Sex hormone-binding globulin antagonizes the anti-apoptotic effect of estradiol in breast cancer cells

SEX HORMONE BINDING GLOBULIN (SHBG), ESTRADIOL AND BREAST CANCER

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1

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SUMMARY

The human serum Sex Hormone-Binding Globulin (SHBG) plays an important role in breast cancer pathophysiology and risk definition, since it regulates the bioavailable fraction of circulating estradiol. We here summarize data reported over the years concerning the involvement of SHBG and SHBG polymorphisms in the definition of breast-cancer risk. We also report what is known about the direct action of SHBG in breast cancer cells, illustrating its interaction with these cells and the subsequent initiation of a specific intracellular pathway leading to cross-talk with the estradiol-activated pathway and, finally, to the inhibition of several effects of estradiol in breast cancer cells. In conclusion, as a result of its unique property of regulating the estrogen free fraction and cross-talking with the estradiol pathways, by inhibiting estradiol-induced breast-cancer cell growth and proliferation, SHBG is associated with a reduced risk of developing the neoplasm after estrogen exposure.

INTRODUCTION

Estradiol, as other sex steroid hormones, circulates in human blood mainly bound to Sex Hormone-Binding Globulin (SHBG), a 373-amino-acid glycoprotein produced by the liver (Hammond 1995, Rosner 1990). The bioavailable fraction of estrogens and their access to target cells are directly regulated by SHBG (Siiteri et al. 1982, Westphal 1986). Sex steroids are known to be involved in several human diseases, including hormone-dependent tumours, e.g. breast cancer (Chen 2008) and prostate cancer (Bosland 2000), obesity, cardiovascular disease and metabolic syndrome (Hennekens 1998, Jones et al. 2005, Bogers et al. 2007, Mattsson & Olsson 2007, Blouin et al. 2008, Renehan et al. 2008). Consequently, factors regulating bioavailable sex steroids are of key importance in the pathophysiologic background of these diseases, and SHBG has thus been the object of several studies about this issue.

We have long focused our interest on SHBG involvement in breast cancer; here we summarize what we and others have reported over the years.

SHBG AND BREAST CANCER RISK

A number of studies have concerned sex hormone and SHBG levels in women in relation to breast cancer risk. In a very early report, Moore and co-workers (Moore et al. 1982) observed that, while SHBG binding capacity was in the normal range in premenopausal breast-cancer patients, in postmenopausal breast-cancer patients it was significantly reduced. The reduction of SHBG levels in postmenopausal breast-cancer patients was later confirmed by other studies (Toniolo et al. 1995, Zeleniuch Jacquotte et al. 2004) suggesting a role for SHBG. Furthermore, in these patients the free fraction of estradiol is increased, in relation to an absolute or relative reduction of SHBG (Lipworth et al. 1996). In addition, in a large study analyzing the individual data from nine prospective studies on 663 women who developed breast cancer and 1765 women who did not, the risk for breast cancer increased in a statistically significant manner with increasing concentrations of all sex

hormones examined, while SHBG was associated with a decrease in breast cancer risk (P_{trend} = .041) (Key et al. 2002). More recently, in a case-controlled study within the European Prospective Investigation into Cancer and Nutrition (EPIC), (Kaaks et al. 2005b), SHBG levels in postmenopausal women who developed breast cancer were confirmed to be significantly lower compared with controls, while no significant difference was observed in premenopausal women (Kaaks et al. 2005a). SHBG, as one of the factors able to modulate estrogen balance, has thus to be taken into account in evaluating a woman's risk of developing breast cancer later in life.

Some early studies also reported an interesting correlation between serum SHBG and estrogen-dependent breast cancer. Murayama and co-workers (Murayama et al. 1978) first observed a correlation between SHBG and estrogen receptor (ER) in postmenopausal breast cancer patients, but not in ER-positive premenopausal breast cancer patients. This finding was later confirmed by the same authors, who observed the simultaneous presence of high plasma SHBG concentrations and estrogen receptors in hormone-dependent breast cancer (Murayama et al. 1979) and by Plymate and co-workers (Plymate et al. 1984) who reported higher SHBG levels in ER-positive than in ER-negative breast-cancer patients.

