GLUCOCORTICOID RECEPTOR ALTERNATIVE SPLCING: KEY PLAYERS & ROLE IN TM AND GLAUCOMA

DISSERTATION

Presented to the Graduate Council of the University of North Texas Health Science Center at Fort Worth in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY By ANKUR JAIN, November 2012.

ACKNOWLEDGMENTS

This project was supported by the grant from the National Institute of Health to Dr. Abbot F Clark (EY016242-06A1).

I would like to express my deepest gratitude to my advisor, Dr. Abe Clark, for his excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research. I would also like to acknowledge my co-mentor Dr. Robert J. Wordinger for his support and additional wisdom in critical problem solving. In addition to guiding this research, the opportunities they provided to broaden my professional experience and prepare me for future challenges are gratefully acknowledged.

I would like to thank Dr. Thomas Yorio from Department of Pharmacology and Neurosciences; Dr. Hassan Alizadeh from Department of Cell Biology and Anatomy; and Dr. Michael L Smith from Department of Integrative Physiology for serving on my dissertation committee and for providing valuable guidance and suggestions. My thanks are extended to the Graduate School of Biomedical Science and the Department of Cell Biology and Anatomy for giving me the opportunity to advance my skills and education in research.

I would also like to offer special thanks to Dr. Yi-Qiang "Eric" Cheng from Department of Biological Sciences in University of Wisconsin – Milwaukee for providing us with sufficient quantities of compounds to perform experiments in a timely manner.

A few Individuals who provided their invaluable continued support with various molecular biology techniques and data interpretation include Dr. John Fuller (John Hopkins University), Dr. Weiming Mao (University of North Texas Health Science Center) and Dr.

Anirudh Sethi (University of Texas Southwestern Medical Center). I am very grateful to them for their support throughout this research.

I would like to thank to Dr. D K Majumdar (University of Delhi) from my undergraduate school who always acted as a motivational catalyst before and throughout this whole process.

I would like to express my appreciation to Syndi Espinoza, who constantly ensured that all the administrative requirements of the department and graduate school were met.

I would also like to thank my grandparents, my parents (Father Vipin Jain and Mother Shobha Jain) and my three sisters (Anu Gupta, Meetu Jain and Neetu Jain) for their continued motivation, sacrifice, and unconditional love.

I would like to express my deep appreciation to the many friends and family who provided so much support and encouragement throughout this process. They include Manoj Kumar, Imit Kaur, Ginni Kumar Wadhwa, Tomi Luan, Rahul Roopwani, Alakananda Mudumby, Rahul Garg, Lokesh Gowda, Yogesh Mishra, Maria Gonzalez, Nishkarsh Likhar, Swati Gulati, Mukul Retdhania, Amit Kumar, Anirudh Sethi, Tasneem Putliwala, Wanda Media-Ortiz, Prateek Pathak, Akhil Gupta, Sean Silverman, Ashley Fitzgerald, Mufaddal Kasim, Rahul Chib, Avani Mody, Urmimala Raychaudhuri, Divya Duggal and each member of Clark-Wordinger family. Your friendship is of indescribable value, not only during the course of this research, but in all that we do.

Finally, I would like to thank my future wife, Monika Mittal. She has always been there cheering me up and stood by me through the good times and bad. No words can express how grateful I am for your love and support and how very much I love and appreciate you.

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CHAPTER1

INTRODUCTION

Glaucoma

Glaucoma is the leading cause of irreversible blindness in the world and affects 1% of the world population that represents about 60-70 million people[1-3]. It represents a group of optic neuropathies that result in death of retinal ganglion cells leading to slow and progressive vision loss[4]. Primary open angle glaucoma (POAG) is the most prevalent form of glaucoma in the United States. It was named because of an open iridocorneal angle and no known causative factors in patients. The loss of vision in POAG is slow, painless and asymptomatic. And unfortunately, it is usually diagnosed at the later stages of the pathology during evaluation of other eye-related disorders by clinicians. Although, no affirmative factors have been designated to POAG pathophysiology, elevation of intraocular pressure (IOP) is regarded as the most significant risk factor[5]. IOP is a measurement of the pressure maintained by the fluid called aqueous humor (AH) in the anterior segment of the eye. The AH is secreted from the ciliary body, flows and nourishes different tissues in the anterior chamber and exits through the trabecular meshwork (TM) outflow pathway into Schlemm's canal and episcleral veins[6-7].

Besides POAG, there is another prevalent form of glaucoma called secondary open angle glaucoma, caused by known factors or exposure to some stimuli. Steroid-induced glaucoma is the major example of secondary open angle glaucoma caused by topical or systemic administration of glucocorticoids (GCs). Although they differ in the onset of disease, POAG and GC-induced glaucoma share similar clinical phenotypes. Both involve similar biochemical and

molecular changes in the TM[8-12], the elevated IOP in both types is due to impaired AH outflow, and if left untreated, both lead to similar optic nerve head disc cupping and characteristic visual field loss (Table 1.1).

Table 1.1: Comparisons between POAG and steroid-induced glaucoma

Feature	**Primary Open Angle Glaucoma**	**Steroid-Induced Glaucoma**
Iridocorneal Angle	Open	Open
Causative Factors	Unknown	Glucocorticoid administration
Elevated IOP as risk Factor	Yes	Yes (in responders)
Associated TM changes	Yes	Yes
Cross-linked actin networks (CLANs)	Present	Present
Optic nerve head disc cupping	Yes, later stages	Yes, if untreated
Current treatment	IOP-lowering agents	Withdraw GCs, IOP-lowering agents

Trabecular Meshwork (TM)

The TM is a specialized tissue present at the junction of iris and corna, rich in extracellular matrix (ECM) beams. Histologically, the TM can be divided into three distinct layers. The outer uveal and corneoscleral portions of the TM are highly fenestrated and are composed of several irregular layers of ECM covered by the TM cells. The trabecular beams become more flattened and sheet-like in the deeper portions of this region[13]. The center or stroma of the beams exhibits typical collagen fibrils, elastic fibers and microfibril sheath-derived material[14]. Between the beams and sheets, the irregular intertrabecular spaces form channels leading to the juxtacanalicular (JCT) region, which lies adjacent to the endothelial lining of Schlemm's canal

(SC)[15-16]. The cells on the outer layers of the TM are actively phagocytic and are thought to act primarily as pre-filters, removing cellular debris from the aqueous humor prior to its passage through the less porous inner JCT and SC regions. The JCT region is composed of an amorphous ECM with a discontinuous scattering of several layers of TM cells embedded within the ECM. Basement membrane proteins, type IV collagen, fibronectin and laminin, and basement membrane proteoglycans have been identified in the JCT region[13,17-22]. The TM tissue (especially JCT region) has been proposed as a major conventional outflow pathway to provide the greatest resistance to AH exit and involved in POAG etiology[7].

Glucocorticoids (GCs) and Glucocorticoid Receptor (GR)

Glucocorticoids (GCs) are a class of steroid hormones that possess anti-inflammatory properties and have a significant role in glucose metabolism. Over decades, they have been ruling the market of anti-inflammatory, anti-allergic and immune-suppressant classes of drugs. However, the prolonged use of these slow acting drugs has been found to increase IOP. There are a number of in vitro, ex vivo and in vivo studies suggesting the role of GCs in glaucoma. GC treatment results in altered TM cell morphology[8] and reduced phagocytic properties of the TM cells. GCs cause reorganization of the cytoskeleton to form cross-linked actin networks (CLANs) in cultured human TM cells and tissue[9-11]. A potent and well-established GC, dexamethasone (DEX) is known to induce the glaucoma gene MYOC or trabecular meshwork inducible glucocorticoid response (TIGR gene) in TM cells[12].

However, there have been several reports about differential GC responsiveness in the human population[23]. The human population can be classified into three categories based on responsiveness to topical ocular administration of DEX. 4-6% of the population were classified as super responders, developing steroid-induced pressure rises of >15 mm Hg following

administration of GC 3-4 times a day for 4-6 weeks. Approximately one- third of the population was moderate responders, developing pressure rises of 6-15 mm Hg. The rest of the population was considered non-responders. It has been reported that almost all POAG patients are moderate to high steroid responders[24]. Strikingly, it has also been observed that steroid-responsive non-glaucomatous individuals are at much higher risk of developing POAG compared with steroid non-responders[25, 26].

GCs bind to glucocorticoid receptors (GR) present in the cytoplasm to exert their actions. After binding to its receptor via the ligand-binding domain, a conformational change occurs in the receptor-ligand complex. The conformational change results in removal of the inhibitory accessory proteins. As a result, the ligand-bound receptor gets translocated to the nucleus. In the nucleus, the ligand-bound receptor homodimer binds to glucocorticoid response elements (GREs) within the promoter region of target genes (Figure 1.1). The GR can positively or negatively regulate gene expression, depending on the response element sequence and the promoter context. The GR also modulates gene expression, independent of GRE, by physically interacting with other transcription factors (e.g. activating protein AP-1 and nuclear factor NF-κB, Figure 1.1)[27-29]. In addition to the variety of genomic signaling mechanisms, there is increasing evidence that glucocorticoids are capable of rapid signaling events independent of transcriptional changes[30-32] (Figure 1.1). This mechanism of action of GCs is very well documented for the classical GRα. However, other isoforms for GR are also present. The GRβ isoform is formed as a result of alternative splicing within exon 9[33]. Exon 9α in GRα codes for the major portion of the ligand binding domain of the GR. Due to inclusion of exon 9β in the GRβ isoform, a unique 15 amino acid sequence is inserted at the C-terminus in contrast to a 50 amino acid sequence in the classical GRα[32]. The remainder of the receptor is identical in both

isoforms. Since the GRβ isoform does not possess ligand-binding capacity, it acts as a dominant negative regulator of GRα-induced transactivation of GC-responsive genes[33] (Figure 1.2). Recent studies also indicate that at least eight different GRα or GRβ N-terminal isoforms are generated from one single GR gene by alternate translation initiation[34]. The unique transcriptional activities and distinct tissue specific patterns of GRα and GRβ isoforms could provide a novel mechanism for tissue specific GC responses. It has been shown that the GRβ/GRα ratio is increased in GC-insensitive diseases like asthma[35], leukemia[36], and rheumatoid arthritis[37] and is decreased in conditions like essential hypertension[38], diabetes mellitus type-2[39], and glaucoma[40]. Our lab has previously published that both GRβ and GRα exist in the human TM[40, 41]. Also, we reported that knocking down GRβ enhances GC responsiveness in human TM cells in terms of increased myocilin and fibronectin production[40] (Figure 1.2). There might be differential regulation of these GR isoforms due to action of accessory proteins and by their nuclear translocation[42, 43].

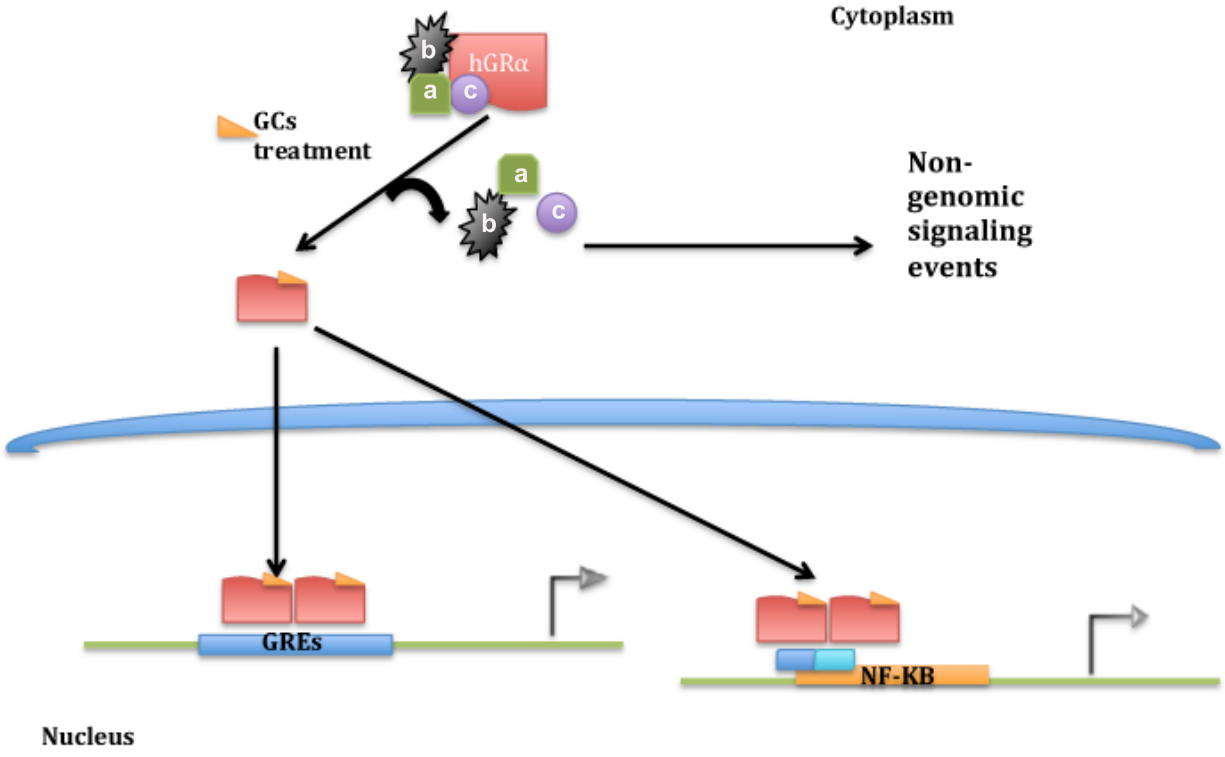

Figure 1.1: Glucocorticoid (GC) treatment leads to the dissociation of inhibitory complex (a, b and c) from GR (hGRα). This triggers either genomic signaling or rapid nongenomic signaling events or both. Ligand bound hGRα can translocate to nucleus where it homodimerizes and interacts with either glucocorticoid response elements (GREs) or with other transcription factors such as NF-KB subunits to inhibit gene expression regulated by these transcription factors.

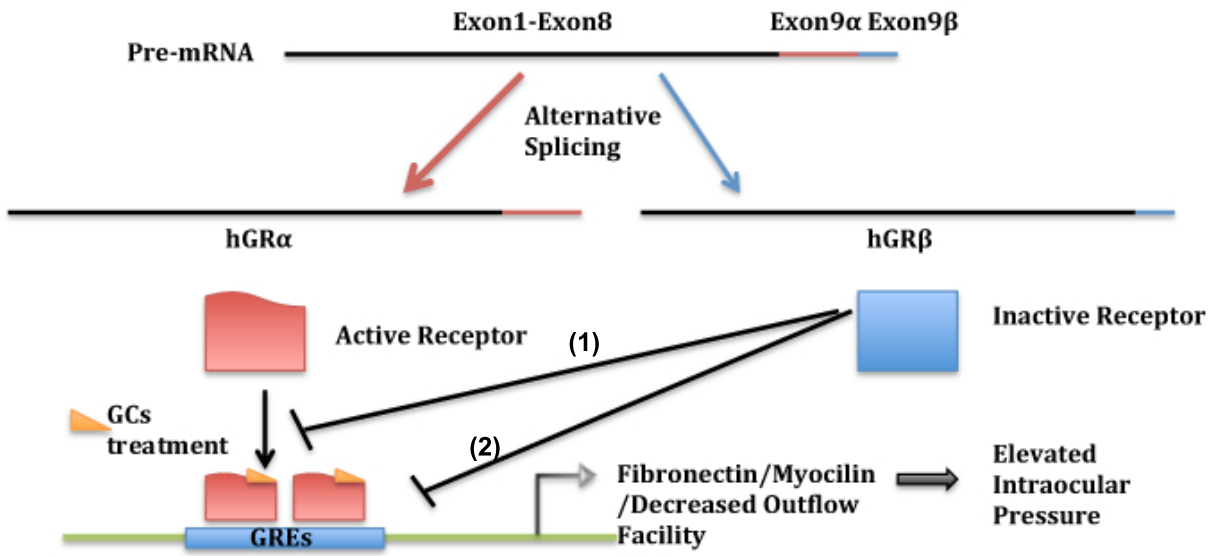

Figure 1.2: Alternative splicing of hGRβ eliminates the GC ligand-binding domain. hGRβ either prevents hGRα homodimer formation {(1) by forming a heterodimer with hGRα} or binding of hGRα homodimer to glucocorticoid response elements (GREs) on DNA (2). As a result, hGRβ acts as a dominant negative inhibitor of hGRα transcriptional activities.

Splicing Factors

Higher GRβ/GRα ratios in normal versus glaucoma patients as well as in other GC-resistant diseases implies their levels are regulated. Splicing and its regulation are disrupted both by mutations within cis-acting elements required for correct pre-mRNA processing as well as mutations that affect trans-acting components. A specialized assembly of proteins known as the spliceosome regulates this process. A spliceosome is composed of about 150 proteins, collectively known as splicing factors, in addition to the five small nuclear ribonucleoprotein particles (snRNPs). The precision of the reaction is accomplished through a coordinated series of

RNA-RNA, RNA-protein and protein-protein interactions[44-49]. Besides three core sequences: 5' and 3' splice sites and the branch point sequence, there are additional sequences located within both introns and exons that recruit trans-acting splicing factors to ensure inclusion of constitutive exons or modulate the efficiency of splice site recognition, promoting alternative splicing. These sequences are referred to as intronic or exonic splicing enhancers (ISE or ESE) or silencers (ISS or ESS). The splicing factors, also known as SR proteins (SRps), have an RNA recognition motif (RRM) and a C-terminal domain rich in arginine–serine (RS) residues. The RRMs determine RNA binding specificity, whereas the RS domain mediates protein-protein interactions that are thought to be essential for the recruitment of the splicing apparatus and for the splice site pairing[44, 45]. These SR proteins are predicted to bind enhancer elements that increase exon inclusion[46-48]. In contrast, negative regulators that decrease exon inclusion, such as the hnRNP proteins, bind silencer elements[49]. The critical balance of these antagonistic regulators is necessary for controlling the level of exon inclusion in the mRNA transcript. Also, such a large number of SR proteins may be well correlated to the requirement of particular SRp/SRps for ESE or ISE selection specificity that in turn, facilitate specific exon splicing pattern in mRNA. However, SR protein function is not restricted to nuclear mRNA splicing, and it is not hard to believe that these proteins, already bound to spliced mRNA and in place to facilitate future interactions, may function in subsequent processing events such as mRNA nuclear transport and translation[50-56].

Spliceosome Modulators

Understanding or optimizing the specificity of effects of the splicing factors will enable future development of small molecule modulators of splicing. One of such molecules is a chemical modulator of splicing called bombesin. Bombesin is a 14-amino acids long peptide, originally

isolated from frog skin, which has been shown to alter GR splicing in PC-3 cell lines[57]. A significant amount of research has been dedicated to develop other novel molecules to affect splicing process. Although, many of these compounds are targeted for the treatment of various cancers, these agents may also be exploited to affect GR splicing and for regulating GC responsiveness in the TM of steroid responders and glaucoma patients. FR901464, Spliceostatin, Pladienolide B, and Sudemycin are microbially derived compounds that have shown promising results as spliceosome inhibitors in various cancer cells[58-64]. Most of these compounds are chemically complex and are challenge to synthesize. Structurally similar compounds called Thailanstatins (TSTs) have recently been isolated from *Burkholderia thailandensis* (Liu, X. et al. and Y.-Q. Cheng, unpublished data). These TSTs show antiproliferative properties in different cancer cell lines with IC50 in low nanomolar concentrations (Liu, X. et al. and Y.-Q. Cheng, unpublished data). These compounds can be bioengineered using the microorganism's various biosynthetic pathways.

Specific Aims:

There have been reports that different SRps such as SRp30c, SRp40 are involved in alternative splicing of GR to increase GRβ cellular levels[57, 65-67]. No one has directly examined the role of these splicing factors in the TM and correlated this with GCs responsiveness. Studying the role of these SRps in the TM will directly answer whether these splicing factors control the GRβ/GRα ratio, which would further explain the heterogeneous response to GCs in terms of elevated IOP and glaucoma pathogenesis. Our lab has previously reported SNP allele frequencies for several of these splicing factors in clinically characterized individuals (normal, POAG patients, and steroid responders); however, these allele differences were not significant[68]. This suggests that inherited differences in these specific SRp genes are not responsible for GC-induced ocular

hypertension and/or glaucoma. However, differential expression of key SRps may determine GRβ/GRα ratio and GC response in such patients. Results from our proposed studies will also tell if measuring the levels or ratio of these SRps can be utilized as screening test for steroid responders and non-responders.

