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14. ABSTRACT

The plasma protein sex hormone-binding globulin (SHBG) not only binds estrogens in plasma, but also is part of an estrogen signal transduction system that starts with a receptor (RSHBG) for SHBG on breast cell membranes. The SHBG-RSHBG complex is activated by an appropriate steroid hormone, eg. estradiol (E2), (forming the new complex, E2-SHBG-RSHBG), to trigger a second messenger system to produce cAMP minutes after steroid binding. Frozen sections of normal and cancerous breast cells stain with anti-SHBG antibodies and these same cells contain SHBG mRNA. MCF-7 cells also make their own SHBG mRNA and protein. We present a complex picture of extra hepatic human SHBG gene expression, including at least 12 different transcripts generated through alternative splicing and alternative promoter utilization. We have devised an inducible system to study SHBG protein expression in MCF-7 cells, and have expressed tagged versions of SHBG in MCF-7 cells that will be useful for future studies on biologic function in breast cells and in breast cancer.

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INTRODUCTION

We propose that the expression of Sex Hormone-Binding Globulin (SHBG) by breast cancer cells is biologically regulated and that this SHBG functions to alter the effects of estrogens within the breast cancer cell. We have shown that the plasma protein sex hormone-binding globulin (SHBG) not only binds estrogens in plasma, but also is part of an estrogen signal transduction system that starts with a receptor (R_{SHBG}) for SHBG on breast cell membranes (1). The SHBG-R_{SHBG} complex is activated by an appropriate steroid hormone, such as estradiol (E_2), (forming the new complex, E_2-SHBG-R_{SHBG}), to trigger a second messenger system to produce cAMP within minutes after the steroid binds. We have shown that frozen sections of normal and cancerous breast cells stain with anti-SHBG antibodies and that these same cells contain SHBG mRNA. Further, the well-known breast cancer cell line, MCF-7, contains both SHBG mRNA and SHBG protein. The expression of SHBG by breast cancer cells raises the important question of how the local regulation of SHBG synthesis and secretion affects both the sequestration of steroid hormones within the breast, and estrogen induced signal transduction at the cell membrane. The local synthesis of SHBG is consistent with an autocrine/paracrine role for this protein in breast cancer.

To this end, we planned studies to conduct genetically and pharmacologically based studies to address our hypothesis. These included: 1) generation and characterization of breast cell lines that inducibly or constitutively overexpress SHBG and condition their medium with secreted SHBG; 2) characterization of SHBG mRNA expression in breast cell lines; 3) characterization of SHBG protein expression in breast cell lines, and normal and tumor tissues. The cell lines generated will be useful for investigating the effects of SHBG expression on selective stimulation of either the estrogen receptor (ER) or the SHBG-receptor based pathways, and the effects of conditioned medium from these cell lines on cell growth. We also have characterized SHBG gene expression in MCF-7 cells and other cell lines and tissues, generating a profile that is much more complex than previously reported. The human SHBG gene contains at least three different promoters, and the transcripts from these three promoters undergo alternative splicing of exons 4, 6, and 7. It was important to note that the secreted form of SHBG was absent in MCF-7 cells, causing us to reevaluate our original hypothesis [e.g. that SHBG exerted paracrine effects in the breast], and focus on the characterization of novel SHBG gene transcripts in breast cancer cell lines and breast tissue. These results compel further study of the biologic properties of novel SHBG isoforms expressed in the breast and how their differential expression contributes to malignant transformation.

BODY

1. Generation of inducible SHBG plasmids. We cloned the human SHBG cDNA into the inducible expression vector, pINDHyg, in both the sense (pIND/Hygro/SHBGsense) and antisense (pIND/Hygro/SHBGantisense) directions, and confirmed the fidelity of the

inserts by sequence analysis. Importantly, we decided that it would be scientifically prudent to establish intermediate MCF-7 and MDA-MB-231 cell lines that incorporated functional pVgRXR sequences so that all derivative cell lines in these studies would be from identical subclones. In this way, we minimize artifacts due to positional effects on pVgRXR genomic insertion points or clonal variance. These cell lines will also be valuable as tools for inducible expression of other proteins.

2. Generation of a nonleaky intermediate inducible MCF-7 cell line. The plasmid, pVgRXR was transfected into MCF7 cells. Stable transfectant colonies were generated by growing the transfected cells in the presence of zeocin. Twenty-four candidate zeocin resistant colonies were isolated and expanded as intermediate clonal cell lines. In transient transfection experiments with pIND/Hygro/LacZ, all clones were tested for their ability to induce expression of the LacZ gene following treatment with the inducing agent, Ponasterone A (Pon A). Three intermediate clones, MCF7-pVgRXR4, 12, and 13, demonstrated inducible LacZ activity (β-galactosidase assay). The MCF7-pVgRXR 13 cell line showed no background and had the highest degree of induction; therefore, we chose it as our intermediate cell line.

3. Generation of M13S14, an MCF-7 cell line with inducible SHBG expression, and M13V7, a vector control cell line.
We stably transfected the inducible plasmids pIND/Hygro/SHBGsense and pIND/Hygro/SHBGantisense, and the control plasmid, pIND/Hygro into MCF7-pVgRXR13 cells. Selection for incorporation of plasmid sequences was in hygromycin B. We isolated and expanded 24 colonies from each of the pIND/Hygro/SHBGsense, pIND/Hygro/SHBGantisense, and pIND/Hygro transfectants.

We tested the positive control, MCF7-pVgRXR13-pIND/Hygro/LacZ, pool of cells with Pon A (10uM), and found that approximately 8% of the induced cells turned blue in a β-galactosidase assay, while no uninduced cells from the same pool turned blue. This indicated that, not only was the transfection process successful, but more importantly that our overall strategy was feasible.

After expansion of the 24 clones from each of the other three transfections, DNA was prepared, and analyzed by PCR for uptake of SHBG sequences (or vector sequences for the control transfectants). Positive pIND/Hygro/SHBGsense clones were analyzed for induced SHBG expression by treating cells with 10 μM ponasterone A, and measuring cellular SHBG. PonA markedly induces SHBG in M13S14 and less markedly in M13S24, which also shows some basal leakiness (**Figure 1**). As expected, PonA did not induce SHBG expression in the vector control cell line, M13V7.

We did not pursue further analysis of the antisense candidates past the PCR analysis step described above because recent work has shown that RNA interference technology provides a more prudent way to silence endogenous gene expression.

Because the inducible overexpression of SHBG worked in MCF-7 cells, we performed parallel experiments in MDA-MB-231 cells. pVgRXR was transfected into MDA-MB-

231 cells, and 24 zeomycin resistant colonies were expanded as cell lines. Unexpectedly, MDA-MB-231 cells were highly resistant to killing by the drug zeocin, including relatively large doses of up to 2 mg/ml. Because we select for pVgRXR uptake with zeocin, we instead attempted to introduce pVgRXR into MDA-MB-231 cells by cotransfecting this plasmid with the vector pCMVFlag (described below), which contains a selectable neomycin resistance gene. Transfections were performed using a 10:1 ratio of pVgRXR to pCMVFlag. We performed PCR analysis on 24 G 418 resistant colonies, and these all proved negative for pVgRXR, as did the pool of remaining colonies. We repeated transfections using even higher ratios of pVgRXR to pCMVFlag and obtained 24 candidate colonies for uptake of functional pVgRXR sequences. Unfortunately, none of these 24 colonies proved to be inducible. We therefore postponed these studies and shifted our efforts to evaluating the novel SHBG expression pattern that we discovered as part of this study (see below).

4. Anti-human SHBG antibodies for Western blot analysis.
A major technical problem that we addressed was identifying an antibody that would be suitable for Western blot analysis of cellular extracts. First, we tested all the polyclonal and monoclonal antibodies that we have generated or obtained from others using HepG2 cell extracts and purified SHBG as test substances. None proved useful for Western blot analysis. We next had custom polyclonal antisera generated against both the secreted form of SHBG (encoded by the cDNA used in these studies) and the alternative form of SHBG first described in the testis (2). Unique peptides, CLRPVLPTQSA and CFSLRLTHPPRTW, corresponding to the respective SHBG isoforms, were synthesized and used to immunize rabbits. Affinity purified antisera were positive by ELISA assay, however they did not prove useful for Western blot analysis. Fortunately, at the beginning of 2004, we obtained a polyclonal antibody, WAK-S102-12-53 (WAK-Chemie, Steinbach, Germany), which is useful for Western blot analysis. As detailed below, we used WAK-S102-12-53 to confirm ELISA and PCR results on cell lines that inducibly or constitutively express various SHBG constructs at elevated levels.

5. Generation of pSHBG-FL and pSHBG-MP, plasmid constructs that direct constitutive expression of SHBG. We synthesized two additional constructs for evaluating the effects of constitutively expressed SHBG in MDA-MB-231 cells. The vector used for these experiments was pCMVFlag, a plasmid containing a CMV promoter upstream of an ATG start codon, immediately followed by three iterations of a Flag tag (4) sequence. We generated: 1) pSHBG-FL, a plasmid containing the full length SHBG cDNA sequence cloned immediately downstream of the Flag tags and in the same reading frame, and 2) pSHBG-MP, a plasmid containing SHBG cDNA lacking the 29 amino acid amino terminal leader sequence which, in the liver, is cleaved from the nascent protein before secretion. Thus, this Flag tagged protein mimics the mature, processed SHBG found in serum. However, since it lacks the leader sequence, we hypothesized that the SHBG-MP protein would remain inside cells. We expected that the Flag tag would allow us to specifically detect the expressed pSHBG-FL and pSHBG-MP proteins in western blots. In addition, the Flag tag would produce different sized SHBG proteins, distinguishable from endogenous SHBG.

We expected that SHBG-FL might be detectable in cells, but it would have the Flag tag cleaved along with the signal peptide prior to secretion. However, if the cleavage system was overloaded with SHBG-FL protein, we might also detect residual full length SHBG-FL protein. We expected that the SHBG-MP construct would remain inside cells because it lacked a leader sequence, and might serve to mimic the fate of SHBG absorbed from outside the cell. The CMV promoter would direct constitutive expression of these proteins at elevated levels.

6. Generation of MCF-7 and MDA-MB-231 clonal cell lines that constitutively overexpress the pSHBG-FL and pSHBG-MP constructs. pSHBG-FL and pSHBG-MP were transfected into MDA-MB-231 cells. Following selection, 12 resistant colonies from each transfection were isolated and expanded. MDA-MB-231-MP7 was positive in Western blot analysis using the anti-Flag monoclonal antibody (**Figure 2A**), and cell extracts from 12 MDA-MB-231-FL clones have recently been prepared for analysis.