SHBG IN BREAST CANCER CELLS: ROLE AND MECHANISM OF ACTION

1. SHBG IS DETECTED IN BREAST CANCER CELLS

The existence/presence of intracellular forms of SHBG has been evaluated in different tissues and cell types. As far as breast cancer is concerned, early immunohistochemical studies, using rabbit polyclonal antisera, showed the presence of SHBG antigen in breast cancer cells and tissue sections (Bordin and Petra 1980; Tardivel-Lacombe et al. 1984; Sinnecker et al. 1990; Meyer et al. 1994; Germain et al. 1997). The source of this immunoreactive SHBG has never been completely clarified; two possible explanations (uptake from the circulation or synthesis in loco) have been suggested but neither has been definitively proved.

Another group of studies focused on the demonstration of SHBG transcripts in tissues other than the liver, where SHBG is normally produced. SHBG mRNA has also been demonstrated in the placenta (Larrea et al. 1993), in the endometrium and endometrial cancer (Misao et al. 1994, Misao et al. 1997). Furthermore, using RT-PCR Moore and co-workers (Moore et al 1996) detected SHBG mRNA in ZR-75-1, MCF-7 and MDA-MB-231 breast cancer cells, and in 11 (out of 30) breast tissue samples. Two different PCR products (300 and 500 bp) were reported and the DNA sequence of the 300 bp PCR product was consistent with alternate splicing of the SHBG mRNA. Although data concerning the cell lines are convincing, no evidence for mRNA translation was presented.

More recently, in tissue sections of human prostate, Hryb and co-workers (Hryb et al. 2002) demonstrated the presence of both SHBG (immunohistochemistry) and SHBG mRNA (*in situ* hybridization), suggesting that SHBG, at least in the prostate, is locally regulated and produced. The same laboratory reported similar findings also in breast cancer (Khan et al. 2008).

Even though considerable effort has been made to understand the significance of the intracellular presence/production of SHBG, no consistent, conclusive and convincing evidence has been provided and this issue is still an open one.

In contrast, two very interesting recent papers provide strong evidence in favour of SHBG uptake from the extracellular space. It has been reported that megalin, an endocytic receptor in the reproductive tissues, promotes cellular uptake of biologically-active androgens and estrogens bound to SHBG (Hammes et al. 2005). Ng and co-workers also found that, in the endometrium, SHBG binds the carboxyl-terminal domains of fibulin-1D and fibulin-2 in a steroid-dependent manner, estradiol being the most effective ligand. In addition, SHBG co-immunoprecipitates with these fibulins in uterine extract; it is thus suggested that these matrix-associated proteins may therefore sequester plasma SHBG and control sex-steroid access to target cells (Ng et al. 2006). The possibility that SHBG can interact with matrix-associated proteins is of considerable interest, since it could explain both previous data concerning the intracellular presence of SHBG and the findings reported in the next section about SHBG interaction with sex steroid target cells.

2. SHBG INTERACTS WITH BREAST CANCER CELL MEMBRANES

Starting from the early 1980s, numerous studies reported the existence of specific and high-affinity binding sites for SHBG on cell membranes from different human tissues. The binding site for SHBG was observed in plasma membranes from decidual endometrium (Strel'chyonok et al. 1984), normal and neoplastic endometrium (Fortunati et al. 1991; Fortunati et al. 1992), prostate (Hryb et al. 1985), human placenta (Avvakumov et al. 1985), and epididymis (Gueant et al. 1991). Binding sites for SHBG have also been observed in the medial preoptic area and the medial basal hypothalamus in rats (Caldwell JD 2001). SHBG has also been reported to interact with membranes of estrogen-dependent MCF-7 breast cancer cells (Porto et al. 1992; Fortunati et al.1993).

In all tissues and cell types, the binding characteristics of SHBG to cell membranes are consistent with the presence of a receptor structure, since all reports have described highly specific binding that is time- and temperature-dependent. The *SHBG receptor* has never actually been purified or cloned, but recent evidence concerning the interaction of SHBG with matrix-associated proteins

(Ng et al. 2006) has opened up a new scenario, that deserves further study. In line with this, we suggest that SHBG interacts with cell membranes through a receptor able to connect extracellular SHBG to intracellular pathways, as will be described in detail.

