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Investigating the dual roles of STRA6 in cell signaling and vitamin A trafficking

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**Investigating the dual roles of STRA6 in cell signaling and vitamin A
trafficking**

by

Christina Elaine Schulte

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Toxicology

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Ames, Iowa

2012

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TABLE OF CONTENTS

LIST OF FIGURES	iv
LIST OF TABLES	v
LIST OF ABBREVIATIONS	vi
ABSTRACT	viii
CHAPTER 1. LITERATURE REVIEW	1
Introduction	1
Diverse Physiological Function of Vitamin A	2
Vitamin A Digestion and Absorption	5
Transport, Metabolism, and Storage	7
Plasma Retinol Binding Protein	8
STRA6, the RBP Receptor that Mediates Vitamin A Uptake	9
Evidence for the existence of an RBP receptor	9
Identification	10
STRA6 topology	12
STRA6 mutations associated with human diseases	13
Retinoid Signaling in the Target Cell	14
Retinoid signaling	17
JAK/STAT Activation	18
Role of Vitamin A in Carcinogenesis	20
Abnormal retinoid metabolism in cancer	22
CHAPTER 2. METHODS	25
Cell Culture	25

Cell Culture Treatments	25
Crystal Violet Assay	26
mRNA Extraction and Reverse Transcriptase-Quantitative PCR	26
Western Blots	28
Fluorescent Microscopy Retinol Uptake Assay	29
Conjugating RBP with Alexa Fluor 488	29
RBP uptake assay	29
Radioactivity Retinol Uptake Assay	30
Conjugating RBP with ³ H-retinol	30
Retinol Uptake assay	30
Statistics	30
CHAPTER 3. RESULTS	32
Retinol uptake over time	32
Effects of Retinoic Acid	33
STRA6 protein expression	33
Expression of cell cycle progression proteins	33
Change in gene expression	33
Cell viability	34
Retinol uptake	35
Effects of Holo-RBP	35
STRA6 expression	36
Effects of holo-RBP on protein expression	36
Change in gene expression	37
Effects on cell viability	37
CHAPTER 4. DISCUSSION AND CONCLUSION	49
BIBLIOGRAPHY	53

LIST OF FIGURES

Figure 1. Chemical Structures of Vitamin A	3
Figure 2. Vitamin A Absorption, Metabolism, and Tissue Delivery.	6
Figure 3. Vitamin A Transport and Activation.	16
Figure 4. Retinol Uptake Over Time.	38
Figure 5. Effects of Retinoic Acid on STRA6 Expression	39
Figure 6. Effects of Retinoic Acid on Cell Cycle Progression Protein Expression	40
Figure 7. Effects of Retinoic Acid on Gene Expression Levels	42
Figure 8. Effects of Retinoic Acid on Cell Viability	43
Figure 9. Effects of Retinoic Acid on Retinol Uptake	44
Figure 10. Effects of Holo-RBP on Protein Expression	45
Figure 11. Effects of Holo-RBP on Gene Expression Levels	47
Figure 12. Effects of Holo-RBP on Cell Viability	48

LIST OF TABLES

Table 1. RT-qPCR Primer Sequences

27

LIST OF ABBREVIATIONS

- RBP – Retinol Binding Protein
STRA6 – Stimulated by Retinoic Acid 6
Jak2 – Janus Kinase 2
Stat5 – Signal Transducer and Activator of Transcription 5
p21 – Cyclin-Dependent Kinase Inhibitor 1
ATRA – All-*trans* Retinoic Acid
9-*cis* RA – 9-*cis* Retinoic Acid
CRBP I – Cellular Retinol Binding Protein I
CRBP II - Cellular Retinol Binding Protein II
LRAT – Lecithin:Retinol Acyltransferase
ARAT – Acyl CoA:Retinol Acyltransferase
RPE – Retinal Pigment Epithelium
VLDL – Very Low-Density Lipoprotein
ATP – Adenosine Triphosphate
TTR – Transthyretin
RAR – Retinoic Acid Receptor
RXR – Retinoid X Receptor
RARE – Retinoic Acid Response Element
CDK – Cyclin Dependent Kinase
p27 – Cyclin-Dependent Kinase Inhibitor 1B
Cav-1 – Caveolin 1
ER – Estrogen Receptor
MAPK – Mitogen-Activated Protein Kinase

cAMP – Cyclic Adenosine Monophosphate

JNK – c-Jun N-Terminal Kinases

p38 – Mitogen-Activated Protein Kinase

c-fos – Cellular Oncogene fos

REH – Retinyl Ester Hydrolase

RA - Retinoic Acid

RDH – Retinol Dehydrogenase

ALDH – Aldehyde Dehydrogenase

AC6 – Adenylyl Cyclase 6

CYP – Cytochrome P450 Superfamily

CD1 – Cyclin D1

CD3 – Cyclin D3

18S – 18S Ribosomal RNA

SH2 – Src Homology 2 Domain

ABSTRACT

The long-term goal of the present study is to understand how cancer incidence and progression can be reduced through the optimization of vitamin A trafficking and signaling in tissues prone to tumor development. However, little is known about vitamin A uptake, transport, or signaling in epithelial cells prone to tumorigenesis. Retinol, the major circulating retinoid, is delivered to peripheral tissues in complex with retinol binding protein (RBP). Recently, a retinoic acid responsive protein with no previous known biological function, STRA6, has been identified as an essential transmembrane receptor that mediates retinol uptake in retinal epithelial cells. More recently, STRA6 was also found to be a cell surface signaling receptor activated by the binding of the RBP-retinol complex (holo-RBP) in HepG2 hepatocarcinoma cells. STRA6 contains an SH2 domain-binding motif in the receptor's cytosolic domain that appears to be activated through tyrosine phosphorylation after binding with the holo-RBP complex. This activation leads to the recruitment and activation of Janus kinase 2 (Jak2) and Signal transducer and activator of transcription 5 (Stat5). Once activated, Stat5 is translocated to the nucleus where it mediates the expression of specific target genes related to differentiation, apoptosis, and anti-proliferation. In the current studies, I have tested my central hypothesis that retinoic acid and/or binding of the holo-RBP complex leads to the anti-proliferative effects observed with retinol treatment in epithelial tissues. The results presented in this thesis show that treatment of cancer cells with all-*trans*-retinoic acid (ATRA) increased levels of STRA6 and p21, a cell cycle inhibitor, as well as down regulated the expression of cell cycle progression proteins. Moreover, our

results indicate a role for holo-RBP in apoptosis. Collectively these studies offer new insight into the role vitamin A trafficking plays in the anti-proliferative actions of vitamin A.

CHAPTER 1. LITERATURE REVIEW

Introduction

Vitamin A is an essential nutrient across species, and plays an important role in vision and cell differentiation (1). Dietary sources of preformed vitamin A include retinol esters, present in foods of animal origin and hydrolyzed in the intestine to form retinol, and provitamin A carotenoids, which are present in plants. In addition to naturally occurring dietary sources, synthetic carotenoids and vitamin A are produced for pharmaceutical and cosmetic uses. Humans require only small amounts of vitamin A in their diets, 400 to 1300 μg daily depending on sex and age (2,3). Vitamin A deficiency is a major issue in many developing countries and can result in blindness, severe infection, and even death (4). The molecular mechanism of vitamin A's physiological function was first discovered for vision by Wald and colleagues in 1933 (5). However, vitamin A's diverse role has been recognized in almost every vertebrate organ system and includes roles in embryonic growth and development, immune response, reproduction, maintenance of epithelial surfaces, and proper functioning of the adult brain (reviewed in 6–10). As a result of its role in cell growth and differentiation, vitamin A plays both positive and negative roles in several pathological conditions, such as visual disorders, cancer, infectious diseases, diabetes, teratogenicity, and skin diseases (reviewed in 10–17). Most of these pathological or physiological functions can be attributed to vitamin A's most biologically active form, retinoic acid, and its effects on nuclear hormone receptors (18).

Following absorption, vitamin A, in the form of retinol, is transported from storage in the liver to target cells via the blood bound to retinol binding protein (RBP). RBP binds with high affinity to retinol and serves as the main transport of vitamin A in the blood. This means of transport allows vertebrates to adapt to fluctuation in vitamin A dietary intake levels and ensures that the blood plasma retinol concentration is close to 2 μM (19). Recently, the cell surface RBP receptor has been identified as STRA6 (stimulated by retinoic acid gene 6 homolog), a widely expressed multitransmembrane domain protein. STRA6 binds to RBP with high affinity, mediates cellular uptake of vitamin A, and is localized to cellular locations expected of the RBP receptor in native tissues (20).

In this literature review, current knowledge of vitamin A and RBP relative to my research is discussed. Additionally, STRA6's identification, function, and unique features as a membrane receptor and transporter are described in more detail, as well as its link to cancer prevention.

Diverse Physiological Function of Vitamin A

The term vitamin A is used to describe the group of compounds that possess the biological activity of all-*trans* retinol (21,22). The term "vitamin A" refers to molecules that are structurally similar and possess vitamin A activity. These molecules include alcohol, aldehyde, acid, and ester forms, as well as synthetic analogues. Structurally, retinoids contain a β -ionone ring and a polyunsaturated side chain, with either an alcohol, an aldehyde, a carboxylic acid, or an ester group (**Figure 1**). Some forms have direct biological activities; where as other forms serve as important reaction intermediates or as the storage form of

vitamin A. Retinol and its derivatives and analogs, either natural or synthetic, with or without biological activity are termed retinoids.

The alcohol form of vitamin A, retinol, serves as a substrate for RBP, which determines the ability of vitamin A to be transported to target tissues. Retinol is less toxic than retinal and retinoic acid and may be the reason it evolved to be the major transport form of vitamin A (23). After delivery to target tissues, retinol can be oxidized to yield retinal, the form essential for vision, or retinoic acid, the most biologically active form of vitamin A.

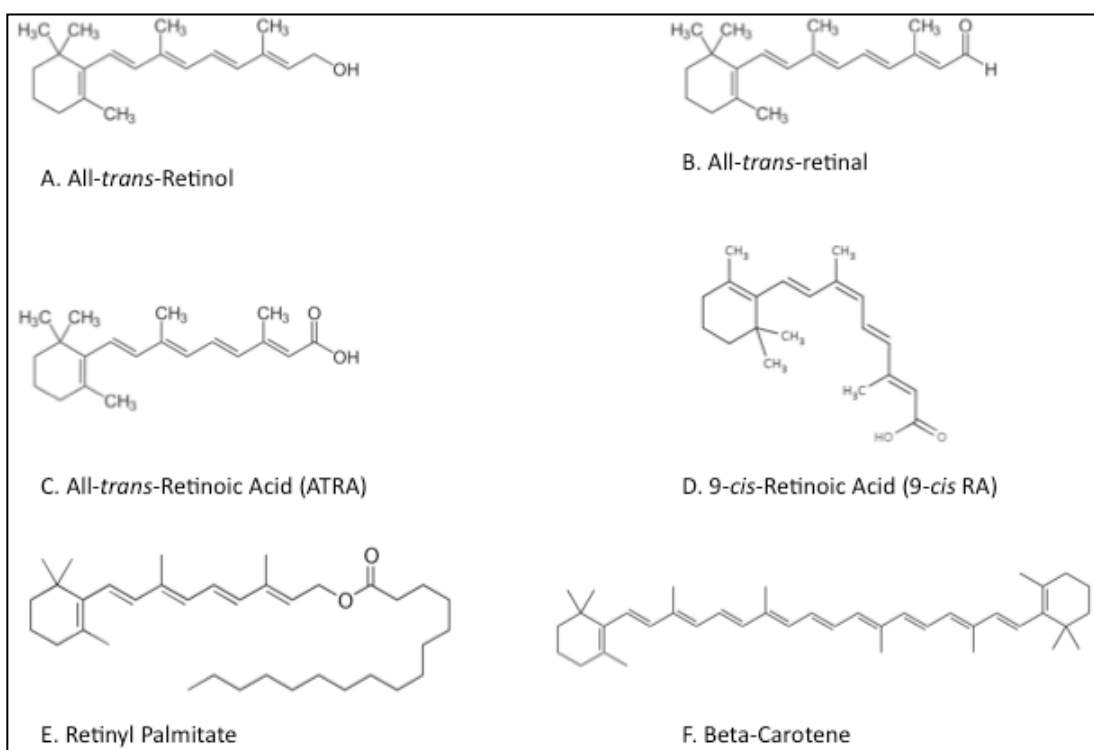


Figure 1. Chemical Structures of Vitamin A

The aldehyde form of vitamin A, 11-cis retinal, is used in photoreceptor cells in the retina as a chromophore to support vision. However, free retinal has been shown to be highly toxic (24). Additionally, the aldehyde form of vitamin A serves as an intermediate in the synthesis of retinoic acid.

Most of vitamin A's numerous activities can be attributed to the action of all-*trans* retinoic acid (ATRA) and its 9-*cis* isomer (9-*cis* RA). These acids exert their actions by binding to nuclear retinoic acid receptors and regulating gene transcription of a large number of genes. It is also the most biologically active form of vitamin A. It can either stimulate or suppress mitogenesis depending on the cellular context (25). Additionally, retinoic acid can mediate its effects by retinoylating proteins (26,27). Retinoic acid is not stored and is metabolized quickly. Furthermore, it cannot support vision because it cannot be reduced to retinaldehyde or retinol.

Free retinol is chemically unstable and does not occur to a significant extent in foods or tissues; rather, it is present as a variety of esters, mainly retinyl palmitate. The retinyl ester form of vitamin A is also the main form of storage in the cells (22,28). It is also an alternative form for vitamin A delivery; however, this delivery system has been associated with toxicity (4,29). Tissues can take up retinyl esters from chylomicrons, however, most is left in the chylomicron remnants to be taken up by the liver. Hypervitaminosis A, a toxic state associated with increased plasma concentrations of vitamin A, particularly retinyl esters, occurs when excessive amounts of vitamin A are presented to cell membranes in association with plasma lipoproteins (29,30). In fact, elevated amounts of retinyl esters have been used as markers for chronic hypervitaminosis A in humans (31–33). Although the mechanism remains inconclusive, a proposed mechanism of toxicity is the leaking of esters into the bloodstream from saturated hepatic stellate cells and the incorporation into LDL particles (31,34,35).

