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Nuclear Receptor Expression and Characterization of the Major Retinoic Acid Target Gene in Melanoma

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NUCLEAR RECEPTOR EXPRESSION AND CHARACTERIZATION OF THE MAJOR RETINOIC ACID TARGET GENE IN MELANOMA

Dissertation

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by
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Dedication

I would like to dedicate this to my family. First, my parents, Eldred Lloyd and Larry Lloyd, who have always encouraged me to pursue an advanced education. I would like to thank my father, who bought my first microscope, and has helped to set the course of my education on a pathway which has led me to where I am today. I also would like to thank my husband Jay Eastham, and my daughters Leslie Ann and Laura Beth, for their understanding, patience and encouragement, during my pursuit to obtain my Ph.D. in Biomedical Sciences. I love my family very much and appreciate the support they have given me throughout the course of my education.

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List of Symbols/Nomenclature

AKAP12	A kinase anchoring protein12
AKT	Ak thymoma, also known as protein kinase B
ANOVA	Analysis of variance
APAF-1	Apoptotic protease activating factor-1
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
atRA	*All trans* retinoic acid
BRAF	Murine sarcoma viral oncogene homolog B1
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CaM	Calmodulin
CDC2	Cell division cycle 2 protein
CDC44L	Cell division cycle 45-like
CDK-1/4	Cyclin dependent kinase 1 and 4
CDKN1A	Cyclin dependent kinase inhibitor 1A
CDKN2A	Cyclin dependent kinase inhibitor 2A
CHEK1	Checkpoint kinase 1
Cig	Ciglitazone
CMM	Cutaneous malignant melanoma
CRBP	Cellular retinol binding protein
CRABP	Cellular retinoic acid binding protein
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CyD	Cyclin D
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTIC	Dacarbizine
EMT	Epithelial mesenchymal transition
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	Food and drug administration
GalTase	β1,4-galactosyltransferase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
HAT	Histone acetyl-transferase
HDAC	Histone deacetylase
HEMn-LP	Human epithelial neonatal melanocytes-lightly pigmented
HIF-1α	Hypoxia-inducible factor -1 alpha

HMGS	Human melanocyte growth serum
HRP	Horseradish peroxidase
IFN-α	Interferon-alpha
IL-2	Interleukin-2
LEF	Lymphoid enhancer factor
LTB_4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MCAD	Medium chain acyl dehydrogenase
MC1R	Melanocortin-1 receptor
MCM6	Minichromosome maintenance deficient protein 6
Met	Metastatic
MITF	Microthalmia transcription factor
MLL	Mat-LyLu prostate cancer cells
N-CoR	Nuclear receptor co-repressor
NF-κB	Nuclear factor-κB
NRAS	Neuroblastoma rat sarcoma
$p16^{INK4a-ARF}$	Cyclin dependent kinase inhibitor 4A-alternate reading frame
PDGFRβ	Platelet-derived growth factor receptor beta
PGC-1α	PPAR gamma co-activator-1 alpha
PGJ2	15-deoxy-Δ-12,14-prostaglandin J2
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PP2B	Phosphatase 2B
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
PSA	Penicillin/streptomycin/amphotericin B
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real time polymerase chain reaction
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinol binding protein
RGP	Radial growth phase
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMRT	Silencing mediator of retinoid and thyroid receptors
SRC-1	Serum receptor co-activator-1
SSeCKS	Src suppressed C kinase substrate
STAT3/5	Signal transducers and activators of

	transcription 3 and 5
TCF4	T cell factor 4
TNFα	Tumor necrosis factor alpha
TR	Thyroid hormone receptor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Tro	Troglitazone
UVR	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
VDR	Vitamin D receptor
VGP	Vertical growth phase
WY	WY14643

Abstract

NUCLEAR RECEPTOR EXPRESSION AND CHARACTERIZATION OF THE MAJOR RETINOIC ACID TARGET GENE IN MELANOMA

Linda L. Eastham

Malignant melanoma is the primary cause of death from all diseases arising from the skin and it is one of the most resistant tumors to standard treatment options. The increasing incidence of melanoma and its poor prognosis makes the understanding of melanoma cell biology very important. Melanoma progression is a complex, multi-step process, and the molecular events leading to melanoma tumor cell proliferation and invasion still need further examination. This study is focused on measuring the expression and activity of two specific genes and their protein counterparts that are altered during melanoma progression. It also examines the mechanism through which Vitamin A induces melanoma cell differentiation and inhibits tumor cell growth.

The first part of this research focused on a family of ligand activated transcription factors, the PPARs (Peroxisome Proliferator-Activated Receptors). I examined the expression and activity of PPARs and the effects of PPARα and γ agonists on the growth of mouse melanocytes and melanoma cells. I found increased expression of PPARα and γ protein levels in B16 mouse melanoma cells compared to mouse melan-a melanocytes. PPARγ-selective agonists,

prostaglandin J2, troglitazone and ciglitazone, were able to inhibit growth of both melan-a and B16 cells. The only effective PPARα agonist was leukotriene B4 and it was only able to inhibit melan-a cell growth. PPAR transcriptional activity was examined by reporter gene activity and I found a correlation between activation of the receptor and its ability to inhibit cell growth.

The second part of my thesis work examines the expression and function of an indirect target gene of atRA stimulation, AKAP12. This protein functions as a scaffold, assembling a multi-protein complex containing enzymes involved in regulating the activity of various signaling pathways. Results show atRA treatment of mouse and human melanocytes and melanoma cells increased AKAP12 expression, and this change was modulated by the transcription factor AP-1. I found a significant correlation between atRA induction of AKAP12 mRNA and the ability of atRA to inhibit growth. Functional studies using AKAP12 siRNA to decrease the level of this protein indicate that AKAP12 contributes to the ability of melanoma cells to invade and grow in an anchorage-independent manner.

Chapter1: Introduction

Melanoma

Cutaneous malignant melanoma (CMM) is a deadly cancer of the skin. It arises from melanocytes, located in the basal layer of the epidermis. These cells produce the pigment melanin, which is not only responsible for the coloration of hair and skin, but has photoprotective qualities enabling it to protect skin cells from UV-induced DNA damage. Melanocytes are derived from cells of the neural crest and are also located in tissues such as brain, inner ears, heart and eyes (Santiago-Walker, *et. al.,* 2008).

The incidence of melanoma worldwide has been rising for the past three decades and it is the sixth most diagnosed cancer in the USA (Boyle, 2011; www.cancer.org). This year (2012), it is estimated that 70,250 Americans will be diagnosed with melanoma. Early diagnosis leads to surgical removal of the tumor and results in an average 5 year survival rate of 93%. However, once the melanoma has metastasized, there is an extraordinarily poor survival rate with <10% of patients surviving after 5 years (Boyle, 2011). Until recently, the only FDA-approved drug for the treatment of malignant melanoma was the alkylating agent dacarbazine (DTIC), which only induced complete remission in 5-10% of patients. Combination therapies using nitrosoureas (carmustine, lomustine), taxanes (taxol, docetaxol), platinum-associated drugs (cisplatin, carboplatin), IL-2 and/or IFN-α have also failed (Boyle, 2011; Soengas and Lowe, 2003). However, within the past year, two new therapeutic agents have been shown to inhibit the growth of metastatic melanoma and extend patient lifespan. The first new FDA-

approved agent is the monoclonal antibody ipilimumab. It is currently given to treat patients with late stage melanoma that has metastasized or cannot be removed by surgery. This antibody binds to cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). It plays an important regulatory role in the immune system. This immunoglobulin is expressed on the surface of helper T cells and transmits an inhibitory signal to T cells, suppressing the immune system's T cell response. The second FDA-approved compound is the BRAF inhibitor PLX4032 (vemurafenib). BRAF is a protein kinase that regulates the mitogen-activated protein kinase (MAPK) pathway involved in regulating cell proliferation. This drug targets the V600E (valine to glutamate) activating mutation commonly seen in this protein. This class of drugs has been shown to delay the progression of advanced melanoma, improving overall patient survival (Guida, *et. al*., 2012).

After melanoma has been diagnosed, tests are done to find out if the melanoma cancer cells have spread within the skin or to other parts of the body. Staging is performed to determine how widespread the melanoma has become. This diagnosis takes into account information on tumor thickness (T) (Breslow measurement), whether the melanoma has spread to the nearby lymph nodes (N), and whether it has metastasized (M) and to which organs it has reached. A biopsy of the tumor is performed and the thickness is measured under a microscope. The Breslow thickness is a major predictor of prognosis in CMM. The thinner the melanoma, the better the prognosis will be (Osborne and Hutchinson, 2001). Melanomas less than 1mm in depth have a very small chance of spreading. As

the melanoma becomes thicker, the chance of spreading increases (www.cancer.org).

Several factors have been identified that play a role in melanoma formation and they can be divided into two categories: environmental factors and host factors (Boyle, 2011). Exposure to UV radiation, usually from the sun, is the most important environmental risk factor for developing malignant melanoma. UV radiation is composed of both UVA (320-400nm) and UVB radiation (280-320nm). Although UVA is the major component of UV radiation and can penetrate the deepest, it is the UVB fraction that is mainly responsible for the development of skin carcinomas (Oikarinen, *et. al.,* 1991; Benjamin, *et. al.*, 2007).

There are also several host factors that are associated with increased risk. These include a family history of melanoma, a large number of benign or dysplastic nevi, mutations in the CDKN2A ($p16^{INK4a-ARF}$) gene and variation in the melanocortin-1 receptor (MC1R) genotype that leads to increased sensitivity to UV radiation (Boyle, 2011; Benjamin, *et. al.*, 2007; Melnikova and Bar-Eli, 2008).

The process of melanoma progression involves the sequential accumulation of a series of genetic alterations that drive cellular transformation. The melanoma tumor cells evolve through three distinct phases of growth. In the radial growth phase (RGP), the tumor grows laterally, and although cells may invade the dermis, they do not form a nodule. In the vertical growth phase (VGP), the cells grow vertically, invading the underlying tissues and forming a true tumor

(Hussein, 2004; Vladislova and Bar-Eli, 2008; Bennett, 2008). In the metastatic phase (Met), the transformed cells are able to invade and spread to distant target organs such as the lungs, liver, gallbladder, brain and bones.

Many signaling pathways also become dysregulated during melanoma development. The most common mutations are those that constitutively activate the proteins NRAS and BRAF. Activating mutations in these proteins are mutually exclusive, with approximately 20% of tumors having NRAS mutations and 50-70% of tumors having BRAF mutations (Wagner and Fisher, 2005; Haluska, *et. al.*, 2006; Benjamin, *et. al.*, 2007; Bennett, 2008; Boyle, 2011). These proteins drive the MAPK pathway, which is a crucial pathway involved in the regulation of cellular proliferation and differentiation. The PTEN/PI3K/AKT pathway is also altered. Inactivating mutations in the tumor suppressor PTEN are common in melanoma and seem to be prevalent in tumors harboring a BRAF mutation (Wagner and Fisher, 2005; Haluska, *et. al.*, 2006; Benjamin, *et. al.*, 2007; Boyle, 2011). The β-catenin pathway is also abnormally regulated in approximately 30% of melanoma tumors (Haluska, *et. al.*, 2006; Bennett, 2008). β-Catenin, complexed with the transcription factors LEF and TCF can upregulate c-myc and cyclin D1, suppress apoptosis and upregulate the transcription factor MITF, a master regulator of melanocyte linage. This factor is responsible for regulating melanocyte proliferation, differentiation and survival (Wagner and Fisher, 2005; Bennett, 2008; Mitra and Fisher, 2009). In addition, up-regulation of the transcription factor NF-κB is also found in melanoma tumors (Bennett, 2008; Melnikova and Bar-Eli, 2008). This factor regulates pathways involved in

inflammation, cell cycle regulation, apoptosis and oncogenesis. Constitutive activation of NF-κB is found in melanoma and supports cancer cell survival because it is involved in reducing the sensitivity of melanoma cells to chemotherapeutic drugs. It is also linked to the constitutive activation of AKT (Bennett, 2008; Melnikova and Bar-Eli, 2008). The transcription factors STAT3 and STAT5 (signal transducers and activators of transcription), have also been shown to respond to oncogenic stimulation by growth factors or non-receptor tyrosine kinases. They are involved in the down-regulation of anti-apoptotic genes such as Bcl-X_L. The activity of STAT3 and 5 are often up-regulated in melanoma (Melnikova and Bar-Eli, 2008). In addition, melanomas are usually resistant to external inducers of apoptosis, including TNF-α, FasL and TRAIL. The intrinsic apoptotic activator APAF-1 is silenced in advanced melanomas (Soengas and Lowe, 2003; Bennett, 2008).

Migration and metastasis of melanoma cells mimics a physiologic process called EMT (epithelial mesenchymal transition). During this process cells lose their cell-cell and cell-matrix connections and acquire the motile, migratory properties of mesenchymal cells. There is also a down-regulation of the cell adhesion protein E-cadherin and an upregulation of N-cadherin. EMT conversion confers metastatic and invasive properties to melanoma cells. The transcription factors SNAIL and SLUG contribute to this process. Over-expression of SNAIL suppresses expression of E-cadherin and SLUG activity is essential for melanoma metastasis (Haass, *et. al.,* 2005; Bennett, 2008; Girouard and Murphy, 2011). This information reveals that there are many pathways that are dysregulated in

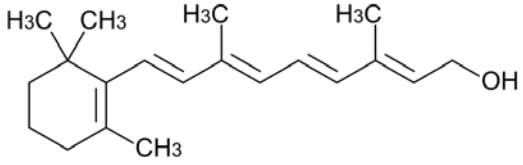
melanoma, and these altered pathways drive the formidable aggressiveness of this cancer.

Vitamin A

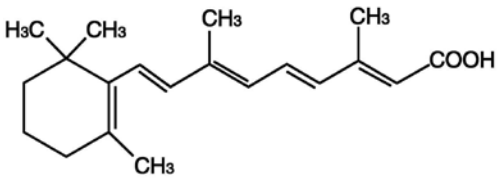
Vitamin A is a fat-soluble co-factor present in food that is essential for life. It plays an important role as a coenzyme in vital processes that the body needs to function normally. The structure of Vitamin A makes it good molecule for trapping light energy. It is the molecule responsible for transferring light energy into a chemical nerve impulse in the eye (Amann, *et. al.*, 2011). It is also important for the regulation of biological activities such as embryogenesis, cell growth, differentiation, reproduction, apoptosis, and bone formation. (Niles, 2000; Sun and Lotan, 2002; Mamede, *et. al.*, 2011).

Physiological metabolites of Vitamin A are collectively known as retinoids. Although the most common dietary form of Vitamin A is retinol, there are over 4000 natural and synthetic molecules that are structurally and/or functionally related to this compound. Vitamin A is only obtained through the diet in the form of retinol, retinyl esters, or proVitamin A (β-carotene). It is found in foods of plant origin such as sweet potatoes and carrots as β-carotene and in animal products such as liver and eggs as retinyl esters (Amann, *et. al.*, 2011).

A. Retinol



B. all-*trans* Retinoic Acid



C. Beta-Carotene

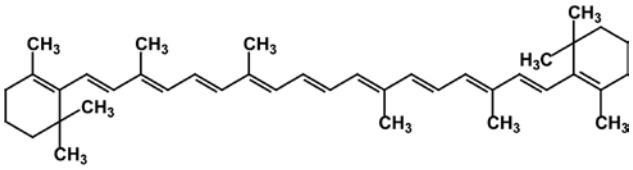


Figure 1.1. Chemical structures of **A)** retinol, **B)** all-*trans* retinoic acid, and **C)** beta-carotene.

Retinol is either stored in the liver as retinyl esters or circulates in the bloodstream complexed with plasma retinol-binding protein (RBP) or transthyretin (pre-albumin). Plasma membrane receptors for the retinol-binding protein mediate the cellular uptake of retinol. After retinol has been taken up by the cell, it becomes bound to cellular retinol-binding protein (CRBP). Retinol is first converted to retinal, before being metabolized into all-*trans* retinoic acid (atRA) by a retinal dehydrogenase. The major physiologically active metabolite of retinol, retinoic acid, becomes bound to cellular retinoic acid-binding protein (CRAPB). The CRABP bound retinoic acid is then delivered to the nucleus of the cell by CRABPII, where it will bind to and activate the retinoic acid receptor. (Niles, 1998).

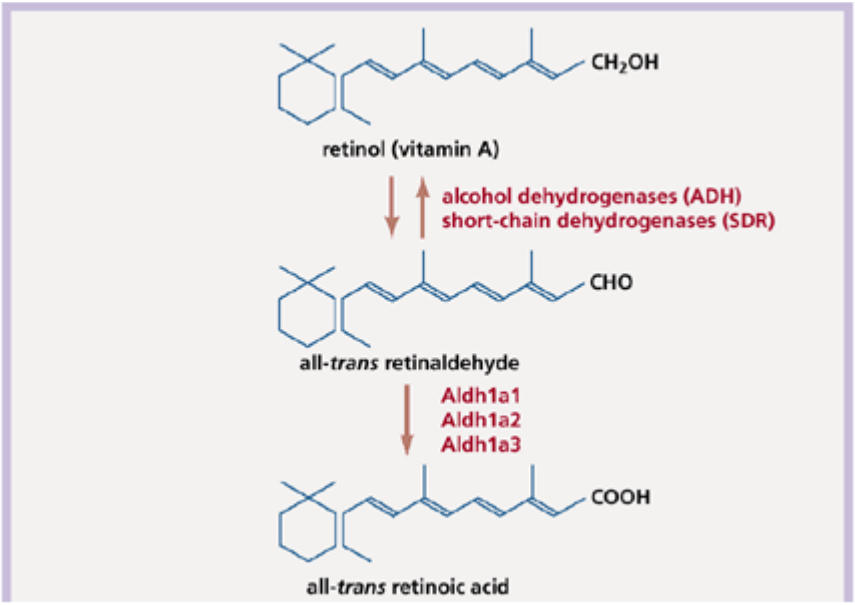


Figure 1.2. Retinol metabolism in the body. Retinol is first converted to all-*trans* retinaldehyde by alcohol dehydrogenases or short-chain dehydrogenases before being converted to all-*trans* retinoic acid by aldehyde dehydrogenases (Perlmann, 2002).

Retinoic Acid Regulated Gene Expression

Retinoids exert their effects on biological processes through the regulation of gene expression, which is due to the ability of atRA to bind and activate specific nuclear receptors. There are two distinct sub-families of nuclear retinoid receptors. The first are the RARs (retinoic acid receptors) that bind all-*trans* retinoic acid and 9-*cis* retinoic acid. The RXRs (retinoid X receptors) bind only 9-*cis* retinoic acid. There are three distinct subtypes of RXRs and RARs (α, β and γ), which are encoded by different and highly conserved genes.

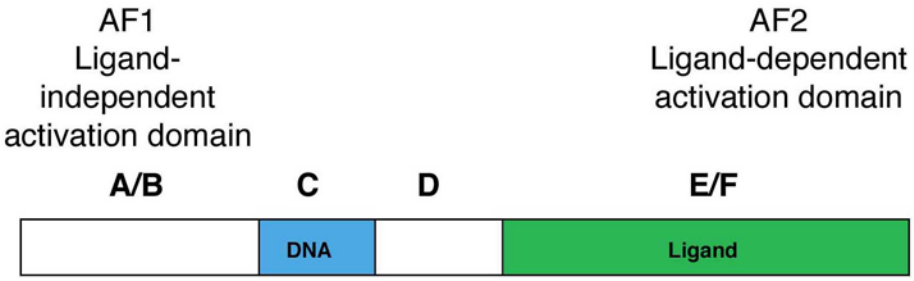


Figure 1.3. Functional domains of steroid receptor proteins. The A/B region is the ligand-independent activation domain, the C region is the DNA-binding domain, the D region acts as a hinge, while the E/F region contains the ligand binding domain.

The RARs share functional domains common to other members of the steroid hormone receptor gene family. The A/B region located in the N-terminal portion functions as a ligand-independent transcriptional activation domain. The C region contains the DNA-binding domain that consists of two zinc-binding, cysteine-rich segments. The D domain acts as a hinge, whereas the E segment acts as a dimerization interface for ligand-dependent transcriptional activation. This region is highly conserved between all of the RAR subtypes. The C-terminal F region is different between the RAR subtypes and at the present time its function is not known. However, it is thought that it may bind the coactivator CBP/p300 (Niles, 1998).

Retinoic acid induces phenotypic changes in target cells by inducing the transcriptional activity of the nuclear retinoid receptors. It is the main activator of RARs whereas the RXR serves as its common heterodimeric partner. The RXRs

have the ability to also heterodimerize with other nuclear receptors. These include the thyroid hormone receptor (TR), Vitamin D receptor (VDR), PPARs (peroxisome-proliferator activated receptors), and numerous orphan receptors. The RAR-RXR heterodimer binds to a specific sequence located in the promoter region of the DNA called a retinoic acid response element (RARE) with a half site sequence similar to AGGTCA. It is a direct repeat (DR) of half sites with a spacer of 2 to 5 nucleotides (DR2-DR5). In the absence of ligand, the RAR-RXR dimer is bound to the RARE, and held inactive by co-repressors, such as SMRT (silencing mediator of retinoid and thyroid signaling) and N-CoR (nuclear receptor co-repressor). These co-repressors bind to the non-liganded receptors resulting in suppression of basal transcriptional activity. The ability of atRA to bind to the RARs results in destabilization of this complex and a conformational change in the receptors that allow for the disassociation of co-repressors and binding of co-activators, such as SRC-1 (steroid receptor co-activator 1) and CBP/p300. Consequently, atRA exerts its effects through changing the structure of the receptor, which allows for control of distinct molecular events at retinoid-regulated promoters (Niles, 1998).

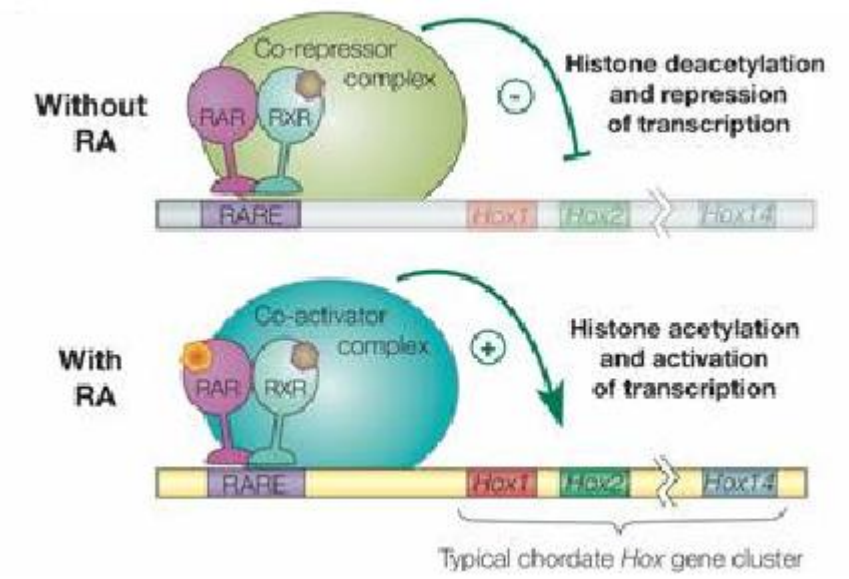


Figure 1.4 Modulation of RA induced gene activity by the RAR. The RAR/RXR heterodimer mediated the effects of RA. In the absence of ligand (RA), the heterodimer complex is bound to DNA at its retinoic acid response element (RARE) and also bound to co-repressors. This complex induces transcriptional repression through histone deacetylation. Binding of ligand (RA) induces a conformational change that leads to binding of co-activators and histone acetylation, which induces gene activation. Hox genes are targets of RA induced activity during mammalian embryonic development. These genes are responsible for the determination of the basic structure and orientation of an organism (Marletaz , *et. al.*,2006).

An important regulatory mechanism that co-activators and co-repressors have is that they harbor histone acetylation/deacetylation enzyme activity. Acetylation of lysine residues on nucleosome tails results in the relaxation of the chromatin structure, allowing access of transcription factors to DNA and therefore, an

increase in transcriptional activity. Co-activators, such as CBP/p300, contain histone acetyl-transferase (HAT) activity while co-repressors recruit enzymes that have histone deacetylase (HDAC) activity. The level of acetylation is controlled by these two opposing families of enzymes, and ultimately, the activity of the RAR/RXR complex.