The modalities of SHBG-membrane interaction have been exhaustively studied and described. As elegantly described by Hryb and co-workers (Hryb et al. 1989; Hryb et al. 1990), only steroid-free SHBG binds to cell membranes, whereas, if sex steroids bind first to SHBG, they prevent the SHBG-cell interaction; the extent of the inhibition is directly proportional to the magnitude of the association constant for the steroid-SHBG interaction. Once bound to the membrane site, SHBG can bind steroids with equal affinity as it does in solution. The SHBG domain interacting with cell membranes has also been identified; it has been localized to a ten-amino-acid stretch (TWDPEGVIFY) at the amino-terminal end of the SHBG molecule (Khan et al. 1990). This region is referred to as the laminin-G domain of human SHBG and is the most highly conserved portion of the molecule, both across species and in related proteins such as protein S, laminin A, merosin, and *Drosophila crumbs* protein (Gershagen et al. 1987; Joseph and Baker 1992; Grishkovskaya et al. 2000). Moreover, the structural integrity of the *O*-glycosylation site in Thr^7 of this SHBG domain is critical for significant biological effects to be achieved in breast cancer cells: it has been observed that mutant SHBG lacking Thr^7 *O*-glycosylation at its amino terminus fails to produce any biological effect, whereas the activity of SHBG mutants lacking the two *N*-glycosylation sites located at the carboxy-terminus of the protein does not differ from that of the *wild type* protein (Raineri et al.2002).

It may thus be concluded that SHBG interacts with cell membranes, binding to matrix-associated proteins. SHBG laminin-G domain, located at the amino-terminus portion of the protein, is the membrane-binding domain, and its carbohydrate chain at Thr^7 contributes to its structural stability, allowing correct interaction with cell membranes and induction of biological effects, at least in breast cancer cells.

3. SHBG CROSS-TALKS WITH ESTRADIOL IN BREAST CANCER CELLS

A number of reports indicate that the interaction of SHBG with breast cancer cell membranes is closely related to cell sensitivity to estrogens. The binding sites for SHBG have been described in MCF-7 breast cancer cells, which are positive for the estrogen receptor α (ERα), (Porto et al 1992; Fortunati et al. 1993), while no binding site was detectable on MDA-MB 231 breast cancer cells, which are ERα-negative and estrogen-insensitive (Fissore et al. 1994). In addition, in tissue samples from breast cancer patients, SHBG binding was detectable in 75% of ERα-positive samples and in 37% of ERα-negative samples. Samples binding SHBG and expressing ERα were also characterized by a significantly lower proliferation rate than samples unable to bind SHBG (Catalano et al. 1997).

The binding of SHBG to MCF-7 cell membranes generates a cascade of signals; the first event to be described was increased intracellular cAMP. The accumulation of cAMP induced by SHBG binding has been described both in prostate cancer cells (Nahkla et al. 1990; Rosner et al. 1992) and in breast cancer cells (Fissore et al. 1994; Fortunati et al. 1999). SHBG caused a significant increase of cAMP in both cell types, this effect being induced by DHT in the former and by estradiol in the latter cells. The elevation in intracellular cAMP suggests that the SHBG binding site is related to a G-protein membrane receptor (Nakhla et al. 1999). The biological effects of SHBG appear to be mediated by cAMP and its target PKA (Protein Kinase A): they are lost in MCF-7 cells if PKA is blocked by the specific inhibitor, PKI (6 - 22), (Fortunati et al. 1996; Fazzari et al. 2001).

The immediate target of PKA after SHBG activation in breast cancer cells has not yet been identified, but it has been suggested that increased PKA levels may inhibit the MAP kinase pathway. It has also been reported that SHBG counteracts estradiol-induced ERK activation (Catalano et al. 2005), which is one of the MAP kinases involved in controlling cell proliferation. ERK activity is suppressed by both cAMP and PKA (Filardo et al.2002) and inhibition of ERK causes inhibition of the anti-apoptotic effects caused by estradiol (Catalano et al. 2005) and reduces ERα transcription efficiency (Kato et al. 1995).

