THE CELL CYCLE REGULATORS, B-CELL TRANSLOCATION
GENE 2 AND CYCLIN D1, ARE TARGETS FOR RETINOIC ACID
RECEPTOR SIGNALING IN HUMAN MAMMARY CARCINOMA
CELLS

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# ABSTRACT

The vitamin A metabolite, retinoic acid (RA), displays pleiotrophic activities by virtue of its ability to activate the ligand-inducible transcription factors known as retinoic acid receptors (RAR), thereby regulating the transcription of multiple target genes. In addition to RAR, RA binds in cells to cellular retinoic acid binding proteins (CRABP-I and CRABP-II), and it has been established that CRABP-II enhances the transcriptional activity of RAR by directly delivering RA from the cytosol to the receptor in the nucleus. Among its other biological activities, RA displays a growth inhibitory effect in a variety of cancer cells. Growth inhibition by this compound may be mediated by three different cellular processes: apoptosis, cell cycle arrest, and differentiation. It has been reported that, in human mammary carcinoma cells, RA induces growth inhibition by triggering apoptosis, cell cycle arrest, or both. The present study was undertaken to identify target genes responsible for RA-induced, RAR-mediated cell cycle arrest in MCF-7 mammary carcinoma cells, and to examine the contribution of CRABP-II to this process. Upon RA treatment, MCF-7 cells were arrested in $G_1$ phase. Affymetrix expression array data revealed that RA upregulates the expression of several genes that control cell cycle progression, including B-cell translocation gene, member 2 (BTG2). In accordance with the role of CRABP-II in enhancing RAR transactivation, overexpression of CRABP-II augmented RA-upregulation of the BTG2 transcript level. BTG2 was found to contain a functional RAR response element 3,250 bp upstream of the transcription start site. Previous studies reported that BTG2 represses transcription of

cyclin D1, a protein that enables $G_1$ to S transition. Indeed, additional experiments showed that RA reduced cyclin D1 expression by 50%. Taken together, the observations suggest that RA induces cell cycle arrest in MCF-7 by directly inducing BTG2 expression. In turn, BTG2 functions to reduce cyclin D1 expression, leading to cell cycle arrest in the $G_1$ phase.

# BIOGRAPHICAL SKETCH

Jean H. Suh was born in Seoul, South Korea, on March 27, 1980 as the younger daughter of Suk C. Suh and Namjin Suh. She immigrated to the United States of America in 1995 with her parents and her older sister, Jane A. Suh, and obtained her U.S. citizenship in 2000. She took up residence in Bridgewater, New Jersey, and received her high school diploma in 1999 from Bridgewater-Raritan Regional High School. She subsequently matriculated at Cornell University to pursue her undergraduate studies in biology. In May 2003, she received a Bachelor of Arts degree *cum laude* in Biological Sciences with double concentrations in Cell & Molecular Biology and Animal Physiology. Shortly thereafter, she entered the Graduate School of Cornell University to pursue further research with her undergraduate honors thesis advisor, Dr. Noa Noy. She is a candidate for a Master of Science degree in Nutritional Biochemistry expected to be conferred in January 2006. Currently, she resides in Watchung, New Jersey, and is a full-time student in the combined MD/PhD program offered through Robert Wood Johnson Medical School, Rutgers University, and Princeton University.

To my family, my mentor, and my Jim

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# CHAPTER ONE
# INTRODUCTION

Retinoic acid (RA) is an active metabolite of vitamin A introduced to the body through diet. Vitamin A in the form of retinyl ester (RE) is stored in liver, forming the biggest vitamin A pool (50-80%) of the organism. RE stored in the liver becomes hydrolyzed into retinol (ROH). Subsequently, retinol-binding protein (RBP) binds ROH intrahepatically for release into the bloodstream. ROH is further coupled to transthyretin (TTR) to avoid renal clearance. The uptake of ROH by target cells may occur via endocytosis through a receptor-independent mechanism of lipid membranes (Biesalski and Nohr, 2004). The majority of cellular retinol is bound by cellular retinol-binding protein (CRBP), a protein that is believed to shuttle retinol to various enzymes that catalyze its metabolism (Spinella and Dmitrovsky, 2000). In cells, ROH can be reesterified into RE for storage in the liver and extrahepatic tissues. ROH can also become oxidized into RA, an active metabolite that serves as a ligand for retinoic acid receptor (RAR) to regulate its target gene transcription. In addition to RAR, two other proteins, termed cellular retinoic acid binding proteins (CRABP-I and CRABP-II) are involved in regulating the biological activities of RA. CRABP-II cooperates with RAR in mediating the transcriptional activities of RA. This protein binds free RA in the cytoplasm and translocates it to the nucleus to channel RA directly to RAR (Budhu and Noy, 2002). CRABP-I, on the other hand, has been implicated in shuttling RA to P450 enzymes, specifically CYP26, for its degradation (Won et al., 2004).

## 1-1 NUCLEAR HORMONE RECEPTORS

The superfamily of nuclear hormone receptors is a group of ligand-inducible transcription factors, which includes receptors for small hydrophobic ligands such as steroids, thyroid hormones, and retinoid receptors, as well as orphan receptors with unknown ligands. Based on evolutionary analysis, the receptors are divided into six different subfamilies (Aranda and Pascual, 2001). Class I includes thyroid hormone receptor (TR), vitamin D receptor (VDR), peroxisome proliferator activated receptor (PPAR), retinoic acid receptor (RAR) and orphan receptors. The second subfamily contains retinoid X receptor (RXR) among others. Class III represents receptors for classic steroid hormones: estrogen, androgen, progesterone, and glucocorticoids. Lastly, the fourth, fifth, and sixth subfamilies contain different orphan receptors.

All nuclear hormone receptors share a common structure, which is divided into five domains: the most variable amino-terminal transactivation region, a highly conserved DNA-binding domain (DBD), a hinge region, a ligand-binding domain (LBD), and a carboxyl-terminal region (Figure 1-1). Each domain confers specific functions in target gene transcription (Bastien and Rochette-Egly, 2004). The amino-terminal transactivation region is involved in ligand-independent transcriptional regulation. The DNA-binding domain binds a specific receptor to particular response elements in the promoters of the target genes. Lastly, the ligand-binding domain at the carboxyl-terminal region is involved in ligand binding, dimerization, and interactions with transcriptional co-activators.

| A/B | C | D | E | F |

**Transactivation Region**      **Hinge Region**      **C-terminal Region**

**DNA-Binding Domain**      **Ligand-Binding Domain**
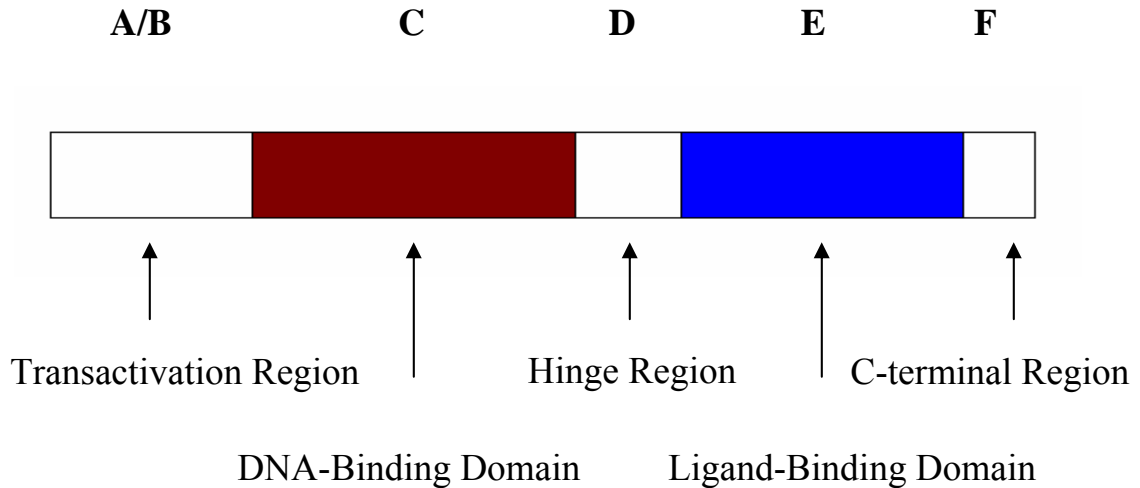
**Figure 1-1 Nuclear Hormone Receptor Structure.** All nuclear hormone receptors share a common structure, which is divided into five domains: a highly variable amino-terminal transactivation region, a well-conserved DNA-binding domain (DBD), a hinge-region, a ligand-binding domain (LBD), and a carboxyl-terminal region.

Nuclear hormone receptors are direct regulators of transcription that function by binding to specific DNA sequences, named hormone response elements (HREs), in the promoters of target genes. These response elements typically consist of two hexameric motifs, PuG(G/T)TCA, spaced by different numbers of nucleotides in different orientations which include direct repeats, inverted repeats, and everted repeats (Leid et al., 1992). Transcriptional activation by these receptors requires either a homodimerization or a heterodimerization (Aranda and Pascual, 2001). The class III receptors bind to their specific HREs as homodimers while the class I receptors bind to their HREs as heterodimers with a common dimerization partner, the retinoid X receptor (RXR). In the absence of their ligands, class III receptors associate with heat-shock proteins and remain in the cytoplasm. Only upon binding of

ligands, these receptors dissociate from the heat-shock proteins and translocate into the nucleus. The activated receptor homodimers then bind to their HREs to recruit a complex of co-activators. In turn, these coactivators modify chromatin and stabilize the preinitiation complex to initiate transcription (Aranda and Pascual, 2001).

