


2010-01-01

A Specific Regulatory Role For Sgta On The Maturation And Activation Of Steroid Hormone Receptors

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A SPECIFIC REGULATORY ROLE FOR SGT α ON THE
MATURATION AND ACTIVATION OF STEROID HORMONE
RECEPTORS

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2010

A SPECIFIC REGULATORY ROLE FOR SGT α ON THE
MATURATION AND ACTIVATION OF STEROID HORMONE
RECEPTORS

by

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THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

May 2010

ACKNOWLEDGEMENTS

This research would not have been possible without the support of many people. Firstly, I would like to express my deepest gratitude and appreciation to Dr. Marc B. Cox for his continuous supervision and guidance from the very early stage of this research when I joined the lab 2+ years ago. His faith in my abilities and patience to tolerate my thousands of questions whilst allowing me the room to work in my own way, not only helped me to learn science but also made me confident to carry out research. One simply could not wish for friendlier mentor. He helped me a lot to develop a positive attitude towards my career.

Secondly, of course the COX lab. Without the help and co-operation of the people around me for most of the day in my schedule, I would not be able to finish this work. Special thanks should go to Heather Balsiger, who is not only our lab technician but also a friend who makes us laugh even when frustrated with the experiments. Thanks Heather for all the laughs. Also, Kimberly, Cheryl, Diondra, Nai, Jackie, Yenni and Johanny for their co-operation and help throughout my stay in the lab. A special thanks should go to Kimberly for letting me use her supplies whenever I needed them, sometimes even without her permission. I am grateful to you guys for all your assistance and back up, without which I would not have been as productive.

A sincere thank to Dr. Rosas. You were not only my thesis committee member but also a friend. Your classes influenced me to choose science as a career. I was a fledgling graduate student when I joined UTEP. The classes I took with you not only helped me to learn science but also motivated me to be in this challenging field of molecular biology. You also helped me a lot to be a successful teacher. The way you

taught us helped me to hone my own teaching skills. And lastly, thanks for putting up with all my questions afterhours.

I must acknowledge all of my thesis committee members: Dr. Cox, Dr. Rosas, Dr. Miranda and Dr. Lee. Your guidance helped me to finish my thesis. Thanks for your co-operation for all the committee meetings we had (some were even with short notice because of me!) and constructive comments you gave me for my thesis. Special thanks to Dr. Rosas and Dr. Miranda for providing me recommendation letters.

My sincere thanks should go to the Core Facilities we have in the Border Biomedical Research Bioscience building. Thanks for technical support Lani. You helped me use some of the instruments that I never used. Also, thanks to you Omar for the sequencing results. It was really nice for us to have you on the third floor.

Also I would like to thank my family members for being with me all the time. Without their help and support, I would not be able to come this far. Their inspiration and encouragement even when far away from home helped me a lot to finish my graduate study.

And last but not least, I am indebted to my fiancée Sangita, who was there all the time with me. Though she may think it is needless to thank her, without her presence in my life, this journey would have been much more difficult and harder for me.

ABSTRACT

Steroid hormone receptors (SHRs) are ligand-dependent transcription factors belonging to the nuclear receptor superfamily. These receptors regulate various physiological functions in higher ordered eukaryotes. In the absence of hormone these receptors form a complex with molecular chaperones such as Hsp90 and Hsp70 and other cochaperones in the cytoplasm. Association with the Hsp90-Hsp70 chaperone machinery leads to the maturation of the Ligand Binding Domain (LBD) of the receptors and enables the receptors to bind hormone with high affinity. Upon binding with a specific hormone the receptors are translocated into the nucleus where they initiate transcription of specific genes. Although the involvement of the Hsp90-Hsp70 chaperone machinery in steroid hormone receptor function is well-characterized, other proteins have also been found in the receptor-chaperone complex which are known as Hsp90 and Hsp70 cochaperones. One such cochaperone is human Small Glutamine Rich Tetratricopeptide repeat (TPR) containing protein α (SGT α).

SGT α is a Tetratricopeptide (TPR) containing protein which belongs to the Hsp90 chaperone family. SGT α consists of three TPR motifs which mediate interaction with the C-terminal EEVD motif of Hsp90, Hsp70 and an N-terminal domain which facilitates self-dimerization of the protein. In addition, recent studies indicated SGT α is a key participant in Androgen receptor (AR) signaling pathway and negatively affects its activity. However, the role of SGT α on other steroid hormone receptors still remains elusive. In this study, we investigated the role of SGT α on other steroid hormone receptors such as Glucocorticoid (GR), Progesterone (PR), Estrogen (ER) and Mineralocorticoid (MR).

In order to determine the possible role played by SGT α in other steroid hormone receptor maturation pathways, we used two model systems, *Saccharomyces cerevisiae* and mammalian cell culture. Our results showed that SGT α is also a regulator of glucocorticoid (GR) and progesterone (PR) receptors' maturation and activation in a ligand-dependent manner. For both GR and PR, SGT α was found to downregulate receptors' activity in yeast in the presence of hormone. However, this cochaperone was not found to be involved with the mineralocorticoid and estrogen receptor maturation pathways. In Hela cells, stable knockdown of endogenous SGT α resulted in an upregulation of the GR and PR-mediated reporter activity in comparison to the stably transfected scrambled shRNA as well as wild type cells. Transfection of SGT α in the stable SGT α knockdown cells resulted in restoration of receptor activity to the wild type level. Furthermore, our studies in the yeast model system demonstrate that SGT α is a competitive inhibitor of FK506 binding protein 52 (FKBP52), a well-characterized immunophilin, which is involved in AR, GR and PR maturation pathways and potentiates their activity. Assays done in yeast showed that SGT α inhibits FKBP52-mediated potentiation of AR, GR and PR. Moreover, *in vitro* studies showed that SGT α and FKBP52 compete for binding on Hsp90. Collectively, our results indicate that SGT α partakes in GR and PR complex maturation and down-regulates these receptors' activity in the presence of hormone. These findings cast new insights on how different cochaperones specifically interact with different steroid hormone receptors and regulate their functions.

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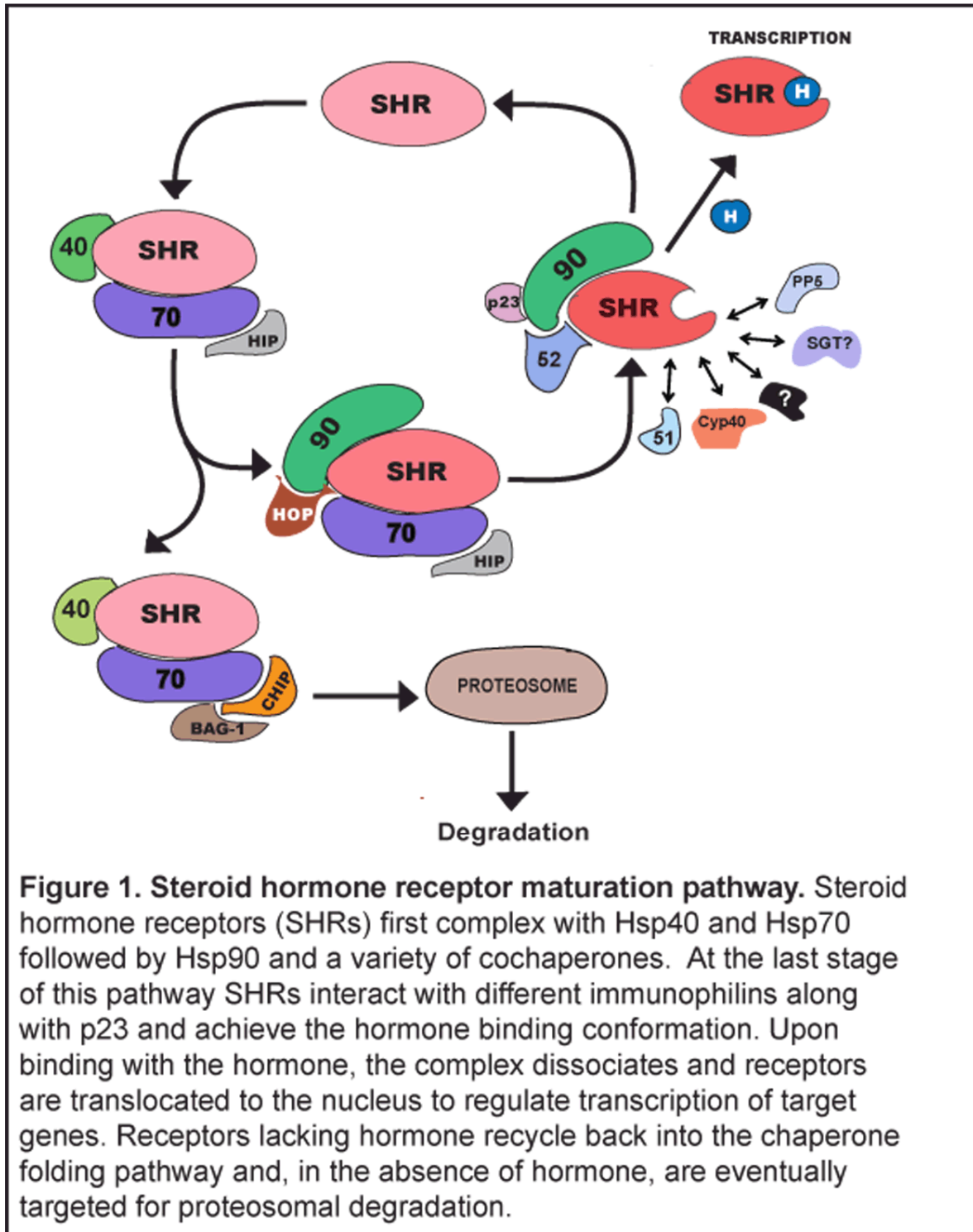
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CHAPTER1: INTRODUCTION

1.1 Steroid Hormone Receptor Maturation.

Steroid hormone receptors, members of the nuclear receptor superfamily, are ligand-dependent transcription factors which mediate various physiological functions in cells including development, homeostasis, reproduction, growth etc. In the absence of the appropriate hormone, the receptors remain in cytoplasm in complex with heat shock protein 90 (Hsp90) (1-4) and other chaperones and co-chaperones as reviewed by Pratt and Toft (5). Molecular chaperones and co-chaperones play an important role in the maturation of the receptors to the hormone binding conformation (6). The receptor-chaperone complexes are assembled in an ordered fashion that dictates the maturation of the receptor. This pathway is depicted in the Figure 1. In the cytoplasm, Hsp40 first interacts with the receptor after being translated on the ribosome (7). This interaction is followed by association with Hsp70 (8) which is mediated in an ATP-dependent manner. ATP binds with the N-terminal domain of Hsp70 while the J-domain of Hsp40 interacts with Hsp70 and stimulates its ATPase activity. This in turn results in tight association of Hsp70 with the receptor (9). The binding of Hsp70 facilitates the folding process and paves the way for association with the Hsp90 complex (10, 11). At this point Hsp70 recruits Hsp interacting protein (Hip), Hsp organizing protein (Hop) and Hsp90. Hop acts as a scaffolding protein which connects Hsp70 with the Hsp90 complex and modulate their activities (12, 13). Association of Hsc70-interacting protein (CHIP) in the complex acts as a quality control monitor, which contains a U-box ubiquitin ligase domain that directs the misfolded receptors to the proteosome for degradation (14, 15). Addition of Hsp90 to the complex stabilizes the hormone binding domain (HBD) of the receptor and



renders the receptor to remain soluble in the cytoplasm (16). Following association of Hsp90, a rearrangement ensues which triggers the dissociation of Hsp70 and recruitment of the p23 cochaperone along with one of several immunophilin proteins as

well as other co-chaperones to the receptor complex (17). This transposition renders the receptors in a structural conformation with hormone binding affinity. This mature conformation of the receptors lasts for a very short time which correlates with the slow ATPase cycle of Hsp90 (18, 19). In the absence of hormone, the receptor is cycled back into the chaperoning pathway. However, the presence of hormone triggers the dissociation of the complex and translocates the receptor into the nucleus where it dimerizes and binds to hormone response elements (HRE) thereby enhancing the expression of the target genes responsible for growth, homeostasis, differentiation, and reproduction in higher ordered eukaryotes (20).

1.2 Association of TPR proteins in Steroid Hormone Receptor Complexes.

Heat shock protein 90 (Hsp90) and heat shock protein 70 (Hsp70) are highly conserved molecular chaperones in eukaryotic cells which help in proper folding of their client proteins like steroid hormone receptors (17). Although Hsp90 and Hsp70 are the key players in steroid hormone receptor maturation and activation, multiple proteins have been found to be associated with this machinery and regulate their chaperone activity. A common feature shared by those proteins involved in this process is the presence of the TPR (Tetratricopeptide repeat) motif which helps in protein-protein interaction (20). Table 1 lists the various TPR-containing proteins known to be involved in steroid hormone receptor maturation along with the domain organization of these proteins. The TPR motif was first identified in proteins involved in the cell division cycle

Table 1. TPR proteins involved in steroid hormone receptor complex. The table indicates different TPR containing proteins in the steroid hormone receptor complex and association of these proteins with Hsp90 and Hsp70. Almost all these proteins interact with Hsp90 and some interact with Hsp70(10).

Name (alternatives)	Hsp70	Hsp90	Domain Organization
Hip (p48)	yes	no	— [TPR] — [DP] —
Hop (Sti1, p60)	yes	yes	— [TPR1] — [TPR2a] — [TPR2b] — [DP] —
Chip	yes	yes	— [TPR] — [U-box] —
Cyp40	no	yes	— [PPase] — [TPR] —
FKBP52 (p59, Hsp56)	no	yes	— [FK1] — [FK2] — [TPR] —
FKBP51 (p54)	no	yes	— [FK1] — [FK2] — [TPR] —
PP5	no	yes	— [TPR] — [Phosphatase] —

Adopted from David Smith, Cell Stress & Chaperones , 2004

(CDC) in yeast (21, 22). The TPR motif consists of 3-16 tandem arrays of 34 amino acids. The different TPR motifs can be distributed in the protein sequence. X-ray crystallography revealed that each of the TPR motifs consists of anti-parallel α helices. Adjacent TPR motifs pack in a parallel arrangement resulting in a spiral of repeating anti-parallel α helices (23). Protein Phosphatase 5 (PP5) was the first identified TPR protein involved in steroid hormone receptor maturation (24). Since then crystallographic analysis of several proteins involved in steroid hormone receptor complexes have been revealed to have one or more TPR domains (e.g. Hip, Hop, Chip, Cyp40, FKBP52, FKBP51) (11). Although all the detectable TPR motifs present in a protein are not necessarily functional, mutation in the TPR region of these proteins not only alters their interaction specificity but also has functional consequences for the cell indicating that the TPR motif is functionally important (23).