We also transfected pSHBG-FL and pSHBG-MP into MCF-7 cells, and expanded 12 individual clones from each transfection. Three MCF-7-FL clones had elevated amounts of SHBG protein (ELISA). Western blot analysis showed that MCF-7-FL10 had high expression of the Flag-tagged SHBG construct (**Figure 2B**). Furthermore, this clone secreted non Flag-tagged SHBG (**Figure 2B**), demonstrating that the leader peptide is properly processed from Flag-tagged SHBG-FL, and that the mature processed protein is secreted just like the secreted form of SHBG in the liver. We isolated 12 MCF-7-MP clones, and Western blot analysis showed that MCF-7-MP11 had high expression of the Flag-tagged SHBG-MP construct.

7. Construction of pSHBG-myc, a plasmid directing constitutive expression of carboxyl terminal myc-tagged SHBG. The SHBG-FL, amino terminal Flag-tagged protein, loses its Flag-tag upon cleavage of the amino terminal SHBG signal peptide prior to secretion. To obtain a secreted tagged-SHBG species that could be immunologically distinguishable from endogenously synthesized SHBG in MCF-7 cells, we synthesized pSHBG-myc. pSHBG-myc encodes a full length SHBG protein whose expression is driven by a constitutively active CMV promoter. The SHBG protein is tagged at its carboxyl terminus with a highly immunogenic peptide sequence derived from the human myc protein. Highly versatile antibodies directed against this myc tag are commercially available making it a valuable reagent for studying SHBG in breast cells.

To make this construct, we used the Xi-Clone system from Gene Therapy Systems (San Diego, Ca.). This system affords sequence specific cloning by taking advantage of the homologous recombination properties of a proprietary E. coli strain, therefore bypassing the need for a cloning strategy dependent on restriction sites. We designed SHBG specific PCR primers with Xi-Clone recombination sequences at their 5' ends. The forward primer had a run of amino terminal SHBG cDNA sequences following the recombination sequences. The reverse primer was longer and more complex. It contained Xi-clone recombination sequences, a stretch encoding the myc peptide, and then carboxyl terminal SHBG sequences. The forward and reverse Xi-Clone/SHBG primers were used to amplify SHBG cDNA sequences. The PCR product was purified to

remove unincorporated primers, and then was mixed with the linearized Xi-clone vector. These DNAs underwent homologous recombination following the transformation of the proprietary E. coli strain. Successful recombinant plasmids afforded kanamycin resistance. Over 30 kanamycin resistant colonies were isolated and plasmid DNAs were prepared and sequenced. pSHBG-myc23 contained proper sequences (often, mutations were found within the long 3' primer sequence, likely due to errors incorporated during synthesis of the primer), as was used for transfection into mammalian cells.

8. Generation of MCF-7 and HepG2 clonal cell lines that constitutively overexpress the pSHBG-myc23. MCF-7 and HepG2 cells were transfected with pSHBG-myc23, and pools of G418 colonies were obtained. The pools were screened by ELISA assay for secreted SHBG, and by Western blot for secreted and intracellular SHBG using both anti SHBG and anti-myc antibodies. Both MCF-7 and HepG2 pools were positive in all cases. To obtain clones that expressed high amounts of the pSHBG-myc23 construct, and that were pure populations of cells, the MCF-7 and HepG2 pools were plated at clonal density in 225mm cell culture dishes. From each dish, 24 individual colonies were isolated and expanded as cell lines. Cell lines were screened by immunostaining to identify those that expressed the SHBG-myc protein at high levels in all cells. We successfully identified MCF-7 SHBG-myc23-2 and HepG2 SHBG-myc23-13 as pure populations of cells that highly express the SHBG-myc construct. **Figure 3** shows the immunofluorescence of SHBG in MCF-7 SHBG-myc23-2 cells. These cells appear to have strong staining in the Golgi, as would be expected of secreted proteins, and also staining along what appears to be microfilaments. Further study of the staining pattern of these overexpressers is in progress. The successful generation of these cell lines will now allow us to use them as powerful reagents for examining SHBG uptake by MCF-7 cells, and for investigating proteins that interact with SHBG in MCF-7 cells.

9. MCF-7 cells express novel SHBG isoforms generated through alternative splicing of exons 4, 6, and 7.

The bulk of published data on SHBG gene expression at the mRNA level are based on work that was performed prior to the advent of PCR. It had been thought from early cDNA cloning experiments that two mRNA species are synthesized from the human SHBG gene (2-3). The first is the secreted form of SHBG, made in the liver. This 8 exon-long species is the isoform we have overexpressed above. The second SHBG isoform was originally described in the testis, and apart from a recent publication that shows it to be expressed in human sperm (5), it remains very poorly characterized. This isoform uses an upstream promoter and therefore has different first exon sequences. Early cDNA studies suggested that this isoform has exon 7 sequences spliced out.

We obtained data suggesting that SHBG gene expression in MCF-7 cells is different from that described above. We prepared first strand cDNA from total cellular MCF-7 RNA using oligo dT primers. Using primers that amplified exon 5-8 sequences, we unexpectedly generated three RT-PCR transcripts (**Figure 4**). Sequence analysis revealed that the largest transcript contained faithful splicing of exon 5,6,7,and 8 sequences. The intermediate transcript was missing all of exon 7 sequences. The smallest transcript was missing both exon 6 and 7 sequences.

We were unable to generate RT-PCR transcripts from MCF-7 cells using secreted SHBG exon 1 specific primers (**Figure 5**). As expected, HepG2 RT-PCR samples were positive for the secreted SHBG exon 1 transcript (6).

Next, we performed RT-PCR experiments using primers specific for SHBG exons 2 and 8. Because the SHBG gene had been reported to use two promoters, this should give us a composite picture of expression of the secreted and testis SHBG mRNA. As we later discovered, the SHBG gene contains a third, novel upstream promoter (see below). All evidence to date points to splicing of all different first exon species directly to exon 2, and termination occurring at exon 8. Therefore, the exon 2-8 RT-PCR amplification is a composite of SHBG expression derived from at least three promoters. **Figure 6, panel A** is an RT-PCR experiment using as template, first strand cDNA from HepG2, LNCaP, and MCF-7 cells, testis tissue, and an MCF-7 transfectant that constitutively overexpresses the full length secreted SHBG cDNA (MCF-7 FL-10 cells, see above). We identified, and confirmed by DNA sequence analysis, SHBG transcripts containing contiguous exon 2-8 sequences, as well as those lacking exon 7, 6 and 7, and 4, 6, and 7 sequences, respectively in HepG2, LNCaP, and MCF-7 cells, and in testis tissue. Only the full length contiguous exon 2-8 species is seen in the MCF-7 transfectant, because of the overwhelming abundance of the overexpressed full length SHBG construct. We are currently characterizing the additional bands present in the HepG2, LNCaP, and MCF-7 cells, and testis tissue lanes.

Next, we examined the expression pattern of exon 1 specific transcripts using exon 1 primers specific for (**Figure 6, panel B**) the secreted form of SHBG and (**Figure 6 Panel C**) the testis form of SHBG. HepG2 cells and normal liver express the contiguous exon 1-8 transcript, as well as those lacking exon 7, 6 and 7, and 4, 6, and 7 (very faint). Testis does not. The additional bands are currently undergoing characterization. The contiguous testis exon 1-8 transcript, as well as transcripts lacking exons 7, 6 and 7, and 4, 6, and 7 are present in LNCaP, MCF-7, and testis tissue. HepG2 mostly expresses the alternatively spliced testis transcript lacking exons 4, 6, and 7.

10. Discovery of a novel third human SHBG gene promoter.

We made an important and unexpected discovery that a novel, third human SHBG gene promoter exists, and is being used by MCF-7 and LNCaP cells. The 5' ends of SHBG gene transcripts were specifically amplified from LNCaP, MCF-7, and HepG2 cells by RT-PCR, through use of the FirstChoice RLM-RACE Kit (Ambion). Briefly, this kit invokes a series of enzymatic steps designed to allow only full length, capped RNA species to undergo ligation to a synthetic RNA adaptor of known sequence. cDNA synthesis is directed by a gene-specific oligonucleotide primer, and subsequent RT-PCR analysis is performed using an upstream adaptor-specific primer, and a nested downstream gene specific primer. In these experiments, an exon 3 specific oligo was used to direct cDNA synthesis for each of the cell lines, and RT-PCR analysis was performed using a primer set that included a nested downstream exon 2 specific primer. The resultant RT-PCR fragments (not shown) were sequenced, and consisted of unique 5'

sequences spliced directly to exon 2 sequences. The 5' sequences were mapped upstream of the prior two reported SHBG gene first exon sequences.

Figure 6, panel D is an RT-PCR experiment performed using a first exon primer specific for the novel upstream first exon sequences, and an exon 8 specific reverse primer. LNCaP cells express the novel exon 1-8 transcript, as well as transcripts lacking exons 7, 6 and 7, and 4, 6, and 7. MCF-7 cells express the transcript lacking exons 6 and 7.

11. Immunohistochemical analysis of SHBG protein expression in human breast cell lines and effects of steroid binding to SHBG on membrane localization.
SHBG is visualized clearly in the cytoplasm, but not the nucleus of MCF-7, MDA-MB-231, and 184-B5 cell lines **(Figure 7)**. Note that most of the heavy staining in MCF-7 cells is perinuclear **(Figure 7A)**. MCF-7 cells stain more intensely than the other lines and also (less apparent) show staining in a greater fraction of cells than do MDA-MB-231 or 184-B5 cells **(Figure 7B and 7C**). Nonpermeabilized MCF-7 cells showed membrane staining for SHBG, consistent with secretion of SHBG followed by binding to R_{SHBG} **(Figure 8A,B)**. How these observations fit with our finding that alternative SHBG transcripts are synthesized in MCF-7 cells is unclear at the moment, but is consistent with a protein that retains its membrane binding site. MDA-MB-231 cells showed little if any membrane binding in nonpermeabilized cells (data not shown).

The current model for steroid signaling through R_{SHBG} includes binding of steroid-free SHBG to R_{SHBG} as an initial step. Biochemical studies have shown that SHBG, prebound to steroid, is unable to bind to R_{SHBG}. To test this model in vivo using MCF-7 cells (known to possess R_{SHBG}), we asked whether specific steroids that bind to SHBG could displace it from the membrane (7). MCF-7 cells treated with purified SHBG showed a marked increase in membrane staining, and in intracellular SHBG **(Figure 8C)**. Cells treated with SHBG that had been preincubated with 2-methoxyestradiol ($2MeOE_2$), a steroid that binds tightly to SHBG, showed a marked decrease in membrane staining **(Figure 8D)**. These results support our observations using biochemical approaches (8). We then tested the effects of MCF-7 cells treated with SHBG that had been preincubated with dihydrotestosterone (DHT) or testosterone (T) (both of which are tightly bound to SHBG) **(Figure 8E and 8F)**. None of the steroids added in conjunction with SHBG appeared to have any effect on intracellular SHBG (permeabilized cells) (data not shown).