Our preliminary bioinformatics studies showed differences in the number of potential binding sites on exon9 of GR gene for SRp20 and SRp40 (out of many SRps examined) with the former having predominance in exon 9β and the later in exon 9α. Our preliminary studies also showed differences in the level of mRNA expression of SRp40 in number of normal and glaucomatous cell lines tested. Utilizing the data from prediction software, previously published data in other cell types, and our preliminary data, we selected three SRps: SRp20 (SFRS3), SRp40 (SFRS5) and SRp30c (SFRS9) to examine their role in alternative splicing of GR and GC responsiveness in primary human TM cells

The overall goal of this proposal is to characterize compounds that will target the molecules involved in regulation of DEX induced ocular hypertension. We also propose to test whether the relative levels of GRα and GRβ in TM are regulated by specific splicing factors. We hypothesize (summarized in Figure 1.3) that different SR proteins in the TM regulate relative levels of GRβ in trabecular meshwork and that TSTs can be utilized to affect this splicing process to increase GRβ and decrease GC response in TM. The following aims have been developed to test the hypothesis:

1. Determine the role of SRps (SRp20, SRp30c and SRp40) in GCs response in human TM cells.

1.1. To determine whether bombesin (chemical modulator of splicing) affects SRps and the GRβ/α ratio in cultured TM cells.

1.2. To determine the effect of bombesin on DEX activity (fibronectin induction, myocilin induction and GRE-luciferase reporter activity assays).

1.3. To evaluate the effects of SRp20, SRp30c and SRp40 over-expression on GRα, GRβ and DEX activity in the TM cells.

2. Determine the role of Thailanstatins (TSTs) as spliceosome modulators to affect GR splicing and glucocorticoid response in human TM cells.

Significance:

This will be the first study focusing on the molecular role of SRps in steroid responsiveness and glaucoma. This study will be useful in better understanding the specific mechanisms involved in GC-mediated glaucoma that can be targeted therapeutically. Although the role of SRps on GR splicing has been studied previously, their role in GC signaling and activity remains unknown. We address the mentioned gaps in current literature through several of our specific aims. We will evaluate the effect of modulating SRps in human TM cells on DEX response. Proving beneficial roles of TSTs first in-vitro and then translation of in vitro studies to the ex vivo model of DEX-induced ocular hypertension in the perfusion organ culture (POC) of bovine eye anterior segments will help in developing molecules that target GR splicing to affect DEX regulated IOP and glaucoma.

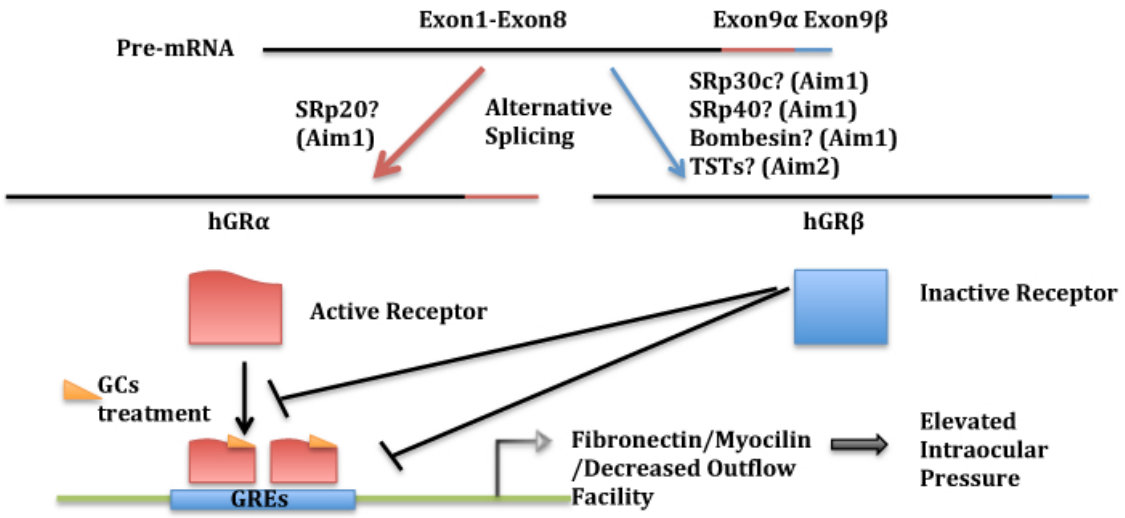

Figure 1.3: Hypothesis. The therapeutic use of glucocorticoids (GCs) can cause elevated intraocular pressure (IOP) and iatrogenic glaucoma in susceptible individuals. GCs have also been implicated in the development of glaucoma. Modulating the levels of Splicing factors (SRps) either by overexpression plasmids (specific aim 1) or by using the bombesin peptide (specific aim 1) or by proposed use of TSTs (specific aim 2) alters GRβ/α ratios in trabecular meshwork (TM) cells. An increased GRβ/GRα ratio decreases GRα-mediated biochemical and cellular changes in the TM and lowers IOP in the eye.

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CHAPTER II

SPLICEOSOME PROTEIN (SRp) REGULATION OF GLUCOCORTICOID RECEPTOR ISOFORMS AND GLUCOCORTICOID RESPONSE IN HUMAN TRABECULAR MESHWORK CELLS*

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* Published *IOVS* 2012 Feb 21;53(2):857-66.

Abstract

Introduction: Glaucoma is a leading cause of visual impairment and blindness, with elevated intraocular pressure (IOP) as a major causative risk factor. Glucocorticoid (GC) therapy causes morphological and biochemical changes in the trabecular meshwork (TM), an ocular tissue involved in regulating IOP, which can lead to the development of glaucoma in susceptible individuals (steroid responders). Steroid responders comprise 40% of the general population and are at higher risk of developing glaucoma. In addition, a majority of glaucoma patients are steroid responders. Differential distribution of various isoforms of GC receptor (GR) may be responsible for this heterogeneity in the steroid response. The alternatively spliced GRβ isoform acts as dominant negative regulator of classical GRα transcriptional activity. mRNA splicing is mediated by spliceosomes, which include SR proteins (SRps). The purpose of our study was to determine whether specific SRps regulate levels of these isoforms and thereby GC response in TM cells.

Methods: Quantitative RT-PCR, western immunoblotting and immunocytochemistry were used to determine the differential expression of different SRps (SRp20, 30c and 40) in human normal

and glaucomatous TM cell strains. Bioinformatics was used to find putative binding sites for SRp20 and SRp40 on exon9 of the GR gene. A peptide modulator of splicing (bombesin) and SRp expression vectors were used to modulate SRps levels and determine their effects on GRα/GRβ ratios as well as dexamethasone (DEX) responsiveness via GRE- luciferase reporter activity, fibronectin, and myocilin induction in TM cells.

Results: SRp20, SRp30c and SRp40 regulate GR splicing and the GC response in TM cells. Modulation of SRps levels altered the GRα/β ratio that correlated with DEX responsiveness. Bombesin decreased SRp20; increased SRp30c, SRp40 levels and GR β/α ratio, and suppressed DEX response in TM cells.

Conclusion: Relative levels of SRp20, SRp30c, and SRp40 in TM cells control differential expression of the two alternatively spliced isoforms of the GR and thereby regulate GC responsiveness. Different levels and/or activities of these SRps may account for differential GC sensitivity among the normal and glaucoma populations.

Key Words: trabecular meshwork, glucocorticoids, glucocorticoid receptor, alternative splicing, glaucoma

Introduction

Glaucoma represents a group of optic neuropathies that result in retinal ganglion cell death leading to a slow and progressive loss of vision and blindness. Glaucoma involves multifactorial etiologies and occurs in many forms, with primary open angle glaucoma (POAG) being the most prevalent form in the United States. Glucocorticoids (GCs) have been suggested to play an important role in pathophysiology of POAG. Elevated levels of cortisol have been reported in

plasma[1-3] and aqueous humor[2] of POAG patients. In addition, altered cortisol metabolism has been reported in the trabecular meshwork (TM)[4-6] and peripheral blood cells[7] in POAG patients.

Steroid-induced glaucoma shares many physiological and clinical symptoms with POAG. GCs cause various morphological and biochemical changes in the TM that are associated with increased aqueous humor outflow resistance leading to ocular hypertension (OHT). GCs inhibit TM cell functions, including phagocytosis[8, 9] and cell migration[10]. GCs also reorganize the cytoskeleton to form cross-linked actin networks (CLANs) in cultured human TM cells[10] and tissues [11] that mimic CLANs observed in cultured glaucoma TM cells [12] as well as TM tissue from glaucoma eyes[13]. A potent and well-established GC, dexamethasone (DEX) is known to induce the glaucoma gene myocilin (MYOC) in TM cells[14, 15].

Physiologically characterized as potent regulators of metabolism, GCs are also used as potent anti-inflammatory, anti-allergic and immune-suppressant agents. However, systemic and ocular side effects, including GC-induced OHT, limit their use. In addition to GC potency, duration and route of administration, GC–induced OHT depends upon the inherent GC sensitivity of the patient [16]. Approximately 4-6% of the general population develop a large increase (>15 mm Hg) in intraocular pressure (IOP) after topical ocular GC administration for 4-6 weeks, while one-third of population develops moderate pressure rise (6-15 mm Hg)[17, 18]. These individuals are categorized as steroid-responders. The rest of the population are considered non-responders. It has been reported that majority of POAG patients are moderate to high steroid responders[17]. In addition, it has also been observed that steroid-responsive non-glaucomatous individuals are at much higher risk of developing POAG compared with steroid non-responders[19, 20]. The molecular mechanisms underlying this differential sensitivity between normal and POAG patients towards GCs are poorly understood.

Alternative splicing of NR3C1, human GR gene, generates two isoforms: GRα and GRβ[21]. GRα is the physiological and pharmacological receptor for GCs and is the classical active isoform through which most GCs work. The GRβ isoform is a truncated form of GRα that lacks the ligand-binding domain and does not transactivate GC-responsive genes. In fact, GRβ functions as dominant negative regulator of GRα transcriptional activity[21]. GRb levels are elevated in a variety of GC resistant diseases, including asthma[22], rheumatoid arthritis[23], inflammatory bowel disease[24], among others[25-30]. We have previously reported that most normal TM cells express relatively higher amounts of GRβ compared to glaucomatous TM cells, making GTM cells more sensitive to GCs [31].

Alternative splicing is one of the many mechanisms that regulate gene expression in eukaryotes, and this process itself must also be regulated. An imbalance or mutation in either trans-acting splicing factors or cis-acting DNA elements might disturb this RNA processing event. A specialized assembly of proteins, known as the spliceosome, mediates this mRNA splicing process. A spliceosome is composed of about 100 proteins, collectively known as splicing factors, in addition to the five small nuclear ribonucleoprotein particles (snRNPs)[32]. The critical balance of these serine/arginine-rich proteins (SRp) and snRNPs is necessary for controlling the level of exon inclusion/exclusion in the mRNA transcript.

There have been reports that different SRps such as SRp30c[33, 34] and SRp40[35] could be involved in alternative splicing of GR, thus altering relative levels of GRa and GRβ. The potential role of these splicing factors in TM and GC responsiveness has not been previously explored. Studying the role of these SRps in the TM will directly answer the heterogeneous response to GCs in terms of elevated IOP and glaucoma pathogenesis. Our lab recently examined SNPs in some of these splicing factors in clinically well-characterized individuals (normal, POAG patients, and

steroid responders) [36]. There was no significant differences in SNP allele frequencies between the 3 cohorts, suggesting that there are no heritable differences in these examined SRp genes associated with steroid responsiveness or POAG. Therefore, differences in the levels and/or activities of these different SRps may determine the GRβ/α ratio and GC response in steroid responders and POAG patients.

Methods

TM Cell Culture

Human TM cells were isolated from carefully dissected human TM tissue explants derived from patients with glaucoma or from normal donors and were characterized as previously described[10, 37-39]. All donor tissues were obtained from regional eye banks and managed according to the guidelines in the Declaration of Helsinki for research involving human tissue. Isolated TM cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) containing L-glutamine (0.292 mg/mL; Gibco BRL Life Technologies), penicillin (100units/ml)/streptomycin (0.1 mg/mL; Gibco BRL Life Technologies), amphotericin B (250 µg/µl; Thermo Scientific ltd.) and 10% fetal bovine serum (Gibco BRL Life Technologies). Stably transformed human TM cell lines GTM3 and NTM5[40] were also used and cultured in the same medium.

TM Cell Treatment

TM cells were grown to 100% confluency in serum containing medium. TM cells were incubated with fresh medium containing different doses (0-10 mM) of bombesin acetate hydrate (Sigma Aldrich; Cat # B4272) for 0.5-48 hrs. In some studies, bombesin (1uM) treatment was

followed by treatment with or without DEX (100 nM) for 6-12 hrs for mRNA isolation and 24-72 hrs for protein isolation.

Bioinformatics Approach

The splicing rainbow (Morais & Valcarcel EMBL 2002 at http://www.ebi.ac.uk/asd-srv/wb.cgi?method=8) bioinformatics software was used to find putative binding sites for various spliceosome proteins in exon 9α and 9β sequences. From the list of binding sites received after the exon9 sequence analysis, we only selected putative binding sites for SRp20 and SRp40 (two splicing factors that have been shown to regulate GR isoforms expression in various cell lines and GC-sensitive diseases). Information on potential SRp30c binding sites was not available in this software package. A sequence was considered a putative binding site for SRp20 if the score (S) was >6 and for SRp40 if S>5. We reduced the stringency for SRp40 to include more sites for analysis.

Over-expression Plasmids and Transfection

Expression plasmids for human SFRS3, SFRS5 and SFRS9 (encoding SRp20, SRp40, and SRp30c, respectively; see Table 1) were purchased from Origene (Rockville, MD). Transfection of plasmids (pCMV6-XL5 was performed as described Origene protocol for transient transfection of mammalian cells. In one tube, Surefect transfection reagent (SA Biosciences, MD) was mixed gently with 250 µl of Opti-MEM medium (Invitrogen) and incubated for 5 min at room temperature. In separate tubes, 1µg (100 ng/µl) of overexpression plasmid or control empty vector plasmid were mixed gently with 250 µL of Opti-MEM medium. These two tubes were combined, gently mixed, and incubated for 20 min at room temperature. After incubation, Opti-MEM without FBS and antibiotics was added to obtain a final volume of 1 mL for each well. 1 ml of GTM-3 cells (dissolved in Opti-MEM medium containing 5% FBS) were plated in

6 well plate ($3X10^5$ cells/ml) and incubated with transfection mixture for 24 h at 37°C. Cells were washed with sterile PBS and incubated with 10% FBS containing DMEM for 24 h (for mRNA) or 36-48 h (for protein) at 37°C. Experiments involving DEX treatment, cells were further kept for 24 h in 10%FBS containing DMEM with or without DEX (100 nM). Cell lysates were analyzed for various proteins by the western immunoblotting (see Table 2 for antibodies used).

RNA isolation, RT-PCR and agarose gel electrophoresis

Total cellular RNA was prepared from cultured TM cells using TRI Reagent RT extraction (Cat. # RL-311, MRC Inc., Cincinnati, OH). The SuperScript VILO cDNA Synthesis kit (Cat. # 11754, Invitrogen) was used for first strand cDNA synthesis. Primers for the various SR proteins were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/). The primer pairs are listed in Table 2.2. The PCR products were loaded and electrophoresis performed on a 1.5% agarose gel containing ethidium bromide to detect DNA bands under UV.

Quantitative Real Time PCR

Real-time PCR was performed as described previously[41]. Briefly, 2.5 µL of cDNA was used in a reaction consisting of 1.5 units per reaction of antibody-bound Taq enzyme (Jump Start; Sigma-Aldrich, St. Louis, MO), 10x PCR buffer, 1.5 mM $MgCl_2$, 200 nM dNTP mix, 100 nM respective primers (Table 2.2), 2.5 µL green nucleic acid dye (EvaGreen; Biotium, Hayward, CA), as well as 30 nM passive reference dye (Rox; USB, Cleveland, OH) per 50-µL reaction. PCR was performed on a real-time thermal cycler (model Mx3000p; Stratagene, La Jolla, CA), with cycling parameters of initial denaturation at 95°C; 40 cycles of 95°C 30 seconds, 60°C 30 seconds, and 72°C 60 seconds, and a denaturation cycle for creation of a dissociation curves. Reactions for each sample and gene of interest were run in duplicate, cycle thresholds (Ct) were

normalized to GAPDH expression as a housekeeping gene, and comparative quantitation was performed using MxPro ver. 4.0 software (Stratagene). Only individual PCR samples with single-peak dissociation curves were selected for data analysis.

Protein Extraction and Western Blot Analysis

Total cellular protein was extracted from the TM cells using mammalian protein extraction buffer (MPER, Cat # 78501; Pierce Biotech, Rockford, IL) containing protease inhibitor (Cat. # 78415, Pierce Biotech) and phosphatase inhibitor (Cat. # 78420, Pierce Biotech) cocktails. Protein concentration was determined using the Bio-Rad Dc protein assay system (Cat. # 500-0111, Bio-Rad Laboratories, Richmond, CA). The cellular proteins were separated on denaturing polyacrylamide gels and then transferred to PVDF membranes by electrophoresis. Blots were blocked with 10% Fat-free Dry Milk in tris-buffered saline tween buffer (TBST) for 1 h and then incubated overnight with primary antibodies (Table 2.3). The membranes were washed with TBST and processed with corresponding horseradish peroxidase-conjugated secondary antibodies. The proteins were then visualized in a Fluor ChemTM 8900 imager (Alpha Innotech, San Leandro, CA) using ECL detection reagent SuperSignal West Femto Maximum Sensitivity Substrate (Cat. # 34096, Pierce Biotechnology, Rockford, IL). To ensure equal protein loading, the same blot was subsequently reprobed for β-actin or GAPDH expression.

Immunocytochemistry of TM Cells

TM cells from 5 different primary cell strains were grown on glass coverslips in 24 well plates. At 100% confluency, cells were fixed with 3.5% formaldehyde (Fisher Scientific, Pittsburgh, PA) in PBS for 20 minutes. Cells were treated with 0.2% Triton X-100 in PBS for 20 minutes, and then incubated for 1 hour with 5% normal blocking serum and 0.3% in PBS. Cells were then incubated with primary antibodies (Table 2.3) overnight at 4º C and secondary antibodies at

1:200 in 1 X PBS for 1 hour at room temperature. Negative controls consisted of omission of primary antibody. To visualize nuclei, cells were treated with 300 nM DAPI nuclear stain and mounted using Aqua-Mount (Lerner Laboratories, Pittsburgh, PA). Slides were stored in the dark at 4º C until visualized on a Zeiss 410 confocal imaging system (Carl Zeiss, Thornwood, N.Y.).

ELISA Immunoassay for FN

Conditioned medium was obtained from three TM cell lines after 24-72 hrs of 1 μM bombesin treatment with or without 100 nM DEX and centrifuged at 2,000 rpm to remove cellular debris. Fifty μL of conditioned medium was diluted to 150 μL with dilution buffer, and soluble FN was quantified using a commercially available ELISA kit (cat no. ECM 300; Chemicon International, Temecula, CA). Amounts of soluble FN (ng/ mL) were plotted for each treatment using Graph-PadPrism 5.

GRE-Luciferase Reporter Assays

In a 96 well opaque plate (BD falcon, New Jersey), $2X10^4$ GTM3 cells were transfected with 100ng cignal GRE reporter plasmid (CCS-006L, SA Biosciences) and 0.6ul Surefect transfection reagent (SA Biosciences, Frederick, MD) with or without 100ng empty vector/pCMV6-XL5, SFRS3/pCMV6-XL5, SFRS5/pCMV6-XL5, or SFRS9/pCMV6-XL5 vectors or 100 nM siRNA against SFRS 3, 5 or 9 (Dharmacon, Lafayette, CO). For experiments to study effect of bombesin on DEX activity, cells transfected with GRE reporter plasmid were treated with or without different doses of bombesin for 24hrs. Forty-eight hours after transfection, cells were treated with or without 100 nM DEX (in ethanol) in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 1% penicillin+ streptomycin and 2mM glutamine (Thermoscientific, Rockford, IL). Six hours later, Dual-Glow substrate (Promega, San Luis Obispo, CA) was added to each well, and the signal was detected with an M200 plate reader (Tecan, Durham, North

Carolina). Firefly luciferase activity was normalized to renilla luciferase activity. Experiments were performed in quintuplets (N=5).

Statistical Analysis

For comparing results between two groups, Student's t test was performed. For comparison of results between more than two groups, One-Way ANOVA was employed. Statistical tests used for each individual experiment are listed in the respective figure legends.

Results

Profiling of SRps in TM cells

The expression of different members of the SR protein family in the TM has not been previously studied. Therefore, we determined whether these SRps are expressed in cultured human TM cells. Utilizing qRT-PCR, we profiled the cDNA samples obtained from four normal and four glaucomatous TM cell strains (Fig. 2.1). Numerous SRps were expressed in multiple TM cell strains. Although there appeared to be differences in their basal expression among the TM cell strains, SFRS5.1 (SRp40) expression was higher in NTM cells ($p< 0.05$). Both GRα and GRβ were expressed in all strains, and GRβ was higher in NTM cell strains, confirming our previous findings (data not shown).