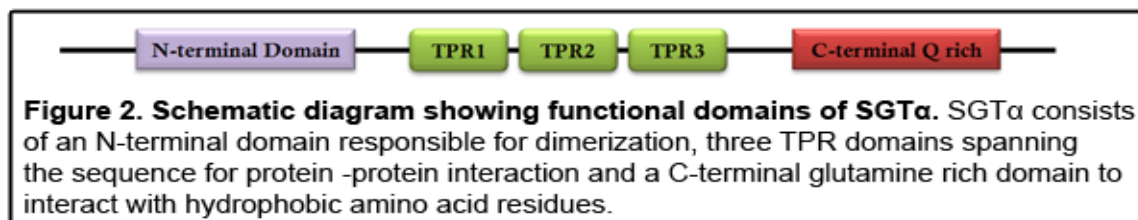
1.3 SGT α as a TPR-containing protein.

Although, association of different TPR proteins in the steroid hormone receptor complex is well characterized, the list of proteins involved in this process continues to expand. One such newly identified TPR protein is human small glutamine-rich TRP containing protein α (SGT α). SGT α was first identified as a TPR containing protein which interacts with Nonstructural Protein NS1 of Parvovirus H-1 and found to be located both in the cytoplasm and nucleus (25). Later yeast two-hybrid screening demonstrated SGT α interacts with severe acute respiratory syndrome coronavirus (SARS-CoV) protein 7a which induces apoptosis when overexpressed in a variety of cell types. Deletion mutant analysis showed TPR2 of SGT α (aa 125-158) was crucial for this interaction (26). In addition, independent studies showed that SGT α interacts with HIV-1 viral encoded protein U (Vpu) the viral core protein precursor Gag (27) and overexpression of the TPR region of SGT α inhibits HIV-1 particle release (27). Dutta et al (2008) showed that the central TPR domain of SGT α is enough to interact with Vpu and overexpression of TPR domain inhibits the HIV-1 particle release (28). Although much remains to be elucidated about the mechanism of how SGT α inhibits viral particle release, together these findings indicate SGT α plays a crucial role during viral infection. Understanding the interaction between viral proteins and SGT α thus could lead to novel therapeutic intervention in treating viral infection.

The first reported homologues of human SGT α were in *Saccharomyces cerevisiae* (SGT2) and *Caenorhabditis elegans* (29). The highest degree of similarity was found to be present in the central region containing three TPR motifs in tandem array. Later Ommen et al (2009) showed SGT α is conserved from invertebrate to the higher ordered

vertebrates such as zebrafish, frog, and chicken and is essential for viability of *Leishmania donovani* (30). Deletion of the yeast homologue (SGT2) of human SGTα in yeast resulted in defects in the ability of the yeast to recover from heat shock (38). *Zhu et al* (2003), showed SGTα interacts with the N-terminal region of myostatin (31). In humans, SGTα was found to be present in all tissues tested and mapped to chromosome 19p13 (29). Mass spectrometry analysis showed SGTα interacts with human β amyloid peptide (Aβ) along with five other chaperone proteins (32). Moreover, 2-D gel electrophoresis analysis identified SGTα as a phosphoprotein pointing out that this protein may be involved in a variety of cellular functions including signal transduction, transcriptional regulation, and enzymatic regulation (33). Furthermore, *Winnefeld et al* (2004) showed that transient transfection with siRNA targeting endogenous SGTα in a variety of cell types results in mitotic arrest which is followed by cell death implying the possible roles of SGTα in cell cycle (34). In brief, these findings indicate that SGTα is an essential protein which is conserved through various organisms and plays crucial roles in various cellular processes.

SGTα consists of three structural units – an N-terminal domain which facilitates



self-association (dimerization) of the protein, three TPR domains for protein-protein interaction and a C-terminal glutamine-rich domain which is able to interact with hydrophobic amino acid segments within polypeptides (Figure 2) (35). Yeast two-hybrid screening showed SGTα interacts with heat shock cognate protein 70 (Hsc70) via c-

terminal GPTIEEVD peptide (36, 37). More recently, by “Far-Western” and pull-down assays, Angeletti *et al* (2002) demonstrated SGT α interacts with Hsp70 and negatively regulates its chaperone activity suggesting its regulatory role in the chaperone complex (38). Furthermore, the TPR domain of SGT α has also been reported to interact with Hsp90. The C-terminal EEVD motif deletion mutant of Hsp90 was found no longer to be associated with SGT α pointing out the fact that SGT α binds with Hsp90 via this domain (39). Together, these findings indicate that SGT α is a molecular cochaperone belonging to the Hsp90-Hsp70 chaperone family.

Recently, GST pull-down assay showed SGT α interacts with growth hormone receptor and the first TPR motif is responsible for interacting with the ubiquitin-dependent endocytosis motif of the receptor (40). However, most importantly, Buchanan *et al* (2007) showed that SGT α plays a role in androgen receptor (AR) mediated prostate cancer indicating the possible involvement of SGT α in the steroid hormone receptor complex. The expression of SGT α in prostate cancer epithelial cells was found to be second only to that of FKBP52, an immunophilin involved in the steroid hormone receptor maturation pathway. Overexpression of SGT α in prostate cancer cells was found to down-regulate DHT (dihydrotestosterone) mediated androgen receptor transactivation activity. Moreover, knocking down SGT α with specific siRNA in C4-2B cells resulted in a marked increase in basal and DHT-mediated AR activity. Taken together; these findings implicate the possible involvement of SGT α in the androgen receptor-chaperone complex and plays a key role in the AR maturation pathway (41).

1.4 Objectives.

To date, Hsp90 and Hsp70 have been identified as the chaperone partner of SGT α . However, interaction of SGT α with Hsp90 and Hsp70 does not provide much insight about its function. The study done by Buchanan *et al* (2007) showed its role as an Androgen receptor (AR) specific Hsp70/Hsp90 cochaperone TPR partner. *In vivo* it was found to promote cytoplasmic retention of the receptor and plays a role in the sensitivity and specificity of the receptor (40). However, the role of SGT α on other steroid hormone receptors (GR, PR, MR, ER) still remains elusive. These steroid hormone receptors play crucial roles in normal growth, development, homeostasis, metabolism, and reproduction. Misfolding of these receptors by the proteins involved in the chaperone complex thus could lead to disease conditions. Therefore, a more detailed understanding of the steroid hormone receptors and the proteins involved in the maturation and activation of these receptors is necessary in order to understand the development and progression of different diseases and to the development of effective treatments. Given that SGT α is a key regulator of AR activity, it remains possible that SGT α is a relevant regulator of other steroid hormone receptors. With recent identification and characterization of different TPR proteins in the receptor-chaperone complex, it is becoming evident that these co-chaperones contribute to and facilitate receptor maturation and regulate receptor activity in a significant manner. **Therefore, the main objective of this study is to characterize the effects of SGT α on different steroid hormone receptors.** This may cast meaningful insights into cochaperone specificity of different steroid hormone receptors and their role in receptor maturation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Yeast Strains and Hormone Induction Assays.

β -Galactosidase reporter assays were used as quantitative measurement of the receptor activity (42). The W303 α (MAT α *leu2-112ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100 GAL SUC2*) yeast strain was used for all the yeast hormone induction assays. For the *SGT2* deletion-complementation assay, BY4742 (MAT α *his3delta1, leu2delta0, lys2delta0, met3delta0, ura3delta0*) as wild type and *SGT2* knock out (MAT α *his3delta1, leu2delta0, lys2delta0, met3delta0, ura3delta0, sgt2::Kan^r*) yeast strains were obtained from Open Biosystems (Huntsville, AL). Yeast cells were transformed by standard lithium acetate procedure (43) and were plated on synthetic complete (SC) medium containing 2% glucose, 0.5% ammonium sulfate, 0.015% adenine sulfate, 0.17% yeast nitrogen base, 2% agar and complete supplemental mixture lacking specific amino acids. Parent strains were co-transformed with three plasmids: a constitutive receptor expression plasmid, a hormone-inducible β -Galactosidase reporter plasmid, and an SGT α (or *SGT2* for Figure 2) expression vector or empty vector as a control. For each receptor-mediated reporter assay, the hormone (ligand) concentrations were optimized in order to maximize the difference between cells carrying an empty vector versus cells carrying SGT α expression vector by performing dose-response curves. Hormone-induced reporter activity was measured from yeast extracts as described previously (42). All assays were performed at least three times with all variables measured in duplicate.

2.2 Generation of Stable SGT α Knock down HeLa Cells.

Plasmid Construction: To obtain SGT α knockdown (SGTKD) HeLa cells, distinct short hairpin RNA (shRNA) sequences containing inverted repeats (sense,

ATAAATAGGCGTTGTGACCTC; antisense, GAGGTCACAACGCCTATTTAT) separated by a loop (TTCGAGACG) were designed. The oligos targeting SGT α were designed as a synthetic duplex with overhanging sites for restriction digestion (BamHI-5' end and HindIII-3' end). The oligos were heated to 90°C for 3 min and then cooled to 37°C for 1 hr for annealing reaction. The ligation reaction was performed with the annealed shRNA and BamHI and HindII digested pSilencer 2.1-U6 vector (Applied Biosystems, Foster City, CA) at room temperature for 2 hr. The ligation product was transformed into DH5 α competent cells. Positive clones were confirmed by sequencing. A scrambled shRNA was also constructed to exclude any shRNA specific effects. A blast search was performed to ensure that the scrambled construct has no significant homology with any known functional mRNA sequence. A second shRNA construct was also made in a similar way (sense, ACTTTGAAGCTGCCGTGCA; antisense, TGCACGGCAGCTTCAAAGT; loop TTCGAGACG) to 'rule-out' any off-target effects.

Stable SGT α Knockdown Cell Line (SGTKD): For stable transfection, HeLa cells were plated in petridishes in Hyclone MEM/EBSS media (Thermo Scientific, Waltham, MA) containing 10% fetal bovine serum (FBS) 24 hrs prior to transfection. At 80% confluence, shRNA targeting SGT α as well as scrambled shRNA were transfected by Lipofectamine 2000 method according to the manufacturers instructions (Invitrogen, Carlsbad, California) followed by selection with Hygromycin (Thermo Scientific, Waltham, MA). Individual colonies were isolated and screened for effective knockdown of endogenous SGT α protein levels by western blot. The selected clone was used to perform the luciferase reporter assays. Also, the scrambled shRNA construct was

stably transfected into HeLa cells to demonstrate the effects were not due to non-specific knockdown of cellular proteins in general.

2.3 Mammalian Cell Lines and Hormone Induction Assays.

For the reporter-mediated receptor assays, wild type HeLa cells, SGT α knockdown (SGTKD) cells and cells with stably transfected scrambled shRNA were cultured at 5% CO₂ in MEM supplemented with 10% charcoal-stripped FBS and essential amino acids. Cells were plated at a cell density of 6×10^5 in 6-well plates. At 80% confluence, the cells were transfected for 4 hours using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a DNA (μ g):Lipofectamine (μ l) ratio of 1:3 in MEM lacking FBS according to manufacturer's instructions. For the reporter activity three plasmids (800 ng each plasmid/well) were transfected - a hormone-responsive firefly luciferase reporter, a mammalian expression vector (pCI-neo; Promega, Madison, WI) carrying steroid hormone receptor, and an empty mammalian expression vector (pCI-neo; Promega, Madison, WI) or a mammalian expression vector (pCI-neo; Promega, Madison, WI) carrying SGT α . As a control for transfection efficiency each well was also transfected with 50 ng of a constitutive β -galactosidase expression plasmid. For the PR assays a mammalian expression vector carrying progesterone receptor was also transfected but not for the GR assays since HeLa cells have endogenous GR. 24 hours post-transfection, cells were treated with appropriate hormone along with vehicle control (ethanol). The cells were lysed 48 hours after transfection in M-PER (Pierce, Rockford, IL). Luciferase activity was measured by adding 40 μ l of cell lysate with 100 μ l of luciferase reagent (Promega, Madison, WI) in an opaque 96-well plate. β -galactosidase activity was assayed by adding 20 μ l of the cell lysate with 100 μ l Tropix Gal-Screen

assay reagent (Applied Biosystems, Foster City, CA) in an opaque 96-well plate. The 96-well plates were incubated at room temperature until maximum luminescence developed (about 5 min for the luciferase assay and 2 hours for the β -galactosidase assays). After normalizing for transfection efficiency (relative light units/ β -galactosidase activity), the data were plotted as fold induction of luciferase activity over background activity observed in the absence of hormone. All assays will be performed at least three times with all variables measured in duplicate.

2.4 Yeast two-hybrid assay.

Yeast two-hybrid assays were performed according to manufacturer's recommendation (Matchmaker GAL4 Two-Hybrid System 3). Briefly, SGT α was used as bait and glucocorticoid (GR) or progesterone (PR) receptors were used as prey. The yeast two-hybrid vectors pGADT7 (encoding Gal4 Activation domain) and pGBKT7 (encoding Gal4 DNA binding domain) were obtained from Clontech. SGT α was fused with Gal4 DNA-binding domain (pGBKT7: SGT α). The progesterone receptor in pGADT7 vector (pGADT7:PR) was a kind gift from Dr. Miguel Beato (Centre de Regulacio' Genomica, Universitat Pompeu Fabra) and the GR in pGADT7 vector (pGADT7:GR) was kindly provided by Dr. Kaju Yanai (Department of Biomolecular Science Faculty of Sciences, Toho University). The resulting plasmids were co-transformed in combinations as indicated into the yeast strain AH109 and plated on Synthetic complete medium lacking leucine and tryptophan (SC/-leu/-trp). Cotransformants were selected to grow to detect HIS3 activation by selecting for on SC plates deficient in Leucine, tryptophan and histidine (SC/-leu/-trp/-his).