Although MDA-MD-231 cells have cytoplasmic SHBG, none is associated with the cell membrane, either from endogenous SHBG or added SHBG. These data indicated an absence of R_{SHBG} in these cells (Data not shown).

12. Immunohistochemical analysis of SHBG and in situ analysis of SHBG mRNA expression in normal human breast tissue and breast tumors.
In normal breast tissue, staining for SHBG is most intense in epithelial cells, predominating in luminal epithelial cells **(Figure 9A)**. Because there is a substantial concentration of SHBG in plasma, the staining seen in sections of human breast could result from simple diffusion of SHBG from plasma with subsequent cellular uptake. To address this issue, we examined sections for SHBG mRNA expression by *in situ*

hybridization. SHBG mRNA **(Figure 10A)** is detected and is located predominantly in luminal epithelial cells. Although SHBG protein and mRNA appear to colocalize in cells, normal breast tissue is heterogeneous, with areas of intense staining and areas of zero to light staining.

SHBG protein expression is abundant in cancerous areas of the breast **(Figure 9B)** with strong staining seen in periglandular epithelium. Epithelial cells, both normal and cancerous, stain strongly for SHBG mRNA, and there is minimal staining in stromal cells **(Figure 10B)**. These observations are consistent with a model in which SHBG is synthesized and secreted primarily by epithelial cells and binds to R_{SHBG} on epithelial and/or stromal cells.

13. Quantitation of SHBG gene expression. Our major work over the past year focused on two major aspects of local SHBG expression in breast cells. To better understand the biologic function of locally expressed SHBG, we devoted much effort to a detailed characterization of SHBG gene expression.

Over the last year, we pursued our initial findings that the SHBG gene has a much more complex transcription pattern than was previously reported. Previously, two major human SHBG genes transcripts had been reported (2,3)(**Figure 11A**), one encoding the secreted form of SHBG and the second encoding an alternative transcript lacking exon 7 that originated from a separate promoter and was most abundant in testis. We now have a more comprehensive overall picture of how the human SHBG gene is expressed (**Figure 11B**).

The SHBG gene has at least three active promoters. The downstream promoter regulates expression of at least eight different SHBG transcripts, including the secreted form of SHBG found in plasma. The intermediate promoter, located 2 kb upstream, regulates expression of at least four main SHBG transcripts, including the major species found in testis. The third, and novel promoter discovered as a part of these studies, is located a further15 kb upstream, and we have found it to regulate expression of at least four main different SHBG transcripts. The two upstream promoters map to promoters for genes that are adjacent to the SHBG gene, and are transcribed in the opposite direction. Apparently, structural and/or transcription factors allow the SHBG gene to utilize these promoters, making them bi-directional. Sequence analysis of the transcripts that originate from the novel, third upstream promoter, reveals a first exon that, after encountering the first ATG codon, contains a nine amino acid open reading frame, followed by a termination codon. It is curious that the termination codon is followed by a long open reading frame that merges within exon 2 with the open reading frame of the secreted form of SHBG. We are very interested in understanding whether translation stops at this termination codon, or if the translation machinery can hiccup past.

Real-time PCR experiments were performed to quantitate SHBG transcriptional activity in the MCF-7, 184, and MB231 cancer cell lines and normal breast tissue. Expression in the prostate was compared to the liver (HepG2 cancer cell line and normal liver tissue), testis (testis tissue), and prostate (LNCaP cancer cell line and normal prostate tissue).

Total SHBG gene transcription was measured by quantitative PCR using primers specific for exons 2 and 3, both of which are conserved amongst all known SHBG gene transcripts (**Figure 12A**). The highest SHBG expression levels were in testis, the HepG2 cell line, and normal liver tissue. Normal breast tissue had just over a two-fold higher expression level than normal prostate tissue, and SHBG expression was also present at similar levels in the MCF-7, and MB231 breast cell lines and slightly higher in the 184 cell line. LNCaP cells had approximately a seven-fold higher expression level than normal prostate tissue. These results are to be expected from the physiology of SHBG. The liver needs to make large amounts of SHBG to populate the plasma; the testes needs to secrete relatively large amounts of SHBG into the tubules; the prostate and breast, on the other hand, need make only small amounts for local, e.g. paracrine or autocrine use.

SHBG gene expression arising from each of the three individual SHBG gene promoters was quantitated by real-time PCR (**Figure 12B**). Three primer sets were designed, each with an upstream primer specific for either exon 1L, 1T, or 1N, and a downstream primer specific for exon 2. Normal breast mostly expresses 1L transcripts, though in much lower (60-fold) amounts when compared to the liver. This result is consistent with our hypothesis that locally produced SHBG has an autocrine or paracrine effect on R_{SHBG} signaling within the breast. In normal breast,1L transcripts are expressed at 5- and 20-fold higher levels, respectively than 1T and 1N transcripts. The MB-231 cell line expresses the highest total amount of SHBG mRNA, 5-fold that of MCF-7 and 8-fold that of 231 cells. MCF-7 cells mostly express1T containing transcripts, 2-fold more than 1N transcripts, and 10-fold more than 1L transcripts. 184 cells express similar amounts of the three, as do MB-231 cells.

Promoter utilization by the SHBG gene can show tissue specificity (**Figure 12B**). HepG2 cells and normal liver tissue have abundant expression of 1L transcripts, and the greatest expression of 1T transcripts was in normal testis tissue. LNCaP cells expressed the highest level of 1N transcripts. Interestingly, 1N transcripts are exceedingly scarce in the liver. It appears that tumor cell lines express more 1N transcripts than the normal tissue from which they were derived. Whether 1N transcripts contribute to the malignant phenotype or whether their increased expression results from general genomic instability and deregulation of gene expression in cancer cell lines remains to be determined.

14. Regulation of gene expression in LNCaP cells by SHBG.

Our second major focus during the last year of this study has been to determine whether SHBG expression can affect overall gene expression in MCF-7 cells. In our initial proposal, we outlined a series of reporter assays that would be used to determine the effects of SHBG expression on a few androgen responsive genes and constructs that were sensitive to PKA activation. The availability of microarray technology at rapidly falling costs, made microarray analysis a preferable approach for determining the effects of SHBG on gene transcription. As reported in section 7, we generated the MCF-7 clonal cell line, MCF-7 SHBG-myc23-2, that constitutively overexpresses high amounts of SHBG that is tagged at its carboxyl terminus with a myc-tag.

Parental MCF-7 and MCF-7 SHBG-myc23-2 cells were seeded in medium containing 10% charcoal stripped fetal calf serum, and incubated for 48 hours. Each cell line was treated in triplicate for four, eight, 12, 24, or 48 hours with 10nM estradiol, or with the carrier ethanol.

This strategy gave us the following treatment conditions (each condition was performed in triplicate)-

1. MCF-7 negative treatment control (carrier treated only)*
2. MCF-7 estradiol treated 4 hours*
3. MCF-7 estradiol treated 8 hours
4. MCF-7 estradiol treated 12 hours
5. MCF-7 estradiol treated 24 hours*
6. MCF-7 estradiol treated 48 hours

7. MCF-7 SHBG-myc23-2 negative treatment control (carrier treated only)*
8. MCF-7 SHBG-myc23-2 estradiol treated 4 hours*
9. MCF-7 SHBG-myc23-2 estradiol treated 8 hours
10. MCF-7 SHBG-myc23-2 estradiol treated 12 hours
11. MCF-7 SHBG-myc23-2 estradiol treated 24 hours*
12. MCF-7 SHBG-myc23-2 estradiol treated 48 hours

Total cellular RNA was prepared from each of the above triplicate samples.

Due to the expense of microarray analysis, we chose to analyze only one sample from each of the treatment conditions denoted above with a "*". In addition, we analyzed single samples from HepG2 liver cancer cells and a constitutive SHBG overexpressing clone, HepG2myc23, as well as from an inducible LNCaP cell line, L5S2 and its sister control, L5V4. breast cancer cells treated with 10nM DHT for 4 or 24 hours. Each total cellular RNA sample was pretreated with RNase-free DNase. Total cellular RNAs were sent to our colleagues at the Yerkes Genomics Core Facility at Emory University. RNAs were analyzed by Agilent Bioanalyzer to check the RNA qualities, all of which passed stringent controls. Samples were labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 Array chips. Raw data was collected by GCOS software and analysis of the microarray data was performed using GeneSpring software. The quality of data, as measured by internal controls and housekeeping genes, was excellent.

A general view of the scope of this work is presented in **Figure 13**, which shows the relationship between the 16 samples based on their gene profiles. As expected, there are clusters between the MCF-7, HepG2, and LNCaP cells. Furthermore, parental MCF-7 samples cluster together, as do the constitutive SHBG overexpressing MCF-7myc23 samples. Similarly, the inducible LNCaP cell line L5S2 samples cluster together, as do the L5V4 vector control samples.

We are currently performing a detailed analysis of the expression data to determine which genes are specifically regulated by SHBG alone, and by activation of the steroid-

SHBG-R_{SHBG} signaling pathway. In **Figures 14A-4C**, we present a preliminary analysis of our raw data, showing those genes whose expression displays the greatest induction, or repression in MCF-7myc23 cells compared to parental MCF-7 cells. From these preliminary studies, we have identified genes whose expression increases or decreases by a factor of at least 15-fold in MCF-7 cells as a result of SHBG expression, in the absence or presence of estrogen. These genes can be grouped into three sets, a) those which respond to SHBG alone (**Figure 14A**), b) those exhibiting a dampened response to ER activation by estrogen (**Figure 14B**), and c) those responsive to estrogen activation of the SHBG-R_{SHBG} signaling pathway (**Figure 14C**). Our preliminary work suggests that, along with novel genes that we have detected, SHBG regulates, known genes that are involved in various aspects of cell signaling, transcription, and breast biology. We are specifically excited about our finding that a G-coupled protein receptor, GPCR, family C group 5, shows reduced expression in SHBG overexpressing cells following treatment with estradiol whereas its expression remains unaffected in parental MCF-7 cells treated with estradiol (**Figure 14C**). This observation is consistent with a scenario in which transient activation of the SHBG-R_{SHBG} signaling pathway downregulate expression of R_{SHBG} through a negative feedback pathway. From these results, we have deemed GPCR, family C group 5 a R_{SHBG} candidate. We have already cloned the cDNA encoding GPCR, family C group 5 R_{SHBG} into a mammalian expression vector. We plan to overexpress this protein in MCF-7 cells to ascertain whether it exhibits properties attributable to R_{SHBG}. In summary, these preliminary microarray results support our hypothesis that SHBG exerts biologic effects in MCF-7 cells, both alone and in response to estrogen treatment through the ER and SHBG-R_{SHBG} signaling pathways. Given the fact that the SHBG gene is located at a locus that is often deleted in breast cancer cells, these findings are both exciting and provocative.