Bioinformatics Identification of Putative SRp Binding Sites

The Splicing Rainbow software predicted more binding sites for SRp20 on exon 9β as compared to exon 9α. In contrast, SRp40 was found to have more binding sites on exon 9α as compared to

exon 9β (Fig. 2.2). Altered levels or function of these SRps might therefore affect GR mRNA slicing and thereby alter levels GRα and GRβ.

Expression of SRp20, 30c and 40 in TM cells

Given the mRNA expression of SRp20, 30c, and 40 (Fig. 2.1), we wanted to determine whether these SRps are expressed at the protein level in five TM cell strains. Our western immunoblot results showed that SRp20, 40 and 30c proteins were expressed in the three normal and two glaucomatous TM cells tested (Fig. 2.3A). Immunocytochemistry analysis showed these SRps mainly present in nucleus co-localized with DAPI staining, although there also was some staining in cytoplasm, particularly for SRp30c (Fig. 2.3B). These results are in agreement with previous studies implicating SRp involvement in post mRNA processing events such as nuclear export of mRNA and translation process[42-45].

Effect of Bombesin on SRps Expression and the GRβ/GRα Ratio

Bombesin has been shown to alter SRp30c levels and affect GR splicing in PC-3 cells[33]. To study the effect of SRps on GR splicing in TM, we treated TM cell lines (n=5) with increasing concentrations of bombesin (0-10 μM) for 24hrs. The protein expression of SRp20, SRp30c, SRp40, GRα and GRβ was determined by western immunoblotting. Bombesin induced SRp30c, SRp40; decreased SRp20; and increased the GRβ/GRα ratio in concentration dependent manner (Fig. 2.4A). TM cell strains (n=3) were treated with 1 μM bombesin for 0.5-48 hours to examine the time dependent effects on SRps expression and GR splicing (Fig. 2.4B). Bombesin induced SRp30c and SRp40 proteins maximally at 12-24 hours. The decrease in SRp20 was seen as soon as 30 minutes after bombesin treatment. Also, the maximum effect on GR splicing (i.e. highest

GRβ/GRα ratio) was observed at approximately 24 hours. Results obtained were comparable among the normal and glaucomatous cell lines tested.

Effect of Bombesin on DEX Responsiveness in TM cells

GTM3 cells were pretreated +/- bombesin (1 mM) for 24 hrs prior to treatment with or without DEX (100 nM) for 12 hours. Total RNA was isolated for RT-PCR analysis. DEX treatment elevated fibronectin expression compared to untreated or bombesin only-treated samples. Pretreatment with bombesin (Fig. 2.5A for regular PCR and 2.5B for real time PCR) completely blocked the DEX- mediated fibronectin induction.

We also treated TM cell strains (n=8) with or without DEX (100 nM) for 1-7 days (1 day for transformed cell lines; 3 and 7 days for primary cell strains to check for DEX induction of fibronectin and myocilin, respectively), with or without 24 hours pre-treatment with 1 μM bombesin. DEX elevated fibronectin and myocilin protein expression compared to untreated or vehicle-treated samples. Bombesin completely inhibited the DEX-mediated fibronectin induction in whole cell lysates (Fig. 2.5C, western immunoblotting) and in conditioned medium (Fig. 2.5D, ELISA). Bombesin alone appeared to affect cell-associated fibronectin (Fig. 2.5C), but not soluble fibronectin in conditioned medium (Fig. 2.5D). This suggests a role for some of these splicing factors, which are affected by bombesin treatment, in alternatively spliced and differentially distributed isoforms of fibronectin. Bombesin also inhibited the DEX induction of myocilin in TM cells (Fig. 2.5E).

In addition to these bombesin effects on the DEX induction of fibronectin and myocilin, we used a GRE-reporter assay to examine the effects of bombesin on DEX-induced GRE activity. GTM3

cells were transfected with a GRE-luciferase vector followed by pretreatment with 0, 0.5, 1 or 2 mM bombesin for 24 hrs. The cells were then treated with or without DEX (100 nM) and luciferase activity was determined 6 hrs later. Consistent with the fibronectin and myocilin results, all three doses of bombesin significantly reduced DEX mediated GRE-luciferase activity as compared to the untreated control (Fig. 2.5F). These results strongly suggest that bombesin affected SRp20, SRp30c and SRp40 levels, which altered GR splicing. The increased GRβ/GRα ratio is associated with decreased DEX responsiveness in TM cells.

Effect of SRp Over-expression on GR Splicing

To study the direct role of SRps in alternative splicing of GR, we over-expressed different SRps in GTM3 cells using expression plasmids. The 3 different SRp expression plasmids increased the expression of their respective SRps (Fig. 2.6A), without affecting levels of other two SRps tested (e.g. SRp20 overexpression did not affect SRp30c and SRp40 levels). SRp20 did not significantly change GRa and GRb levels, therefore not changing the overall GRb/GRa ratio (Fig. 2.6B and 2.6C). SRp30c and SRp40 overexpression resulted in higher GRβ and lower GRα levels compared to empty vector control (Fig. 2.6B and 2.6C), thereby increasing the GRβ/GRα ratio.

Effect of SRp30c and SRp40 Over-expression on DEX Activity

We examined the effect of SRp over-expression on GC activity by determining DEX induction of fibronectin protein levels in TM cells. GTM3 cells were transfected with SRp20, 30c, or 40 expression vectors followed by treatment with or without 100 nM DEX for 24 hours. DEX significantly increased fibronectin expression in the empty vector control compared to untreated

samples. In contrast, SRp30 and SRp40 overexpression significantly blocked this DEX induction ($p<0.001$) (Fig. 2.7A).

We also employed the GRE-luciferase reporter assay as an independent way to assess GC activity in TM cells. GTM3 and HTM5 cell lines (n=5) overexpressing individual SRps were also transfected with the GRE promotor constructs followed by DEX (100 nM) or vehicle (0.1% ethanol) treatment for 6 hours. DEX increased luciferase activity four-fold in the empty vector control. SRp30c and SRp40 over-expression significantly reduced DEX mediated luciferase activity as compared to empty vector control in GTM3 cells (Fig. 2.7B). SRp20 overexpression modestly reduced the DEX activity, but this effect was not that statistically significant. As expected, the basal induction of luciferase activity by DEX in HTM5 cells, which already have a high GRβ/GRα ratio, was not as high compared to GTM3 cells. However, SRp20 over-expression significantly increased DEX-mediated luciferase activity, whereas SRp30c and SRp40 did not have significant effects (Fig. 2.7C).

Effect of SRp Knockdown on DEX Activity

To complement our SRp over-expression results on DEX activity, we also knocked down expression of different SRps in GTM3 cells using specific SRp siRNAs. These siRNA treated cells were cotransfected with GRE-promotor plasmids for 48 hours followed by 6 hours of treatment with or without DEX (100 nM). DEX increased luciferase activity in untransfected as well as in the non-targeting siRNA and RNAi mediated silencing complex (RISC)-free siRNA controls (negative controls used to test for possible nonspecific siRNA effects). This increased activity was further enhanced with SRp30c knock down (Fig. 2.8), which would alter the splicing of GR, favoring GRa expression. These results along with the over-expression data

strongly suggest that SRp20, SRp30c and SRp40 are involved in GR alternative splicing in TM cells, with the later two increasing the GRβ/GRα ratio and thus increasing GC resistance.

Discussion

The association between GC therapy, OHT, steroid glaucoma, and POAG has been known for >60 years[16, 18]. A number of laboratories have studied the effects of GCs on the TM to better understand steroid glaucoma and POAG because of the similarities in clinical phenotypes as well as the similar morphological and biochemical changes in the TM[46]. There is considerable heterogeneity in an individual's response to GCs, and approximately a third of the population develop GC-induced OHT compared to most POAG patients, who are steroid responders. We previously suggested that the molecular mechanism responsible for these differences in steroid responsiveness is due to differential expression of the GRβ isoform between normal and glaucoma TM cells[9, 31]. However, the mechanisms responsible for this differential expression of GRβ in TM cells had not been previously explored.

The relative levels of the two alternatively spliced isoforms of the GR, GRα and GRβ, regulate GC responsiveness. Increased expression of the dominant negative GRβ isoform has been associated with numerous steroid resistant diseases[16, 22, 24-27, 30]. We previously demonstrated lower levels of GRβ in GTM cells, making these cells more sensitive to GCs. Increased expression of GRβ made the TM cells more resistant to GCs[9, 31].

We have now shown that TM cells express a number of different SRp splicing proteins and that the three SRps tested are involved in the alternative splicing of GR in TM cells. It would be

interesting to explore whether additional SRps expressed in the TM would also modify GR splicing. The peptide bombesin increased the expression of SRp30c and SRp40 as well as decreased SRp20 levels. Both SRp30c and SRp40 increased the expression of GRβ, whereas SRp20 did not alter the GRβ/GRα ratio. Increased expression of either SRp30c or SRp40 with SRp expression vectors decreased the DEX induction of FN and myocilin as well as blocked the DEX induction of a GRE-luciferase reporter gene. These results demonstrate one important mechanism regulating GR isoform expression and GC response in TM cells (Fig. 2.9).

There are emerging data that establish an association between altered expression of normal or mutant SR proteins and a number of human diseases[47-53]. Involvement of SRp30c in alternative splicing of GR in neutrophils[34] and of SRp40 but in HeLa cells[35] show the cell/tissue-specific effect of SR proteins. There was no significant difference of protein expression of SRp20 and SRp30c between normal and glaucomatous cell strains tested. However, expression of SFRS5.1 (SRp40) was significantly higher in NTM compared to GTM cells. This would favor the alternative splicing and expression of GRβ, making NTM cells more resistant to GCs, as we previously demonstrated[31]. To confirm this, it would necessary to perform large population-based studies and measure the mRNA levels of these SRps in normal, glaucomatous, and steroid responders. Alternatively, it might be the stoichiometry of SR proteins and/or their relative activities that regulate GR splicing in particular cell type.

There appeared to be a discrepancy on the effects of over or under expression of SRp40 in our GRE-luciferase assays. It is possible that SRp40 knock down results in compensatory effects on other SR proteins. This possibility needs to be considered in our other experiments with

bombesin or SRp overexpression, which are indirect measures of the DEX response executed by changes in GR levels. It is possible that changes associated with other components of GR signaling that are involved in regulating the GC response.

In conclusion, this is the first report demonstrating the role of SR proteins in GR splicing in human TM cells. To best of our knowledge, no study had previously correlated the effect of GR splicing with functional GC response assays. In the future, translation of our in vitro results to an ex vivo anterior segment perfusion organ culture (POC) model and/or an *in vivo* model will help better understand the role of GR splicing in GC regulation of IOP.

Figure Legends

Figure 2.1: Expression of SRp genes in TM cells. qRT-PCR was done on RNA samples from four normal (NTM) and four glaucomatous TM (GTM) cell strains for 12 different SRp genes that are involved in constitutive and alternative splicing. GAPDH was used as a housekeeping gene. SFRS5.1 (SRp40) mRNA levels are significantly higher in NTM as compared to GTM cells. Mean +/- SEM; *$p < 0.05$ using unpaired t-test

Figure 2.2: Bioinformatics prediction of SRp binding. Exon 9α was predicted to have more binding sites for SRp40 (blue dots), while exon 9b had more binding sites for SRp20 (brown dots). More binding sites on exon 9α predicts elevated activity of SRp40 that would increase exon9β/GRβ mRNA splicing. Similarly, more SRp20 binding sites on exon 9β predicted increased chances of exon9α/GRα mRNA splicing.

Figure 2.3: Protein expression of SRp20, 30c and 40 in TM cells. A) Western immunoblot shows SRp20, SRp30c and SRp40 protein expression in five TM cell strains with GAPDH as a loading control. B) Immunocytochemistry data showing levels and localization of SRp20, SRp30c and SRp40 in NTM-210-04 at 20X. Insets show 40X images of cells marked by arrowheads. These are representative data from five different TM cell strains.

Figure 2.4: Bombesin concentration and time-dependent increases in SRp30c, SRp40 & GRβ/GRα ratio with decreases in SRp20. A) Representative data showing dose-dependent induction of SRp30c, SRp40 and GRβ as well as decreased expression of SRp20 and GRα proteins by 0-10 μM bombesin in cultured TM cells (n=5 TM cell strains). B) Time course induction (0.5-48 hours) of SRp30c, SRp40 and GRβ proteins by 1 μM bombesin in cultured TM cells (n=3 TM cell strains). Maximum induction was seen with 1 μM bombesin at 12-24 hours for SRp30c and SRp40 and 24 hours for GRβ.

Figure 2.5: Bombesin decreases DEX responsiveness in TM cells. An increased GRβ/GRα ratio by bombesin is associated with decreased DEX activity in TM cells. 1-7 days treatment with DEX (100 nM) increased fibronectin mRNA (5A and 5B, n=3), cell associated fibronectin protein (5C, western immunoblots, n=8) and soluble fibronectin in conditioned medium (5D, ELISA, n=8) of cultured TM cells. 7 days treatment of DEX (100 nM) also increased myocilin in conditioned medium of cultured TM cells (5E, n=5; a,b and c denote biological replicates). 1 μM bombesin pretreatment for 24 hours decreased DEX- mediated induction of FN mRNA (5A, 5B), FN protein (5C, 5D) and myocilin protein (5E) levels. 0.5-2 μM bombesin pretreatment for 24

hours significantly decreased DEX-mediated GRE-luciferase reporter activity in GTM3 (5F, n=5). Mean +/- SEM *p<0.05, **p<0.01 and *** p<0.001 (One- way ANOVA)

Figure 2.6: SRp30c and SRp40 overexpression increases GRβ/GRα ratio in GTM3. A) Western-immunoblot confirming overexpression of SRp20, SRp30c and SRp40 after transfection. B) Representative western immunoblot showing that SRp30c and SRp40 overexpression decreased GRα and increased GRβ levels whereas SRp20 overexpression did not significantly change GRα and GRβ levels in GTM3 cells (n=3). C) Densitometric analysis of data shown in Figure 6B; mean +/- SEM

Figure 2.7: SRp30c and SRp40 over-expression decreases DEX activity in TM cells.

A) SRp30c and SRp40 overexpression decreased DEX-mediated FN induction as compared to empty vector control in GTM-3 cells, corresponding to an increased GRβ/GR ratio. The western immunoblot is representative of three experiments. B, C) SRp40 and SRp30c over-expression significantly decreased DEX mediated GRE-luciferase activity as compared to empty vector control (CT) in GTM3 cells (n=5), whereas SRp20 over-expression significantly increased GRE-luciferase activity compared to empty vector CT in HTM5 (7C, n=5). Mean +/- SEM, *** p<0.001 (one-way ANOVA)

Figure 2.8: SRp30c knockdown increases DEX-activity in GTM3 cells.

Six hours of 100 nM DEX treatment increases GRE-luciferase activity in untreated, non-targeting siRNA treated and RISC-free siRNA treated GTM3 cells compared to the corresponding vehicle (ethanol) controls (n=5). SRp30c knockdown significantly augmented

DEX activity when compared to no siRNA, no-targeting siRNA, and RISC-free siRNA treated samples. Mean +/- SEM; *** $p<0.001$ (one-way ANOVA)

Figure 2.9: Modulation of levels of Splicing factors either by overexpression plasmids or bombesin peptide alters GRβ/α ratio in trabecular meshwork (TM) cells. Increased GRβ/GRα ratio decreases GRα mediated responses in presence of DEX. Increased GRβ/GRα ratio also decreases DEX- mediated ECM changes in TM and elevated intraocular pressure in eye. Image modified from Revollo & Cidlowski [54].

Table 2.1. Summary of SFRS genes and SR proteins and their effects on GR splicing and DEX effects in TM cells

Gene Name	**Protein Name**	**Effect on GRβ/GRα Ratio**	**Effect on DEX mediated GRE-luciferase activity**
SFRS3	SRp20	No significant change In GTM3	No significant change in GTM3; increase in HTM5
SFRS5 (SFRS5.1 and SFRS5.2 differs in 5' UTR)	Both isoforms encode SRp40	Increase in GTM3	Decrease in GTM3; No change in HTM5
SFRS9	SRp30c	Increase in GTM3	Decrease in GTM3; No change in HTM5

Table 2.2. List of PCR Primers

Gene Name	Left Primer sequence (5' to 3')	Right Primer Sequence (5' to 3')
SFRS1.1	GAAGACGCGGTGTATGGTC	GATCTGCTATGACGGGGAGA
SFRS2	GACCGCTACACCAAGGAGTC	TTGGATTCCCTCTTGGACAC
SFRS3	ATGCATCGTGATTCCTGTCC	ACCCTTAAACTGGCAGGACA
SFRS4	CTCACAAGGGACGCAAAAAT	CTCACACTCCCTCGCTTCTC
SFRS5.1	AGTGCGTCAGTTGTGGAGTG	CCACGTTTCTGGCTCTTCTC
SFRS5.2	GCTGCTAAGTGCGTCAGTTG	CCACGTTTCTGGCTCTTCTC
SFRS6	TATGCGACAAGCAGGTGAAG	TTGAGGGTGGAACAGGTAGC
SFRS7	GTTGGTAACCTGGGAACTGG	CCATTCTTTCAGGACTTGCAC
SFRS8	GGCGGATCTCACTACAGCTC	AGACCGAGGAGGACTTGGAT
SFRS9	ACAATGCAGTGCGGCTGA	TTCTGAGCACAAAGCAGCTC
SFRS12	ACGGGAAAAAGAGCATGAGA	TCCAACTTCTCCTTCCCATC
SFRS17A	GCTTCTCCGACATCCTGAAG	CCTTCTCTCTGCGCTTTTGT
NR3C1 (GR alpha)	TACCCTGCATGTACGACCAA	TCATCCAGCCAACTGTGAAA
NR3C1 (GR beta)	CTTCCAGAACCATGGTAGCC	TACGAAACTCCACCCAAAGG
FN1 (Fibronectin)	ACCAACCTACGGATGACTCG	GCTCATCATCTGGCCATTTT
MYOC (Myocilin)	CTGGAAACCCAAACCAGAGA	CATTGGCGACTGACTGCTTA
GAPDH	GGGAGCCAAAAGGGTCAT	TTCTAGACGGCAGGTCAGGT
ACTA1 (b-Actin)	GGCATGGAGTCCTGTGG	GAAGCATTTGCGGTGG

Table 2.3. List of Antibodies

Protein Name	Primary Antibody	Dilution
SRp20	Santa Cruz Biotech, CA SC-73059; Mouse Monoclonal	1:200 for WB 1:50 for ICC
SRp30c	Santa Cruz Biotech, CA SC134036; Rabbit Polyclonal	1:200 for WB 1:50 for ICC
SRp40	Novus Biologicals, CO H00006430-B01P Mouse Polyclonal	1:500 for WB 1:100 for ICC
GR alpha	Santa Cruz Biotech, CA SC-1002; Rabbit Polyclonal	1:300 for WB 1:50 for ICC
GR beta	Custom made Rabbit Polyclonal	1:2000 for WB 1:500 for ICC
Fibronectin	Millipore, MA AB1945; Rabbit Polyclonal	1:500 for WB 1:500 for ELISA
Myoclin	Santa Cruz Biotech, CA SC-20976; Goat Polyclonal	1:500 for WB
GAPDH	Cell signaling, MA 14C10, Rabbit monoclonal	1:1000 for WB
B-Actin	Millipore, MA MAB 1501; Mouse Monoclonal	1:500 for WB