Provitamin A carotenoids represent a group of compounds that are precursors of vitamin A and are synthesized by a wide variety of fruits and vegetables, including carrots,

apricots, mangoes, sweet potatoes, spinach, kale, and tomatoes. Structurally, carotenoids have an expanded carbon chain containing conjugated double bonds that usually, but not always, contain an unsubstituted β -ionone ring at one or both ends of the chain. Although there are over 600 carotenoids produced by plants, fewer than 10% are thought to exhibit vitamin A activity. These active carotenoids are called provitamin A, and their metabolism yields biologically active retinoids (retinol, retinal, retinoic acid) (36). β -carotene, α -carotene, and β -cryptoxanthin are three dietary provitamin A carotenoids that are found most often in the *all-trans* form (21).

Vitamin A Digestion and Absorption

Prior to absorption, vitamin A requires digestion, given that it is bound to other food components. In fact, retinol is typically bound to fatty acid esters, most commonly retinyl palmitate, or complexed with protein from which it must be released (37). Hydrolysis of carotenoids and retinyl esters from proteins begins in the stomach and is initiated by pepsin (**Figure 2**). Due to their innate fat solubility, free retinyl esters and carotenoids join together to form fat globules in the stomach. These fat globules are emulsified later in the duodenum into smaller droplets by bile. Micelles containing bile salts, phospholipids, triglycerides, and retinyl and carotenoid esters form within the lumen of the small intestine. Retinyl and carotenoid esters are further hydrolyzed by various hydrolases and esterases and pancreatic lipases and remain within the micellar solution. The micellar solution is absorbed across the microvilli brush border membrane of the duodenum and jejunum and into the enterocyte (38).

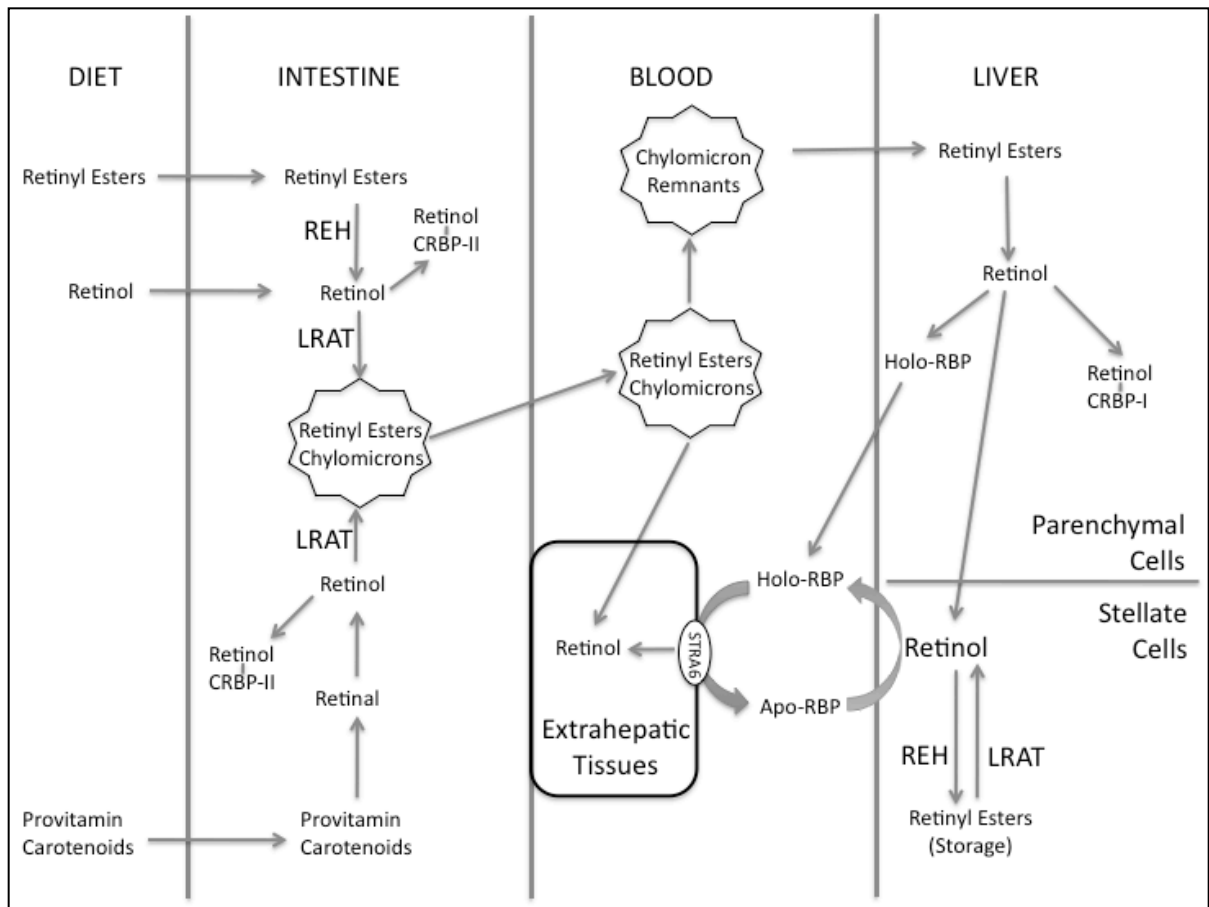


Figure 2. Vitamin A Absorption, Metabolism, and Tissue Delivery.

The principle dietary sources of vitamin A include retinol, retinyl esters, and provitamin A carotenoids, such as β -carotene. Within the enterocytes, retinyl esters are hydrolyzed to retinol through retinyl ester hydrolases (REHs), whereas the provitamin A carotenoids are cleaved to retinal and metabolized to retinol. Retinol is then bound to cellular retinol binding protein II (CRBP-II) and esterified to retinyl esters through the action of lecithin:retinol acyltransferase (LRAT). The retinyl esters are secreted into the lymphatic system through the incorporation with other lipids into chylomicrons. The chylomicrons are taken up by hepatocytes, where the retinyl esters are hydrolyzed by REH to retinol, where it undergoes one of several fates. Retinol can combine with cellular retinol binding protein I (CRBP-I) for hepatocellular use, conjugate with retinol binding protein (apo-RBP) to form holo-RBP for delivery to other tissues, or it can be transferred to the stellate cells to be stored as retinyl esters until required.

Approximately 70-90% of dietary preformed vitamin A and 5-60% of carotenoids, depending on the cooking process, are absorbed (39). Once within the intestinal mucosal cell, carotenoids undergo metabolism and can be hydrolyzed into retinaldehyde, or retinal, by 15,15'-carotene dioxygenase. Retinal can be further converted to retinol by retinal reductase or oxidized to form retinoic acid. Retinol is bound to cellular retinol binding protein II (CRBP-II) and then esterified to form mainly retinyl palmitate, although small amounts of retinyl stearate and retinyl oleate are also formed, by lecithin:retinol acyl transferase (LRAT) using phosphatidylcholine as the fatty acid donor. A minor secondary pathway involves acyl CoA:retinol acyltransferase (ARAT), esterifying the free retinol (37). Retinyl esters are then incorporated into a chylomicron, which can travel to other tissues via the lymphatic circulation and then the bloodstream, together with dietary lipids and carotenoids. In contrast, retinoic acid is transported directly from the small intestine to the liver via portal circulation, and then transported in the plasma bound tightly to albumin.

Transport, Metabolism, and Storage

Chylomicrons deliver retinyl esters and carotenoids to many extrahepatic tissues, including bone marrow, blood cells, spleen, adipose tissue, muscle, lungs, and kidneys (40). However, most remain in the chylomicron remnants and are taken up into the liver through endocytosis. Upon reaching the liver, vitamin A is taken up by parenchymal cells. Carotenoids are cleaved into retinol, incorporated into very low density lipoproteins (VLDLs) for transport to other tissues, or stored in the liver as retinyl esters. Retinyl esters are cleaved by hydrolases into retinol and bound to CRBP-I. CRBP-I is thought to function

both to help control concentrations of free retinol within the cell and prevent its oxidation, as well as to direct the vitamin to specific enzymes of metabolism (41,42).

Finally, retinol is esterified by LRAT or ARAT and stored with lipid droplets in stellate cells of the liver, oxidized to retinal by dehydrogenase, or phosphorylated to retinyl phosphate by ATP for glycoprotein functions (43). Hydrolysis can release the retinol from storage as needed for use. Hence, plasma vitamin A concentrations remain relatively constant over a large range of dietary intakes (39), and only after the hepatic storage can no longer accept more retinol does hypervitaminosis A occur (44).

Plasma Retinol Binding Protein

Retinol is released from the liver bound to RBP and transthyretin (TTR). Both proteins are synthesized in the liver by the hepatocytes. Because free retinol is insoluble in aqueous media, chemically unstable, and relatively toxic to cells, RBP serves to solubilize retinol, protect it against oxidation, and deliver it to target tissues (4,45). RBP binds 1 mol of retinol per mol of protein, which also interacts with 1 mol of TTR. This complex containing TTR, RBP, and retinol is referred to as holo-RBP-TTR complex (21). TTR is critical because it increases the molecular weight of the holo-RBP complex and reduces its loss through glomerular filtration in the kidneys.

In the liver, apo-RBP-TTR complex, where no retinol is present, is formed in the rough endoplasmic reticulum (ER), then migrates through the smooth ER and binds to retinol. The holo-RBP complex is then free to migrate to the Golgi for secretion (46). RBP is the principle means of transporting vitamin A in the blood and allows for plasma concentrations to be 1000-fold higher than would occur for free retinol (4). Though RBP is

mainly produced in the liver, it can be produced in many other tissues. For example, RBP is highly expressed in adipose tissue. However, extrahepatic RBP cannot mobilize vitamin A stores from the liver (47). The role of RBP produced by tissues other than the liver is not completely understood. However recently, RBP produced by adipose tissue has been found to be an adipokine for insulin resistance (16). Additionally, adipose tissues contain 15% to 20% of total body vitamin A, mostly taken up from chylomicrons by lipoprotein lipases in the form of retinyl esters (48). The synthesis of RBP by adipose tissue may provide a mechanism for return of excess retinol.

With respect to vitamin A homeostasis, RBP is critical. RBP knockout mice could not mobilize the hepatic vitamin A stores and were extremely sensitive to vitamin A deficiency. These mice had dramatically lower serum vitamin A levels, even with nutritionally complete diets. Additionally, these mice had classic symptoms of vitamin A deficiency, including lower levels of circulating immunoglobulin, abnormal heart development, and impaired vision.

STRA6, the RBP Receptor that Mediates Vitamin A Uptake

Evidence for the existence of an RBP receptor

Evidence for the existence of a specific cell-surface receptor for RBP first emerged in the 1970's and was first revealed in retinal pigment epithelium cells and mucosal epithelial cells (49–53). In fact, since the 1970's there has been strong evidence for the existence of a cell-surface receptor for RBP in not only retinal pigment epithelium (RPE) cells but also in other tissues including placenta, choroid plexus, and macrophages (54–57). The observed tissue distribution of the RBP receptor parallels with what we know about vitamin A function

and metabolism. In a systematic comparison of RBP binding in different tissues, the highest RBP-binding activities were found in membranes from the RPE, placenta, bone marrow, choroid plexus, and undifferentiated keratinocytes, which has been correlated with vitamin A function in vision, fetal development, immune response, brain development, and cell differentiation, respectively (58).

There are several key indicators that suggested an RBP receptor is required for retinol uptake. Evidence for a requirement of an RBP receptor includes the inhibition of retinol uptake from RBP by an excess of unlabeled RBP, as well as by antibodies to RBP, and by a cysteine modification compound (49,53,54,59). Also, ^{125}I -RBP was shown to have saturable binding to the cell membrane (60) and upon injection of ^{125}I -RBP into rats, specific labeling was observed on the basolateral membrane of the RPE and in the choroid plexus (49,56). Finally, specific mutations in RBP or a monoclonal antibody against a specific region of RBP can terminate its interactions with the receptor and abolish uptake of vitamin A into the cells (61–63).

Interestingly, the cell-surface RBP receptor was shown to mediate uptake of vitamin A from holo-RBP before its identification (50,52,60). In fact, vitamin A uptake from the holo-RBP complex has been found in several tissues including mucosal epithelial cells, human placenta, Sertoli cells of the testis, human skin, and macrophages before the RBP receptor was elucidated (53,55,59,64,65).

Identification

Even with the growing body of evidence of a cell-surface RBP receptor that mediates vitamin A uptake and/or downstream signaling, it has been challenging to purify and identify

the RBP receptor using traditional biochemical approaches due to the fragility of the receptor protein and the transient nature of the binding of RBP to its receptor. In 2007, Kawaguchi and his colleagues designed a strategy to stabilize the RBP-receptor interaction, permitting high affinity purification of the RBP-receptor complex (20). Using this strategy, the RBP receptor was identified as STRA6, a multitransmembrane protein of previously unknown function. STRA6 binds to RBP with high affinity and mediates uptake of vitamin A into the cell from the holo-RBP complex. Moreover, STRA6's mechanism of retinol uptake does not depend on endocytosis and does not require active transport or an electrochemical gradient of the free substrate.