A more detailed analysis of our raw data should reveal whether members of the PKA pathway, specifically, and other signaling pathways in general are affected by conditions that activate R_{SHBG}. Furthermore, we are in the process of comparing those genes that are induced by SHBG alone in MCF-7 cells are also affected in HepG2 or LNCaP cells. These findings will be confirmed by quantitative PCR experiments.

We delayed our functional analysis of the effects of SHBG expression in breast cells on steroid signaling because of our novel findings that MCF-7 cells display complex expression of the SHBG gene. MCF-7 cells do not appear to make the mRNA encoding the secreted isoform that we proposed as an integral part of this study. Though we made inducible and constitutively active SHBG constructs that were successfully introduced and expressed in MCF-7 cells, our novel results suggest this does not reflect the physiologic state of SHBG expression in the human breast. Therefore, despite the availability of these cell lines, we focused on fully characterizing SHBG gene expression in MCF-7 cells and in the breast at the mRNA level. The inducible and constitutive cell lines that we produced for this study will be highly useful for modeling experiments on SHBG binding to R_{SHBG} and internalization of plasma SHBG. These cell lines secrete SHBG into the media at high concentrations (over 200 nM, ELISA results, data not shown).

Our immunohistochemistry data showing SHBG staining in normal breast, breast tumor tissue, and breast cancer cell lines begs the question of what SHBG isoform is being recognized. We know from staining of MCF-7 cells that they are expressing their own SHBG. However, we do not know the identity of the SHBG isoform(s) being expressed at the protein level. Although in breast tissue we detect both SHBG mRNA and protein, we may need to modify our original hypothesis that the secreted form of SHBG is being synthesized endogenously. We are currently addressing the question of which SHBG isoform is being detected by our antibodies in these experiments. If it is one or more of the novel SHBG isoforms, we will address its biology. If it is the secreted form of SHBG, then we have to address the possibility that breast tissue actively internalizes SHBG from plasma.

Our results from this study have painted a new and exciting picture of extrahepatic expression of the SHBG gene. Before, it was assumed that two major SHBG transcripts existed, that which encodes the secreted form of SHBG and that which encodes the exon 7 lacking testis form. We now know that there are at least 16 different SHBG transcripts made by human cells that result from alternative splicing and alternative promoter utilization. We have shown that our system of overexpression works, and we are now set up to perform similar experiments to address the functions of these additional SHBG gene transcripts. We expect that because these novel, alternatively spliced transcripts lack certain structural domains, they may not bind steroid, dimerize, or be secreted. It is important to note, however, that they do retain reported R_{SHBG} binding domain sequences. Our future studies and grant applications will address these biologic questions. Furthermore, because the SHBG gene lies in a hotspot for chromosomal deletion in breast cancer cells, we will address how decreased expression might be involved with cancer progression.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of plasmid constructs encoding the full length human SHBG cDNA, in the sense and antisense orientations
- Performed immunohistochemical and in situ analyses of SHBG protein and mRNA expression in breast cell lines, normal breast tissue, and breast tumors.
- Generation of the intermediate cell line MCF7-pVgRXR 13, which has been stably transfected with the plasmid, pVgRXR. This cell line shows no leakiness and has a high degree of induction when transiently transfected with the β-galactosidase reporter plasmid, pIND/Hygro/LacZ.
- Identification of the anti-human SHBG polyclonal antibody, WAK-S102-12-53, for use in Western blot analysis.
- Generation of M13S14, an MCF-7 subclone engineered to inducibly express the hepatic, secreted form of SHBG. Generation of M13V7, a control MCF-7 subclone.

- Synthesis of pSHBG-FL and pSHBG-MP, flag tagged full length and mature processed SHBG constructs for constitutive expression. Generation of 12 candidate MDA-MB-231-FL clonal cell lines, and MDA-MB-231-MP7 that constitutively expresses a stably incorporated flag tagged mature processed SHBG construct. Generation of MCF-7-FL10 which constitutively expresses a stably incorporated flag tagged full length SHBG. Generation of MCF-7-MP11 which constitutively expresses a stably incorporated flag tagged mature processed SHBG construct.
- Demonstration that elevated amounts of SHBG can be made in MCF-7 and MDA-MB-231 cells, and that MCF-7 overexpressing cells secrete SHBG. Demonstration that the flag tagged mature processed SHBG protein is stable and remains intracellular.
- Synthesis of pSHBG-myc, a carboxyl terminal myc-tagged SHBG construct.
- Generation of MCF-7-SHBG-myc23-2 and HepG2-SHBG-myc23-13, clonal cell lines that express and secrete myc-tagged SHBG.
- MCF-7 cells exhibit a complex alternative splicing pattern, thereby generating multiple SHBG mRNA species. We also discovered a third, novel upstream SHBG gene promoter.
- Each of the three human SHBG gene promoters gives rise to four main SHBG transcripts, the largest containing contiguous exon 1-8 sequences, and alternative splicing resulting in transcripts that lack exons 7, exons 6 and 7, and exons 4, 6, and 7. Expression of these transcripts appears to be tissue dependent, and cell line dependent.
- Sequence analysis revealed that transcripts derived from the novel upstream promoter encode a short, nine amino acid long peptide, unless these transcripts undergo a novel form of translation.
- The discovery of 1L-containing transcripts that lack exon 4, which contains steroid binding and dimerization domains, is significant. This transcript retains the secretion signal peptide in exon 1L, and the receptor-binding domain within exon 3. If translated, this isoform could be involved in the local regulation of steroid signaling through R_{SHBG}.
- SHBG protein is expressed in the cytoplasm of the MCF-7, MDA-MB-231, and 184-B5 cell lines. In MCF-7 cells, SHBG protein is strikingly perinuclear.
- Immunohistochemical results demonstrate SHBG binding to the membrane of MCF-7 cells, but not MDA-MB-231 cells, suggesting the latter lack R_{SHBG}. MCF-7 membrane binding can be displaced by preincubation of SHBG with 2-methoxyestradiol, T, and DHT.
- In normal breast tissue, staining for SHBG is most intense in epithelial cells, predominating in luminal epithelial cells. SHBG mRNA colocalizes to these same cells, suggesting that in vivo, endogenously synthesized SHBG mRNA is translated and expressed. Normal breast tissue itself appears heterogeneous, with areas of intense staining and areas of zero to light staining.
- SHBG protein expression is abundant in cancerous areas of the breast with strong staining seen in periglandular epithelia. Strong staining for the SHBG mRNA was seen in epithelial cells as well as in carcinoma cells infiltrating the stroma adjacent to the epithelial cells.

- Demonstration that human SHBG gene expression differs between liver, testis, prostate and breast tissue.
- Demonstration that human SHBG gene expression differs between HepG2 liver, LNCaP prostate, and MCF-7 breast cancer cell lines.
- Demonstration that SHBG expression in cancer cell lines can differ from corresponding normal tissue.
- Quantitation of SHBG gene expression arising from each of three human SHBG gene promoters in LNCaP, HepG2, MCF-7, 184, MB231 cell lines and in normal prostate, testis, liver and breast tissue.
- SHBG alone is a potent stimulator/repressor of a number of genes. This new and exciting finding opens up new ways in which to think about how signaling through this pathway occurs.
- SHBG affects DHT induction of genes in LNCaP cells after both 4 hours and 24 hours of DHT treatment. These result are consistent with our main hypotheses posed at the beginning of these studies, namely that SHBG modulates steroid signaling through R_{SHBG} and that SHBG can act as an intracellular buffer for steroids. We are analyzing our microarray data in detail to determine which genes may be modulated by R_{SHBG}, and which androgen responsive genes may be affected by the presence of SHBG in LNCaP cells.

REPORTABLE OUTCOMES:

1. Fourth International Symposium on Hormonal Carcinogenesis. Valencia, Spain, June 21-25, 2003

Poster presentation and book chapter:

IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

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Immunohistochemical and in situ detection of sex hormone-binding globulin (SHBG) expression in breast and prostate cancer: Implications for hormone regulation. "Hormonal Carcinogenesis, vol. 4." (2005) Jonathan Li, Sara Li, Antonio Llombart-Bosch, editors. Springer, publisher. pp.508-514

2. Third International Meeting- Rapid Responses to Steroid Hormones. Florence, Italy, Sept. 12-14, 2003

Poster presentation:

IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

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3. Poster Presentation- Era of Hope Conference. Philadelphia, Pa. June 8-11, 2005

HUMAN SEX HORMONE-BINDING GLOBULIN (SHBG) GENE EXPRESSION: UTILIZATION OF MULTIPLE PROMOTERS AND COMPLEX ALTERNATIVE SPLICING OF TRANSCRIPTS

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4. Poster Presentation- Society for Basic Urologic Research Conference, Miami, Fla. December 1-4, 2005

HUMAN SEX HORMONE-BINDING GLOBULIN (SHBG) GENE EXPRESSION: UTILIZATION OF MULTIPLE PROMOTERS AND COMPLEX ALTERNATIVE SPLICING OF TRANSCRIPTS

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CONCLUSIONS:

We have shown that MCF-7 and MDA-MB-231 cells can be used to produce the hepatically secreted isoform of SHBG. From our initial RT-PCR results, we expected that this was the major isoform expressed in breast cells. However, based on our newer RT-PCR results, this is not the case. Alternative splicing and alternative promoter utilization results in the expression of mRNAs encoding potentially novel SHBG isoform(s) in MCF-7 cells. If this is also the case in normal breast epithelial cells in general, this could change our prior hypothesis of how allelic deletions of the SHBG gene locus could contribute to breast cancer. The distinct perinuclear immunohistochemical staining of SHBG seen in MCF-7 cells is striking, and raises new questions about SHBG function. SHBG also affects overall gene expression in MCF-7 cells, and we have data that suggests it can do this on its own, through R_{SHBG}, or through modulation of ER activation.

