doi:10.4149/endo_2011_03_125

The ability of hydroxylated estrogens (2-OH-E2 and 4-OH-E2) to increase of SHBG gene, protein expression and intracellular levels in MCF-7 cells line

Gregoraszczuk EL, Ptak A, Wrobel A

Department of Physiology and Toxicology of Reproduction, Chair of Animal Physiology, Institute of Zoology, Jagiellonian University, 30-060 Kraków, Ingardena 6, Poland
e-mail: ewa.gregoraszczuk@uj.edu.pl

Objective. Sex Hormone-Binding Globulin (SHBG) – specific carrier for sex steroids – regulates hormone bioavailable fraction and estrogen signaling system in breast cancer cells. This study was conducted to elucidate the effects of hydroxylated estrogen (E2) metabolites (2-OH-E2 and 4-OH-E2) on sex hormone binding globulin (SHBG) mRNA and protein expression as well as on intracellular and extracellular SHBG levels.

Methods. MCF-7 human breast cancer cells were cultured with 2-OH-E2 or 4-OH-E2 in concentration of 1, 10 and 100 nM for 24 h, 17β-estradiol being used as a positive control. SHBG levels were measured in medium and cells by ELISA, SHBG mRNA expression was evaluated by real-time-PCR and protein expression by Western blot analysis.

Results. 4-OH-E2 in high doses and 2-OH-E2 in the highest dose, while 17β-estradiol in all doses used increased intracellular but not extracellular SHBG levels. Both metabolites increased SHBG mRNA expression, the rank order of potency being E2 > 4-OH-E2 > 2-OH-E2. Both E2 and its metabolites increased SHBG protein expression.

Conclusion. Although the metabolites showed a lower potency than 17β-estradiol, further studies are needed to clarify whether hydroxylated metabolites of E2 are potent ligands for SHBG.

Keywords: 17β-estradiol, 2-OH-E2, 4-OH-E2, SHBG mRNA and protein expression, MCF-7

Evidence is accumulating that not only estradiol (E2), but also its metabolites may be involved in breast cancer development. Our previously published data showed that local activation of cytochrome P450 enzyme CYP1B1 by E2 may change the local metabolic activation pathway into 4-OH-E2 (Gregoraszczuk et al. 2011). In other study we also found that E2 and its metabolite 4-OH-E2 are potent mitogens in breast cancer cells and also demonstrated a stimulatory effect of 2-OH-E2 during long time exposure (Gregoraszczuk et al. 2008).

Sex Hormone-Binding Globulin (SHBG) – specific carrier for sex steroids – regulates hormone bioavailable fraction and estrogen signaling system in breast cancer cells. Using reverse transcriptase-polymerase chain reaction (RT-PCR) Moor et al. (1996) detected SHBG mRNA in ZR-75-1, MCF-7 and MDA-MB-231 cells, and also in 11 breast tissue samples. With the use of Western blot analysis Nakhla et al. (2009) showed that wild type SHBG in MCF-7 cell line was characterized by two bands (51-53 and 48-50 kDa). However, to our knowledge, no evidence for mRNA translation has been presented so far.

Corresponding author: Prof. Ewa L. Gregoraszczuk, PhD, Department of Physiology and Toxicology of Reproduction, Chair of Animal Physiology, Institute of Zoology, Jagiellonian University, 30-060 Kraków, Ingardena 6, Poland; phone: +48 12 6632615; fax: +48 12 6343716; e-mail: ewa.gregoraszczuk@uj.edu.pl

Moreover, the action of hydroxylated metabolites of E2 (2-OH-E2 and 4-OH-E2) on SHBG intra/extracellular secretion as well as on gene and protein expression in extra hepatic tissue has not yet been investigated.