It has also been reported that membrane-associated ER-α is responsible for rapid E_2-induced cAMP accumulation and subsequent activation of the downstream PKA pathway, and that cAMP-activated PKA activity is associated with inhibition of breast cancer cell proliferation (Zivadinovic et al. 2005). Besides the classical pathway mediated by nuclear ER, estradiol it thought to bind to a putative membrane ER (Marquez & Pietras, 2001) and to trigger specific intracellular signal transduction pathways (Razandi et al. 2000) leading to activation of MAP kinase, that transmits and amplifies signals involved in both cell proliferation and apoptosis (Pearson et al., 2001; Santen et al. 2002; Freeman & Whartenby 2004). Furthermore, MAP kinase can directly catalyze the phosphorylation of serine 118 of nuclear ERα, thus increasing ER transcriptional efficiency (Kato et al. 1995).

Estradiol and SHBG membrane-initiated pathways can thus cross-talk at different levels (at the membrane or at the ERK site) with the ultimate result of inhibiting cell proliferation and inducing apoptosis (Catalano et al. 2005; Fortunati & Catalano, 2006).

The mechanism of action of SHBG in breast cancer cell is summarized in Figure 1.

4. SHBG INHIBITS ESTRADIOL EFFECTS IN BREAST CANCER CELLS

SHBG-estradiol cross-talk is thus likely to interfere with the biological effects of estradiol in breast cancer cells, and several lines of evidence reported over the years substantiate this suggestion.

Firstly, SHBG has been widely demonstrated to inhibit estrogen-dependent cell growth. Estradiol is known to induce and maintain the proliferation of breast cancer cells (Dickson et a. 1989). Through its nuclear receptor ERα, it regulates the transcription and expression of many genes involved in cell proliferation, inducing positive regulators of cell growth and inhibiting negative regulators (Frasor et al. 2003; Thiantanawat et al. 2003). In addition, by interacting with its membrane receptor (Marquez & Pietras, 2001), it activates ERK and inhibits Jun kinase, causing the induction of bcl-2 and thus the inhibition of apoptosis in MCF-7 breast cancer cells (Razandi et al., 2000). The ability of SHBG to inhibit estradiol-induced cell proliferation was first reported in 1993,

together with one of the first descriptions of the SHBG binding site on MCF-7 cells (Fortunati et al. 1993). This observation was subsequently further confirmed in our laboratory and this effect of SHBG in MCF-7 cells was described in detail. The antiproliferative effect of SHBG is detectable when the correct sequence of binding is followed; that is, SHBG first binds to its membrane on MCF-7 cells, and then estradiol binds at the steroid-binding site of SHBG; the inhibition of estradiol-induced proliferation of MCF-7 cells is mediated by increasing intracellular cAMP and activating its target, PKA (Fortunati et al. 1996). SHBG is also reported to completely reverse the anti-apoptotic effect of estradiol (Catalano et al. 2005) and the facilitating effect of SHBG on apoptosis is one of the mechanisms involved in the inhibition of MCF-7 cell proliferation. Moreover, the structural integrity of the *O*-glycosylation site in Thr^{7} at the amino-terminal end of SHBG is required to block estradiol antiapoptotic effect and to induce cell proliferation (Raineri et al. 2002).

In view of estradiol's central role in gene expression, the ability of SHBG to modulate estradiol-controlled genes in breast cancer cells was also evaluated (Catalano et al. 2007). SHBG is effective on a small number of genes, all involved in cell growth, apoptosis control and cell estrogen-dependence. In detail, SHBG inhibits estradiol up-regulation of bcl-2, c-myc, EGF-R, PR, and its down-regulation of ERα. The effect on bcl-2 expression may be one of the mechanisms elicited by SHBG to restore apoptosis in breast cancer cells under the action of estrogen. The proto-oncogene bcl-2 inhibits apoptosis (Thompson 1995; Craig 1995) and is over-expressed in breast cancer (Nahta & Esteva, 2003). The ability of SHBG to down-regulate bcl-2 can counteract its effects, normally amplified by estradiol, in breast cancer cells. As reported for bcl-2, SHBG also inhibits estradiol induction of c-myc expression. In breast cancer cells the proto-oncogene c-myc, which is up-regulated by estradiol (Shang & Brown , 2002), acts on multiple targets that are key cell-cycle regulators; in particular, it increases the expression of cyclin E and CDK4 (Santoni-Rugiu 2000; Hermeking 2000). Also in this case SHBG can abrogate the magnifying effect of estradiol on positive regulators of cell cycle and growth. It is also reported that SHBG inhibits estradiol