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APPENDICES:

Figure 1. SHBG overexpression is induced by PonA in M13S14 cells

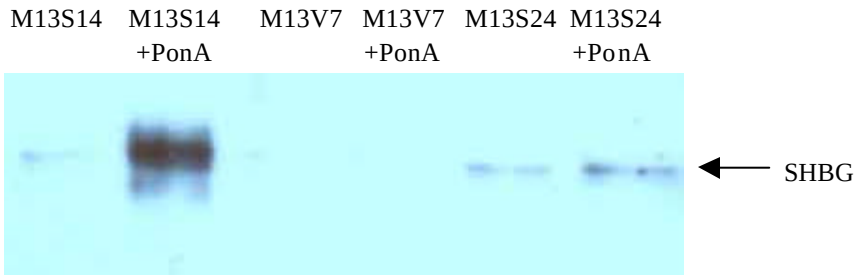


Figure 1: PonA induces SHBG protein expression in MCF-7 cells stably transfected with pVgRXR (a plasmid encoding the PonA-activatable transactivator), and the inducible construct, pIND/Hygro/SHBGsense (a plasmid that expresses the secreted form of SHBG in response to the activated transactivator). M13S14, M13V7, and M13S24 cells were plated in duplicate in 6 well dishes at 75% confluence. After incubating for 48 hours, cells in one well were exposed to 10uM PonA for 24 hours, while control, unexposed cells were mock treated with solvent. Total cellular protein was prepared and analyzed by Western blot. Low amounts of SHBG were visible on the original film in the M13V7 lanes. Parental M13 cells showed similarly low expression of endogenous SHBG, on a par with untreated M13V7 cells, and were not affected by PonA treament

Figure 2. Western blot analysis of SHBG overexpression in MB-MDA-231 and MCF-7 clonal cell lines

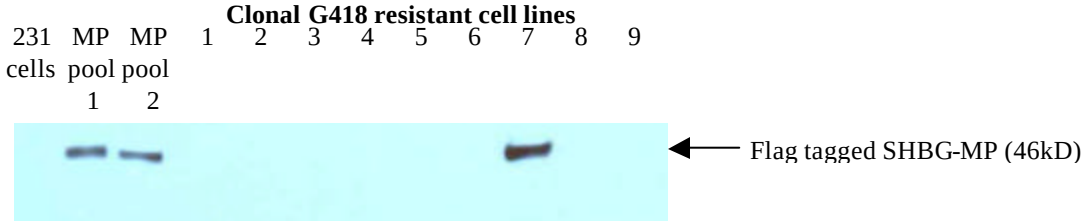


Figure 2A: MB-MDA-231-MP7 cells (MB-MDA-231cells stably transfected with pSHBG-MP, a construct that encodes a Flag-tagged, mature, processed form of SHBG) constitutively overexpress Flag-tagged SHBG-MP. Total cellular protein extracts were prepared from two MB-MDA-231 transfectant pools and candidate MB-MDA-231-MP clonal cell lines. Western blot analysis, shown above, was used to detect Flag tagged SHBG-MP expression using the Anti-Flag M2 monoclonal antibody (Sigma).

Figure 2B: M13FL10 (MB-MDA-231 cells stably transfected with pSHBG-FL, a construct that encodes a Flag tagged, full length secreted form of SHBG) cells secrete SHBG. M13, M13FL5, M13FL6, and M13FL10 cells were grown in 6 well dishes for 4 days in 1ml of serum-free medium. Cells appeared viable after this period of time. Conditioned medium was collected and centrifuged to remove cells and cellular debris. 30ul aliquots of conditioned medium were analyzed by Western blot, left, using the anti-human SHBG WAK-S102-12-53 antibody. Right, Western blot analysis of M13FL10 using the Anti-Flag M2 monoclonal antibody (no band was apparent). Sample on the right was from the same original Western blot shown as in Figure 2A.

Figure 3. Expression of SHBG-myc in MCF-7-SHBG-myc23-2 cells

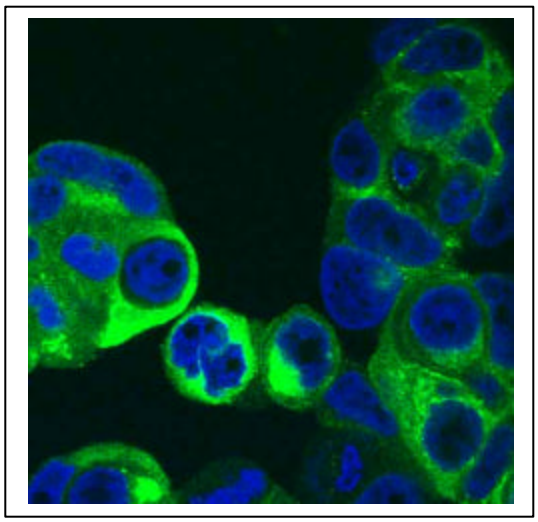


Figure 3. Immunohistochemical detection of SHBG in MCF-7 SHBG-myc23-2 cells. Cells were plated on glass slides, fixed, and exposed to a polyclonal antibody (WAK-S102-12-53) to SHBG and developed with a donkey anti-rabbit IgG1 linked to the green fluor, Alexa -488. Nuclei were stained with the blue dye, DAPI. Shown is a representative 1 micron confocal section. Note, the staining of these overexpressing cells for SHBG-myc is much more intense than the above staining for endogenous SHBG.

Figure 4. SHBG is alternatively spliced: RT-PCR analysis of MCF-7 cells.

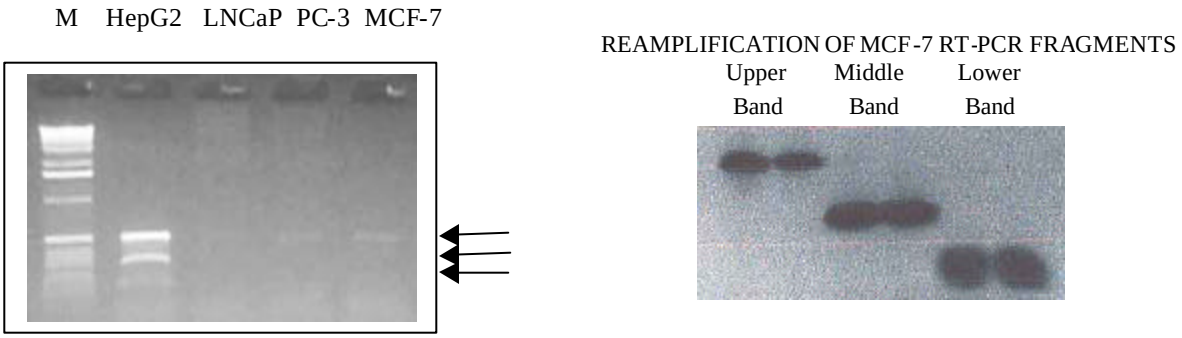


Figure 4. RT-PCR analysis reveals alternative splicing in MCF-7 cells. Left: Total cellular mRNA was prepared from exponentially growing MCF-7, HepG2 (a human hepatic cell line that secretes SHBG and was included as a positive control), LNCaP, and PC3 cells. First strand cDNAs were generated using an oligo dT primer. PCR was then performed using primers predicted to amplify exon 5-8 sequences. Three RT-PCR transcripts were generated in all samples, the predicted 521nt band, and two smaller bands (three light MCF-7 bands (arrows) were visible in the original gel). We have reproduced this alternative splicing pattern three times, using different MCF-7 total cellular RNA preparations. Right: MCF-7 RT-PCR products were electrophoresed alone in a single gel (not shown). DNA from each MCF-7 band was reamplified using the same exon 5 and 8 primers. Duplicate lanes were run for each sample (right), and DNA was extracted from the gel. DNA sequence analysis showed the 521nt band to contain contiguous exon 5-8 sequences. The middle band is mis sing all of exon 7, and the small band is missing exons 6 and 7. M: DNA size marker.

Figure 5. MCF-7 cell SHBG mRNAs lack hepatically secreted isoform exon 1.

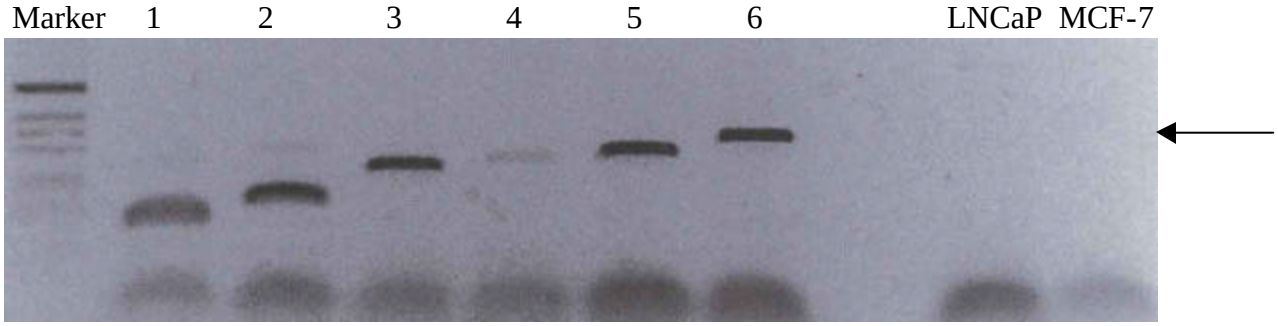


Figure 5. RT-PCR analysis of SHBG exon 1 sequences. The same first strand cDNA samples from Figure 4 were amplified by PCR. Control HepG2 RT-PCR amplifications were performed using a single exon 1 forward primer and six different exon 2 (lanes 1 and 2) or exon 3 (lanes 3-6) reverse primers in order of increasing fragment size. LNCaP and MCF-7 RT-PCR amplifications were performed using the same exon 1 forward and a single exon 3 reverse primer (the same set as in HepG2 lane 6). RT-PCR transcripts of expected sizes are present in all HepG2 amplifications. The exon 1-3 RT-PCR fragment is absent in MCF-7 and LNCaP cells (arrow).

Figure 6. Alternative SHBG gene promoter utilization and alternative splicing of SHBG exons 4, 6, and 7 in MCF-7, HepG2, and LNCaP cells, liver and testis.

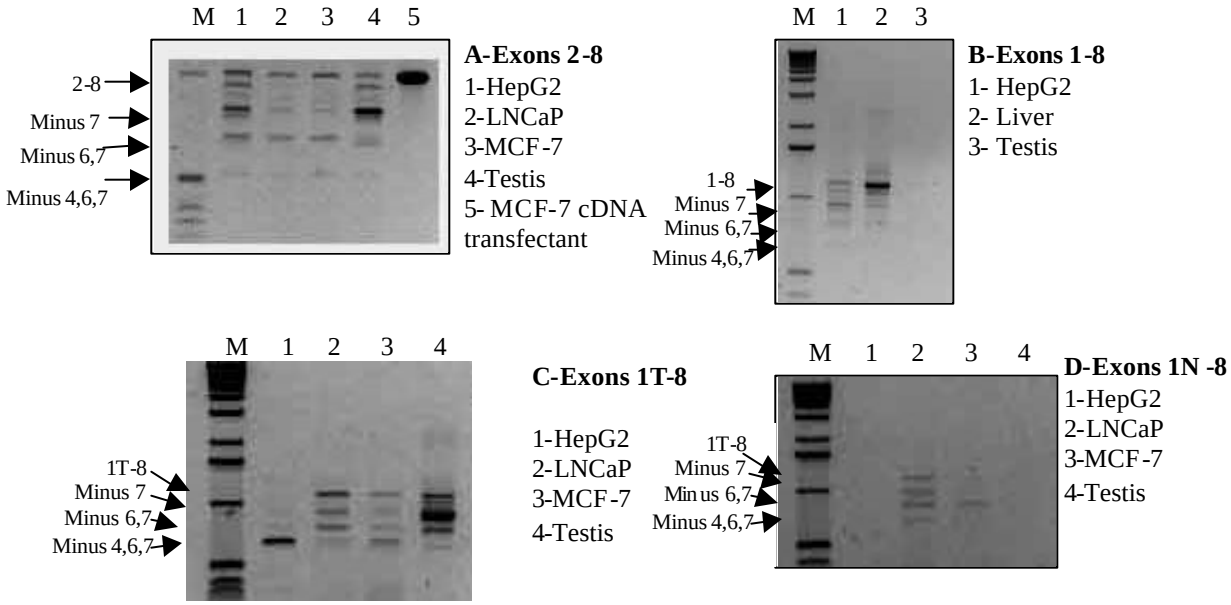
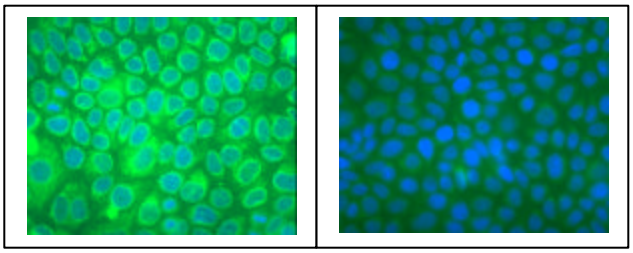
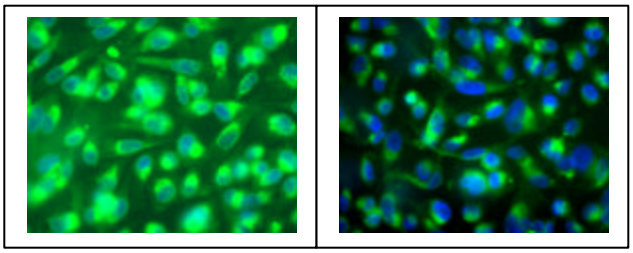


