG2, it is thought that ABCG2 plays an important role in the protective mechanism against exogenous substances [4, 5]. On the other hand, it has been shown that anticancer drugs, such as methotrexate and SN-38, are transported by ABCG2. Therefore, it is thought that ABCG2 is also involved in acquisition of multi-drug resistance in carcinoma cells [6].

There have been some reports on regulation systems of ABCG2 expression. The structure and characteristics of the ABCG2 promoter have been described, and the presence of an estrogen response element (ERE) in the ABCG2 promoter has been reported [7]. However, it has been reported that estrogen also down-regulates ABCG2 expression by a post-transcriptional mechanism in MCF-7 cells [8]. Furthermore, we have reported that estrogen increases the expression level of ABCG2 in the placental cell line BeWo but that the expression level of Abcg2 in the rat placenta decreases with advance of gestation, although secretion of estrogen from the placenta increases with advance of gestation [9, 10]. Nakanishi et al. [11] reported that ABCG2 mRNA has variants of 5'-UTR, and this might be the reason for the difference in ABCG2 expression in various cell lines and tissues and for the different responses to sex hormones and other transcription factors. However, the differences in responses of the ABCG2 promoter to transcription factors in various cell lines and tissues have not been investigated, and further investigation is therefore needed to clarify these differences.

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T47D cells and BeWo cells are known to be estrogen receptor-positive cell lines and to express ABCG2 [7, 10, 12]. Moreover, it has been shown using these cell lines that sex hormones are involved in the regulation system of ABCG2 [10, 13]. However, it has not been determined whether sex hormones affect ABCG2 expression by response of the ABCG2 promoter in these cell lines to sex hormones. In this study, we investigated the effects of sex hormones on the ABCG2 promoter in T47D cells and BeWo cells using an ABCG2 promoter–luciferase construct.

## Materials and methods

### Chemicals

Estradiol and progesterone were purchased from Wako (Osaka, Japan). All other reagents were of the highest grade available and used without further purification.

### Cell culture

T47D cells and BeWo cells were obtained from American Type Culture Collection (Manassas, VA) and Riken Cell Bank (Saitama, Japan), respectively. T47D cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH) and 1% penicillin–streptomycin. BeWo cells were cultivated in the nutrient mixture F-12 Ham Kaighn's modification (Sigma) supplemented with 15% fetal bovine serum and 1% penicillin–streptomycin. Both T47D cells and BeWo cells were cultivated at 37°C under 95% air–5% CO<sub>2</sub>. Cells were grown for 4–5 days and after reaching confluency were washed with PBS and harvested by exposure to a trypsin–EDTA solution and then passed into new flasks.

### Plasmid construct

The human ABCG2 promoter (–1221/+298; Accession No. NM\_004827) was amplified from a BAC clone (RP11-368G2, Invitrogen) by PCR using primers with the sense sequence of 5'-TGT CTC GAG AGT AGA GGC AGG GTT TCA CCA TGT TG-3' and antisense sequence of 5'-GCA AAG CTT GTT CCT AAA TCC TAC CCA GTT CCT C-3' and then subcloned into a pGL3-basic vector (Promega, Madison, WI). Deletion fragments (–636/+298, –311/+298, and –16/+298) were generated from –1221/+298 by PCR steps and subcloned into a pGL3-basic vector. The vectors of ERE\_mut1 and ERE\_mut2 were generated from –1221/+298 by megaprimer PCR methods using sense and antisense primers for the ABCG2 promoter and primers with a sense sequence of 5'-GTG TCA CGG

CAG GGT TAC CCT AGC CCC GAG-3' for ERE\_mut1 and a sense sequence of 5'-CTG CGT GTC ACT TCA GGG TTA CCC TAG CC-3' for ERE\_mut2. The open reading frame (ORF) of ER $\alpha$  was cloned by PCR using cDNA of T47D cells with primers having a sense sequence of 5'-CAA TGA CCA TGA CCA TGA CCC TCC AC-3' and antisense sequence of 5'-CAG TGA TTA TCT GAA CCG TGT GGG AG-3'. The amplified ORF of ER $\alpha$  was subcloned into a pcDNA3.1-zeo(+) vector (Invitrogen). Expression of ER $\alpha$  using the pcDNA3.1-ER $\alpha$  construct was confirmed by immunocytochemistry using a specific antibody of ER $\alpha$  (Santa Cruz, CA; data not shown). All of the constructs were verified by sequencing.