Retinoids and Cancer

Retinoids have been used as both chemopreventative and chemotherapeutic agents for the treatment of many types of cancers. Studies using Vitamin A-deficient animals showed that there was a strong connection between deficiency and the susceptibility to cancer (Chambon, 1994; Sporn, *et. al.,* 1976). The addition of retinoic acid to the media of various types of cancer cells grown in culture was able to significantly reduce proliferation (Niles, 2000; Bushue, *et. al.*, 2010). Retinoic acid can induce differentiation in tumor cells such as neuroblastoma (Sidell, 1982), melanoma (Wood, *et. al.*, 1990; Fligiel, *et. al.*, 1992; Jacob, *et. al.,* 1998; Niles, 2003), and promyelocytic leukemia (Breitman, *et. al.,* 1980). Retinoids are able to elicit their effects on malignant cell growth through the regulation of cell growth, differentiation, and apoptosis (Lotan, 1995; Bushue, *et. al.,* 2010; Mamede, *et. al.,* 2011).

There is evidence that demonstrates a connection between cellular transformation and altered expression of RARs. It is believed that the RAR-β2 receptor is critical for mediating the growth inhibitory effects of retinoids and its down-regulation is correlated with the loss of atRA's ability to inhibit cell growth

(Lotan, 1996; Sun, 2004; Pavan, *et. al.,* 2006; Bushue, *et. al.,* 2010). The expression of this receptor is down-regulated in head and neck cancer, melanoma, breast, and oral cancers (Lotan, 1995; Fan, *et. al.,* 2010). The reactivation of RAR-β2 expression leads to reacquisition of atRA's ability to inhibit cell growth (Xu, 2007), therefore it has been considered as a tumor suppressor gene. The silencing of RAR-β2 expression is due to epigenetic changes, mainly DNA methylation (Virmani, *et. al.,* 2001; Shaw, *et. al.,* 2006). Current research from our laboratory indicates that, while DNA methylation appears to be the predominant mechanism for silencing of RAR-β2 expression, there are other mechanisms by which this occurs, such as modulating the levels of acetylation of histones H3 and H4 (Fan, *et. al.,* 2010).

Retinoids and Melanoma

A number of synthetic and natural occurring retinoids are available for the treatment of many skin diseases, including skin cancer (Niles 2002). Several studies have shown that retinoids, especially atRA, are able to induce differentiation and growth arrest in both mouse and human melanoma cells (Jacob, *et. al.,* 1998; Fligiel, *et. al.,* 1992; Huang, *et. al.,* 2002; Niles, 2003). These effects can happen through a number of mechanisms. In B16 mouse melanoma cells, atRA inhibits both anchorage-dependent and –independent growth through inhibition of cell growth at the G1 phase of the cell cycle (Niles, 1987). It also stimulates the production of melanin and induces an increase in nerve growth factor receptors on the cell membrane. Through suppression of the

transformed phenotype, retinoids have also been shown to inhibit invasion and metastasis in human melanoma cells (Wood, *et., al.,* 1990; Jacob, *et. al.,* 1998).

Numerous studies have shown that retinoids have potential in the prevention and treatment of many cancers. The ability of retinoids to inhibit tumor growth may be related to changes in specific signaling pathways. However, the complete mechanism by which they exert their effects still remains poorly understood, and needs to be studied in greater detail.

PPARs

Peroxisome proliferator-activated receptors (PPARs) are a group of ligand-activated transcription factors and members of the nuclear hormone receptor superfamily that include the thyroid, Vitamin D, and retinoid receptors. There are three separate genes that give rise to the different isoforms of PPAR, termed PPARα, β/δ, γ. Due to differential splicing and alternate promoter usage there are three isoforms of gamma - γ_1, γ_2, γ_3 (Fajas, *et. al.,* 1998; Desvergne and Wahli, 1999; Roberts-Thomson, 2000). Each of the PPAR isoforms has a different tissue distribution, function, and to some extent, different ligand specificity. PPARα is expressed primarily in the liver, heart, kidney, skeletal muscle and brown adipose tissue. PPARβ/δ expression is ubiquitous, while PPARγ is primarily found in white and brown adipose tissue, large intestine and spleen (Desvergne and Wahli, 1999).

PPARs function as the body's fatty acid sensors, regulating genes involved in lipid and glucose metabolism (Green, 1995; Desvergne and Wahli, 1999; Escher

and Wahli, 2000; Berger and Moller, 2002). Endogenous ligands include palmitic, oleic, linoleic, arachidonic fatty acids, as well as the eicosanoids, including prostaglandins and leukotriene B_4. The synthetic ligands include the fibrate class of compounds used to treat hyperlipidemia and the thiazolidinediones that are used to treat type II diabetes (Roberts-Thomson, 2000; Michalik, *et. al.*, 2004).

Upon activation, PPARs form a heterodimer with the retinoid X receptor. This complex binds a specific sequence in the promoter region of target genes called a peroxisome proliferator response element (PPRE). This sequence is a direct repeat of a hexameric nucleotide sequence – AGGTCA – separated by one nucleotide, called a DR-1. This sequence is important in determining PPAR/RXR binding specificity (Isseman, *et. al.*, 1993; Roberts-Thomson, 2000). PPRE elements have been identified in the regulatory regions of many genes involved in carbohydrate and lipid metabolism (Tontonoz, *et. al.*, 1994; Tontonoz, *et. al.*, 1995, Schoonjans, *et. al.*, 1996; Fajas, *et. al.*, 1997; Kliewer, *et. al.*, 2001).

PPAR activation up-regulates genes involved in β and ω fatty acid oxidation, such as acyl CoA oxidase and cytochrome P450 isotypes (Tugwood, *et. al.*, 1992; Aldridge, *et. al.*, 1995). These receptors are also the target for the fibrate class of drugs used to treat hyperlipidemia (Forman, *et. al.*, 1997; Kliewer, *et. al.*, 2001). PPARγ activation is involved in adipocyte differentiation, as well as glucose and fatty acid storage (Rosen, *et. al.*, 1999; Rosen and Spiegelman, 2000; Rosen, *et. al.*, 2000). This receptor is the target for the thiazolidinedione drugs that are used as insulin sensitizers for the treatment of type II diabetes (Kletzien, *et. al.*, 1992,

Rosen and Spiegelman, 2000; Kliewer, *et. al.*, 2001, Rosen and Spiegelman, 2001). The specific role of PPARβ/δ is not defined, but emerging evidence suggests that it may play a role in other processes, such as promotion of lipid accumulation in macrophages (Nagy, *et. al.*, 1998; Tontonoz, *et. al.*, 1998; Vosper, *et. al.*, 2001), differentiation of epithelial cells (Rosenfield, *et. al.*, 1999; Matsuura, *et. al.*, 1999), keratinocytes (Westergaard, *et. al.*, 2001), adipocytes (Bastie, *et. al.*, 1999; Bastie, *et. al.*, 2000; Hasen, *et. al.*, 2000), cholesterol homeostasis (Leibowitz, *et. al.*, 2000; Oliver Jr., *et. al.*, 2001), bone metabolism (Mano, *et. al.*, 2000), skin wound healing (Michalik, *et. al.*, 2001; Wahli, 2002), and embryo implantation and decidualization (Lim, *et. al.*, 1999). PPARβ/δ can be activated by many of the compounds that activate PPARα (Roberts-Thomson, 2000). Specific endogenous ligands that activate this receptor remain unknown, although there are data to support that the arachadonic acid metabolite, prostacyclin, can act as an endogenous ligand for PPARβ/δ (Shao, *et. al.*, 2002; Michalik, *et. al.*, 2003).

Aberrant expression of PPAR receptors has been found in several types of cancers. These include colon, prostate, lung, skin, breast, and liposarcomas (Sarraf, *et. al.*, 1995; Tontonoz, *et. al.*, 1997; Elstner, *et. al.*, 1998; Mueller, *et. al.*, 1998; Mueller, *et. al.*, 2000; Motomura, *et. al.*, 2000; Thuillier, *et. al.*, 2000; Suchanek, *et. al.*, 2002; Gupta, *et. al.*, 2004). Involvement of PPARα in tumorigenesis is thought to be through induction of cell cycle regulatory genes such as CDK-1 and 4 and c-myc (Peters, *et. al.*, 1998; Roberts-Thomson, 2000). Furthermore, there is evidence to suggest that this receptor is involved in

suppression of apoptosis, which also promotes carcinogenesis (Desvergne and Wahli, 1999; Roberts-Thomson, 2000). PPARβ/δ has been found to be over-expressed in many human and rodent colorectal cancers and the expression of this receptor has been shown to be regulated by the adenomatous polyposis coli (APC)/β-catenin/TCF-4 pathway (He, *et. al.,* 1999; Gupta, *et. al.,* 2000; Park, *et. al.,* 2001). PPARγ has powerful effects on cellular differentiation and is thought to act as a tumor suppressor gene (Sarraf, *et. al.,* 1999; Ikezoe, *et. al.,* 2001). Activation of PPARγ causes inhibition of cell growth and promotes differentiation in many epithelial derived cancer cell lines (Sarraf, *et. al.,* 1995; Tontonoz, *et. al.,* 1997; Elstner, *et. al.,* 1998; Mueller, *et. al.,* 1998; Kubota, *et. al.,* 1998; Mueller, *et. al.,* 2000; Chang and Szabo, 2000).

AKAP12

A kinase anchoring proteins (AKAPs) are a class of structurally diverse but functionally similar scaffolding proteins. They were discovered due to their ability to bind the regulatory subunit of Protein Kinase A (PKA) (Rubin, 1994; Dell'Aqua and Scott, 1997; Colledge and Scott, 1999; Feliciello, *et. al.,* 2001; Carlisle-Michel and Scott, 2002). The ability of AKAPs to bind PKA allows the holoenzyme to be sequestered at specific regions within the cell where it is exposed to increased levels of cAMP. This allows more efficient activation of the enzyme and more precise substrate selection. AKAP proteins are involved in assembling multi-protein signaling complexes that function to promote efficient

and specific enzyme/substrate reactions in a spatial and temporal manner (Gelman, 2002).

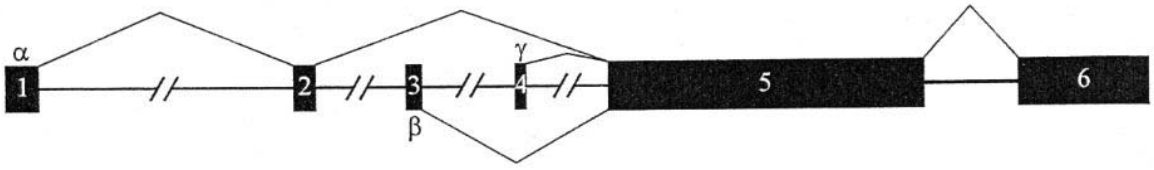


Figure 1.5. Organization of the AKAP12 gene. The generic gene structure of mammalian AKAP12 locus. The arched lines connecting the exons represent the splicing of the three mRNAs encoded by the AKAP12 gene (Streb, *et., al.*, 2004).

Human AKAP12 is also known as AKAP250 or Gravin. Gravin was identified as the target of an autoantibody in patients with myasthenia gravis and targets the PKA binding domain of AKAP12 (Nauert, *et. al.*, 1997). The rodent orthologue is termed SSeCKS (Src-Suppressed C Kinase Substrate) and was discovered in rat fibroblasts to be down-regulated upon transformation by oncogenic forms of Src and Ras (Lin, *et. al.*, 1996). The hAKAP12 gene is located on chromosome 6q24-25.2, which is a deletion hotspot in advanced prostate, breast and ovarian cancer (Noviello, *et. al.*, 1996; Cooney, *et. al.*, 1996; Tibilette, *et. al.*, 2000; Tibilette, *et. al.*, 2001; Gelman, 2002; Abdollahi, *et. al.*, 2003; Gorringe, *et. al.*, 2009). Therefore, it is believed that the AKAP12 locus may encode a tumor suppressor gene (Lin, *et. al.*, 2000). The AKAP12 gene is approximately 100kb long and contains 6 exons. There are 3 distinct gene products, AKAP12α, β, and γ, each with their own promoter allowing their expression to be independently regulated.

AKAP12α is the main isoform and the protein contains a myristoylation sequence that can target it to the cell membrane or endoplasmic reticulum. In addition to binding PKA, AKAP12 proteins also have docking sites for many other kinases and adaptor molecules. These include protein kinase C, phosphatase 2B, calmodulin, cyclin D and F-actin (Gelman, 2002). Mechanisms regulating AKAP12 function are still being explored.

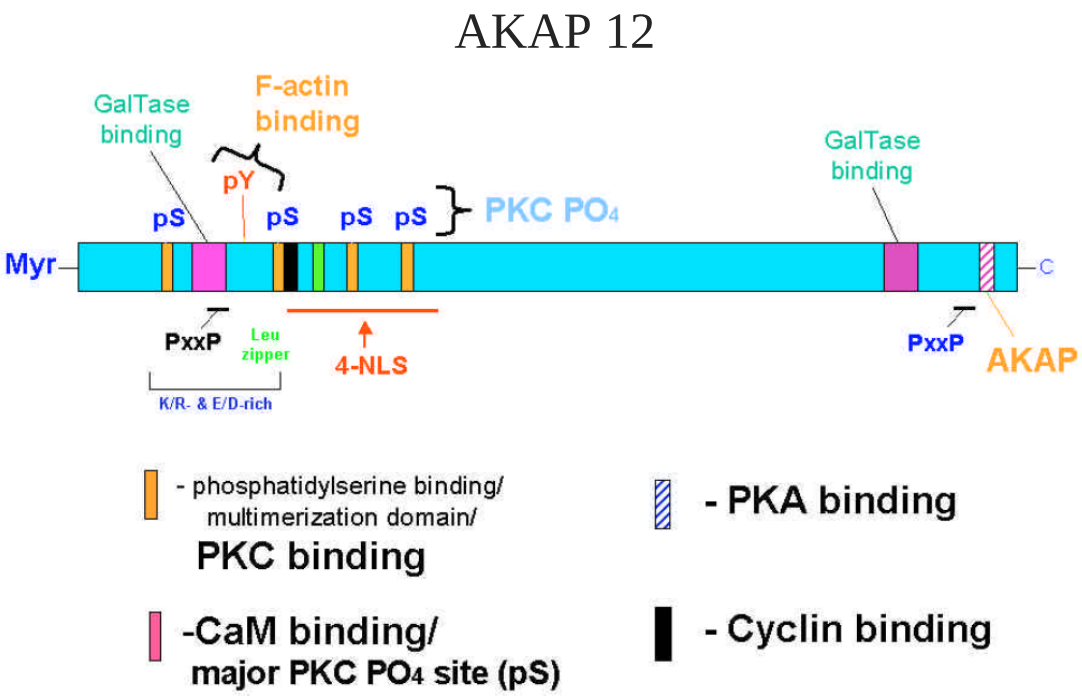


Figure 1.6. Structure of the AKAP12 scaffolding protein. AKAP12 contains several PKC phosphorylation sites (pS) as well as various protein binding domains, including protein kinase A (PKA), protein kinase C (PKC), calmodulin (CaM), cyclin D, F-actin, and GalTase (β1,4-galactosyltransferase) a

transmembrane receptor for laminin. PxxP represents potential SH3 binding sites. AKAP12 also encodes several nuclear localization sites (NLS) (Gelman, 2002).

The over-expression of AKAP12/SSeCKS in NIH3T3 cells causes inhibition of cell growth by promoting G1 phase arrest through binding and regulation of cyclin D activity (Lin, *et. al.*, 2000; Gelman, 2002). It also promotes re-organization of the actin-based cytoskeleton that induces cell flattening (Nelson and Gelman 1997; Gelman, *et. al.*, 1998, Gelman, *et. al.*, 2000; Xia and Gelman, 2002; Cheng, *et. al.*, 2007). This restructuring seems to be modulated through focal adhesion kinase-dependent tyrosine phosphorylation of AKAP12 (Xia and Gelman, 2002). In addition, re-expression of AKAP12 causes a down-regulation of proliferative and pro-angiogenic gene expression, such as VEGF (Su, *et. al.*, 2006; Liu, *et. al.*, 2006), and also inhibits podosome formation and metastatic progression in human prostatic cancer (Lin and Gelman, 1997; Xia, *et. al.*, 2001; Gao and Gelman, 2006). Further evidence for the involvement of this protein in cancer is provided by the SSeCKS null mice that develop prostatic hyperplasia (Akakura, *et. al.*, 2008).

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Rationale for Thesis Projects

Melanocytes play a very important role in the normal physiology of the skin. These cells produce the pigment melanin, which not only affects the visible color of our skin, but also functions as a photoprotective substance that is able to dissipate more than 99.9% of absorbed UV radiation as heat. UV radiation is a potent DNA damaging agent and is a causation of skin carcinogenesis. UV-induced DNA damage in primary cultures of melanocytes showed an increased frequency of pyrimidine dimers, which are signatures of UV mutation (Mouret, *et. al.*, 2012). The genes that are principal targets of mutations in melanoma are commonly oncogenes and tumor suppressor genes. The most frequent mutations include genes that cause constitutive activation of RAS and RAF proteins, $p16^{INK4a}$ gene deletions, mutations in the melanocortin 1 receptor (MC1R), PTEN, and PI3K genes. Mutations in these genes can change the expression and/or activity of transcription factors. The transcription factor AP-1, as well as the ligand activated nuclear receptors RARs, RXRs and PPARs have exhibited altered expression and activity in melanoma cells.

The purpose of my thesis research was to study specific genes and proteins that changed during melanoma progression. Specifically, I was interested in determining whether these altered pathways could be ameliorated by the dietary component Vitamin A resulting in cancer cell differentiation and loss of malignancy. I focused initially on a family of ligand activated nuclear receptors, the PPARs, due to their interaction with RXRs. Subsequently, I studied the gene AKAP12, which was identified through microarray analysis as exhibiting the

highest degree of stimulation in atRA-treated melanoma cells. The following chapters explain the results from these two studies.

Chapter 2: PPARα/γ Expression and Activity in Mouse Melanocytes and Melanoma Cells

The information presented in this chapter is the author's contribution to the following manuscript "PPARα/γ Expression and Activity in Mouse and Human Melanocytes and Melanoma Cells." This manuscript was accepted for publication in *Pharmaceutical Research* (2008) 25:1326-1333. Reprinting for dissertation is part of the author's rights and permission is not required from Springer Science Business Media, LLC.

Abstract

The purpose of this research was to examine the expression of PPARs and the effects of PPARα and PPARγ agonists on the growth of mouse melanocytes and melanoma cells. Expression of PPARα, β, and γ mRNA levels in melan-a mouse melanocytes and B16 mouse melanoma cells was assayed quantitatively by RT-PCR, while quantitative PPARα mRNA levels were measured by QuantiGene assay. The effects of natural (PGJ2, leukotriene B4) and synthetic (ciglitazone, troglitazone, WY14643) PPAR ligands on cell growth were also examined, while PPAR transcriptional activity was determined by a PPRE-reporter gene assay. B16 melanoma cells produced more PPARα and PPARγ protein than melan-a melanocytes. However, B16 PPARα mRNA levels were similar to those of melan-a melanocytes. The PPARγ-selective agonists were able to significantly inhibit the cell growth of both melan-a melanocytes and B16 mouse melanoma cells, while PPARα-selective agonists had limited effects on melanoma growth. The increased expression of PPARα and γ protein in B16 cells compared to melan-a melanocytes may be a common occurrence in melanoma, however, its biological significance remains to be determined. Selective PPARγ agonists may prove useful to arrest the growth of some melanomas.

Introduction

PPARs are members of the nuclear receptor superfamily of ligand-activated transcription factors. There are three subtypes-PPARα, PPARβ/δ, and PPARγ.

Endogenous fatty acids and synthetic agonists activate these receptors. Upon activation, PPARs regulate genes involved in lipid and glucose homeostasis (Green, 1995; Eschler and Wahli, 2000; Berger and Moller, 2002). These receptors also regulate normal and disease related processes, including embryo implantation, inflammation, cell cycle progression, differentiation and tumorigenesis (Berger and Moller, 2002).

Activation of PPARs decreases cell growth and induces differentiation in many cancer cell lines (Mueller, *et. al.*, 1998; Kawa, *et. al.*, 2002; He, *et. al.*, 2005). However, there is little information on the PPAR subtypes and relative levels of PPAR expressed in melanocytes and melanoma cells (Kang, *et. al.*, 2004). We therefore characterized the types of PPARs, level of PPARα and PPARγ mRNA and protein, and the ability of PPAR-selective agonists to inhibit proliferation in mouse melanocytes and melanoma cells.

Materials and Methods

PPAR Agonists

Leukotriene B_4 was obtained from Sigma Chemical Co. (St. Louis MO). Ciglitazone, 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ2), and WY14643 were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Troglitazone was obtained from Axxora, LLC (San Diego, CA). A stock solution of LTB_4 was made using 102 μg/ml in 100% ethanol and diluted to 0.01, 0.05 and 0.1 μM concentrations in DMEM complete media. For ciglitazone, troglitazone,

and WY14643, 10^{-2}M stock solutions were made using DMSO and diluted to 1, 5, and 10 μM concentrations in DMEM complete media.

Cell Culture

B16 mouse melanoma cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO/Invitrogen, Carlsbad, CA), as described previously (Boskovic, 2004). Melan-a cells (Dr. D. Bennett, Department of Anatomy and Developmental Biology, St. George's Hospital Medical School, London, England) were grown in RPMI 1640 medium as previously described (Boskovic, 2004). Cells were maintained in a 10% CO_2/90% air humidified atmosphere. Both the B16 (ATCC, Manassas, VA) and melan-a cell lines were derived from the C57/BL6 mouse.

Western Blots and Antibodies

Equal amounts of protein (50μg) were separated using 7.5% SDS-PAGE gels and transferred to blotting membranes. The blots were blocked with 5% non-fat dry milk overnight at 4°C and probed with the following antibodies: monoclonal mouse PPARα for mouse melanocytes and melanoma 1:250 (Affinity Bioreagents, Golden CO); and polyclonal PPARγ2 1:500 (Cell Signaling Technology, Beverly, MA). Secondary antibodies were polyclonal HRP-linked anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) and monoclonal anti-mouse IgG (Amersham, Chicago, IL) used at 1:3000. Blots were developed with

ECL reagents (Amersham, Chicago, IL), and the chemiluminescent signal quantified with a Chemi-doc Imager (Bio-Rad).

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted using Tri-Reagent (Sigma Chemical Co.) and reverse transcribed using the RT for PCR kit (Clontech, Palo Alto, CA). PCR was performed using the following primers: mouse; PPARα forward 5'-AGCTGGTGTAGCAAGTGT-3', reverse 5'-TCTGCTTTCAGTTTTGCTTT-3' product 163 bp; PPARβ/δ forward 5'-CCCGGGAAGAGGAGAAAGAG-3', reverse 5'-AAAGCGGATAGCGTTGTGC-3' product 402 bp; and PPARγ2 forward 5'-CCAGTGTGAATTACAGAAATCTCTGTTTTATGCTG-3', reverse 5'-AGAACGTGATTTCTCAGCC-3' product 120 bp. The PCR was performed with the AccuPrime *Taq* DNA Polymerase System (Invitrogen) with the following conditions: PPARα and PPARγ, 40 cycles of 94° C, 30 s; 65° C, 30 s; 68° C, 1minute; PPARβ/δ, 40 cycles of 94° C, 30 s; 65° C, 30 s; 68° C, 1 minute. PCR products were electrophoresed through 1% agarose gels and visualized with ethidium bromide.

QuantiGene Assay

RNA from cells was extracted with Tri-Reagent. QuantiGene assays (Panomics, Inc., Freemont, CA) were performed according to the manufacturer's protocol. Briefly, 2 μg of RNA in 10 μl was loaded into each well of a 96 well plate and incubated with PPARα, β-actin target probes, in conjunction with

dendrimer DNA amplifier and labeled probes at 56° C overnight. Following several washes with QuantiGene buffer, chemiluminescent substrate was added to the wells and incubated at 56° C for 30 minutes. The reaction signals (relative light units – RLU) were measured using a Centro LB 960 luminometric plate reader (W. Nuhsbaum Inc., McHenry, IL).