Figure 6. Expression of SHBG transcripts in human cancer cell lines, testis and liver. First strand oligo-dT primed cDNAs from the indicated cell lines and tissues were subject to RT-PCR amplification using primers specific for the SHBG exons shown in boldface (forward primers specific for, A- exon2, B- secreted isoform exon 1, C- testis isoform exon 1, D- novel exon 1 discovered in this study. The reverse primer for all amplifications was specific for exon 8. M- DNA Marker. Arrows point to RT-PCR fragments which have undergone sequence analysis and lack the exons denoted on the left.

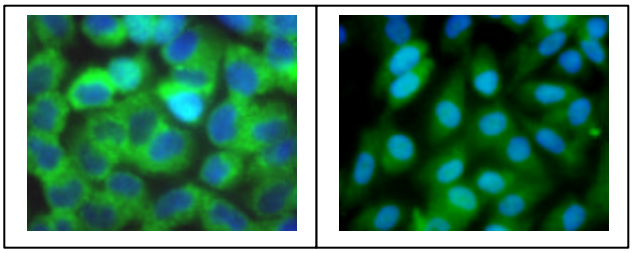
Figure 7. Immunohistochemical detection of SHBG in MCF-7, MD-MBA-231, and 184-B5 cells



A: MCF-7 cells - SHBG MCF-7 cells - IgG1



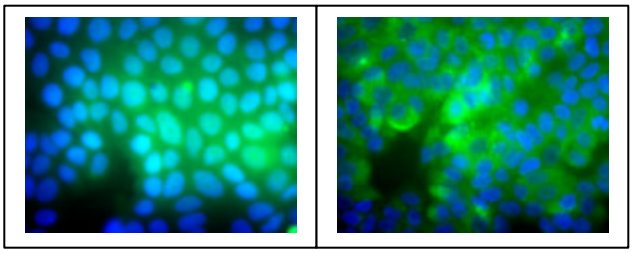
B: MD-MBA-231 cells - SHBG Ab MD-MBA-231 cells - IgG1 control



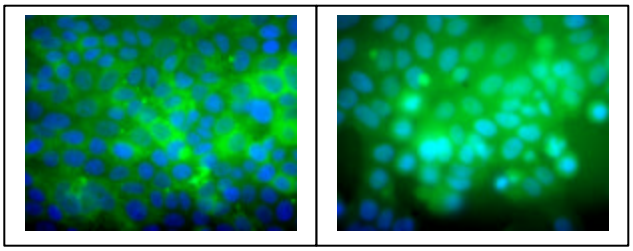
C: 184-B5 cells - SHBG Ab 184-B5 cells - IgG1 control

Figure 7. Immunohistochemical detection of SHBG in MCF-7, MD-MBA-231, and 184-B5 cells. Cells were plated on glass slides, fixed, and exposed to a monoclonal antibody (5B2) to SHBG (left panels) or mouse IgG1 (right panels), and developed with a rabbit anti-mouse IgG1 linked to the green fluor, Alexa - 488. Nuclei were stained with the blue dye, DAPI.

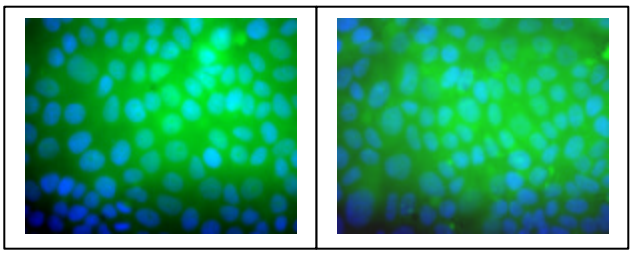
Figure 8. SHBG binding to the MCF-7 cell surface is displaced by specific steroids



A: nonpermeabilized: B: nonpermeabilized: SHBG Ab



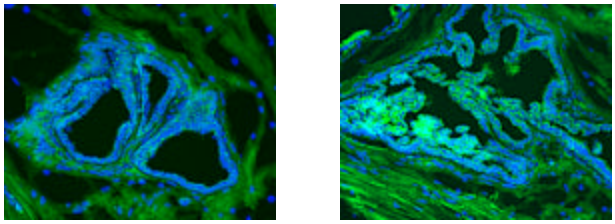
C: + added SHBG D: + added SHBG/ +2MeOE2



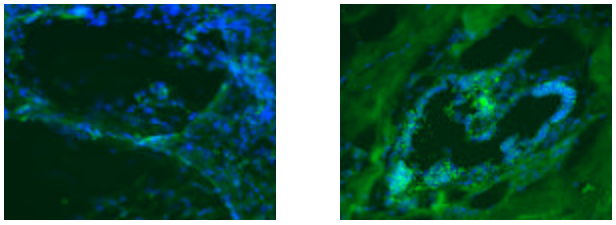
E: + added SHBG/ +DHT F: + added SHBG/ +T

Figure 8. SHBG binding to the MCF-7 cell surface is displaced by steroids that bind to SHBG. MCF-7 cells, grown in serum free medium, were fixed and treated in the absence of the permeabilizing agent, Triton X-100. Cells were incubated in the absence of added SHBG (A, B), in the presence of added SHBG alone (C), or with SHBG and the indicated steroid (D-H). Cells were visualized with a monoclonal antibody (5B2) to SHBG (green) and were counterstained with DAPI (blue). The antibody control was mouse IgG1.

Figure 9. Immunohistochemical analysis of SHBG protein in normal breast and tumor tissue



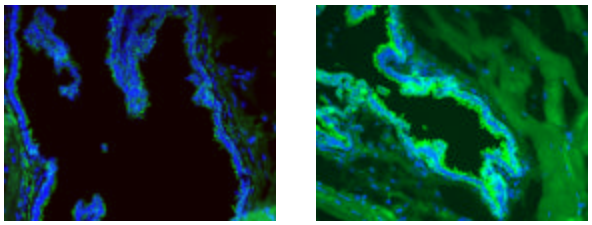
A: Normal tissue- IgG1 control Normal tissue- SHBG Ab



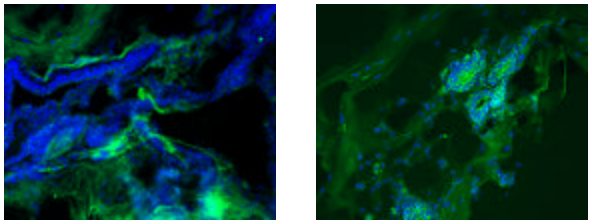
B: Tumor tissue- IgG1 Tumor tissue- SHBG Ab

Figure 9. Immunohistochemical analysis of SHBG protein in normal breast and breast tumors. Sections of frozen samples of normal human breast tissue (A), and tumor tissue (B) stained with either IgG1 (control-left panels), or with rabbit anti-SHBG (FITC, green-right panels). Sections were counterstained with the nuclear stain, DAPI (blue).

Figure 10. In situ analysis of SHBG mRNA in normal breast and tumor tissue



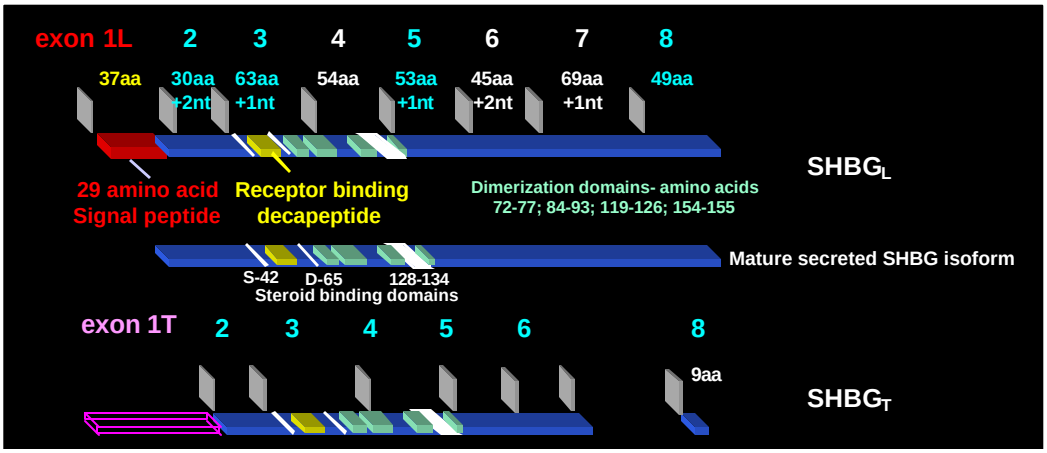
A: Normal tissue- control Normal tissue- SHBG



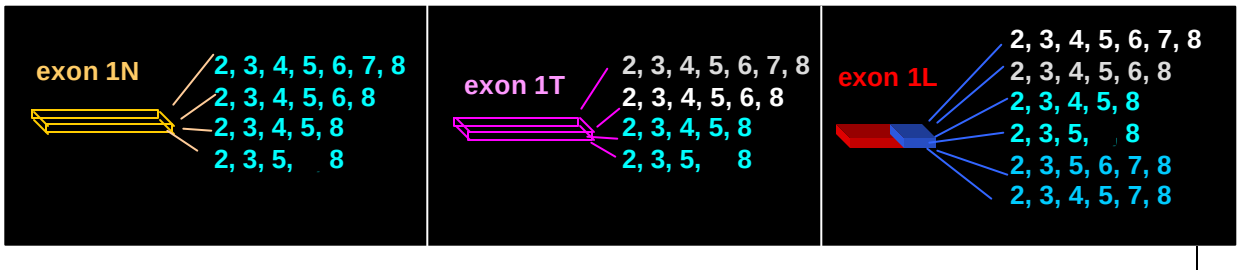
B: Tumor tissue- control Tumor tissue- SHBG probe

Figure 10. In Situ hybridization of SHBG mRNA in normal breast and tumor tissue. Sections were incubated with a 521 bp (corresponding to the secreted SHBG cDNA fragment from nt.628-1148) SHBG probe. SHBG-FITC probe, green; nuclear counterstain, DAPI, blue.