In contrast, class I receptors bind to their HREs as heterodimers. In the absence of ligands, receptor dimers recruit co-repressors containing histone deacetylase activity, which silences transcription by promoting a closed chromatin structure. Upon binding of the ligands, the receptors undergo conformational changes resulting in dissociation of the co-repressors and association of co-activators containing histone acetyltransferase activity, which facilitates transcription by opening the chromatin structure. In addition, co-activators stabilize the preinitiation complex which, in turn, binds to RNA polymerase II to initiate transcription of target genes (Aranda and Pascual, 2001).

## 1-2    RETINOIC ACID RECEPTORS

The term "retinoids" is used to describe active natural and synthetic derivatives of vitamin A. They serve as ligands for the retinoic acid receptors (RAR) to regulate target gene transcription. There are three separate genes encoding three isotypes of retinoic acid receptor: RARα, RARβ, and RARγ. For each receptor isotype, there exist at least two isoforms generated by alternative splicing and differential promoter usage (Freemantle et al., 2003). RARs display an expression pattern that is both developmentally and tissue specific, allowing for the pleiotropic effects of retinoids, which include key roles in embryonic development, postnatal

development, and in adult life, growth, reproduction, and homeostasis in diverse tissues (Freemantle et al., 2003).

RAR functions as a heterodimer with RXR. The RAR/RXR heterodimer binds to the specific DNA sequences referred to as retinoic acid response elements (RAREs). RAREs are typically composed of two direct repeats of the hexameric motif PuG(G/T)TCA, separated by either 2 (DR-2) or 5 (DR-5) basepairs (Leid et al., 1992). RAREs have been identified in the promoters of a large number of retinoid-target genes (Bastien and Rochette-Egly, 2004). For example, the classical DR-5 was found in the promoters of RARβ, CYP26, and several Hox and HNF genes. DR-2 elements were identified in the CRABPI and CRABPII promoters (Smith et al., 1991; Durand et al., 1992).

As a class I receptor, RAR in its heterodimer form with RXR binds to DNA and regulates transcription through its association with coactivators and repressors depending on the status of its ligand availability. Though both RAR and RXR have the ability to recruit coactivators on their own, it has been shown that RXR is subordinate to its RAR partner in RAR/RXR heterodimers (Roy et al., 1995). This phenomenon has been attributed to the inability of RXR to dissociate corepressors unless its partner is also liganded. Therefore, in the presence of both RAR and RXR ligands, there is a synergy in transcriptional activation resulting from the RAR agonist-induced dissociation of corepressors and the subsequent binding of coactivators by both partners (Bastien and Rochette-Egly, 2004).

Once the chromatin decondenses, coactivators dissociate or become degraded by the proteasome, thereby allowing retinoid receptors to recruit

the transcription machinery via SMCC (Srb and Mediator protein containing complex) mediator complex (Malik and Roeder, 2000). The mediator then facilitates the formation of preinitiation complex consisting of RNA polymerase II and the general transcription factors to begin transcription of target genes (Bastien and Rochette-Egly, 2004). The ubiquitin-protease system presents another level of control in RAR/RXR transactivation. In response to appropriate signals, the substrates such as coactivators and the receptors themselves undergo multi-ubiquitination, which then become targeted for destruction by the proteasome. The system therefore provides a mechanism not only to shut off the RAR/RXR activation, but also to modulate the magnitude and the duration of retinoid-mediated transcription (Bastien and Rochette-Egly, 2004).

## 1-3    CELLULAR RETINOIC ACID BINDING PROTEINS

Cellular retinoic acid-binding proteins (CRABPs) are small intracellular lipid-binding proteins of approximately 15 kDa in size, which bind RA with high affinity and selectivity (Noy, 2000). There are two CRABPs, designated as CRABP I and CRABP II. The two CRABPs display distinct expression profiles across tissues and development stages despite a high degree of homology between them. This suggests that they do in fact serve their own unique roles in mediating retinoid functions (Manor et al., 2003).

The specific function of CRABP I in the context of retinoids remains to be elucidated. However, it can be inferred from previous studies that it may function in concert with cytochrome P450 enzyme,

CYP26, to send RA towards its degradation pathway, thereby decreasing the intracellular RA concentration. The physiological consequences of decreased RA have been studied in the retinoic acid-sensitive human squamous cell carcinoma cell line HTB35 by ectopic overexpression of CRABP I (Blaese et al., 2003). The study demonstrated that overexpression of CRABP I induced a retinoic acid-insensitive phenotype resistant to combined treatment of retinoic acid and radiation in contrast to the cells expressing a basal level of CRABP I (Blaese et al., 2003). A similar study was performed by another group in a head and neck squamous cell carcinoma (HNSCC) cell line and demonstrated that the expression of the CRABP I resulted in a lower sensitivity to RA treatment in comparison with the control in a clonogenic assay (Won et al., 2004). Moreover, it showed an increased level of polar metabolites of RA in thin-layer chromatography, suggesting RA metabolism. Several groups showed in different cell lines that CRABP I expression either decreased or did not enhance RAR transactivation (Won et al., 2004; Venepally et al., 1996). Despite much effort, no direct evidence showing that CRABP I expression indeed decreases intracellular RA concentration has been reported. Moreover, the mechanism of how CRABP I delivers RA to CYP26, if it does, remains to be investigated.

On the other hand, the role of CRABP II in context of RAR activation has been studied extensively. First, it has been shown that CRABP II expression significantly enhances RAR-mediated transcriptional activation of a reporter gene in COS-7 cells (Dong et al., 1999). The study also demonstrated that despite similar binding affinity of RA to both CRABP I and CRABP II, functional differences exist between

the two binding proteins due to the direct physical interaction between CRABP II and RAR. This direct interaction allows RA to be channeled from CRABP II to RAR and facilitates the formation of holo-RAR, thereby enhancing the transcriptional activity of RAR in cells (Budhu et al., 2001). The region of CRABP II that mediates this interaction with RAR has been localized to three spatially-aligned amino acid residues, Gln75, Pro81, and Lys102, around the ligand-binding pocket of the protein (Budhu et al., 2001). The three residues were identified through comparison between CRABP I and CRABP II which revealed a sole region displaying a dramatic change in electrostatic surface potential. Moreover, it has been shown that conferring these three key residues onto CRABP I resulted in its ability to channel RA to RAR. Most recently, CRABP II has been shown to contain a ligand-responsive nuclear localization signal (NLS) in its tertiary structure, which was not apparent in its primary sequence (Sessler and Noy, 2004), further supporting the idea that CRABP II travels to the nucleus upon ligand binding to channel RA to RAR in order to enhance the transcriptional activity of RAR.

In accordance of the ability of CRABP II to channel RA to RAR, CRABP II has been shown to enhance retinoid function through RAR. The functional consequences of CRABP II therefore lie in its ability to sensitize cells to retinoic acid. CRABP II overexpression in MCF-7 mammary carcinoma cells has been shown to dramatically increase their sensitivity to retinoic acid-induced growth arrest (Budhu and Noy, 2002). An *in vivo* study was performed using immunodeficient mice, whereby the injection of mammary carcinoma SC115 cells stably expressing CRABP II was shown to suppress their tumorogenicity compared to the

control (Manor et al., 2003). In a similar fashion, the injection of an adenovirus expressing CRABP II into mammary carcinomas that spontaneously develop in TgN(*MMTV*$_{neu}$)202Mul mice exhibited a slowed progression in tumor growth and prolonged survival rates (Manor et al., 2003). Taken together, these studies demonstrate that CRABP II sensitizes cells to RA both *in vitro* and *in vivo* for RAR activation, thereby resulting in enhanced RAR target gene transcription.

## 1-4 RAR/RXR HETERODIMER AND ITS IMPLICATIONS IN CANCER

Retinoid-mediated activation of the RAR/RXR heterodimer and subsequent activation of target gene transcription play critical roles in the development and homeostasis of virtually all vertebrate tissues. The regulatory effects of RAR are accomplished through modulating cell differentiation, proliferation, and apoptosis.

Inhibition of cell growth results from a number of different cellular processes, including cell differentiation, which is accompanied by a cell cycle exit. When cells exit the cell cycle and enter the stage known as $G_0$, they no longer divide. Instead, they may undergo differentiation to serve their intended purposes within the organism. In some instances, cells receive signals to re-enter the cell cycle and proliferate. Cancer cells, however, are characterized by inappropriate cell division even in the presence of differentiation signals. They lack the ability to sense these signals in order to exit the cell cycle and differentiate, and continue to go through cell cycle repeatedly. A better understanding of the mechanism

by which cells decide to enter or exit the cell cycle would provide significant benefits to human cancer research.