Reporter Gene Assays

B16 cells were seeded at a density of $3x10^5$ per 60 mm tissue culture dish. Each assay was performed in triplicate. The mouse melanoma cells were transfected with β-galactosidase (β-galactosidase Enzyme Assay System, Promega Corp., Madison, WI); pGL-2x-PPRE-Luciferase reporter plasmid (Dr. John Capone, Department of Biochemistry, McMaster University, Hamilton, ON, Canada); pSV sport PPARα or pSV sport-PPARγ expression vectors (Dr. Bruce Spiegelman, Dana-Farber Cancer Institute, Boston, MA). The plasmid DNA ratio was 1:10:10 respectively. Lipofectamine plus reagent kit was used to transfect the cells (Invitrogen). After transfection, media, serum and appropriate agonists were added to the cells. Cells were harvested after 48h, and assayed for β-galactosidase and luciferase activity using kits from Promega Corp. Chemiluminescence was determined using a Lumat LB 9501 Luminometer. Transfection efficiency was corrected by measuring the amount of β-galactosidase activity.

Statistics

Samples for each experiment were performed in triplicate (n=3). Biological repeats for each experiment were performed at least three times, or more if necessary. Statistical significance determined using the SigmaStat analysis program. Sample comparisons were made using One Way ANOVA (analysis of variance). Probability of significance was determined using the Student-Knewman Keuls t-test. Standard error bars are shown.

Results

Expression of PPAR subtypes

The expression of PPAR subtypes in mouse melan-a melanocytes and B16 mouse melanoma cancer cells was examined using RT-PCR. Figure 2.1 shows that mouse melanocytes and melanoma cells express mRNA for PPARα, PPARβ/δ, and PPARγ2.

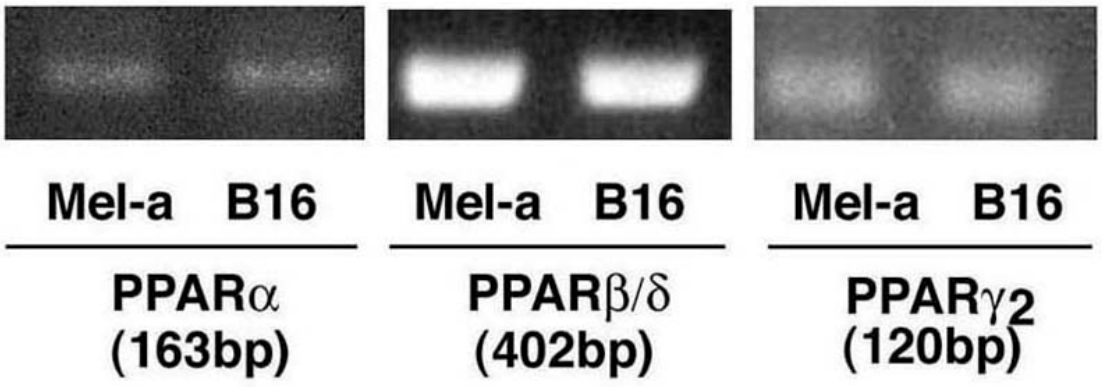

Figure 2.1. Expression of PPAR subtype mRNA in mouse melanocytes and melanoma cells. RNA was isolated, purified and reverse transcribed using the Advantage RT-for PCR kit (Clontech, Palo Alto, CA). PCR analysis was performed using the specific primers for each subtype listed in the "Materials and Methods".

Because RT-PCR provides only qualitative results, we also measured the relative amount of PPARα and PPARγ protein and PPARα mRNA in these cells using Western blotting and QuantiGene assays respectively. Unfortunately, we could not Quantitate PPARγ mRNA levels because there are no capture probes for this RNA species. Results show that B16 melanoma cells expressed higher levels of PPARα (52 kD) and PPARγ (56 kD) protein compared to non-malignant melan-a cells (Figure 2.2). Quantitation of the immunoblots revealed that B16 mouse melanoma cells have threefold more PPARα and 40% more PPARγ protein than melan-a mouse melanocytes (Figure 2.2A and B).

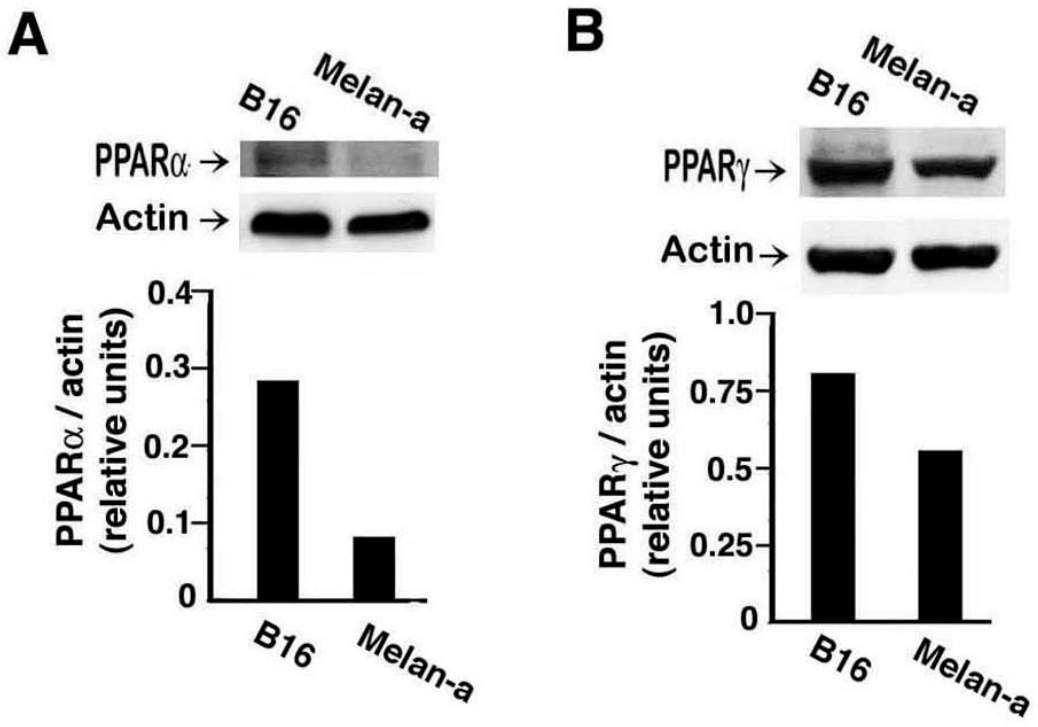

Figure 2.2. Expression of PPAR subtype protein in mouse melanocytes and melanoma cells. Proteins were separated and PPARs detected by selected antibodies as outlined in "Materials and Methods". Relative amounts of PPARα and PPARγ were determined by densitometry (bar diagram) and corrected for the amount of actin in each sample. **A** and **B** represent PPARα and PPARγ protein expression respectively in mouse melan-a melanocytes and B16 melanoma cells. A representative analysis is shown. The experiments were repeated three additional times with the same qualitative results.

QuantiGene assays (Panomics) revealed that mouse B16 melanoma cells had 60% less PPARα mRNA compared to the melan-a melanocytes (Figure 2.3). This is in stark contrast to the level of PPARα protein, which is threefold higher

in B16 relative to melan-a cells. While there is not a strong correlation between mRNA and protein levels for this receptor in the mouse melanoma cells, it does suggest that the expression of PPARα it regulated at the translational level.

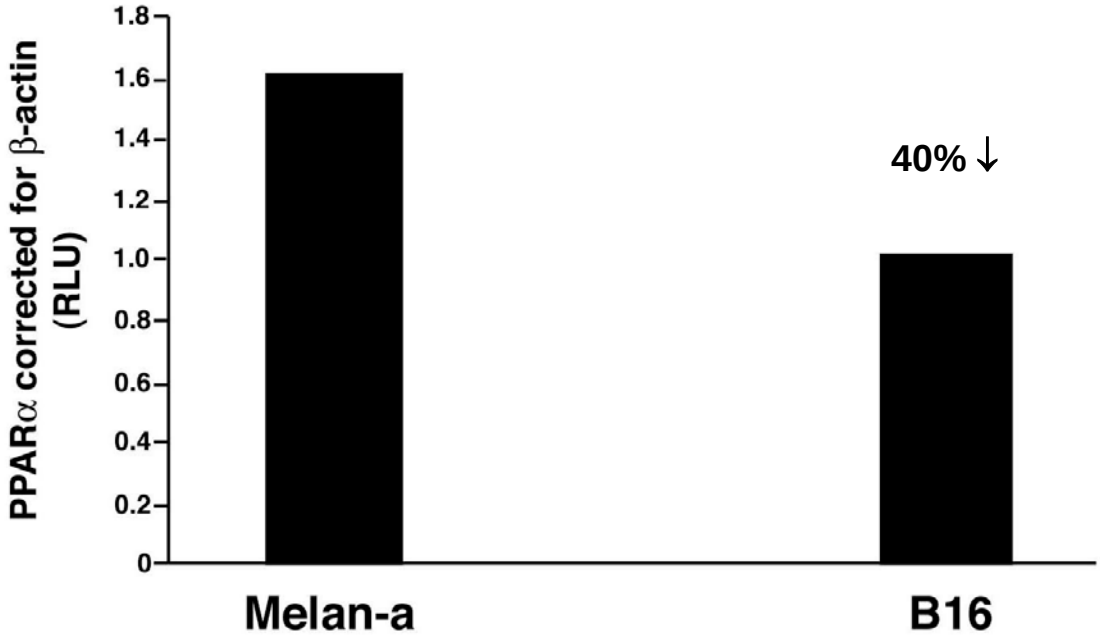

Figure 2.3. Quantitation of PPARα mRNA levels in mouse melan-a melanocytes and B16 melanoma cells. The QuantiGene assay system was used to determine relative amounts of PPARα mRNA. Cell lysates were loaded into wells of a QuantiGene capture plate and hybridized to probes specific for mouse PPARα mRNA. A representative experiment representing the ratio of PPARα expression, corrected for β-actin is shown. This experiment was repeated twice with similar results.

Effect of PPARα and PPARγ Agonists on Cell Proliferation

We measured the effect of PPAR-selective agonists on melanocyte and melanoma cell proliferation. Agonist concentrations were chosen that would maximize receptor subtype specificity. The natural PPARγ agonist, PGJ2, inhibited the growth of both melan-a melanocytes and B16 melanoma cells (27% and 33% respectively) (Figure 2.4A). The synthetic PPARγ agonist ciglitazone also inhibited the growth of melan-a and B16 cells (33% and 22% respectively) (Figure 2.4B), while troglitazone, a synthetic and highly selective PPARγ agonist, was only able to inhibit the growth of B16 melanoma cells (36%) (Figure 2.4C).

The synthetic PPARα agonist, WY14643, had no significant effect on proliferation of melan-a and B16 cells (Figure 2.4D), while the natural PPARα agonist LTB_4, inhibited the growth of the melan-a cells but not B16 cells (Figure 2.4E).

Figure 2.4A.

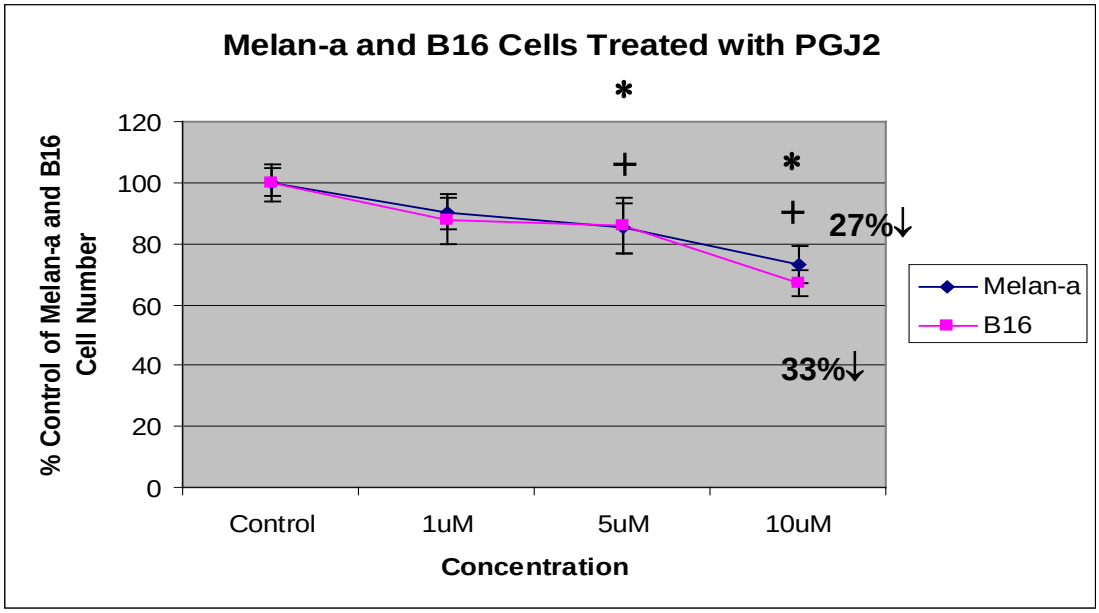

Figure 2.4B.

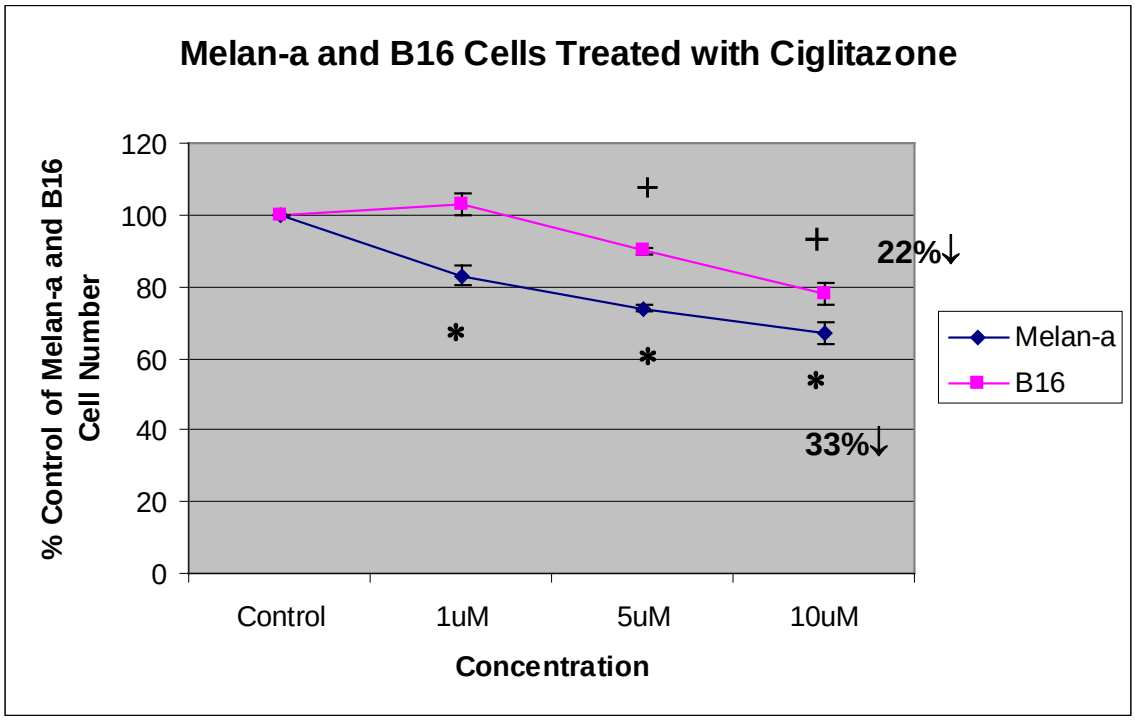

Figure 2.4C.

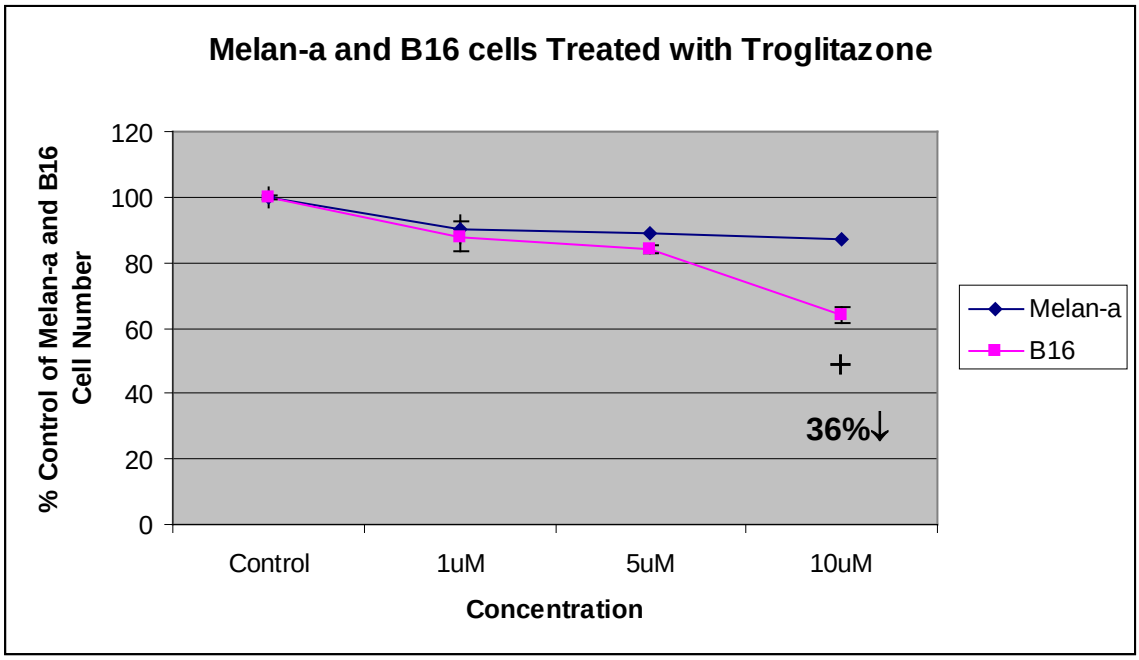

Figure 2.4D.

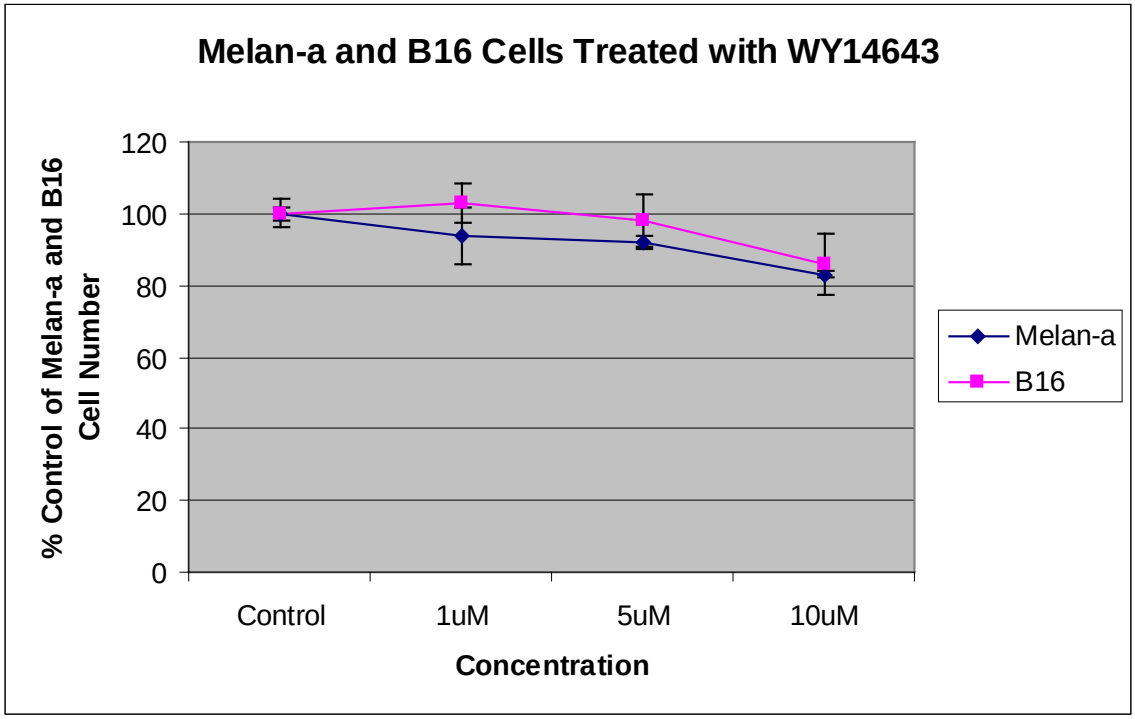

Figure 2.4E.

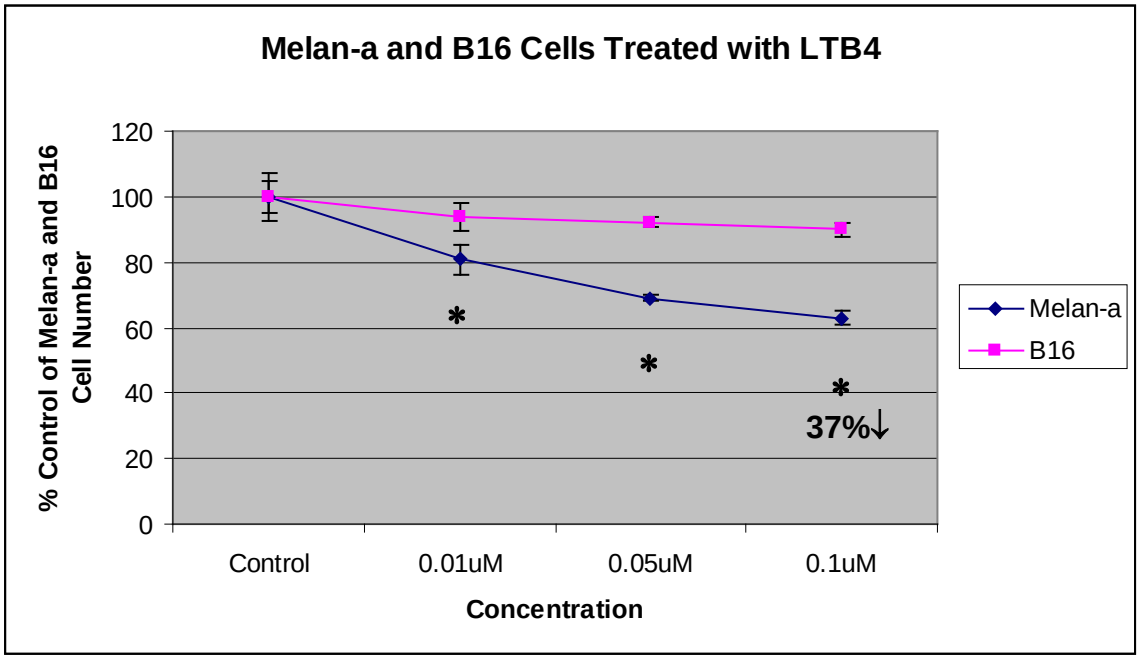

Figure 2.4. Effect of PPAR agonists on mouse melan-a melanocytes and B16 melanoma cell proliferation. (**A,** PGJ2; **B,** ciglitazone; **C,** troglitazone; **D,** WY14643; and **E,** LTB_4) Triplicate dishes of cells were treated with the indicated concentration of agonist for 48h (previously determined to be the most effective treatment time), then harvested and cell number determined by cell counts with a hemocytometer. Data are presented as cell number after 48h treatment with appropriate agonist. The *error bars* represent the SEM of triplicate dishes at each concentration of agonist tested. All experiments were repeated a minimum of three times with similar results. (**∗** = Melan-a; **+** = B16; significantly different relative to control cells $p<0.05$)

PPAR Trans-Activation

PPAR agonists have non-receptor-mediated effects. For example, PGJ2 alters NF-κB activity (Okano, *et. al.*, 2003). Therefore, we compared the concentration-dependent ability of the three PPARγ agonists PGJ2, Ciglitazone, and Troglitazone, as well as the PPARα agonist WY14643, to stimulate PPRE reporter gene activity in B16 mouse melanoma cells and correlated this activity with their ability to inhibit the growth of the same cell line. B16 cells were co-transfected with either the PPARγ or PPARα expression plasmid since endogenous PPRE reporter gene activity was too low to be accurately detected. The PPARγ agonists, PGJ2 and ciglitazone stimulated PPRE reporter gene activity in a dose dependent manner (Figure 2.5A and B). There was also significant reporter gene activity when B16 cells were treated with troglitazone or

WY14643 (Figure 2.5C and D). The ability of these agonists to stimulate the reporter gene correlated with their ability to inhibit B16 cell proliferation (Figure 2.4). However, the amount of growth activity was small (22-36%), relative to the increase in reporter gene activity, which ranged from 2-fold (WY), to 5-fold (Cig).