induction of EGF-R, a member of the epidermal growth factor family of trans-membrane receptors (Roskoski 2004). Over-expression of EGF-R in breast cancer, which might be induced by estradiol (Berthois 1989), is a negative prognostic factor (Spyratos 1990) and, in addition, is involved in a bidirectional cross-talk with estrogen receptors; activation of the EGF-R-derived signalling pathway could amplify the effect of estradiol in breast cancer (Levin 2003). Therefore, by abrogating estradiol induction of EGF-R expression, SHBG breaks the loop between the two pathways, reducing breast cancer cell growth.

Furthermore, SHBG is also effective on both ERα and PR expression. First, it prevents estradiol-induced down-regulation of ERα, an effect that could also be related to apoptosis induction and cell growth inhibition, since both phenomena peak when ERα expression is increased and bcl-2 reduced (Truchet 2000; Detre 1999). With regard to PR modulation, in MCF-7 cells, estradiol strongly up-regulates PR levels, measured either by gene expression or by functioning protein (Berkenstam et al. 1989; Jensen et al. 1999; Frasor et al. 2003). The estradiol-induction of PR gene expression and protein levels is inhibited by SHBG (Fazzari et al. 2001; Catalano et al. 2007).

Finally, the effect of SHBG on gene expression is highly selective, depending on its interaction with cells, and restricted to genes associated to cell growth and estrogen-sensitivity.

SHBG POLYMORPHISMS IN BREAST CANCER

Some nucleotide variations in the human *SHBG* gene have been described, in both coding and regulatory sequences. Two different mutations (P156L and Δ326) leading to an apparent absence of SHBG in one individual with symptoms of androgen excess have been reported (Hogeveen KN et al. 2002), but these mutations appear to be extremely rare.

On the contrary, a single-nucleotide polymorphism, Asp327Asn (D327N), which introduces an additional consensus site for N-glycosylation, is present in the *SHBG* gene exon 8 (Power SG et al. 1992) and is found worldwide (Van Baelen et al. 1992). This substitution leads to an increase in the

half-life of human SHBG (Cousin P et al. 1998) and may be associated with higher SHBG levels in variant allele carriers.

A further polymorphism in the *SHBG* gene 5'-flanking region has been reported (Hogeveen KN et al. 2001). It is a pentanucleotide repeat polymorphism [PNRP $(TAAAA)_n$] within the human *SHBG* promoter that has a marked effect on its transcriptional activity *in vitro* in HepG2 cells. The number of TAAAA repeat elements ranged from 6 to 10 in the original report by Hogeveen and co-workers, but later studies found 11 repeats (Xita et al. 2003, Cousin et al. 2004). This polymorphism has been studied in depth in women affected with Polycystic Ovary Syndrome (PCOS) who presented a significantly greater frequency of longer (TAAAA)n alleles (more than eight repeats) than normal women who had shorter alleles (less than eight repeats); carriers of the longer allele genotypes had lower SHBG levels than those with shorter alleles (Xita et al. 2003, Xita et al. 2008). Moreover, in these patients a strong disequilibrium linkage between the D327N *v* allele and the eight-TAAAA repeat was observed (Cousin et al. 2004). Longer (TAAAA)n repeats in the SHBG gene promoter are also reported to be associated with more severe CAD in women undergoing coronary angiography (Alevizaki M et al. 2008).

As far as the general population is concerned, Haiman and co-workers (Haiman et al. 2005) evaluated the association between the (TAAAA)n repeat polymorphism, Asp327Asn polymorphism, and SHBG levels in a population of African-American, Native Hawaiian, Japanese, Latina, and Caucasian healthy postmenopausal women from the Multiethnic Cohort Study (n = 372). They found suggestive evidence of linkage disequilibrium between the Asn327 allele and the eight-repeat allele in all populations except the African-Americans. The individual genotypic classes contributed modestly to the overall prediction of SHBG levels, but carriers of the six-repeat allele were found to have significantly lower SHBG levels than non-carriers.