Despite the fact that STRA6's function in vitamin A uptake was recently elucidated, it was first characterized in 1995 as a retinoic acid induced gene in P19 embryonic carcinoma cells (66) and stands for "stimulated by retinoic acid 6." It can be induced by Wnt-1 and retinoic acid synergistically (67). STRA6 is also widely expressed during embryonic development and in adult organ systems. In adult tissues, STRA6 is expressed in blood-organ barriers including the RPE (blood-retina barrier), the placenta (maternal-fetal barrier), the choroid plexus (blood-brain barrier), and the Sertoli cells of the testis (blood-testis barrier) (56), as well as non-blood-organ barriers such as the eye, both male and female reproductive system, the nervous system, the lymphoid organs, the skin, the lungs, the kidneys, and the heart, which correspond to its recently discovered function as the RBP receptor (14,68–73).

STRA6 topology

STRA6's function was especially hard to indentify because it has no homology similar to any known transport proteins. STRA6 is a 74-kDa protein that has a transmembrane topology determined experimentally by Kawaguchi and his colleagues (74).

The topology model for STRA6 indicates that it has 19 distinct domains comprising of five extracellular domains, nine transmembrane domains, and five intracellular domains.

Although STRA6 represents a new membrane transport system, many membrane transporters have 8-12 transmembrane domains and its number of these domains lies within this range (75). It is thought that the large number of transmembrane domains allows for STRA6 to form a pore through which retinol can pass into the cytoplasm of the target cell (75), though this hypothesis has yet to be proven.

RBP-binding domain

Since STRA6 is not homologous to any known membrane receptor, transporter, or channel protein, the identification of the RBP-binding domain on STRA6, was accomplished by the creation and analysis of over 900 random mutants of STRA6 that elucidated its structure and function (76). The mutation of three key residues in the RBP binding domain was sufficient to eliminate RBP binding and vitamin A uptake activities of STRA6. These three residues are thought to be located in the contact site for STRA6-RBP interaction without affecting its cell-surface expression. In fact, one of the key residues for RBP binding indentified is also the residue affected by a polymorphism in human STRA6 leading to embryonic lethality or severe birth defects in many organ systems.

STRA6 mutations associated with human diseases

Mutations in STRA6 have been associated with severe pathological phenotypes in human genetic studies including mental retardation, congenital heart defects, lung hypoplasia, duodenal stenosis, pancreatic malformations, and inhibition of intrauterine growth (77,78). One of the most prominent features of the loss of STRA6 function in humans is anophthalmia, the absence of one or both eyes. The loss of retinoid uptake in the eye due to the knockdown of STRA6 has been shown in zebrafish models, as well as developmental defects due to the loss of STRA6 (79).

In mammals, STRA6 is expressed in a variety of embryonic and adult tissues. Mutations in STRA6 in humans cause the fatal Matthew-Wood syndrome, which is characterized by severe microphthalmia, pulmonary agenesis, bilateral diaphragmatic eventration, duodenal stenosis, and pancreatic malformations (78,80–82). An analysis of STRA6 in two human fetuses from consanguineous families with Matthew-Wood syndrome showed a homozygous insertion/deletion in exon 2 or a homozygous insertion in exon 7 predicting a premature stop codon in STRA6 transcripts (77). In a separate study, STRA6 molecular analysis was performed in three fetuses and one child diagnosed with Matthew-Wood syndrome and in three siblings affected with combinations of clinical anophthalmia, tetralogy of Fallot, and mental retardation. Among these individuals, six novel mutations in STRA6 were identified, bringing the current total of known mutations to seventeen (81). Loss-of function analysis in zebrafish embryos revealed that STRA6 deficiency caused vitamin A deprivation of the developing eyes and provokes nonspecific vitamin A excess in several embryonic tissues, thereby impairing normal retinoic acid receptor signaling and gene

regulation (79). However, reducing RBP levels largely alleviated these fatal consequences due to nonspecific retinoic acid excess in the absence of STRA6.

Retinoid Signaling in the Target Cell

Upon binding of the holo-RBP complex to STRA6 of target cell, STRA6 effectively mediates vitamin A uptake from the holo-RBP-TTR complex by catalyzing retinol release from holo-RBP (**Figure 3**) (20). Lecithin:retinol acyltransferase (LRAT) stimulates retinol uptake activity from STRA6 (83). Within the cytoplasm of the target cell, LRAT converts retinol to retinyl esters, the storage form of vitamin A. Retinol can also be stored by binding to cellular retinol binding proteins (CRBPs), such as CRBP-I and CRBP-II. In fact, CRBP-I also enhances retinol uptake from holo-RBP by STRA6, where as CRBP-II was much less effective at similar expression levels (83). The dependence of STRA6 on LRAT or CRBP-I for retinol uptake suggests that STRA6 is tightly coupled to these proteins. It has been hypothesized that retinol released by STRA6 inhibits further retinol release, but LRAT or CRBP-I relieves the inhibition by converting retinol to retinyl esters (83). STRA6 was also found to load retinol back onto RBP. LRAT and CRBP-I effectively prevents STRA6 catalyzed retinol loading and can enhance STRA6's retinol uptake (83). This mechanism offers control of vitamin A uptake and prevents toxicity due to excessive accumulation of free vitamin A and the potent activities of retinoids in regulating gene transcription during development and in adult tissues. Finally, apo-RBP dissociates from STRA6 and will be lost to kidney filtration.

Vitamin A helps maintain both the normal structure and functions of the epithelial cells (43). Retinoic Acid is needed for cellular differentiation by epithelial cells, found as part

of the skin and the respiratory, gastrointestinal, and urogenital tracts. Retinoic acid is also thought to act as a signal to switch on the genes for keratin proteins of keratinocytes into mature epidermal cells. For example, retinoic acid directs the differentiation of keratinocytes, immature skin cells, into mature epidermal cells (84,85). However, vitamin A deficiency causes keratin-producing cells to lose retinoic acid's transcriptional regulation and to be replaced with mucus-secreting cells in many body tissues.

The mechanism by which retinoic acid is thought to affect cell differentiation, at least in part, is due to its effects on gene expression. A series of events occur where the retinol is converted into retinoic acid and moved into the nucleus, where it can interact with the DNA. Once in the target cell, retinol is converted to ATRA and/or 9-cRA by alcohol dehydrogenase to retinaldehyde, then it is further converted to retinoic acid by retinaldehyde dehydrogenase. Retinoic acid is then transported into the nucleus bound to CRBP-I and presented to retinoic acid receptors. The two classes of receptors convey the activity of retinoic acid. The retinoic acid receptors (RARs) bind ATRA and 9-cRA and the retinoid X receptors (RXRs) binds solely 9-cRA. Liganded RXR homodimerizes or heterodimerizes with RAR and participates in tissue specific gene regulation by binding to specific DNA nucleotide sequences, referred to as retinoic acid response elements (RARE) in the promoter region of specific genes permitting the direct regulation of retinoid-dependent transcriptional responses (22,86,87). Both RAR and RXR have multiple isotypes that have been identified including α , β , and γ . RAR α , RAR β , and RAR γ are activated by both ATRA and 9-cRA, whereas RXR α , RXR β , and RXR γ have only been shown to be activated by 9-cRA (8,88–93). Furthermore, each isotype has been found to generate several isoforms with distinct regulatory and functional

properties, due to alternative splicing. RAR α and RAR γ have two major isoforms ($\alpha 1$, $\alpha 2$, $\gamma 1$ and $\gamma 2$), whereas RAR β has at least four forms ($\beta 1-4$) (94).

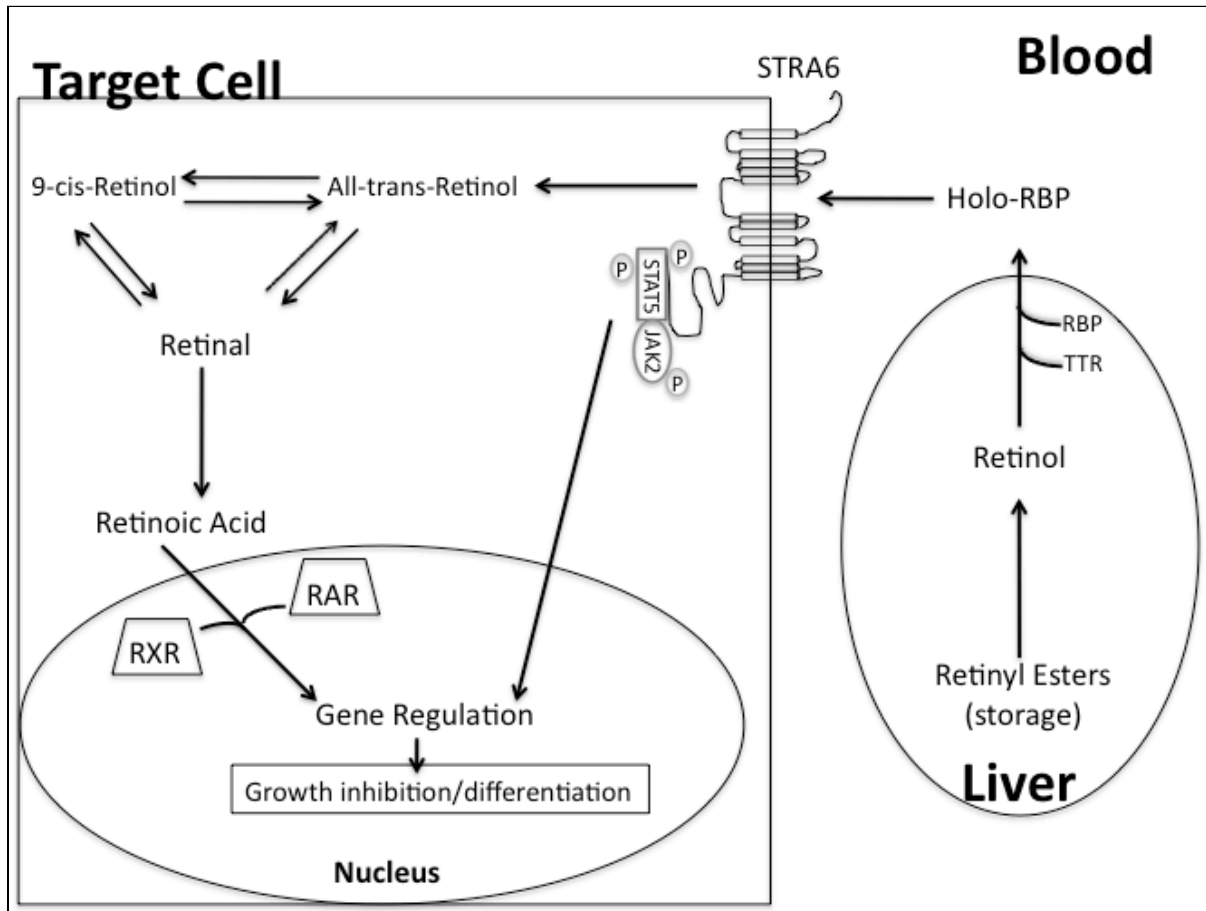


Figure 3. Vitamin A Transport and Activation.

When vitamin A stores are mobilized from the liver for use in peripheral tissues, retinyl esters are converted to retinol and bound by retinol binding protein (RBP). The holo-RBP complex is bound by transthyretin (TTR), then secreted into the plasma and delivered to target tissues. Uptake of retinol from RBP into the cell is mediated by STRA6. Once in the target cell, the retinol is activated to ATRA and/or 9-cRA, which is translocated into the nucleus, and then binds to retinoic acid receptors (RARs and RXRs). Liganded RAR dimerizes with RXR and participates in tissue specific gene regulation, specifically transcription of anti-proliferative proteins. Alternatively, the binding of holo-RBP to the extracellular moiety of STRA6 triggers tyrosine phosphorylation of the SH2 binding domain. Phosphorylated STRA6 recruits and activates Jak2, which, in turn, phosphorylates Stat5. Upon activation, Stat5 translocates to the nucleus to regulate the expression of target genes.

Retinoid signaling

Progression through the cell cycle is driven by complexes between cyclin dependent kinases (CDKs) and cyclins. In cancer cells, mutations leading to irregular cell proliferation, DNA instability, and chromosome instability accumulate through the uncontrolled regulation of CDK activities(95). Therefore, cyclins, CDKs, and factors that regulate CDKs are potential targets for inhibition of cancer cell proliferation.

Retinoids, in particular retinoic acid, have been shown to inhibit cell cycle progression in a variety of human cancer cells, including promyelocytic leukemia, lung squamous carcinoma, embryonal carcinoma, bronchial epithelial, and breast cancer cell lines, by directly or indirectly modulating cyclins, CDKs, and cell-cycle inhibitors (96–100). Retinoic acid, specifically, causes a block in the G1 phase of the cell cycle, with an increase in the proportion of cells in the G0/G1 phase and a decrease in the proportion of cells in the S phase (101).

Although there is some data suggesting that RAR α and RAR γ play a role in growth inhibition, RAR β has emerged as a critical mediator of the growth and proliferation-suppressing actions of retinoic acid (102). In fact, RAR β_2 expression is retinoic acid inducible and is lost early in the process of tumorigenesis in F9 mouse teratocarcinoma cells, as well as human hepatoma cells and human breast cancer cells (103–106). RAR β_2 , which is often lost or epigenetically silenced in human cancers, stimulates the induction of cell cycle inhibitors including p21 and p27 (105,107). More importantly, restoration of RAR β_2 gene expression reactivates the retinoic acid dependent growth control (108,109). In fact, ectopic

restoration of RAR β_2 expression in MCF7 and MDA-MB-231 breast cancer cell lines has been shown to restore the ability of retinoic acid to induce both growth arrest and apoptosis (110). During carcinogenesis, the loss of RAR β_2 expression leads to retinoid resistance and loss of retinoic acid induced growth arrest, suggesting that retinoic acid acts through RAR β_2 to mediate its effects on cellular proliferation and differentiation (103).