### Reporter assays

T47D cells were grown at a density of  $1.0 \times 10^5$  cells/well in 12-well plates and cotransfected with 0.6  $\mu$ g of the luciferase constructs and 0.1  $\mu$ g pRL-SV40 vector (Promega) with/without pcDNA3.1-ER $\alpha$  by Lipofectin (Invitrogen) according to the manufacturer's protocol. BeWo cells were grown at a density of  $2.0 \times 10^5$  cells/well in 12-well plates and cotransfected with 0.6  $\mu$ g of the luciferase construct and 0.1  $\mu$ g pRL-SV40 vector with/without pcDNA3.1-ER $\alpha$  by Lipofectamine (Invitrogen) according to the manufacturer's protocol. After transfection, T47D cells and BeWo cells were cultured in phenol red-free DMEM (Sigma) supplemented with serum replacement 2 (Sigma) and in phenol red-free DMEM/F-12 (GIBCO) supplemented with charcoal/dextran-stripped serum (GIBCO), respectively. After 24 h, fresh medium containing each concentration of sex hormones dissolved in methanol or methanol alone (0.1%) was added to the transfected cells. The cells were harvested 24 or 72 h later and analyzed for both firefly luciferase and renilla luciferase activities using assay kits from Promega. Relative firefly luciferase activities were normalized with renilla luciferase activities.

### Western blot analysis

Total protein extracts were prepared from BeWo cells. Cells were suspended in lysis buffer containing 1.0% Triton X-100, 0.1% SDS, and 4.5 M urea. The suspension was left to stand for 5 min and sonicated for 15 min at 4°C. Then it was centrifuged at 12,000 rpm for 15 min at 4°C, and the protein concentration in the clear supernatant was determined by the method of Lowry. The samples were denatured at 85°C for 3 min in loading buffer containing 50 mM Tris–HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% BPB, and 3.6 M urea and then separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically onto nitrocellulose membranes (Trans-Blot; Bio-Rad) at 15 V for

90 min. The membranes were blocked with PBS containing 0.05% Tween 20 (PBS/T) and 10% non-fat dry milk for 1 h at room temperature. After being washed with PBS/T, the membranes were incubated with monoclonal anti-progesterone receptor (AB-52, Santa Cruz, CA) (dilution of 1:100) for 1 h at room temperature and then washed with PBS/T ( $3 \times 10$  min). The membranes were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz) at a dilution of 1:2000 and washed with PBS/T ( $3 \times 10$  min). The bands were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Biosciences Corp., Piscataway, NJ).

## Results

### Effects of sex hormones on response of ABCG2 promoter

To investigate the responses of the ABCG2 promoter in T47D cells and BeWo cells, reporter assays using an ABCG2 promoter–luciferase plasmid construct were