Another mechanism through which cells can accomplish growth inhibition is by controlling the cell cycle (Vermeulen et al., 2003; Levine, 1997). The cell cycle is divided into three phases consisting of $G_1$, S, and $G_2$/M, with $G_0$ accounting for non-cycling cells. $G_1$ is a phase in which cells commit to DNA replication in S phase before the cell division that occurs in $G_2$/M phase. There exist many checkpoints within the cell cycle. In response to DNA damage, checkpoints serve to arrest the cell cycle in order to provide time for DNA repair. DNA damage checkpoints are positioned before the cell enters S phase ($G_1$ to S checkpoint) or after DNA replication ($G_2$ to M checkpoint). Further, there appear to be other checkpoints during S and M phases. These checkpoints involve different proteins that regulate the cell cycle such as cyclins, cyclin-dependent kinases and their inhibitors, and tumor suppressor proteins (p53 and pRb). These proteins have been shown to contain inactivating mutations in human cancers. The cell cycle, therefore, presents a complex array of regulating processes which could serve as a point of interest for studying mechanisms of cancer, the unrestrained proliferation of cells.

Lastly, cells can accomplish growth inhibition simply by undergoing programmed cell death, also called apoptosis. Apoptosis is a specific type of cell death, which describes, in a sequential manner, shrinkage of the cell, hypercondensation of chromatin, cleavage of chromosomes into nucleosomes, violent blebbing of the plasma membrane, and packaging of cellular contents into membrane-enclosed vesicles called 'apoptotic bodies' (Shi, 2001). Apoptosis is executed by a

family of cysteine proteases that cleave their substrates after an aspartate residue. The cleavage activates the substrates which then go on to ultimately cleave a number of cellular targets leading to cell death (Shi, 2001). The significance of apoptosis recently gained attention as an important contributor to cancer.

In most RA-sensitive tumor cells, RA causes inhibition of cell growth, resulting from different combinations of the three cellular processes discussed above: cell differentiation, cell cycle arrest, and apoptosis (Strickland and Mahdavi, 1978; Battle et al., 2001; Breitman et al., 1980; Altucci et al., 2001). In mammary carcinoma, the focus of this study, RA has been shown to mediate its growth inhibitory effect through cell cycle arrest, apoptosis, or both (Elstner et al., 1998; Toma et al., 1998; Mangiarotti et al., 1998). Therefore, RA presents itself as an efficacious cancer therapy. The biological functions of RA are primarily exerted through its ability to induce target gene transcription via RAR in the form of a RAR/RXR heterodimer. However, limited information is currently available as to direct target genes that mediate the antiproliferative activities of RAR. Three such genes were reported to be involved in RA-induced apoptosis and differentiation in NB4 promyelocytic leukemia cells, namely, ubiquitin-activating enzyme E1-like protein (UBE1L), CCAAT/enhancer binding protein $\varepsilon$ (C/EBP$\varepsilon$), and TRAIL (Altucci et al., 2001; Kitareewan et al., 2002; Park et al., 1999).

The RAR isotype RAR$\beta$ is also an RAR target gene. In several cancer cells, a strong correlation has been demonstrated between the ability of cells to increase RAR$\beta$ levels upon RA treatment and RA-dependent growth inhibition (Soprano et al., 2004). In RA-sensitive

cancer cells, an increase in RARβ expression was observed upon RA treatment as opposed to the RA-resistance cancer cells that failed to elevate RARβ level. Overexpression of RARβ in the RA-resistant cells rescued the ability of these cells to inhibit growth upon RA treatment. These findings further suggest that RAR signaling indeed plays a key role in inhibiting growth of cancer cells. Therefore, there still exists the need to identify and further characterize direct target genes of RAR in order to fully elucidate the mechanism by which RA induces growth inhibition in the mammary carcinoma MCF-7 cells.

## 1-5   CELL CYCLE ARREST IN $G_1$

One of the key check points in the cell cycle resides in $G_1$. There exist many regulatory proteins in $G_1$ that control cell cycle progression. Two tumor suppressor proteins are extensively studied for their role in the control of $G_1$ to S progression: pRb (retinoblastoma protein) and p53. pRb is a nuclear phosphoprotein whose phosphorylation state oscillates during the cell cycle. The hypophosphorylated form predominates in $G_0$ and $G_1$, while its hyperphosphorylated form exists in S and $G_2$/M phases (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989). The primary biological role of its hypophosphorylated form is to serve as a check point in late $G_1$ phase. The hypophosphorlyated pRb associates with members of the E2F family of transcription factors and prevents their ability to initiate transcription, thereby leading to a cell cycle block in $G_1$. In contrast, when pRb becomes phosphorylated, it releases E2Fs allowing them to transactivate genes required for $G_1$ to S progression (LaThangue et al., 1994; Sanchez and Dynlacht, 1996; Weinberg, 1995).

Cyclin-dependent kinases (CDKs) are responsible for pRb phosphorylation. Each CDK has its own functional specificity based on the period of activity during the cell cycle and on the specific cyclin partner. D-type cyclins predominate during $G_1$ phase to associate with appropriate CDKs to activate their ability to phosphorylate the target proteins including pRb. The expression of D-type cyclins and their ability to assemble with respective CDKs are heavily dependent on stimulation by growth factors (Sherr, 1995; Sherr, 1996). When the stimulation for growth ceases, the expression of D-type cyclins has been shown to decrease dramatically with consequent impairment of S-phase entry (Pagano et al., 1992; Baldin et al., 1993).

A further level of cell cycle control is mediated by another member of the tumor suppressor protein family. Unlike pRb, p53 is a transcription factor that enhances the rate of transcription of its target genes, which are involved in DNA damage repair, cell cycle arrest, and apoptosis (Levine, 1997). These target genes, therefore, contain p53-dependent, *cis*-acting, DNA-responsive elements. p21 is one of the p53-induced target genes that binds to CDKs to directly inhibit their function, resulting in cell cycle arrest (Levine, 1997). More specifically, p21 binds to CDK4, a partner of cyclin D1 in $G_1$ phase. The inhibition conferred by p21 on CDK4 prevents the assembly with cyclin D1, thereby preventing pRb phosphorylation. The hypophosphorylated pRb, then associates with E2Fs to prevent their transcription of target genes involved in $G_1$ to S progression. GADD45 and Bax are other known p53-inducible genes that are involved in DNA repair and apoptosis respectively (Levine, 1997). With the pleiotrophic

effects of p53, it can be appreciated how p53 has become the center of intensive study for human cancers.

Recently, B-cell translocation gene, member 2 (BTG2) has been shown to be stimulated by p53 as well as by another transcription factor, nuclear factor kappa B (NF-κB) in breast cancer (Kawakubo et al., 2004). The promoter of BTG2 indeed has been shown to contain several putative binding sites for p53, NF-κB, and other transcription factors (Duriez et al., 2002). BTG2 is a member of the PC3 family. The function of BTG2 was originally identified through studies of neural differentiation induced in the rat PC12 cell line by nerve growth factor (Guardavaccaro et al., 1999). Subsequently, the antiproliferative activity of this protein has been demonstrated in other cells (Kawamura-Tsuzuku et al., 2004). In NIH 3T3 cells, overexpression of PC3 has been shown to induce accumulation of the hypophosphorylated form of pRb, leading to $G_1$ arrest (Guardavaccaro et al., 1999). The arrest could be reversed upon coexpression of cyclin D1, but not by cyclin A or E. Indeed, PC3 expression was found to decrease the expression levels of cyclin D1 transcript and protein and to repress cyclin D1 promoter activity. Cyclin D1 serves the critical role in activating kinase activity of its partner CDK, which leads to pRb phosphorylation. Subsequently, the phosphorylated pRb releases the growth inhibitory effect on E2Fs, thereby resulting in $G_1$ to S progression. Taken together, these findings pointed to an important role of PC3 as an anti-proliferative gene through reduction of cyclin D1 expression, which inhibits pRb function to arrest cells in $G_1$.

# CHAPTER TWO
# MATERIALS AND METHODS

**2-1    Cell Line.** MCF-7 cells were a gift from Dr. Zhen Fan (M.D. Anderson Cancer Center of the University of Texas, Houston, Texas). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

**2-2    Ligand.** *trans*-retinoic acid (RA) was purchased from CalBiochem and stored at -80˚C. A fresh dilution of RA was prepared in ethanol for each experiment.

**2-3    Oligonucleotide and primers.** A 95 bp oligonucleotide, containing the putative BTG2 RARE, was synthesized with Hind III and Bam HI restriction site overhangs at 5' and 3'-ends, respectively, and PAGE-purified (www.idtdna.com). The oligonucleotide was used to perform Electrophoretic Mobility Shift Assay (EMSA) directly or cloned into tk-luciferase reporter vector using the restriction sites for transactivation assays. All primers used in this study were synthesized by www.idtdna.com.

**2-4    Antibodies.** The following antibodies were used to perform chromatin immunoprecipitation: rabbit IgG (Santa Cruz 2027), RAR (Santa Cruz 773), RARα (Santa Cruz 551), and RXRα (Santa Cruz 553).

**2-5   Proteins.** Recombinant histidine-tagged RARα and RXRα lacking the amino terminal A/B domains (RARαΔAB and RXRαΔAB) were gifts from Leslie Donato and Rubina Yasmin respectively (Cornell University, Ithaca, NY). Both proteins were expressed in E. coli and purified as previously described (Kersten et al., 1996).