JAK/STAT Activation

Recently, STRA6 was shown to not only regulate uptake and reloading of retinol from RBP, but also act as a cell surface signaling receptor activated by holo-RBP binding (111). STRA6 contains an SH2 domain-binding motif in the receptor's cytosolic domain that can be activated through tyrosine phosphorylation after binding with the RBP-retinol complex (**Figure 3**). This activation leads to the recruitment and activation of Janus kinase 2 (Jak2) and Signal transducer and activator of transcription 5 (Stat5). Once activated, Stat5 is translocated to the nucleus where it regulates the expression of specific target genes related to proliferation and differentiation. Studies in mice support a role for Stat5 as a proto-oncogene in mammary tumors; however, the analysis of Stat5 in human breast malignancies suggests a role of Jak2/Stat5 pathway in the restriction of carcinogenesis (112).

When Jak2 or Stat5 are dysregulated, two important hallmarks of cancer are stimulated: evasion from apoptosis and self-sufficiency in growth signals (113). In fact, hyperactive Stat5 has been detected in a subset of human breast cancer cases, even though no genomic alterations in Jak2 and Stat5 were reported (114). Stat5a was found to be activated in a high proportion of breast cancers. Approximately 76% of human breast tumors displayed nuclear localized, tyrosine phosphorylated Stat5a (114).

This notion that hyperactive Jak2/Stat5 pathway leads to neoplastic formation is supported by studies in caveolin-1 (Cav-1) knockout mice (115–121). The loss of Cav-1 eliminates the inhibition of Jak2 signaling, resulting in hyperactivation of Stat5a and hyperplastic, well-differentiated mammary tumors. The hyperactivation of Stat5a was also associated with increased expression of ER α and Cyclin D1 (115,116,118,120). Recently, constitutively active and over expression of the wildtype Stat5 has been shown to promote the occurrence of sporadic mammary cancers in mice (122). Both animal models developed highly differentiated micropapillary and papillary adenocarcinomas after 8 to 12 months. However, Stat5 cannot be considered a potent proto-oncogene because only 10% of these transgenic mice exhibited mammary cancer within two years.

In a larger study investigating three separate clinical materials consisting of healthy breast specimens, as well as a progression series of primary lymph node-negative, primary lymph node-positive, and metastatic breast cancer totaling more than 1,100 breast specimens, immunohistochemistry was utilized to detect active, tyrosine-phosphorylated Stat5 (123). They found that Stat5 activation, but not Stat5 protein expression, was gradually lost during cancer progression, with detectable activation in 100% of healthy breast specimens compared with less than 20% of node-positive breast cancer and metastases. In fact, Stat5 activation in tumors was associated with favorable prognosis. These findings were further supported through work done by Yamashita and colleagues. They found in ER-positive breast cancer, Stat5 protein levels were associated with favorable prognosis and were also predictive of therapeutic response to antiestrogen therapy (124).

The current working hypothesis for the biological mechanism to explain the favorable prognostic effects associated with activated Stat5 is that it promotes differentiation and

inhibits invasive characteristics (125). This hypothesis has been supported by several independent reports that demonstrate a positive correlation between breast tumor histological differentiation and activation of Stat5 (114,124,126).

Stat5 may exert its effects on the Mitogen-activated protein kinase (MAPK) pathway. MAPKs are common intra-cellular signaling networks in response to cytokines or stress on the cell (127,128). The cAMP-stimulated MAPK pathway activates apoptosis, inflammation, and fibrosis in the kidney (128). Specifically, MAPK family members, c-Jun N-terminal kinase (JNK) and p38, are known to be involved in the regulation of apoptosis (127,128). JNK and p38 activation also repress RAR expression, leading to apoptosis. Jak2/Stat5 pathway has been shown to affect the JNK/p38 cascade in hepatocytes, macrophages, and adipocytes (129–131). Chen et al (132) showed that an increase in the ratio of serum apo-RBP to holo-RBP influenced the binding of RBP to STRA6. Interactions of holo-RBP with its receptor increased, leading to enhanced phosphorylation of Jak2 and Stat5 initiating an increase in the production of AC6-catalyzed cAMP. This, taken together, prompts apoptosis through the suppression of CRBP-I and RAR α and through the activation of JNK and p38.

Role of Vitamin A in Carcinogenesis

Given that vitamin A deficiency characteristically results in a failure of differentiation of epithelial cells without the impairment of proliferation, it is reasonable to question the possible role of vitamin A in the etiology of carcinomas. In fact, the metaplastic changes in squamous cells seen in vitamin A deficiency are morphologically similar to precancerous lesions induced experimentally. It has been proposed that retinoic acid can upregulate RARs in columnar and squamous epithelial cells of the skin, which can, in turn, complex with

proto-oncogenes, such as c-fos, to prevent malignant transformation (133). Studies with retinoic acid indicate that it could, to some extent, reduce several types of tumors in mammals (134–137).

Although the use of retinoic acid, which is rapidly degraded and eliminated from the body, avoids the problem of chronic hypervitaminosis, its substantial toxicity makes it unsuitable for regular clinical use. Therefore, more than 1,500 retinoids have been synthesized and tested for potential anti-carcinogenicity. Several have been found to effectively inhibit tumor growth and appearance in several organs of animals. These retinoids include 13-*cis*-retinoic acid, *N*-ethylretinamide, *N*-(2-hydroxyethyl)-retinamide, etretinate, and *N*-(2,3-dihydroxypropyl)-retinamide (138–142). Although some of these synthetic retinoids have yielded hopeful results in clinical trials, the consensus is that although retinoids currently available can delay tumorigenesis, they cannot do so at doses that are not toxic (143).

Epidemiological studies investigating the relationship between vitamin A intake and human cancer support the hypothesis that low vitamin A status may increase cancer risk, as well as suggest that individuals who consume three or more servings of fruits and vegetables each day, particularly those rich in carotenoids, have lower risks of several types of cancer in comparison with those who do not (144). These findings come from both case control and cohort studies. They have shown inverse associations between the consumption of the provitamin A, β -carotene, and the risk of lung and stomach cancer (145,146). However, these studies only indicate modest protective effect against breast and colon cancer, and such associations with prostate cancer have been inconsistent (147–149). Unfortunately, problems that often occur with epidemiological studies and may produce confounding variables

include inconsistent and often imprecise ways of reporting the dietary intake of vitamin A in free-living people, the difficulty in differentiating preformed vitamin A and carotenoids, and the difficulties in interpreting serum retinol concentrations.

Abnormal retinoid metabolism in cancer

The concentration of retinol is primarily regulated by STRA6, LRAT, and retinol ester hydrolase (REH) (19,93). Alterations in retinol metabolism, which occurs in many types of cancers, leads to lower retinol uptake and intracellular levels of retinol, a lower conversion of retinol to retinoic acid, and finally, a greater conversion of retinoic acid to its less biologically active metabolites ultimately resulting in vitamin A deficiency.

A reduction or loss of retinol esterification, the storage form of vitamin A in the cells, produces a reduction in total retinol accumulation and retinyl ester storage in the cells. An example of this is LRAT knockout mice, where the lack of esterification of retinol makes the mice more susceptible to vitamin A deficiency (150,151). LRAT is responsible for converting retinol to retinyl esters and also plays a role in the uptake of retinol through metabolism to the storage form of vitamin A. Various human cancers, both in cell lines and in tissue samples from tumors in patients, including cancers of the oral cavity, skin, bladder, kidney, prostate, and breast, have abnormally low intracellular retinyl ester levels and decreased LRAT expression (150,152–156). Therefore, low levels of LRAT expression results in lower concentrations of retinol in the cell due to a decrease in uptake of retinol from the holo-RBP complex and, in fact, this outcome was observed in renal cancer cells (153).

Once in the cell, retinol is first oxidized to retinal by retinol dehydrogenases (RDHs), and then retinal is metabolized further to retinoic acid by retinaldehyde dehydrogenases (ALDHs). ALDH1a2 is expressed at aberrantly low levels in many human prostate and breast cancers compared to their normal tissue counterparts, as well as in the TRAMP mouse model of prostate cancer (157–159). In fact, retinoic acid levels are high in normal colorectal cells, because RDH is highly expressed in normal epithelial cells. In normal cells, the protein adenomatous polyposis coli (APC) promotes the expression of RDH by degrading its transcriptional repressor, as well as degrading β -catenin. β -catenin can activate the cytochrome P450 enzyme, CYP26A1. Members of the cytochrome P450 family, including CYP26A1, can degrade retinoic acid to less biologically active metabolites. Therefore with the presence of normal APC function, CYP26A1 is present at low levels and degradation of retinoic acid into less biologically active metabolites is regulated. However, in tumorigenesis, APC function is lost and β -catenin accumulates. Enhanced activation of β -catenin pathways leads to enhanced expression of CYP26A1 and increased expression of RDH's transcriptional repressor. In fact, CYP26A1 has been shown to be highly expressed in human primary breast cancers, promoting cell survival and tumorigenesis (160). Also, the loss of RDH expression leads to a lower levels of retinoic acid in the cell due to degradation. Thus, impairment of both the synthesis and the metabolism of the bioactive metabolites of retinol can be observed in cancer cells relative to normal cells.

Additionally, the mechanism by which retinoid signaling pathway may become abnormal in carcinomas is through the silencing of RAR β_2 due to hypermethylation of the RAR β_2 gene and its promoter. The promoter is retinoic acid responsive in normal cells; however, in carcinoma cells the retinoid responsiveness is often lost (161). This observation

has been made in oral and epidermal human squamous cell carcinomas, head and neck squamous cell carcinoma, as well as breast, lung, and prostate cancer (161–165). The fact that the RAR β_2 is not mutated or deleted suggests that epigenetic events are taking place. Several studies have shown that RAR β_2 RARE is epigenetically silenced in many cancer types (108,166–169). RAR β_2 gene repression is due to a combination of local histone deacetylation and CpG island methylation resulting in resistance to the growth inhibitory effects of retinoic acid.

Naturally occurring derivatives of vitamin A, especially retinoic acid, are potent inhibitors of tumor growth in the mammary gland. Hence, developing dietary strategies that optimize tissue concentrations of vitamin A may be critical in reducing the incidence of cancer. We believe the first step toward this goal is to discover the mechanism by which vitamin A is transported into cells. This will assist in establishing how vitamin A levels can be modulated in target tissue, and how optimal vitamin A nutrition can reduce the incidence of cancer.

In our laboratory, I have examined the role of STRA6 in mediating cell cycle arrest and apoptosis by the addition of retinoic acid and the holo-RBP complex due, in part, at the transcriptional/translational levels of vitamin A tissue specific gene expression, specifically several cell cycle progression proteins. In the following chapters, original research is presented, as is a discussion based on my observations.

CHAPTER 2. METHODS

Cell Culture

The human colon adenocarcinoma cell line, Caco-2, the human mammary gland ductal carcinoma cell line, T-47D, the human prostate adenocarcinoma cell line, PC-3, and the human ovary adenocarcinoma cell line, OVCAR-3 were obtained from the American Type Culture Collection (Rockville, MD, USA). Mediatech cellgro* Minimum Essential Medium Eagle (MEM), RPMI 1640 Medium, and F12K Medium, Fetal Bovine Serum, and Penicillin Streptomycin Solution (5000 IU Penicillin, 5000 μ g/mL Streptomycin) were all purchased from Fisher Scientific (Pittsburgh, PA, USA). Plastic dishes, plates, and flasks were obtained from Corning (Corning, NY).

Cell Culture Treatments

Cells were plated in 6 well plates or in cell culture petri dishes at a cell density of 2×10^5 or 1×10^6 cells, respectively. After 48 hours of incubation at 37°C and 5% CO₂, the growth medium was replaced with fresh, serum-free medium containing all-*trans* retinoic acid at the final desired concentration (0, 0.1, 1, or 10 μ M) and cells were incubated for an additional 48 hours.

In a separate experiment, after 48 hours of incubation with retinoic acid, medium was replaced with serum-free medium containing 2 μ M holo-RBP complex and incubated for an additional 8 hours.

Crystal Violet Assay

Caco-2, PC-3, and T-47D were plated in 6 well plates at a cell density of 2×10^5 cells per well. Cells were treated as described above. The cell supernatant was aspirated and 1% glutaraldehyde was added to each well and incubated for 15 minutes at room temperature. The glutaraldehyde was discarded and the wells were blotted gently with a paper towel. In each well 0.1% crystal violet dye was added and incubated for 30 minutes at room temperature. The dye was discarded and the wells were again blotted gently with a paper towel. The plates were then placed in a container filled with running tap water and destained for several minutes. The plates were allowed to air-dry overnight. 0.2% Triton X-100 was added to each well and the plates were incubated for 30 minutes on a shaker at room temperature. From each well, Triton X-100 was transferred to a 96 well plate. Optical density was measured reading the absorbance of each well at 562nm. Three wells were measured for each treatment group.

mRNA Extraction and Reverse Transcriptase-Quantitative PCR

Cells were plated in 6 well plates at a cell density of 2×10^5 cells per well, and treated as described above. Recovery of total RNA was performed as described by the Trizol reagent (Invitrogen, Carlsbad, CA, USA) protocol. The RNA was quantified by optical density reading using a NanoDrop ND-1000 Spectrophotometer v.3.3.0 (NanoDrop products, Wilmington, DE, USA). Reverse transcription of 2 μ g of purified RNA was performed using iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). All primers were designed for use with the following thermal cycler parameters: 95°C for 3 min, followed by 40 cycles of 95°C for 15s, 60°C for 30s, and 72°C for 30s. The primer sequences for STRA6, CD3, CDK4,

p21, CDK2, CD1, and 18S are reported in Table 1. Reverse transcriptase-quantitative PCR reactions were performed on an iCycler using IQ SYBR Green Supermix (BioRad, Hercules, CA, USA). Data was normalized against 18S RNA.