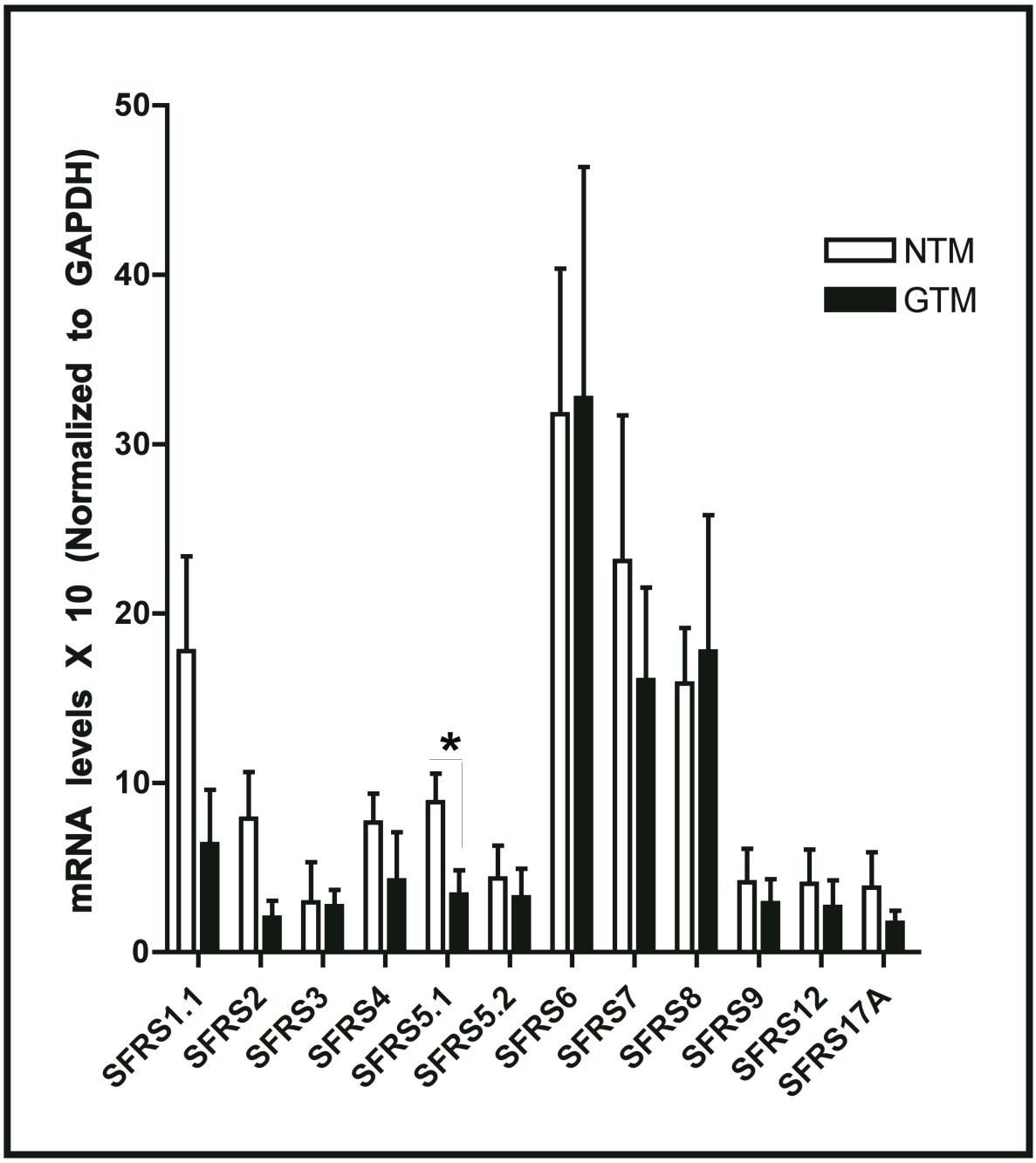
mRNA levels X 10 (Normalized to GAPDH)
50
40
30
20
10
0
NTM
GTM
*
SFRS1.1
SFRS2
SFRS3
SFRS4
SFRS5.1
SFRS5.2
SFRS6
SFRS7
SFRS8
SFRS9
SFRS12
SFRS17A

Figure 2.1

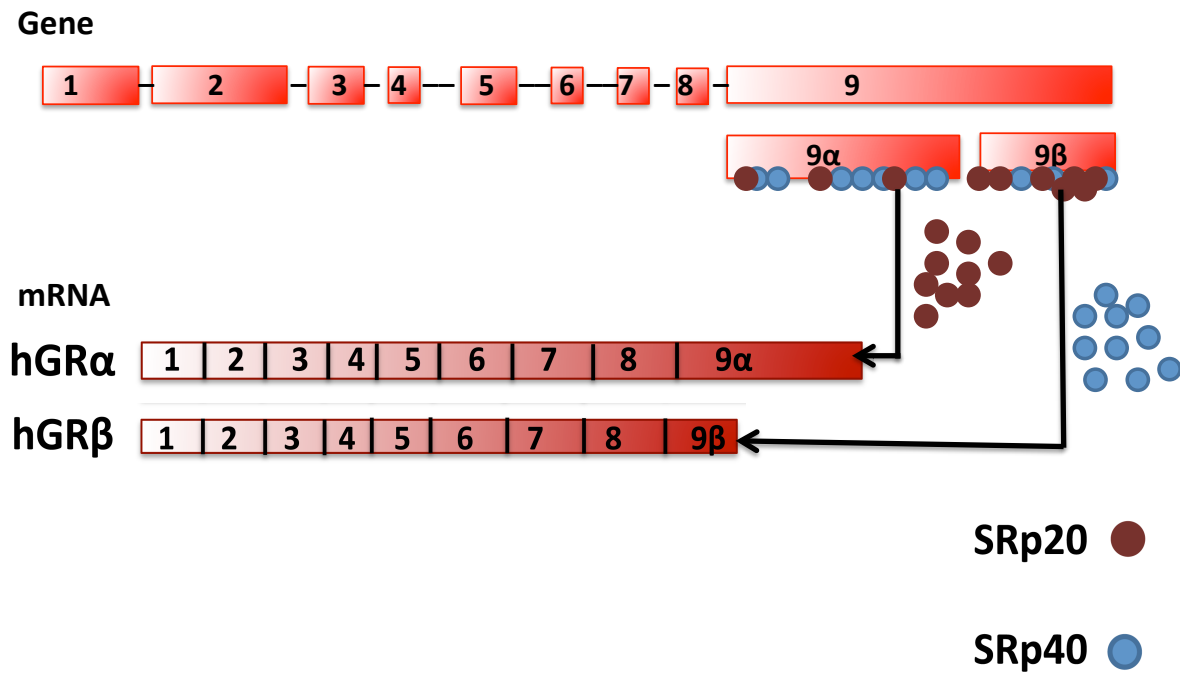
Gene
1
2
3
4
5
6
7
8
9
9α
9β
mRNA
hGRα
1
2
3
4
5
6
7
8
9α
hGRβ
1
2
3
4
5
6
7
8
9β
SRp20
SRp40

Figure 2.2

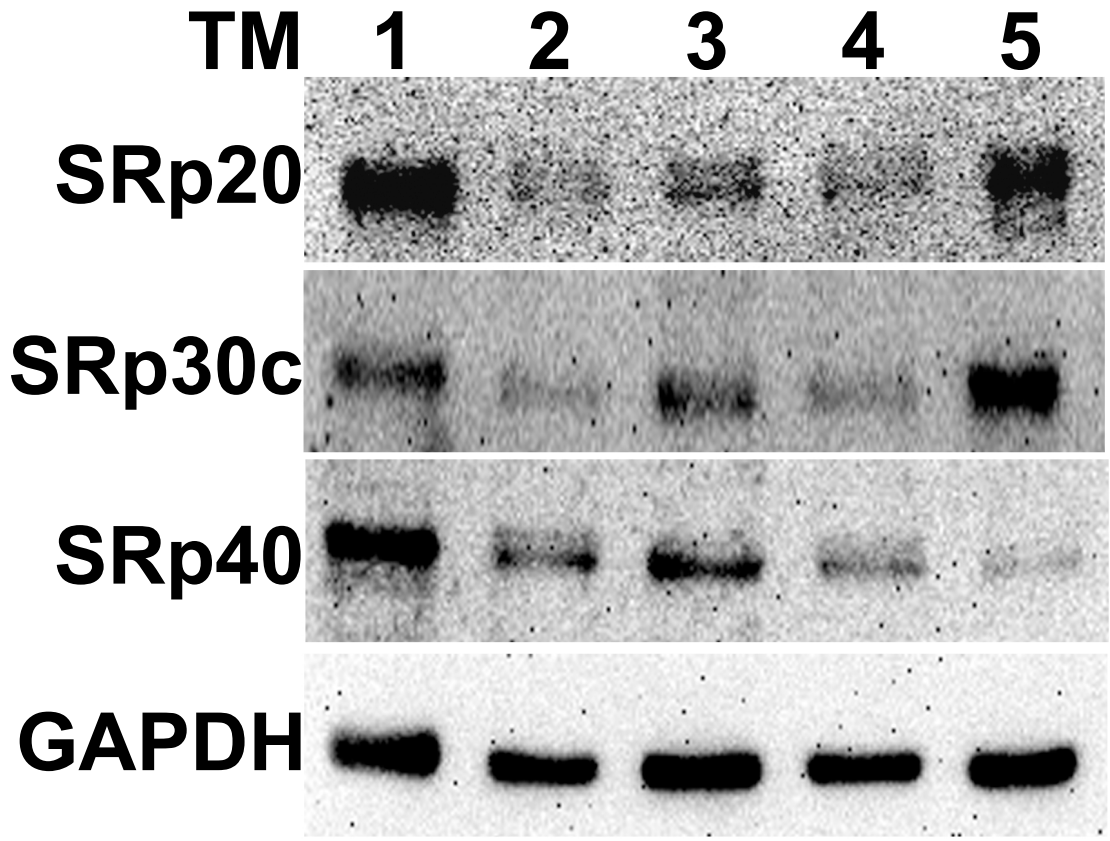

Figure 2.3A

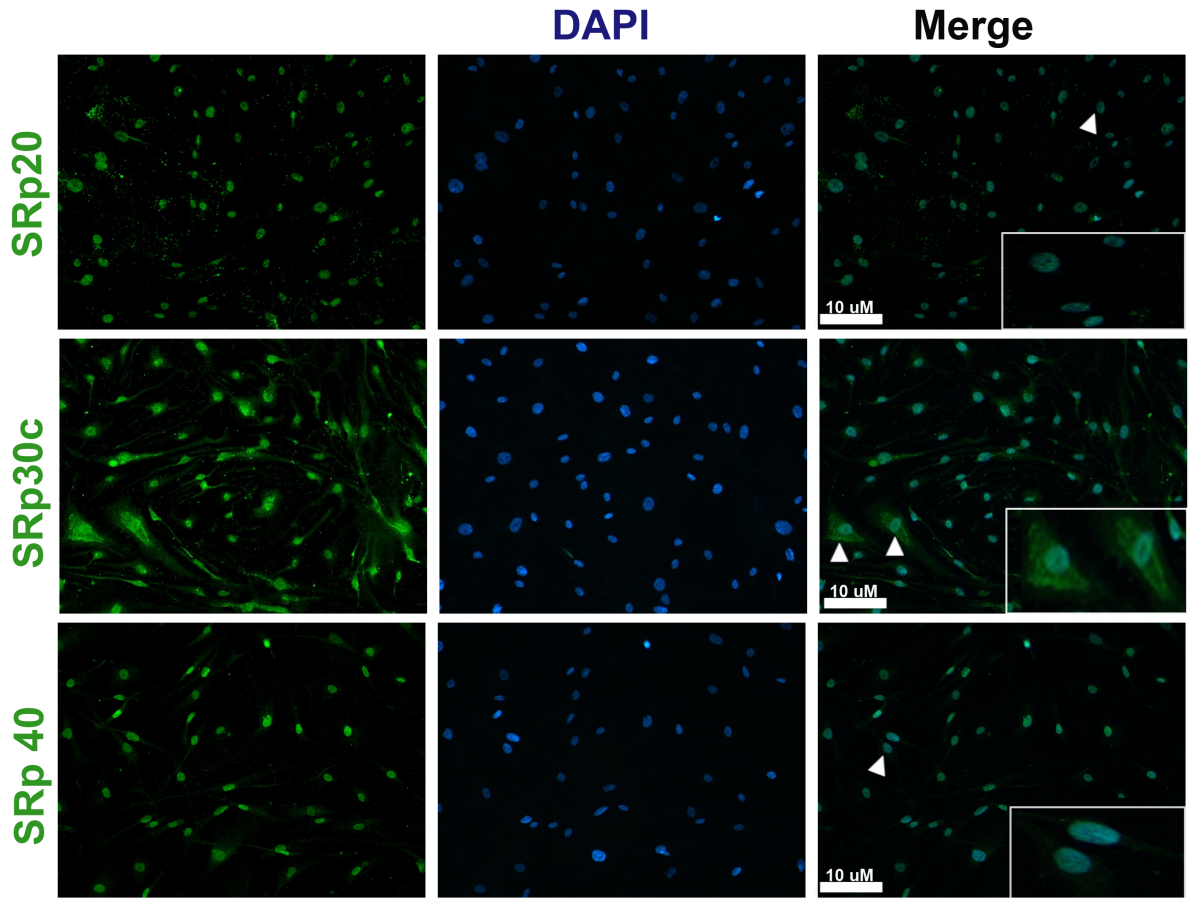

Figure 2.3B

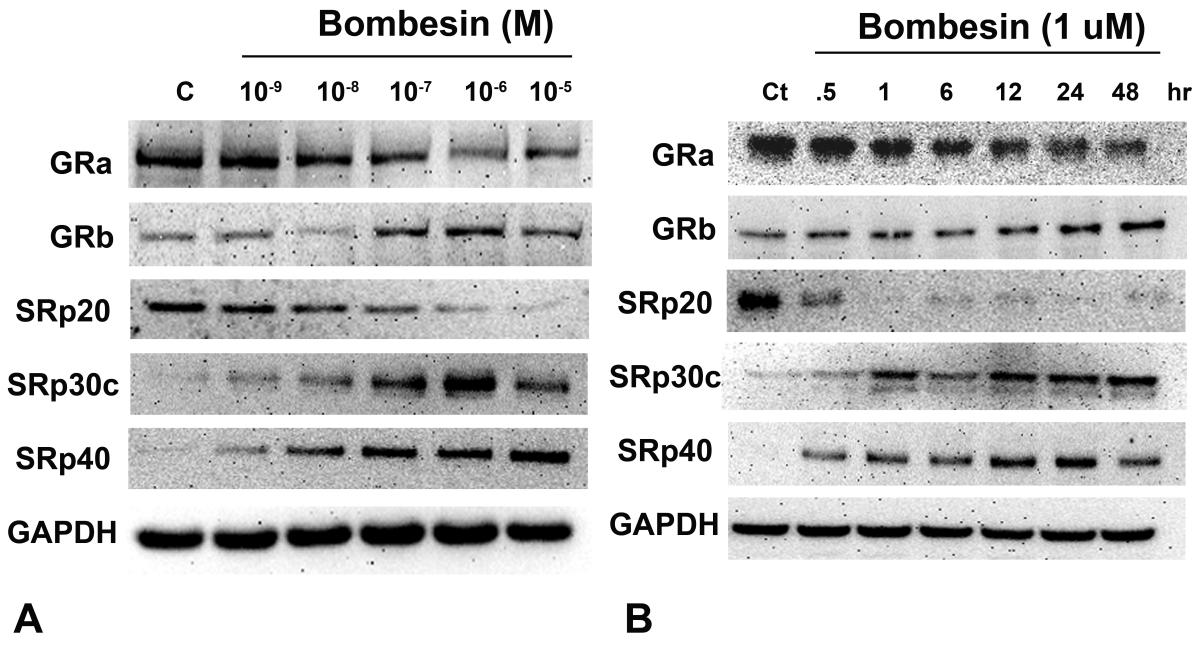
Bombesin (M)
C
10-9
10-8
10-7
10-6
10-5
GRa
GRb
SRp20
SRp30c
SRp40
GAPDH
A
Bombesin (1 uM)
Ct
.5
1
6
12
24
48
hr
GRa
GRb
SRp20
SRp30c
SRp40
GAPDH
B

Figure 2.4

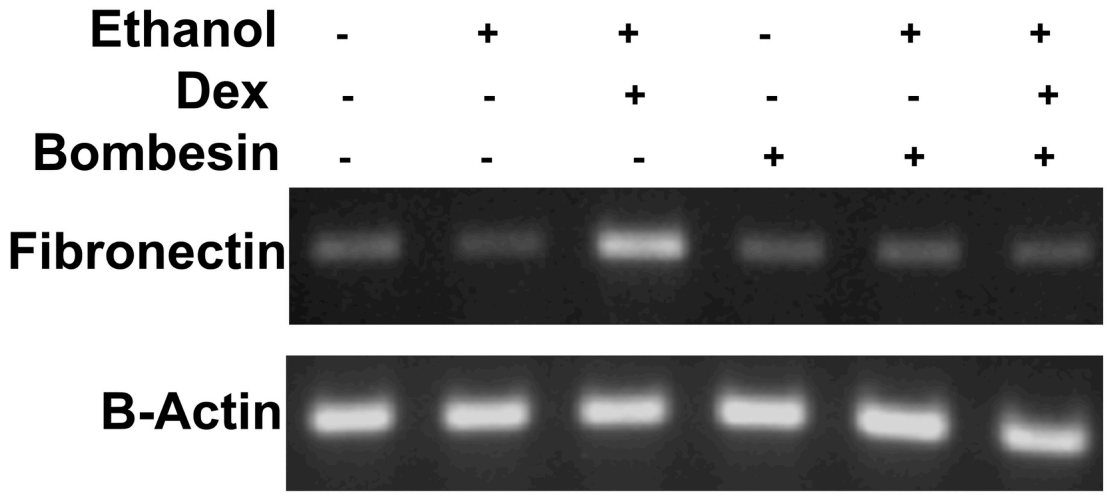

Figure 2.5A

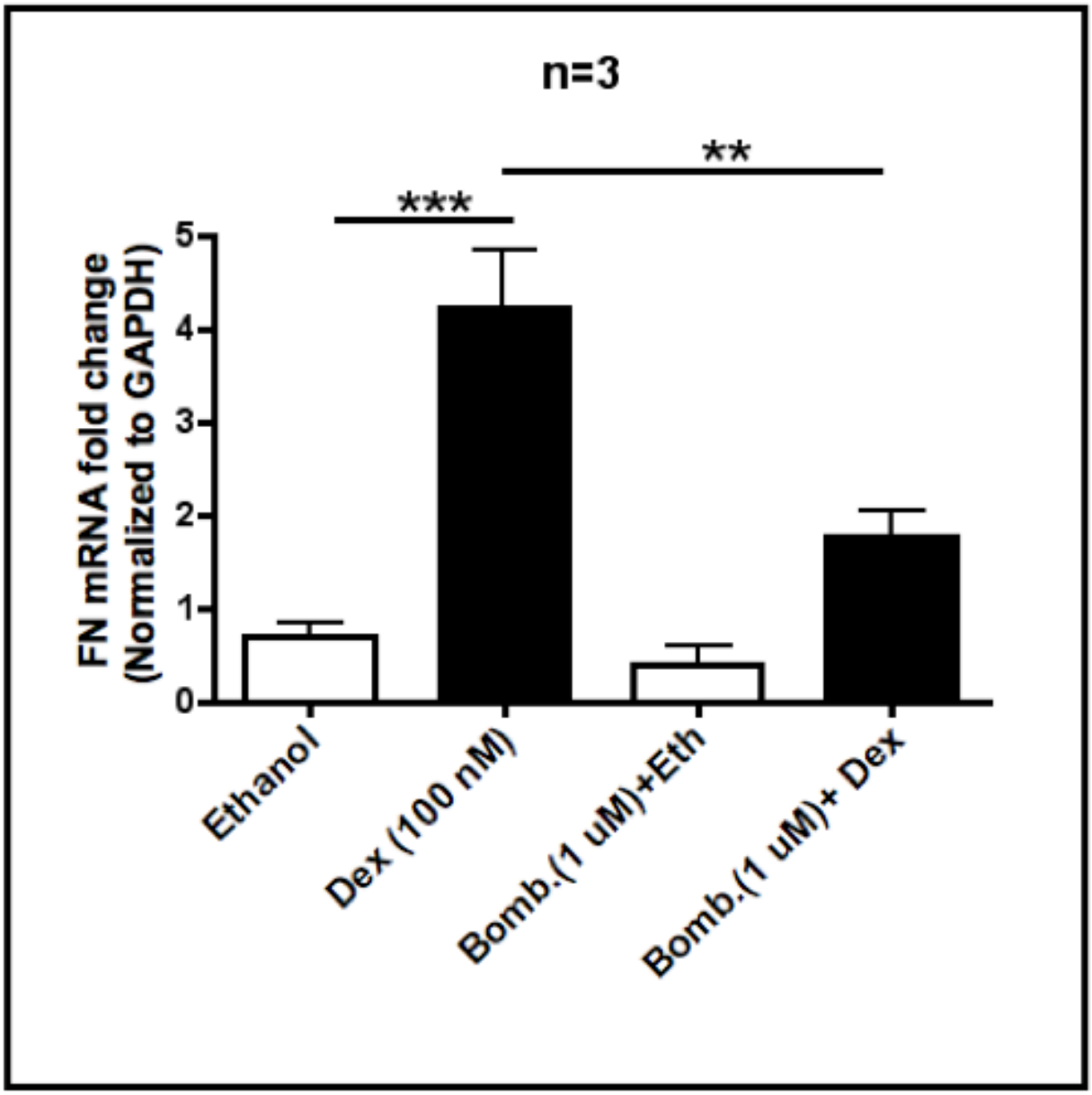
n=3
**
***
FN mRNA fold change
(Normalized to GAPDH)
5
4
3
2
1
0
Ethanol
Dex (100 nM)
Bomb.(1 uM)+Eth
Bomb.(1 uM)+ Dex