**2-6   Luciferase Reporter Construct.** 1 μg of tk-luciferase reporter vector was digested with Hind III and Bam HI restriction enzymes (Promega) and purified using the gel extraction kit (Qiagen). The 95 bp oligonucleotide was subcloned into the luciferase reporter vector using T4 ligase. The construct was verified by automated DNA sequencing performed at Cornell Biotechnology Center, Ithaca, NY.

**2-7   Fluorescence Activated Cell Sorter (FACS).** MCF-7 cells were seeded in 6-well plates (120,000 cells per plate) in DMEM containing 10% FBS and grown overnight. Cells were treated with RA in DMEM containing 1% FBS for 24 or 72 hours prior to incubation at 37˚C with 5'-Bromo-2'-deoxyuridine (Sigma B5002) at a final concentration of 30 μg/ml for 20 minutes. The media, two PBS washes, and trypsinized cells were collected into the same conical tube. A cell pellet was obtained after a quick spin (1,000 rpm, 5 min, room temperature (RT)). The pellet was then resuspended in 1 ml of PBS, rapidly injected into 10 ml of 70% ethanol stored at -20˚C, and incubated overnight at 4˚C. Cells were then centrifuged (2,500 rpm, 5 min, 10 ºC) and the supernatant aspirated. To the pellet, 1 ml of 2N HCl/Triton X-100 was added and incubated at room temperature for 30 minutes to denature the DNA. Cells were again

centrifuged (2,500 rpm, 5 min, RT) and the supernatant aspirated. To the pellet, 1 ml of 0.1M $Na_2B_4O_7 \cdot 10H_2O$ at pH 8.5 was added to neutralize the acid. Cells were centrifuged as above to obtain the pellet to which 1 ml of dilution buffer (0.5% Tween 20 and 1% BSA in PBS) was added. To the cell suspension, anti-BrdU FITC antibody (Becton Dickinson Catalog No. 347583) at 20 μl per $10^6$ cells was added and incubated at room temperature for 30 minutes. Stained cells were washed with 5 ml of dilution buffer and resuspended in 1 ml of PBS containing 5 μg/ml of propidium iodide (Sigma P4864). The stained cells were sent to Biomedical Sciences Flow Cytometry Core Laboratory at Cornell University, Ithaca, NY to obtain percentages of cells in different phases of cell cycle according to their FITC and PI signals.

**2-8    Chromatin Immunoprecipitation (ChIP).** MCF-7 cells were seeded in three 100 mm plates (2,000,000 cells per plate) and grown to confluency. Proteins were cross-linked to DNA (10% formaldehyde, 20 min, 37°C) for 20 minutes and the reaction was quenched by glycine (125mM, 5 min, 4°C). Cells were washed twice with PBS, scraped, lysed (1% SDS, 10mM EDTA, 50mM Tris at pH 7.9, 1mM DTT, and protease inhibitors (Roche 1836153)), and incubated on ice for 45 minutes. Lysed cells were then sonicated to obtain average DNA fragment size between 300 to 700 bp (10 sec on and 1 min off cycle twice at 27% power). At this point, some of the sonicated materials (1/5 volume of samples used in IP) were stored at 4°C as an input. The rest were diluted (0.5% Triton X-100, 2mM EDTA, 20mM Tris at pH 7.9, 150mM NaCl, 1mM DTT, protease inhibitors, salmon sperm DNA) and pre-cleared for 1 hour at 4°C with

protein A beads. After a quick spin, the supernatant was transferred to new tubes and 3.5 μg of appropriate antibodies were added and incubated overnight at 4˚C. The next day, protein A beads were added and incubated (2 hours, 4 ºC) to immunoprecipitate antibodies bound to chromatin. Beads were washed three times in wash buffer (0.25% NP-40, 0.05% SDS, 2mM EDTA, 20mM Tris at pH 8.0, 250mM NaCl, leupeptin, and aprotinin) and once in TE buffer. Beads were then resuspended in elution buffer (100mM $NaHCO_3$, 1% SDS) and incubated for 4 hours at 65˚C to reverse cross-link proteins from DNA. Proteins were digested with Proteinase K for 1 hour and the DNA was purified using a nucleotide removal kit (Qiagen). The putative RARE containing region in the BTG2 promoter was amplified by PCR using the forward primer of 5'-cccggctacactgtatattgacttgg-3' and the reverse primer of 5'-gggtttcatcacgttggtcaggat-3'.

**2-9    Transactivation assays.** MCF-7 cells were seeded in 12-well plates (75,000 cells per well) in DMEM containing 10% FBS and grown overnight. Cells were then transfected using Fugene (Roche) in DMEM containing 1% FBS with 400 ηg of tk-luciferase reporter vector containing BTG2 RARE and 300 ηg of pCH110. After an overnight incubation, cells were treated with RA in serum-free DMEM for 24 hours and lysed. Luciferase activity was measured using the luciferase assay system (Promega) and corrected for transfection efficiency by β-galactosidase activity.

**2-10   Quantitative Real Time PCR.** Total RNA was extracted using a RNeasy kit (Qiagen). From the RNA, cDNA was generated using GeneAmp RNA PCR (Applied Biosystems). Real-Time PCR was performed on the cDNA in quadruplicates using TaqMan chemistry and Assays on Demand probes (Applied Biosystems) for B-cell translocation gene 2 (Hs00198887_m1) and cyclin D1 (Hs00277039_m1). 18S ribosomal RNA (4319413E-0312010) was used as a loading control. Analysis was carried out using the relative standard method (Applied Biosystems Technical Bulletin No. 2).

**2-11   Electrophoretic Mobility Shift Assay (EMSA).** The 95 bp oligonucleotide containing BTG2 RARE was end-labeled with $[^{32}P]$-dCTP by filling in fragments with Klenow and free nucleotides were removed with the nucleotide removal kit (Qiagen). The labeled probe (~1 ηg) was incubated with 100 ηM of appropriate receptors for 20 minutes at room temperature in HEDGK buffer (10mM Hepes at pH 8.0, 0.1mM EDTA, 0.4mM DTT, 100mM KCl, 15% glycerol). In the competition assays, either the cold competitor DNA (unlabeled probe) or a 95 bp oligonucleotide of the same sequence with BTG2 RARE mutated to GGAGGGcgAGGGGGagAGAGGG was introduced at an increasing dose (100 to 800 ηg) along with the labeled probe DNA and receptors. Protein-DNA complexes were resolved on 5% polyacrylamide gel and visualized by autoradiography.

**2-12   Affymetrix Expression Array.** The array conditions and results were provided by Leslie Donato (Cornell University, Ithaca, NY).

Iobion's Gene traffic was used to perform Robust Multi-Chip Analysis (RMA) and cluster genes with similar activity by summary function. An unpaired $t$-test giving $p$ values of <0.1 were defined as significantly changed and reported.

# RESULTS

## 3-1 MCF-7 cells arrest in $G_1$ in response to RA.

Previous studies have demonstrated that human mammary carcinoma cells increase the $G_1$ cell population and apoptosis upon RA treatment (Elstner et al., 1998; Toma et al., 1998; Mangiarotti et al., 1998). To verify that indeed MCF-7 cells respond to RA by arresting in $G_1$, cells were treated with vehicle or 1 µM of RA for either 24 or 72 hours prior to incubation with BrdU. BrdU becomes selectively incorporated into replicating DNA of cells in S phase. Then, cells were incubated with an anti-BrdU FITC antibody to stain BrdU in nucleus. In addition, propidium iodide (PI) was used to stain the entire DNA content of cells. PI stain differentiates between cells in $G_1$ with DNA content of 1X versus cells in $G_2$/M with DNA content of 2X. A Fluorescence-Activated-Cell Sorter (FACS) was used to sort cells according to their FITC and PI signals and the percentage of cell population in each phase was calculated and analyzed. As shown in Figure 3-1, MCF-7 cells increased their cell population in $G_1$ by ~10% with 1 µM RA by 24 hours and this increase was further enhanced to ~15% after 72 hours. The cell population in apoptosis was also increased at both 24 and 72 hours after RA treatment. The increase of cell population in $G_1$ corresponded to the decrease in S and $G_2$/M phases. Hence, in agreement with previous findings, RA prevented the cells from undergoing $G_1$ to S transition, thereby arresting cells in $G_1$.