2.5 Protein Purification.

Plasmids carrying Hsp90, FKBP52 and SGT α were cloned into pET28a(+) vector and transformed individually into BL21(DE3) *E. coli*. The colonies were picked and grown overnight. 30 ml of each overnight culture were grown in 1000 ml media in shaking incubator at 37°C until the OD₆₀₀ reached 0.6. Protein expression was induced with Isopropyl β -D-1-thiogalactoside (IPTG) to a final concentration of 1mM. The cultures were grown for an additional 4 hrs and centrifuged at 6000 rpm for 20 min. The pellets were resuspended in 12 ml lysis buffer (20 mM Tris HCl, 300 mM NaCl, 10 mM Imidazole, pH 8) and divided into 3, 4 ml aliquots. 40 μ l of 100 mg/ml Lysozyme was then added to each 4 ml cell suspension and incubated for 20 minutes at 30°C. To lyse the cells, each of the 4 samples were then sonicated and kept on ice. The 4 aliquotes were pulled together and centrifuged at 15,000 rpm for 20 mins at 4°C. To purify the protein, the 12 ml lysate was loaded onto the Ni-NTA column pre-equilibrated with lysis buffer and mixed for 1 hour at 4°C. The resin was then washed with buffer (20 mM Tris HCl, 300 mM NaCl, 20 mM Imidazole, pH 8) and transferred to a 2 ml gravity flow column. 10 ml of elution buffer (20 mM Tris HCl, 300 mM NaCl, 250 mM Imidazole, pH 8) was used to elute the proteins. All proteins were dialyzed extensively against 50mM Hepes, 50mM KCl, 10mM MgCl₂, 1mM DTT (pH 7.4) prior to sample concentration and storage at -80°C.

2.6 *In vitro* binding assay.

To carry out the *in vitro* binding assay, 10 μ l of protein-A-sepharose beads was incubated with anti- SGT α antibody (Protein Tech Group, Chicago, IL) or rabbit non-immune serum (Santa Cruz Biotechnology, Santa Cruz, CA) in 460 μ l of Binding buffer

at room temperature for 30 min with gentle shaking. The antibody bound resins were then washed 3 times with binding buffer. 20 µg of purified SGTα was added in each reaction as bait for the pull-down assay to a final volume of 400 µl with binding buffer. The reactions were then incubated on ice for one hour with gentle mixing. The reactions were washed 3 times with binding buffer. Equimolar concentrations of Hsp90 and FKBP52 were then added to the reaction and incubated at 30°C for 1 hour to a final volume of 400 µl with binding buffer. The reactions were washed 3 times with binding buffer and eluted with 2X sample buffer. The samples were then heat denatured and run on 10-20% Criterion gels (Bio-Rad, Hercules, CA) for western blot analysis.

2.6 Westernblot.

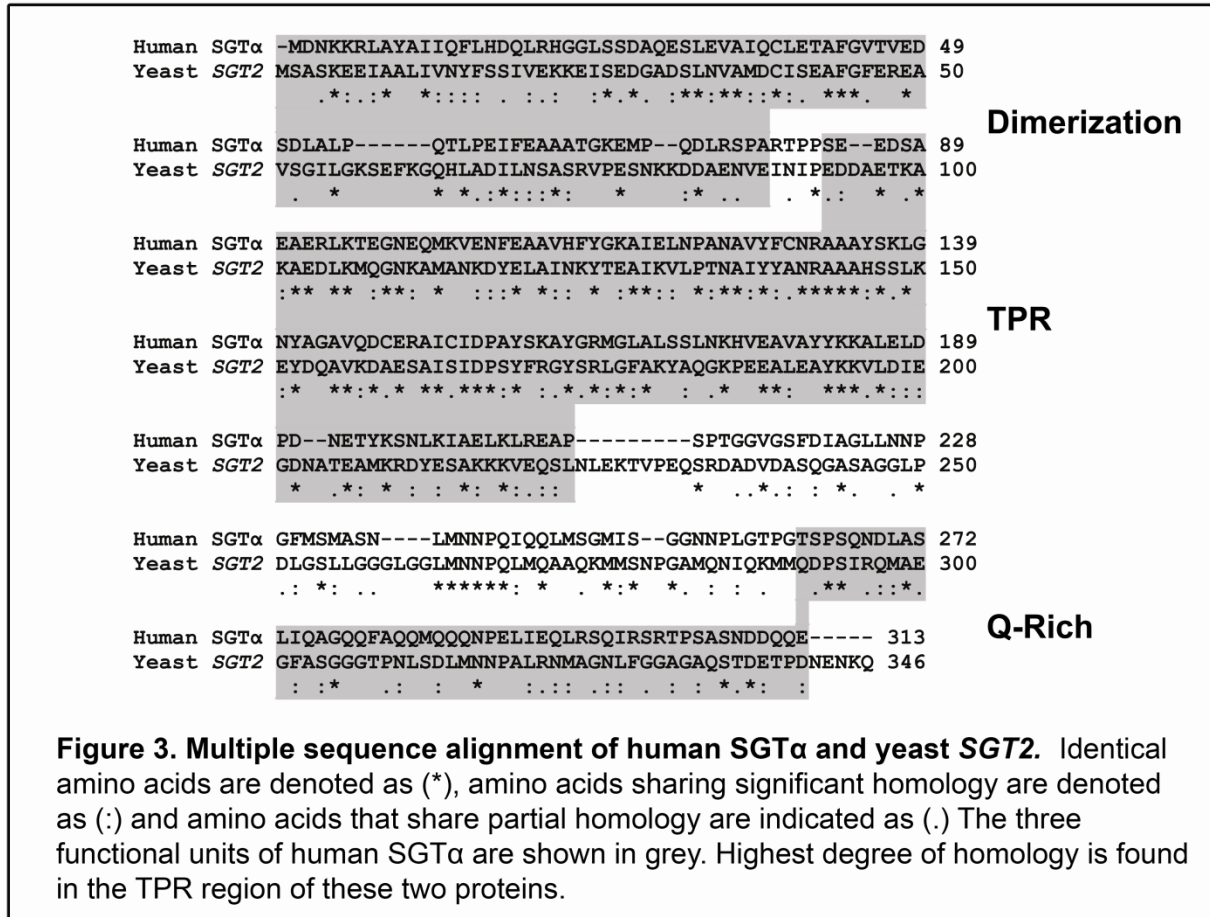
Yeast cells growing overnight were diluted (OD₆₀₀ approximately to 0.2) and grown until the OD₆₀₀ reached 0.8. Yeast cells were then pelleted, resuspended in 2X SDS-PAGE sample buffer and vortexed vigorously in the presence of glass beads. Cells were then centrifuged at 13,200 rpm for 20 min at 4°C. For mammalian cell lysis, cells were washed with 1X PBS and lysed with mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL) supplemented with protease inhibitors (Complete mini EDTA-free; Roche, Indianapolis, IN). Protein concentrations for mammalian extracts were determined by Coomassie Plus Protein Assay (Pierce, Rockford, IL). Approximately 20 µg total cellular protein was added with 2X SDS-PAGE sample buffer, heat denatured at 95°C for 5 minutes and separated on a 10-20% Criterion gel (Bio-Rad, Hercules, CA). Separated protein was transferred to PVDF membranes. The following antibodies were used: rabbit α-human SGTα (Protein Tech Group, Chicago, IL), rabbit α-human AR (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit α-human

GR (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-chicken PR (PR-22). Antibodies detecting yeast ribosomal protein L3 (44) and Glyceraldehyde-3-phosphate dehydrogenase (6C5; Biodesign International, Saco, MN) were used as loading controls. The secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibodies and bands were visualized with Immune-Star AP substrate (Bio-Rad, Hercules, CA) and exposed to X-ray film.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Comparative Analysis of human SGT α and yeast SGT2.

As identified by *Kordes et al*, 1998 (25), SGT2 is the yeast homologue of human SGT α . Amino acid sequence analysis of revealed that these two proteins share 29.8% identity and 45.3% similarity at the amino acid level (Figure. 3). The highest degree of homology was observed in the centrally located TPR domain which consists of three TPR motifs. However, yeast SGT2 does not have a glutamine rich C-terminal domain



and the level of homology in the N-terminal domain is also not significant (17.7% identity, 35.4% similarity). Although yeasts lack steroid hormone receptors, homology in the TPR region of SGT α and SGT2 suggests that the TPR domain is the functional

domain for both these proteins and that these two proteins may work in a similar fashion.

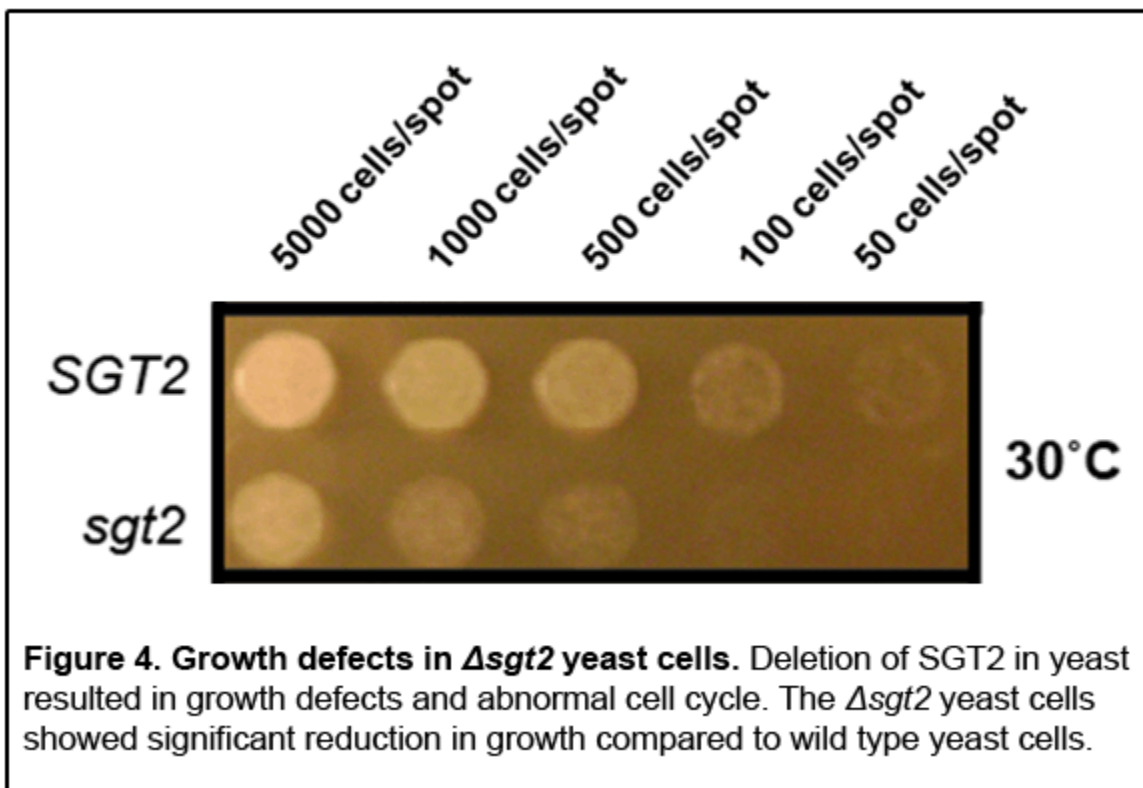
3.2 Functional Homology between human SGT α and yeast SGT2.

To this end, virtually all steroid hormone receptors have been reconstituted in the *S. cerevisiae* (43). Therefore, co-expression of the receptor and a reporter gene (β -galactosidase) carrying hormone response elements (HRE) along with SGT α may indicate the possible involvement of this protein in the steroid hormone receptor signaling pathways. In this study we used the yeast homologue of human SGT α , SGT2, which share 29.8% identity. The SGT2 deletion yeast strain ($\Delta sgt2$) serves as negative background in which SGT α and SGT2 can be studied.

In order to determine the functional homology between SGT α and SGT2, wild type and $\Delta sgt2$ yeast strains carrying androgen receptor (AR) and β -galactosidase reporter expression vectors were transformed with empty vector or vector carrying SGT α or SGT2. The findings indicate that deletion of SGT2 from yeast resulted in 2-fold increase in the androgen receptor-mediated reporter activity in comparison to the wild type cells. However, overexpression of SGT α resulted in a decrease in the receptor-mediated reporter activity to the wild type level (data not shown). These assays suggest that human SGT α and yeast SGT2 are functional homologs. In addition, the effects of SGT α observed on androgen receptor function are similar to what would be expected from previously published data in a mammalian cell system (41).

3.3 Defects in Cell growth in $\Delta sgt2$ Yeast Cells.

Although the $\Delta sgt2$ strain serves as a negative background to study the effects of SGT α and SGT2 in yeast, deletion of SGT2 results in defects in yeast which prevents further characterization of SGT2. Deletion of the *S. cerevisiae* homolog, SGT2, has been reported to result in a cell wall defect and abnormal cell cycle progression (46, 47). Thus, over a short period of time the deletion strain becomes sick and the data obtained from those cells becomes inconsistent. In order to confirm this, both wild type and $\Delta sgt2$ yeast strains were grown at 30°C (Figure 4) and diluted serially on Yeast extract-peptone-dextrose (YEPD plate). The results indicate the SGT2 deletion cells display a