The Asp327Asn polymorphism has also been widely studied in relationship to breast cancer risk, and results from different studies are to some extent divergent, but nevertheless intriguing. In a study on both familial and sporadic breast cancer in Polish and Nordic populations (Försti et al.

2002) the *327Asn* allele carriers correlated to an overall reduced breast cancer risk, but statistical significance was not attained. Dunning and co-workers (Dunning et al. 2004) reported no significant association between the *Asn* variant and breast cancer risk, although they observed increased serum SHBG levels and a reduced estradiol to SHBG ratio in the same subjects. In a large population-based case-control study (1,106 cases, 1,180 controls) in postmenopausal women, Cui et al. (Cui et al. 2005) observed a significant association of the Asp327Asn polymorphism with reduced breast cancer risk. Furthermore, a significantly higher frequency of the polymorphism was observed in postmenopausal patients with ER-positive breast cancer than in ER-negative (Becchis et al. 1999) and more recently Costantino and co-workers (Costantino et al. 2009) observed a significantly higher frequency of the polymorphism in postmenopausal women taking Hormone Replacement Therapy (HRT) who did not develop breast cancer than in their counterparts who did, suggesting a protective role of D327N SHBG in estrogen-dependent breast cancer. The protective role of D327N SHBG in estrogen-dependent breast cancer was further supported by data obtained *in vitro* (Costantino et al. 2009). In MCF-7 cells, D327N SHBG is more effective than *wild type* protein in inhibiting estradiol-induced cell proliferation and anti-apoptosis, and this is due to the fact that D327N SHBG binds to MCF-7 cells to a greater extent than does *wild type* protein. Lastly, D327N causes greater induction of the second messenger cAMP and stronger inhibition of the estradiol-induced Erk ½ phosphorylation, and these data provide evidence for the mechanism of D327N SHBG protective action in breast cancer.

In a recent very large study (Thompson et al. 2008) ten single nucleotide polymorphisms (SNPs) within or close to the SHBG gene were found to be significantly associated with SHBG levels, as was the $(TAAAA)_n$ polymorphism. At least 3 SNPs showed associations with SHBG levels that were highly significant, though relatively small in magnitude. In particular, rs6257 (here referred as to D356N, but normally referred as to D327N) is a potential breast cancer susceptibility variant. In addition, there was no evidence of any association between the $(TAAAA)_n$ polymorphism and breast cancer risk.

A study is currently in progress in our laboratory on the $(TAAAA)_n$ repeat distribution and role in breast cancer patients (Piccioni et al. 2009). We have found that the distribution of the $(TAAAA)_n$ alleles differs significantly between breast cancer patients (79.3% with ≤ 8 repeats; 20.7% > 8 repeats) and healthy controls (59.8 % with ≤ 8 repeats; 40.2 % > 8 repeats). There is also a strong disequilibrium linkage between D327N polymorphism and the 8 TAAAA repeat in breast cancer patients, confirming other studies.

Overall, it can be concluded that relationships between the genetic determinants of SHBG and breast cancer are complex, but are worthy of considerable research effort since genetic SHBG variations in breast cancer may account for different risk profiles.

CONCLUSIONS AND FUTURE PERSPECTIVES

The body of information about the involvement of SHBG in breast cancer has been growing since the very first reports in the early 1980s. At the same time, our knowledge about the role and the mechanisms of action of the protein in breast cancer cells has been increasing and, although it is still far from exhaustive, we can now delineate quite a detailed scenario.

SHBG plays a protective role in the exposure of breast cells to estrogens. Thanks to its unique property of regulating estrogen free fraction and to cross-talk with estradiol pathways in breast cancer cells, SHBG smoothes estrogen effects and has a pivotal effect in reducing breast cancer cell growth and proliferation (Figure 2). Structural alterations to the protein, such as the introduction of an additional carbohydrate chain in D327N SHBG, can modify its functional properties, in some cases increasing its protective function. Finally, SHBG and its polymorphism may correspond to different profiles of breast cancer risk that are not still fully understood.