**A)**
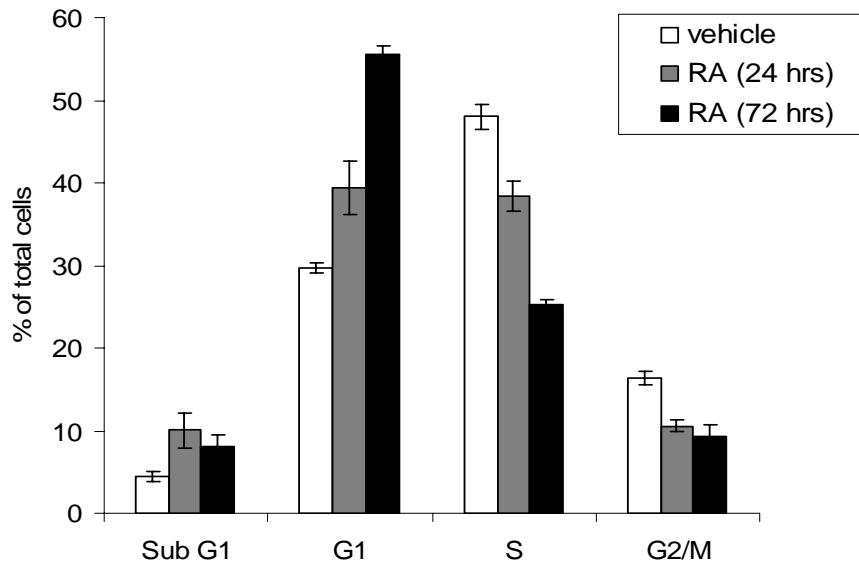


**B)**

|  | Sub G1 | G1 | S | G2/M |
|---|---|---|---|---|
| vehicle-24 hrs | $4.5 \pm 0.6$ | $30.0 \pm 0.6$ | $48.0 \pm 1.5$ | $16.0 \pm 0.8$ |
| RA-24 hrs | $10.0 \pm 2.2$ | $39.0 \pm 3.3$ | $38.0 \pm 1.8$ | $11.0 \pm 0.7$ |
| vehicle-72 hrs | $0.73 \pm 0.2$ | $41.0 \pm 1.2$ | $45.0 \pm 3.9$ | $12.0 \pm 2.0$ |
| RA-72 hrs | $8.1 \pm 1.4$ | $56.0 \pm 1.1$ | $25.0 \pm 0.7$ | $9.4 \pm 1.4$ |

* mean ± standard deviation, n=3

**Figure 3-1 MCF-7 cells arrest in G1 in response to RA.** A) MCF-7
cells were treated with vehicle or RA (1 μM) for 24 or 72 hours prior to
incubation with BrdU for 20 minutes. Cells were then fixed in ethanol and
double-stained with both anti-BrdU FITC antibody and propidium iodide
(PI). FACS was used to sort cells in different phases of cell cycle
according to their FITC and PI signal. B) % of total cells in each phase is
presented.

## 3-2    RA induces expression of cell cycle regulating genes in MCF-7 cells.

Affymetrix Expression Array data was provided by Leslie Donato (Cornell University, Ithaca, NY). Data were collected as follows: MCF-7 cells were treated with vehicle or 50 $\eta$M RA for 4 hours and total RNA isolated. Probes were generated and hybridized to Affymetrix human U133 A/B arrays, monitoring more than 40,000 genes and ESTs. The differences in the gene expression profiles between untreated and RA-treated cells were analyzed. Changes in gene expression were observed in triplicate for each condition and the genes with $p$ values of <0.1 (unpaired $t$-test) were considered significant. RA induced genes were clustered by similar biological functions using the GeneTraffic software system (Iobion).

Several genes that are known to be involved in cell cycle regulation were identified (Table 3-1). Among these, B-cell translocation gene, member 2 (BTG2) known to function in $G_1$ to S progression, displayed a prominent RA-induced increase in expression. RA responsive genes can be directly regulated by RAR through an RA response element in their promoters. Alternatively, these genes can be indirectly regulated by other direct target genes of RAR. With limited information on RA-responsive genes that are directly regulated by RAR, it became pertinent to investigate BTG2 as a direct target of RAR that arrests MCF-7 cells in $G_1$ phase.

**Table 3-1 MCF-7 cells induce cell cycle regulating genes in response to RA.** MCF-7 cells were treated with vehicle or RA (50 ηM) for 4 hours. Affymetrix Expression Array was used to compare the mRNA expression of untreated versus treated cells. Fold inductions of genes involved in cell cycle regulation are presented.

| Gene | Fold activation | Known function |
|---|---|---|
| B-cell translocation gene 2 | $2.3 \pm 0.1$ | G1/S transition |
| Prostaglandin E synthase | $1.6 \pm 0.0$ | induce p53 expression |
| E2F transcription factor 3 | $1.3 \pm 0.1$ | specifically binds to pRb |
| Cyclin-dependent kinase 8 | $1.3 \pm 0.1$ | cell cycle progression |
| Cyclin E2 | $1.2 \pm 0.0$ | interact with CDK inhibitors |

* mean ± standard deviation, n=3

### 3-3   BTG2 expression is upregulated in response to RA.

To verify the array data, TaqMan Real-Time PCR was carried out to quantitate the mRNA level of BTG2. Data were normalized to 18S rRNA. MCF-7 cells were treated with 50 ηM RA for 4 hours, lysed, and total RNA extracted. cDNA was generated from the RNA and Real-Time PCR was used to quantitate BTG2 mRNA level (Figure 3-2). In good agreement with the Affymetrix data, RA treatment increased BTG2 level by ~4 fold.
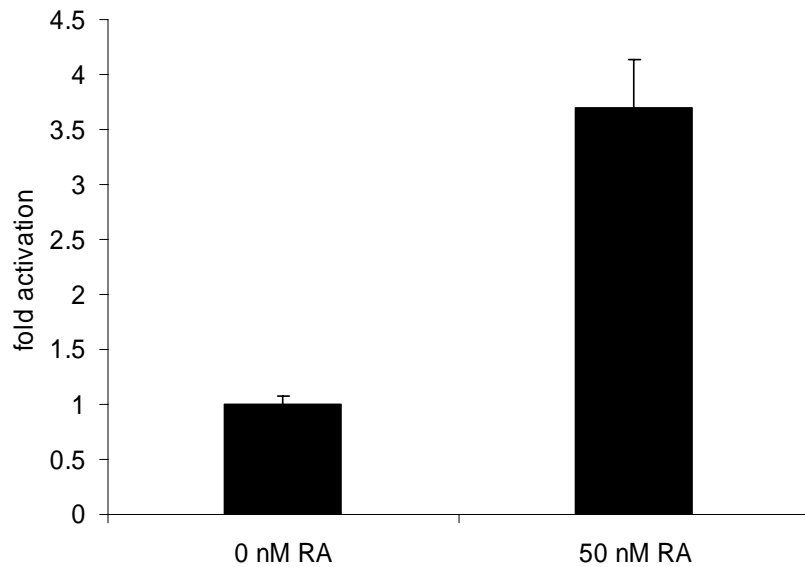


**Figure 3-2 BTG2 mRNA level is upregulated in response to RA.**
MCF-7 cells were treated with vehicle or RA (50 ηM) for 4 hours, then lysed for total RNA. cDNA was synthesized from the total RNA and used to measure BTG2 mRNA level by TaqMan Real-Time PCR. The reading was normalized to 18S rRNA level and fold activation is presented.

### 3-4   BTG2 is a direct target of RAR.

To determine whether BTG2 is a direct target for RAR, the effect of cycloheximide treatment on BTG2 level was examined. Cycloheximide is an inhibitor for protein synthesis, but it does not affect transcriptional activity of genes that do not depend on new protein synthesis. Cells were pre-treated with cycloheximide for 10 minutes, then treated with 50 $\eta$M RA for indicated time periods (4 hours for caspase-7 and 30 minutes for BTG2). Cells were lysed to obtain total RNA which was used to generate cDNA. TaqMan Real-Time PCR was performed on the cDNA to quantitate BTG2 mRNA expression level normalized to 18S rRNA. The analysis (Figure 3-3) showed that inhibition of protein synthesis did not hinder RA-induced upregulation of BTG2 expression, demonstrating that this gene is directly regulated by RAR. Caspase-7, which has been previously shown to be an indirect target of RAR (Donato and Noy, 2005), was used in this analysis as a negative control. Unlike with BTG2, upregulation of caspase-7 in response to RA was completely abolished upon cycloheximide treatment, indicating that caspase-7 requires secondary responses of RAR.
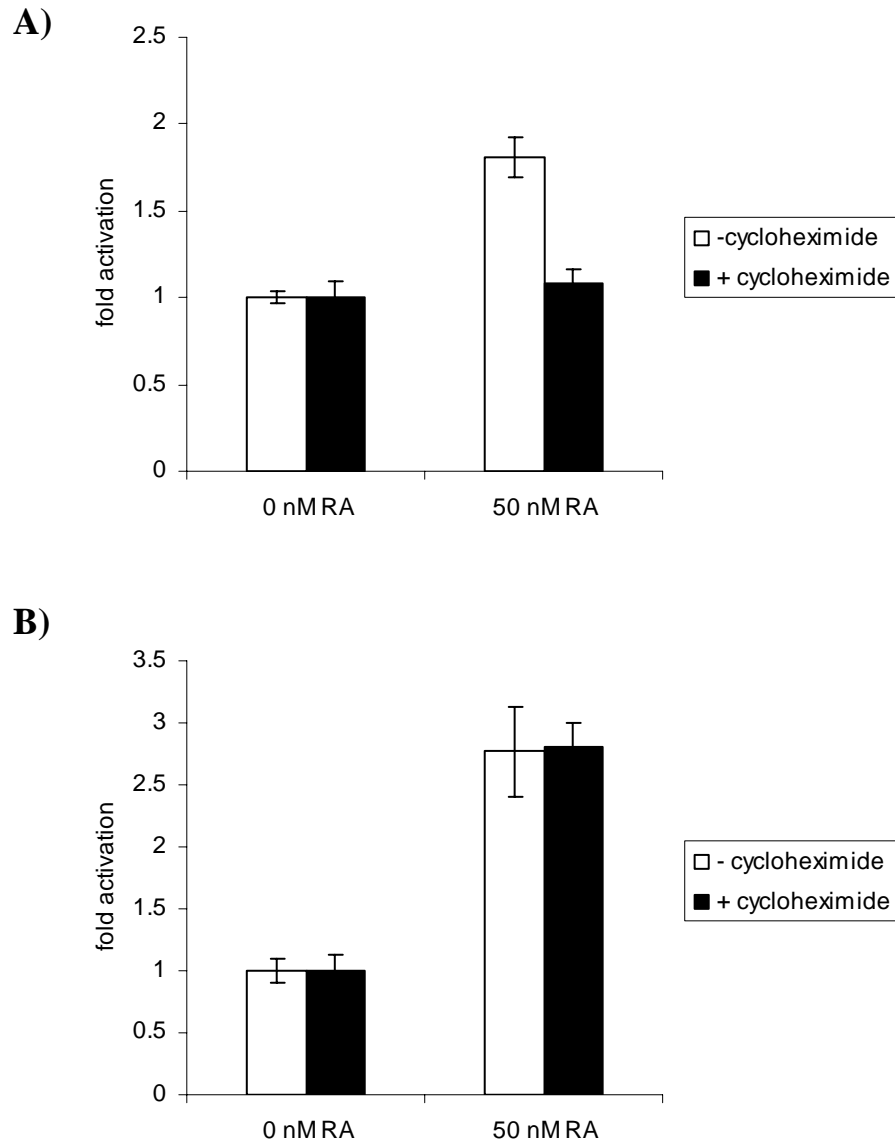
A)