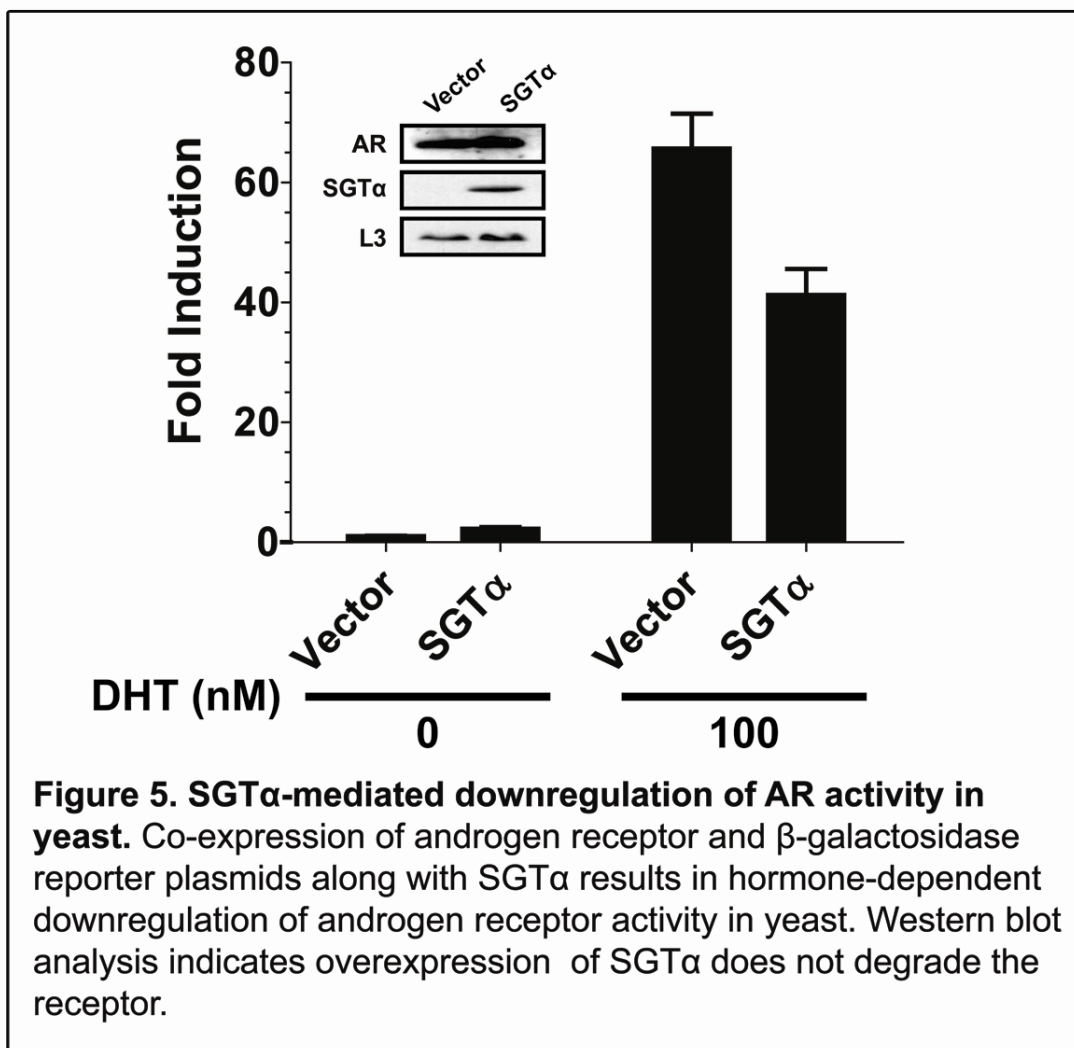
growth defect at optimal growth temperature. This finding was similar with results shown by Angeletti *et al* (2002) where $\Delta sgt2$ strain showed to have defects in recovery from

heat shock (37). Given the abnormal growth phenotypes displayed by the deletion strain, this particular strain is not ideal for the functional study of SGT α . However, SGT α consistently and significantly reduces AR function in the wild type parent strain. Thus, overexpression of human SGT α in the wild type yeast strain carrying a receptor expression plasmid and the receptor-responsive reporter plasmid can serve as an exploratory system for the functional characterization of SGT α effects in steroid hormone receptor signaling pathways.

3.4 Role of SGT α on Different Steroid Hormone Receptor Signaling Pathways in a Yeast Model System.

As discussed above, SGT α has been reported to be AR-specific. However, effects on receptors other than AR and ER have not been explored. Thus, it is possible that SGT α functionally affects other steroid hormone receptors. To determine the possible role of SGT α in different steroid hormone receptor pathways, wild type yeast carrying a steroid hormone receptor expression vector and a β -galactosidase reporter were transformed with an SGT α expression vector. Addition of specific ligand (hormone) resulted in ligand-dependent, receptor-mediated expression of the lacZ reporter gene. The readout of β -galactosidase activity (lacZ units) indicates the functional status of the steroid receptors. Our results suggest that SGT α is a potential regulator of the androgen, glucocorticoid and progesterone receptor signaling pathways. However, no effects were observed on the estrogen and mineralocorticoid receptors upon SGT α overexpression.

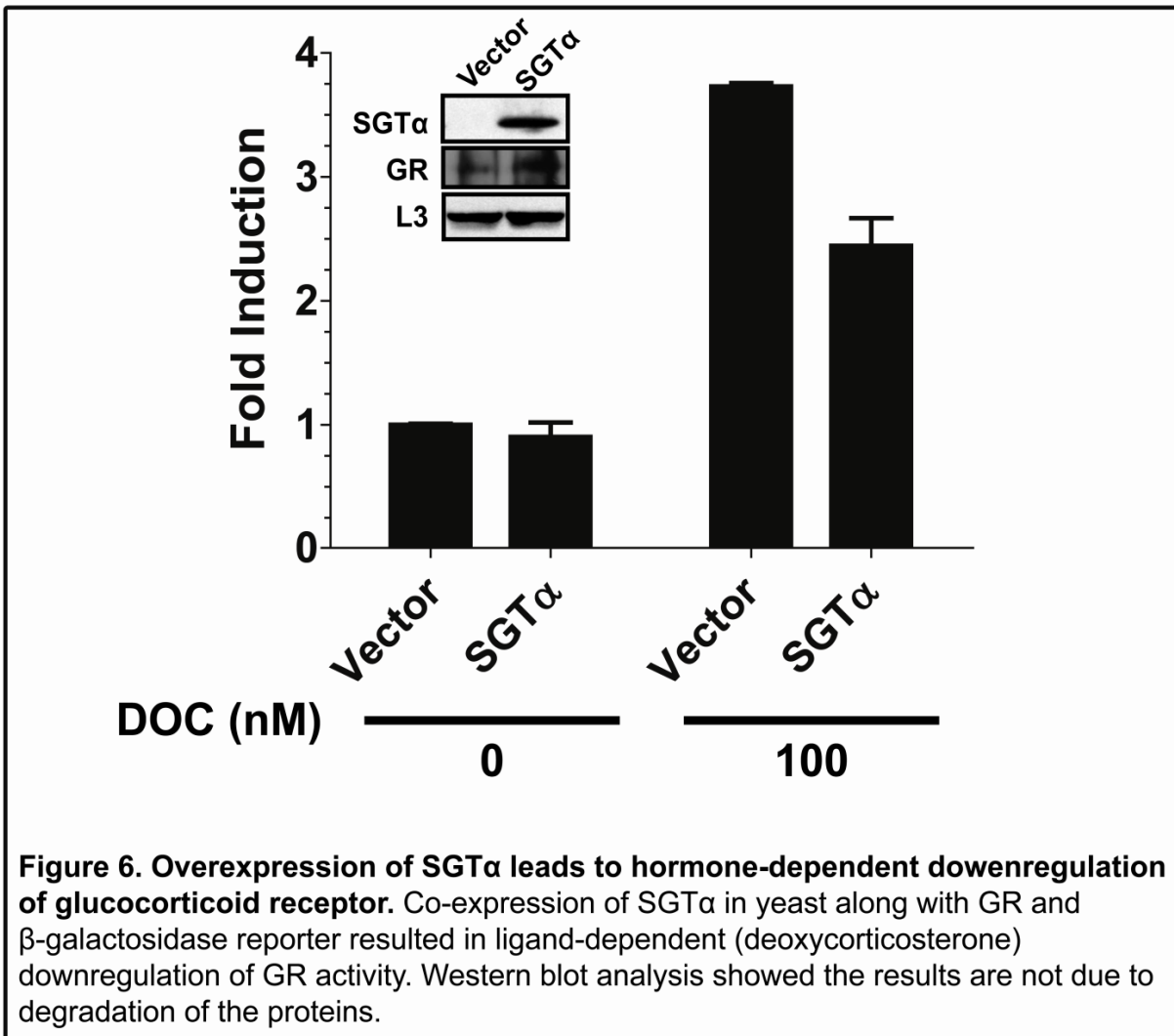
Androgen Receptor (AR): The W303 α yeast cells were transformed with an expression vector carrying the androgen receptor, a β -galactosidase reporter plasmid,



and the SGTα expression vector or empty parent vector. The transformed strains were assayed for receptor-mediated expression of the β-galactosidase reporter gene. The results in Figure 5 indicate SGTα negatively regulates androgen receptor activity in comparison to empty vector control. Western blot analysis showed that all proteins are expressed properly and that the reduction in receptor activity observed is not due to reduced receptor expression and/or stability. The yeast ribosomal protein L3 was used as a loading control.

Glucocorticoid Receptor (GR): In order to determine the effects of SGTα on glucocorticoid receptor the W303α yeast cells were transformed with an expression

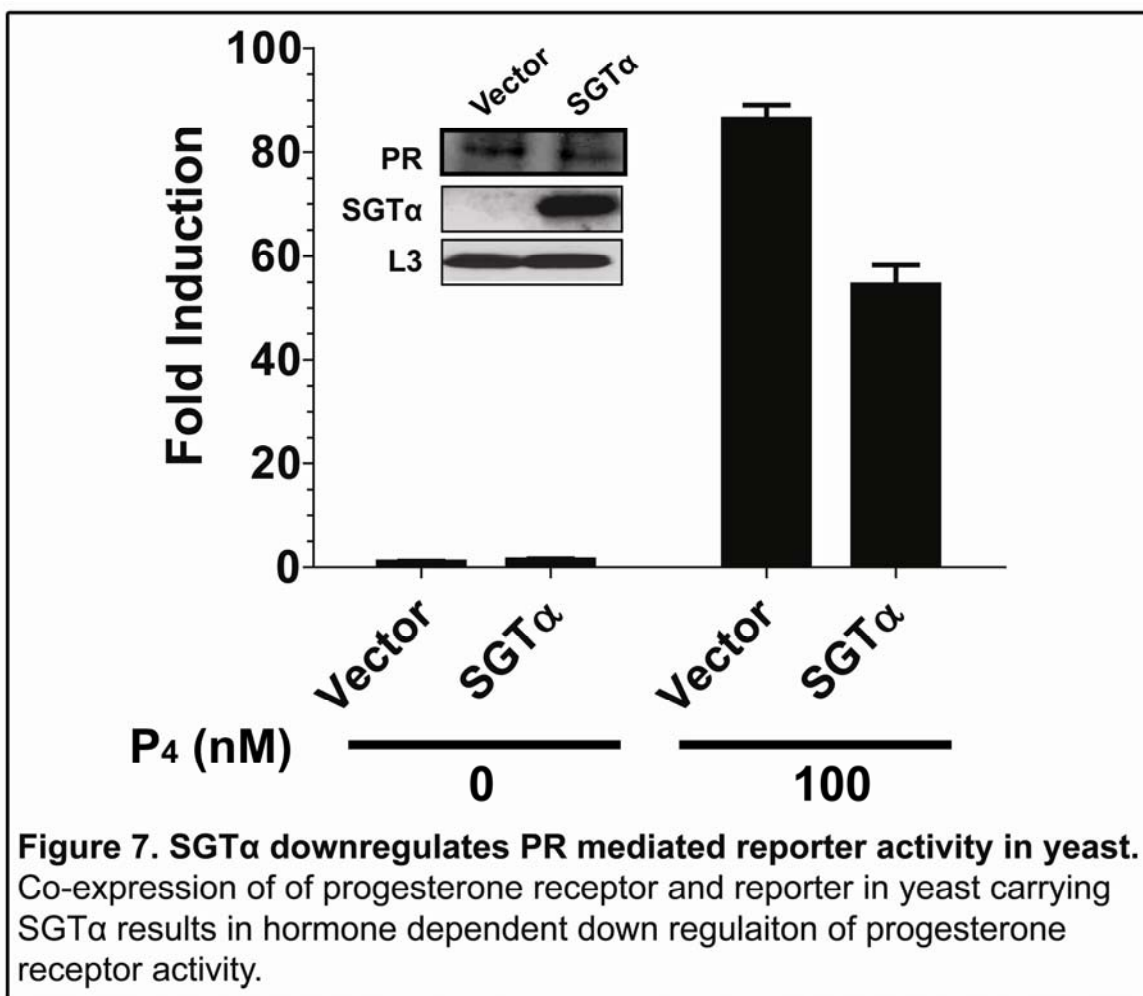
vector carrying the glucocorticoid receptor, a β -galactosidase reporter plasmid, and the SGT α expression vector or empty parent vector. The results showed in Figure 6_



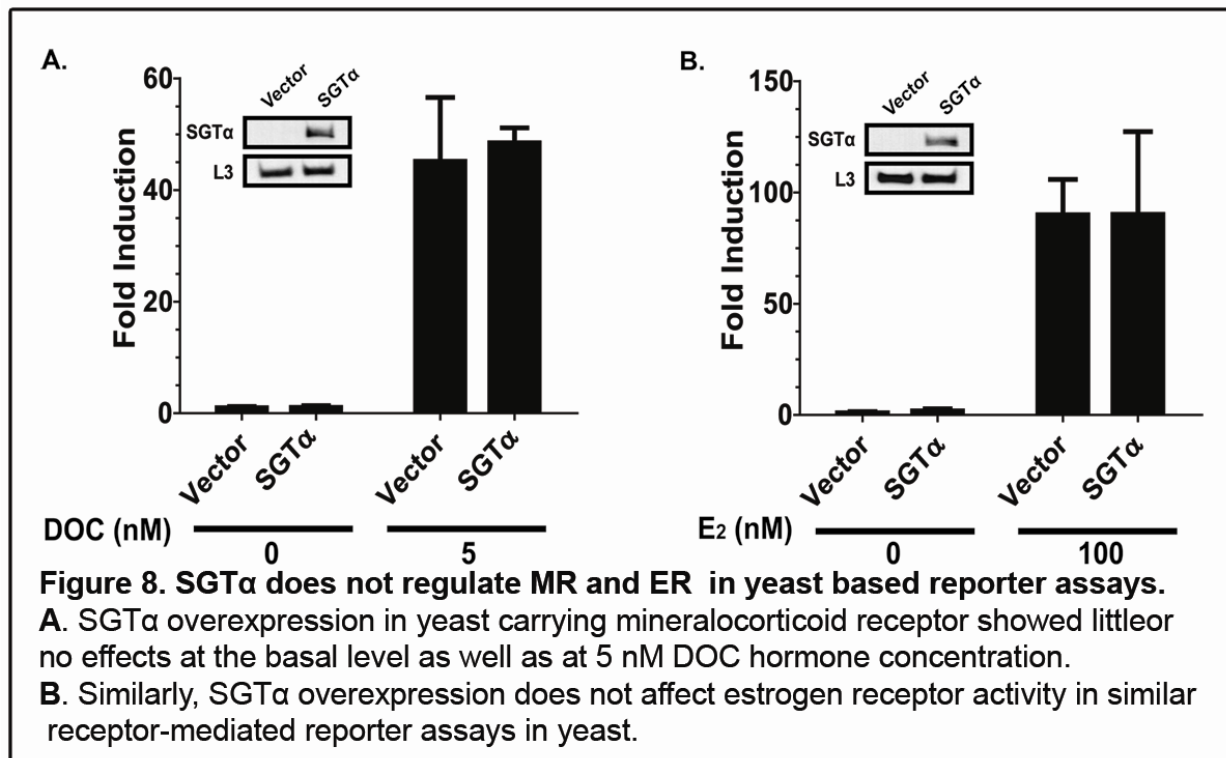
indicate that SGT α is also a modulator of glucocorticoid receptor in the yeast model system. Overexpression of SGT α resulted in downregulation of GR activity in a ligand-dependent manner. Although, hormone-independent receptor activity was seen, in most of the cases the results were not statistically significant. Therefore, it is difficult to draw any conclusion of hormone-independent effects of SGT α on glucocorticoid receptor.