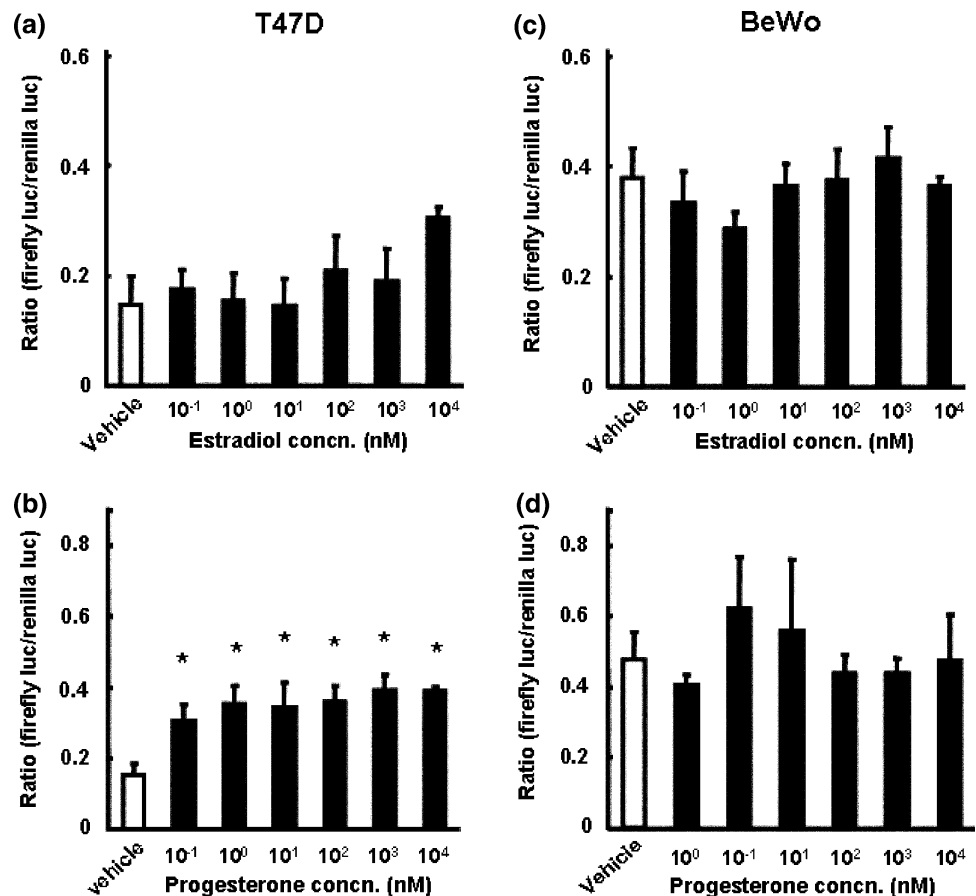
performed. BeWo cells transfected with  $-1221/+221$  showed an approximately twofold higher ratio of luciferase activity than that of T47D cells (Fig. 1a, c).

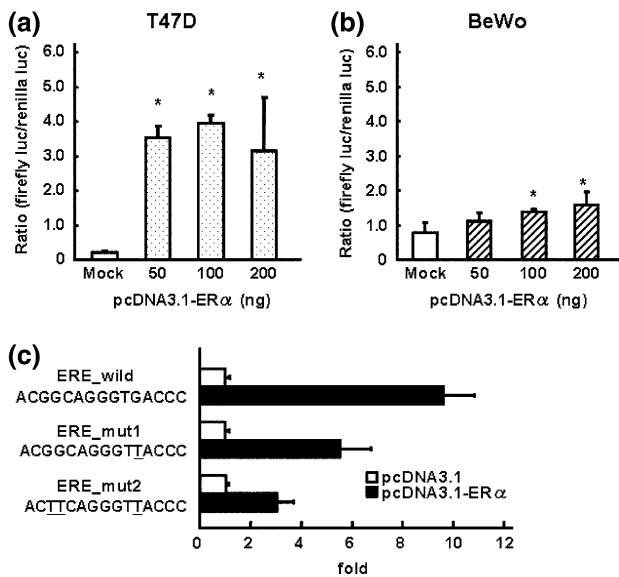
Since it has been reported that expression of ABCG2 is affected by sex hormones [7–10], we investigated the effects of estradiol (Fig. 1a, c) and progesterone (Fig. 1b, d) at concentrations of  $10^{-1}$ – $10^4$  nM on the ratio of luciferase activity. Although it has been reported that an estrogen receptor response element is present in the ABCG2 promoter [7], an effect of estradiol was not observed. On the other hand, progesterone showed different effects on T47D and BeWo cells. Although the ratio of luciferase activity was not changed in progesterone-treated BeWo cells, the ratio in T47D cells was increased significantly. We also performed the same experiment in PA-1 cells, ovarian cancer cells that also express ABCG2 [7], but the ratio of luciferase activity was not changed by sex hormone treatment (data not shown).

### Effect of overexpression of estrogen receptor $\alpha$ (ER $\alpha$ ) on response of ABCG2 promoter

Although the function of the ERE in the ABCG2 promoter has been reported [7], estradiol treatment had no effect on

**Fig. 1** Effect of sex hormones on response of the ABCG2 promoter. Cells were transfected with an ABCG2 promoter–luciferase construct and pRL-SV40 vector as described in section “Materials and methods”. Before performing reporter assays, T47D cells (a, b) and BeWo cells (c, d) were cultured in a medium containing various concentrations of estradiol (a, c) or progesterone (b, d) for 24 h. Relative firefly luciferase activities normalized with renilla luciferase activities of three independent experiments are presented as the mean with SD. \*  $P < 0.05$  compared to vehicle, using Student’s unpaired *t*-test