Figure 2.5B

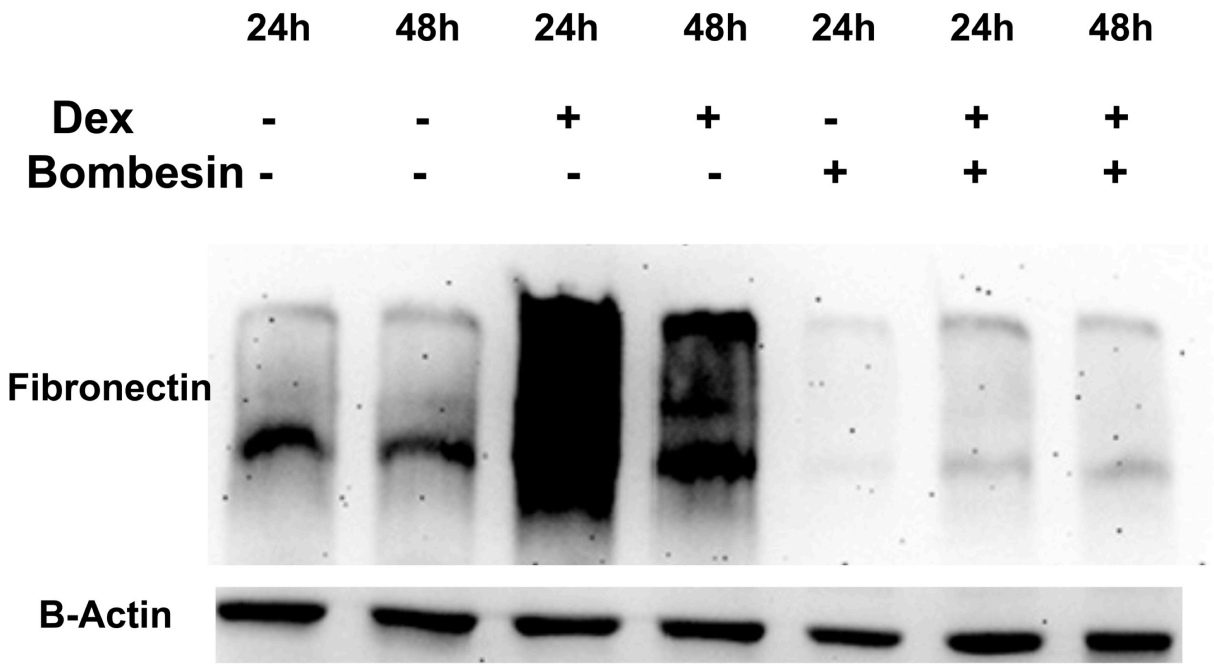

Figure 2.5C

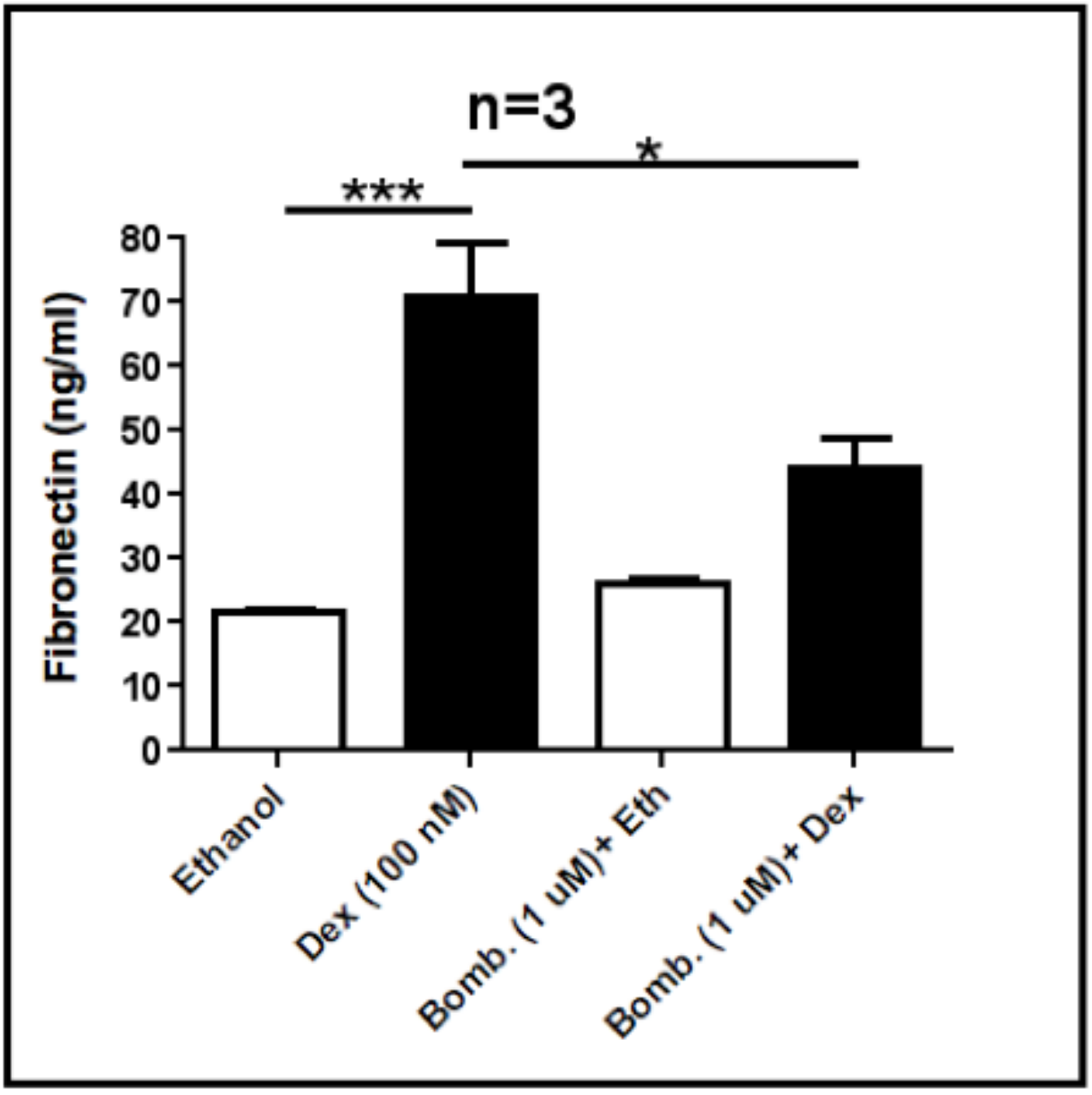
n=3
*
***
Fibronectin (ng/ml)
80
70
60
50
40
30
20
10
0
Ethanol
Dex (100 nM)
Bomb. (1 uM)+ Eth
Bomb. (1 uM)+ Dex

Figure 2.5D

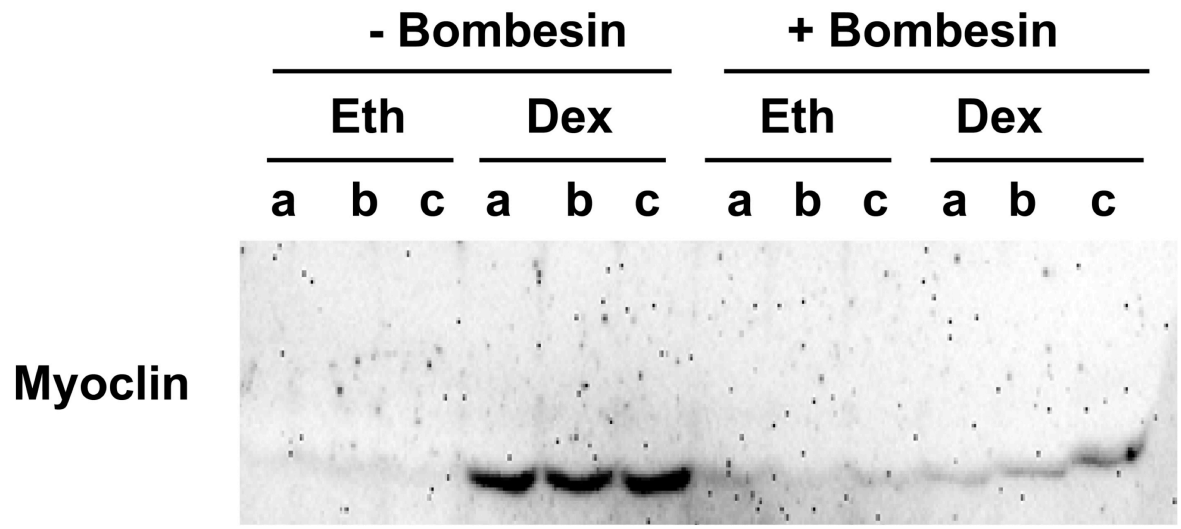

Figure 2.5E

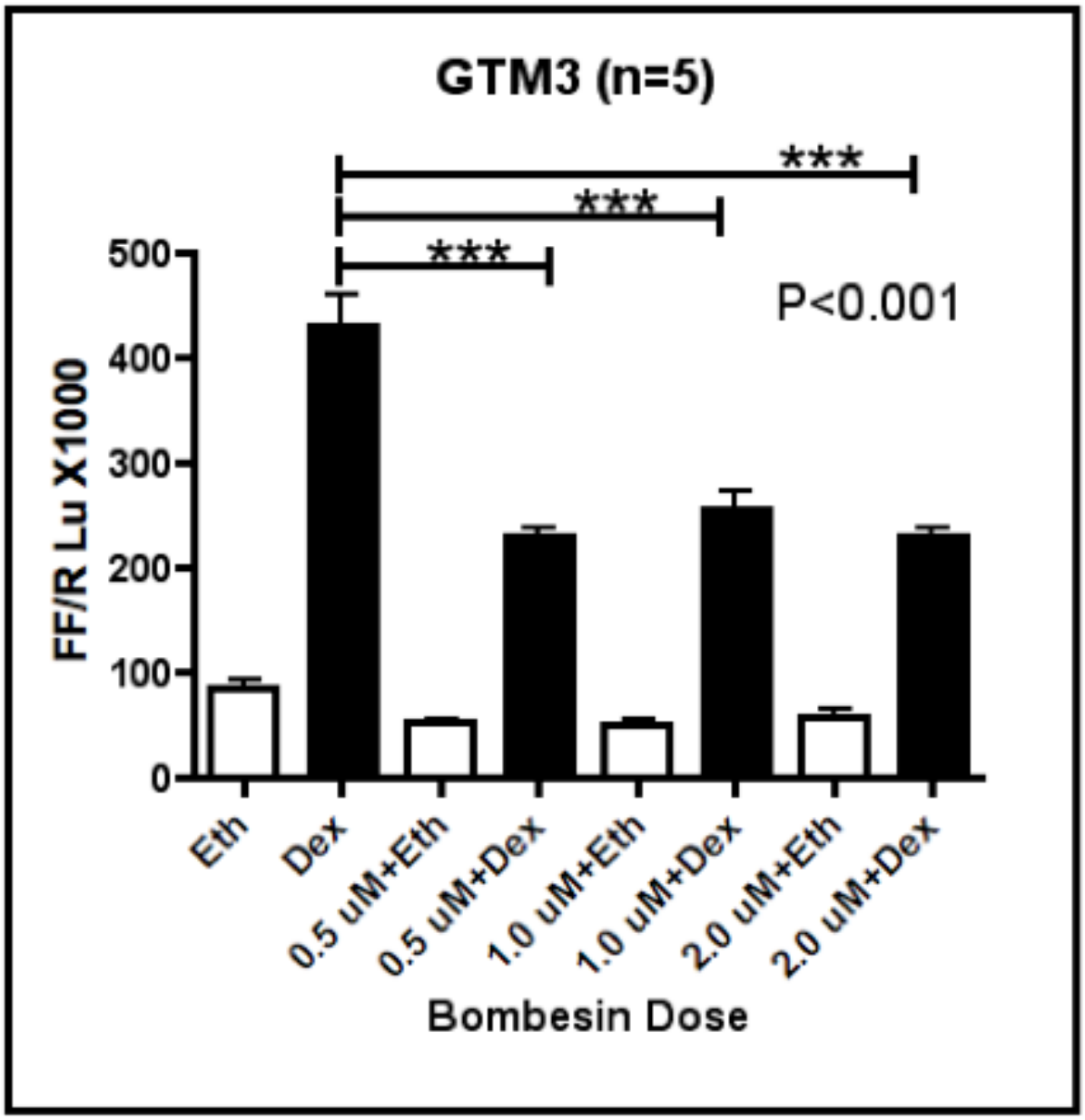
GTM3 (n=5)
***
***
***
P<0.001
FF/R Lu X1000
500
400
300
200
100
0
Eth
Dex
0.5 uM+Eth
0.5 uM+Dex
1.0 uM+Eth
1.0 uM+Dex
2.0 uM+Eth
2.0 uM+Dex
Bombesin Dose

Figure 2.5F

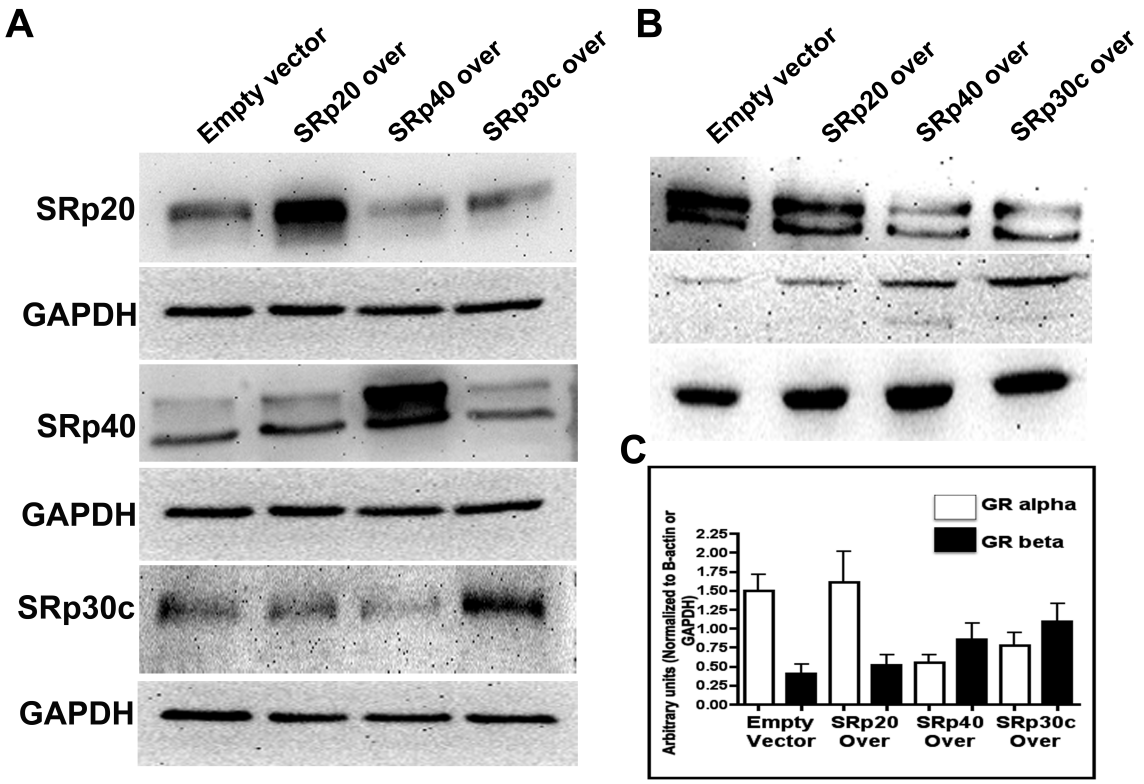
A
Empty vector
SRp20 over
SRp40 over
SRp30c over
SRp20
GAPDH
SRp40
GAPDH
SRp30c
GAPDH
B
Empty vector
SRp20 over
SRp40 over
SRp30c over
C
GR alpha
GR beta
Arbitrary units (Normalized to B-actin or GAPDH)
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Empty Vector
SRp20 Over
SRp40 Over
SRp30c Over

Figure 2.6

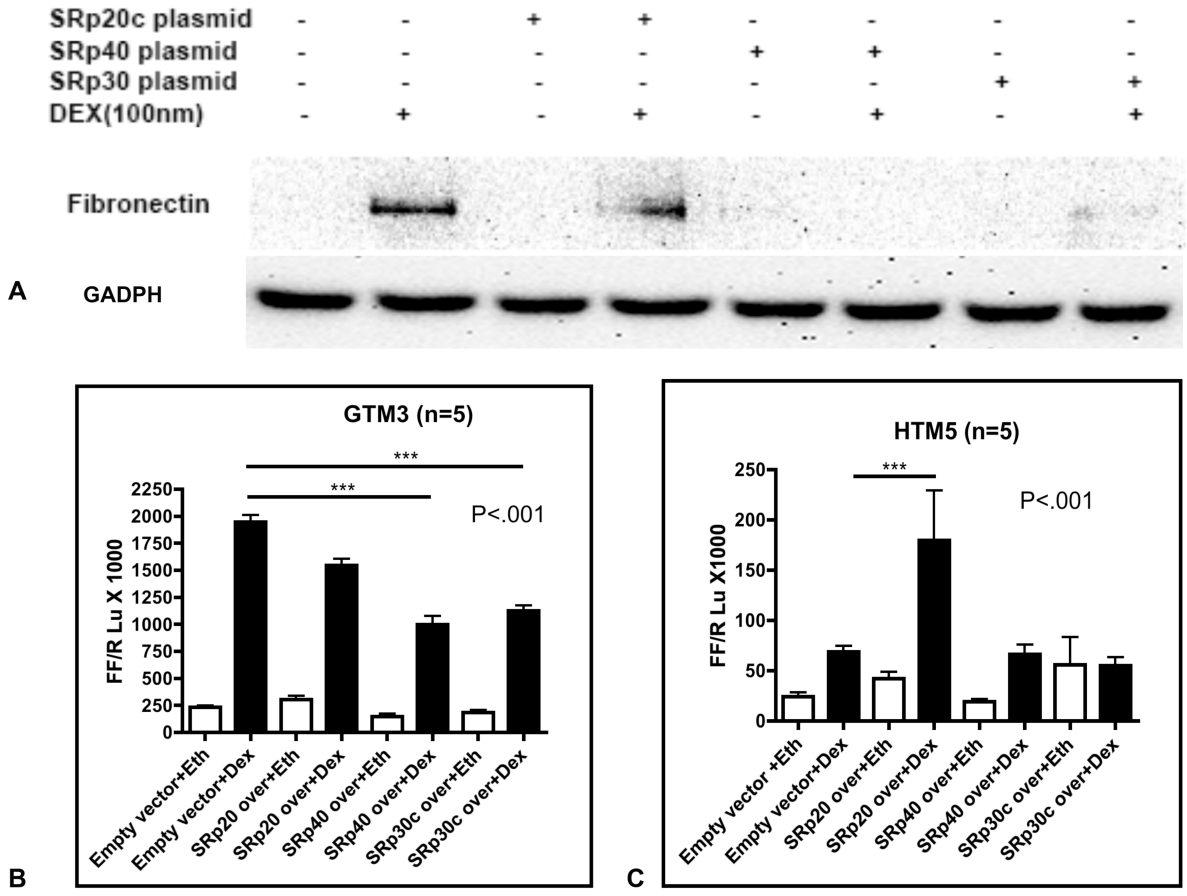
SRp20c plasmid - - + + - - - -
SRp40 plasmid - - - - + + - -
SRp30 plasmid - - - - - - + +
DEX(100nm) - + - + - + - +
Fibronectin
A
GADPH
GTM3 (n=5)
***
***
P<.001
FF/R Lu X 1000
2250
2000
1750
1500
1250
1000
750
500
250
0
Empty vector+Eth
Empty vector+Dex
SRp20 over+Eth
SRp20 over+Dex
SRp40 over+Eth
SRp40 over+Dex
SRp30c over+Eth
SRp30c over+Dex
B
HTM5 (n=5)
***
P<.001
FF/R Lu X1000
250
200
150
100
50
0
Empty vector +Eth
Empty vector+Dex
SRp20 over+Eth
SRp20 over+Dex
SRp40 over+Eth
SRp40 over+Dex
SRp30c over+Eth
SRp30c over+Dex
C