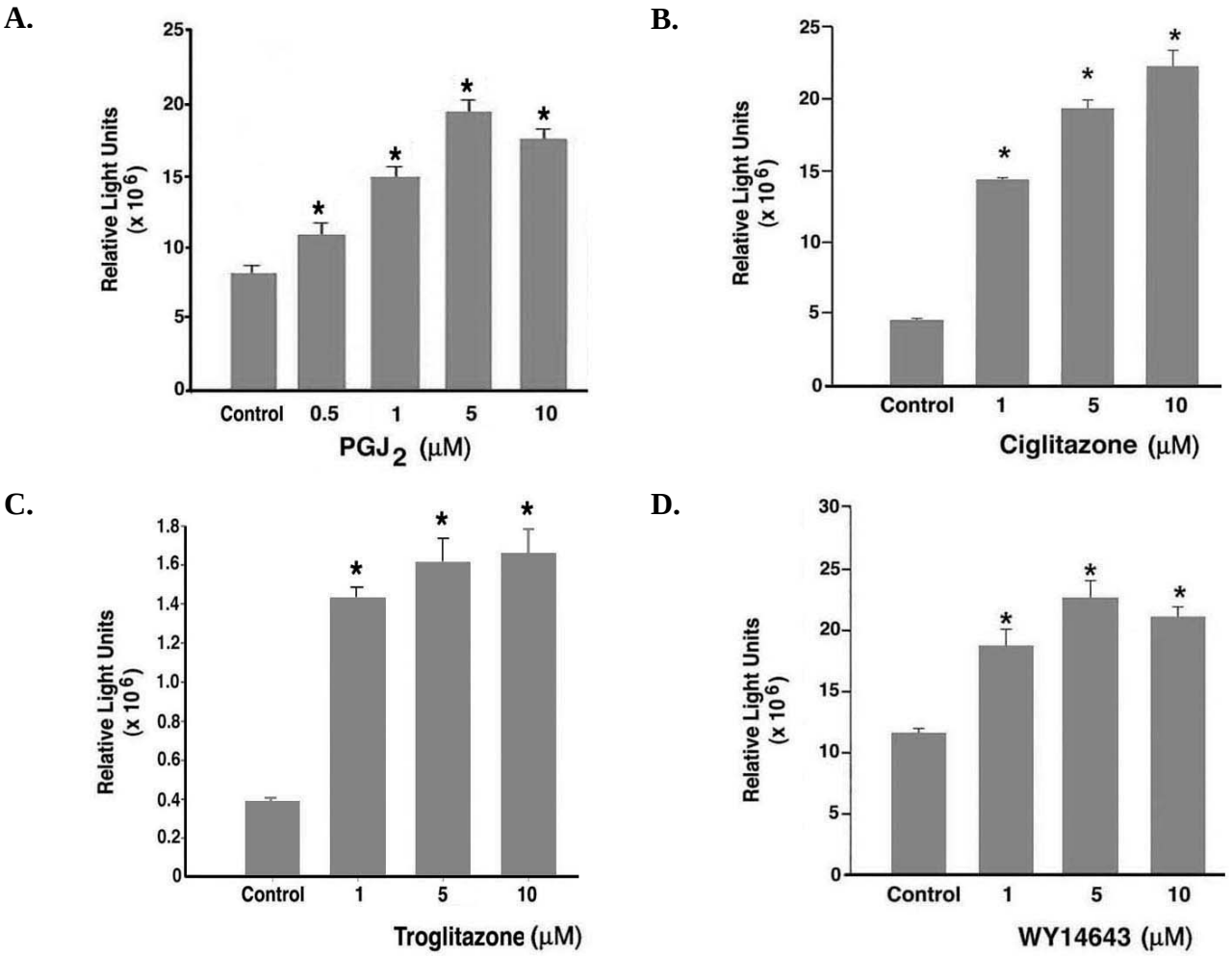

Figure 2.5. Ability of PPAR agonists to increase PPRE reporter gene expression. A β-galactosidase expression plasmid and a 2x PPRE pSG5-luciferase plasmid were transiently co-transfected into B16 cells. The transfection mixture also

contained either pSV-PPARγ (A, B, and C) or pSV-PPARα (D), with a plasmid DNA ratio of 1:10:10 respectively. Cells were harvested and assayed for β-galactosidase and luciferase activity 48h after agonist addition. The error bars represent the SEM of triplicate dishes of transfected cells. The experiment was repeated three times with similar results. (∗$p<0.001$) Significantly different relative to transfected cells without agonist. (**A** PGJ2; **B** Ciglitazone; **C** Troglitazone; **D** WY14643).

Discussion

Aberrant expression of PPARs has been found in several types of cancers such as colon, prostate, lung, skin, breast, and liposarcomas (Tontonoz, *et. al.*, 1997; Inoue, *et. al.*, 2001; Suchanek, *et. al.*, 2002; Segawa, *et. al.*, 2003; Aung, *et. al.*, 2005; Nijsten, *et. al.*, 2005). Overexpression of PPARα might contribute to tumorigenesis through its ability to stimulate proliferation by enhancing expression of genes encoding CDK-1 and CDK-4 and c-myc (Miller, *et. al.*, 1996; Zang, *et. al.*, 2006). In contrast, expression and activation of PPARγ is reported to stimulate differentiation and inhibit cell proliferation of several carcinoma-derived cell lines (Mueller, *et. al.*, 1998; Kawa, *et. al.*, 2002; Park, *et. al.*, 2005). In the skin, PPARα is involved in maintaining epidermal barrier function as well as differentiation and wound healing (Di-Poi, *et. al.*, 2004; Michalik, *et. al.*, 2005). The types of PPAR expressed in normal melanocytes and whether their expression is altered in melanoma cells has not been well studied. We found that

all of the PPAR subtypes, including PPARβ/δ are expressed at the mRNA level by mouse melanocytes and melanoma cells. Quantitative assay of mRNA levels revealed that melan-a melanocytes had more PPARα mRNA than B16 melanoma cells. In contrast, B16 cells had much higher amounts of PPARα protein relative to melan-a melanocytes. These findings suggest there is significant regulation of PPARα at the protein level, possibly through efficiency of translation. The amount of PPARγ was also higher in B16 melanoma cells. Unfortunately, due to a lack of a PPARγ capture probe for the QuantiGene assay, we could not quantitatively compare the mRNA and protein levels for this PPAR subtype to see whether it also might be subjected to regulation at the protein level.

Several groups have reported that PPARγ-selective ligands can inhibit the growth of human melanoma cells (Plachaa, *et. al.,* 2003; Freudlsperger, *et. al.,* 2006; Lee, *et. al.,* 2007). However, in several of these reports, significant inhibition of proliferation did not occur until 20 μM or higher concentrations of ligand. Many natural and synthetic PPAR ligands lose their selectivity at these concentrations (Selmandi, *et. al.,* 2005). We therefore limited our ligand concentrations to a maximum of 10 μM. While the effects on cell growth were significant, the compounds were only moderately effective at inhibiting proliferation using this concentration.

It is curious that B16 mouse melanoma cells express high levels of both PPARα and γ relative to their non-transformed counterpart, melan-a melanocytes, yet are either not growth inhibited by receptor-selective ligands or their inhibition is similar to normal melanocytes. One possible reason for this may be due to

changes in expression or activity of PGC-1α (PPARγ Co-activator 1α). This cofactor is involved in regulating the activity and binding specificity of PPAR receptors to their response elements. This process is important for activation of specific target genes. The reduction of PGC-1α expression has been implicated in type II diabetes; however, I found very little information on PGC-1α expression and activity in melanoma.

Because it has been reported that PPAR ligands can have receptor-independent effects (Okano, *et. al.*, 2003; Chaffer, *et. al.*, 2006), we compared their concentration-dependent ability to activate PPRE reporter gene activity with their concentration-dependent ability to inhibit the growth of B16 mouse melanoma cells. In general, we found a good correlation between these two activities in B16 cells. However, the degree of growth inhibition did not correlate as well with the level of gene activity. For the reporter gene assays, we co-transfected plasmids encoding the PPARγ and PPARα proteins into the B16 cells, since it was difficult to detect endogenous ligand-activated reporter gene activity. Therefore, it is possible that the endogenous PPARα and γ receptors in the B16 melanoma cells are defect in transcriptional activation despite being over-expressed relative to normal melan-a melanocytes. One reason for this may be due to the constitutive activation of the MAPK pathway. The B16 cells contain an NRAS mutation that drives the activation of ERK1/2. The phosphorylation of specific serines in the N-terminal region, regulate the activity of the receptors in a ligand-independent manner. This leads to a change in its conformation and a decrease in its transcriptional activity.

Conclusion

In summary, we have found that mouse melan-a melanocytes and B16 melanoma cells express PPARα, PPARβ/δ, and PPARγ2 receptors. The melanoma cells overexpress PPARα and PPARγ protein relative to melanocytes, however, there is a discordance between RNA and protein levels for these receptors. The ability of PPAR ligands, at concentrations where selectivity was maintained, to inhibit replication of these cells was variable and did not correlate as well with the expression level of the relevant PPAR. We did not find convincing evidence that PPARs are involved in development or maintenance of the melanoma phenotype as there was no observable change in their physical characteristics. However, PPARγ ligands at pharmacological concentrations might be useful in the treatment of certain melanoma lesions (Liu, *et. al.*, 2006).

Acknowledgments

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Rationale for Discontinuing PPAR Project and Initiating a New Project Involving Retinoic Acid and AKAP12

Data I acquired in the PPAR part of my thesis work were published together with data obtained by co-author Dr. Caroline Mills and her work with PPARs in human melanoma cells.

To ascertain the effect of PPARα down-regulation on melanoma cell biology, Dr. Mills transfected SK-mel28 human melanoma cells with siRNA to PPARα in order to down-regulate the expression of this gene. Even though QuantiGene assays determined that there was >80% knockdown of PPARα after 96 hours after transfection, there was no change in any biological parameter that was measured. Cell growth, morphology and viability of SK-mel28 melanoma cells were examined in these cells and no changes in the cell phenotype were observed. In addition, a known transcriptional target of PPARα, medium chain acyl CoA dehydrogenase (MCAD) protein was measured by Western blotting to determine whether PPARα knockdown decreased the amount of this protein. There was no change in MCAD protein levels between SK-mel28 cells transfected with PPARα siRNA or control siRNA, which could be because there was not a complete knockdown of protein expression. The amount of remaining protein would still be able to activate gene transcription. Also, these receptors have redundant functions; therefore, the PPARβ/δ or γ proteins may act as a substitute for PPARα. Due to the inability of PPARα downregulation to induce changes in the melanoma phenotype, we decided to end this project and search for another

avenue of research that might lead to an understanding of how retinoids can cause growth arrest and differentiation in responsive melanoma cells.

During this same time frame, our laboratory was using microarrays for gene expression profiling to determine the effect of atRA treatment on B16 melanoma gene expression. The results of the microarray analysis showed that after 48 hours of atRA treatment, there was one gene, AKAP12 whose expression was increased 28 fold relative to control cells. A thorough literature search did not yield any publications describing this gene or its regulation in melanocytes or melanoma cells. Therefore, with the permission and encouragement of my thesis advisor, Dr. Richard Niles, I initiated a study of the expression, atRA regulation and possible role of AKAP12 in melanoma.

Chapter 3: Retinoic Acid Regulated AKAP12 Expression in Mouse and Human Melanocytes and Melanoma Cells

Abstract

Retinoic Acid (*at*RA) is the most biologically active form of Vitamin A and inhibits the growth of mouse and human melanoma cells. Profiling of *at*RA regulated genes in B16 melanoma identified AKAP12, a scaffolding protein involved in assembling multi-protein signaling complexes, as the most highly induced gene at 48 h of *at*RA treatment. In this chapter I report that *at*RA also regulates AKAP12 expression in human melanocytes and melanoma cells and provide evidence for the involvement of AP-1 in the regulation of AKAP12 gene expression. Functional studies involving knockdown of AKAP12 expression using siRNA showed that AKAP12 was involved in the regulation of invasive and anchorage-independent growth properties. The basal mRNA and protein levels of AKAP12 differed among human melanocytes and six human melanoma cell lines. Also, AKAP12 mRNA and protein was differentially induced by *at*RA in the different human melanoma cell lines. There was a significant correlation between *at*RA induced increases in AKAP12 levels among the melanoma cell lines and the ability of *at*RA to inhibit proliferation. *In silico* analysis of the AKAP12 promoter region revealed 3 activator protein (AP-1) transcription factor binding sites. Since *at*RA stimulates AP-1 activity in B16 melanoma cells (Desai and Niles, 1997; Huang, *et. al.,* 2002), I examined whether AP-1 mediated the atRA induction of AKAP12. Reporter gene assays showed that *at*RA treatment

increased AP-1 activity in WM3248 and WM239 human melanoma cells. Co-transfection of these melanoma cells with an AP-1 reporter plasmid and a dominant-negative c-fos (A-fos) resulted in inhibition of *at*RA induced AP-1 activity. Unexpectedly, transfection of the A-fos plasmid into human melanoma cells enhanced rather than inhibited *at*RA induced AKAP12 expression. These data suggest that rather than mediating the stimulation of AKAP12 expression by atRA, AP-1 attenuates the induction of this gene.

Introduction

The most aggressive form of skin cancer is cutaneous malignant melanoma (CMM). Although it accounts for only 4% of all skin cancers, it is responsible for nearly 80% of the total number of skin cancer related deaths in the United States. The incidence of melanoma is steadily growing, becoming one of the fastest growing cancers worldwide (Boyle, 2011; www.cancer.org). Melanoma progression undergoes distinct phases (Urso, 2004; Melinkova and Bar-Eli, 2008). In the radial growth phase (RGP), the cancer cells spread horizontally and are confined to the epidermis. If detected during this phase of development surgical excision leads to a very high cure rate. In the vertical growth phase (VGP), the melanoma cells penetrate vertically through the basement membrane and invade the dermis and underlying tissues. The metastatic phase (Met) is characterized by the ability of melanoma cells to spread to distant organs and at this stage the disease is usually incurable. Melanoma is among the most aggressive tumor types because of its high capacity to metastasize and its resistance to treatment.

Currently, there are few effective therapies available for the treatment of metastatic melanoma (Lens, 2008; Boyle, 2011).

Retinoids are natural or synthetic derivatives of Vitamin A. All-*trans* retinoic acid (atRA) is the active metabolite of Vitamin A and inhibits growth and/or induces differentiation in a variety of tumors (Nettesheim, 1980; Garattini, *et. al.*, 2008), including mouse and human melanoma (Fliegel, *et. al.,* 1992; Desai, *et. al.*, 2000; Niles, 2003). Our laboratory studies the mechanism by which atRA exerts its biological effects in melanoma cells. Gene expression profiling during a 4-48 hour treatment of B16 mouse melanoma cells with atRA identified the gene coding for A-Kinase Anchoring Protein 12 (AKAP12) as the highest induced gene at the 48 hour time point.

AKAP12, also known as AKAP250 or Gravin, is a member of a family of structurally diverse but functionally similar scaffolding proteins. These proteins are involved in assembling multi-protein signaling complexes that function to promote efficient and specific enzyme/substrate reactions in a spatial and temporal manner (Gelman, 2002). The hAKAP12 gene is located on chromosome 6q24-25.2, which has been found to be a deletion hotspot in advanced prostate, breast and ovarian cancer, and may encode one or more tumor suppressor genes (Cooney, *et. al.,* 1996; Noviello, *et. al.,* 1996; Tibiletti, *et. al.*, 2000; Tibiletti, *et. al.*, 2001; Gelman, 2002; Abdollahi, *et. al.*, 2003; Gorringe, *et. al.,* 2009).

The objective for this study was to determine whether atRA also stimulated AKAP12 protein and RNA expression in human melanocytes and a series of

human melanoma cell lines established from different stages of malignant progression. I also examined the possible intermediary role of RA-induced AP-1 activity on AKAP12 gene regulation. AKAP12 was differentially expressed and induced by atRA in melanocytes and in six different melanoma cell lines. There was a significant correlation between atRA's ability to induce AKAP12 expression and its ability to inhibit growth. Unexpectedly, AP-1 was demonstrated to be a negative regulator of AKAP12 mRNA expression. Down-regulation of AKAP12 expression in mouse and human melanoma cells led to an increase in invasiveness and an increased ability to grow in an anchorage-independent manner.

Materials and Methods

Cell Culture

Samples of human epidermal neonatal lightly pigmented melanocytes (HEMn-LP) were obtained from Cascade Biologics, Portland, OR, and maintained in Medium 254 supplemented with 5 ml human melanocyte growth serum (HMGS) and 1 ml penicillin, streptomycin and amphotericin B (PSA) solution per 500 ml bottle. SbCl2 and WM3211 cells were isolated from radial growth phase melanomas, WM1366 and WM3248 were isolated from vertical growth phase melanomas, and WM9 and WM239 cells were isolated from metastatic melanomas. These cell lines were obtained from Meenhard Herlyn, D.V.M., D.Sc., Professor and Director of the Molecular and Cellular Oncogenesis Program at The Wistar Institute, Philadelphia, PA. Cells were grown as described (Mills,

et. al., 2009). The B16 mouse melanoma cells (ATCC, Manassas, VA) were grown in RPMI 1640 media (Gibco/Invitrogen, Carlsbad, CA) with 10% FBS and 1% pen/strep. All cells were maintained at 37º in cell culture incubators with a 5% CO_2/95% air humidified atmosphere.

Retinoic Acid

All-*trans* retinoic acid was purchased from Sigma Chemical Co. (St. Louis, MO). All experiments involving the use of RA were conducted under yellow light to prevent photo-oxidation of this retinoid. A 10 mM stock solution was prepared in DMSO. Control cells received the same volume of vehicle. Stock solution was diluted to the desired final concentration in tissue culture medium.

RNA Extraction and DNA Microarray Analysis

Control and 10 μM atRA treated B16 cells were harvested at 4, 10, 24, and 48 hours after treatment. RNA was extracted using TRI Reagent (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's suggested protocol. Microarray gene profiling was performed as previously described (Estler, *et. al.*, 2008). The calculated log ratios were compared for significant deviation from zero using one-class Significance Analysis of Microarrays (SAM) (Tusher, *et. al.*, 2001). SAM was performed with the maximum number of unique permutations available. Delta values were chosen to give a median False Discovery Rate of 10%. This setting allowed for the detection of RARβ expression, which is used as a control and is a direct target gene of RA.

QuantiGene Analysis

RNA from control and 10 μM atRA stimulated B16 cells was extracted using TRI Reagent (Sigma Chemical Co., St. Louis, MO). QuantiGene assays were performed according to the manufacturer's protocol. Briefly, 2 μg/10 μl RNA was loaded into each well of the 96 well plate and incubated overnight at 56° C with AKAP12 and β-actin target probes, in conjunction with dendrimer DNA amplifier. Following several washes with supplied buffer, chemiluminescent substrate was added to the wells and the plates incubated at 56° C for 30 minutes, and luminescence measured on a Centro LB 960 luminometric plate reader (W. Nuhsbaum Inc., McHenry, IL).

Western Blot Analysis

HEMn-LP, SbCl2, WM3211, WM3248, WM1366, WM239, and WM9 cells were treated +/- 10 μM atRA for 48 hours and harvested in lysis buffer containing Complete Mini Protease Inhibitor (Roche Diagnostics, Mannheim, Germany). Protein concentration was measured with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and 30 μg of total cellular protein from control and treated cells was separated on 5% gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA at 4° C overnight and then probed with the pAKAP12 antibody (1:500) (AbCam, Cambridge, MA) at 4° C overnight, followed by an incubation with HRP-linked anti-goat IgG (1:1000) (Vector Laboratories, Burlington, CA) for 1 hour at room temperature. Since this antibody was discontinued, Western blot analysis of

protein levels after siRNA transfection used a new pAKAP12 antibody (1:1000) (Novus Biologicals), followed by an incubation with HRP-linked anti-rabbit IgG (1:1000) (Cell Signaling). The membranes were stripped using Re-Blot Plus antibody stripping solution (Chemicon International, Temecula, CA). Next, the membranes were incubated with polyclonal β-Catenin antibody (1:500) (Sigma Chemical Co., St. Louis, MO) at 4°C overnight, followed by an incubation with HRP-linked anti-rabbit IgG (1:500) (Cell Signaling Technology, Beverly, MA). Blots were developed with ECL reagent (Amersham, Chicago, IL) and the chemiluminescent signal was quantified using a Chemi-Doc Imager (Bio-Rad). Protein loading was normalized using β-catenin expression. The use of 5% gels only retains proteins that have a molecular weight of approximately 70kD or higher. Most conventional proteins used to control for protein loading are lower than 70kD and therefore not appropriate for this type of gel. Levels of β-catenin protein expression are similar in each of the cell lines used and determined to be a good housekeeping protein for these Western Blots.

Quantitative RT-PCR

HEMn-LP, $SbCl_2$, WM3211, WM3248, WM1366, WM239, and WM9 cells were treated with +/- 10μM atRA for 48 hours. RNA was extracted using the RNeasy Mini kit (#74104, Qiagen, Valencia, CA). Purified RNA samples were dissolved in RNase–free water and quantified using a Nanodrop Spectrophotometer (Nanodrop Technology Inc., Wilmington, DE). Each sample had a 260/280 ratio of greater than or equal to 1.8. RNA quality was assessed by electrophoretic

analysis on an Agilent Model 2100 Bioanalyzer (Santa Clara, CA). All RNA samples used had RNA Integrity Numbers greater than 9. RNA was reverse transcribed to cDNA using an RT-for-PCR kit (Clontech, Palo Alto, CA) and random hexamer primers. QPCR analysis was performed using ABI TaqMan probes for human or mouse isoforms of AKAP12 and an 18S probe was used as a control. Assays were performed under conditions specified in the ABI TaqMan Gene Quantitation assay protocol. For functional assays using siRNA, RNA was isolated 96 and 120 hours after transfection using the method stated above. Data is expressed as ΔΔCT and normalized for 18S expression. This method (ΔΔCT) is used to measure relative changes in gene expression of a sample compared to that of a control. Experiments were repeated a minimum of three times.

Plasmid DNA Constructs

The pGL2-4X-AP-1 –luciferase reporter plasmid was constructed as previously described (Desai and Niles, 1997). The pCMV500 A-fos plasmid was kindly provided by Dr. Charles Vinson at the National Cancer Institute.

Transient Transfections and Reporter Gene Assays

B16 cells were seeded at a density of $1.5x10^5$, while WM3248 and WM239 cells were seeded at a density of $3x10^5$ in 60 mm tissue culture dishes. Two groups of three plates each were co-transfected with the β-galactosidase (Promega Corp., Madison, WI) and the AP-1 luciferase reporter plasmids, while an additional two groups of three plates each were co-transfected with the β-galactosidase, AP-1

reporter and A-fos plasmids. Fugene 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) was used to transfect cells at a ratio of 1:3, Fugene 6 to μg of DNA. After 5 hours of incubation with the transfection mixture, the media was changed to the regular growth media and the next morning 10 μM atRA was added to appropriate plates. Cells were harvested after 48 hours of atRA treatment and β-galactosidase and luciferase activity assayed (Promega Corp., Madison, WI). Chemiluminescence was measured using a Lumat LB 9501 Luminometer and corrected for transfection efficiency as estimated by β-gal activity.

siRNA Transfection

AKAP12 expression was knocked down by treating cells with siRNA directed against AKAP12. WM3248 and B16 cells were seeded at $3.5x10^5$ and $1.5x10^5$ respectively into 60 mm tissue culture dishes. After 24 hours, the medium was changed to 1.6 ml RPMI 1640 complete (seeding medium). The transfection solution consisted of 800 μl ECR buffer with 50nM siGLO transfection indicator, and either 50nM control siRNA or 50 nM AKAP12 siRNA. This mixture was incubated for 5 minutes at room temperature (RT). Next, 30 μl of RNAifect reagent was added to the mixture and incubated for an additional 15 minutes at RT. Lastly, 400 μl of the transfection mixture was added to each of the appropriate dishes and cells were incubated overnight. The following morning the medium was changed to 4 ml RPMI 1640. Control siRNA (siGenome Non-targeting siRNA #1), mouse and human AKAP12 siRNA, and siGLO green

transfection reagents were purchased from Dharmacon/ThermoScientific, Lafayette, CO. RNAifect reagents were purchased from Qiagen (Valencia, CA).