**Fig. 2** Effect of overexpression of ER $\alpha$  on response of the ABCG2 promoter. Cells were transfected with an ABCG2 promoter–luciferase construct and pRL-SV40 vector with pcDNA3.1 vector or pcDNA3.1-ER $\alpha$  construct as described in section “Materials and methods”. Relative firefly luciferase activities normalized with renilla luciferase activities of *three independent experiments* are presented as the mean with SD (a, b). T47D cells were transfected with ERE\_mut1 or ERE\_mut2 and pRL-SV40 vector with pcDNA3.1 vector or pcDNA3.1-ER $\alpha$  construct. Fold induction over pcDNA3.1 vector-transfected cells is presented as the mean with SD (c). \*  $P < 0.05$  compared to mock, using Student’s unpaired *t*-test

promoter activity in either T47D cells or BeWo cells (Fig. 1a, c). Therefore, we investigated the effect of additional expression of ER $\alpha$  on response of the ABCG2 promoter in both cell lines. Ratios of luciferase activity were increased by expression of ER $\alpha$ , by more than 20-fold in T47D cells and 1.5–2-fold in BeWo cells (Fig. 2a, b). Furthermore, mutation in the ERE decreased the response of the ABCG2 promoter in T47D cells to ER $\alpha$  (Fig. 2c).

Effects of RU486 and mithramycin A on response of ABCG2 promoter in T47D cells to progesterone

Since T47D cells are known to respond to progesterone [14], it is thought that the response of the ABCG2 promoter to progesterone treatment would be enhanced by the progesterone receptor (PR) pathway. Therefore, we investigated the mechanism of activation of the ABCG2 promoter by progesterone in T47D cells. Figure 3a shows that RU486, an inhibitor of the PR, inhibited the response of the ABCG2 promoter to progesterone at a concentration of 100 nM, suggesting that progesterone activates ABCG2 promoter by binding to the PR. On the other hand, basal ABCG2 promoter activity was increased upon treatment with the RU486. It has been known that RU486 works as not only antagonist but partial agonist of steroid hormone

receptor [15]. That is the reason of inductive effect of RU486 on the response of ABCG2 promoter. Although the response of the ABCG2 promoter was changed by using upstream-deleted ABCG2 promoters (–636/+298, –311/+298 and –16/+298), all of their luciferase activities were increased by progesterone treatment. Furthermore, RU486 inhibited all of the responses to progesterone (Fig. 3b).

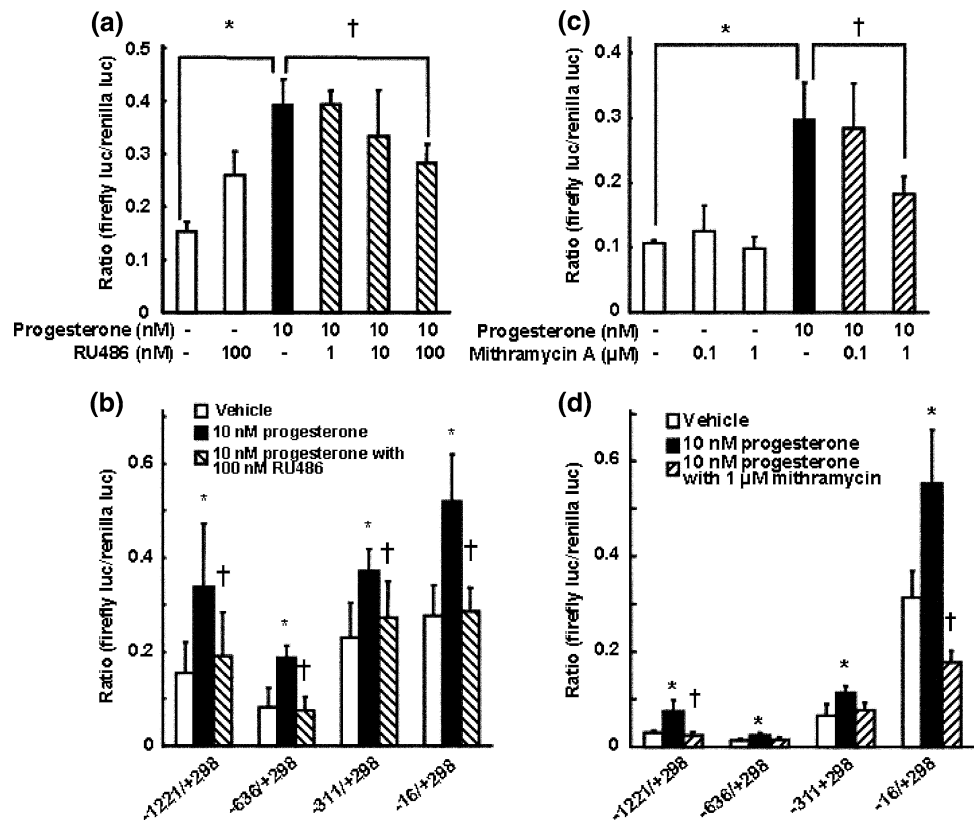
It has been reported that PR regulates promoter activity by interacting with another transcription factor, such as specific transcription factor 1 (Sp1) and activator protein-1 (AP1) [16, 17]. Hence, the response of the ABCG2 promoter to progesterone might involve these transcription factors in T47D cells. We therefore investigated effect of mithramycin A, an inhibitor of Sp1 [18, 19], on the response to progesterone treatment. An inhibitory effect of mithramycin A on the response of the ABCG2 promoter to progesterone was observed (Fig. 3c), suggesting that the PR-Sp1 pathway is involved in the regulation of activation of the ABCG2 promoter in T47D cells. Furthermore, an inhibitory effect of mithramycin A was observed in an assay using deleted ABCG2 promoter–luciferase constructs (Fig. 3d). Although we also investigated the effect of curcumin as an AP1 inhibitor [20, 21], no notable effect was observed (data not shown).