B)



**Figure 3-3 BTG2 mRNA upregulation in response to RA is not affected by cycloheximide.** A) MCF-7 cells were pre-treated with cycloheximide (20 μg/ml) for 10 minutes, then treated with RA (50 ηM) for 4 hours in the presence of cycloheximide. Caspase-7 (negative control) mRNA level was measured with TaqMan Real-Time PCR and normalized to 18S rRNA. Fold activation is presented. B) Same treatment condition was followed as in A) to measure BTG2 mRNA level except that cells were treated with RA for 30 minutes.

**3-5    CRABP II further enhances BTG2 upregulation by RA.**

Previous studies demonstrated that CRABP II directly delivers RA from the cytosol to RAR in the nucleus, thereby enhancing the transcriptional activity of the receptor (Budhu and Noy, 2002). These observations suggest that the RA-induced upregulation of direct RAR target genes will be enhanced by CRABP II. Indeed, it was previously shown that CRABP-II enhances RA-induced upregulation of another direct RAR target gene, caspase-9 (Donato and Noy, 2005).  Therefore, it was of an interest to investigate the effect of CRABP II overexpression on the expression BTG2 in the absence and presence of RA.

MCF-7 cells were transfected with an expression vector encoding CRABP-II for 24 hours, then treating with vehicle or RA for 4 hours. The mRNA level of BTG2 was then examined by TaqMan Real-Time PCR. Figure 3-4 shows that RA treatment upregulated BTG2 expression by ~2.2 fold. With CRABP II overexpression, BTG2 expression was further enhanced to ~3.0 fold. Notably, CRABP II overexpression alone did not increase the expression of BTG2, indicating that CRABP II does not regulate BTG2 independently of RA.
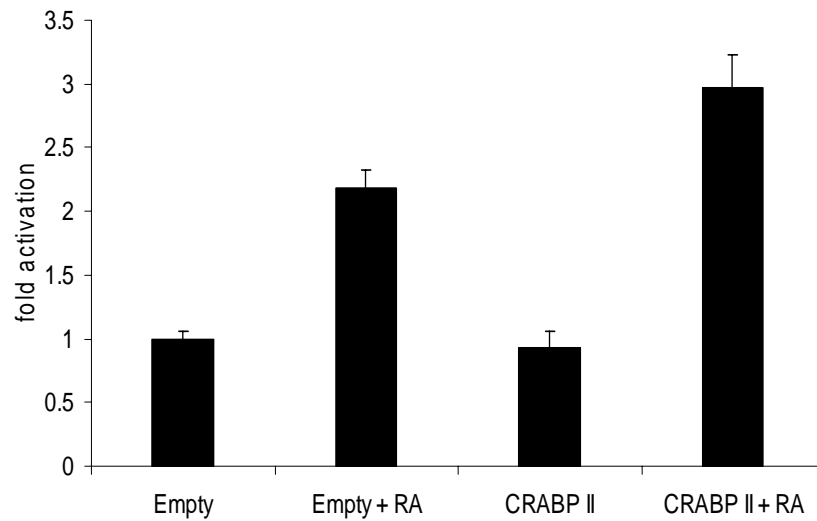
**Figure 3-4 CRABP II overexpression further enhances BTG2 mRNA level in response to RA.** MCF-7 cells were transfected with either an empty vector (pSG5) or a CRABP II expression vector. Cells were treated with vehicle or RA (50 ηM) for 4 hours, then processed for TaqMan Real-Time PCR to measure BTG2 mRNA level. The reading was normalized to 18S rRNA level and fold activation is presented.

**3-6    A functional RARE is present in the promoter of BTG2.**

Consensus RAREs consist of two direct hexameric repeats of PuG(G/T)TCA spaced by either 2 or 5 bp, DR2 and DR5 respectively. TransFac (www.gene-regulation.com) was used to screen 4,000 bp upstream of the transcription start site of BTG2 for putative RAREs. DR2s consisting of three half sites were detected at 3,250 bp upstream of the start site (Figure 3-5). The putative BTG2 RARE sequence is as follows: GGATCAcgAGGTCAagAGATCA. This element along with flanking sequences on both sides (95 bp in length) was used to perform EMSA to determine if RARα and RXRα can bind to the RARE *in vitro*.

RAR and RXR lacking their N-terminal A/B domain (RARαΔAB and RXRαΔAB) were provided by Leslie Donato and Rubina Yasmin respectively. These proteins, fused with a hexahistidine tag, were expressed in E. coli using the bacterial expression vector pET18, and purified by affinity chromatography (Kersten et al., 1996). The 95 bp oligonucleotide containing BTG2 RARE was end-labeled with [$^{32}$P]-dCTP by filling in fragments with Klenow, and used as a probe in the experiment. The labeled probe (~1 ηg) was incubated with 100 ηM of appropriate receptor proteins for 20 minutes at room temperature in HEDGK buffer. In the competition assays, either the cold competitor DNA (unlabeled probe) or a 95 bp oligonucleotide of the same sequence with a mutated BTG2 RARE was introduced at an increasing dose (100 to 800 ηg) along with the labeled probe DNA and receptors. Protein-DNA complexes were resolved on 5% polyacrylamide gel and visualized by autoradiography.

The data (Figure 3-6C: Lane 1-4) showed that RAR and RXR bind to the RARE as a heterodimer. Competition assay was performed with increasing amounts of the cold probe to observe that the intensity of the heterodimer band decreased in a dose-dependent manner (Figure 3-6C: Lane 5-8). In contrast, addition of increasing amounts of the mutated RARE (Figure 3-6C: Lane 9-12) did not inhibit DNA binding by the heterodimer. Taken together, the data demonstrated that RAR and RXR as a heterodimer specifically binds to the BTG2 RARE *in vitro*.

Next, chromatin immunoprecipitation assay (ChIP) was performed to determine if RAR/RXR heterodimer binds to the BTG2 RARE in cells. Proteins were cross-linked to chromatin in MCF-7 cells, sonicated, then immunoprecipitated using antibodies for IgG, RAR, and RXR. Finally, the crosslink was reversed, DNA isolated, and a 200 bp region flanking the BTG2 RARE was amplified by PCR. The data (Figure 3-7) showed that antibodies for RAR or RXR precipitated the putative BTG2 RARE, indicating that the RARE is occupied by the heterodimers in cells.

Since RAR/RXR heterodimer binds to the putative RARE both *in vitro* and in cells, the functionality of the element was examined by transactivation assays performed in MCF-7 cells. First, the 95 bp oligonucleotide containing the element used in EMSA was cloned into the tk-luciferase reporter vector. This vector was transfected into MCF-7 cells along with an expression vector for β-galactosidase and incubated overnight. Cells were then treated with RA for 24 hours prior to lysis. Cell lysates were used to measure the luciferase activity and the measurements were normalized to β-galactosidase activity. The luciferase activity from a vector containing the element, as expected, displayed RA-dose

responsiveness (Figure 3-8). These observations showed that the response element found in BTG2 promoter indeed binds RAR/RXR heterodimers in cells and that the element comprises a functional RARE, as demonstrated by its RA dose-responsiveness. Hence, BTG2 is a direct target for RAR signaling.

**5'-**
TACATTAAGTTTCCTCTCCCTAGGCTTGTGTATAGAAAGAAAAG
AGGAGAGGCTGGGCACAATGGCTCATGCCTGTAATCCCAGCAC
TTTGGGAAGCCAAGGTGGGT**GGATCA**CG**AGGTCA**AG**AGATCA**
AGACCATCCTGACCAACGTGATGAAACCCCACCTCTACTAAAA
ATACAAAAATAGCTGGGCATGGTGGTGCATGCCTGCAGTCCCA
GCTACTCGGGAGGCTGAAGCAG
**-3'**

**Figure 3-5 BTG2 upstream sequence from -3,357 to -3,142 bp with respect to transcription start site.** The sequence contains three RARE half sites starting at -3,250 bp. RAREs are boldfaced and underlined.