Cell Invasion Assay

Cytoselect™ 24-well Cell Invasion Assay kit was purchased from Cell Biolabs, Inc., San Diego, CA. A suspension containing $5x10^5$ cells/ml in serum-free RPMI 1640 media was used for WM3248 (human) and B16 (mouse) melanoma cells. For B16 cells, the medium also contained 30 mg/ml serum albumin and, when appropriate, $1x10^{-7}$M atRA. Inserts containing the dried basement membrane matrix were placed in appropriate wells of the plate and rehydrated with 300 μl of serum-free RPMI 1640 media for 1 hour at RT. Next, the rehydration medium was removed and 500 μl of RPMI 1640 containing 10% FBS as a chemo-attractant was added to the lower chamber and 300 μl ($1.66x10^5$) of cells were added to the upper chamber. For B16 cells, control samples contained 30 mg/ml serum albumin, while test samples contained 30mg/ml albumin and $1x10^{-7}$M atRA. Cells were incubated at 37° C for 48 hours. To harvest invasive cells, non-invasive cells were first removed from the top of the insert with a cotton swab. The inserts were then stained with cell stain dye solution for 10 minutes, washed, air dried and photographed (Olympus DP12 digital microscope camera system, Melville NY). Next, the stained cells were solubilized with 200 μl extraction solution, and 100 μl from each sample transferred to a 96 well plate and OD measured at 560nM (Molecular Devices, Vmax kinetic microplate reader**)**.

Anchorage-Independent Growth Assay

CytoSelect™ 96-well Cell Transformation Assay (Soft Agar Colony Formation) kit was purchased from Cell Biolabs, Inc., San Diego, CA. For the first layer of agar, a 1.2% agar solution was produced and solubilized using a microwave on high for 1 minute 15 seconds and allowed to cool in a 37° C water bath. The, 2xDMEM/20% FBS media was also warmed to 37° C in a water bath. Equal volumes of these solutions were mixed (1:1) and 50μl of this mixture was added to each sample well of a sterile, 96-well, flat-bottomed, black microplate. The plate was then transferred to 4°C for 30 minutes to allow the base layer to solidify. For the second layer, cells were harvested and resuspended at $4x10^5$ in RPMI 1640 media. Control samples contained a similar volume of DMSO while test samples contained $3x10^{-7}$M atRA. Cell suspensions were mixed 1:1:1 with 1.2% agar and 2xDMEM/20% FBS media. The final concentration of atRA was $1x10^{-7}$M. Control or atRA treated cells (10,000 cells/75 μl) were added to appropriate wells already containing the base agar layer. The microwell plate was transferred to 4° C for 15 minutes to solidify the top agar layer. Last, 100 ul of RPMI 1640 complete plus 10% FBS with either DMSO (Control) or $1x10^{-7}$M atRA was added to the appropriate wells. The 96-well plate was incubated for either 48 or 72 hours at 37° C and 5% CO_2. Cells were examined and photographed under an Olympus DP12 digital microscope camera system. The agar was solubilized and the cells lysed and combined to ensure a homogeneous mixture. A 10 μl sample from each well was transferred to a black, 96 well plate for fluorescence measurement. Just before use, the CyQuant working solution

was made by diluting CyQuant CR Dye 1:400 with 1X PBS. A 90 µl aliquot of CyQuant working solution was added to each well. The plates were immediately wrapped in foil and kept under low lighting conditions during a 10 minute incubation at room temperature. Fluorescence was measured using a SpectraMAX GeminiEM fluorometer with a 485/538 nm filter set. Fluorescence is proportional to the amount of DNA present in the cells.

Statistics

Samples for each experiment were performed in triplicate (n=3). Biological repeats for each experiment were performed at least three times, or more if necessary. Statistical significance determined using the SigmaStat analysis program. Sample comparisons were made using One Way ANOVA (analysis of variance). Probability of significance was determined using the Student-Knewman Keuls t-test. Standard error bars are shown.

Linear Regression Analysis

Linear regression analysis attempts to examine and model the relationship between two or more variables using a straight line. A GraphPad Prism 5 program was used to analyze data for this comparison. The effect of atRA on AKAP12 mRNA expression (x) was compared to RA's ability to inhibit growth (y) and analyzed for statistical significance.

Results

Microarray analysis identifies AKAP12 as an atRA induced gene

In a previous study (Estler, *et. al.*, 2008) we used gene expression profiling to identify genes in B16 melanoma cells whose expression is altered as a function of different times of atRA exposure. The goal was to understand how a series of activated/repressed genes might contribute to the ability of atRA to inhibit proliferation and stimulate differentiation of this cell line. This microarray analysis showed that AKAP12 expression is induced 25.6 fold (Table 3.1) in B16 cells treated with atRA for 48 hours. AKAP12's degree of induction was the highest among the 717 genes whose expression was significantly increased by atRA at this time point. To verify these microarray results, QuantaGene analysis was performed on RNA extracted from B16 melanoma cells treated for 24, 48 and 72 hours with 10 μM atRA. Retinoic acid induced a 6-fold and a 9-fold increase in AKAP12 mRNA at 48 and 72 hours respectively (Figure 3.2).

Table 3.1. Identification of AKAP12 as an atRA induced gene.

Fold Increase in mRNA Expression

	4 Hours	10 Hours	24 Hours	48 Hours
AKAP12	—	—	13.6	**25.6**
RAR β2	3.73	6.85	3.84	6.4

Table 3.1. Gene expression profiling was performed on control and 10 μM atRA treated B16 mouse melanoma cells (Estler, *et. al.*, 2008). Microarray analysis identified AKAP12 as the highest induced gene at 48 hours of atRA treatment. The expression of AKAP12, as well as the control gene RARβ2, is shown after 4, 10, 24, and 48 hours of RA treatment.

Pathway analysis of microarray data

Data from the microarray were analyzed by the Pathway Studio v5.0 (Ariadne Genomics) program to identify and illustrate molecular connections between certain proteins encoded by the genes whose expression was significantly increased by atRA. This program searches through the ResNet database for all known interactions between genes/proteins, which includes physical interactions, regulation of expression and protein modification, and expresses the result in graphical form (Figure 3.1). Four major hubs for connectivity were identified. These were cell division cycle 2 protein (CDC2), checkpoint kinase 1 (CHEK1), CDC45 cell division cycle 45-like (CDC45L) and minichromosome maintenance deficient protein 6 (MCM6). There were also three additional hubs that were identified which had relatively high numbers of interactions as well. These were p53 protein (TP53), cyclin-dependent kinase inhibitor 1A protein (CDKN1A/p21/WAF1/CIP1) and cMYC (Estler, *et. al.*, 2008).

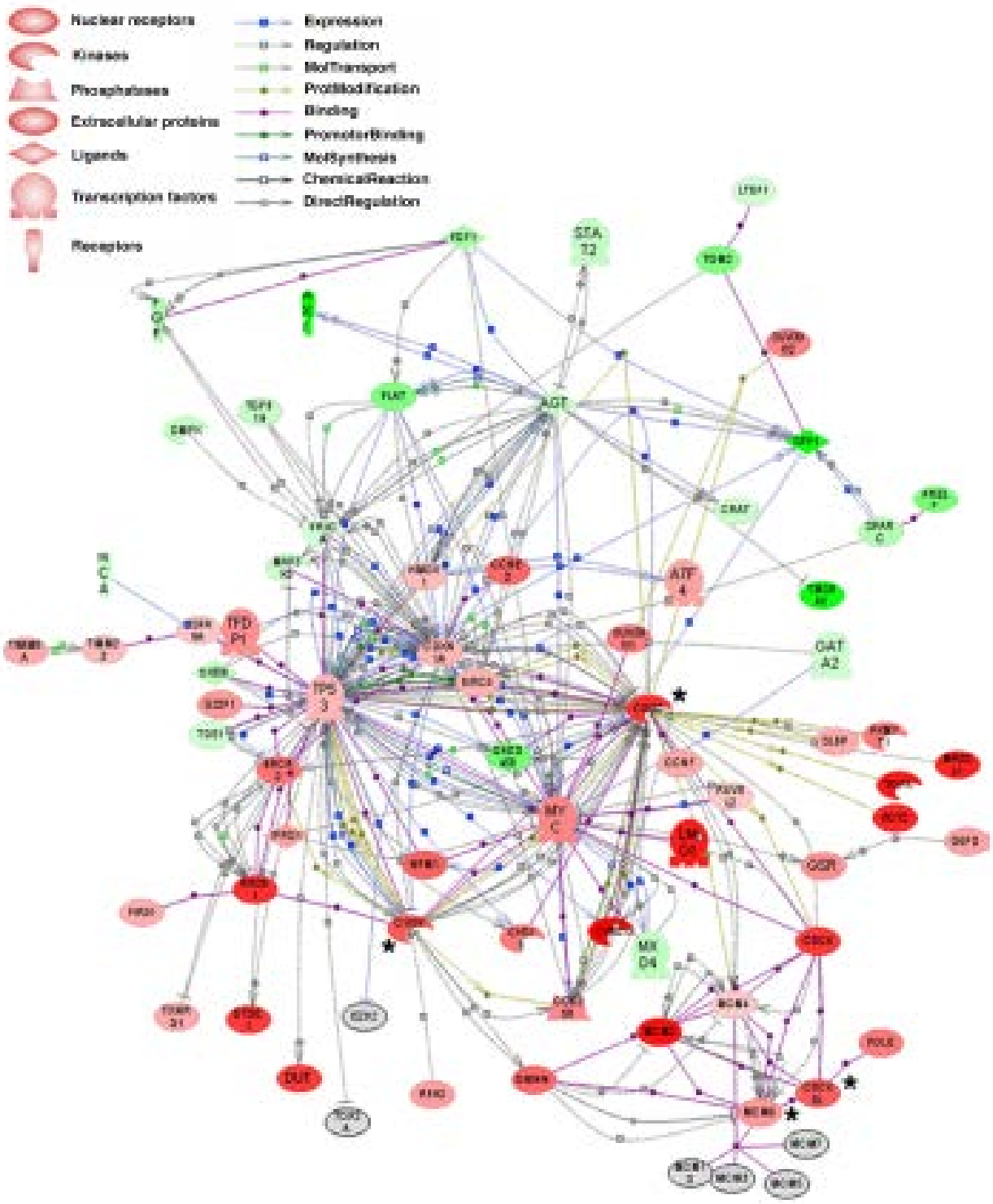

Nuclear receptors
Kinases
Phosphatases
Extracellular proteins
Ligands
Transcription factors
Receptors
Expression
Regulation
MolTransport
ProtModification
Binding
PromoterBinding
MolSynthesis
ChemicalReaction
DirectRegulation

Figure 3.1 Pathway Studio Analysis of Microarray Gene Set. Protein hubs that have the most significant interactions (CDC2, CHEK1, CDC45L and MCM6) are marked with an asterisk (∗). Although there are relatively large interactions stemming from TP53, p21 and cMYC, these proteins were not among the most statistically significant hubs. Green color indicates a gene is upregulated whereas red color indicates that a gene is downregulated. Color intensity reflects the expression ratio.

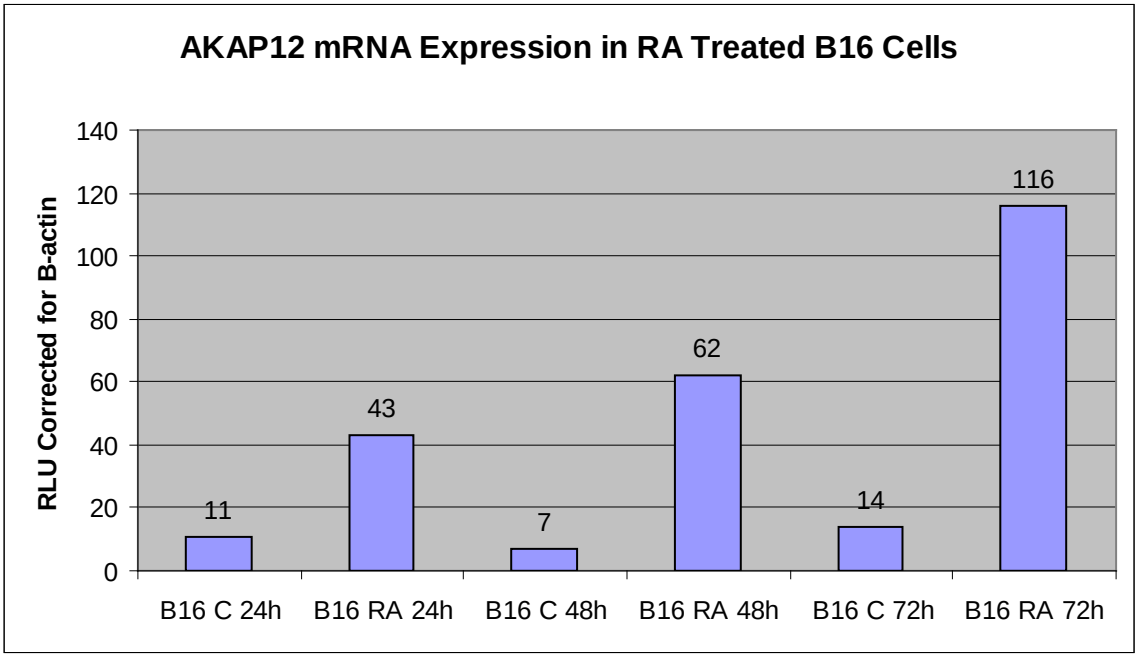


Figure 3.2. Quantification of AKAP12 mRNA in B16 Mouse Melanoma Cells. Amount of AKAP12 mRNA expression in B16 cells treated +/- 10μM atRA. The QuantaGene Assay system was used to determine relative amounts of AKAP12 mRNA. Cell lysates were loaded into 96 well capture plates and hybridized to a probe specific for mouse AKAP12 mRNA. Relative light units were measured on a luminometer. The amount of AKAP12 mRNA expression, corrected for β-

actin, is shown. {(AKAP12 untreated/β-actin)/(AKAP12 + RA/β-actin + RA)}. This experiment was repeated twice with similar results.

AKAP12 Protein and mRNA Expression after atRA Stimulation

To establish if AKAP12 expression is also regulated by atRA in human melanocytes and melanoma cells, I examined the level of AKAP12 mRNA and protein from control and atRA treated normal human melanocytes (HEMn-LP), and the human melanoma cell lines SbCl2 & WM3211 (RGP); WM1366 & WM3248 (VGP); and WM239 & WM9 (Met). These melanoma cell lines were chosen because they were established from tumors representing different stages of malignant progression.

All cell lines expressed AKAP12 protein and its expression was differentially induced after atRA treatment (Figure 3.3A). The major isotype of AKAP12 in these cells is a 305/287kD doublet, with the top band designated as the α isoform and the lower band the β isoform (Gelman, 2002; Streb, *et. al.*, 2004). In untreated HEMn-LP, WM3211 and WM3248 cells, AKAP12α was the main isotype while untreated WM9 and WM1366 cells expressed equal amounts of the α and β isoforms. Treatment of these cells with atRA for 48h induced expression of both isoforms, except in SbCl2 cells where the predominant isoform was AKAP12β. Quantification of the protein expression revealed the following order of AKAP12 induction: SbCl2 (RGP) (3.54 fold), HEMn-LP human melanocytes (3.38 fold), WM3248 (VGP) (2.72 fold), WM239 (Met) (2.4 fold), WM3211

(RGP) (2.29 fold), WM9 (Met) (2.09 fold) and WM1366 (VGP) (0.9 fold) (Figure 3.3B).

Figure 3.3A

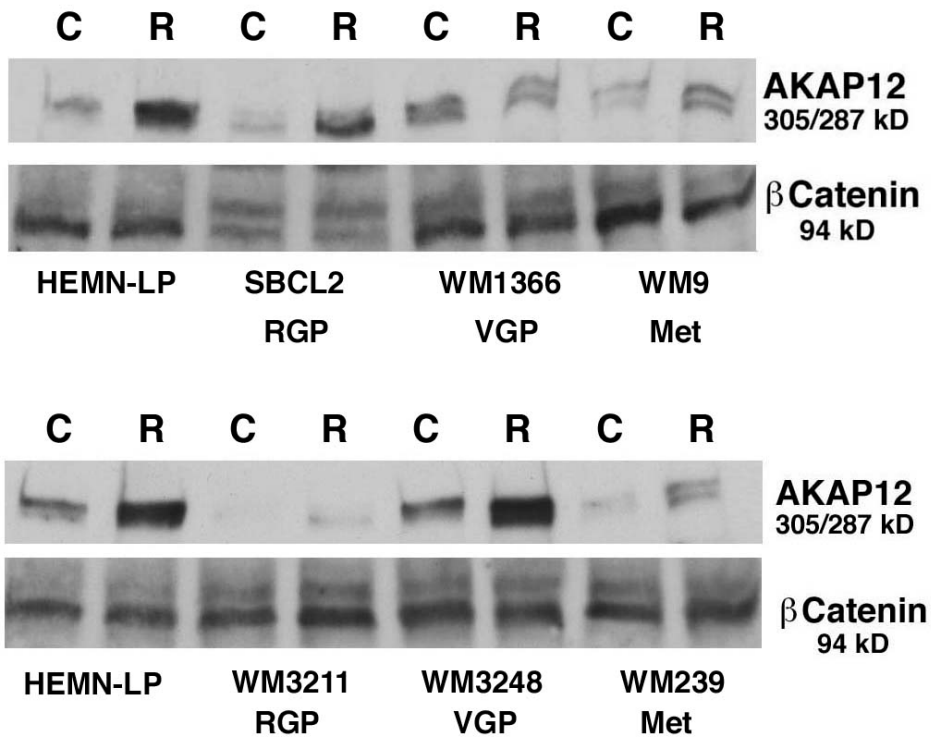


Figure 3.3B

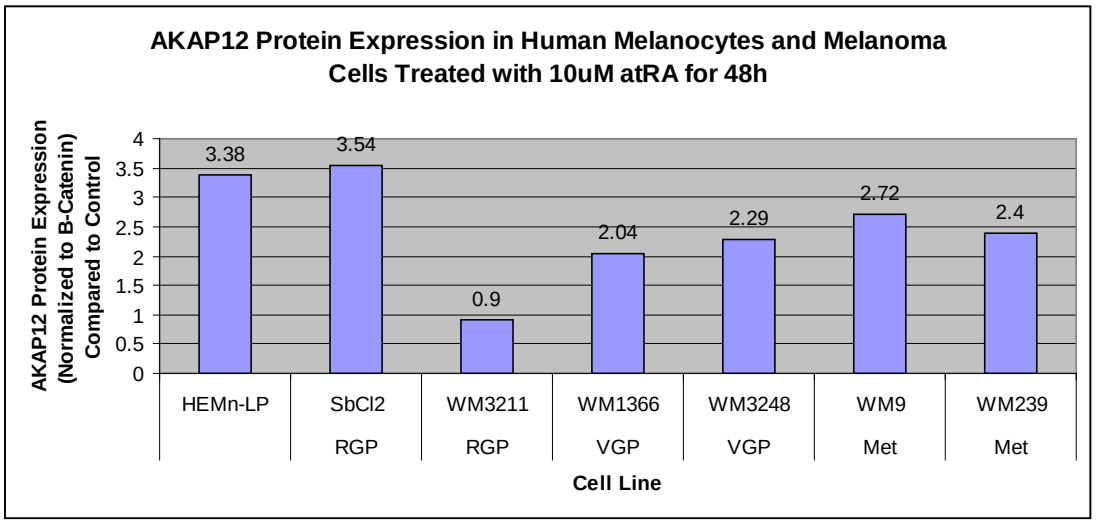


Figure 3.3. AKAP12 Protein Expression in Human Melanocytes and Melanoma Cells. The amount of AKAP12 protein in control (C) and 10 μM atRA treated (R) cells was determined by immunoblotting as described in Materials and Methods. (**A**) Immunoblot of AKAP12 protein levels in SbCl2 (RGP), WM1366 (VGP), and WM9 (Met) human melanoma cells compared to HEMn-LP human melanocytes. Immunoblot of AKAP12 protein levels in WM3211 (RGP), WM3248 (VGP), and WM239 (Met) human melanoma cells compared to HEMn-LP human melanocytes. (**B**) Densitometry of immunoblots (A&B) showing the relative changes in AKAP12 protein expression in human melanocytes and melanoma cells after 48 hours of atRA treatment. A value of 1.0 corresponds to the level of AKAP12 protein in control cells. These experiments were repeated two additional times with similar results.

QRT-PCR was used to measure the amount of AKAP12 mRNA in human melanocytes and melanoma cells treated +/- 10 μM atRA for 48 hours. All cell lines expressed AKAP12 mRNA and its level was significantly increased relative to control in all cell lines after atRA treatment except for WM1366 (Figure 3.4A). There was a strong correlation between the degree of atRA induction of AKAP12 mRNA and protein expression in these cell lines. Only one cell line (WM3248-VGP) had basal levels of AKAP12 mRNA that were higher than human melanocytes. The remaining cell lines have the same or significantly lower amounts of AKAP12 mRNA relative to normal human melanocytes (Figure 3.4B).

Figure 3.4A

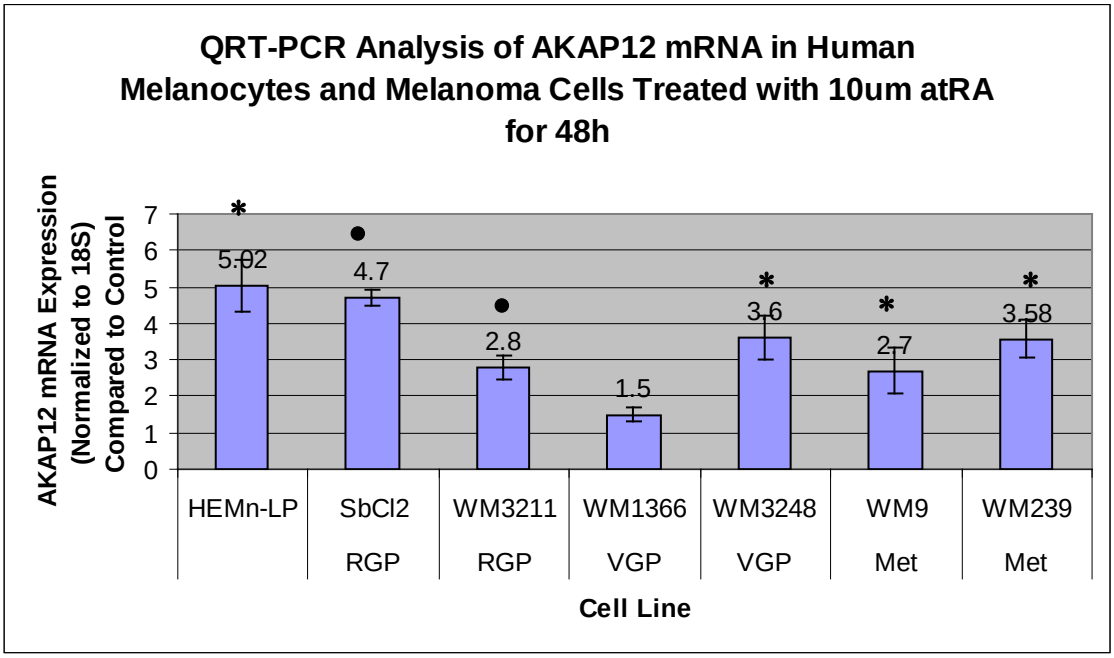


Figure 3.4B

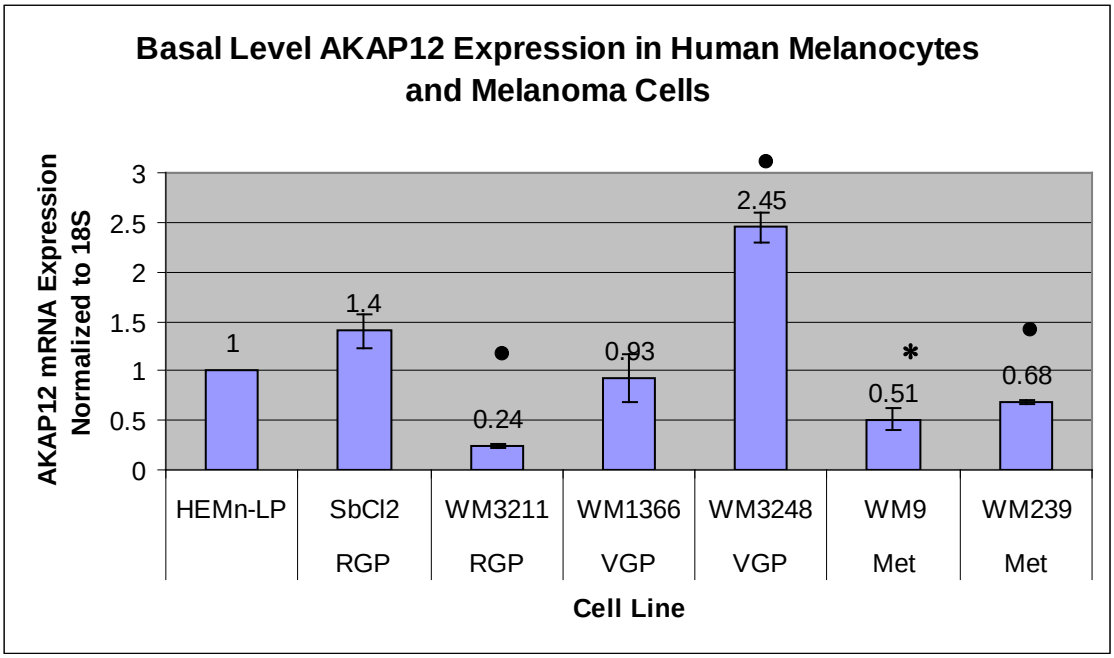


Figure 3.4. atRA Stimulation of AKAP12 mRNA Expression in Human Melanocytes and Melanoma Cells. QRT-PCR was performed on total RNA extracted from each of the cell lines after 48 hours of +/- 10μM atRA treatment. (**A**) Changes in AKAP12 expression after atRA treatment. Data are expressed as fold change relative to control cells and normalized using 18S ribosomal RNA expression. (**B**) Basal level AKAP12 expression. Data are expressed as amount of AKAP12 mRNA relative to HEMn-LP cells. Results are the average of 4 separate experiments. (∗ = $p < 0.05$, • = $p < 0.001$ compared to control)

Effect of atRA on melanoma cell growth and correlation with induction of AKAP12 expression

Our laboratory has previously found that atRA inhibits the proliferation of mouse and human melanoma cells (Desai and Niles 1997; Huang, *et. al.*, 2002; Niles, 2003; Estler, *et. al.*, 2008; Fan, *et., al.*, 2010). A 72 hour atRA treatment of the human melanocyte and melanoma cell lines caused varying amounts of inhibition of their proliferation (Table 3.2). SbCl2 (RGP) cells were the most sensitive with a 70% decrease in cell number. SbCl2 (RGP), HEMn-LP human melanocytes, WM3211 (RGP) and WM3248 (VGP) cell proliferation was significantly inhibited by atRA. WM1366 (VGP), WM9 (Met) and WM239 (Met) melanoma cell proliferation was not significantly inhibited.