Table 1. RT-qPCR Primer Sequences

Primer	Sequence
STRA6 Forward	5'-TGC CAC CTT TCT GAG ACC GTT TCT-3'
STRA6 Reverse	5'-ATG TGC CAG GTT CTA GGT GGT GAA-3'
CD3 Forward	5'-TGT CCA CAC ACG CAT TCA GAT – 3'
CD3 Reverse	5'-CTG GCA CCA AAC GAT CCC TT-3'
CDK4 Forward	5'-TTT GTG GCC CTC AAG AGT GTG AGA-3'
CDK4 Reverse	5'-AAA GCC TCC AGT CGC CTC AGT AAA-3'
p21 Forward	5'-TTA GCA GCG GAA CAA GGA GTC AGA-3'
p21 Reverse	5'-ACA CTA AGC ACT TCA GTG CCT CCA-3'
CDK2 Forward	5'-AGA TGG ACG GAG CTT GTT ATC GCA-3'
CDK2 Reverse	5'-TGG CTT GGT CAC ATC CTG GAA GAA-3'
CD1 Forward	5'-GGC TGG GAA TAT GGC CAA GCA TTT-3'
CD1 Reverse	5'-TGG GCA GGT GTC ACT GAG AAG ATT-3'
18S Forward	5'-CCA GAG CGA AAG CAT TTG CCA AGA-3'
18S Reverse	5'-AAT CAA CGC AAG CTT ATG ACC CGC-3'

Western Blots

Cells were plated in cell culture petri dishes at 1×10^6 cells per dish, and treated as described above. To harvest protein for analysis, media from cells was aspirated, and cells were rinsed once with sterilized 1x phosphate buffered saline (1x PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.47 mM KH_2PO_4). Lysis buffer containing 2x Laemlli buffer and protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) was added to each petri dish. The petri dishes were then scraped using a tissue culture cell scraper (Fisher Scientific, Pittsburgh, PA, USA). To determine protein concentration, a bicinchoninic acid (BCA) assay was conducted using BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) for each well against an albumin standard. Total cellular proteins were separated by electrophoresis using 10% SDS–polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane for western blot analyses. After application of Ponceau stain to determine loading lanes, the membranes were blocked with 5% skim milk in 1x PBS for one hour at room temperature, and then incubated with monoclonal antibody specific for p21 (1:200) (BD Pharmingen), CD1 (1:500), CD3 (1:100), CDK2 (1:400), CDK4 (1:500), STRA6 (1:100), or β -actin (1:1000) (abcam, Cambridge, MA, USA) overnight at 4°C. After washing three times with 0.1% Tween-1x PBS for 5 min, the membranes were incubated for 1 hour with anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (1:5000) or with anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (1:5000) (SouthernBiotech, Birmingham, AL, USA), accordingly. After washing the membranes with 0.1% Tween-1x PBS twice for 10 minutes and twice with 1x PBS for 5 minutes, the signal was detected using the SuperSignal West Dura Extended Duration Substrate or Supersignal West Femto Extended Duration Substrate (Thermo Scientific,

Rockford, IL, USA). Relative densities were determined using ImageJ software obtained from the National Institute of Health (Bethesda, MD, USA).

Fluorescent Microscopy Retinol Uptake Assay

Conjugating RBP with Alexa Fluor 488

500 μ L 1x PBS was added to Human Retinol Binding Protein crystals (Scripps Laboratories, San Diego, CA, USA) for a final concentration of 2 mg/ml, and conjugated with Alexa Fluor 488 following Invitrogen labeling kit protocol (Invitrogen, Eugene, OR, USA). The Alexa Fluor 488-RBP complex was saturated with molar excess of all-*trans* retinol (Sigma-Aldrich, St. Louis, MO, USA), and placed in a 37°C water bath for one hour. Column separation was used to purify the Alexa Fluor 488-holo-RBP following the Econo-Pac 10DG Columns Instruction Manual (Bio-Rad, Hercules, CA, USA).

RBP uptake assay

2×10^5 cells were plated in Lab-Tek II Chamber slide system (Nalge Nunc Internationals, Naperville, IL, USA) and incubated for 48 hours, and treated as described above. Media was aspirated and serum-free media containing 1 μ M Alexa-488-labeled RBP conjugated to retinol and incubated for 30 minutes. Media containing fluorescent probe was aspirated and ice-cold formaldehyde was added to each well, covered with aluminum foil, and incubated at room temperature for 6 minutes. The formaldehyde was removed and the slides were washed five times with 1x PBS. Wells were then removed from the slides, and slides were mounted using Prolong Gold Anti-fade with DAPI (Invitrogen, Eugene, OR, USA). Fluorescent images were captured on an Olympus AX70 microscope with a Spot RT digital camera.

Radioactivity Retinol Uptake Assay

Conjugating RBP with ³H-retinol

500 μ L 1x PBS was added to Human Retinol Binding Protein crystals for a final concentration of 2 mg/ml, saturated with [11,12-³H(N)]-retinol (PerkinElmer, Wiltham, MA, USA), and placed in a 37°C water bath for one hour. Column separation was used to purify the holo-RBP following Econo-Pac 10DG Columns Instruction Manual.

Retinol Uptake assay

Cells were plated in 6 well plates at 2×10^5 cells per well and incubated and treated as described above. Media was aspirated from wells and fresh media containing 1μ M ³H-retinol-RBP was added to each well and incubated for one hour. Media containing ³H-retinol-RBP was removed and wells were washed twice with ice cold 1x PBS. Lysis buffer (40 mM Tris pH 7.4, 120 mM NaCl, 0.5% Triton X-100, 0.3% 1x SDS, and 3x dH₂O) was added to the wells and placed on a shaker for 15 minutes. Lysate was transferred into scintillation vials containing 5ml of Scintiverse™ BD Cocktail (Fisher Scientific, Pittsburgh, PA, USA). Cellular lysate levels of radioactivity were measured in disintegrations per minute (DPM) using Packard 1900 TR Liquid Scintillation Analyzer.

Statistics

Data was analyzed using one-way analysis of variance (ANOVA), followed by multiple comparison tests (Dunnett or Tukey as appropriate), two-way ANOVA, or linear regression with Graph Pad Prism software version 3.0 for Windows (GraphPad Software,

San Diego, California USA). Differences between means and linear relationships were considered significant with p values < 0.05 were obtained.

CHAPTER 3. RESULTS

Retinoic acid has been shown to enhance the expression of STRA6 in F9 cells (67). To ascertain the effects of retinoic acid on T-47D, PC-3, Caco-2, and OVCAR-3 cell lines, we treated these cells with 0, 0.1, 1, or 10 μM concentrations of all-*trans* retinoic acid. We utilized uptake assays, including radioactivity and fluorescent microscopy uptake assays, to determine the effect of retinoic acid treatment on retinol uptake from the holo-RBP complex, as well as RT-qPCR and western blot analysis to determine the effect retinoic acid had on gene and protein expression. Finally, we utilized the crystal violet assay to determine retinoic acid's effect on cell viability.

Retinol uptake over time

To determine the extent of retinol uptake from holo-RBP over time, we treated cells with a tritiated retinol (^3H -retinol) bound to RBP as described above. T-47D, Caco-2, and PC-3 cells were grown in 6-well plates until confluent, and then treated with the ^3H -retinol-RBP complex. The plates were harvested for cellular lysate at 0, 5, 15, 30, 60, 90, and 120 minutes (**Figure 4**). Our results showed a significant linear relationship between retinol uptake from the holo-RBP complex and time in both T-47D and PC-3 cell lines, where the correlation coefficient was 0.9859 and 0.9327 (p values for non-zero slope were <0.0001 and 0.0022, respectively) over a two-hour time course. However, Caco-2 cells did not have a significant correlation between retinol uptake and time and the slope was not significantly non-zero.

Effects of Retinoic Acid

STRA6 protein expression

To determine the effect retinoic acid treatment had on STRA6 expression, PC-3 and Caco-2 cells were treated with all-*trans* retinoic acid (0, .1, 1, or 10 μ M) in media for 48 hours, and then harvested for protein as described above. Our western blot analysis shows that STRA6 expression, in both cell lines, increased with increasing concentration of retinoic acid treatment (**Figure 5**).

Expression of cell cycle progression proteins

To determine the effects all-*trans* retinoic acid had on the expression of several other cell cycle progression proteins, we treated PC-3 and Caco-2 cells with 0, 0.1, 1, or 10 μ M retinoic acid. Our western blot analysis shows a clear inhibition of the expression of CDK2, CDK4, CD1, and CD3 at the 10 μ M retinoic acid treatment for both PC-3 and Caco-2 (**Figure 6**). We also found that CDK2 in both cell lines and CD3 in Caco-2 cells were inhibited at the 1 μ M RA treatment. Conversely, the expression of the cyclin dependent kinase inhibitor, p21, was increased in both cell lines. Unfortunately, we were unable to fully elucidate CD1 and CDK4 protein expression in Caco-2 cell line at this time.

Change in gene expression

To quantify gene expression of STRA6 and several cell cycle progression proteins after treatment with retinoic acid, we determined the abundance of specific mRNA molecules utilizing reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). In Caco-2 cells, we saw an increase in the expression of STRA6 gene with increasing concentrations of

retinoic acid treatment, however this was not significant (**Figure 7A**). However, CDK2 and p21 expression stayed relatively the same throughout the treatment groups.

In OVCAR-3 cells, we saw a similar increase in STRA6 expression with increasing concentrations of retinoic acid treatment. In fact, STRA6 expression was significantly different in 1 μ M and 10 μ M concentrations compared to the control group (**Figure 7B**). However, CDK2, CDK4, p21, and CD1 expressions did not change throughout the retinoic acid treatments compared to the control group (**Figure 7C**).

We saw a similar, however non-significant, increase in STRA6 gene expression with increasing concentrations of retinoic acid in PC-3 cells (**Figure 7D**). CDK2 gene expression was significantly lower in 1 μ M retinoic acid treatment group compared to the control group. However, once again, CDK4, p21, and CD1 had no significant change in gene expression with retinoic acid treatment (**Figure 7E**).

In T-47D cells, we saw an increase in STRA6 expression with increasing concentrations of retinoic acid (**Figure 7F**). The 1 μ M retinoic acid treatment group was significantly different from the control group; however, the 10 μ M group was not significantly different from the control group. Although there was no significant difference seen in CD1, CDK2, and p21 gene expression, we did see a significant decreasing trend in CDK4 gene expression with increasing concentrations of retinoic acid (**Figure 7G**).

Cell viability

To determine cell viability, we utilized crystal violet assay using PC-3, T-47D, and Caco-2 cells lines treated with 0, .1, 1, or 10 μ M retinoic acid. Our results indicated a decrease in cell viability with increasing concentrations of retinoic acid in PC-3 cells (**Figure**

8). As expected, PC-3 cells treated with 10 μ M retinoic acid were significantly different than the control group. However, we did not see a significant change in viability in T47-D and Caco-2 cells treated with retinoic acid compared to controls.

Retinol uptake

To determine the extent of retinol uptake from the holo-RBP complex, we treated PC-3, Caco-2, and T-47D cells with ^3H -retinol bound to RBP after treatment with 0, 1, or 10 μ M all-*trans* retinoic acid and quantified the disintegrations per minute (dpm) using a scintillation counter. Our results show that PC-3 and T-47D cell lines increased the uptake of ^3H -retinol from the holo-RBP complex with increased concentrations of retinoic acid (**Figure 9A**). PC-3 cells treated with 10 μ M RA had a significantly higher uptake of ^3H -retinol compared to the control group and T-47D cells treated with 1 and 10 μ M RA had significantly higher ^3H -retinol uptake compared to the control group. However, we did not see a significant change in ^3H -retinol uptake in Caco-2 cells with RA treatment.

We also utilized fluorescent microscopy to determine retinol uptake from the holo-RBP complex. We treated PC-3, Caco-2, and T-47D cells with Alexa Fluor-488 conjugated holo-RBP complex after treatment with 0 or 1 μ M retinoic acid. Our results show that RBP bound to the cell surface increased with retinoic acid treatment in all cell lines (**Figure 9B**).

Effects of Holo-RBP

Holo-RBP complex has recently been shown to phosphorylate the SH2 binding domain on STRA6, leading to phosphorylation and activation of the Jak2/Stat5 pathway (170). This complex has also been shown to mediate apoptosis through the enhancement of Jak2/Stat5 cascade by up-regulating adenylate cyclase 6, increasing cAMP, enhancing the

JNK/p38 cascade, and suppressing the CRBP-I/RAR α expression in HK-2 and human umbilical vein endothelial cells (132). To determine the effect of the holo-RBP complex on the expression of several cell cycle progression proteins in T-47D, PC-3, and Caco-2 cell, we treated these cell lines with the holo-RBP complex in the presence or absence of 1 μ M RA. To determine holo-RBP's effects on STRA6 gene and protein expression, as well as cell cycle progression proteins expression, we utilized RT-qPCR and western blot analysis. Finally, we utilized the crystal violet assay to determine holo-RBP effect on cell viability.

STRA6 expression

We utilized western blot analysis to determine the expression of STRA6 in PC-3 and Caco-2 cells treated holo-RBP complex in the presence or absence of 1 μ M RA. STRA6 expression was inhibited in groups treated with holo-RBP, with or without retinoic acid treatment in PC-3 cells. In fact, expression levels are completely inhibited when treated with both retinoic acid and holo-RBP. However, we were unable to obtain data for Caco-2 cells due to poor antibody signaling and background noise (**Figure 10**).

Effects of holo-RBP on protein expression

We saw similar results for the expression levels of cell cycle progression proteins CD1, CDK2, and CDK4, as well as the cyclin-dependent kinase inhibitor, p21. When PC-3 cells were treated with holo-RBP, regardless of the retinoic acid treatment, protein expression levels were inhibited (**Figure 10**). CDK4 and p21 were fully inhibited when PC-3 cells were treated with both retinoic acid and holo-RBP complex. In PC-3 and Caco-2 cells, CDK2's expression levels were fully inhibited by holo-RBP treatment, with or without retinoic acid

treatment. In both cell lines, CD1 expression levels, as well as CDK4 levels in Caco-2 cells, were down regulated by the addition of the holo-RBP complex.