Materials and Methods

Reagents. E2 (17-β-estradiol), 2-OH-E2 (2-hydroxy-17-β-estradiol), and 4-OH-E2 (4-hydroxy-17-β-estradiol) (Steraloids, Inc., Newport, RI) were dissolved in absolute ethanol. The final concentration of ethanol in the medium in each case was 0.1 %. DMEM medium without phenol red, Fetal Bovine Serum (FBS, heat inactivated), insulin-transferrin-sodium selenite media supplement (ITS), penicillin, streptomycin, trypan blue, charcoal-dextran and human sex hormone binding globulin (SHBG) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture. MCF-7 human breast cancer cells (ATCC, Manassas, VA, USA) were routinely cultured in DMEM supplemented with 10 % heat inactivated FBS, 100 IU/ml of penicillin and 100 μg of streptomycin. Forty-eight hours before experiments, the medium was removed and replaced by DMEM without phenol red supplemented with 5 % dextran-coated, charcoal treated FBS (5 % DC-FBS). The cells were plated in the same medium and allowed to attach overnight. The next day the medium was replaced with phenol red free DMEM supplemented with ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, using the stock preparation from Sigma) to remove SHBG from the medium. After 24 h the cells were treated with 1, 10 and 100 nM E2, 2-OH-E2 or 4-OH-E2 for next 24 hrs.

Measurement of SHBG intra/extracellular level. The cells were seeded in 12-well culture plates at a density of 5×10^5 cells/well and then incubated either in DMEM supplemented with ITS as a control medium or in the medium supplemented with 1, 10 and 100 nM E2 or its metabolites (2-OH-E2 and 4-OH-E2) for 24 h. After 24 h, the medium was collected and the cells were harvested mechanically in phosphate buffered saline PBS, and stored at –70 °C until homogenized and assayed. The SHBG concentrations were determined using the enzyme immunoassay SHBG ELISA (DRG Instruments GmbH, Marburg, Germany), according to the manufacturer's instructions. The sensitivity of assay was 0.2 nM/l, the intra-assay variation was 3.0–8.6 % and the inter-assay variation of 7.2–11.6 %, while the linear measuring range was 0–260 nM/l. Absorbance values were measured at 450 nm using ELISA reader ELx808 (BIO-TEK Instruments, Vinnooski, VT, USA).

Measurement of SHBG gene expression. The cells were seeded in 96-well culture plates at a density of 2×10^4 cells/well and then incubated in DMEM supplemented with ITS as a control medium or in medium supplemented with 100 nM E2, 2-OH-E2, or 4-OH-E2 for 24 h. The dose of E2 and metabolites were chosen as based on the results of Exp 1. RNA isolation and cDNA synthesis was performed using TaqMan Gene Expression Cells-to-CT kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The lysis solution contains DNAse I to remove genomic DNA during the cell lysis. Resulting pre-amplified cDNA preparations were analyzed by real-time PCR in a StepOnePlus Real-time PCR System (Applied Biosystems, Foster city, CA, USA) using the TaqMan Gene Expression Assay in combination with TaqMan Gene Expression Master Mix containing ROX (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's instructions. Following PCR conditions were used: incubation for 2 min at 50 °C, followed by incubation for 10 min at 95 °C, 40 cycles (denaturation step: 15 sec at 95 °C; annealing/elongation step: 60 sec at 60 °C). Duplicate samples without cDNA for each gene showed no DNA contamination. The relative expression of SHBG (Hs00168927_m1) was normalized to 18S rRNA (Hs99999901_s1) (ΔC_t) to compensate for differences in the amount of cDNA (assay identification number) and converted to a relative expression quantity using the 2(-Delta Delta C(T)) ($\Delta\Delta C_t$) method (Livak and Schmittgen 2001). TaqMan Gene Expression Assay (Hs00168927_m1) coding *homo sapiens* sex hormone-binding globulin (SHBG), transcript variant 1, mRNA (NCBI Reference Sequence: NM_001040) and transcript variant 2, mRNA (NM_001146279).