**A)**

AGCTTTCTAATCCCAGCACTTTGGGAAGCCAAGGTGGGT**GGAT CA**CG**AGGTCA**AG**AGATCA**AGACCATCCTGACCAACGTGATGA AACCCCACCG

**B)**

AGCTTTCTAATCCCAGCACTTTGGGAAGCCAAGGTGGGT**GGAG GG**CG**AGGGGGG**AG**AGAGGG**AGACCATCCTGACCAACGTGATG AAACCCCACCG
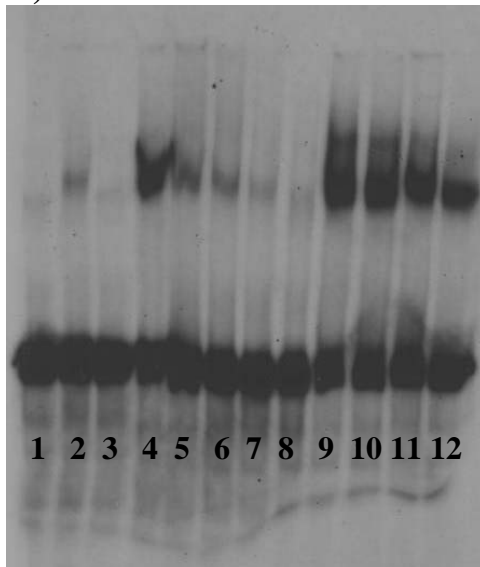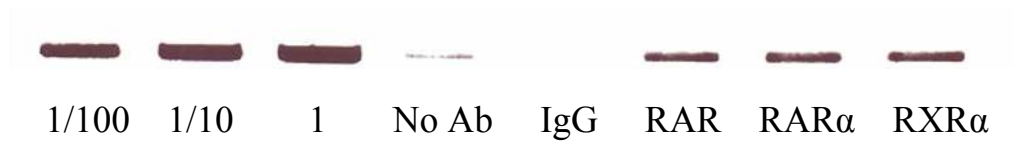
**C)**



**Figure 3-6 RAR-RXR heterodimer binds to BTG2 RARE *in vitro*.** A) 95 bp oligonucleotide sequence containing BTG2 RARE and the flanking region. RAREs are boldfaced and underlined. B) 95 bp oligonucleotide sequence containing mutated BTG2 RARE and the flanking region. Mutated RAREs are boldfaced and underlined. C) EMSA was carried out using bacterially expressed RARαΔAB (100 ηM), RXRαΔAB (100 ηM), and the oligonucleotide (1 ηg) from A) as a radioactive-labeled probe. Lanes (1-4): None; RAR alone; RXR alone; RAR and RXR together. Lanes (5-8): Both RAR and RXR in all lanes with increasing amounts of cold oligonucleotide from A) (100, 200, 400, and 800 ηg respectively). Lanes (9-12): Both RAR and RXR in all lanes with increasing amounts of cold mutated oligonucleotide from B) (100, 200, 400, and 800 ηg respectively).

**A)**



1/100   1/10   1   No Ab   IgG   RAR   RARα   RXRα

**B)**

BTG2 RARE

5'- ————————————————————————— -3'

Primer (26 bp)              Primer (26 bp)
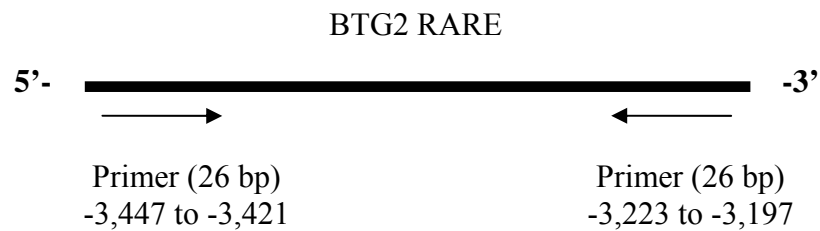-3,447 to -3,421           -3,223 to -3,197

**Figure 3-7 RAR-RXR heterodimer binds to BTG2 RARE *in vivo*.** A) MCF-7 cells were fixed with 1% formaldehyde prior to lysis. Cells were then sonicated to obtain DNA fragment size between 300 and 700 bp, which served as an input. The remaining chromatin was immunoprecipitated with appropriate antibodies as indicated. PCR was performed on the immunoprecipitated materials to amplify the BTG2 RARE containing region as shown in B).
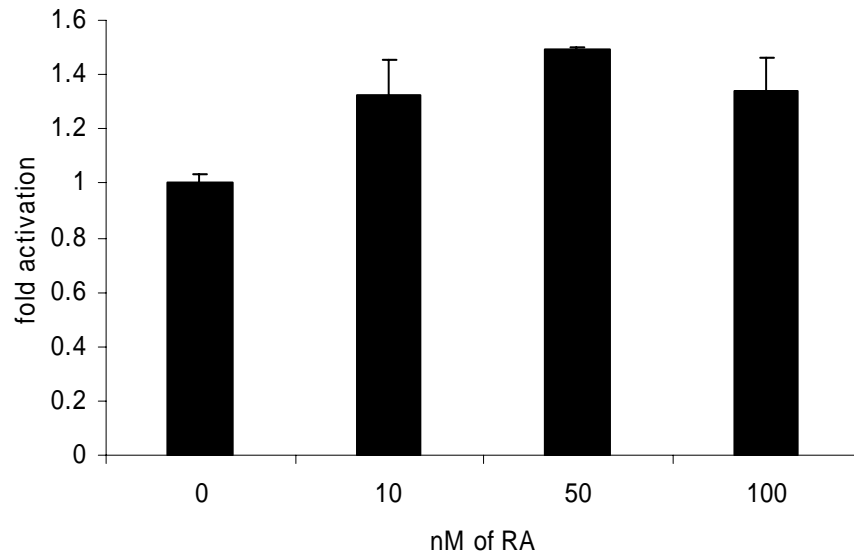
**Figure 3-8 BTG2 RARE is RA-dose responsive.** The 95 bp oligonucleotide containing BTG2 RARE and the flanking region (sequence shown in Figure 3-6A) was cloned into a tk-luciferase reporter vector. MCF-7 cells were transfected with the reporter vector and an expression vector for β-galactosidase. Cells were then treated with increasing doses of RA (from 0 ηM to 100 ηM) for 24 hours prior to measurement of luciferase activity. Luciferase activity was normalized to β-galactosidase activity and fold activation is presented.

**3-7    RA downregulates the cyclin D1 mRNA expression.**

Previous studies demonstrated that BTG2 confers its anti-proliferative activity through downregulating cyclin D1 expression. Since the data so far have demonstrated that BTG2 is a direct target of RAR and that its expression upregulated by RA, it became of an interest to examine whether cyclin D1 expression responds to RA. Figure 3-9 showed that cyclin D1 expression was indeed downregulated by ~50% in response to 1 μM RA treatment for 24 hours. As cyclin D1 is not a direct target for RAR, the data strongly suggest that BTG2 induction by RA leads to decrease in cyclin D1 expression, thereby arresting cells in $G_1$ phase.
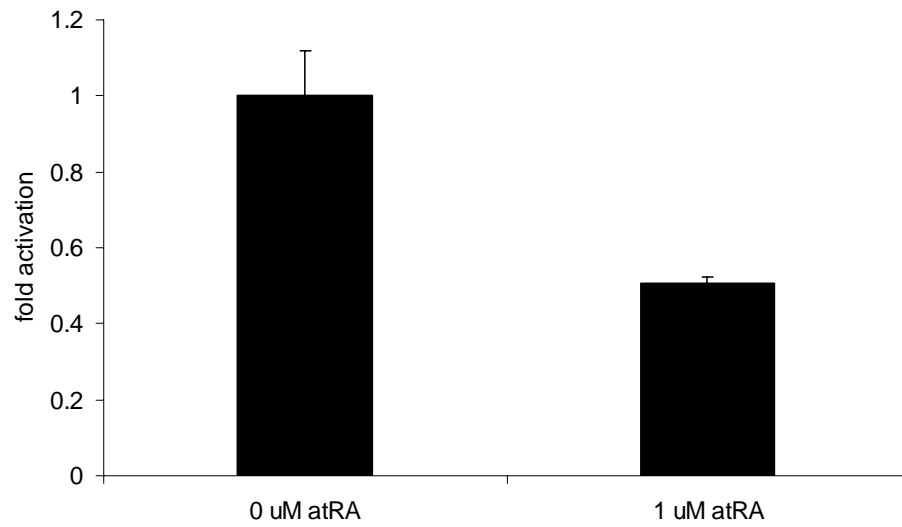
**Figure 3-9 Cyclin D1 mRNA level is downregulated in response to RA.** MCF-7 cells were treated with vehicle or RA (1 µM) for 24 hours, then processed for TaqMan Real-Time PCR to measure cyclin D1 mRNA level. The reading was normalized to 18S rRNA level and fold activation is presented.

# CHAPTER FOUR
## DISCUSSION

RA displays a growth inhibitory effect on human mammary carcinoma cells through apoptosis, cell cycle arrest, or both (Elstner et al., 1998; Toma et al., 1998; Mangiarotti et al., 1998), rendering RA as a sensible agent for a breast cancer therapy. However, the exact mechanism of inhibition and the genes involved in the growth inhibition are poorly understood. The present study was undertaken to identify target genes responsible for RA-induced, RAR-mediated cell cycle arrest, and the contribution of CRABP II to the process.