Figure 2.7

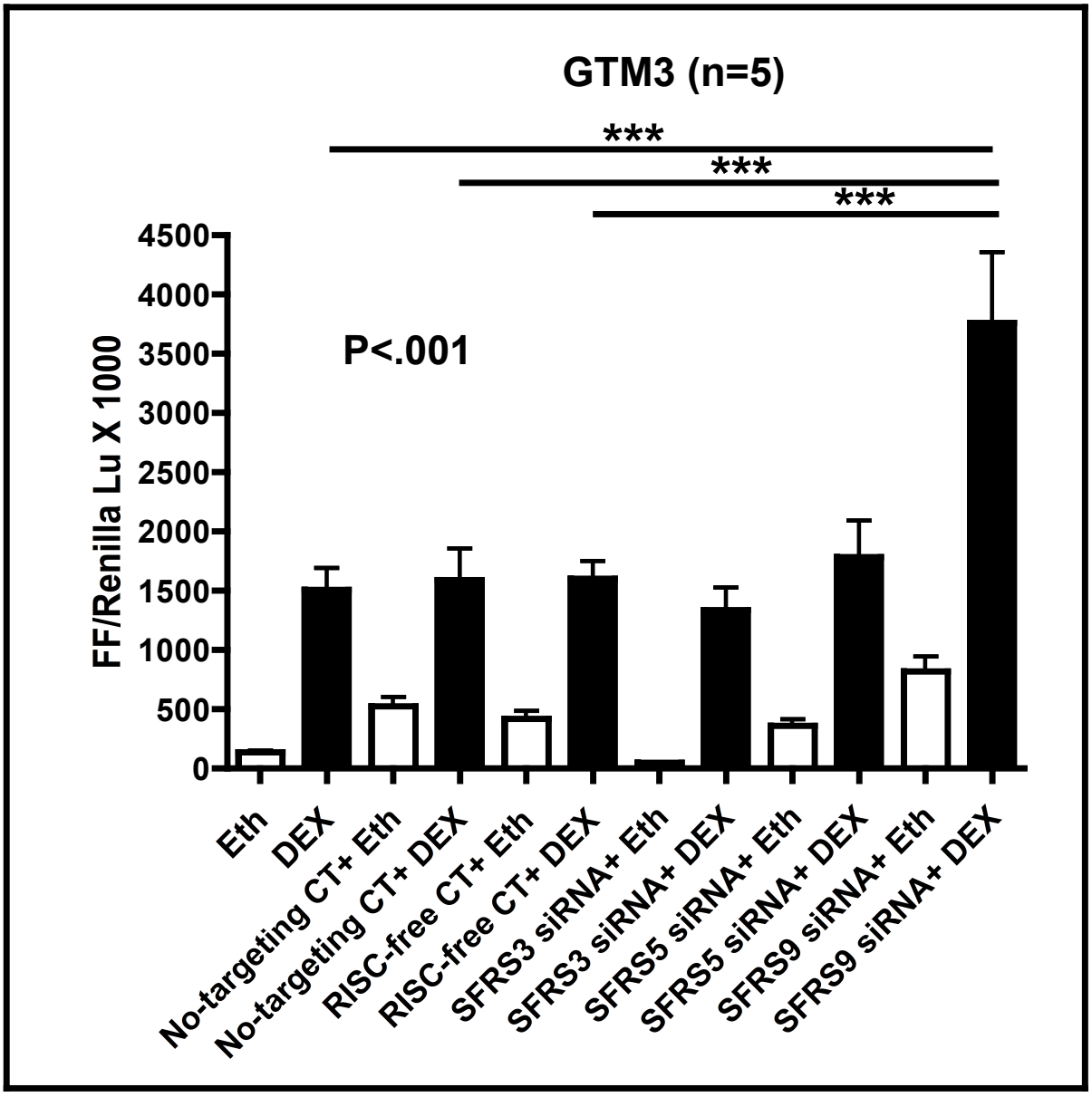
GTM3 (n=5)
***
***
***
P<.001
FF/Renilla Lu X 1000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Eth
DEX
No-targeting CT+ Eth
No-targeting CT+ DEX
RISC-free CT+ Eth
RISC-free CT+ DEX
SFRS3 siRNA+ Eth
SFRS3 siRNA+ DEX
SFRS5 siRNA+ Eth
SFRS5 siRNA+ DEX
SFRS9 siRNA+ Eth
SFRS9 siRNA+ DEX

Figure 2.8

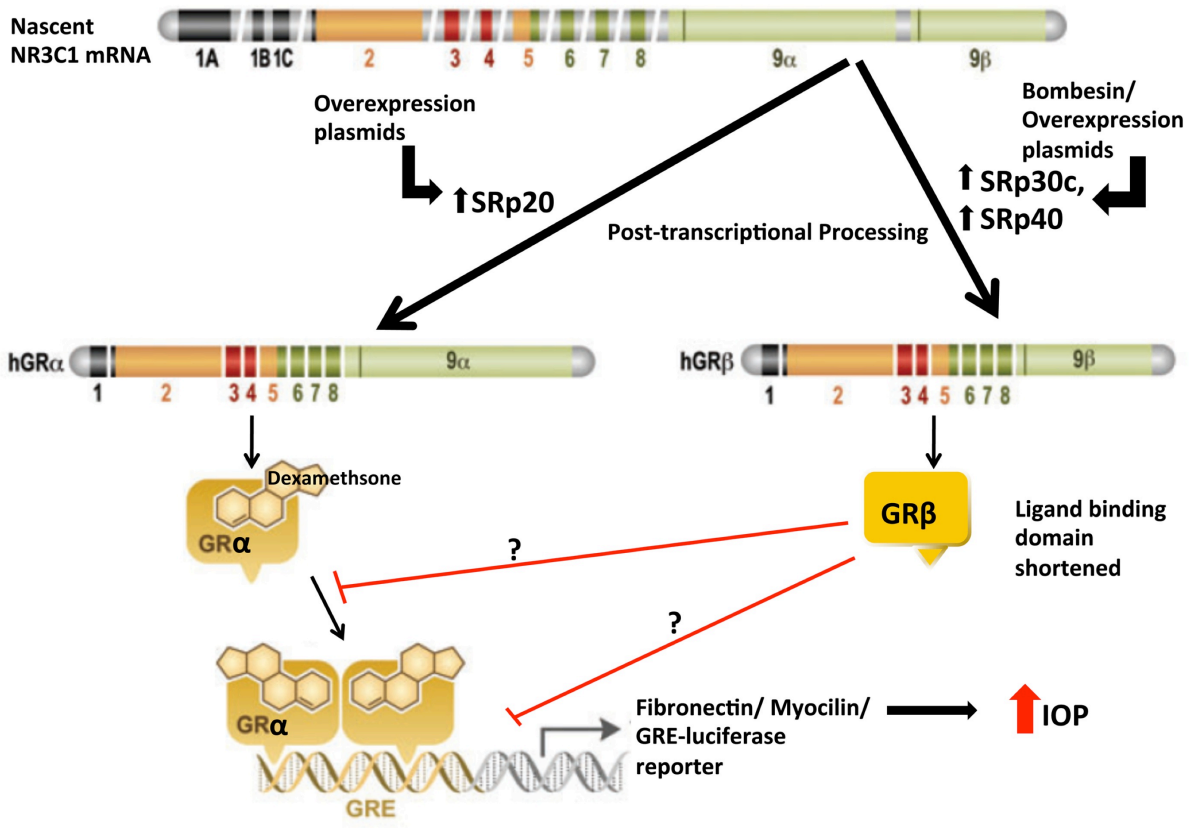
Nascent
NR3C1 mRNA
1A
1B1C
2
3
4
5
6
7
8
9α
9β
Overexpression
plasmids
↑SRp20
Post-transcriptional Processing
Bombesin/
Overexpression
plasmids
↑SRp30c,
↑SRp40
hGRα
hGRβ
Dexamethsone
GRα
GRβ
Ligand binding
domain
shortened
?
?
Fibronectin/ Myocilin/
GRE-luciferase
reporter
↑IOP
GRE

Figure 2.9

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CHAPTER III

ROLE OF THAILANSTATINS IN GLUCOCORTICOID RESPONSE IN TRABECULAR MESHWORK AND STEROID-INDUCED GLAUCOMA*

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* Under review

Abstract:

Elevated intraocular pressure (IOP) is the primary risk factor in the development of glaucoma, a leading cause of irreversible blindness. Various morphological and biochemical changes in the trabecular meshwork (TM) appear to be responsible for blocking aqueous humor outflow, thereby elevating IOP. Glucocorticoids (GCs) are known to induce ocular hypertension and mediate biochemical changes in the TM, similar to those associated with glaucoma. Interestingly, there are differences in steroid responsiveness among the population, with approximately 40% of individuals who are responders with significantly elevated IOP and others being classified as nonresponders. The steroid-responders are at higher risk of developing primary open angle glaucoma (POAG) as compared to the steroid nonresponders. At the same time, almost all POAG patients are moderate to high steroid responders. GC responsiveness is regulated by the relative ratios of the GC activated transcription factor GC receptor alpha (GRα) and the alternatively spliced dominant negative regulator isoform of this receptor (GRβ). Glaucomatous TM cell strains have higher GRα/GRβ ratio as compared to normal TM cells, making them more sensitive to GCs. The role of splicing factors that regulate spliceosome

assembly seems to be one of the key factors regulating the process of alternative splicing, but the overall regulation of the GRα/GRβ splicing is not very well documented. We have previously shown that the relative levels of the different SR proteins in the TM regulate the differential expression of these two alternatively spliced isoforms of GR, and thereby regulate TM sensitivity to GCs. We have tested a special class of compounds, thailanstatins, in cultured human TM cells. These compounds modulated the GR splicing process to enhance GRβ levels, thereby increasing the GRβ/GRα ratio and decreasing the GC response in TM cells. These thailanstatins, or similar compounds, may provide new potential glaucoma therapeutic agents.

Introduction:

Steroid-induced glaucoma and POAG share many phenotypes. They both involve increased ECM deposition in the TM[1-5], TM cell cytoskeletal reorganization[2, 6-8], and elevated IOP due to impaired aqueous humor outflow[2, 9]. Approximately 40% of the general population are steroid responders and are at a higher risk for developing either of these forms of glaucoma[1, 2, 10, 11]. In addition, the majority of people suffering from POAG develop elevated IOP after GC treatment[10]. This limits the anti-inflammatory and anti-allergic use of GCs in these patients. Several different mechanisms may be responsible for differential GC sensitivities among populations, including polymorphisms in the GR gene (NR3C1)[12] and differences in the GR isoform expression due to alternative splicing of the GR gene[13-20]. GRβ lacks the GC binding domain and acts as a dominant negative regulator of GC activity[21] and is expressed at higher levels in TM cells of the normal/non-responder population compared to glaucoma TM cells[19, 22]. Thus normal TM cells are less responsive to GCs compared to glaucoma TM cells. We have also

previously shown that the process of GR alternative splicing in TM cells can be regulated by the levels and/or activities of different spliceosome proteins[23].

For the past decade, there has been a significant amount of research to discover and develop novel spliceosome inhibitors and modulators. Although many of these compounds are targeted for the treatment of various cancers[24-30], these agents may also be useful in regulating GC responsiveness in the TM of steroid responders and glaucoma patients. FR901464, Spliceostatin, Pladienolide B, and Sudemycin are microbially derived compounds that have shown promising results as spliceosome inhibitors in various cancer cells[24-30]. Most of these compounds are chemically complex and are a challenge to synthesize. Structurally similar compounds called Thailanstatins (TSTs) (Fig.3.1) have recently been isolated from *Burkholderia thailandensis* (Liu, X. et al. and Y.-Q. Cheng).

Three TSTs (TST A, TST B and TST C) are spliceosome inhibitors that have antiproliferative properties in number of human cancer cell lines (Liu, X. et al. and Y.-Q. Cheng. 2013. Genomics-guided discovery of cytotoxic thailanstatins A, B and C as potent spliceosome inhibitors from *Burkholderia thailandensis* MSMB43). These TSTs show half-maximal growth inhibition concentrations in low nanomolar concentrations. These compounds can be bioengineered using the microorganism's various biosynthetic pathways. The overall goal of our study is to investigate the role of this new class of spliceosome modulators in GR alternative splicing and regulation of the GC response in TM cells.

Methods:

TM Cell Culture

Human TM cells were isolated from carefully dissected human TM tissue explants derived from patients with glaucoma or from normal donors, and the TM cells were characterized as previously described[5, 6, 31, 32]. All donor tissues were obtained from regional eye banks and managed according to the guidelines in the Declaration of Helsinki for research involving human tissue. Isolated TM cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) containing L-glutamine (0.292 mg/mL; Gibco BRL Life Technologies), penicillin (100units/ml)/streptomycin (0.1 mg/mL; Gibco BRL Life Technologies), amphotericin B (250 μg/μl; Thermo Scientific ltd.) and 10% fetal bovine serum (Gibco BRL Life Technologies). The stably transformed human TM cell line GTM3[33] was also used and cultured in the same medium.

TM Cell Treatment

TM cells were grown to 100% confluency in serum containing medium. TM cells were incubated with fresh medium containing different doses (10nM, 100nM and 1mM) of TST A, B or C for 6-24 hours (GTM3 cells) or for 24-48 hours (primary TM cell strains). In some studies, TST (100 nM or 1 uM) treatment was followed by treatment with or without DEX (100 nM) for 24-72 hrs for protein isolation.

RNA isolation, Reverse transcription and Quantitative Real Time PCR

Total cellular RNA was prepared from cultured TM cells using TRI Reagent RT extraction (Cat. # RL-311, MRC Inc., Cincinnati, OH). iScript™ Reverse Transcription Supermix for RT-qPCR (Cat. # 170-8840, Bio-Rad, Hercules, CA) was used for first strand cDNA synthesis. Primers for the various SR proteins, GRα, GRβ and GAPDH were designed using Primer3 software

(http://frodo.wi.mit.edu/primer3/). The primer pairs are listed in the Table 3.1. qPCR was performed using the BioRad CFX96 real time system (Bio-Rad Laboratories, Hercules, CA) with the SSoAdvanced™ SYBR Green master mix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction for 20ul of sample (95^0 C for 3 min; 40 cycles 95^0 C for 10 sec, 62^0 C for 30 sec) The cycle threshold (Ct) was assigned as log2 of PCR amplification. Technical triplicates for each sample were averaged and each sample was normalized to internal GAPDH Ct values to give ΔCt values. The difference between the experimental and control ΔCt values were used to determine the relative fold change in gene expression for each sample based on a 2-fold exponential.

Protein Extraction and Western Blot Analysis

Total cellular protein was extracted from the TM cells using mammalian protein extraction buffer (MPER, Cat # 78501; Pierce Biotech, Rockford, IL) containing protease inhibitor (Cat. # 78415, Pierce Biotech) and phosphatase inhibitor (Cat. # 78420, Pierce Biotech) cocktails. Protein concentration was determined using the Bio-Rad Dc protein assay system (Cat. # 500-0111, Bio-Rad Laboratories, Richmond, CA). The cellular proteins were separated on denaturing polyacrylamide gels and then transferred to PVDF membranes by electrophoresis. Blots were blocked with 10% Fat-free Dry Milk in tris-buffered saline tween buffer (TBST) for 1 hr and then incubated overnight with primary antibodies (Table 3.2). The membranes were washed with TBST and processed with corresponding horseradish peroxidase-conjugated secondary antibodies. The proteins were then visualized in a Fluor ChemTM 8900 imager (Alpha Innotech, San Leandro, CA) using ECL detection reagent SuperSignal West Femto Maximum Sensitivity Substrate (Cat. # 34096, Pierce Biotechnology, Rockford, IL). To ensure equal protein loading, the same blot was subsequently reprobed for GAPDH expression.

Cell-viability Assays

To study effect of TSTs on cell viability, Cell-titer Glo (Cat. # G7570, Promega, Madison, WI) was used according to the manufacture's protocol. Briefly, primary TM cell strains (5000 cells/well; n=4) were grown in 96-well opaque bottom plates. Cells were treated with three concentrations of TSTs (10 nM, 100 nM and 1 uM) for 24 hours. DMSO vehicle was used as a negative control. Medium was removed, 100 uL of Cell titer-Glo reagent was added (containing 1% Triton X-100), incubated for 10 minutes at room temperature and luminescence signals were recorded using a M200 plate reader (Tecan, Durham, North Carolina).

GRE-Luciferase Reporter Assays

In a 96 well opaque plate (BD falcon, New Jersey), $2X10^4$ GTM3 cells/well were transfected with 100ng cignal GRE reporter plasmid (CCS-006L, SA Biosciences) and 0.6ul Surefect transfection reagent (SA Biosciences, Frederick, MD) with or without TSTs for 24hrs. Forty-eight hours after transfection, cells were treated with or without 100 nM DEX (in ethanol) in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 1% penicillin+ streptomycin and 2mM glutamine (Thermoscientific, Rockford, IL). Six hours later, Dual-Glow substrate (Promega, San Luis Obispo, CA) was added to each well, and the signal was detected with an M200 plate reader (Tecan, Durham, North Carolina). Firefly luciferase activity was normalized to renilla luciferase activity. Experiments were performed in triplicates (N=3).

Statistical Analysis

For comparing results between two groups, Student's t test was performed. For comparison of results between more than two groups, One-Way ANOVA was employed. Statistical tests used for each individual experiment are listed in the respective figure legends. A p value of <0.05 was considered statistically significant (* = $p<0.05$; ** = $p<0.001$; *** = $p<0.001$).

RESULTS:

Effect of TST A, B & C on GRα and GRβ levels in TM cells

To investigate the role of TSTs on GR splicing, we treated TM cells (n=4) with different doses of TST A/B/C for 24hrs (mRNA) or 48hrs (protein) with DMSO vehicle being used as control. All three TSTs significantly increased GRβ mRNA levels. The concentration for maximum induction depended upon the specific TST. TST A had maximum effect at 1 mM (Fig. 3.2A), while TST B and TST C induced maximally at 100 nM (Fig. 3.2B and 3.2C). Also, 1 mM concentrations of TST B or TST C significantly decreased GRα expression as compared to the DMSO vehicle control. Forty-eight hours treatment with TST A (1 mM), TST B (100 nM), and TST C (100 nM) also increased GRβ protein levels (Fig. 3.2D) without affecting the levels of GRα.

Effects of TSTs on TM cell viability

To ensure that the observed changes in the GRα and GRβ mRNA levels were the result of alternative splicing and not due to changes in cell viability/cell-death, three primary TM cells were treated with 10 nM, 100 nM and 1 mM of each TST for 24 hours compared to the DMSO vehicle control. Cell viability was assessed by the amount of ATP produced by metabolically active cells using the CellTiter-Glo system (Cat. # G7570, Promega). The ATPs released convert luciferin substrate to luciferin oxide, and released luminescence signals were recorded. Luminescent signals are directly proportional to cell numbers. No significant differences were observed among any treatment (at any concentration) and were similar to the DMSO control (Fig. 3.3).

Effect of TSTs on DEX activity in TM cells

TM cells were pretreated +/- TSTs (1 mM for TST A, 100 nM for TST B and C) for 48 hours prior to treatment with or without DEX (100 nM) for 3 days. DEX treatment elevated fibronectin expression compared to its ethanol vehicle control (Fig. 3.4A and 3.4B). Pretreatment with TSTs completely blocked the DEX- mediated fibronectin induction both in whole cell lysate (Fig. 3.4A) and in conditioned medium (Fig. 3.4B).

Effect of TSTs on GRE-Luciferase Reporter Activity

In addition to evaluating the effects of TSTs on the DEX induction of fibronectin, we used a GRE-reporter assay to examine the effects of TSTs on DEX-induced GRE activity. GTM3 cells (n=3) were transfected with a GRE-luciferase vector followed by pretreatment with TST A (1 mM), TST B (1 mM), TST C (100 nM), or DMSO vehicle control for 24 hours. The cells were then treated with or without DEX (100 nM), and luciferase activity was determined 6 hrs later. Consistent with the fibronectin results, all three TSTs significantly reduced DEX mediated GRE-luciferase activity as compared to the untreated control (Fig. 3.5). These results strongly suggest that the TST-mediated increase in GRβ/GRα ratio is associated with decreased DEX responsiveness in TM cells.

DISCUSSION:

Long-term GC therapy can lead to ocular hypertension and iatrogenic glaucoma[1, 2]. However, there are significant differences in GC sensitivities between individuals. Approximately sixty percent of the population does not develop elevated IOP with GC-therapy[10, 11]. In contrast, almost all glaucoma patients are steroid responders. Expression of the dominant negative isoform GRβ has been associated with a number of steroid resistant diseases[14-18, 34]. We have previously shown that GRβ is expressed at lower levels in GTM cells compared to NTM cells, GTM cells are more responsive to GCs[19]. Also, TM cells become more resistant to GCs with increased GRβ expression.

We have also shown that spliceosome SR proteins are involved in GR alternative splicing and GC responsiveness in human TM cells (Jain et al. 2011)[23]. Increased SRp30c and SRp40 expression increased the GRβ/GRα ratio and decreased GC responsiveness in TM cells. The significant decrease in SFRS5.1 (SRp40) gene expression in GTM cells may be responsible for the decreased expression of GRb in GTM cells[23]. Therefore, it may be possible to exploit alternative splicing of GR to make TM cells more resistant to GCs for therapeutic intervention in steroid-induced glaucoma and POAG.

In our current study, we investigated the role of a special class of spliceosome modulators, the thailanstatins, in GR splicing and GC response in human TM cells. All three TSTs significantly increased GRβ levels in transformed as well as in primary human TM cell strains. In our previous studies, we employed the synthetic bombesin peptide to alter GR splicing[23]. However, we now find that the TST compounds are much more potent and efficacious in mediating GRβ expression compared to bombesin. The TSTs work in nanomolar concentrations as compared to

micromolar concentrations of bombesin. Increased GRβ levels with these TSTs are associated with decreased DEX response for the induction of fibronectin as well as for GRE-luciferase reporter activity.