Measurement of SHBG protein expression. The cells were seeded in 60 mm dishes at a density of 2×10^6 cells/dish and then incubated in DMEM supplemented with ITS as a control medium or in medium supplemented with 100 nM E2, 2-OH-E2, or 4-OH-E2 for 24 hrs. For Western blot analysis, the cells were transferred into ice-cold lysis buffer after 24 h of exposure to test compounds and stored at –20 °C. The lysate protein concentrations were determined by the Bradford assay (Bio-Rad Protein). Equal amounts of protein (100 μg) from each treatment group were separated by 12 % SDS-PAGE and transferred to PVDF membranes using a Bio-Rad Mini-Protean 3 apparatus (Bio-Rad Laboratories, Inc., USA). The blots were blocked for 2

h with 5 % dry milk and 0.1% Tween 20 in 0.02 M TBS buffer. Blots were incubated overnight at 4 °C with polyclonal antibodies specific for human SHBG (sc-32890) (diluted1/1000) (Santa Cruz Biotechnology Inc, CA) and β-actin (A5316) (diluted 1/3000) (Sigma Chemical Co., MO, USA). After the incubation with primary antibody, the membranes were washed three times and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody, sc-2004 for SHBG (dilution 1/5000) (Santa Cruz Biotechnology Inc., CA, USA) and P0447 for β-actin (diluted 1/5000) (Dako-Cytomation, Denmark). Sixty micrograms of purified human SHBG protein (Sigma) were used as a positive control. Immunopositive bands were visualized using the Amplified Opti-4CN Kit (Bio-Rad Laboratories, Inc., USA) and were quantified using a densitometry analysis (EasyDens, Cortex Nowa, Poland).

Statistical evaluation. Each experiment was repeated three times (n=3), and each sample was run in quadruplicate. Data were plotted as mean ± S.E.M. Statistical analysis was performed using Stastistica 6.0. Data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant differences (HSD) multiple range test. Groups that are significantly different from each other are indicated in the figures as: *(p<0.05), ** (p<0.01) *** (p<0.001).

Results

Action of E2, 2-OH-E2 and 4-OH-E2 on extracellular and intracellular SHBG levels. In the control culture, intracellular SHBG level was 4.5 fold higher than that of extracellular SHBG (4.55±0.34 nM/l vs. 1.03±0.01 nM/l). In all doses used, 17β-estradiol (1, 10 and 100 nM) increased intracellular SHBG levels (6.09±0.08; 7.50±0.37 and 7.05±0.18 nM/l vs. 4.55±0.34 nM/l in untreated cells, respectively) (p<0.001). Stimulatory action on SHBG levels was noted under the influence of 10 and 100 nM of 4-OH-E2 (6.34±0.30 and 5.18±0.05 vs. 4.55±0.34 nM/l for control, respectively) (p<0.01 and p<0.05) and only the highest doses (100 nM) of 2-OH-E2 (6.39±0.48 vs. 4.55±0.34 nM/l for control, respectively) (p<0.01). However, no increased release of SHBG into the culture medium was observed under the influence of all reagents (Fig. 1).

Action of E2, 2-OH-E2 and 4-OH-E2 on SHBG gene expression. SHBG mRNA was detected in MCF-7 cancer cells. The expression of SHBG mRNA was 4-fold higher (p<0.001) in cells exposed to E2, 1.5-fold in cells exposed to 2-OH-E2 (p<0.05) and 2.8-fold (p<0.01) in cells exposed to 4-OH-E2. (Fig. 2)

Action of E2, 2-OH-E2 and 4-OH-E2 on SHBG protein expression. An immunorelated protein for