Change in gene expression

To quantify the change in gene expression for the cell cycle progression proteins, as well as for STRA6 and p21, we utilized RT-qPCR for PC-3 and T-47D cells were treated with the holo-RBP complex, with or without 1 μ M all-*trans* retinoic acid treatment. Expression levels for CDK2 and CDK4 significantly decreased with holo-RBP complex treatment, regardless of retinoic acid treatment (**Figure 11A and 11B**). CD1, in both cell lines, and p21 expression levels in PC-3 cells decreased with the addition of holo-RBP, however these results were not significant (**Figure 11C, 11D, and 11E**, respectively). Interestingly, STRA6 expression levels increased with the addition of holo-RBP in both PC-3 and T47-D cells, however these results are not significant (**Figure 11F and 11G**) For all proteins, in PC3 cells, there was no interaction between the retinoic acid treatment and the holo-RBP treatment. However, we did see an interaction between retinoic acid and holo-RBP in Caco-2 cells with STRA6 mRNA abundance ($p = 0.0032$).

Effects on cell viability

To determine cell viability, we utilized crystal violet assay with PC-3, Caco-2, and T-47D cells treated with holo-RBP complex in the presence or absence of retinoic acid. For all cell lines, cell viability was significantly disrupted with the addition of the holo-RBP complex, with or without retinoic acid treatment (**Figure 12**).

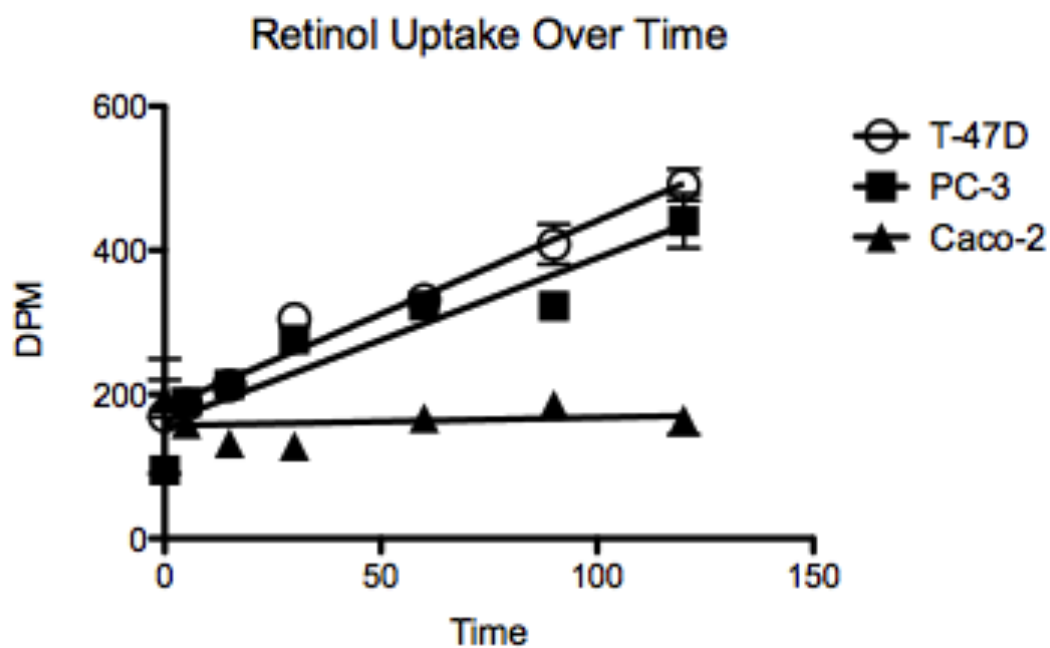
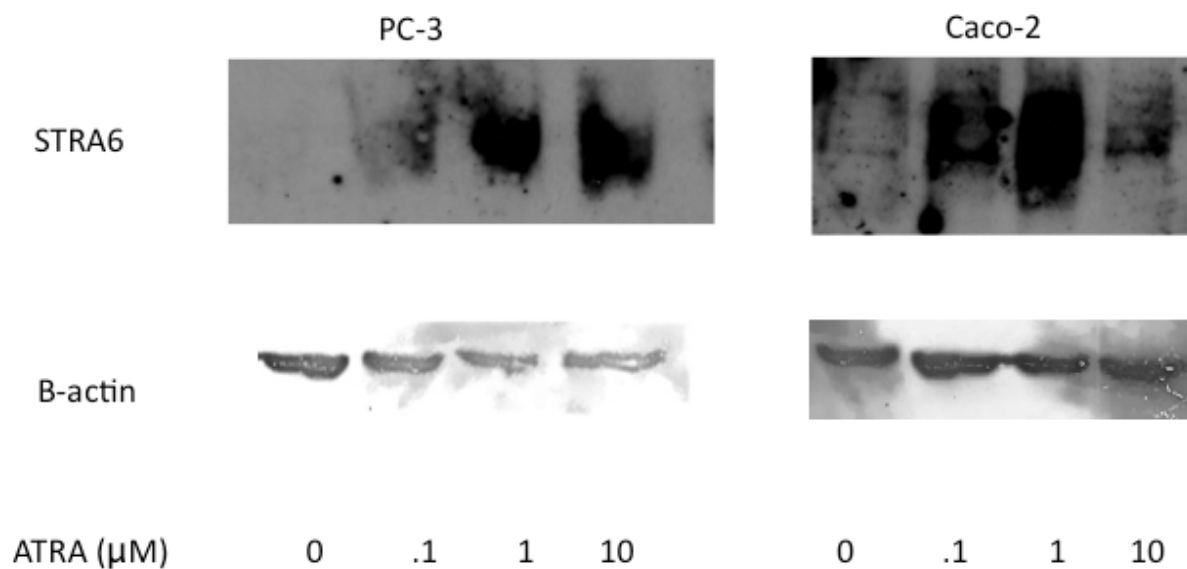


Figure 4. Retinol Uptake Over Time.

T-47D, PC-3, and Caco-2 cells were treated with ^3H -retinol bound to RBP. Cells were harvested at 0, 5, 15, 30, 60, 90, and 120 minutes time points. Retinol uptake was determined in disintegrations per minute (DPM) using a scintillation counter. T-47D and PC-3 cells had a significant linear regression (p value < 0.01), whereas Caco-2 did not have a significant correlation between time and retinol uptake. Figure shows means with SEM and linear regression ($n=3$).

A.



B.

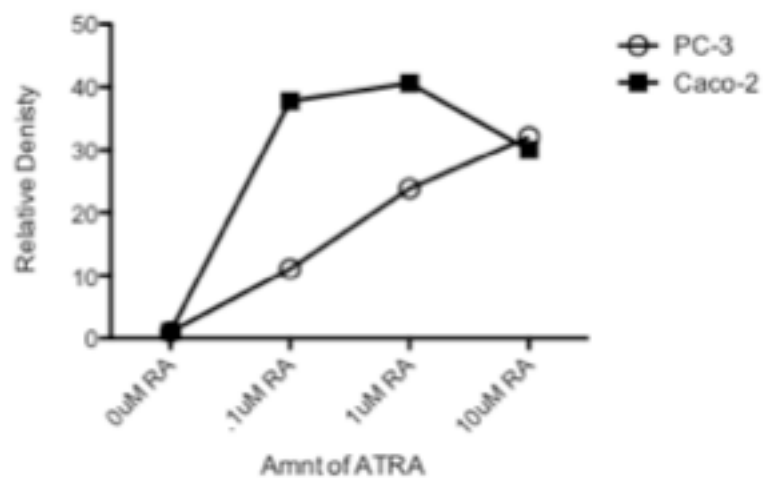
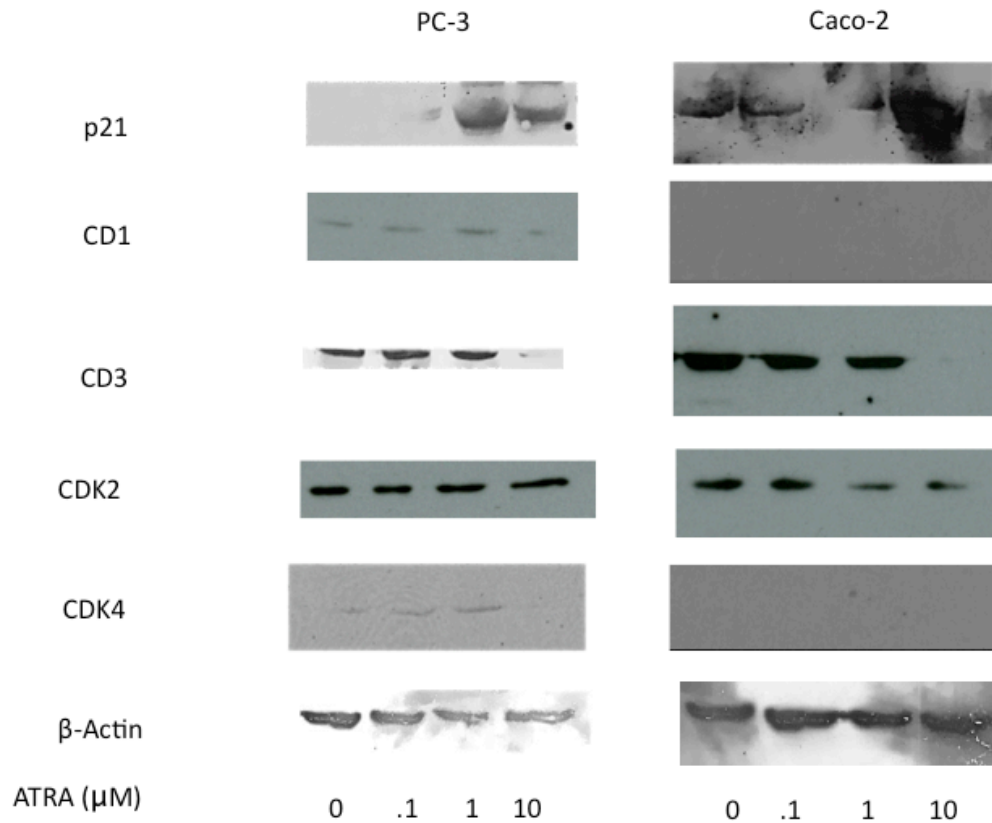


Figure 5. Effects of Retinoic Acid on STRA6 Expression

PC-3 and Caco-2 cells were treated with 0, .1, 1, or 10 μM retinoic acid. Western blot analysis was utilized (A) and quantified (B) using ImageJ software to determine protein expression.

A.



B.

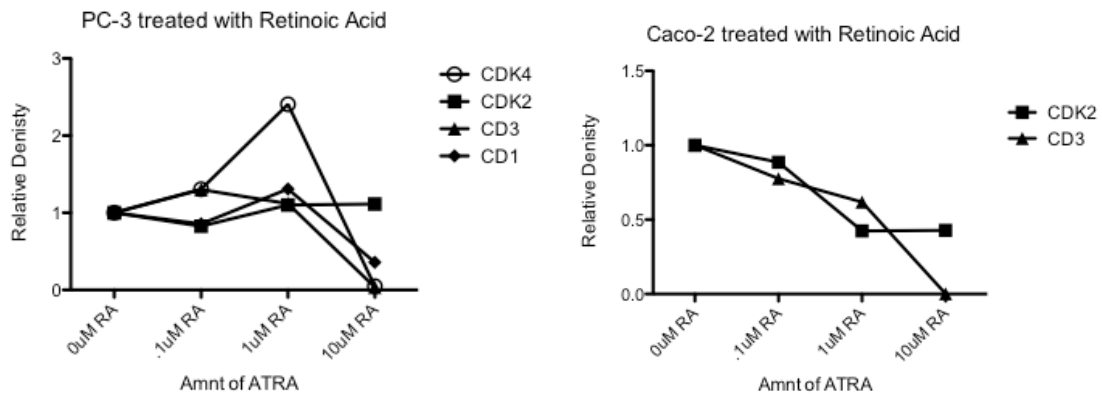
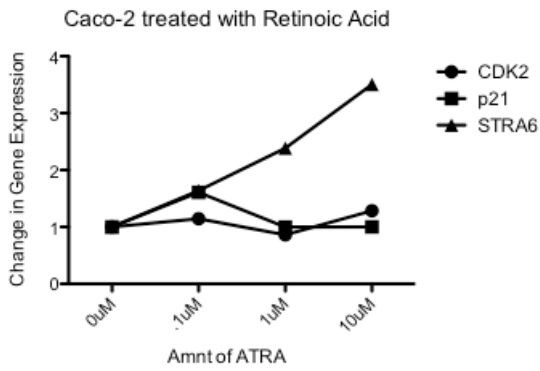


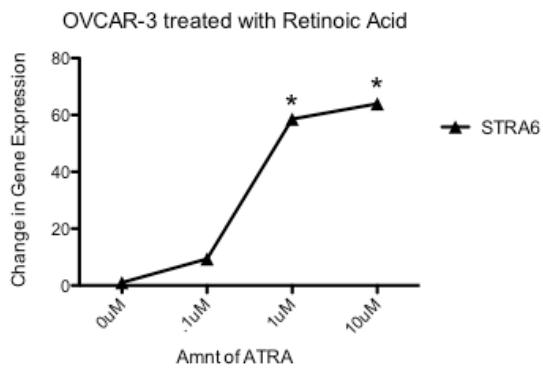
Figure 6. Effects of Retinoic Acid on Cell Cycle Progression Protein Expression

PC-3 and Caco-2 cells were treated with 0, 0.1, 1, or 10 μM retinoic acid. Western blot analysis was utilized (A) and quantified (B) using ImageJ software to determine protein expression.