The western blot analysis showed the effects were not due to degradation in the protein level.

Progesterone Receptor (PR): As seen in glucocorticoid and androgen receptor, overexpression of progesterone receptor and reporter in yeast cells carrying SGT α results in hormone (progesterone) dependent downregulation of progesterone receptor activity (Figure7). The presence of SGT α resulted in a two-fold decrease in the receptor activity. As described above the Western blot showed that the proteins were expressed properly.

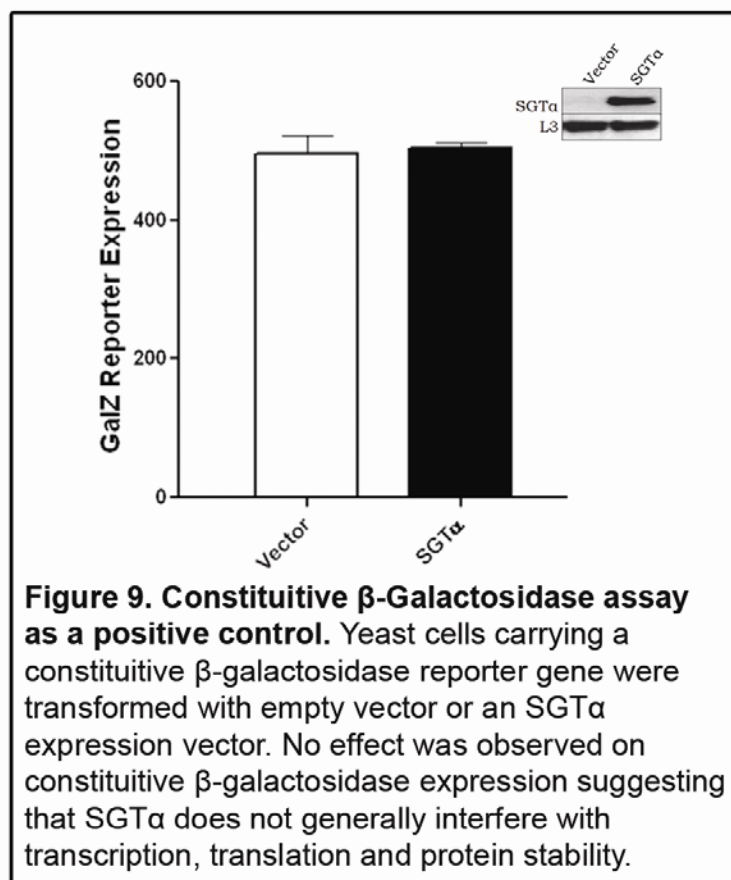


Estrogen and Mineralocorticoid Receptors (ER & MR): In contrary to androgen, glucocorticoid and progesterone receptors, SGT α was found to have no effect on the estrogen and mineralocorticoid receptors in receptor-mediated β -galactosidase reporter assays in the yeast model system. As depicted in Figure 8 for both MR and ER SGT α



was not found to affect the activity of these receptors both in hormone-dependent as well as in a hormone-independent manner. These data suggest that SGT α does not regulate the estrogen and mineralocorticoid receptors.

Constitutive β -Gal Assay: In order to demonstrate that the effects observed on steroid hormone receptor signaling were not due to a general alteration in overall cellular transcription, translation, and protein stability, yeast cells carrying a constitutive β -galactosidase reporter were transformed with empty vector and SGT α . Both the vector and SGT α transformed yeast cells showed the same level of activity which validates the



fact that SGT α does not generally affect cellular transcription or translation but it specifically mediates steroid hormone receptor signaling (Figure 9).

3.5 Validation of Yeast Assay Results in a Mammalian Cell Culture System.

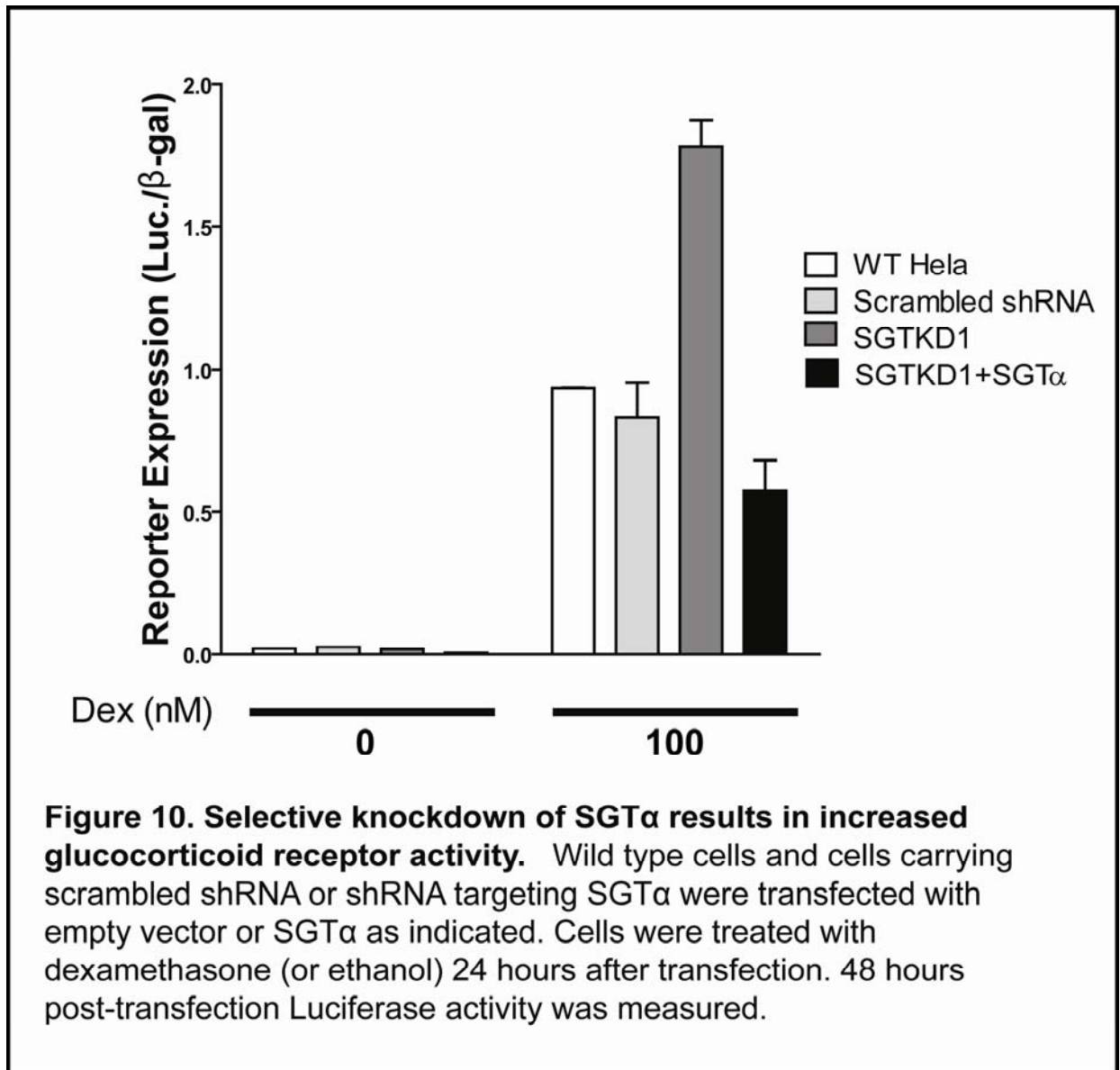
The yeast-based assays provided much evidence regarding the possible involvement of SGT α in androgen, progesterone and glucocorticoid receptors maturation and function. Although yeast genetics provides a powerful tool for characterizing steroid hormone receptor modulators, the yeast system must be viewed as an exploratory system. The data gleaned from yeast should therefore be validated in higher vertebrate model systems to ascertain physiological relevance. HeLa cells have been viewed as an excellent model system in steroid hormone receptor studies. Along with their high transfection efficiency and high growth rate, HeLa cells have endogenous

glucocorticoid receptor at a level appropriate to carry out receptor-mediated reporter assays. Together, all these attributes make HeLa cells a suitable model system to confirm physiological relevance of SGT α in steroid hormone receptor signaling.

Buchanan *et al* (2007) has already demonstrated that SGT α is a key modulator in androgen receptor signaling in mammalian cell lines (41). Therefore, emphasis was placed on assessing the possible interaction and involvement of SGT α in glucocorticoid and progesterone receptor signaling pathways. Western blot analysis revealed that SGT α is present in all human cell lines that we have tested including HeLa cells. Thus, knockdown/complementation experiments were performed in HeLa cells in order to assess the importance of SGT α

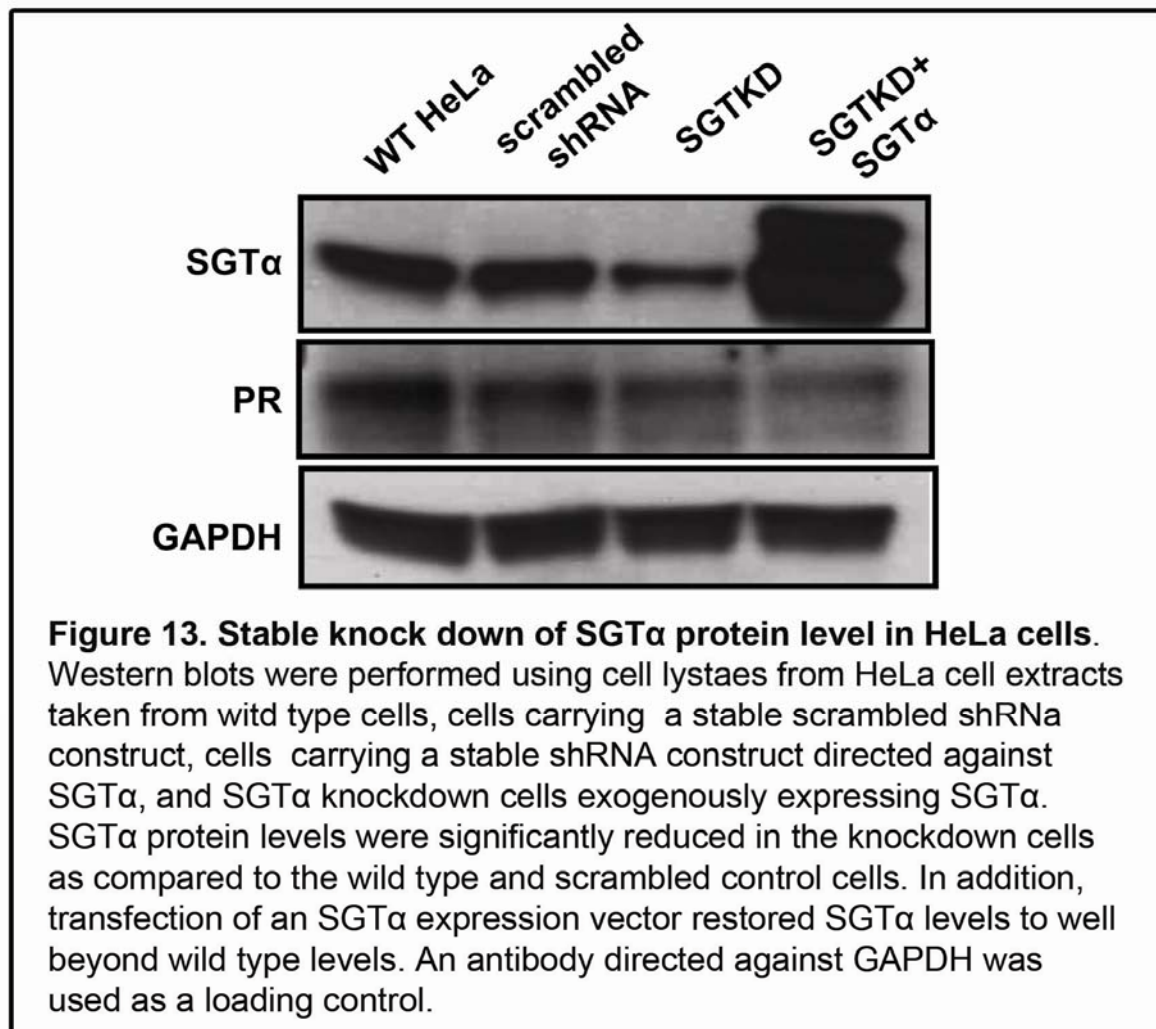
As described in Chapter 2, HeLa cells were stably transfected with a specific shRNA expression plasmid targeting endogenous SGT α . Plasmids carrying the shRNA have a hygromycin antibiotic resistance gene. Therefore, 48 hrs post-transfection cells were maintained in media containing hygromycin antibiotic. This allowed for the isolation and clonal selection of cells carrying a stably integrated copy of the shRNA for SGT α . Western blot analysis confirmed the reduction in protein level in the screened clones and the clone with the highest degree of knockdown was chosen to perform the luciferase assays. Additionally, control HeLa cells carrying a stable copy of a randomly scrambled shRNA was constructed to control for knockdown specificity.