Effect of 72 h of atRA treatment on human melanocyte and melanoma cell growth

CELL LINE	↓ IN CELL GROWTH
HEMn-LP	46 % * p < .001
SbCl2	70 % * p < .001
WM3211	24 % * p < .05
WM3248	20 % * p < .05
WM9	16 %
WM239	14 %
WM1366	12 %

Table 3.2. Effect of 72 hours of atRA Treatment on the Proliferation of Human Melanocytes and Melanoma Cells. Cells were seeded, and after a 24 hour growth period the media was changed. Medium contained either DMSO (control) or 10 μM atRA. Triplicate dishes from each treatment group were harvested after 24, 48 and 72 hours of treatment and the number of cells determined by hemocytometer counts. Data are expressed as percent decrease in cell number compared to control for each cell line 72 hours after treatment. ($^{\%}$ = significantly different from control; $p<0.05$)

Linear regression analysis revealed that there was a significant correlation ($p<0.05$) between the ability of atRA to inhibit proliferation and its ability to increase AKAP12 mRNA levels (Figure 3.5).

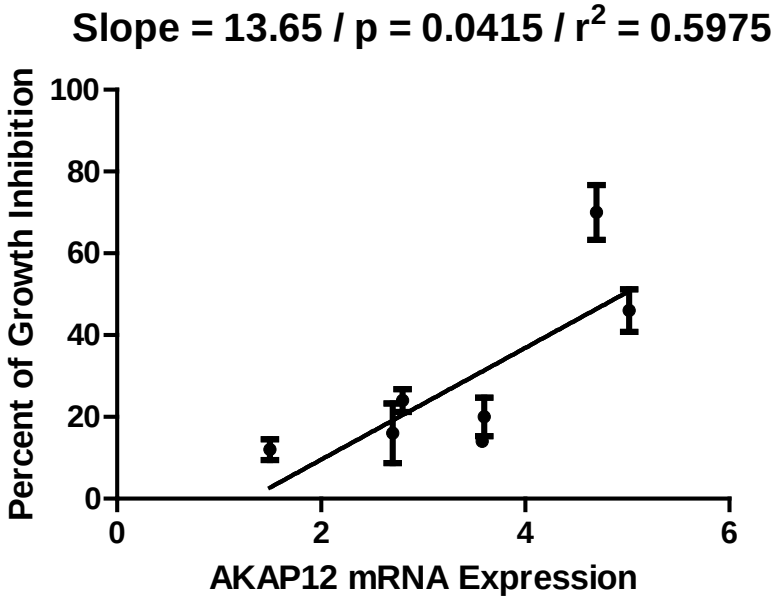


Figure 3.5. Linear Regression Analysis of atRA Induced Increase for AKAP12 mRNA Expression and its Ability to Inhibit Proliferation of the Human Melanocyte and Melanoma Cell Lines. Changes in mRNA expression were determined by QRT-PCR and compared to the ability of atRA to inhibit proliferation of human melanocytes and melanoma cells. Data were analyzed using the Prism 5 software program. Analysis shows a statistically significant correlation between atRA's ability to induce AKAP12 expression and its ability to

inhibit proliferation, with a slope of 13.65, a p value of 0.0415 and an r^2 of 0.5975.

Induction of AP-1 activity by atRA

To understand how AKAP12 gene expression is regulated by atRA we conducted an *in silico* analysis of the AKAP12α promoter region in order to identify putative transcription factor binding sites. This analysis searches highly correlated sequence fragments versus TFMATRIX transcription factor binding site database by Wingender E, Knueppel R, Dietze P, and Karas H (www.cbrc.jp/htbin/nph-tfsearch). Threshold was set at 85%. We found three putative Activator Protein 1 (AP-1) sites located at positions -3305 (AGCTCA), -4558 (GAGTCA) and -4879 (AGTTCG) upstream of the AKAP12α start site. Previous research in our laboratory has shown that atRA treatment in B16 cells induces a slow but sustained increase in AP-1 transcriptional activity (Desai and Niles, 1997; Huang, *et. al.*, 2002). Therefore, it was hypothesized that AP-1 may be involved in mediating the atRA induction of AKAP12 gene expression. Treatment of B16 mouse melanoma cells, WM3248 (VGP) and WM239 (Met) human melanoma cells containing an AP-1 reporter gene with atRA resulted in a significant increase in reporter gene activity relative to control treated cells (Figure 3.6). The effect of atRA on AP-1 activity was specific, since adding a dominant negative c-fos plasmid (A-fos) to the transfection mixture attenuated both basal and atRA stimulated AP-1 activity in all the cell lines (Figure 3.6, columns 3&4, 7&8, 11&12).

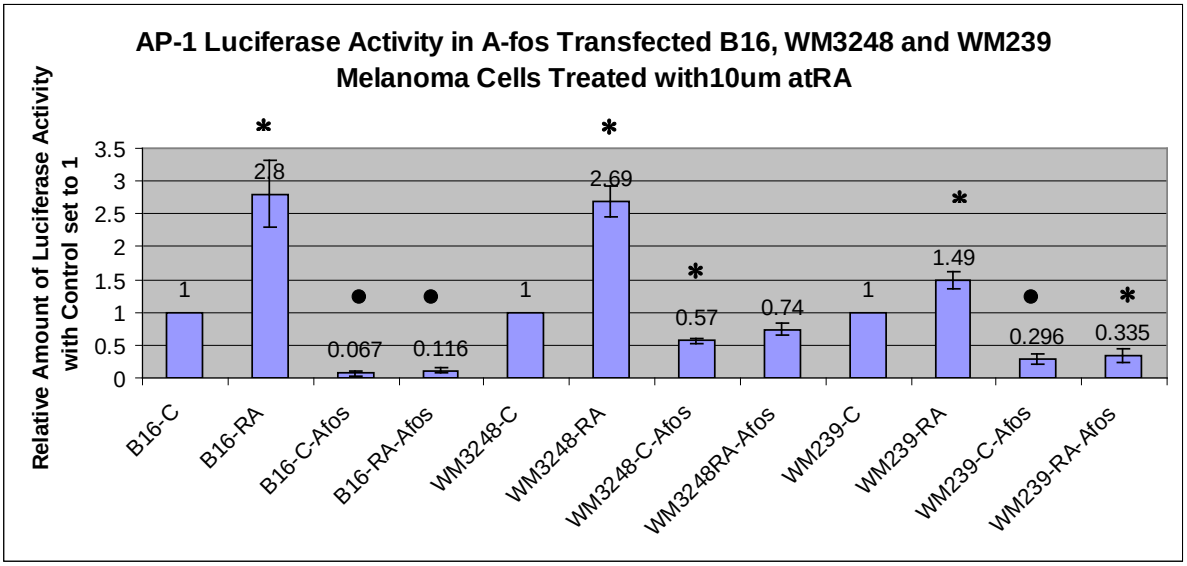


Figure 3.6. AP-1 Activity in A-fos Transfected and atRA Treated Melanoma Cells. Luciferase activity was measured in cell lysates that were harvested from control and atRA treated B16, WM3248 and WM239 melanoma cells that were co-transfected with the β-Gal, AP-1 luciferase vector and either the pCMV control vector (columns 1, 2, 5, 6, 9, 10) or the dominant negative c-fos (A-fos) vector (columns 3, 4, 7, 8, 11, 12). Cells were treated for 48 hours with 10 μM atRA. Data is expressed as relative amount of luciferase activity compared to control. Results are the average of 3 separate experiments. (∗ = $p < 0.05$, • = $p < 0.001$ compared to control)

Inhibition of AP-1 activity by the dominant-negative A-fos causes a further increase in AKAP12 expression

Having shown that the AKAP12 promoter has putative AP-1 binding sites and that atRA stimulates AP-1 activity in both mouse and human melanoma cells, we tested whether the dominant-negative A-fos would antagonize atRA induction of

AKAP12 mRNA levels (Figure 3.7). Contrary to expectations, A-fos antagonism of AP-1 activity enhanced the ability of atRA to increase AKAP12 mRNA levels. In A-Fos transfected B16 cells, AKAP12 expression increased from nearly 29 fold to over 45 fold (an additional 55%). A-fos expression increased atRA stimulated AKAP12 mRNA levels from 6.5 fold to 9 fold (an additional 38%) in WM3248 cells, and an additional 43% in WM239 cells. (Figure 3.7). These data suggest that AP-1 is a negative regulator of AKAP12 mRNA expression.

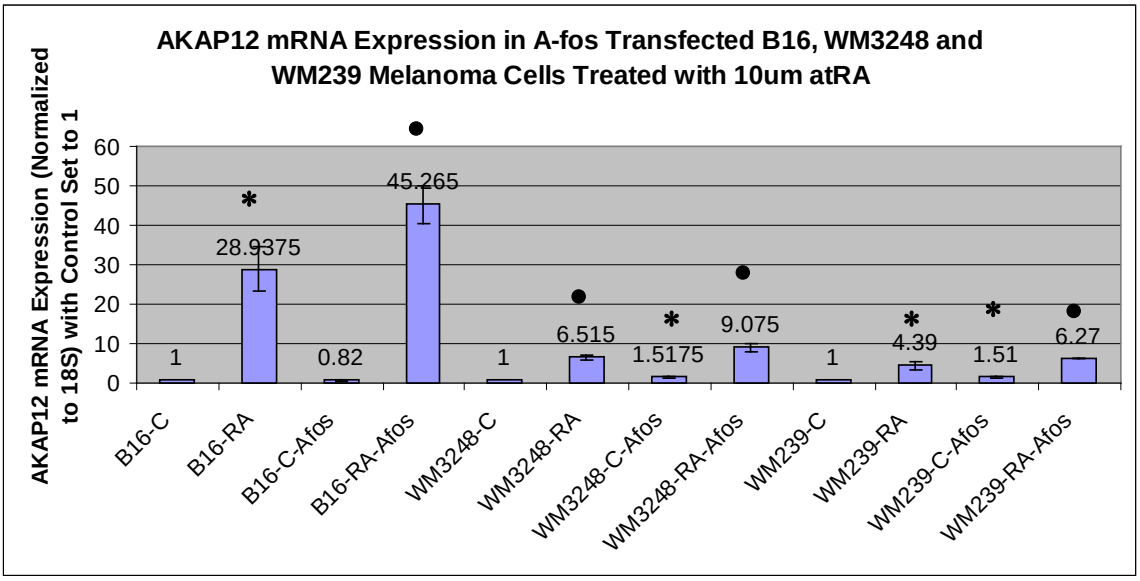


Figure 3.7. AKAP12 mRNA Expression Levels After Down-regulation of AP-1 Activity. QRT-PCR was performed on total RNA extracted from Control and atRA treated B16, WM3248 and WM239 melanoma cells that were transfected with either pCMV control vector (columns 1, 2, 5, 6, 9, 10) or the A-fos plasmid (columns 3, 4, 7, 8, 11, 12). Cells were treated for 48h with 10 μM atRA. Data is expressed as fold change relative to control cells and normalized using 18S ribosomal RNA expression. Results are the average of four separate experiments.

(∗ = p <0.05, • = p <0.001 compared to control) Statistical analysis was performed comparing columns 2&4, 6&8, and 10&12; however, they were not statistically significant, most likely due to the variation between the four experiments.

Effect of AKAP12 downregulation on invasion in WM3248 (human) and B16 (mouse) melanoma cells.

To determine the effect of AKAP12 down-regulation on WM3248 and B16 melanoma cell biology, cells were transfected with siRNA to AKAP12 and analyzed using CytoSelect™ Invasion Assay. These cell lines were used because of their high induction of AKAP12 expression after atRA treatment. Initially, I established the amount of AKAP12 RNA and protein down-regulation after siRNA transfection in these cell lines. At 96 hours of transfection with control siRNA and AKAP12 siRNA, total RNA and protein was isolated and AKAP12 levels examined by QRT-PCR and Western blotting. Figure 3.8 A&B shows that in WM3248 cells treated with AKAP12 siRNA there was a 76% decrease in mRNA and a 44% decrease in protein expression relative to cells treated with control siRNA. In B16 cells (Figure 3.8 C&D), atRA ($1x10^{-7}$M) induced a 4.7-fold increase in AKAP12 mRNA and a 98% increase in protein expression in control siRNA transfected cells, while in AKAP12 siRNA transfected cells, mRNA expression decreased by 38% and protein levels decreased by 32%. However, atRA treatment of AKAP12 siRNA transfected cells still caused a 4.4 fold increase in AKAP12 mRNA levels compared to control siRNA transfected

cells without RA treatment. Interestingly, treatment of AKAP12 siRNA transfected cells treated with atRA resulted in a 51% increase in AKAP12 protein compared to control siRNA transfected cells; however, when this is change is compared to cells transfected with siRNA to AKAP12, there is a 2.2 fold change in protein levels.

Figure 3.8A.

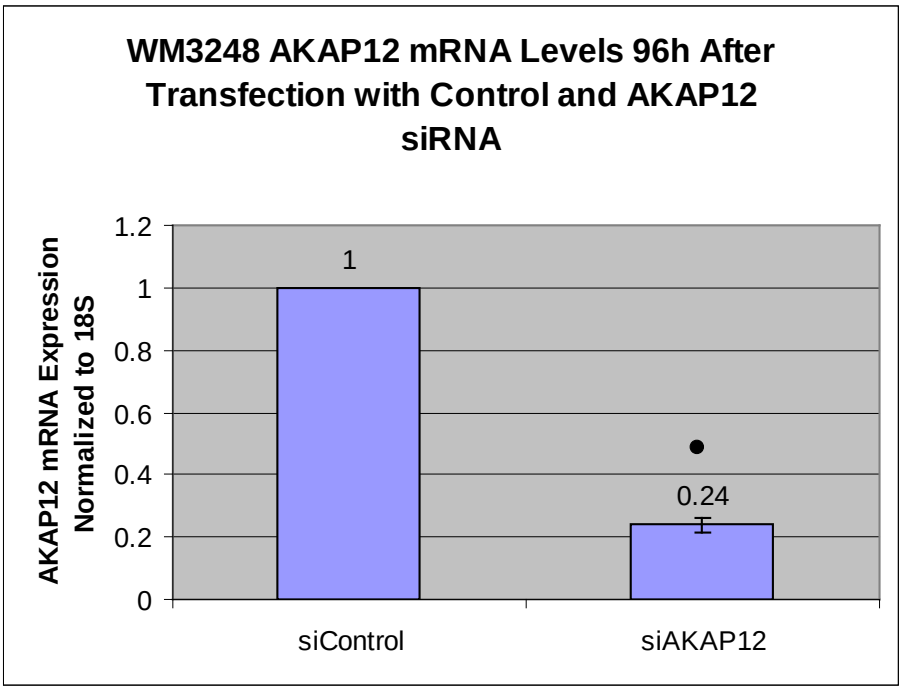


Figure 3.8B.

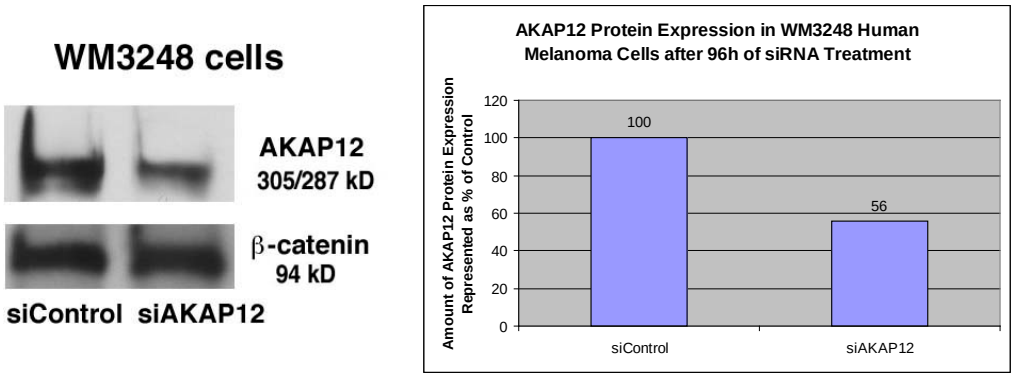


Figure 3.8C.

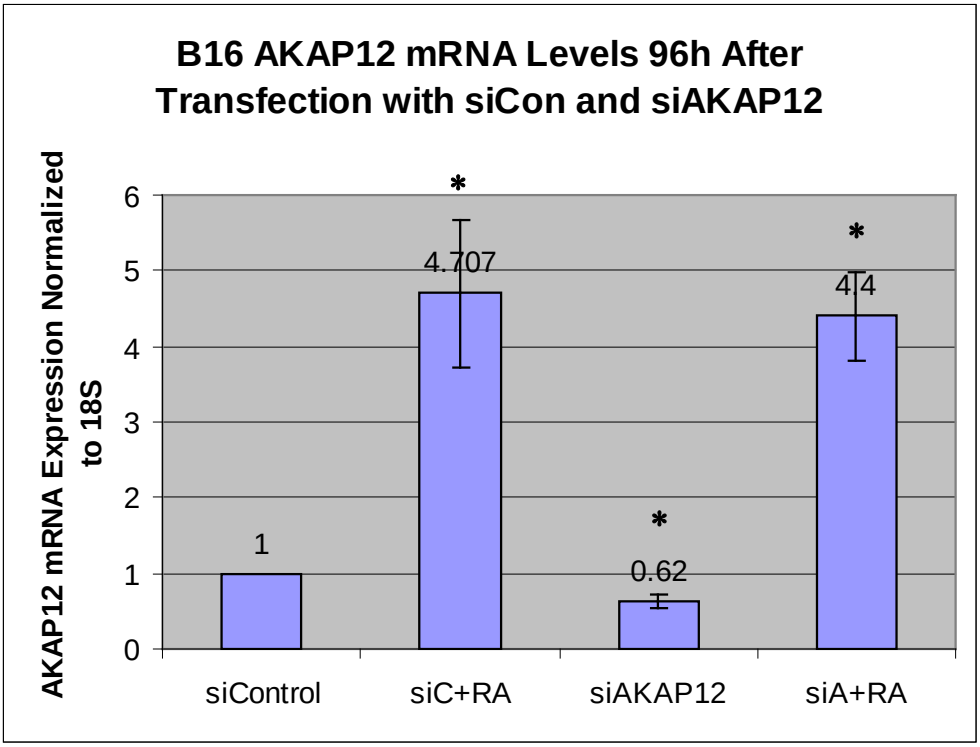


Figure 3.8D.

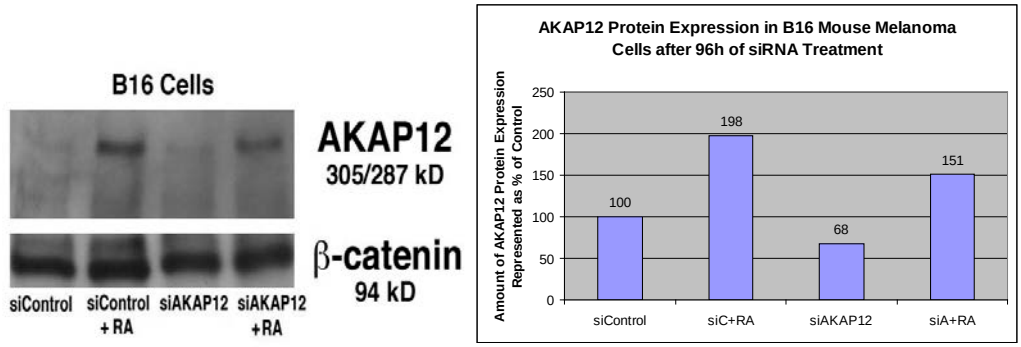
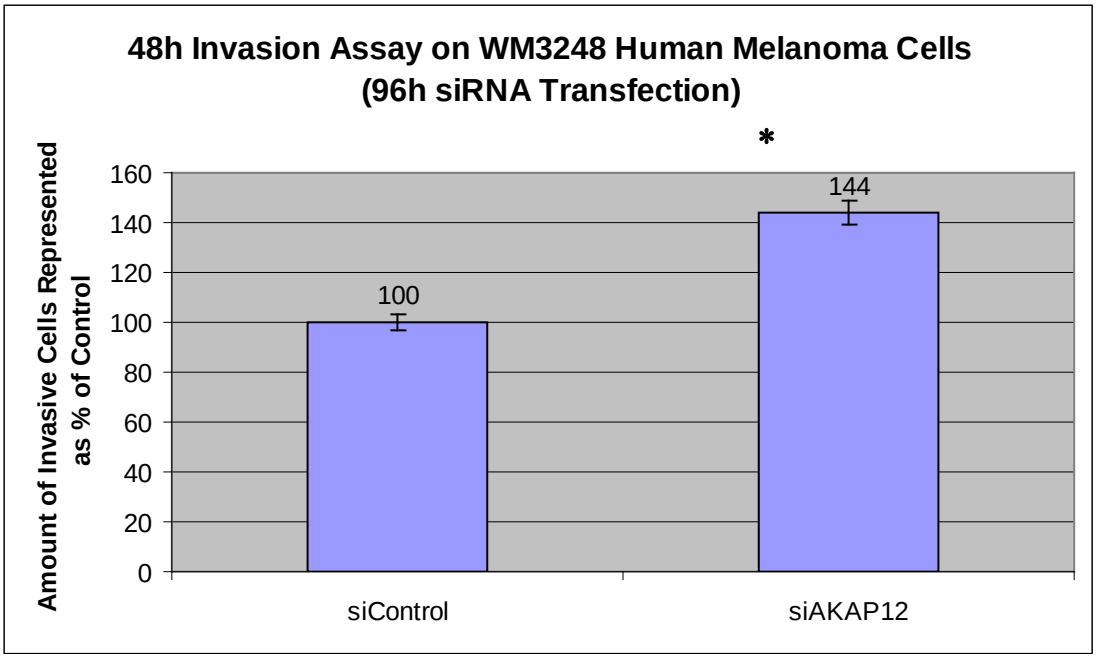


Figure 3.8. Ability of AKAP12 siRNA to decrease AKAP12 mRNA and protein. WM3248 and B16 cells were transfected with control siRNA and AKAP12 siRNA for 48h then split, reseeded, and allowed to grow for another 48 hours. For B16 cells, appropriate plates of cells were treated with $1x10^{-7}$M atRA 6 hours after seeding. After 48h of growth, triplicate plates of cells were harvested for RNA and protein measurements. A representative experiment is shown. Experiments were performed a minimum of three times with similar results. **A)** WM3248 AKAP12 mRNA levels, **B)** WM3248 protein levels, **C)** B16 AKAP12 mRNA levels, **D)** B16 AKAP12 protein levels. (* = $p < 0.05$, • = $p < 0.001$ compared to control)

Next, WM3248 human melanoma cells were assayed for their invasive properties. After 48 hours of transfection with control siRNA and AKAP12 siRNA to allow for a decrease in the levels of AKAP12 RNA and protein, cells were split and reseeded for the invasion assay. After 48 hours, there was a 44% increase in the amount of invasive cells in the AKAP12 siRNA treated cells

compared to control siRNA transfected cells (Figure 3.9A). Images clearly show the increase in invasive cells. Figure 3.9B shows the amount of invasive cells in the control siRNA transfected cells, while figure 3.9C shows the amount of invasive cells in siAKAP12 transfected cells.