Figure 11. Discovery of novel SHBG gene mRNA transcripts.



A. Exon structure and functional domains of the two previously described major human SHBG transcripts, SHBG$_L$ and SHBG$_T$. SHBG$_L$ is the major mRNA synthesized in the liver, and encodes the secreted form of SHBG found in the plasma. SHBG$_L$ is eight exons long (exon number and length in amino acids is given above) and contains a 29 amino acid leader sequence (red) which is encoded within exon 1L. The leader sequence is cleaved prior to the secretion of the mature form of SHBG. Mature SHBG exists in the plasma as a homodimer, which is mediated by sequences within exons 3 and 4 (green). Steroid binding occurs in a pocket bounded by amino acids within exons 3 and 4 (white). The R_{SHBG} binding site is within the decapeptide sequence found in exon 3 (yellow). SHBG$_T$ is the major SHBG transcript found in the testis. It is transcribed from a promoter that lies approximately 2kb upstream of the SHBG$_L$ promoter. SHBG$_T$ contains different exon 1 sequences (purple) and lacks a signal peptide. Because SHBG$_T$ lacks exon 7 sequences, a frameshift occurs within exon 8, thereby creating an alternative reading frame at the carboxyl terminus consisting of nine amino acids.



B. Schematic representation of 14 currently known human SHBG mRNAs. Shown are the exon structures of SHBG transcripts derived from the novel upstream promoter (left), the SHBG$_T$ promoter (middle), and the SHBG$_L$ promoter (right). In total, we have detected 14 SHBG gene transcripts, four of which have been described in the literature, and ten of which are novel. Four novel SHBG transcripts contain exon 1N (left, light blue), a full length transcript containing contiguous exon 2-8 sequences, and three additional alternatively spliced transcripts lacking exons 7, 6 and 7, and 4, 6, and 7, respectively. Four SHBG transcripts contain exon 1T (middle), including the major exon 7-lacking transcript (white), a previously described full length minor transcript (gray) and two novel alternatively spliced transcripts that lack exons 6 and 7, and 4, 6, and 7, respectively (light blue). Six SHBG transcripts contain exon 1L, including the major full length transcript encoding SHBG$_L$ (white), a previously described minor transcript lacking exon 7(gray), two novel transcripts which have been confirmed by DNA sequence analysis to lack exons 6 and 7, and 4, 6, and 7, respectively (light blue), and two additional minor transcripts that we have detected by RT-PCR analysis and are currently presumed to lack exons 4 and 6, respectively (blue).

Figure 12. Quantitation of Human SHBG Expression in Cell Lines and Tissues

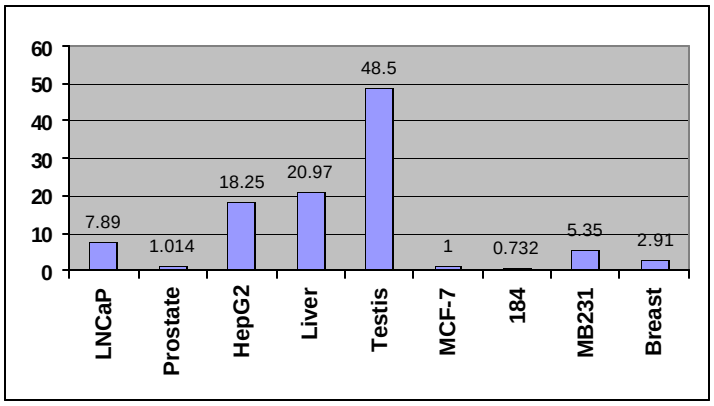


Figure 12A- Relative overall SHBG expression levels in human cell lines and tissues. Quantitative RT-PCR was performed using primers specific for conserved SHBG transcript regions within exons 2 and 3. Average expression levels are given above the data bars, and are normalized to MCF-7 expression levels. Experiments were performed in triplicate, on either two or three RNA samples.

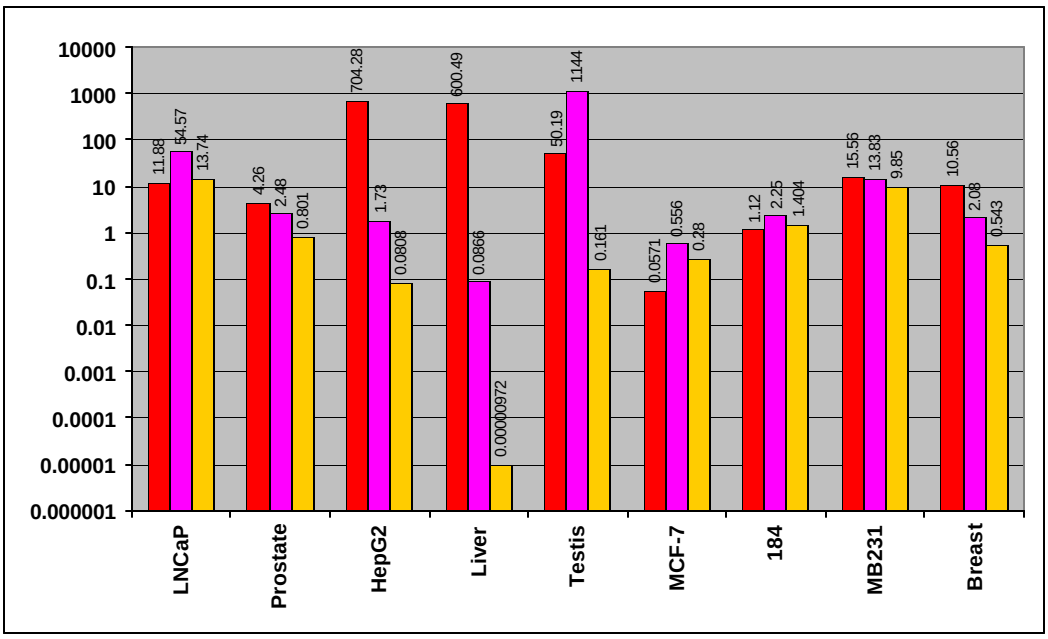
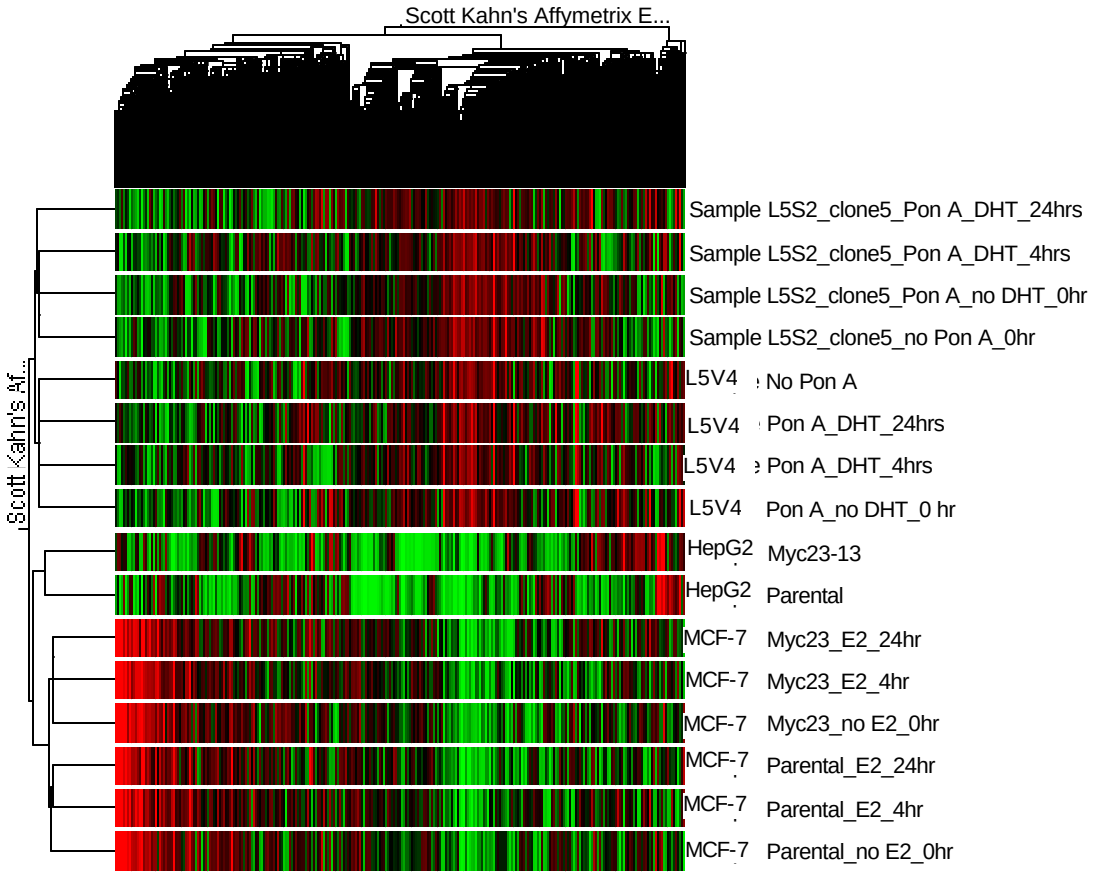


Figure 12B SHBG gene promoter utilization. Quantitative RT-PCR was performed on human cell lines and tissues to determine relative expression of SHBG gene transcripts originating from the downstream promoter (PL, red), the intermediate promoter (PT, pink), and the novel upstream promoter (PN, orange). Primer sets were specific for SHBG exons 1L and 2, 1T and 2, and 1N and 2, respectively. Experiments were performed in triplicate, on either two or three RNA samples. Expression levels are normalized to overall MCF-7 expression levels from Figure 12A. Note- this is a logarithmic scale. Expression levels are denoted above each data bar.