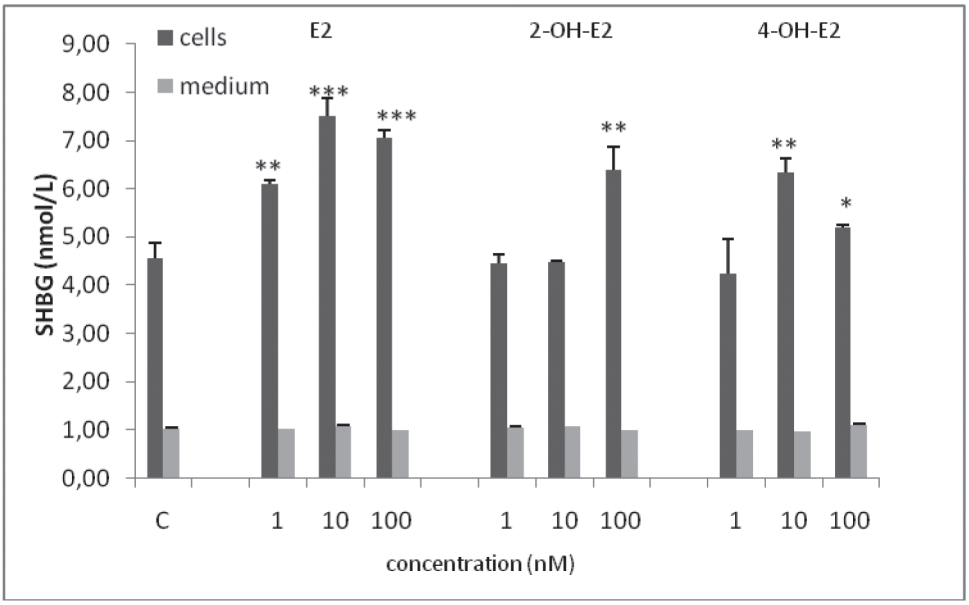

Fig 1 The effect of E2, 2-OH-E2 and 4-OH-E2 on intracellular and extracellular SHBG levels. Control cells obtained in 0.1% ethanol only. Each point represents the mean ± S.E.M. of results from three independent experiments, each of which consisted of four replicates per treatment group. All means marked with *(p<0.05), **(p<0.01), *(p<0.001), are significantly different from the control.**

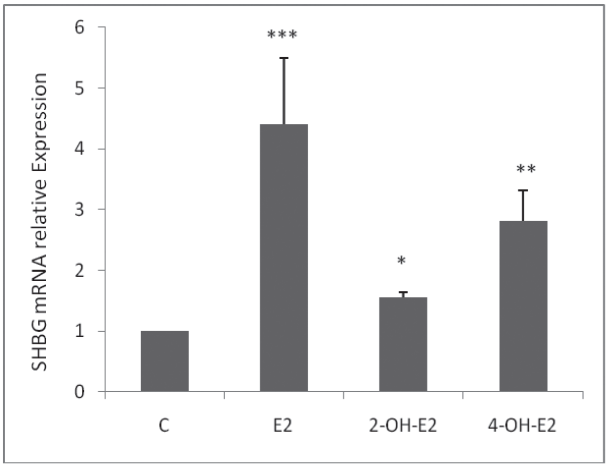

Fig 2 The effect of E2, 2-OH-E2 and 4-OH-E2 on SHBG mRNA expression. SHBG mRNA determined by real time PCR and expressed as relative values to basal conditions. All data were derived from a minimum of three independent experiments using different cell preparations. All means marked with *(p<0.05), **(p<0.01), *(p<0.001), are significantly different from the control.**

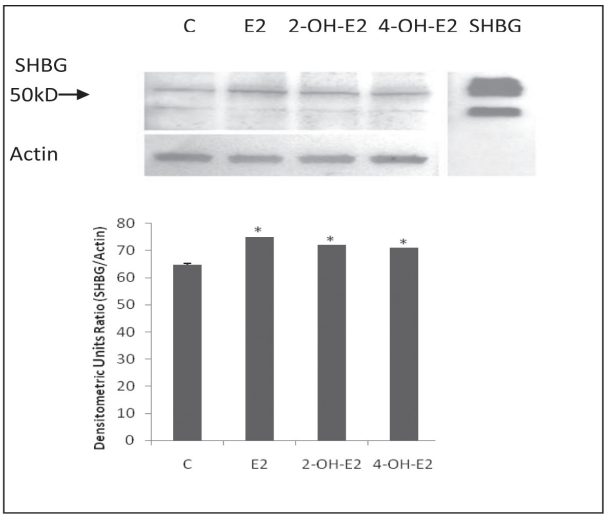

Fig 3 The effect of E2, 2-OH-E2 and 4-OH-E2 on intracellular SHBG protein expression. β-Actin used as loading control for western blot determinations. Blot is representative of three experiments. SHBG densitometry results were normalized to β-actin loading controls to obtain (SHBG/ β-actin ratio). 60 micrograms of purified human SHBG protein (Sigma) were used as a positive control. SHBG during electrophoresis migrate into two bands in MCF-7 cells. All means marked with *(p<0.05) are significantly different from the control.