Effect of 72 h of treatment of sex hormone on response of ABCG2 promoter in BeWo cells

In a previous study, we found that expression of ABCG2 in BeWo cells was induced by estradiol treatment and suppressed by progesterone treatment [10]. However, the ABCG2 promoter did not respond to either hormone treatment for 24 h in this study (Fig. 1). It has been reported that various functions of BeWo cells, such as hormone secretion, are affected by estradiol treatment for more than 24 h [22]. Furthermore, since our previous study showed that a long time was needed for the expression level of ABCG2 be affected by sex hormone treatment, we investigated the response of the ABCG2 promoter to 72 h of treatment with estradiol or progesterone. Estradiol had no effect on the response of the ABCG2 promoter, but treatment with progesterone at a concentration of 10  $\mu$ M significantly suppressed the response of the ABCG2 promoter (Fig. 4a, b). Moreover, the progesterone effect on the ABCG2 promoter was canceled by using a deleted ABCG2 promoter–luciferase construct of –16/+298 in BeWo cells (Fig. 4c).

Although it has been reported that the effect of progesterone on transcription is mediated by the PR pathway [14], various effects by using different subtypes of PR have been shown [23]. Therefore, we also investigated the expression of PR-A and PR-B in progesterone-treated BeWo cells. Figure 5 shows that progesterone treatment

**Fig. 3** Effects of RU486 and mithramycin A on response of the ABCG2 promoter to progesterone. Cells were transfected with an ABCG2 promoter–luciferase construct and pRL-SV40 vector as described in section “Materials and methods”. Before performing reporter assays, T47D cells were cultured in a medium containing 10 nM of progesterone with or without RU486 (a, b) or mithramycin A (c, d) for 24 h. Relative firefly luciferase activities normalized with renilla luciferase activities of *three independent experiments* are presented as the mean with SD. \*  $P < 0.05$  compared to vehicle, †  $P < 0.05$  compared to 10 nM progesterone, using Student’s unpaired *t*-test



for 24 h increased the expression levels of both PR-A (about 90 kDa) and PR-B (about 120 kDa).