Glucocorticoid Receptor: In order to determine the role of SGT α in the glucocorticoid receptor signaling pathway, wild type, scrambled shRNA and SGT α knock down (SGTKD) HeLa cell lines were transfected with a luciferase reporter plasmid, a constitutive β -galactosidase reporter plasmid to control for transfection efficiency, and



an SGTα expression vector or empty control vector. Since HeLa cells express high levels of the glucocorticoid receptor endogenously, there was no need to transfect in a plasmid for receptor expression in this instance. The data in Figure 10 indicates that knockdown of endogenous SGTα resulted in an upregulation of glucocorticoid receptor activity in comparison to the wild type and scrambled shRNA transfected HeLa cells. However, overexpression of SGTα in the SGTKD resulted in restoration of the receptor activity to the wild type level. In addition, cells carrying the scrambled shRNA did not

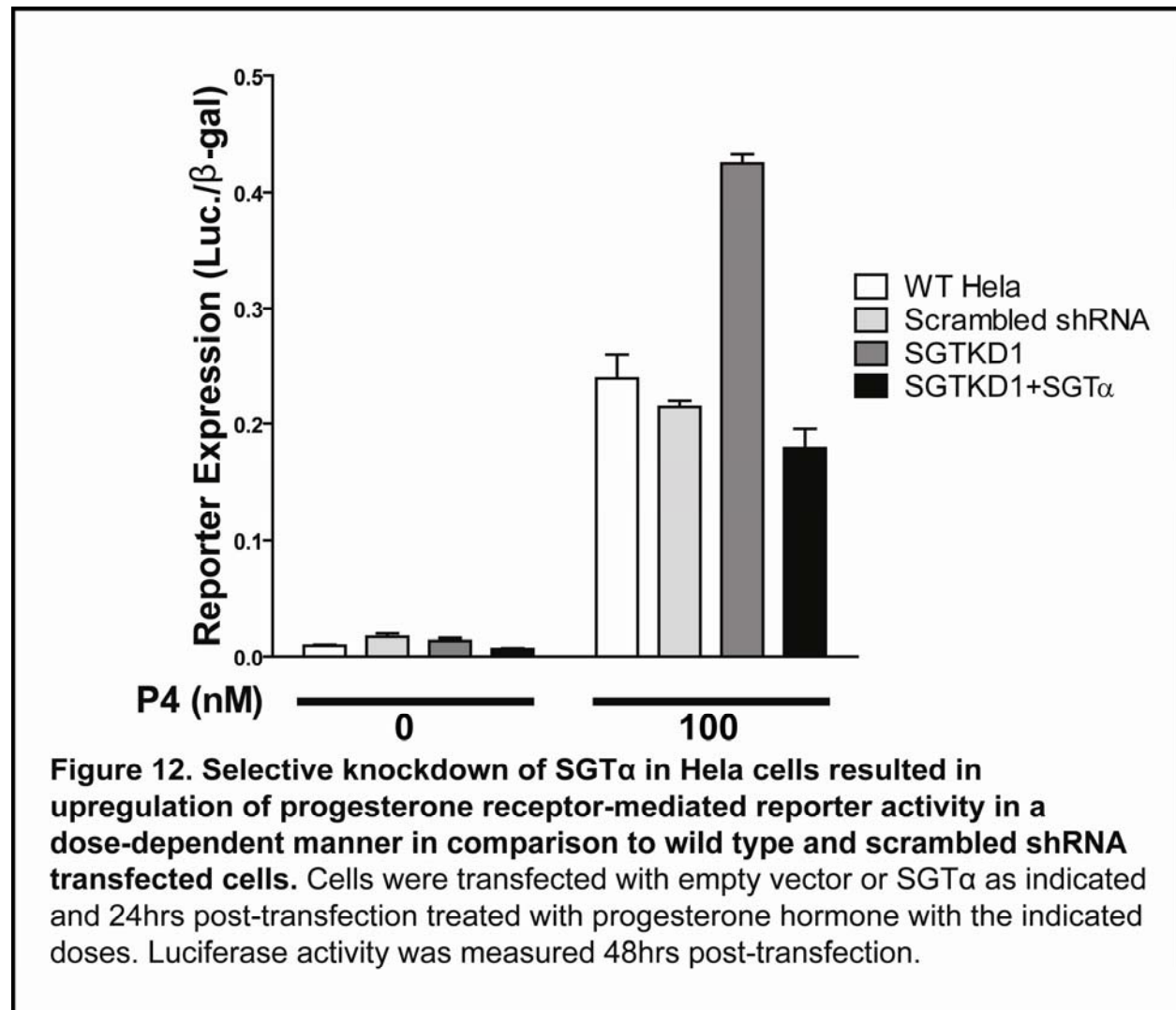
differ in activity from the wild type cells. As shown in Figure 11 GAPDH expression was unaffected by the treatment with either SGT α shRNA or scrambled shRNA, indicating that non-specific downregulation of protein expression was not induced by SGT α



shRNA treatment. In brief, this data suggest that SGT α is involved in the GR signaling pathway and downregulates receptor activity.

Progesterone Receptor: Similar assays were performed in wild type HeLa cells and SGTKD cell lines to resolve the possible involvement of SGT α in the progesterone receptor (PR) signaling pathway. Since HeLa cells do not have endogenous

progesterone receptor, wild type cells, scrambled shRNA and SGT KD HeLa cell lines were transfected with plasmids carrying a luciferase reporter gene, a β -galactosidase expression vector and empty vector along with a progesterone receptor expression



vector. Also, to determine if overexpression of SGT α in the SGT KD cells can lead to restoration of the receptor activity, cells were transfected with an SGT α expression vector. As demonstrated by the data presented in Figure 12 it is clear that upon SGT α knock down, progesterone receptor activity was upregulated in contrast to wild type and scramble shRNA transfected cells in a hormone-dependent manner while

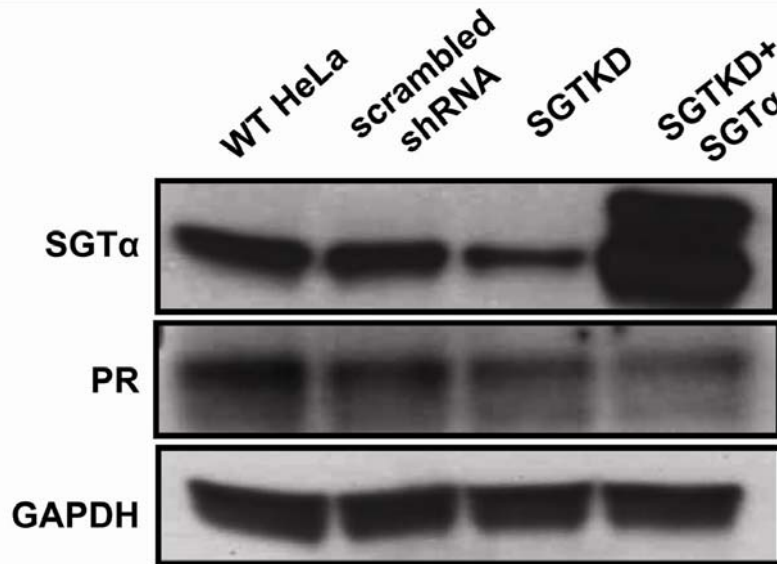


Figure 13. Stable knock down of SGTα protein level in HeLa cells.

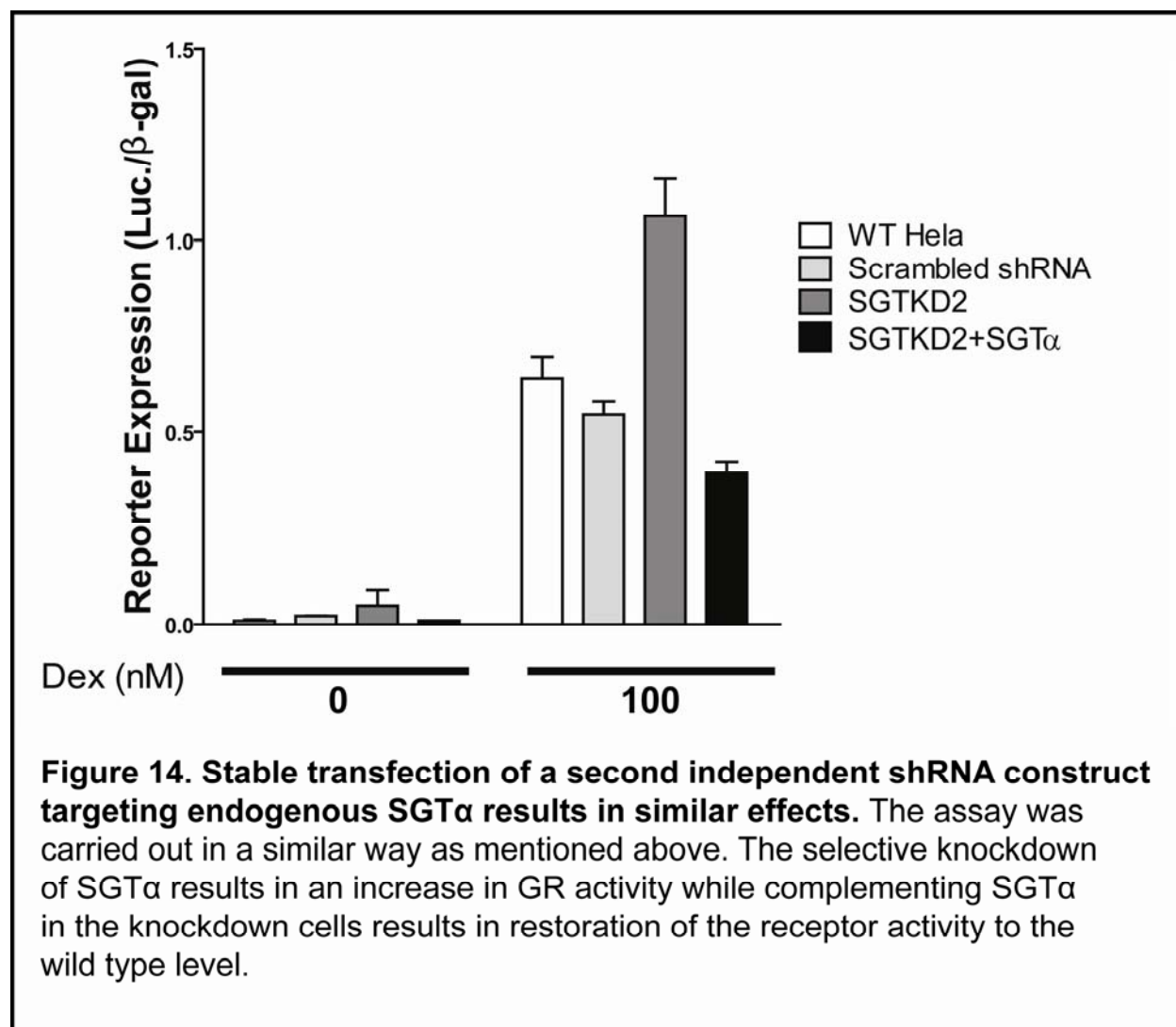
Western blots were performed using cell lysates from HeLa cell extracts taken from wild type cells, cells carrying a stable scrambled shRNA construct, cells carrying a stable shRNA construct directed against SGTα, and SGTα knockdown cells exogenously expressing SGTα. SGTα protein levels were significantly reduced in the knockdown cells as compared to the wild type and scrambled control cells. In addition, transfection of an SGTα expression vector restored SGTα levels to well beyond wild type levels. An antibody directed against GAPDH was used as a loading control.

overexpression of SGTα restored the receptor activity to the wild type level. Figure 13 illustrates the western blot analysis of the assay. Notably, the GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) level was unaffected due to stable transfection of SGTα shRNA or scrambled shRNA.

Taken together, these results indicate SGTα is a key participant in the glucocorticoid and progesterone receptor maturation pathways and downregulates receptor activity.

Alternative shRNA construct to validate knock down effects:

To rule out potential “off-target” effects due to shRNA-mediated knockdown, an additional shRNA construct was made and stably transfected into the HeLa cell line. Glucocorticoid receptor-mediated reporter assays were carried out in a similar way.



The findings shown in Figure 14 confirmed the effects observed on both the glucocorticoid and progesterone receptors are due to specific knockdown of SGTα in the cells.

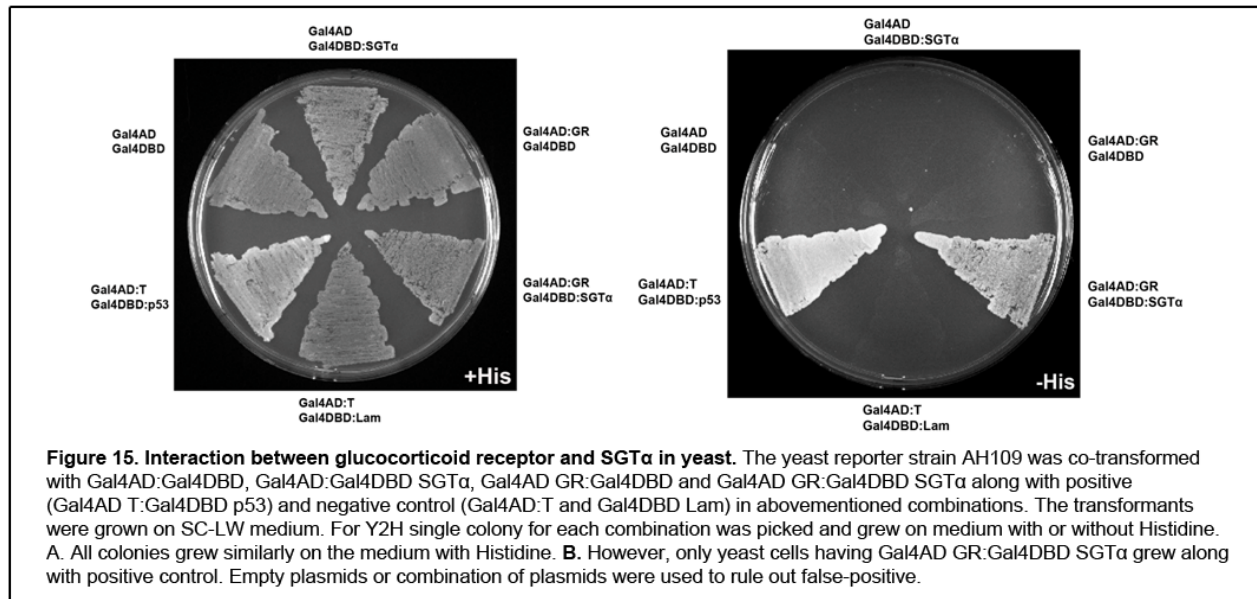
3.6 Determination of Physical Interaction between SGT α and the Receptor-Chaperone Complex.

The assays performed in both yeast and mammalian cell lines suggest SGT α is a potential modulator in glucocorticoid and Progesterone receptor signaling pathways. Previous studies demonstrated that SGT α interacts with Hsp90 and Hsp70 (21, 22) and regulates their activity. However, it is still unclear whether SGT α directly interacts with the receptor-complex. Therefore, the next logical step would be to determine if SGT α regulates the receptor activity by directly interacting with the receptor-chaperone complex.