Sharing the similar pharmacophore as FR901464 and spliceostatin, we suspect the TSTs may also affect alternative splicing by direct interaction with SF3b subunit of the spliceosome[24, 25]. Although several of these agents including FR901464, Pladienolide B, and Sudemycin have been proposed to affect mRNA splicing, there may be mechanisms other than direct interaction with the RNA splicing machinery involved in their activities such as increased mRNA stability, altering the rate of transcription, and/or affecting levels of snRNAs. mRNA splicing is tightly coordinated with mRNA transport and translation, and these agents may also affect these processes. Further studies involving arrays with all known splicing factors, exon junction splice arrays, knock down studies, and interactions with other known spliceosome inhibitors will help to better understand the mechanisms of action of these TSTs compounds. Additional studies will also determine whether these compounds can help mitigate GC-induced ocular hypertension and glaucoma.

Figure Legends:

Figure 3.1: Structure of TSTs.

Figure 3.2: TST A, B, C increase GRβ levels in TM cells. Primary TM cell strains (n=4) were treated with DMSO, 10 nM, 100 nM, and 1 μM concentrations of TST A, B, or C for 24 hours. All three TSTs increase GRβ mRNA levels in TM cells. TST A show significant induction at 1 μM as compared to the DMSO vehicle control (Fig. 3.2A, $p<.001$). TST B significantly induces

GRβ mRNA at 100 nM and 1 μM (Fig 2B, p<.01) and decreases GRα mRNA levels (Fig. 3.2B, p<.001). TST C increases GRβ mRNA levels at 100 nM (Fig. 3.2C, p<.05) and decreases GRα mRNA levels (Fig. 2C, p<.01). One-way ANOVA was used for statistical analysis. All three TSTs (representative data n=4, 48 hours) also increase GRβ protein levels (Fig. 3.2D)

Figure 3.3: TSTs do not affect TM cell viability. 24 hours of TST treatment (10 nM, 100 nM or 1 μM) do not affect TM cell viability as compared to DMSO vehicle control. Luminescent readings (y-axis) are directly proportional to cell numbers in a given well.

Figure 3.4: TSTs decrease DEX-mediated FN induction in TM. Representative data showing 3 days of DEX (100 nM) treatment increases cell-associated fibronectin (n=4, Fig. 3.4A), soluble fibronectin in conditioned medium (n=4, Fig. 3.4B). Pretreatment with TST A (1 μM) or TST B (100 nM) or TST C (100 nM) for 48 hours decreases DEX-mediated FN (Fig. 3.4A and 3.4B) induction. Commassie blue stain was used to stain gel to ensure equal loading in conditioned medium samples.

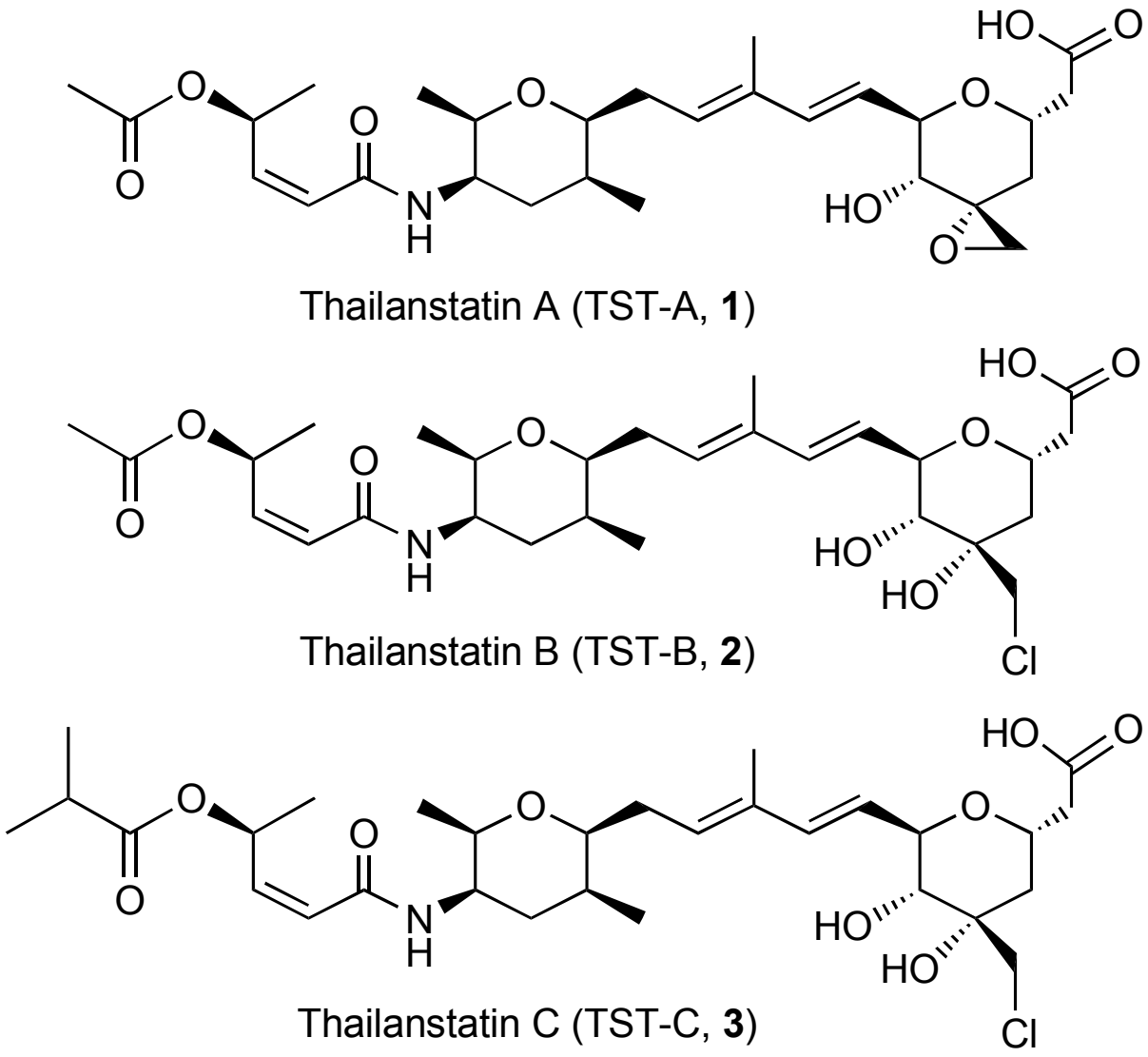
Figure 3.5: TSTs decreases DEX-mediated GRE-Luciferase promotor activity. 1 μM of TST A, 1 μM of TST B and 100 nM of TST C pretreatment for 24 hours significantly decreased DEX (100 nM, 6hrs)-mediated GRE-luciferase reporter activity in GTM3 cells (Fig. 3.5, n=3). Mean +/- SEM *** p<0.001 (One-way ANOVA).

Table 3.1. List of PCR Primers

Gene Name	Left Primer sequence (5' to 3')	Right Primer Sequence (5' to 3')
NR3C1 (GR alpha)	GAACTGGCAGCGGTTTTATC	TTTTGGTATCTGATTGGTGATGA
NR3C1 (GR beta)	GAACTGGCAGCGGTTTTATC	TCAGATTAATGTGTGAGATGTGC TT
GAPDH	GGGAGCCAAAAGGGTCAT	TTCTAGACGGCAGGTCAGGT

Table 3.2. List of Antibodies

Protein Name	Primary Antibody	Dilution
GR alpha	Custom made Rabbit Polyclonal	1:2000 for WB
GR beta	Custom made Rabbit Polyclonal	1:5000 for WB
Fibronectin	Millipore, MA AB1945; Rabbit Polyclonal	1:500 for WB
Myoclin	Santa Cruz Biotech, CA SC-20976; Goat Polyclonal	1:500 for WB
GAPDH	Cell signaling, MA 14C10, Rabbit monoclonal	1:1000 for WB

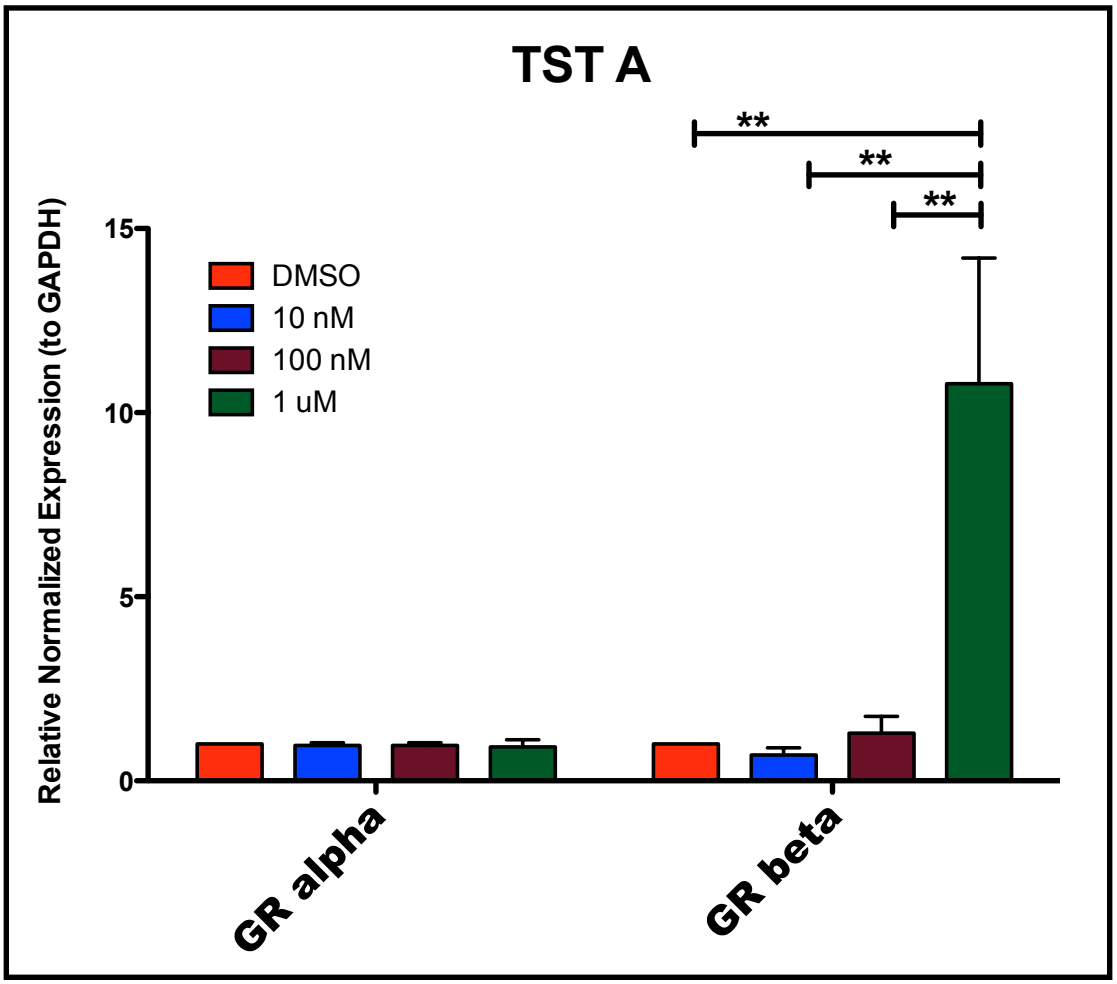


Thailanstatin A (TST-A, **1**)

Thailanstatin B (TST-B, **2**)

Thailanstatin C (TST-C, **3**)

Figure 3.1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**
**
**
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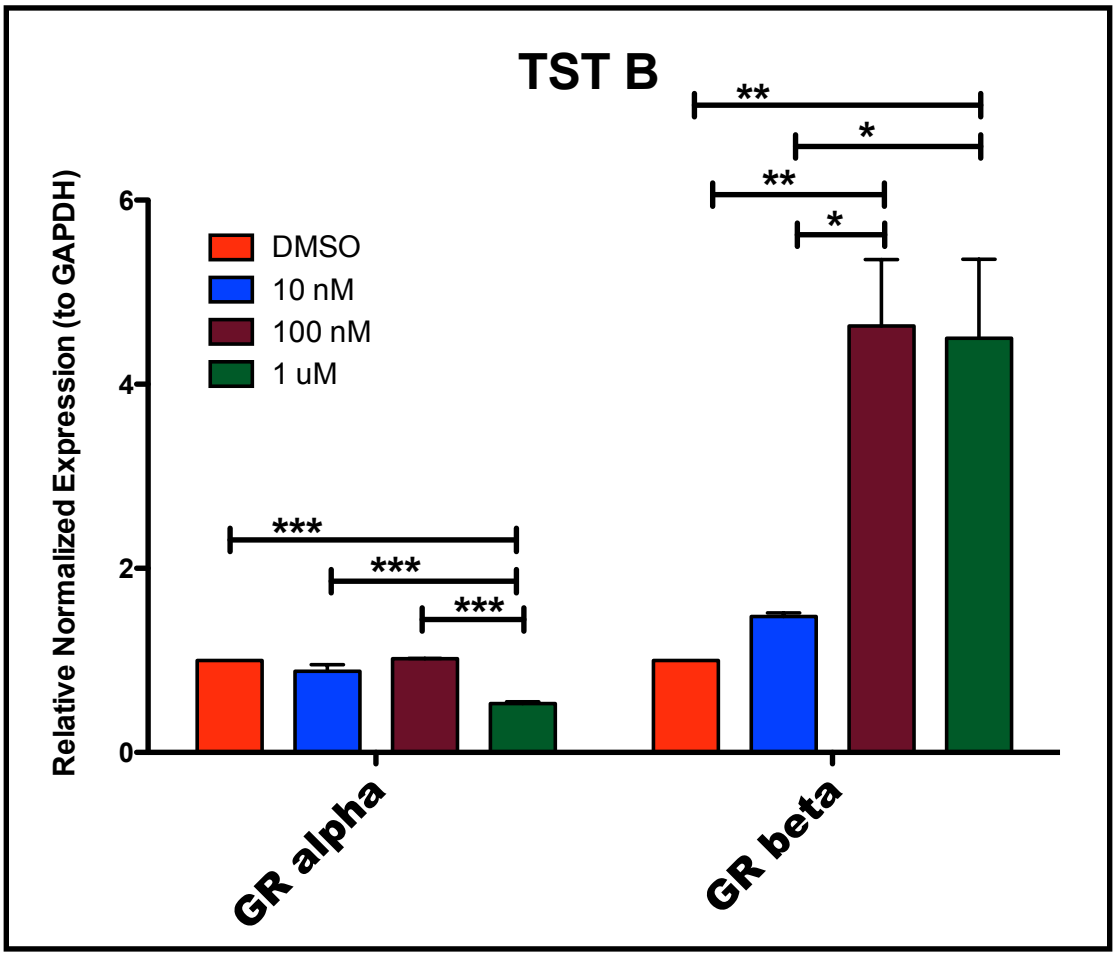
10
5
0
DMSO
10 nM
100 nM
1 uM
GR alpha
GR beta

Figure 3.2A


TST B
Relative Normalized Expression (to GAPDH)
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4
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100 nM
1 uM
***
***
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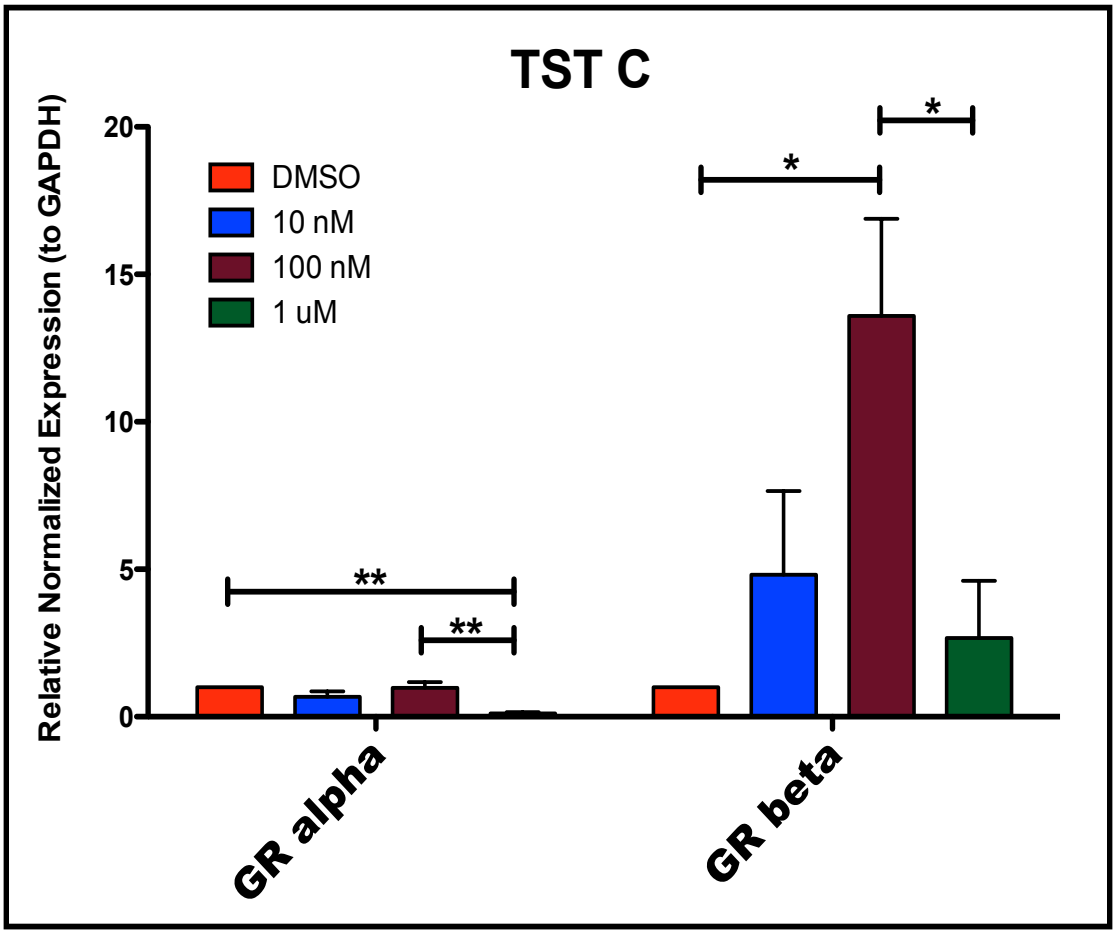
*
**
*
GR alpha
GR beta

Figure 3.2B


TST C
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100 nM
1 uM
**
**
*
*
GR alpha
GR beta