## Discussion

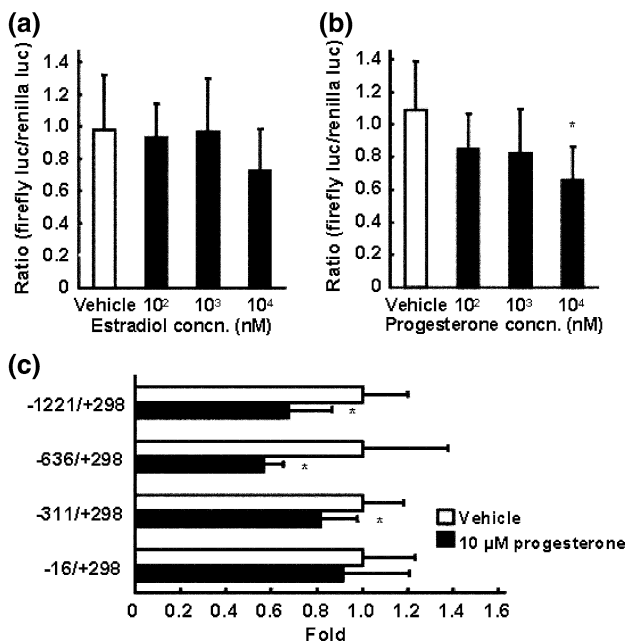
Since ABCG2 was first identified from breast cancer cells [24] and since the placenta is an organ with a high expression level of ABCG2 [5], we investigated ABCG2 promoter activity using T47D cells, a breast cancer cell line, and BeWo cells, choriocarcinoma cell line, as typical models for organs that express ABCG2.

First, it was shown in this study that the response of the ABCG2 promoter with no exogenous stimulation in BeWo cells was higher than that in T47D cells. Bailey-Dell et al. [25] also reported that promoter activity in BeWo cells is higher than that in MCF-7 cells, suggesting that BeWo cells have a greater ability to naturally express ABCG2 than do breast cancer cell lines, including T47D cells. However, the ABCG2 promoter did not respond to 24 h of estradiol treatment in either cell line and responded to progesterone only in T47D cells. Since it has been reported that the ABCG2 promoter responds to estrogen via the ERE–ER pathway [7], we had not expected these results.

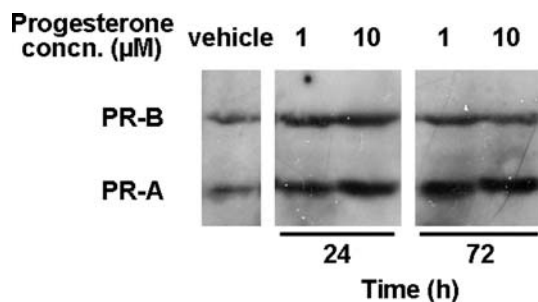
Hence, we evaluated the effect of overexpression of ER $\alpha$  on the response of the ABCG2 promoter in T47D cells

and BeWo cells. The response induced by the expression of ER $\alpha$  was stronger in T47D cells than in BeWo cells. Moreover, the effect of overexpression of ER $\alpha$  was lost when a mutated ERE was used. These results suggested that ER $\alpha$  binds to the ERE to enhance the response of the ABCG2 promoter as already reported, but the results also suggested that the ABCG2 promoter needs ER $\alpha$  expression above a certain level to increase the activity in T47D cells and BeWo cells. However, since the sensitivities to expression of ER $\alpha$  in T47D cells and BeWo cells were different, ER $\alpha$  is not the only factor involved in the response of the ABCG2 promoter and other cofactors might play an important role in the expression of ABCG2 in these cell lines [26]. Further investigations are needed to clarify their involvement.

Since a response by the ABCG2 promoter to progesterone treatment was observed in T47D cells but not in BeWo cells, it was thought that T47D cells might have a different pathway for regulation of ABCG2 from that in BeWo cells. It has also been reported that promoters containing AP1 and Sp1 sites are regulated by steroid receptor in a cell-specific manner [27]. Our results showed that progesterone increases the response of the ABCG2 promoter by the PR–Sp1 pathway in T47D cells. It has been reported that the ABCG2 promoter is TATA-less and contains a CAAT box and several putative Sp1 sites



**Fig. 4** Effects of treatment of estradiol and progesterone for 72 h on response of the ABCG2 promoter in BeWo cells. Cells were transfected with an ABCG2 promoter–luciferase construct and pRL-SV40 vector as described in section “Materials and methods”. Before performing reporter assays, cells were cultured in a medium containing various concentrations of estradiol (a) and progesterone (b) for 72 h. Cells were transfected with a deleted-ABCG2 promoter–luciferase construct and pRL-SV40 vector (c). Before performing reporter assays, BeWo cells were cultured in a medium containing 10 μM of progesterone for 72 h. Relative firefly luciferase activities normalized with renilla luciferase activities of *three independent experiments* are presented as the mean with SD. \*  $P < 0.05$  compared to vehicle, using Student’s unpaired *t*-test