We believe that this field deserves further study, especially with the aim of clarifying the putative interaction of SHBG with matrix associated proteins in breast cancer, and the role of SHBG in the definition of estrogen-dependence of breast cancer.

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LEGEND TO FIGURES

Figure 1 – Mechanism of action of SHBG in breast cancer cells and cross-talk with estradiol pathway.

Figure 2 – SHBG, estradiol and breast cancer.

Figure

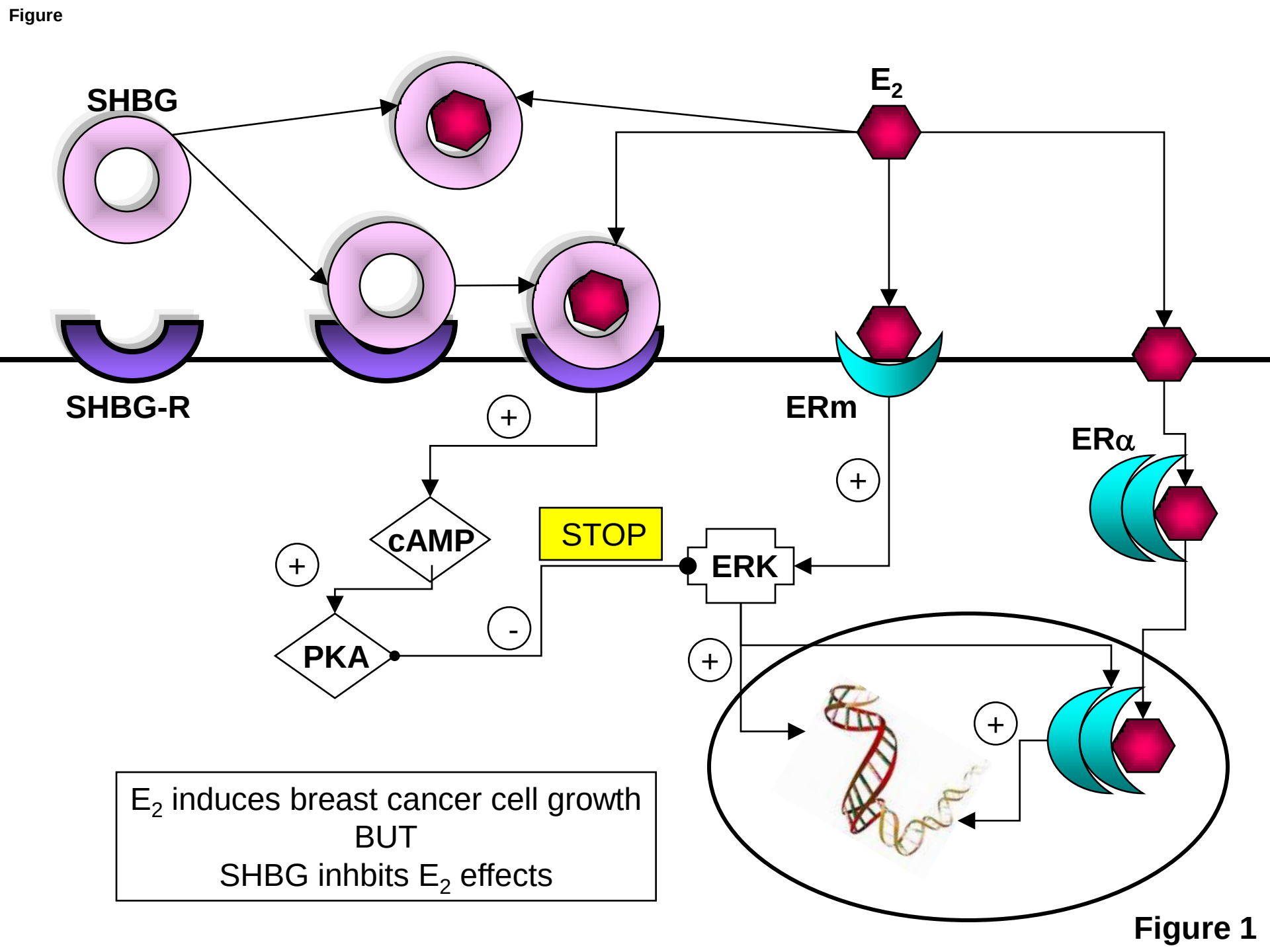

Figure 1

Figure

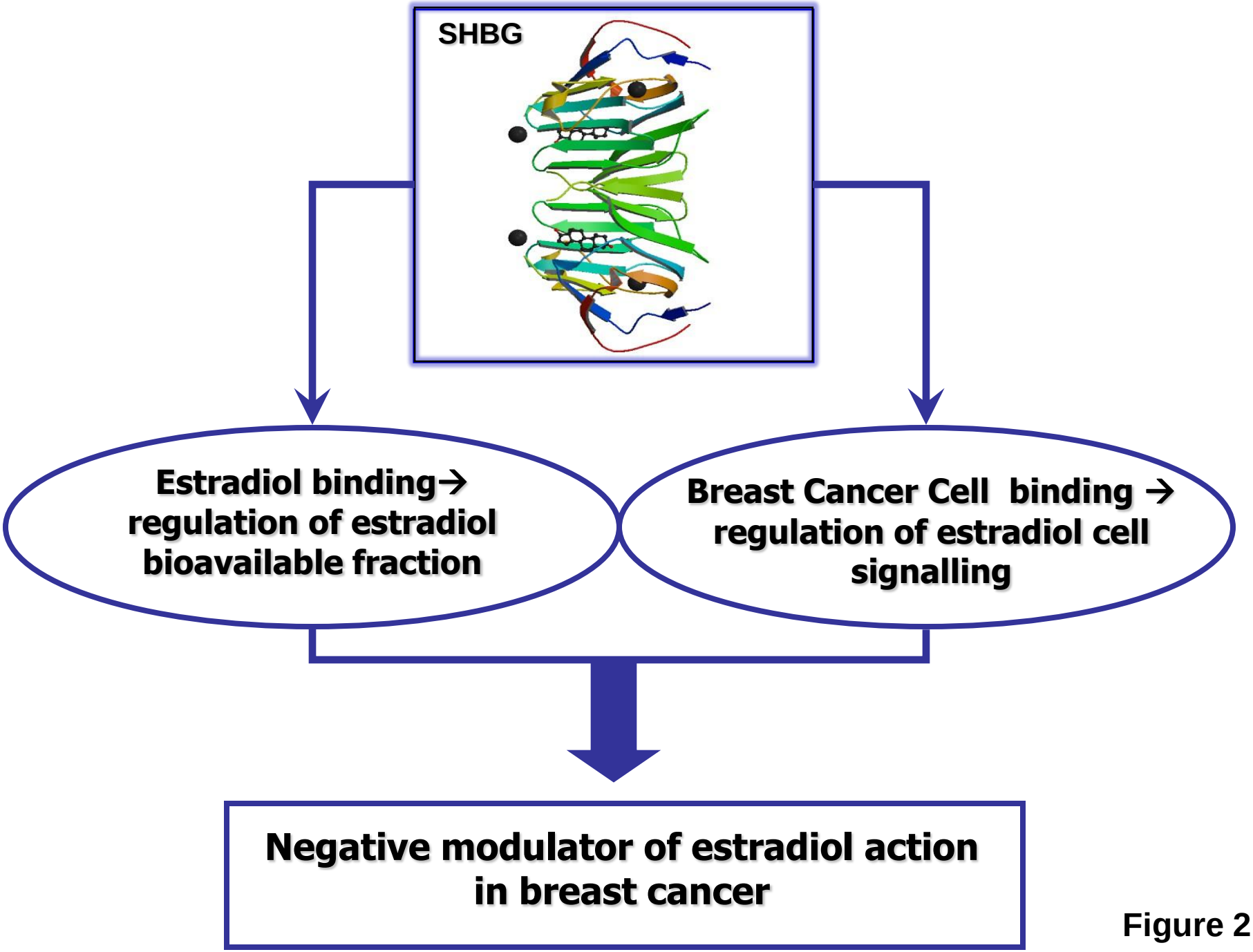

Figure 2