SHBG was detected in untreated control cells. SHBG protein expression was slightly increased following 24 h of exposure to 17β-estradiol and both its metabolites (2-OH-E2 and 4-OH-E2) at a concentration of 100 nM ($p<0.05$). SHBG during electrophoresis migrate into two bands in MCF-7 cells (Fig. 3).

Discussion

Presented data showed that hydroxylated metabolites of E2 with potency of E2 > 4-OH-E2 > 2-OH-E2 increased intracellular and had no effect on extracellular SHBG levels. To our knowledge, this data is the first showing the action of estradiol and its hydroxylated metabolites (2-OH-E2 and 4-OH-E2) on SHBG levels in extrahepatic tissue.

Recently, SHBG mRNA has been demonstrated in a number of non-hepatic tissues and cell lines (Larrea at al. 1993; Misao et al. 1994; Moore et al. 1996). Several findings also showed increased SHBG production *in vitro* by E2, particularly in the intracellular compartment (Loukovaara et al. 1995; Kalme et al. 1999). Raineri et al. (2002) mentioned that SHBG is undetectable in culture medium from untransfected MCF-7 cells, thus confirming our observation. However, according to our knowledge there are no data in the literature showing the increase of SHBG production *in vitro* by E2 hydroxylated metabolites.

Using HepG2 cells, Loukovaara et al. (1995) reported the action of isoflavonoids daidzein and equol on a parallel increase if intracellular and extracellular SHBG levels, while genistein, in contrast had a significant effect on intracellular SHBG levels only, thus resembling the effect of 17β-estradiol. The authors suggested that such difference may originate from the fact that genistein has more hydroxyl groups than daidzein and equol. In the data presented, we showed a difference in the action depended on the substitution of a hydroxyl group in the molecule. Data presented by Nader et al. (2006) showed that in the HepG2 cell line lacking estrogen receptor no effect of 17β-estradiol (E2) or o,p'-DDD on SHBG concentrations appeared. In contrast, in the Hep89 cell line stably transfected by ER, both E2 and o, p'-DDD increased the concentrations of SHBG secreted into culture media. However, whereas the effect of E2 was dose dependent, high doses of o, p'-DDD were required. This data is in agreement with our observation of MCF-7 ER+ cells. We noted dose dependent action of E2 in all doses used, 4-OH-E2 in medium and large doses and 2-OH-E2 only in the highest dose.

Summarizing this part of the discussion, we concluded that not only E2 but also its hydroxylated metabolites increased intracellular SHBG accumulation. The effect

of the former became evident at a higher concentration range, and was of a smaller magnitude. It should be noted, however, that the stimulatory effect of hydroxylated metabolites on MCF-7 cell proliferation appears at similar concentrations and is of a similar magnitude (Gregoraszczuk et al. 2008).

Using real-time PCR and Western blot analysis, we showed SHBG mRNA and protein expression in the MCF-7 cell line. Additionally, we showed that not only E2, but also 2-OH-E2 and 4-OH-E2 increased SHBG mRNA and protein expression. These results are in agreement with data of Hryb et al. (2002), who showed prostate stromal cell cultures, epithelial cell lines, and prostatic tissue all express SHBG mRNA and protein. Similar findings have been reported in breast cancer by Kahn et al. (2008). Nakhla et al. (2009) using RT PCR based mapping assays, determined transcriptional start sites for three distinct human SHBG gene promoters. Moreover, they detected a smaller sized immunoreactive SHBG species in LNCaP, MCF-7 and HepG2 cells, raising the possibility at least one of these transcripts is translated. Moreover, using Western blot analysis these authors identified an immunoreactive protein of the same molecular weight as purified SHBG.