Figure 3.9A.



B. siControl transfected WM3248 cells **C.** siAKAP12 transfected WM3248 cells

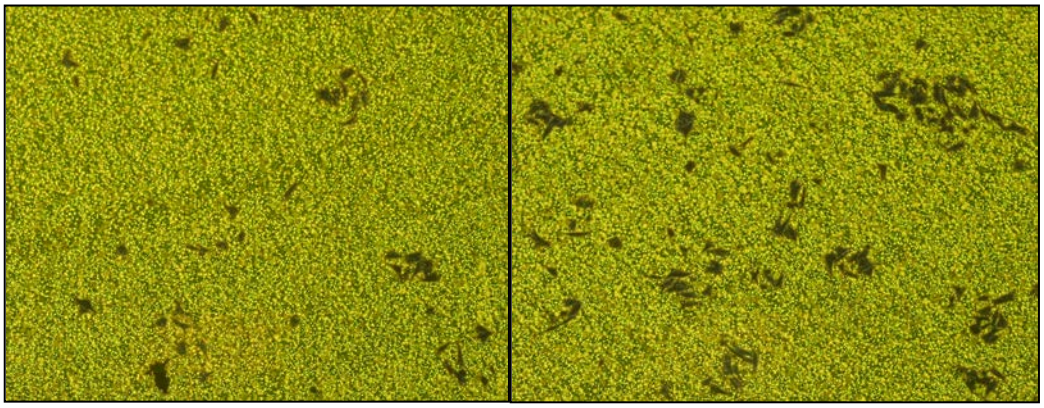
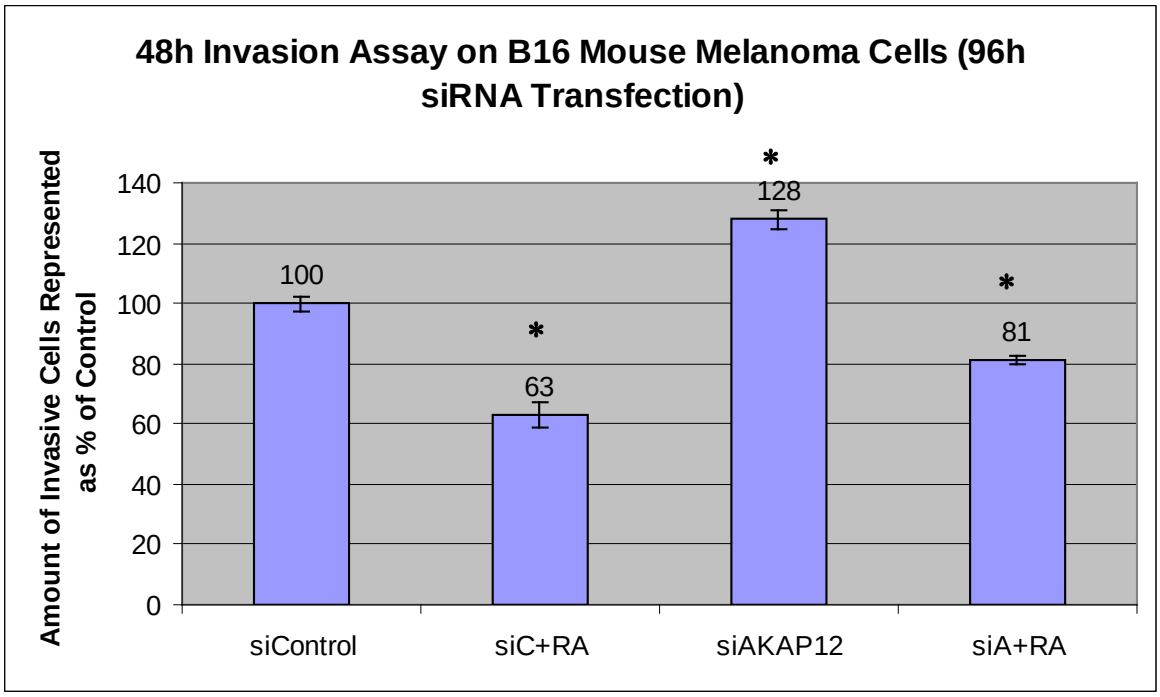


Figure 3.9. Effect of decreasing AKAP12 levels on invasion in WM3248 human melanoma cells. Cells were transfected with control siRNA and AKAP12 siRNA for 48h, then split and reseeded into triplicate sets of wells for the CytoSelect™ Invasion assay, which was performed according to manufacturer's protocol. At 48 hours of incubation, invasive cells were photographed, harvested and counted. A representative experiment is shown. These experiments were repeated a minimum of three times with similar results. **A)** Relative number of invasive cells expressed as a percent of cells transfected with control siRNA **B)** Image of WM3248 cells transfected with siControl, **C)** Image of WM3248 cells transfected with siAKAP12. (∗ = $p < 0.05$ compared to control)

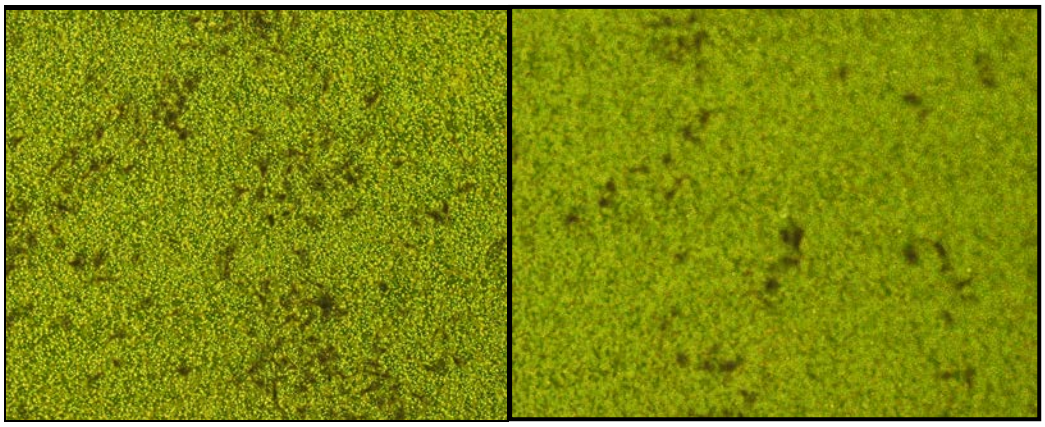
Next, the effect of decreasing AKAP12 expression on the invasive properties of B16 melanoma cells was determined. Cells were transfected with control siRNA or AKAP12 siRNA 48h before reseeding the cells into the invasion assay. Treatment of cells transfected with control siRNA plus atRA had a 37% decrease in the number of invasive cells compared to control siRNA transfected cells receiving vehicle (Figure 3.10A). However, when AKAP12 levels were decreased due to transfection with AKAP12 siRNA, there was a 28% increase in the amount of invasive cells relative to control siRNA transfected cells. In siAKAP12 transfected cells treated with atRA, there was a 19% decrease in the number of invasive cells compared to control siRNA transfected cells. Comparing control siRNA transfected cells treated with atRA to AKAP12 siRNA transfected cells treated with atRA there were 18% more invasive cells when

AKAP12 levels are decreased. Photographic images also show the change in the amount of invasive B16 cells. The image in Figure 3.10B shows the amount of invasive cells after transfection with control siRNA, Figure 3.10C is an image of the invasive cells after transfection with control siRNA and treatment with $1x10^{-7}$M atRA. Note the reduced number of invasive cells. Figure 3.10D is an image of invasive cells after transfection with siAKAP12, note the larger number compared to image B. Figure 3.10E is an image of invasive cells after transfection with siAKAP12 and treatment with $1x10^{-7}$M atRA.

Figure 3.10A.



B. siControl transfected B16 cells **C.** siControl transfected B16 cells + RA



D. siAKAP12 transfected B16 cells **E.** siAKAP12 transfected B16 cells + RA

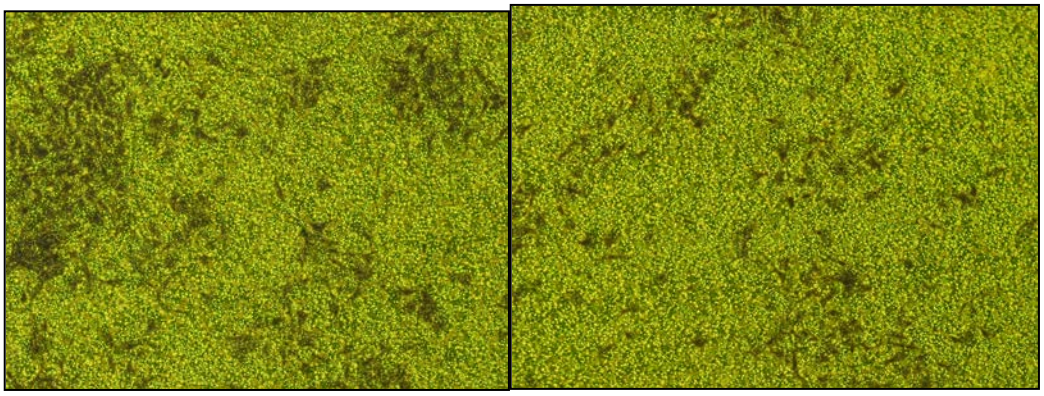


Figure 3.10. Effect of decreased AKAP12 levels on B16 mouse melanoma cells. B16 cells were transfected with control siRNA or AKAP12 siRNA for 48h, then split and reseeded into triplicate wells of the CytoSelect™ Invasion Assay, which was performed according to manufacturer's protocol. Control samples were treated with DMSO and RA treated samples received $1x10^{-7}$M atRA. After 48 hours, invasive cells were photographed, harvested and enumerated. A representative experiment is shown. Experiments were repeated a minimum of three times with similar results. **A)** Number of invasive cells relative to control,

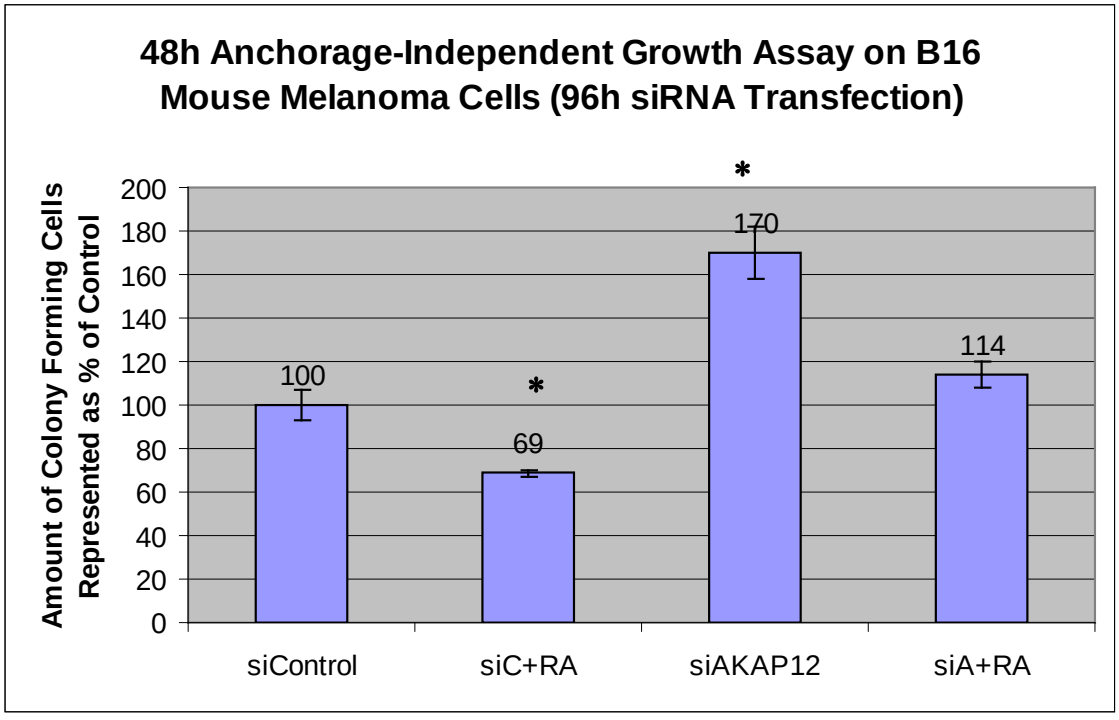
B) cells transfected with control siRNA, **C)**, cells transfected with control siRNA then treated with $1x10^{-7}$M atRA, **D)** cells transfected with AKAP12 siRNA, **E)** cells transfected with AKAP12 siRNA then treated with $1x10^{-7}$M atRA. (* = $p < 0.05$ compared to control)

Effect of AKAP12 downregulation on anchorage-independent growth in B16 mouse melanoma cells.

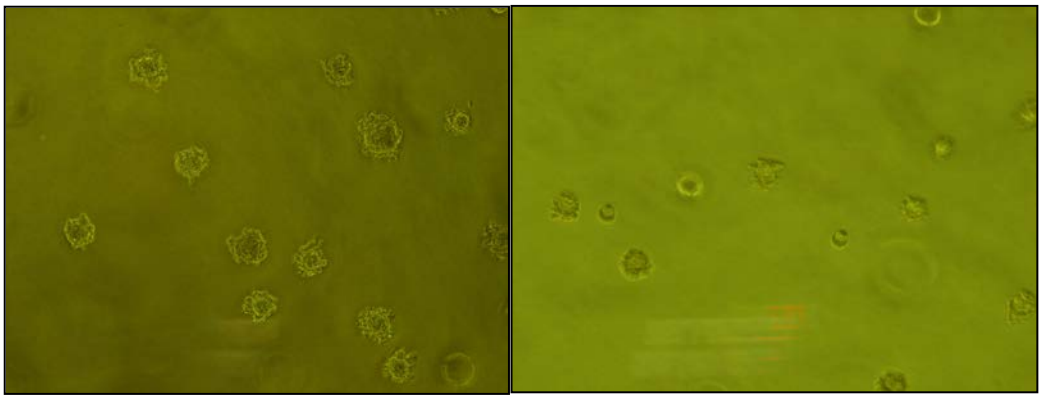
B16 cells were used to investigate the potential involvement of AKAP12 in regulating anchorage-independent growth due to their atRA sensitivity and their ability to form numerous colonies quickly in soft agar. WM3248 human melanoma cells were not used for this assay because they are unable to form colonies until at least 7 days after the start of the assay, a time at which the effect of AKAP12 siRNA was lost. Anchorage-independent growth was measured using the Cyto-Select™ 96-Well Cell Transformation Assay. B16 cells were transfected with control siRNA and AKAP12 siRNA for 48 hours before reseeding into the transformation assay. At 48 hours of incubation in this assay there was a 31% decrease in colony formation in cells transfected with control siRNA plus treatment with $1x10^{-7}$M atRA relative to cells transfected with control siRNA, but not treated with atRA (Figure 3.11A). In cells transfected with AKAP12 siRNA, colony formation increases 70% compared to cells transfected with control siRNA. The cells transfected with AKAP12 siRNA and then treated with $1x10^{-7}$M atRA had a similar amount of anchorage-independent growth relative to control siRNA transfected cells. Photographic images show the

changes in soft agar colony formation. Figure 3.11B is an image of colony formation in control siRNA transfected cells and Figure 3.11C is an image of colony formation after transfection of control siRNA and a 48h treatment with $1x10^{-7}$M atRA. Note the reduced number and smaller colonies. Figure 3.11D is an image of colony formation after 48h transfection with AKAP12 siRNA. This image shows larger and more abundant colony formation. Figure 3.11E is an image of colony formation in AKAP12 siRNA transfected cells and treated for 48h with $1x10^{-7}$M atRA. This image verifies that colony formation is still reduced in these cells.

Figure 3.11A.



B. siControl transfected B16 cells **C.** siControl transfected B16 cells + RA



D. siAKAP12 transfected B16 cells **E.** siAKAP12 transfected B16 cells + RA

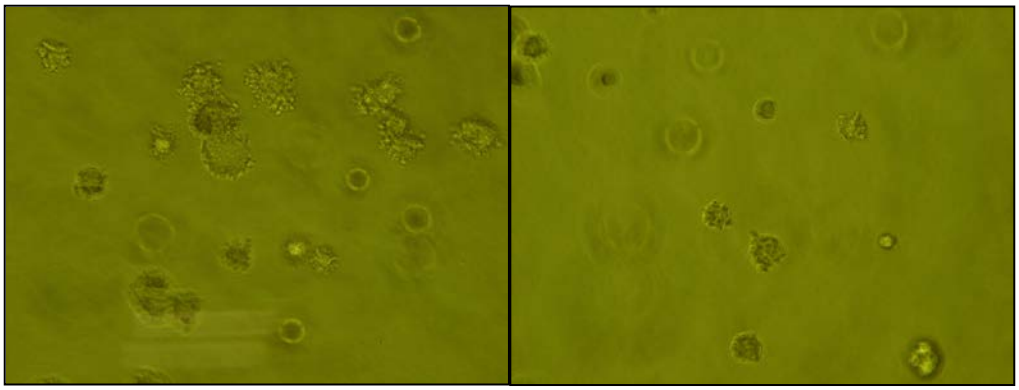


Figure 3.11. Effect of decreased levels of AKAP12 on anchorage-independent growth. B16 cells were transfected with control siRNA and AKAP12 siRNA for 48h, then split and reseeded into triplicate wells for the CytoSelect™ 96-Well Cell Transformation Assay, performed according to manufacturer's protocol. Control transfected siRNA cells and AKAP12 siRNA transfected cells received either DMSO or $1x10^{-7}$M atRA. After 48h of growth, cells were photographed, harvested and assayed for anchorage-independent growth. A representative experiment is shown. Experiments were repeated a minimum of three times.

Each repeat contained a minimum of 3 sample repeats per assay. **A)** Amount of soft agar colony growth relative to control cells, **B)** cells transfected with control siRNA, **C)** cells transfected with control siRNA and treated with $1x10^{-7}$M atRA, **D)** cells transfected with AKAP12 siRNA, **E)** cells transfected with AKAP12 siRNA and treated with $1x10^{-7}$M atRA. (* = $p < 0.05$ compared to control)

We also examined anchorage-independent colony formation after 72 hours of incubation (120 hours after transfection with control siRNA and AKAP12 siRNA). Cells transfected with control siRNA and treated with $1x10^{-7}$M atRA for 72 hours still showed a 40% decrease in colony formation (Figure 3.11A). In AKP12 siRNA transfected cells, colony formation was only increased by 25% (Figure 3.12A), while a 72 hours treatment of AKAP12 siRNA transfected cells with $1x10^{-7}$M atRA causes a 28% decrease in colony formation, compared to cells transfected with control siRNA. However, if this change is compared to what is seen in AKAP12 siRNA transfected cells, the decrease in colony formation is 58%, which is a greater decrease than what is seen in control siRNA transfected cells treated with atRA.

We also examined RNA and protein levels in B16 cells 120 hours after transfection with control siRNA or AKAP12 siRNA. QRT-PCR analysis shows that after 72 hours of $1x10^{-7}$M atRA treatment in control siRNA transfected cells there is a 9.62 fold induction of AKAP12 mRNA (Figure 3.12B). Cells transfected with AKAP12 siRNA show only a 22% decrease in AKAP12 mRNA relative to cells receiving control siRNA, indicating that the siRNA is losing its

effect. However, AKAP12 siRNA transfected cells treated with $1x10^{-7}$M atRA had a 5.93 fold increase in AKAP12 mRNA, a reduction of almost 3.7 fold relative to control cells treated with atRA. Western blot analysis of protein levels show that while mRNA levels are very high in siControl+atRA treated cells, protein levels are only increased 60%. In cells transfected with AKAP12 siRNA there is surprisingly an increase in AKAP12 protein relative to control siRNA transfected cells (49%) (Figure 3.12C). Even though atRA could only increase AKAP12 protein levels by 60% in control cells, in cells receiving AKAP12 siRNA prior to atRA treatment there is a 3.1 fold increase in AKAP12 protein levels.

Figure 3.12A.

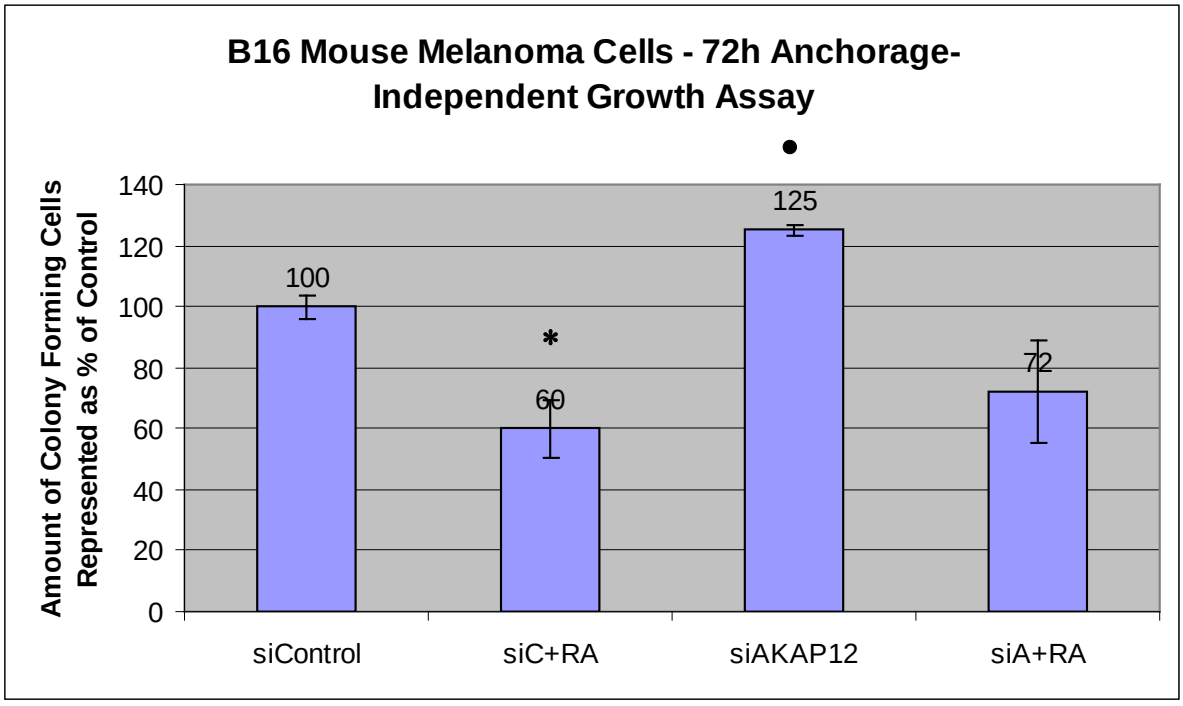


Figure 3.12B.