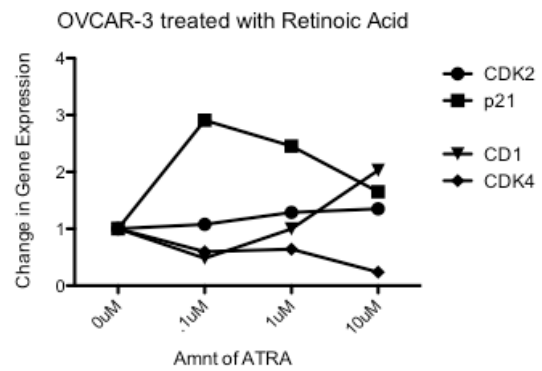
A.



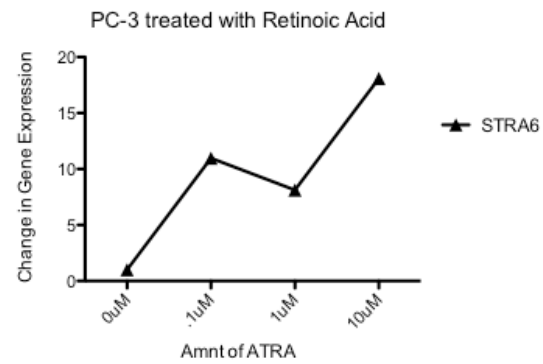
B.



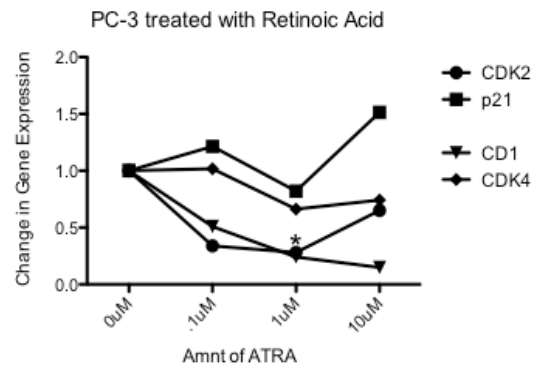
C.



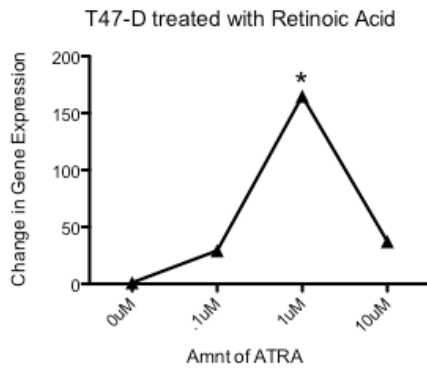
D.



E.



F.



G.

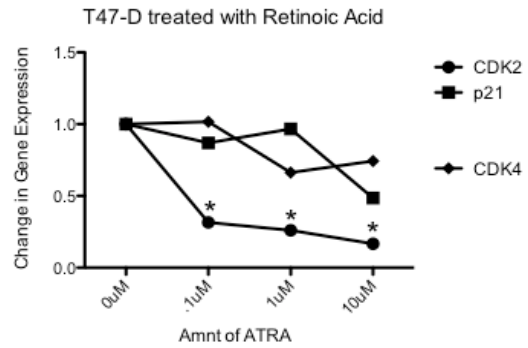


Figure 7. Effects of Retinoic Acid on Gene Expression Levels

PC-3, Caco-2, OVCAR-3, and T-47D cells were treated with 0, 0.1, 1, or 10µM retinoic acid. Total mRNA was quantified using RT-qPCR methods and normalized against 18S expression levels. (* = $p < 0.05$ compared to 0µM RA) (A) Caco-2 gene expression, (B and C) OVCAR-3 gene expression, (D and E) PC-3 gene expression, and (F and G) T47-D gene expression after treatment with all-*trans* retinoic acid.

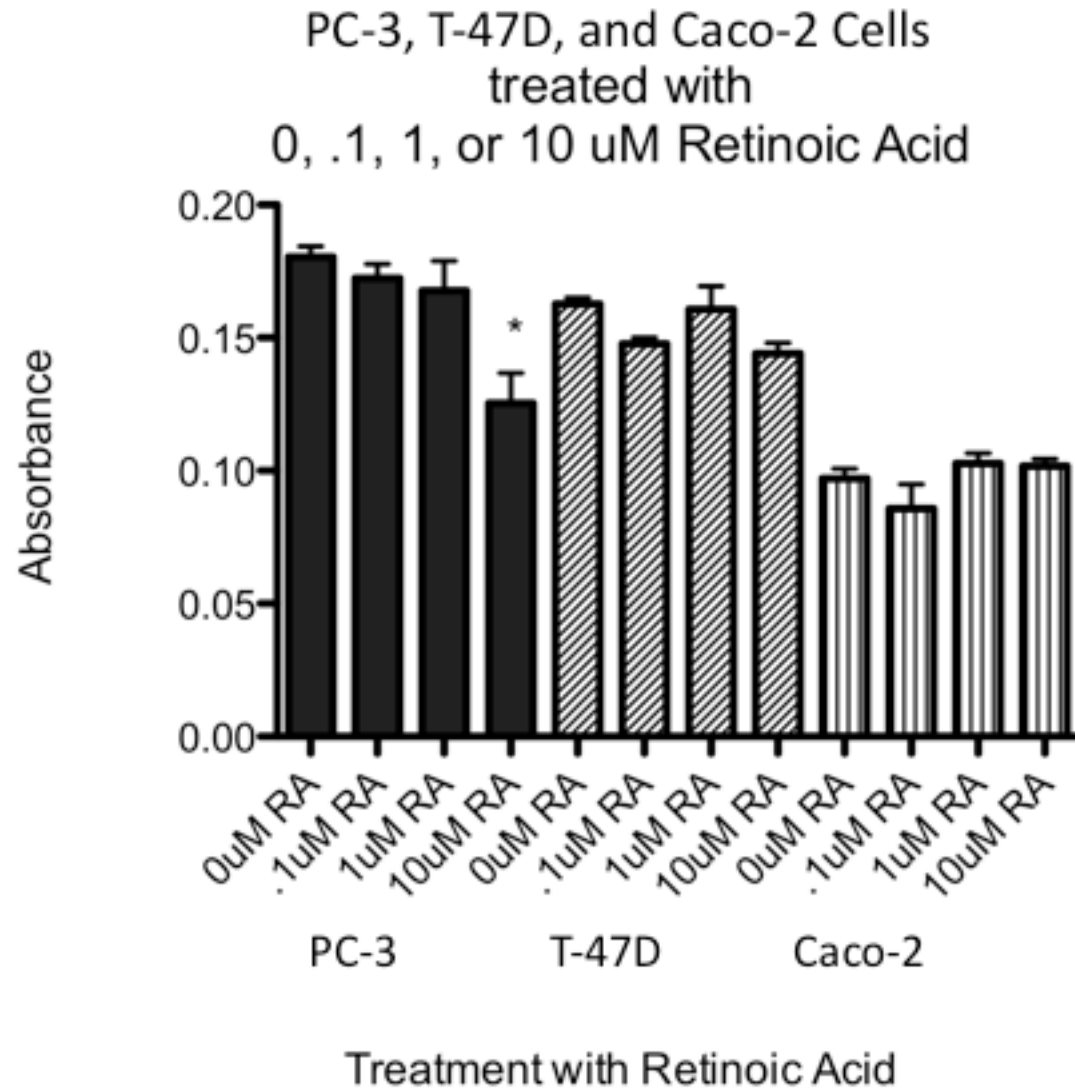
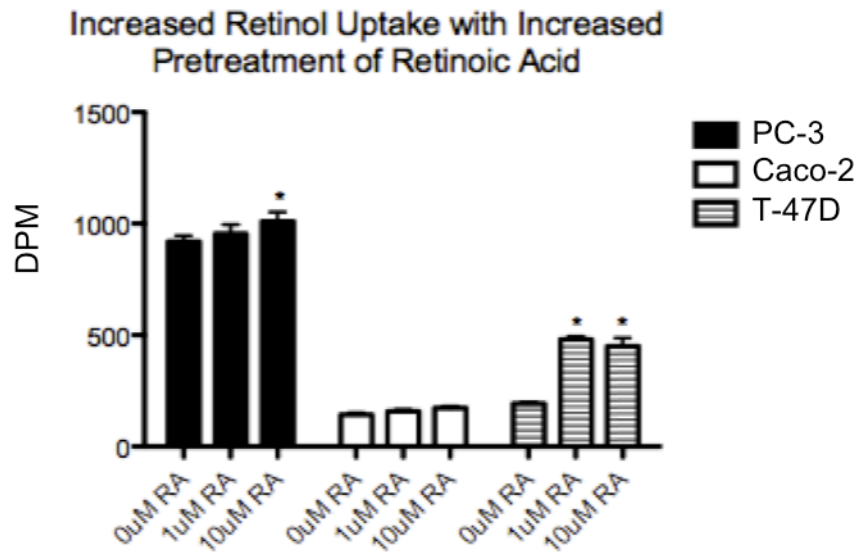


Figure 8. Effects of Retinoic Acid on Cell Viability

PC-3, T-47D, and Caco-2 cells were treated with 0, 0.1, 1, or 10 μ M retinoic acid. Cell viability was measured using crystal violet methods. (* = $p < 0.05$ compared to 0 μ M RA)

A.



B.

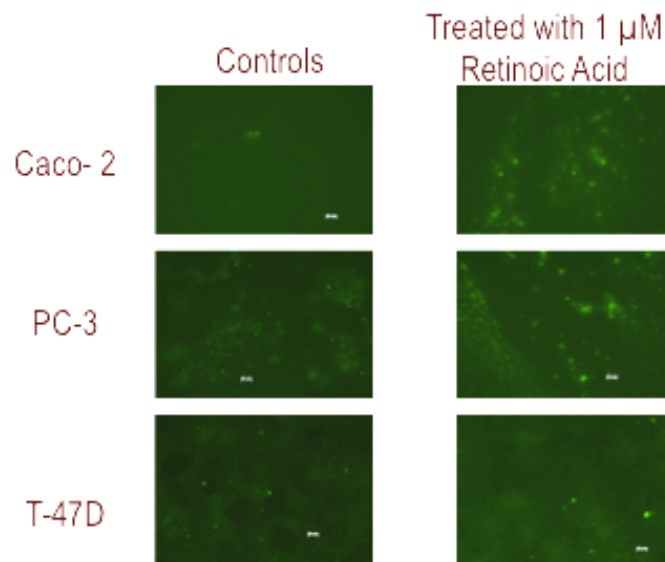
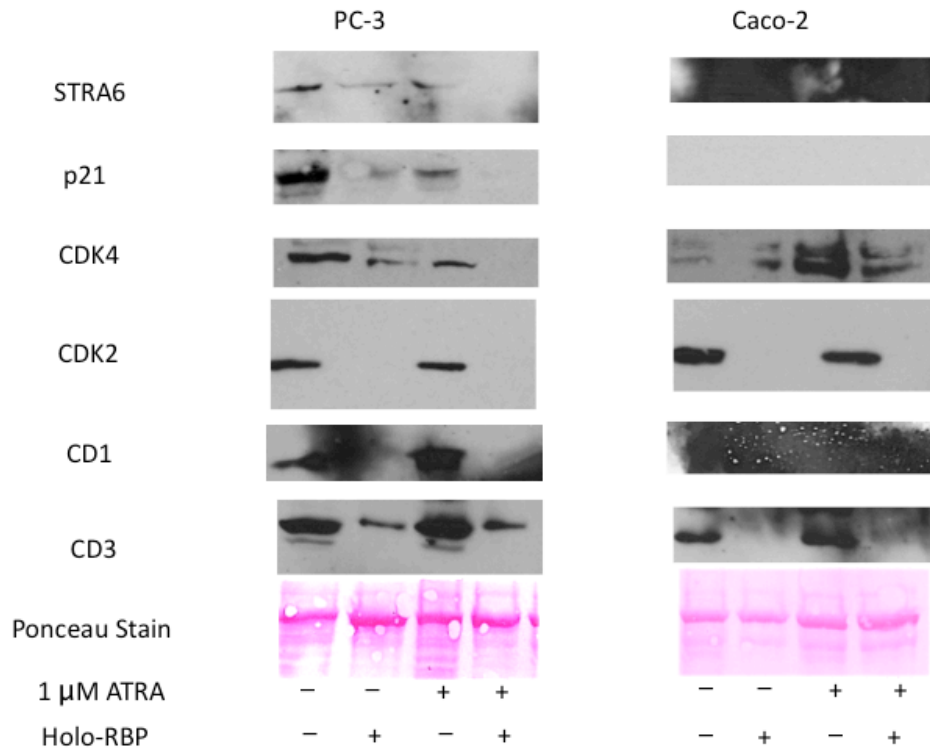


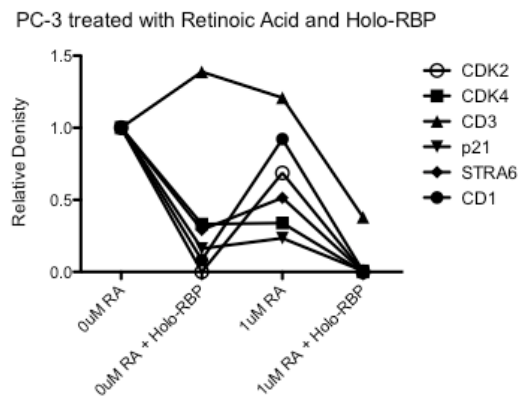
Figure 9. Effects of Retinoic Acid on Retinol Uptake

PC-3, Caco-2, and T-47D cells were treated with 0, 0.1, 1, or 10 μM retinoic acid. Retinol uptake from the holo-RBP complex was determined using a scintillation counter to measure the amount of ³H-retinol uptake (A) and fluorescent microscopy to measure the amount of Alexa-488-holo-RBP bound to the surface (B). (* = $p < 0.05$ compared to 0 μM RA)

A.