To our knowledge, there is a first data showing the potential action of hydroxylated metabolites of E2 on SHBG mRNA and protein expression in MCF-7 cell lines.

The role of SHBG gene expression in estrogen-dependent cell growth of MCF-7 breast cancer was investigated using PCR, RT-PCR and Southern blotting (Murayama et al. 1999). Authors showed that E2 (17β-estradiol) induced the expression of the wild type SHBG gene that in MCF-7 cells showed high expression.

Indirect confirmation of the results presented, is seen in published data by Loukovaara et al. (1995) who showed phytoestrogens, daidzein, equol, or genistein, independent of the hydroxyl groups in the molecules, did not have a clear effect on the steady-state SHBG mRNA levels in HepG2 cells. However, no effect on SHBG gene methylation was observed except under the influence of genistein and 17β-estradiol, which have more hydroxyl groups, than daidzein and equol. The authors suggest the regulation of SHBG production by estrogens occurs at the posttranscriptional level. Metabolism of isoflavonoids in HepG2 cells produces mainly unconjugated and sulfated compounds. Locally regulated intracellular SHBG, in target cells for estradiol and its metabolites, could alter the intracellular free concentration of these hormones. It is possible the source of SHBG membrane signaling in the breast is synthesized in the breast itself, also suggested by Hryb et al. (2002). Thus, the regulation of its synthesis by E2 and its hydroxylated metabolites may play an important role in signaling. Clearly, this is an area requiring further investigation.

Catalano et al. (2005) suggested that the interaction of SHBG with MCF-7 cell membranes causes inhibition of the anti-apoptotic effect of estradiol, which might account for SHBG's inhibitory effect on breast cancer cell growth. Constantino et al. (2009) compared the ability of recombinant wild type and variant (D327N) SHBG to influence estradiol effects in MCF-7 breast cancer cells. They showed that. D327N SHBG was more effective than wild type protein in inhibiting estradiol induced cell proliferation and anti-apoptosis.

Using panel of polychlorinated biphenyls (PCBs) and hydroxy-polychlorinated biphenyls (HO-PCBs) in the screening assay, Jury et al. (2000) showed that although the PCBs have little or no ability to interact with SHBG, at least three HO-PCBs (HO-PCB2, HO-PCB3 and HO-PCB4) were potential ligands for SHBG.

In conclusion, the presented data showed, for the first time, the ability of hydroxylated estrogens to mimic the effects of estradiol, on SHBG gene, protein expression and intracellular levels in MCF-7 cells line.

Acknowledgement

This work was supported by the Polish Committee for Scientific Research from 2006-2009 as a project 1929/PO1/06/31 and partly by K/ZDS/001715/2010.

References

Catalano MG, Frairia R, Boccuzzi G, Fortunati N: Sex hormone-binding globulin antagonizes the anti-apoptotic effect of estradiol in breast cancer cells. Mol Cell Endocrinol 230, 31-37, 2005. doi:10.1016/j.mce.2004.11.005

Costantino L, Catalano MG, Frairia R, Carmazzi CM, Barbero M, Coluccia C, Donadio M, Genta F, Drogo M, Boccuzzi G, Fortunati N: Molecular mechanisms of the D327N SHBG protective role on breast cancer development after estrogen exposure. Breast Cancer Res Treat 114, 449–456, 2009. doi:10.1007/s10549-008-0031-4

Gregoraszczuk EL, Rak A, Ludewig G, Gasinska A: Effect of estradiol, PCB3 and their hydroxylated metabolitem on proliferation, cell cycle and apoptosis of human breast cancer cells. Environ Toxicology and Pharmacol 25, 227-233, 2008. doi:10.1016/j.etap.2007.10.004

Gregoraszczuk E, Ptak A: Involvement of caspase-9 but not caspase-8 in the anti-apoptotic effects of estradiol and 4-OH-Estradiol in MCF-7 human breast cancer cells. Endocrine Regul 45, 3-8, 2011.