Figure 3.2C

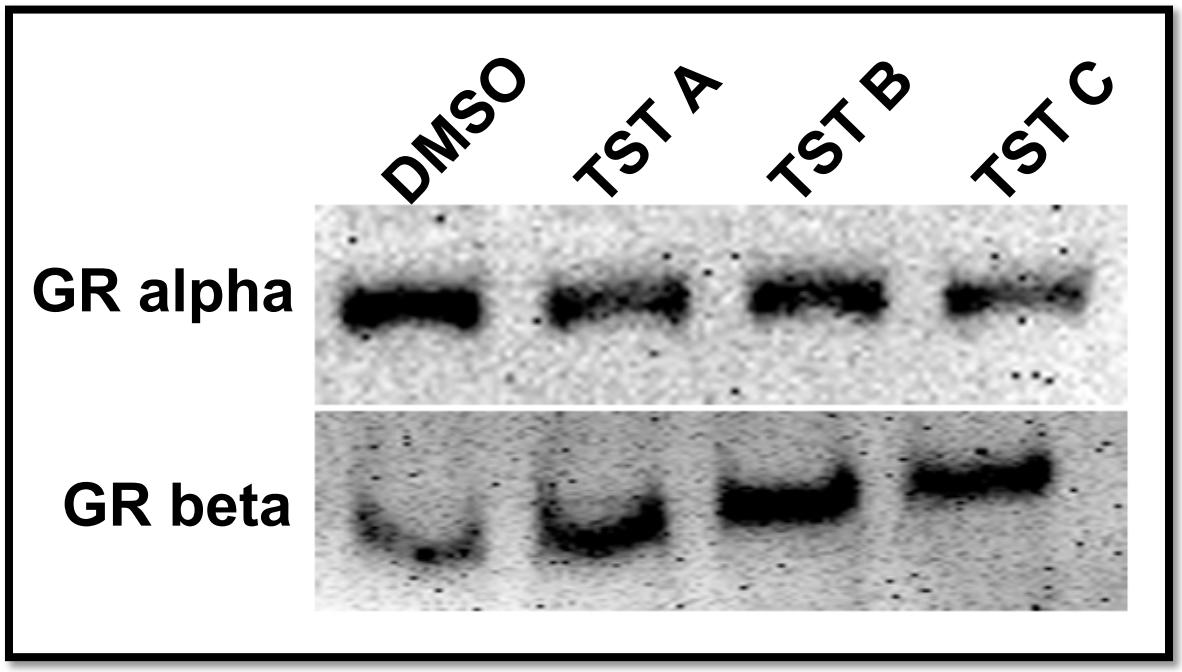
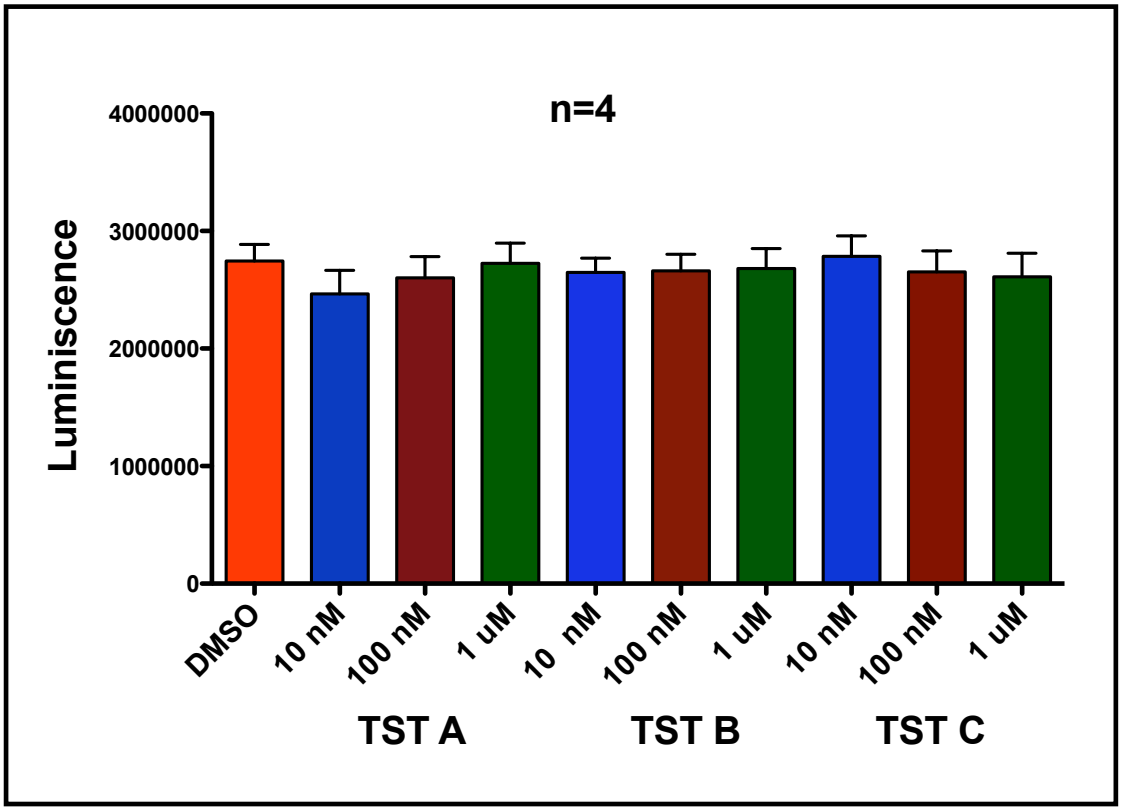


Figure 3.2D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Luminiscence
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3000000
2000000
1000000
0
DMSO
10 nM
100 nM
1 uM
10 nM
100 nM
1 uM
10 nM
100 nM
1 uM
TST A
TST B
TST C

Figure 3.3

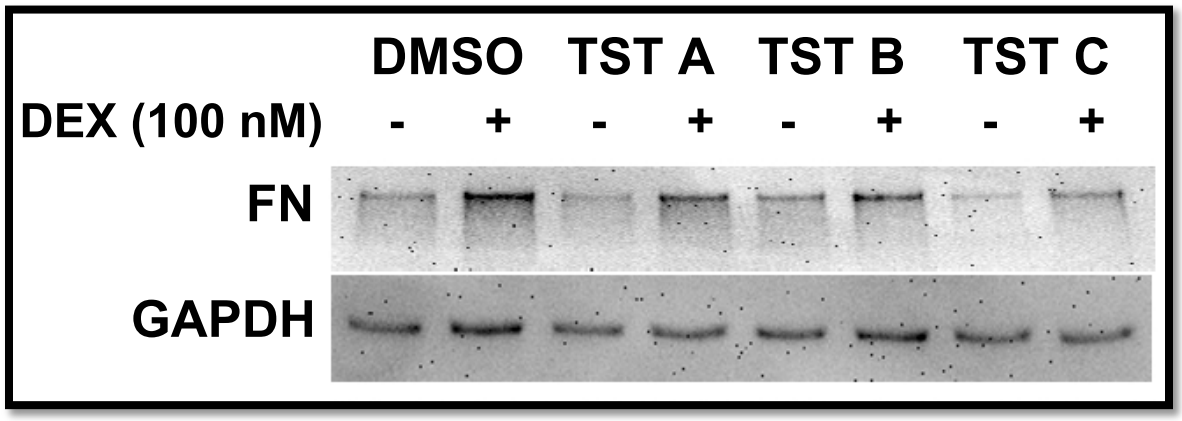


Figure 3.4A

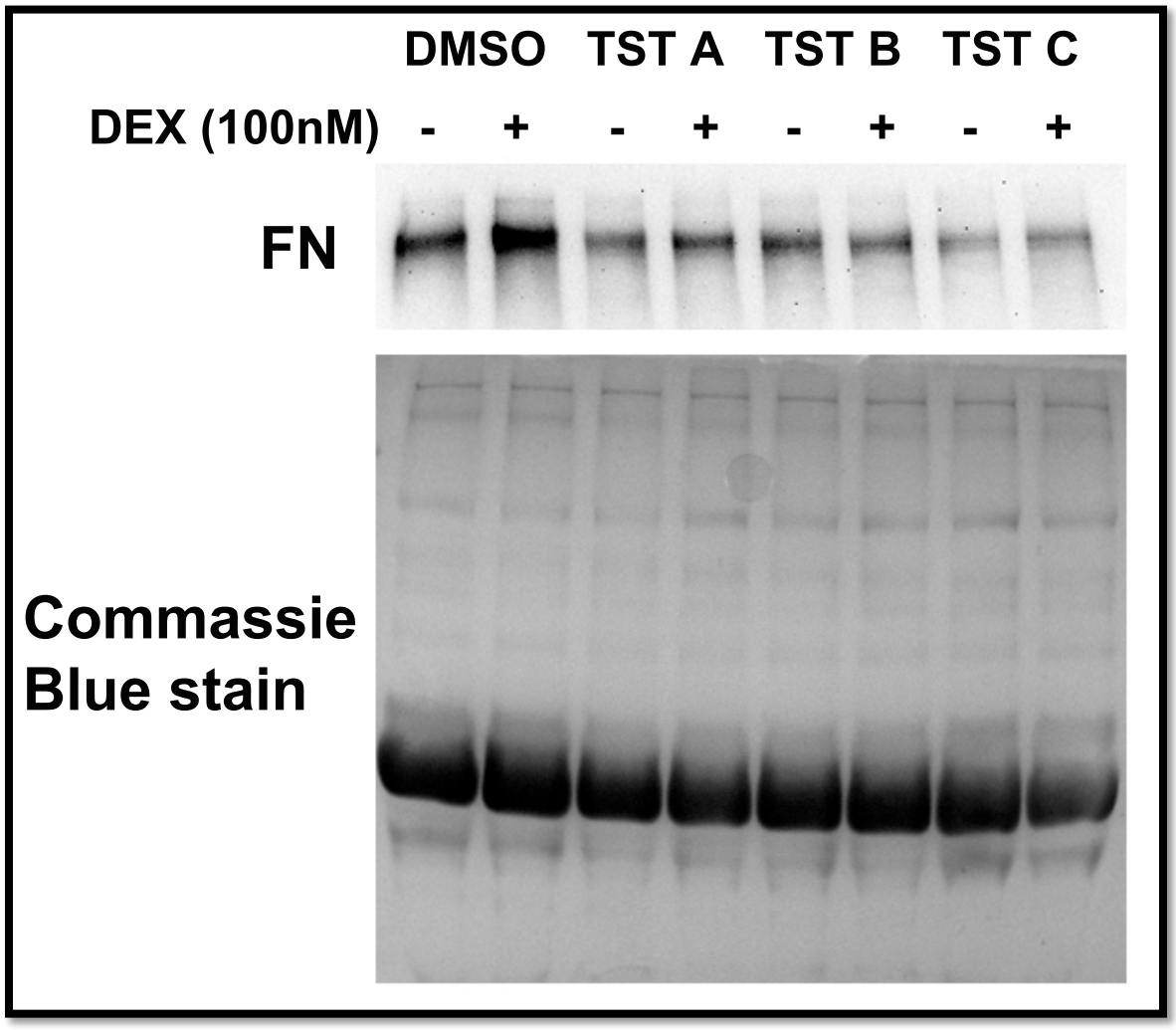
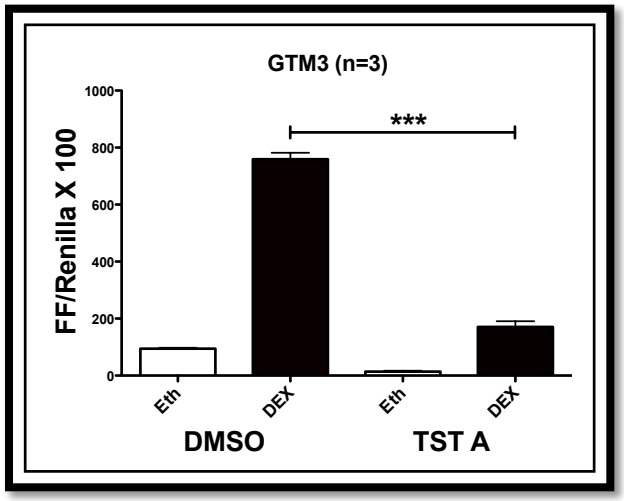


Figure 3.4B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200
0
***
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DEX
Eth
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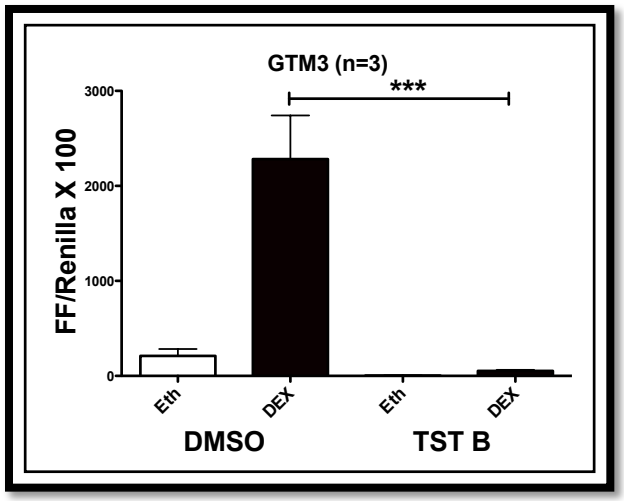
DMSO
TST A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2000
1000
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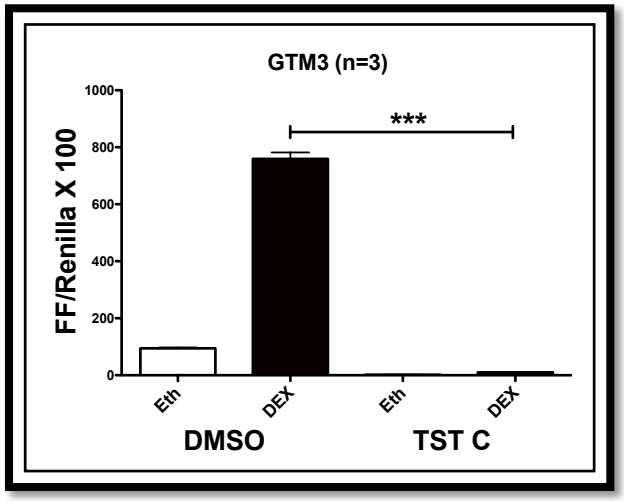
Eth
DEX
DMSO
TST B


GTM3 (n=3)
FF/Renilla X 100
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800
600
400
200
0
***
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DEX
Eth
DEX
DMSO
TST C

Figure 3.5

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CHAPTER IV

CONCLUSIONS

The results from these studies support our hypothesis that different SR proteins regulate relative levels of GRα and GRβ in trabecular meshwork (TM) cells. And, a special class of spliceosome inhibitors called thailanstatins (TSTs) affects GR splicing, increase the GRβ/GRα ratio and decreases the dexamethasone (DEX) response in TM cells.

In the first set of studies (chapter II), we report that various SR proteins are expressed in TM cells with SFRS5.1 being differentially expressed among normal and glaucomatous cell strains. We also observed that both SRp30c and SRp40 increased the expression of GRβ, whereas SRp20 did not alter the GRβ/GRα ratio. Increased expression of either SRp30c or SRp40 with SRp expression vectors decreased the DEX induction of FN as well as blocked the DEX induction of a GRE-luciferase reporter gene. We also report that the peptide bombesin increased the expression of SRp30c and SRp40, decreased SRp20 levels, as well as increased GRβ/GRα ratios. This increased ratio was associated with decreased GC (DEX) response in TM cells.

Significantly, we have now shown that TM cells express a number of different SRp splicing proteins and that the three SRps tested are involved in the alternative splicing of GR in TM cells. These results demonstrate one important mechanism regulating GR isoform expression and GC response in TM cells.

In the second set of experiments (chapter III), we show that TSTs (spliceosome inhibitors, originally isolated *Burkholderia thailandensis*, Liu, X. et al. and Y.-Q. Cheng) affect GR splicing. All three TSTs: TST A, TST B and TST C, increase GRβ/GRα ratios in human TM

cell strains. Increased GRβ levels with these TSTs are associated with decreased DEX response in terms of fibronectin induction and GRE-luciferase reporter activity.

We have shown the correlation between SR proteins, GR alternative splicing and GC responsiveness in human TM cells. Regulation of GR alternative splicing may potentially be exploited (via expression vectors, bombesin or TSTs) for therapeutic intervention in patients with steroid-induced glaucoma and POAG.

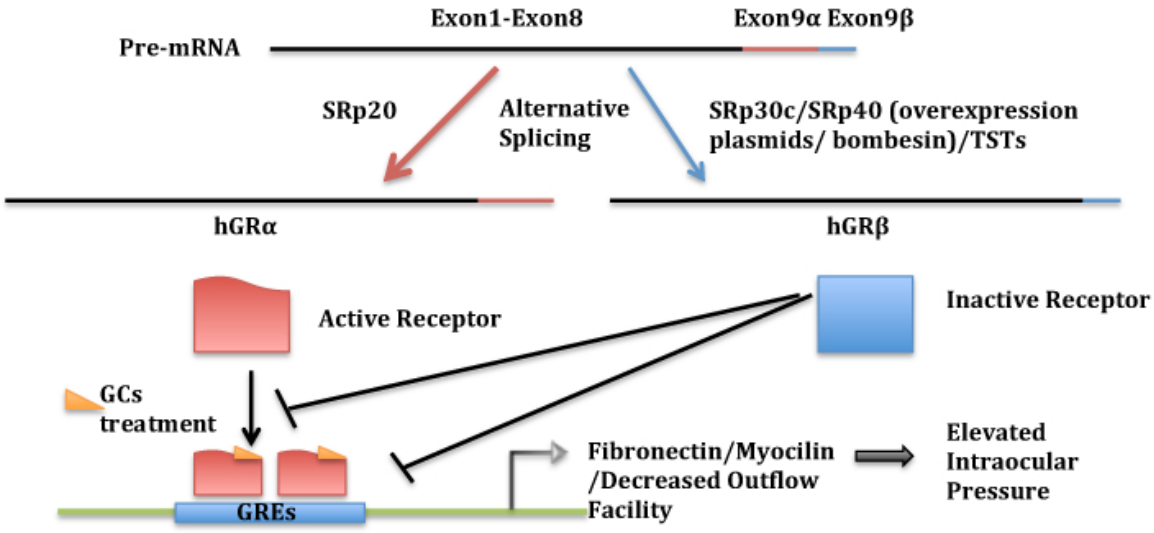

Figure 4.1 Conclusions. The therapeutic use of glucocorticoids (GCs) can cause elevated intraocular pressure (IOP) and iatrogenic glaucoma in susceptible individuals. Modulating the levels of Splicing factors (SRps) either by overexpression plasmids or by using the bombesin peptide or by proposed use of TSTs alters GRβ/GRα ratios in trabecular meshwork (TM) cells. An increased GRβ/GRα ratio decreases GRα-mediated biochemical and cellular changes in the TM and lowers IOP in the eye.

CHAPTER V

FUTURE DIRECTIONS

Our studies elucidate an important mechanism of regulation of glucocorticoid receptor alternative splicing in trabecular meshwork cells via SR proteins. These studies help to answer some important gaps in current literature yet raise very pertinent questions for future experiments. The physiological role of alternatively spliced product GRβ in IOP regulation is one of the most important questions. The role of SRps in DEX-mediated IOP regulation remains unanswered. The role of bombesin and TSTs in regulating DEX induced IOP elevation also warrants further investigation. The following are some of the potential experiments designed to address these concerns and questions.

1. Ex-vivo examination of the role of GRβ in regulating IOP.

To determine the role of GRβ in regulating DEX-mediated IOP elevation, we will employ the human/bovine anterior eye segment perfusion organ culture model (POC). We will use adenoviral vectors to overexpress the human GRβ in the anterior eye segments. The IOP of the eyes will be constantly monitored. We will also treat the anterior eye segments with DEX to observe the role of GRβ on DEX-induced IOP. We will also determine the levels of the GRα and GRβ in TM tissues at the end of each experiment.

2. In-vivo examination of the role of GRβ in regulating IOP in normal as well as GC-induced ocular hypertensive mice.

Ad5-hGRβ viral vectors can be used to transduce mouse eye TM. IOPs will be measured (using TonoLab tonometer) after subconjunctival injections of DEX.

3. Ex-vivo examination of the role of SRps in DEX-mediated IOP.

a) We will over-express SRp 20/30c/40 each in human/bovine anterior eye segments in POC model. While one eye of each pair will be transduced with Ad5-SRp, the contralateral eye will be transduced with the empty viral vector. The IOP will be constantly monitored for a period of 3-4 days. Both eyes of each pair will be perfused with medium containing 100 nM DEX. The change in IOP will be monitored constantly and compared at the end of the 4-7 days treatment period. TM tissue from these eyes will be obtained post perfusion and analyzed for viability and changes in GRβ/GRα ratio using western blotting.

b) We will knockdown SRp 20/30c/40 via shRNAs in human/bovine anterior eye segments in POC model. While one eye of each pair will be transduced with Ad5-shSRp, the contralateral eye will be transduced with the empty viral vector. The IOP will be constantly monitored for a period of 3-4 days. Both eyes of each pair will be perfused with medium containing 100 nM DEX. The change in IOP will be monitored constantly and compared at the end of the 4-7 days treatment period. TM tissue from these eyes will be obtained post perfusion and analyzed for viability and changes in GRβ/GRα ratio using western blotting.

4. Ex vivo examination of role of bombesin in DEX-mediated IOP elevation.

Human/bovine anterior eye segments in POC model will be employed. While one eye of each pair will be transfused with bombesin (1 μM), the contralateral eye will be with the vehicle (normal DMEM/high glucose media). The IOP will be constantly monitored for a period of 3-4 days. Both eyes of each pair will be perfused with medium containing 100 nM DEX. The change in IOP will be monitored constantly and compared at the end of the 4-7 days treatment period. TM tissue from these eyes will be obtained post perfusion and analyzed for viability and changes in GRβ/GRα ratio using western blotting.

5. Ex vivo examination of role of TSTs in DEX-mediated IOP elevation.

Human/bovine anterior eye segments in POC model will be employed. While one eye of each pair will be transfused with different TSTs, the contralateral eye will be with the vehicle (DMSO). The IOP will be constantly monitored for a period of 3-4 days. Both eyes of each pair will be perfused with medium containing 100 nM DEX. The change in IOP will be monitored constantly and compared at the end of the 4-7 days treatment period. TM tissue from these eyes will be obtained post perfusion and analyzed for viability and changes in GRβ/GRα ratio using western blotting.

6. Deduce the mechanisms of action of TSTs

Studies involving arrays with all known splicing factors, exon junction splice arrays and knock down studies will help in better understanding the mechanisms of action of these TSTs compounds and other known spliceosome inhibitors.

These proposed studies would help us better understand the physiological and pathological role of alternative GR splicing in regulating IOP. The studies will also aid in the discovery of novel disease modifying therapeutic targets and strategies.