Yeast two-hybrid screening is a powerful tool to screen possible protein-protein interactions. Many weak protein-protein interactions can be abolished with the use of increasingly stringent selection conditions. Since the proteins are maintained in their native conformation in yeast cells, it is possible to identify even the weak interactions between different proteins by yeast two-hybrid screening. Albers *et al* (2004) have demonstrated yeast-two hybrid screening as a potential approach to assess nuclear receptor interacting proteins (48). We are using the SGT α gene as bait and expressing as a fusion to the GAL4 DNA-binding domain (pGBKT7: SGT α). Glucocorticoid and progesterone receptors were expressed as a fusion to the GAL4 activation domain as prey (pGADT7:GR and pGADT7:PR). Transforming these plasmids into specific yeast strains will bring the activation and DNA binding domain in close proximity and if SGT α interacts with GR or PR, will trigger the transcription of GAL4 reporter gene which will allow the yeast cells to grow in specific amino acid-deficient medium. Therefore, yeast

two-hybrid screening should be considered as an ideal system to analyze the interaction between SGT α and GR and PR.

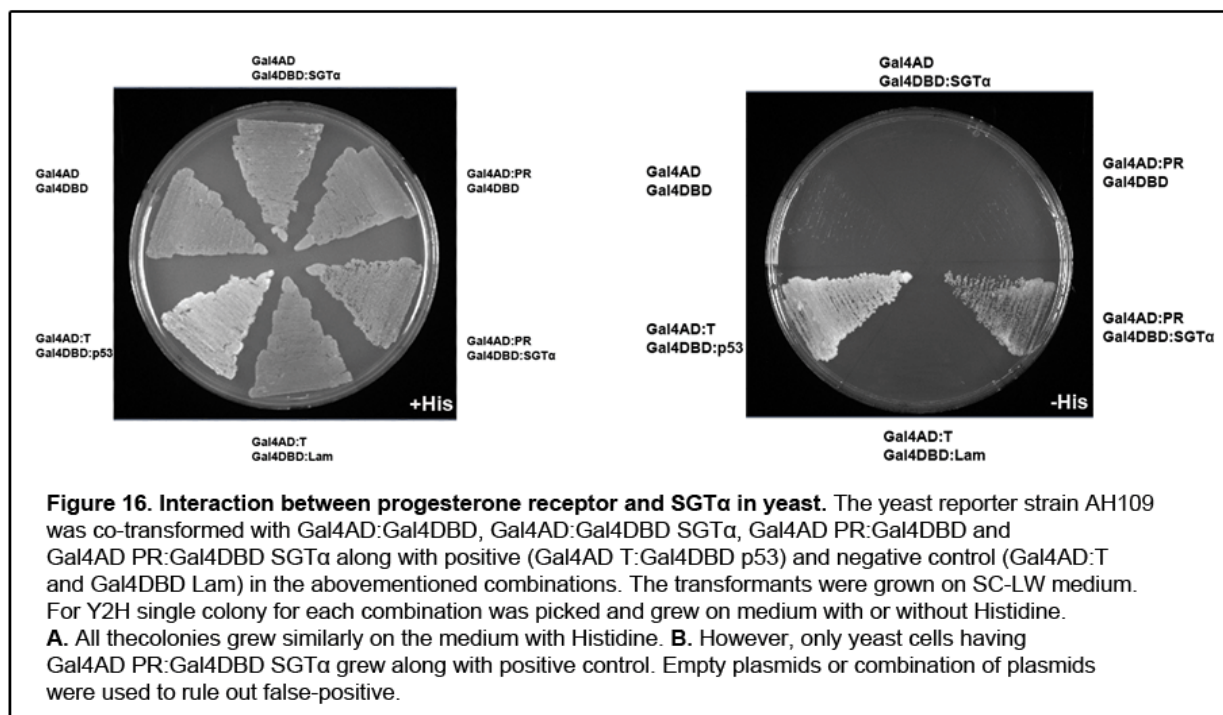
pGADT7:GR and pGBKT7: SGT α plasmids were co-transformed into the yeast strain AH109 obtained from Clontech. Empty plasmids lacking either GR or SGT α were also transformed as a control where indicated. (pGADT7:pGBKT7, pGADT7: SGT α , pGBKT7:GR). In addition, pGBKT7-53 (murine p53 in pGBKT7) and pGADT7-T (SV40 large T-antigen in pGADT7) were also co-transformed as a positive control. Murine p53



and SV40 large T-antigen are known interacting partners in the yeast two-hybrid system. As a negative control, human lamin C fused with DNA-BD (pGBKT7-Lam) was co-transformed with pGADT7-T in AH109 strain. Human lamin C and SV40 large T-antigen do not interact with each other. Therefore, this ruled out fortuitous interaction between two proteins. As shown in Figure 15 co-transformed colonies were grown in medium with or without histidine. All co-transformants grew similarly in non-selective medium (Figure 15. A). However, when plated on selective medium (SC-LWH), only yeast cells expressing GAL4DBD- SGT α and GAL4AD-GR grew (Figure 15. B) along

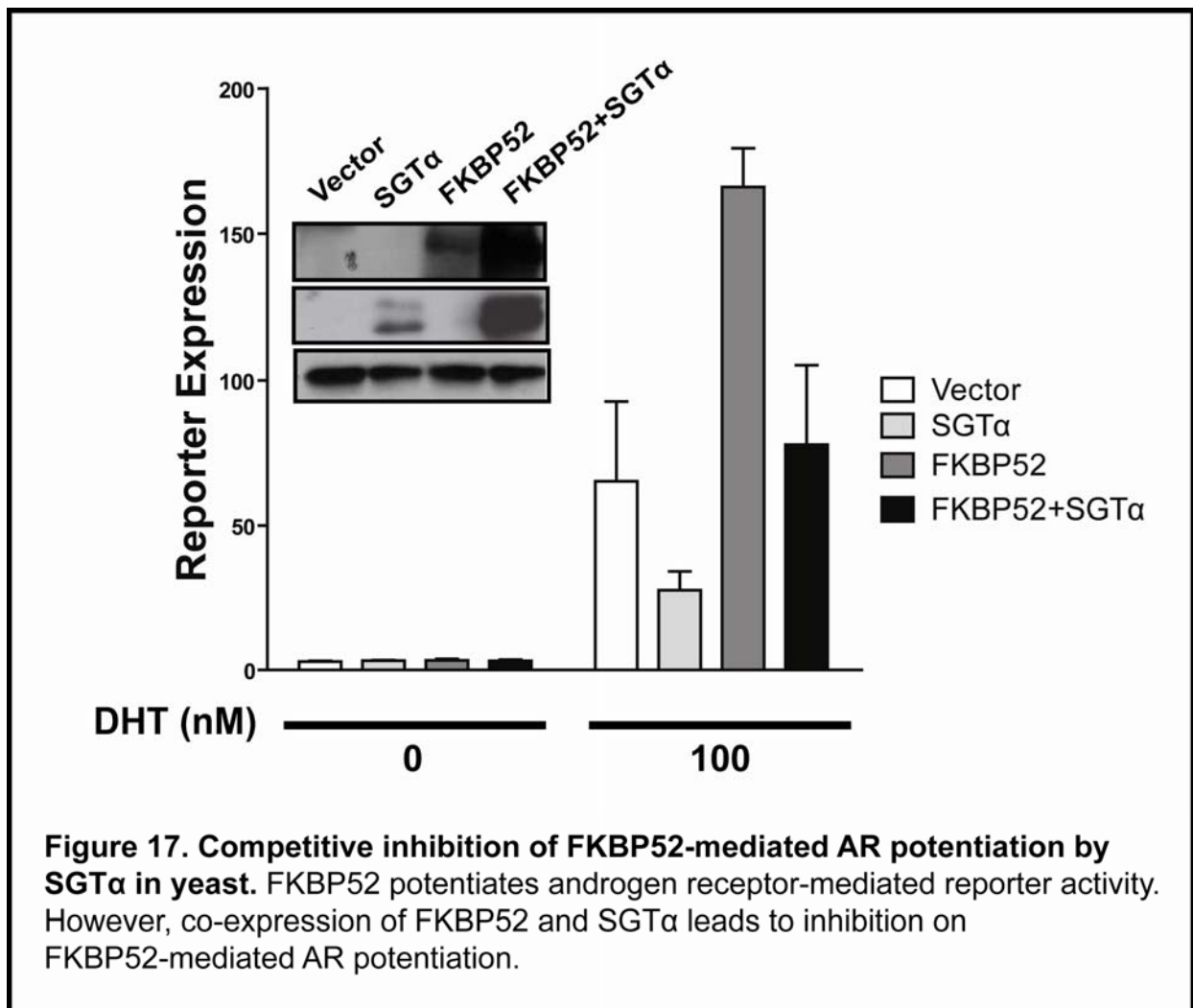
with the positive control (GAL4DBD-53 and GAL4AD-T). Together, these results indicate that SGT α interacts with the GR-Hsp90 complex in a yeast two-hybrid system.

To investigate the possible interaction between human PR and SGT α , similar transformations were carried out along with the abovementioned positive and negative controls. As shown in Figure 16. A, all co-transformed colonies grew similarly in the non-selective medium. However, when they were grown in selective medium lacking histidine (SC-LWH), as shown in Figure 16. B, yeast cells expressing GAL4DBD- SGT α and GAL4AD-PR grew along with the positive control (GAL4DBD-53 and GAL4AD-T). Absence of growth in the negative control (GAL4DBD-Lam and GAL4AD-T) as well as in the cells transformed with empty vectors with or without receptor and SGT α combinations indicate this is not a fortuitous interaction between the receptor and SGT α . Together, these finding indicate that SGT α interacts specifically with glucocorticoid and progesterone receptor complexes.



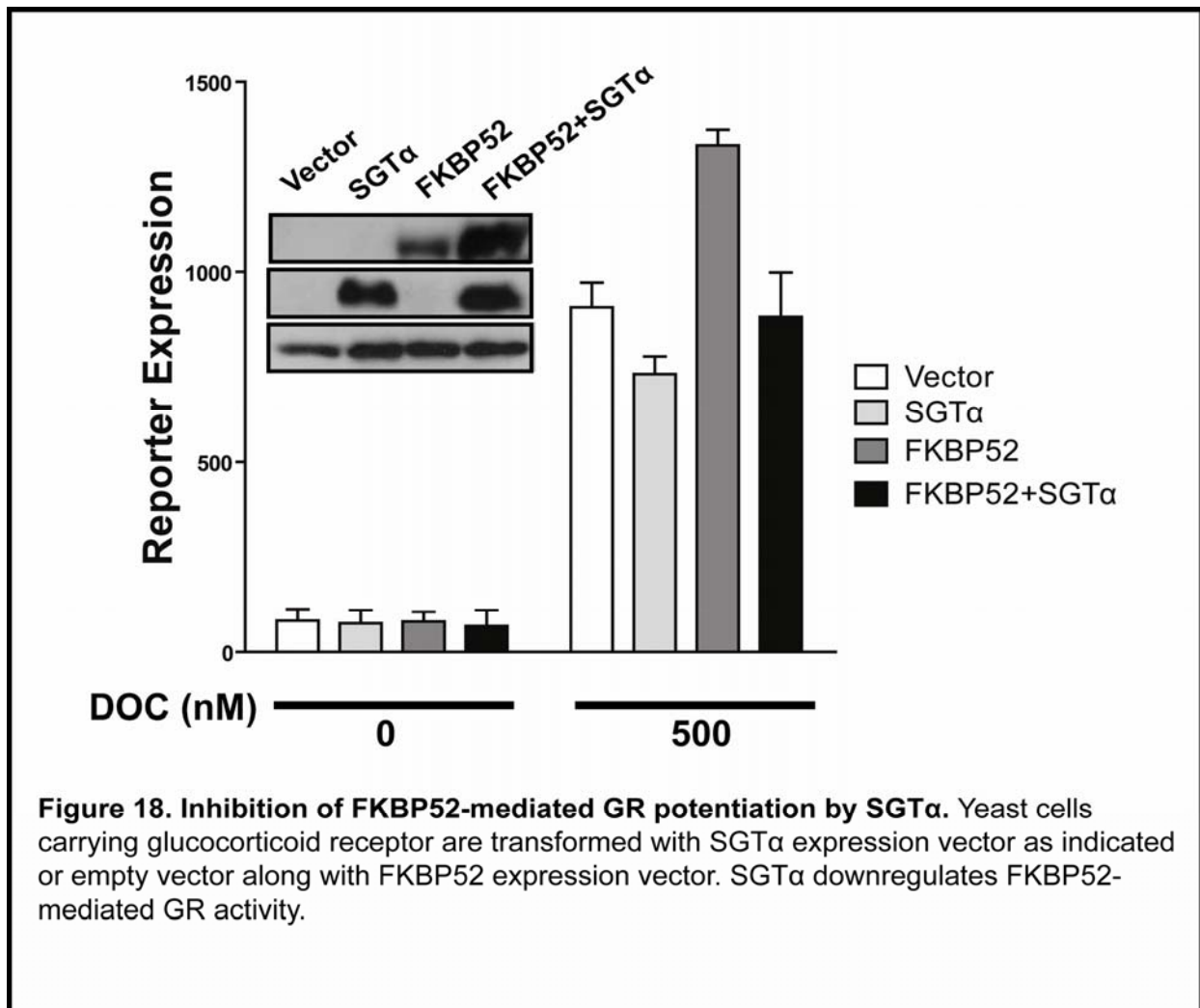
3.7 SGT α inhibits FKBP52-mediated potentiation of AR and GR in yeast model system.