The initial FACS data (Figure 3-1) demonstrated that RA treatment induced cell cycle arrest in the $G_1$ phase as shown by the increase in percentage of cells in $G_1$ and the corresponding decrease in S and $G_2/M$ phases. This is in agreement with previous studies. In this experiment, the percentage of cells in each phase was analyzed after 24 and 72 hours following a 24 hour pretreatment with 1 μM RA. RA-sensitive cells, however, have been shown to display a high rate of RA metabolism. For example, in MCF-7 cells, 1 μM RA was metabolized to its polar metabolites with a corresponding decrease in RA to a trace amount after only ~5 hours (van der Leede et al., 1997). According to this reported rate, RA used in this experiment degrades rapidly. However, the percentage of cells in $G_1$ was further increased by about 15% after 72 hours compared to 24 hours post the 24 hour RA pretreatment. These observations suggest that RA triggers cellular pathways that remain active even long after the pretreated RA has been completely metabolized.

RA exerts its action through RAR by activating the transcription of target genes. Therefore, it became important to examine RA-responsive genes that might be involved in cell cycle regulation. Several RA-responsive genes were identified that control the $G_1$ to S transition (Table 3-1). Among them, B-cell translocation gene, member 2 (BTG2) displayed the most prominent induction in its expression upon 4 hour treatment with RA (Figure 3-2). At this short time period, it can be reasonably expected that some of these genes are direct targets of RAR. In Figure 3-3, MCF-7 cells were pretreated with cycloheximide prior to RA treatment. Cycloheximide stops new protein synthesis, thereby negating any secondary effects from other RA-responsive proteins. The data showed that the upregulation of BTG2 expression by RA was unaffected by cycloheximide treatment, indicating that BTG2 is indeed directly regulated by RAR (Figure 3-3B). In contrast, cycloheximide abolished the RA effect on caspase-7, a known indirect target of RAR, as it depends on RA to upregulate the expression of direct RAR target genes, which in turn induce caspase-7 expression (Figure 3-3A).

The RA action depends on CRABP II in addition to RAR. The previous studies demonstrated that CRABP II binds to RA and directly associates itself with RAR, thereby allowing RA to be channeled to RAR. Therefore, CRABP II expression can further enhance the effect of RA on direct target genes of RAR. The data (Figure 3-4) showed that CRABP II overexpression indeed further enhanced BTG2 expression in the presence of RA, indicating once again that BTG2 is a direct target of RAR. In addition, it should be noted that transfection efficiency of MCF-7 cells with Fugene is relatively low. Therefore, the effect of CRABP II on

BTG2 expression in this study is an underestimation as not all cells are expressing CRABP II. Lastly, CRABP II expression alone did not enhance the basal level of BTG2 expression. This demonstrates that CRABP II does not have a function of its own in regulating BTG2 expression. Instead, CRABP II cooperates with RAR in activating transcription of BTG2. The observations thus far demonstrate that BTG2 is an endogenous gene that is under the direct control of RAR.

As a possible direct target of RAR, 4,000 bp upstream from the start site of BTG2 was screened for potential RAREs. At 3,250 bp upstream of the start site, a putative RARE was located (Figure 3-5). The RARE has three half sites spaced by 2 bp. An EMSA was performed to confirm that RAR and RXR together bind to the element (Figure 3-6: Lane 1-4). The binding was further tested with both a wild-type and a mutant cold competitor. The binding to the element by RAR and RXR together decreased in a dose-dependent manner with the wild-type competitor (Figure 3-6: Lane 5-8). In contrast, the binding was not affected by the mutant competitor (Figure 3-6: Lane 9-12). These observations demonstrate that the RAR/RXR heterodimer binds to the element *in vitro*. The binding was further examined with chromatin immunoprecipitation (ChIP) to show that both RAR and RXR bind to the element in MCF-7 cells (Figure 3-7). Lastly, the functionality of the element was tested by RA-induced transcription of a luciferase reporter vector. The element along with the flanking regions on both sides (95 bp in length) was cloned into the vector and used in a transactivation assay. The element responded to RA in a dose-dependent fashion (Figure 3-8).

Taken together, the RARE located at 3,250 bp upstream of the start site of BTG2 binds RAR/RXR heterodimer and is RA-dose responsive.

The findings demonstrate that BTG2 is a direct target of RAR, which is involved in arresting MCF-7 cells in $G_1$ phase. The downstream targets of BTG2, therefore, became of interest to examine. Several studies have previously reported that BTG2 represses the transcription of cyclin D1, a critical protein in cell cycle regulation. Cyclin D1 serves to activate cyclin-dependent kinase (CDK). The activation induces the kinase activity of CDK to phosphorylate pRb, a $G_1$ checkpoint protein. The phosphorylated form of pRb then releases E2Fs, members of the family of transcription factors that activate transcription of genes involved in $G_1$ to S transition. This release, therefore, allows for the cell cycle progression. Consequently, cyclin D1 expression, when reduced, plays an important role in arresting cells in $G_1$ phase. Therefore, the cyclin D1 expression level was examined to understand the mechanism by which BTG2 induces cell cycle arrest. The data demonstrated that RA induced the decrease in cyclin D1 expression in MCF-7 cells by ~50% (Figure 3-9). This suggests that BTG2, a novel direct target of RAR reported in this study, induces cell cycle arrest by decreasing cyclin D1 expression. Interestingly, previous studies of possible effects of RA on cyclin D1 expression in other cell lines such as human bronchial epithelial cells and lymphomas indicated that cyclin D1 expression was not RA-responsive (Ma et al., 2005; Guidoboni et al., 2005). Therefore, the data presented here in MCF-7 cells are the first demonstration of RA regulating cyclin D1 expression.

Taken together, the findings described here demonstrate that, in MCF-7 cells, RA induces cell cycle arrest in $G_1$ phase by directly

regulating BTG2 expression through RAR, and that CRABP II participates in further upregulation of BTG2 expression. The data further indicate that the most downstream target of RA-induced growth inhibition through this path is cyclin D1 whose expression is, in turn, downregulated by BTG2. This decrease in cyclin D1 expression prevents $G_1$ to S transition, thereby arresting MCF-7 cells in $G_1$. These findings are summarized in Figure 3-10.

RA    CRABP II

↑ BTG2

↓ Cyclin D1

CDK4

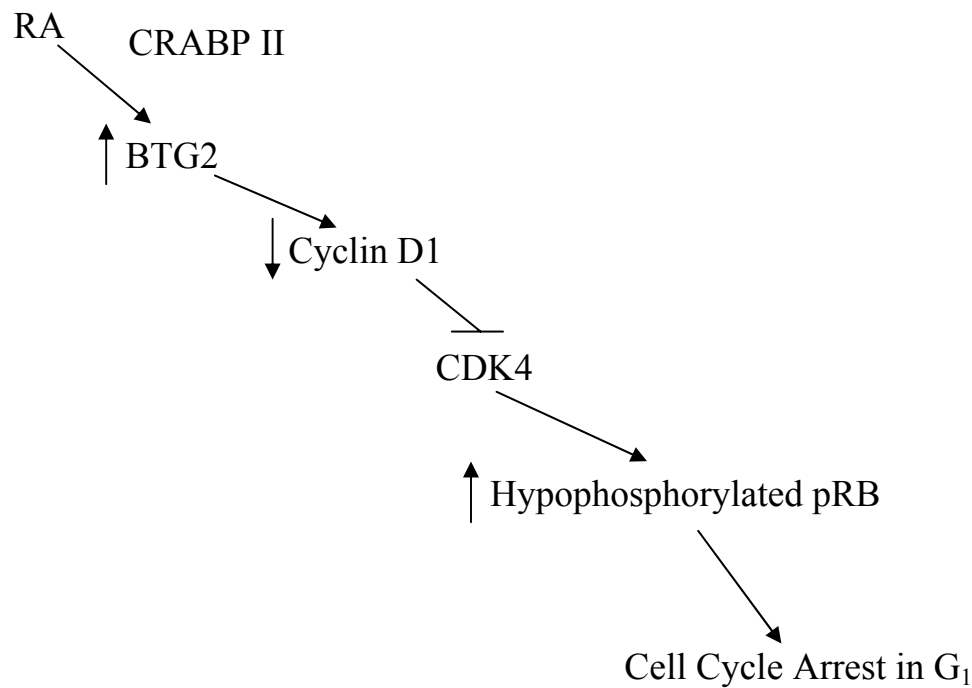↑ Hypophosphorylated pRB

Cell Cycle Arrest in $G_1$

**Figure 3-10 The RA signaling pathway results in cell cycle arrest.**

As for future directions, the rate of RA degradation can be investigated in MCF-7 cells to ascertain the effect of RA on the initiation of cellular pathways. In this study, RA enhanced its effect on cell cycle arrest with corresponding increase in the treatment time. This observation

suggests that RA can trigger a growth inhibitory pathway in MCF-7 cells that can remain active in the absence of a constant supply of RA to the cells. Quantitative measurements of RA concentrations in cells can help to pinpoint a time course during which the effect of RA persists after RA has metabolized to a basal physiological level.

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