Hryb DJ, Nakhla AM, Kahn SM, St George J, Levy NC, Romas NA, Rosner W: Sex hormone-binding globulin in the human prostate is locally synthesized and may act as an autocrine/paracrine effector. J Biol Chem 27, 26618-26622, 2002. doi:10.1074/jbc.M202495200

Jury H, Zacharewski TR, Hammond GL: Interactions between human plasma sex hormone-binding globulin and xenobiotic ligands. J Steroid Biochem Mol Biol 75, 167-176, 2000. doi:10.1016/S0960-0760(00)00168-0

Kahn SM, Li YH, Hryb DJ, Nakhla AM, Romas NA, Cheong J, Rosner W: Sex hormone-binding globulin influences gene expression of LNCaP and MCF-7 cells in response to androgen and estrogen treatment. Adv Exp Med Biol 617, 557-564, 2008. doi:10.1007/978-0-387-69080-3_57

Kalme T, Loukovaara M, Koistinen R, Koistinen H, Angervo M, Leinonen P, Seppala M: Estradiol increases the production of sex hormone-binding globulin but not insulin-like growth factor binding protein-1 in cultured human hepatoma cells. Fertil Steril 72, 325-329, 1999. doi:10.1016/S0015-0282(99)00229-0

Larrea F, Díaz L, Cariño C, Larriva-Sahd J, Carrillo L, Orozco H, Ulloa-Aguirre A: Evidence that human placenta is a site of sex hormone-binding globulin gene expression. J Steroid Biochem Mol Biol 46, 497-505, 1993. doi:10.1016/0960-0760(93)90104-5

Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408, 2001.

Loukovaara M, Carson M, Palotie A, Adlercreutz H: Regulation of sex hormone-binding globulin production by isoflavonoids and patterns of isoflavonoid conjugation in HepG2 cell cultures. Steroids 60, 656-661, 1995. doi:10.1016/0039-128X(95)00089-9

Misao R, Itoh N, Mori H, Fujimoto J, Tamaya T: Sex hormone-binding globulin mRNA levels in human uterine endometrium. Eur J Endocrinol 131, 623-629, 1994. doi:10.1530/eje.0.1310623

Moore KH, Bertram KA, Gomez RR, Styner MJ, Matej LA: Sex hormone binding globulin mRNA in human breast cancer: detection in cell lines and tumor samples. J Steroid Biochem Mol Biol 59, 297-304, 1996. doi:10.1016/S0960-0760(96)00121-5

Murayama Y, Hammond GL, Sugihara K: The SHBG Gene and Hormone Dependence of Breast Cancer: A Novel Mechanism of Hormone Dependence of MCF-7 Human Breast Cancer Cells Based upon SHBG. Breast Cancer 6, 338-343, 1999. doi:10.1007/BF02966450

Nader N, Raverot G, Emptoz-Bonneton A, Déchaud H, Bonnay M, Baudin E, Pugeat M: Mitotane has an estrogenic effect on sex hormone-binding globulin and corticosteroid-binding globulin in humans. J Clin Endocrinol Metab 91, 2165-2170, 2006. doi:10.1210/jc.2005-2157

Nakhla AM, Hryb D, Rosner W, Romas NA, Xiang Z, Kahn SM: Human sex hormone-binding globulin gene expression- multiple promoters and complex alternative splicing. BMC Mol Biol 10, 37, 2009. doi:10.1186/1471-2199-10-37

Raineri M, Catalano MG, Hammond GL, Avvakumov GV, Frairia R, Fortunati N: O-Glycosylation of human sex hormone-binding globulin is essential for inhibition of estradiol-induced MCF-7 breast cancer cell proliferation. Mol Cell Endocrinol 189, 135–143, 2002. doi:10.1016/S0303-7207(01)00725-0