One of the best characterized co-chaperones (immunophilin) in the steroid hormone receptor complex is FK506 binding protein 52 (FKBP52) which potentiates androgen, progesterone, and glucocorticoid receptor function (5). FKBP52 is a TPR protein that acts on the receptor-Hsp90 complex through binding to the C-terminal



EEVD motif on Hsp90. Given that SGT α is a TPR protein that also interacts with Hsp90 through the C-terminal EEVD motif on Hsp90, we investigated if the mechanism by

which SGT α negatively regulates the receptors is at least partially through the competitive inhibition of FKBP52 binding to the complex. In support of this idea is the

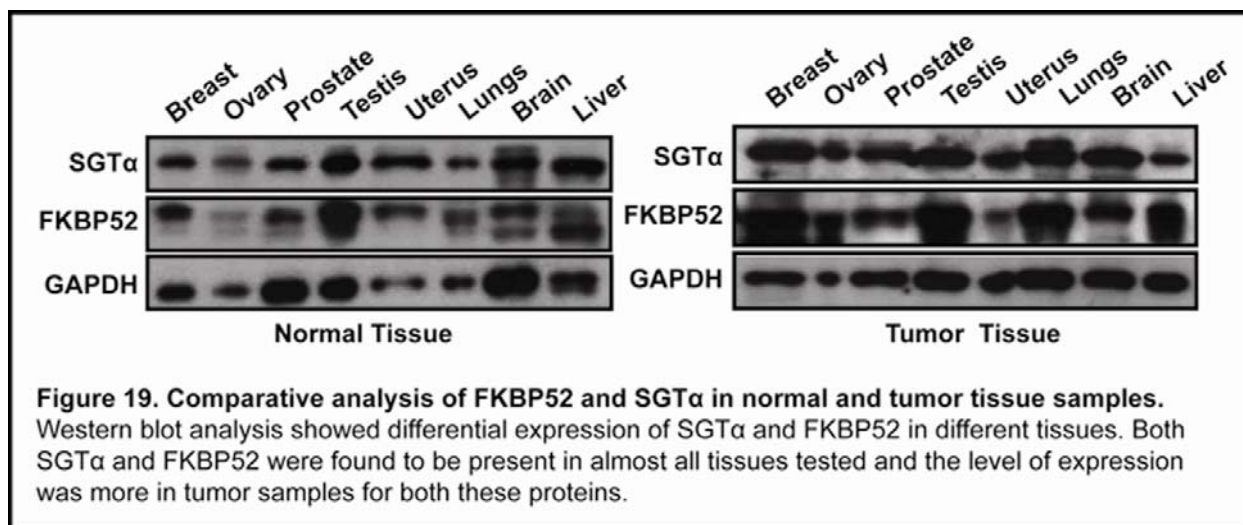


fact that SGT α is a specific negative regulator of the same receptors regulated by FKBP52. To test this idea we assayed receptor function with FKBP52 and SGT α alone or in combination in yeast-based assays. Since mammalian cells have both endogenous SGT α and FKBP52, yeast appeared to be as the ideal model system to perform these assays. As shown in Figures 17 and 18, these data demonstrate that co-expression of SGT α can effectively shutdown the ability of FKBP52 to potentiate androgen and glucocorticoid receptor function in a dose-dependent manner. Western

blot analysis showed that the effects observed in AR and GR activity were not due to degradation of the proteins. Interestingly, co-transformation of FKBP52 and SGT α was found to make these proteins more stable than when transformed individually. The yeast L3 ribosomal protein level was same for all the transformants validating the finding that both FKBP52 and SGT α were more stable when co-transformed. However, it is important to note that SGT α alone can negatively affect receptor function as FKBP52 is not present in the yeast system. Thus, although SGT α may be able to competitively inhibit FKBP52 it can also affect receptor function alone.

3.8. Comparative Analysis of FKBP52 and SGT α in normal and tumor tissues.

Western blot analysis of normal and tumor tissue samples showed that both FKBP52 and SGT α protein level were present in almost all the reproductive tissues testes along with brain, lung and liver (Figure 19). Importantly, the expression of these two proteins was higher in tumor samples than in normal human tissue samples. This is indicative of the fact that these two proteins playing roles in normal physiological conditions as well as during carcinogenesis.



3.9. In vitro competitive inhibition of FKBP52 and SGTα for Hsp90 binding.

Furthermore, we investigated the competition between FKBP52 and SGTα in *in vitro* co-immunoprecipitations. Since both FKBP52 and SGTα compete for binding on the C-terminal EEVD motif on Hsp90, purified Hsp90, FKBP52 and SGTα were used for analysis by co-immunoprecipitation. Briefly, anti-SGTα antibody (Santa Cruz Biotechnology) was used to pull down Hsp90 and FKBP52 along with a rabbit non-immune serum antibody as control. To do this experiment we used equimolar

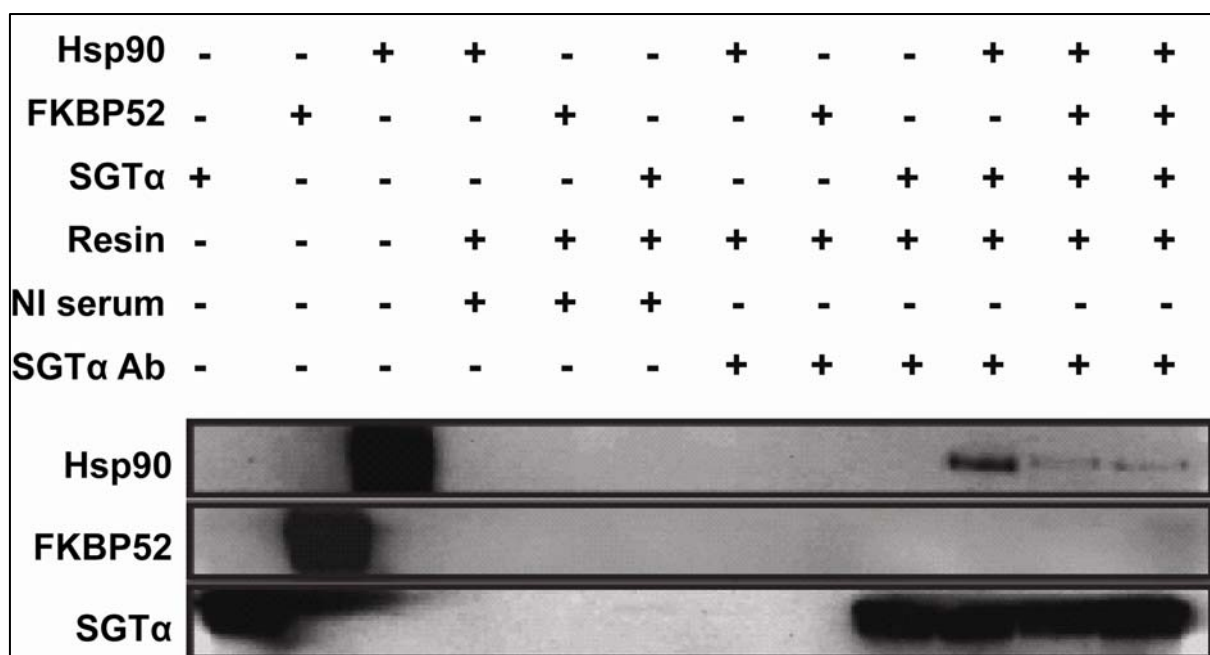


Figure 20. FKBP52 and SGTα compete for binding on Hsp90.

Co-immunoprecipitation was performed with purified Hsp90, FKBP52 and SGTα. Neither of the proteins pulled down with rabbit non-immune serum (lanes 4, 5, 6). Also treatment with anti-SGTα antibody could not detect any bands for Hsp90 and FKBP52 (lanes 7, 8). However, anti-SGTα antibody was used, Hsp90 was detected in absence of FKBP52 (lane 10). This interaction however diminished in presence of equimolar (lane 11) or higher concentration of FKBP52 (lane 12). Lane 9 showing anti-SGTα antibody detecting purified anti-SGTα. Lanes 1, 2 and 3 were positive controls for SGTα, FKBP52 and Hsp90 respectively.

concentrations of Hsp90 and SGT α along with either equimolar or higher concentrations of FKBP52 (where indicated). As shown in Figure 20, neither of the proteins immunoprecipitated with the rabbit non-immune serum or anti-SGT α antibody when used to pull-pull down individual proteins. However, when with anti-SGT α antibody in the reaction containing Hsp90 and SGT α , Hsp90 was detected as its interacting partner. This interaction however diminished when we used equimolar or higher concentration of FKBP52 to the same reaction. The first three lanes showed the purified proteins as positive control. These results demonstrate that FKBP52 and SGT α compete for binding Hsp90 and that FKBP52 has a stronger binding affinity for Hsp90 than SGT α .

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1. Discussion

Hsp70 and Hsp90 are well-characterized molecular chaperones that partake in a myriad of cellular functions (50-55). Steroid hormone receptors are among the first identified client proteins for the Hsp90 chaperone (20). In addition to their effects on steroid hormone receptor folding, both Hsp70 and Hsp90 concentrate the cochaperone activity within the complex (49). Among the well-characterized co-chaperones involved in the steroid hormone receptor complex are the large members of the FK506 binding family of immunophilins (FKBP51 and FKBP52). FKBP52 and FKBP51 were shown to share approximately 70% amino acid identity. FKBP52 was found to influence receptor hormone binding and potentiate receptor activity for AR, GR and PR (56-60). Although FKBP51 was known to antagonize the action of FKBP52 for a considerable period, recent studies done by Ni et al, 2010 cast new insights of how FKBP51 promotes androgen receptor signaling in prostate cancer cells. The findings indicate that FKBP51 is overexpressed in hormone refractory prostate cancer (HRPC) cells and regulates AR-dependent transcription and cell growth (61). Interestingly, using biomolecular complementation and coimmunoprecipitation techniques another group identified a novel FK506 binding protein (FKBPL) that interacts with the glucocorticoid receptor (GR) complex and regulates its activity. FKBPL was classified as a newly identified member in the receptor-chaperone complex (62). In addition, *Chadli et al* (2006) identified a novel cochaperone, GCUNC-45 which was considered to act in folding and activity of myosin (63), as a novel positive regulator for progesterone receptor (64). Moreover, Okada et al (2004) identified S100A1 as a novel cochaperone that interacts with Hsp90,

Hsp70, Cyp40 and FKBP52 (65). Later studies showed that the TPR domain in both Cyp40 and FKBP52 were the domain for interaction with the S100A1 protein (66). Collectively, these findings indicate that we are about to understand biochemical and molecular interactions between different known as well as previously unknown co-chaperones and steroid hormone receptor-chaperone complex. Interestingly, as reviewed by Didier Picard, different steroid hormone receptors have different cochaperone specificity (16) and thus regulate receptor activity in different ways. Therefore, a detail understanding of the co-chaperones involved in different steroid hormone receptor maturation pathways is crucial to understand the roles of these co-chaperones on steroid hormone receptor maturation and activity.

In this study, we investigated the possible roles played by SGT α in different steroid hormone receptor signaling pathways. SGT α was previously known to interact with Hsp90 and Hsp70 and found to be involved only in the androgen receptor signaling pathway (41) among all steroid hormone receptors. Here we report SGT α is also a potential modulator of GR and PR signaling pathways. We exploited yeast as an exploratory model system to investigate the role of SGT α in different steroid hormone receptor maturation pathways. Also, our findings in the mammalian cell culture system confirmed that SGT α downregulates GR and PR activity in a dose-dependent manner. Since, both SGT α and FKBP52 have specificity for the same receptors (AR, GR, PR) and SGT α interacts with C-terminal EEVD motif on Hsp90, which also serves as interacting domain for FKBP52, we examined the presence of FKBP52 and SGT α in the reproductive tissues. Western blot showed both proteins are present in all human reproductive tissues tested and their levels were increased in the tumor samples.

Although, it is difficult to hypothesize the mechanisms of how the effects of FKBP52 and SGT α on SHRs alter during carcinogenesis in different tissues, it is clear that the elevated level of expression observed in our study indicate these two co-chaperones may play some roles during cancer progression. However, further studies must be conducted to confirm the changes that take place during carcinogenesis.

Although Hsp70 and Hsp90 do not have considerable amino acid similarity, they share a consensus motif at the C-terminus. Both of these proteins terminate with an EEVD motif which is recognized by most of the TPR containing proteins (20). Studies performed with an EEVD deletion mutant demonstrated that the EEVD motif is required for interaction with TPR proteins (36, 37, 39). However, the affinity of different TPR proteins for binding with Hsp90 is not well-known. Nevertheless, it always remains a possibility that some proteins interact with the chaperone complex with higher affinity than others. Although, it is difficult to pursue this question *in vivo*, *in vitro* analysis between SGT α and FKBP52 showed FKBP52 has a stronger binding affinity for Hsp90 and knocks off SGT α from the complex (Figure 16). In spite of the inhibitory effects of FKBP52 on SGT α for binding on Hsp90 and thereby dissociating SGT α from the complex, it is important to note that SGT α can downregulate receptor activity even in absence of FKBP52 (Figure 3, 4,5). Since yeasts don't have either SGT α or FKBP52, this served as an ideal model to examine the inhibitory effects of SGT α and FKBP52 on each other in terms of receptor activity. The assays showed functional competition between these two proteins on AR and GR activity (Figures 14 and 15). As postulated by Davies et al, (2005), FKBP52 has a higher affinity for association with the GR-Hsp90 complex followed by protein phosphatase 5 and FKBP51 (67). Although our *in vitro*

studies showed competition between FKBP52 and SGT α , further studies must be pursued to confirm these findings in physiological conditions.

In summary, our findings demonstrate that SGT α is a potential modulator of the GR and PR maturation pathways that directly interacts with the receptor-chaperone complex and alters receptor activity. With these findings, SGT α can be classified as a new cochaperone to the burgeoning cochaperone diversity of the steroid hormone receptor complex.

4.2. Future Directions

Over the past couple of decades significant progress have been made in understanding the role of molecular chaperones and co-chaperones , especially TPR proteins. These co-chaperones were not only found to have a role in client protein folding but also to be involved in various cellular and biochemical processes. Recent findings showed that Fkbp52 $^{-/-}$ female mice have compromised PR function and fail to support blastocyst implantation (68). Also a case-control study showed that alteration in TPR cochaperone FKBP1 leads to male infertility (69). In addition, two other recently identified Hsp70-associated TPR containing co-chaperones HBP21 and DYX1C1 have been implicated in breast cancer progression (70). Collectively, these findings indicate that TPR co-chaperones play significant roles in normal cellular processes as well as during cancer progression and, therefore, can be viewed as potential therapeutic targets. Interestingly, SGT α has recently been identified as a candidate gene for polycystic ovary syndrome (PCOS) (71). Moreover, studies done by *Buchanan et al*, (2007) showed that SGT α downregulates AR activity in a hormone-independent manner and helps in cytoplasmic retention of AR in prostate cancer cells (41). The presence of

SGTα in all human tissues tested and the elevated levels in tumor samples imply its putative involvement in normal signaling cascades as well in tumorigenesis. Our data suggests SGTα is also a possible regulator of GR and PR. Additionally, our data indicates SGTα inhibits FKBP52-mediated potentiation of AR and GR in a yeast model system. Collectively, these data signify SGTα as a potential modulator of steroid hormone receptor maturation and signaling pathways. Receptor specificity for both SGTα and FKBP52 and competitive inhibition between these two TPR proteins as found in both the yeast model system as well as in *in vitro* studies point out that SGTα could be targeted as a potential biological inhibitor for FKBP52. Although the precise mechanism still remains elusive, it is tempting to speculate that SGTα may serve as a molecular buffer in controlling the aberrant gene expression mediated by steroid hormone receptors during cancer. Further studies should be directed to gain knowledge about the mechanism of how SGTα regulates receptor maturation and how this functioning is altered during tumorigenesis. This will not only help to understand the molecular events behind the receptor maturation but will also provide valuable insights to target proteins like SGTα to specifically disrupt the receptor-chaperone complex.

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