B.



C.

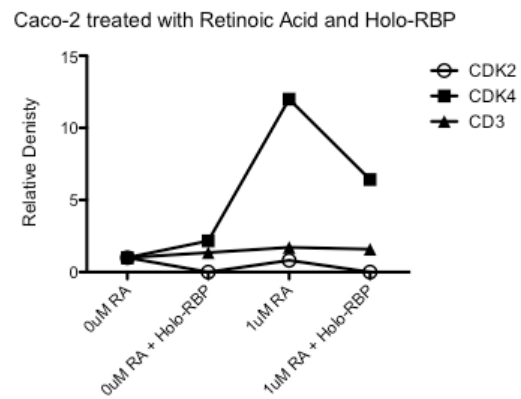
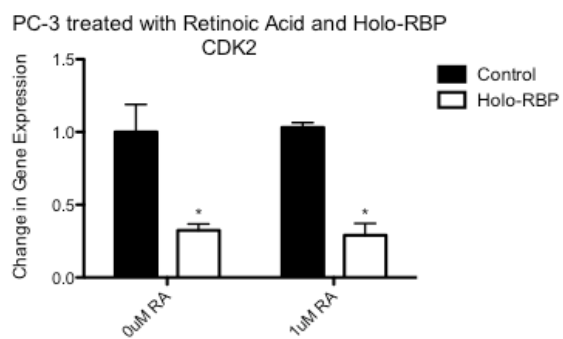


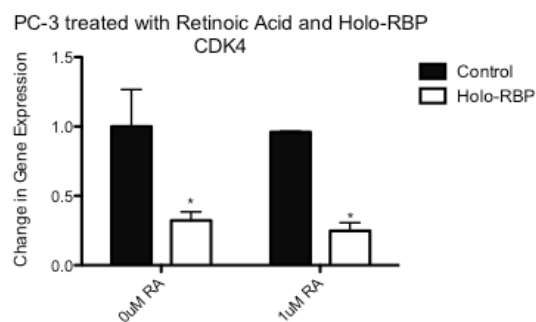
Figure 10. Effects of Holo-RBP on Protein Expression

PC-3 and Caco-2 cells were treated with 0 or 1 μM retinoic acid with or without 2 μM holo-RBP. Western blot analysis was utilized (A) and quantified (B) using ImageJ software to determine protein expression.

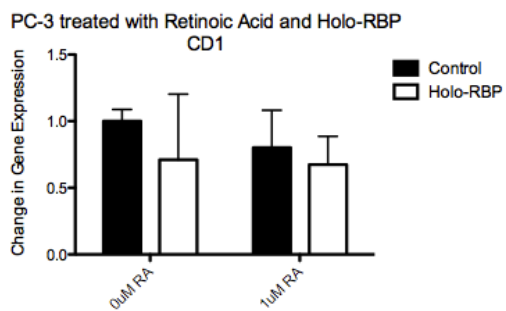
A.



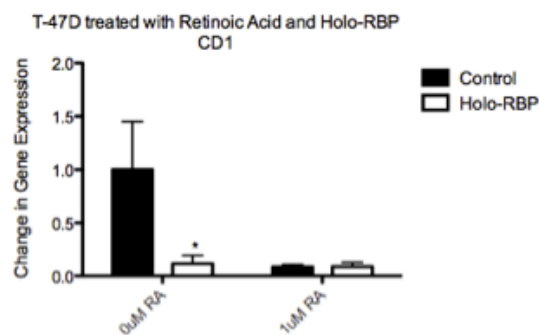
B.



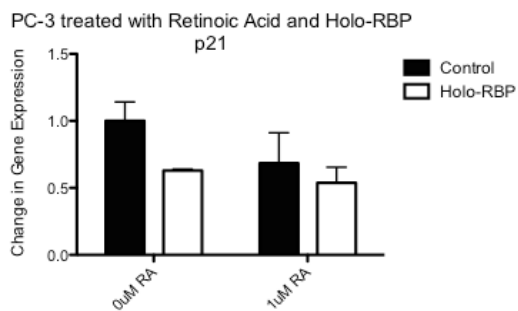
C.



D.



E.



F.

G.

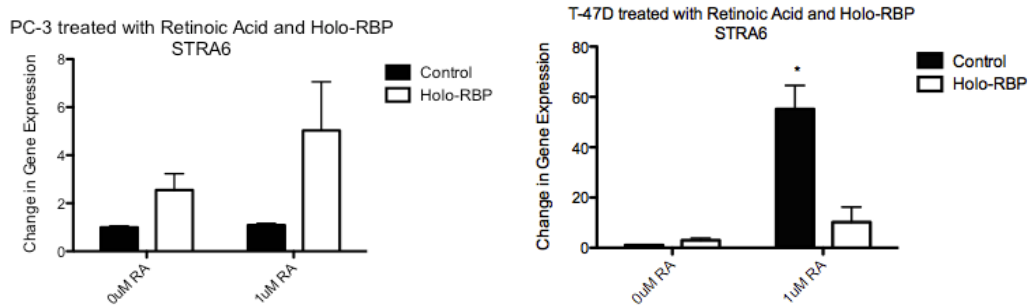


Figure 11. Effects of Holo-RBP on Gene Expression Levels

PC-3 and T-47D cells were treated with 0 or 1 μ M all-*trans* retinoic acid with or without 2 μ M holo-RBP. Total mRNA was quantified using RT-qPCR methods and normalized against 18S expression levels. (* = $p < 0.05$ compared to 0 μ M RA) (A) PC-3 CDK2 gene expression, (B) PC-3 CDK4 expression, (C) PC-3 CD1 expression, (D) T-47D CD1 expression, (E) PC-3 p21 expression, (F) PC-3 STRA6 expression, and (G) T-47D STRA6 expression.

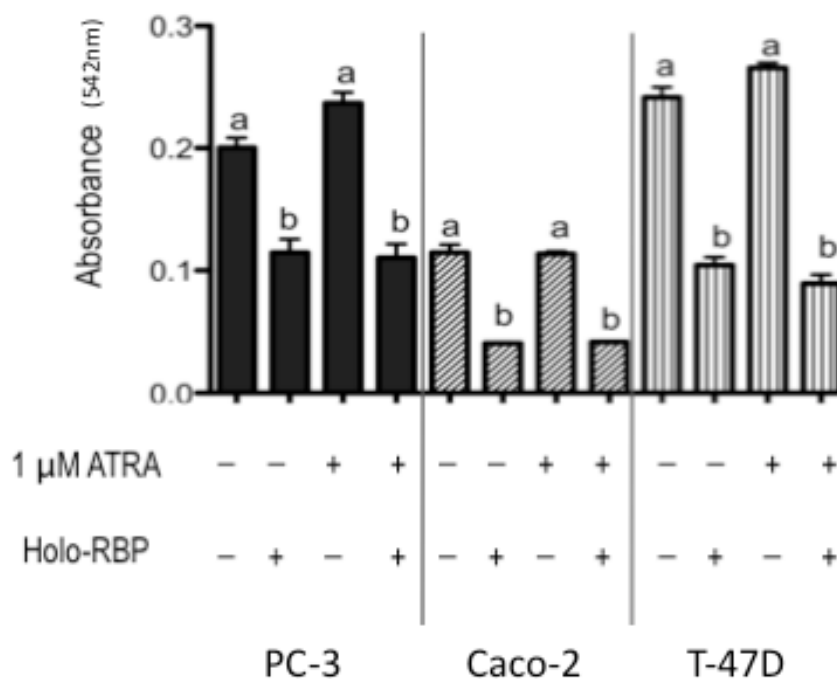


Figure 12. Effects of Holo-RBP on Cell Viability

PC-3, T-47D, and Caco-2 cells were treated with 0 or 1 μ M retinoic acid and 2 μ M holo-RBP. Cell viability was measured using crystal violet methods. (* = $p < 0.05$ compared to 0 μ M RA)

CHAPTER 4. DISCUSSION AND CONCLUSION

STRA6 is expressed in various tissues, including the eye, brain, kidney, reproductive systems, skins, lungs, and heart (56,68–73). Kawaguchi et al. confirmed that STRA6 transports retinol into the cell from the holo-RBP complex and that its mechanism of uptake is not dependant on endocytosis (20). Recently, it was also shown that the binding of holo-RBP complex to STRA6 can induce phosphorylation at the SH2 binding domain on STRA6, leading to the activation of Jak2/Stat5 signaling cascade (170).

STRA6 was initially isolated as a retinoic acid-inducible gene in P19 embryonal carcinoma cells (171), as well as in HCT116 and WiDr colon adenocarcinoma cells (67). In the current study, we confirmed that STRA6 protein expression is inducible by ATRA treatment in pancreatic and colon carcinoma cell lines. We also show that STRA6 mRNA expression increases with ATRA treatment in PC-3, Caco-2, T-47D, and OVCAR-3 cell lines (8, 2, 165, and 59 fold increase in STRA6 mRNA expression levels after treatment with 1 μ M ATRA treatment, respectively).

This increase in STRA6 expression would allow for increased binding of the holo-RBP complex allowing for increased 1:1 molar binding of the two constituents, as well as increased uptake of retinol into these cells by STRA6. In fact, our results show that retinol uptake from the holo-RBP complex is not only time dependent, but also increases with ATRA treatment in PC-3 and T-47D cells.

It has been previously shown that a novel retinoid, AHPN, was able to induce p21 expression, as well as inhibit cyclin dependent kinases, CDK2 and CDK4 in several lung

carcinoma cell lines (172). All-*trans* retinoic acid was also shown to promote degradation of cyclin D1 protein through ubiquitin-mediated proteasomal targeting in human bronchial epithelial cells and human Ntera2 teratocarcinoma cells (173–175). In addition, all-*trans* retinoic acid was found to inhibit cyclin D1 and cyclin D3 expression levels and inhibit the expression levels of CDK2 and CDK4 in MCF-7, ZR-75, and T-47D breast carcinoma cells (173,176). 9-*cis*-retinoic acid was found to increase the amount of the CDK inhibitor, p21 protein in MKN-7 cell and U937 cells (177,178). In our current study, we hypothesized that ATRA would stimulate the expression of p21, the cyclin kinase inhibitor, as well as a decrease in the expression of several cell cycle progression proteins, CDK2, CDK4, CD1, and CD3. Our results show a down regulation of the cell cycle progression proteins in PC-3 and Caco-2 cells, as well as an increase in the expression of p21 with retinoic acid treatment. However, our results for the change in gene expression using RT-qPCR analysis for the cell cycle progression proteins did not significantly change with the addition of retinoic acid at any concentration, with the exception of CDK4 in T-47D and PC-3 cells. Our results are similar to several studies, finding that although retinoic acid does not alter cyclin D1 mRNA expression, it does increase the rate of ubiquitin-mediated proteasomal targeting of cyclin D1 (173–175). However, more information is needed to conclude that these proteins are in fact being ubiquitinated at a higher rate with the treatment of retinoic acid in our experimental model.

Cell viability decreased slightly with the addition of ATRA in PC-3, Caco-2, and T-47D cells and was only significantly decreased at the highest treatment level in T-47D cells. However, treatment with holo-RBP caused significant cell death and down regulation of all the proteins we examined. Chen and his colleagues recently showed that an increase in the

ratio of apo- to holo-RBP may influence the binding activity with STRA6 in HK-2 and human umbilical vein endothelial cells (132). The increased ratio triggered Jak2/Stat5 signaling, which sequentially activated AC6-catalyzed cAMP/PKA/JNK/p38 pathway to suppress CRBP-I and RAR α expression, ultimately causing apoptosis in renal and endothelial cells. Although our results are inconclusive, we predict that the apoptosis we saw in PC-3, Caco-2, and T-47D cells after treatment with holo-RBP may be in part due to this cascade of events and not due solely to increased uptake of retinol from the holo-RBP complex.

The identification of the novel signaling cascade mediated by holo-RBP complex, STRA6, Jak2, and Stat5 by Berry, Noy, and their colleagues establish that STRA6 is not only a vitamin A transporter but also a cell surface signaling receptor (111,170). However, little is known whether these two functions are inter-related, and whether STRA6 signaling modulates STRA6-mediated retinol uptake and if this uptake is necessary for signaling. In our current study, we examined the interactions between holo-RBP and all-*trans* retinoic acid. In PC-3 cell, we did not find an interaction and the apoptotic effects seem to be attributed to the holo-RBP treatment and not retinoic acid. In T-47D cells, we did see an interaction for STRA6 mRNA expression ($p=0.0032$). However, the mechanism for interaction needs further investigation.

Cytokine receptors frequently communicate with multiple signaling cascades. Although STRA6 has been shown to activate a Jak2/Stat5 pathway, it is possible that the receptor could function via other cascades. However, signaling through multiple pathways generated by STRA6's SH2 binding domain remains to be clarified.

Additionally, the involvement of holo-RBP and STRA6 in other biological functions remains to be investigated. Although available data demonstrates that STRA6 regulates the expression of genes involved in insulin responses and lipid homeostasis (111,170), the pathway must also control the expression of other, tissue- and cell-specific genes. Stat5 activation has been shown to promote cell cycle progression, angiogenesis, and survival (179,180). The observations that the expression of STRA6 is upregulated in several cancers and that holo-RBP induced signaling activates Stat5 suggests that the newly found cascade may play a role in cancer development (67). However, the exact role that STRA6 plays in tumor initiation and growth remains to be elucidated.

Conversely, an increase of apo-RBP/holo-RBP concentration may influence the binding of RBP to STRA6, enhance Jak2 and Stat5 phosphorylation, as well as increase AC6-catalyzed camp production leading to apoptosis through suppression of CRBP I and RAR α and activation of JNK and p38 (132). Our results indicate that the addition of the holo-RBP complex leads to apoptosis. However, the mechanism of apoptosis needs further investigation.

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