**Fig. 5** Effect of treatment with progesterone on the expression levels of PR-A and PR-B in BeWo cells. BeWo cells were cultured in a medium containing one or 10 μM of progesterone for 72 h. Methanol vehicle was used as a control. The expression levels of PR-A and PR-B were determined by Western blot analysis. Fifty microgram of cell lysate was applied in each lane. Data shown are typical results from three independent experiments

downstream from a putative CpG island [25]. We also scanned the ABCG2 promoter (−1221/+298) for elements of a specific transcription factor using MatInspector (<http://www.genomatix.de/>). These results of screening suggested that the ABCG2 promoter has highly matched

four elements of Sp1-binding site (−217/−203, −107/−93, −67/−53 and +84/+99). Since Nakanishi et al. [11] reported variants of 5′UTR of ABCG2 mRNA and suggested that ABCG2 has a number of transcription start sites, these Sp1-binding site might activate the ABCG2 promoter in T47D cells. Moreover, treatment with progesterone also increased the response of the ABCG2 promoter and RU486 inhibited its effects in ERα-overexpressed T47D cells, suggesting that progesterone affects the ABCG2 promoter by a pathway different from the ERα-ERE pathway (data not shown).

Although it has been revealed that the expression level of ABCG2 in BeWo cells was regulated by sex hormone treatment [10], treatment with estradiol and progesterone for 24 h had no effect on response of the ABCG2 promoter in BeWo cells in this study. Hence, it was hypothesized that treatment for longer time is needed to evaluate the effects of sex hormones on the response of the ABCG2 promoter, including not only effects on the regions of the ABCG2 promoter but also effects on the function of BeWo cells. It was found that response of the ABCG2 promoter was not changed even when BeWo cells were treated with estradiol for 72 h. Since we previously reported that the expression level of ABCG2 in BeWo cells was increased by estradiol, this result suggested that region of the ABCG2 promoter used in this study did not have sufficient function for regulation of ABCG2. Since the effect of overexpression of ERα on response of the ABCG2 promoter are also weak in BeWo cells, it was suggested that other transcriptional factors or a region upstream of promoter used in this study are needed for regulation of ABCG2 transcription by estradiol in BeWo cells. On the other hand, treatment with progesterone for 72 h significantly suppressed the response of the ABCG2 promoter. This finding is in agreement with the results of our previous study showing that progesterone decreases the expression level of ABCG2 in BeWo cells [10]. Recently, Wang et al. [28] reported that the progesterone receptor response element (PRE) in the ABCG2 promoter is in the same region as that of the ERE and that PR-B increases the activity of the ABCG2 promoter, while PR-A suppresses it. Our results also showed that progesterone had no suppressive effect when an ABCG2 promoter in which the ERE region was deleted was used. The expression levels of both PR-A and PR-B were increased by progesterone treatment for 72 h. These results are consistent with a model in which the suppressive effect of progesterone on the response of the ABCG2 promoter is mediated by PR, the expression level of which was increased by progesterone treatment. We also previously reported that the expression level of ABCG2 in the rat placenta decreases during pregnancy even though secretion of sex hormones from the placenta increases with advance of gestation [9]. These findings suggested that

progesterone secreted from the placenta regulates the expression levels of PR-A and PR-B in the placenta, and that progesterone could thereby down-regulate the expression of ABCG2 during pregnancy.

In summary, BeWo cells have a greater ability to naturally express ABCG2 than do T47D cells. Estradiol had no effect on response of the ABCG2 promoter in either T47D cells or BeWo cells. Progesterone could enhance the response of the ABCG2 promoter through the PR-Sp1 pathway in T47D cells but not in BeWo cells. Furthermore, progesterone suppresses the response of the ABCG2 promoter by changing the expression levels of PR-A and PR-B in BeWo cells. These findings suggest that there are differences in the regulation mechanism of ABCG2 expression by sex hormones in T47D cells and BeWo cells. These fact suggests that the expression level of ABCG2 were regulated by sex hormone differently in various organs but further study is needed to clarify in detail.

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