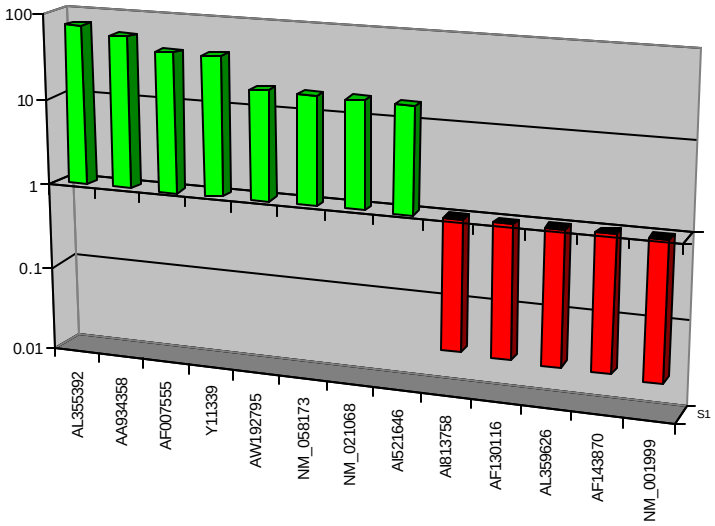
Figure 13. Effects of SHBG Induction on Overall Gene Expression in MCF-7 Cells.



Colored by: Scott Kahn's Affymetrix Experiment (Default Interpretation)
Gene List: all genes (54675)

Figure 13. Similarity Tree of Cell Lines and Treatment Conditions Based on Gene Expression Profiles. Sixteen different LNCaP, HepG2, and MCF-7 samples were analyzed by microarray analysis. Effects of SHBG induction in MCF-7 cells were investigated using the parental MCF-7 cell line and a clone the constitutive SHBG overexpressing clone, MCF-7myc23. Total cellular RNA was prepared from MCF-7 and MCF-7myc23 cells, as well as from the same two cell lines treated with 10nM estradiol for 4 or 24 hours. Total cellular RNA was prepared from L5S2 cells, and its sister vector control cell line, L5V4, as well as from cells that had been treated with the inducing agent, PonA (10uM) for 24 hours, PonA and then 10nM DHT for 4 hours, and PonA and then 10nM DHT for 24 hours. Total cellular RNA was also prepared from HepG2 liver cancer cells, and a constitutive SHBG overexpressing clone, HepG2myc23. In addition, Total cellular RNA was pretreated with RNase-free DNase. Following this, the samples were cellular RNAs were sent to our colleagues at the Yerkes Genomics Core Facility at Emory University. RNAs were analyzed by Agilent Bioanalyzer to check the RNA quality. Samples were labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 Array chips. Raw data was collected by GCOS software and analysis of the microarray data was performed using GeneSpring software. Shown is an expression tree which compares the 16 different samples analyzed in this study, with respect to overall gene expression profiles.

Figure 14. Overexpression of SHBG in MCF-7 Cells Influences Endogenous Gene Expression and Modulates Estrogen Signaling Through the Estrogen Receptor and through R_{SHBG}.



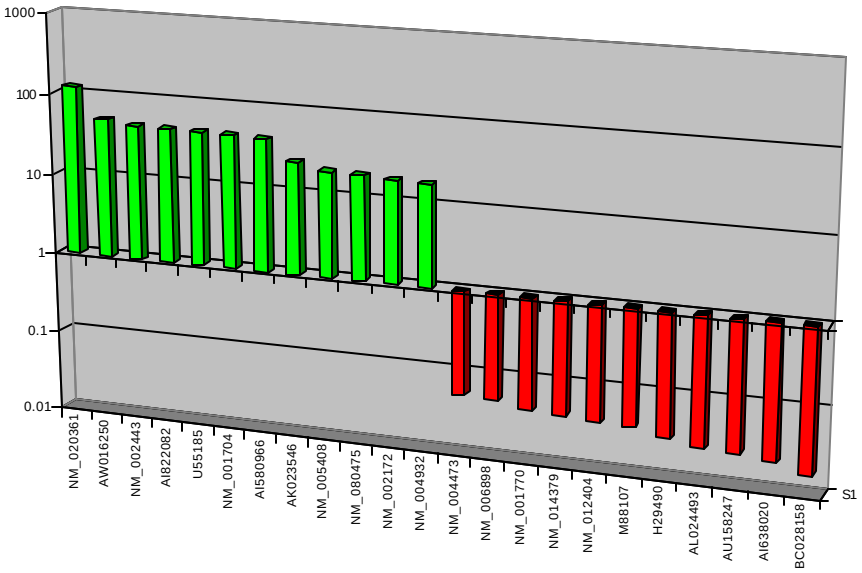
A. SHBG alone affects gene expression in MCF-7 breast cancer cells. Shown are those genes showing the highest induction of expression (15-fold or greater, green) and the greatest suppression of expression (30-fold or greater, red) by SHBG in MCF-7 cells. Methodology- Total cellular RNA was prepared from parental MCF-7 cells and the SHBG overexpressing cell line, MCF-7-SHBG-myc23-2 (>1000-fold SHBG overexpression). Microarray analysis was performed using Affymetrix human genome U133 plus 2.0 gene chips.

Genes displaying induced expression in MCF-7 SHBG-myc23-2 cells

AL355392: novel unknown gene
AA934358: novel unknown gene
AF007555: protein tyrosine phosphatase, receptor type, N polypeptide 2
Y11339: GalNAc-alpha 2,6 sial transferase 1
AW192795: apomucin
NM_058173: small breast epithelial mucin
NM_021068: interferon alpha 14
AI521646: mucin 5B

Genes displaying reduced expression in MCF-7 SHBG-myc23-2 cells

AI813758: collagen type 3-alpha
AF130116: novel unknown gene
AL359626: novel unknown gene
AF143870: novel unknown gene
NM_001999: fibrillin2



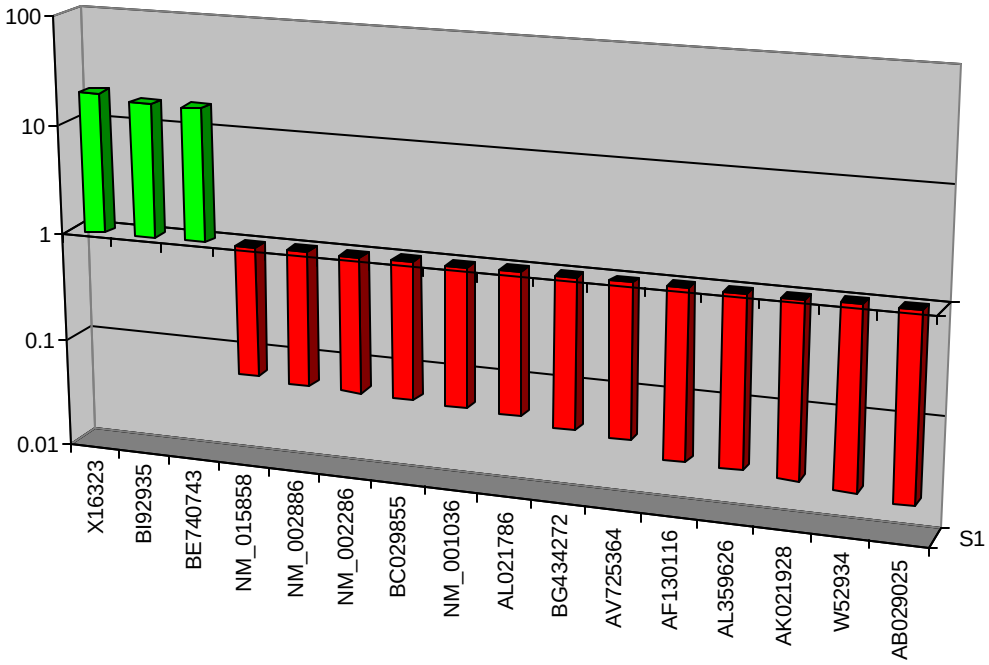
B. SHBG modulates the expression of E2 responsive genes in MCF-7 breast cancer cells.
Shown are those E2-repressed genes in MCF-7 cells showing the greatest suppression of repression (17-fold or greater, green) by SHBG, and E2-induced genes in MCF-7 cells showing the greatest suppression of induction (19-fold or greater, red) by SHBG. Methodology- Parental MCF-7 cells and the SHBG overexpressing cell line, MCF-7-SHBG-myc23-2 (>1000-fold SHBG overexpression) were treated with 10nM E2 for 24 hours. Total cellular RNA was prepared, and microarray analysis was performed as before using Affymetrix human genome U133 plus 2.0 gene chips.

Estrogen mediated repression of the following genes is blocked in SHBG-myc23-2 cells

NM_020361: Carboxypeptidase beta precursor
AW016250: novel unknown gene
NM_002443: microseminoprotein, beta- (MSMB)
AI822082: similar to synaptic glycoprotein SC2
U55185: oral cancer candidate gene
NM_001704: brain specific angiogenesis inhibitor 3
AI580966: novel unknown gene
AK023546: phospholipase C epsilon 2
NM_005408: small inducible cytokine family A member 13
NM_080475: serpin B11
NM_002172: interferon alpha14
NM_004932: cadherin 6 (k-cadherin)

Estrogen mediated induction of the following genes is blocked in SHBG-myc23-2 cells

NM_004473: forkhead box E1 thyroid transcription factor 2
NM_006898: homeobox D3
NM_001770: CD19
NM_014379: Neuronal potassium channel subunit alpha
NM_012404: related to pp32
M88107: formyl peptide receptor
AL024493: novel unknown gene
AU158247: novel unknown gene
AI638020: novel unknown gene
BC028158: novel unknown gene



C. E2-SHBG modulates the expression of genes in MCF-7 breast cancer cells. Shown are those genes whose expression remains unchanged in parental MCF-7 cells following E2 treatment, and which show the greatest degree of induction (16-fold or greater, green) or repression (16-fold or greater, red) in E2 treated SHBG overexpressing cells. Methodology- Parental MCF-7 cells and the SHBG overexpressing cell line, MCF-7-SHBG-myc23-2 (>1000-fold SHBG overexp ression) were treated with 10nM E2 for 24 hours. Total cellular RNA was prepared, and microarray analysis was performed as before using Affymetrix human genome U133 plus 2.0 gene chips.

Genes induced by E2 in SHBG-myc23-2 cells but unaffected by E2 in MCF-7 cells

X16323: hepatocyte growth factor
BI092935: zinc finger protein 42
BE740743: thyroid stimulating hormone receptor

Genes repressed by E2 in SHBG-myc23-2 cells but unaffected by E2 in MCF-7 cells

NM_015858: cellular growth regulating protein
NM_002886: RAP 2B
NM_002286: lymphocyte activating precursor 3
BC029855: similar to zinc finger protein, KR18
NM_001036: ryanodine receptor 3
AL021786: internal membrane protein
BG434272: prothymosin alpha
AV725364: G Protein Coupled Receptor, family C group 5
AF130116: novel unknown gene
AL359626: novel unknown gene
AK021928: rab GTPase activating protein, noncatalytic subunit
W52934: similar to serine threonine kinase
AB029025: novel unknown gene

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