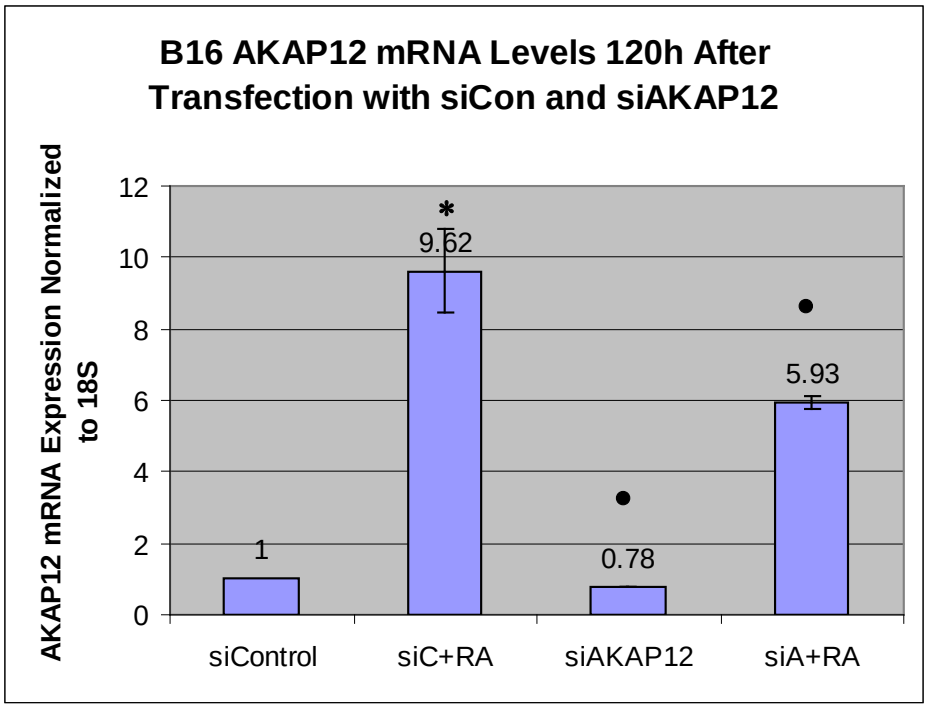


Figure 3.12C.

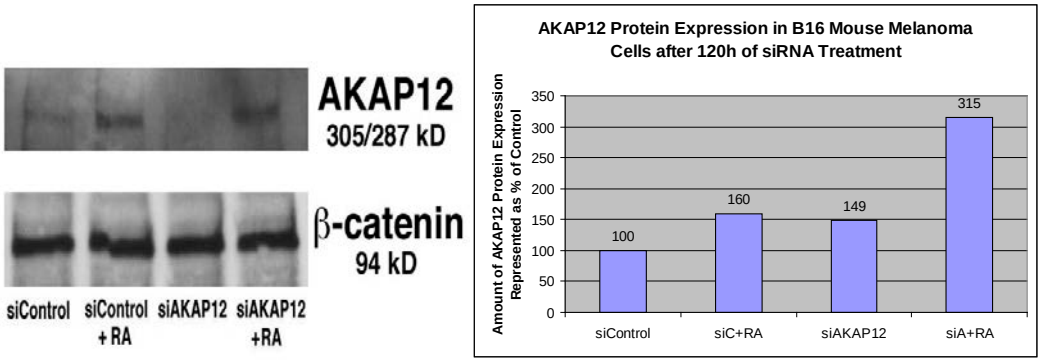


Figure 3.12. Effect of AKAP12 siRNA treatment on B16 mouse melanoma anchorage-independent growth 72 hours after treatment (mRNA and protein

levels are also included). B16 cells were transfected with control siRNA or AKAP12 siRNA for 72 hours, then split and reseeded into triplicate wells per treatment group for the CytoSelect™ 96-Well Cell Transformation Assay, which was performed according to manufacturer's protocol. Cells were treated with DMSO or $1x10^{-7}$M atRA. After 72h of growth, cells were photographed, harvested and assayed for anchorage-independent growth. A representative assay is shown. The experiment was repeated two additional times with similar results. **A)** 72hour anchorage-independent growth assay, **B)** AKAP12 mRNA levels 120 hours after transfection, **C)** AKAP12 protein levels 120 hours after transfection. ($* = p < 0.05$, $\bullet = p < 0.001$ compared to control)

Discussion

Cutaneous malignant melanoma (CMM) is the most serious form of skin cancer and its incidence is steadily increasing. The ability of retinoids to regulate melanoma cell growth and differentiation has been well documented, yet research has also shown that as melanoma progresses from RGP to VGP to Met, cells lose their responsiveness to atRA (Jacob, *et. al.*, 1998; Niles, *et. al.*, 2003; Fan, *et. al.*, 2010). We lack a complete understanding as to how atRA exerts its anti-cancer effects and don't fully understand why advanced stage melanomas become so resistant to the effects of atRA.

Gene expression profiling using DNA microarrays was performed on control and atRA treated B16 mouse melanoma cells to help discover new target genes of atRA. We identified AKAP12 as a novel, atRA target gene.

AKAP12 is an anchoring protein that is involved in assembly and compartmentalization of multi-protein complexes, thus coordinating and directing the transduction of multiple signaling pathways. The development and progression of CMM involves many molecular and genetic changes affecting multiple signaling pathways that regulate growth, metabolism, motility and apoptosis. AKAP12 may play a pivotal role in regulating these changes as it lies at a central junction where the regulation of these pathways converges. However, the role of AKAP12 in the function of melanocyte and melanoma biology is currently not known.

AKAP12/Gravin/SSeCKS is believed to be a Type II tumor suppressor gene, because its protein and RNA expression are downregulated in many epithelial derived tumors, and its re-expression in cancer cells has been demonstrated to suppress various parameters of tumor function. Research from Dr. Irwin Gelman's laboratory has shown that over-expression of AKAP12/SSeCKS in NIH3T3 cells causes inhibition of cell growth by promoting G1 phase arrest through binding and regulation of cyclin D activity (Gelman, 2002; Lin, *et. al.*, 2000). It also promotes re-organization of the actin-based cytoskeleton and induces cell flattening (Nelson and Gelman, 1997; Gelman, *et. al.*, 1998; Gelman, *et. al.*, 2000; Xia and Gelman, 2002; Cheng, *et. al.*, 2007). In addition, ectopic AKAP12 expression inhibits podosome formation and metastatic progression in human prostatic cancer (Lin and Gelman, 1997; Xia, *et. al.*, 2001; Gao and Gelman, 2006). The AKAP12 gene locus on chromosome 6 contains a deletion hotspot and aberrations on chromosome 6 are important in CMM progression. Vertical growth phase and metastatic melanomas also have nonrandom abnormalities on chromosome 6 (Liotta, *et. al.*, 1987). These observations suggest that AKAP12 may play a role in melanoma progression.

There are only two published reports that have examined AKAP12 expression in melanoma cells. Hacker *et al* (2008) compared spontaneous vs UVR-induced mouse melanoma. There were 264 genes differentially expressed between these two groups. qRT-PCR verified that AKAP12 expression was downregulated in the UVR-induced mouse melanomas. In the second report, 5-aza-2-deoxycytidine, a DNA methylation inhibitor and trichostatin A, an inhibitor of

histone deacetylase were used to determine genes that were reactivated in melanoma cell lines (Bonazzi, *et. al.,* 2009). Although AKAP12 was one of these genes, its re-expression did not correlate with a high proportion of promoter demethylation.

Recently Streb *et al* (2011) found that atRA stimulates AKAP12 expression in vascular smooth muscle cells. In this study, atRA specifically targeted the induction of the AKAP12β isoform. Based upon an early induction of the AKAP12 mRNA (3h) and the presence of a RARE in the promoter of AKAP12, it was concluded that AKAP12β is a retinoid responsive immediate-early gene. This finding is in contrast to our results in melanoma cells, where AKAP12 expression was not induced until 24 hours after atRA treatment and no RARE's were identified in the AKAP12α promoter region.

The induction of AKAP12 by atRA is significantly correlated with the ability of this retinoid to inhibit melanoma cell proliferation. There is limited information on the ability of AKAP12 to regulate proliferation in various cancer cell lines. However, several studies suggest that it plays an important role in other aspects of tumor biology, such as cancer cell invasiveness and metastatic properties (Lin and Gelman, 1997; Gao and Gelman, 2006; Su, *et. al.,* 2010). Failure of atRA to increase AKAP12 expression is associated with an inability to inhibit melanoma cell growth. Although these data suggest that high levels of AKAP12 may also mediate atRA induced growth inhibition of melanoma cells, some cell lines such as WM3248 can be induced by atRA to produce high levels of AKAP12, but are relatively unaffected by the growth inhibitory effects of

atRA. One possibility for this anomaly is that the retinoic acid nuclear receptor that mediates the induction of AKAP12 expression is different from the one that mediates inhibition of proliferation.

To address how atRA was inducing the expression of AKAP12, I performed a computer analysis of transcription factor binding sites within the AKAP12 promoter. While I did not find any RARE site, I did identify several putative AP-1 sites. Since our laboratory previously reported that atRA increases AP-1 activity in mouse melanoma cells (Desai and Niles, 1997; Huang, *et. al.*, 2002), I tested the hypothesis that atRA was increasing AKAP12 mRNA expression through its ability to stimulate AP-1 activity. AP-1 activity was increased in several of our human melanoma cell lines when treated with atRA. This finding reproduces what we previously reported in B16 melanoma cells.

I tested whether AP-1 mediates the atRA induction of AKAP12 in B16 cells by inhibiting AP-1 activity through the use of the dominant negative fos (A-fos). The role of AP-1 in the regulation of AKAP12 expression in melanoma is opposite to what we had hypothesized. Instead of mediating the increase in AKAP12 in atRA-mediated melanoma cells, AP-1 acts as a negative regulator. This conclusion is based on the findings that inhibition of AP-1 activity through expression of a dominant-negative A-fos plasmid led to a higher degree of atRA-induced AKAP12 stimulation compared to cells with normal AP-1 activity. This negative regulation of AKAP12 expression by AP-1 may be explained by the location of its binding sites. The AKAP12 promoter can be subdivided into three sections that contain a basal promoter region (-173 to +1), a modulator region (-

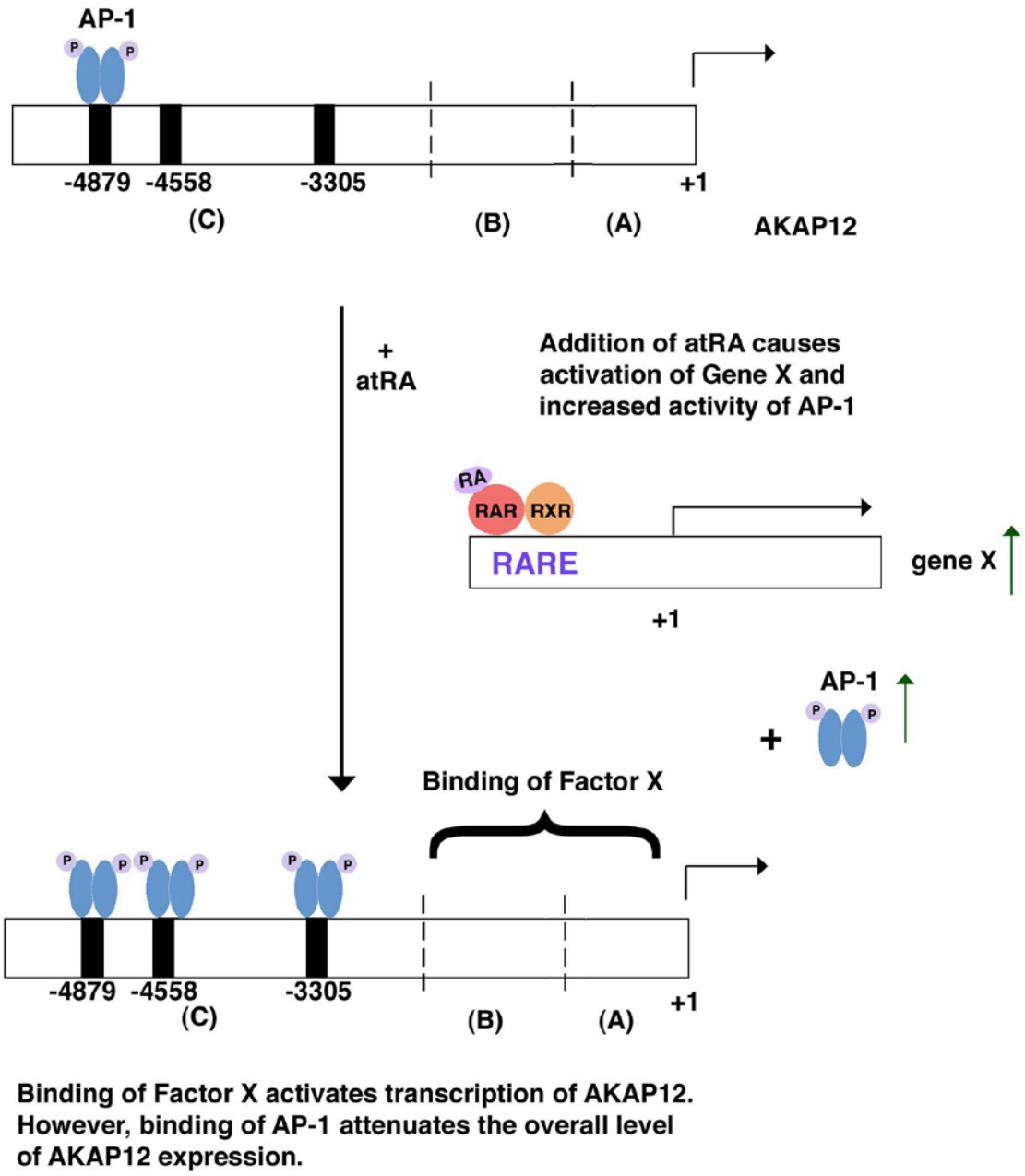
588 to -174) and a negative control region (-4764 to -589) (Streb 2004). My *in silico* analysis of the AKAP12 promoter revealed that the putative AP-1 binding sites lie within the negative regulatory region (-4764 to -589). I postulated that specific deletion or mutation of these sites will lead to enhanced stimulation of AKAP12 expression by atRA.

This paradox of atRA-induced increase of a negative regulator of AKAP12 expression suggests that very high levels of AKAP12 might be detrimental to its normal functioning in the regulation of signaling processes. I hypothesized that if cellular AKAP12 levels remain too low, this may predispose the cell to an enhanced proliferative state, augmenting the loss of controls on processes such as cell adhesion and motility. In contrast, if AKAP12 levels are too high, signaling molecules such as protein kinases are tightly sequestered by the AKAP12 scaffold, thus preventing the normal functioning of pathways. If this latter situation is not reversed it could lead to cell death.

Epigenetic factors are also involved in the control of AKAP12 expression. Methylation of the AKAP12 promoter has been described and associated with silenced or reduced expression of AKAP12 mRNA (Jin, *et. al.,* 2008; Mardin, *et. al.,* 2010). This mechanism may explain the lack of AKAP12 induction in response to atRA treatment seen in WM1366 (VGP) melanoma cells.

Because AKAP12 is a “late” atRA regulated gene and does not contain any discernable retinoic acid response elements, it is likely that an “early” atRA directly-regulated gene is responsible for the stimulation of AKAP12 mRNA and subsequently protein levels in mouse and human melanoma cells. My current

working model for atRA regulation of AKAP12 is shown in Figure 13. In the unstimulated state, there is basal expression of the AKAP12 gene and AP-1 may be bound to some of its consensus sequences. Upon addition of atRA, the RAR (retinoic acid receptor): RXR (retinoid X receptor) heterodimer complex is activated and stimulates the expression of gene "X". In addition, atRA stimulates increased AP-1 activity. The expression of Gene "X" increases and binds to its consensus sequence, most likely located in the basal promoter region or the modulator region of the AKAP12 gene. While the binding of factor "X" induces transcriptional activity of the AKAP12 gene, the binding of AP-1 works to repress expression, restraining the overall production of AKAP12 mRNA and protein.



Binding of Factor X activates transcription of AKAP12. However, binding of AP-1 attenuates the overall level of AKAP12 expression.

Figure 13. Working model for atRA induction of AKAP12 gene activity in melanoma cells. This diagram shows the promoter region of the AKAP12α gene. A is the basal promoter region, B is the modulator region and C is the negative control region. Numbers indicate the location of the AP-1 DNA binding sites.

After addition of atRA, the activated RAR/RXR heterodimer binds to the retinoic acid response element located in Gene X and induces its expression. In addition, atRA also stimulates increased activity of the transcription factor AP-1. Gene X may bind to a site located in the basal promoter or modulator region to induce the expression of AKAP12. However, the increased activity and subsequent binding of AP-1 to the regions located in the negative control region modulates the overall level of AKAP12 expression.

To ascertain AKAP12 function in melanoma cell biology, WM3248 (human) and B16 (mouse) melanoma cells were transfected with siRNA to AKAP12 in order to decrease its expression. These treated cells and appropriate controls were assayed for their ability to invade Matrigel basement membranes and grow in an anchorage-independent manner. AKAP12/SSeCKS/Gravin re-expression has been shown to inhibit Matrigel invasion and soft agar colony formation in NIH-3T3 fibroblasts and MLL prostate cancer cells (Lin 1997, Gao 2006, Su 2010). The effect of AKAP12's decreased expression on atRA's ability to regulate melanoma cell invasion and proliferation was also examined. AKAP12 knock-down in non-atRA treated WM3248 and B16 cells results in increased invasive properties. AKAP12 knock-down also increased anchorage-independent growth in B16 melanoma cells. Although atRA treatment inhibits invasion and soft agar colony formation in B16 cells, and the down-regulation of AKAP12 expression does somewhat antagonize the ability of atRA to inhibit invasive and proliferative properties, results were not as significant as we expected. One possibility to

explain these findings is the amount of time (4-5 days after siRNA transfection) required to measure anchorage-independent growth. This prolonged time leads to decreased capacity of the siRNA to downregulate AKAP12 expression, allowing atRA to overcome the effects of the silencing RNA.

It would be interesting to determine the biological effect of over-expressing AKAP12 in melanoma cells, such as WM1366, which are unresponsive to the effects of atRA and exhibit only low levels of AKAP12 induction after atRA treatment. Overexpression of AKAP12 was attempted using two different plasmids; pEGFP-Gravin and pcDNA3.1/V5/His-Gravin. These plasmids were submitted to us by Dr Bryon Grove, at the University of North Dakota, School of Medicine. The vectors were 4.7 and 5.4kb respectively, while the Gravin insert was 5.343kb. Due to the large size of the Gravin insert, I found it very difficult to achieve a reasonable amount of expression in any of the melanoma cell lines tested with these plasmids.

In conclusion, I have demonstrated that atRA regulates the expression of AKAP12 in mouse melanoma and in human melanocytes, and to varying degrees, human melanoma cells. Further, the induction of AKAP12 is tightly linked to inhibition of melanoma cell proliferation by atRA. However, additional experiments are needed in order to understand how AKAP12 expression is being regulated. My studies also suggest that AKAP12 has a tumor suppressive role in melanoma biology, regulating metastatic and proliferative properties of melanoma cells.

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Chapter 4: Conclusion

During the process of melanoma tumor growth and progression, many genes become deregulated and contribute to its lethality. The regulation of gene expression requires a complex network of positive and negative growth factors, signaling pathways, and transcription factors. Transcriptional regulation of genes in melanoma is a multifaceted process that over-rides the normal melanocyte signaling pathways and there is a powerful association between signal transduction pathways and the activation of transcription factors that are involved in coordinating gene transcription. Transcription factors play a fundamental role in controlling the activity of genes that regulate physiological functions such as cell growth, survival, apoptosis, energy metabolism, inflammation, differentiation, invasion, and metastasis in melanoma cells (Poser and Bosserhoff, 2004).

To help identify the key regulatory factors that contribute to melanoma aggressiveness, a comparative analysis of gene expression data sets from the NCBI GEO database using melanoma, basal cell carcinoma and seven other cancer types was performed. In this analysis, advanced VGP melanoma had the highest number of differentially expressed genes (Xu, *et. al.,* 2012). There is also an upregulation of key genes involved in fatty acid metabolism (β-oxidation), cell invasion and metastasis.

Although available technology can identify the changes in gene expression taking place during melanoma progression, as well as during specific treatment protocols, it is important to address the cause of these gene expression changes.

Because transcription factors are major players in this mechanism, we need to know what role they play in altering the expression of targeted genes during the progression of melanoma.

My research centered on the activity of two transcription factors that regulate key genes involved in melanoma progression. They are members of a super-family of ligand-activated transcription factors that include the RAR's, RXR's, PPAR's, LXR's, FXR's, thyroid receptor and Vitamin D receptor. The first part of my thesis work investigated the biological effect of PPARα/γ activation in B16 mouse melanoma cells, and the second part examined the expression and function of an indirect target gene of RAR activation, AKAP12. Both PPARs and RARs induce gene activity through a similar mechanism and have been shown to regulate the expression of genes involved in cell growth and differentiation.

PPARs are transcription factors that regulate genes involved in glucose and fatty acid metabolism. They also regulate pathways controlling cellular proliferation, differentiation, tumor promotion, apoptosis and the immune response. Ligands that activate PPARs have chemoprevention effects and inhibit growth in several different types of cancer cells (Sertznig, *et. al.*, 2007; Sertznig, *et. al.*, 2010). I therefore investigated the role of PPARs in regulating melanoma cell growth.

The results of my experiments show that B16 mouse melanoma cells have increased expression of PPARα and γ protein, but the growth of melanoma cells treated with PPAR agonists was only moderately inhibited and PPARγ agonists were more effective at inhibiting proliferation than PPARα agonists. In addition,

there is some problem with detecting the ability of PPARs to initiate gene transcription. I was not able to detect a measurable level (lack of reporter gene activity) of endogenous receptor, indicating that there is some problem with regulating the expression of target genes. This decrease in transcriptional activity might be due to the constitutive activation of the mitogen-activated kinase pathway, inducing phosphorylation of specific serines by ERK1/2, located in the N-terminal ligand-independent activation domain of the PPARs. This phosphorylation induces a conformational change in the protein that hinders its ligand binding ability and downregulates its activity. The reason for this level of regulation could be due to PPARs ability to activate the expression of genes involved in inhibition of the cell cycle. More investigation is needed to determine how alteration in PPARs may contribute to melanoma proliferation. Unfortunately, there are few new articles that have addressed the issue of PPAR function in melanoma since the publication of our work.

There are reports of PPAR agonists having effects on metastatic properties and tumor proliferation. Grabacka M, *et.al.,* (2006), demonstrated that activation of PPARα with fenofibrate inhibited mouse and human melanoma cell invasion and soft agar growth. Also, in mouse xenograft models of melanoma, mice were given either the PPARγ agonist rosiglitazone; a RXR agonist; or a combination of the two. Melanoma tumor growth was significantly inhibited by either ligand alone and the combination had an additive effect (Klopper, *et. al.,* 2009). These results show that PPARγ agonists can still be effective inhibitors of melanoma cell proliferation.

For the second part of this project, my research was focused on a newly identified indirect target gene of RAR activation that had not been previously examined in melanoma cells. This gene, AKAP12, has been characterized in other cancer cell lines, especially prostate (Xia, *et. al.*,2001), as being a major regulator of signal transduction pathways that control cell adhesion and oncogenic growth through its binding and sequestering of many different second messengers. Currently, there are no publications reporting the atRA regulation of AKAP12 expression in melanoma cells, or that address its role in melanoma cell biology.

The majority of information on AKAP12 and cancer has come from work done by Dr. Irwin Gelman's laboratory at the Roswell Park Cancer Institute, through the use of fibroblasts and prostate cancer cell lines. In Ras-transformed NIH3T3 fibroblasts, downregulation of SSeCKS increased soft agar colony formation and Matrigel invasiveness (Lin and Gelman, 1997). Re-expression of SSeCKS in MLL prostate cancer cells suppresses formation of lung metastasis and suppresses the expression of vascular endothelial growth factor (VEGF), hypoxia-inducible factor-1α (HIF-1α), and platelet-derived growth factor receptor β (PDGFRβ). SSeCKS was shown to suppress the formation of metastatic lesions through inhibition of VEGF expression and induction of soluble anti-angiogenic factors. This inhibits neovascular formation induced by tumor cells at peripheral sites (Su, *et. al.,* 2006). SSeCKS-knockout mice develop prostate hyperplasia, suggesting that these mice have increased susceptibility for oncogenic transformation (Akakura, *et. al.*,2008). In nude mice injected with MLL cells containing a

tetracycline regulated SSeCKS expression vector, re-expression severely decreased the formation of secondary lung metastasis, indicating that loss of SSeCKS plays a major role in the metastatic progression of human prostate cancer cells (Xia, *et. al.*, 2001). In addition, the re-expression of SSeCKS in NIH3T3 cells suppresses podosome formation and decreases Matrigel invasiveness. Pososomes are actin-rich structures commonly found in cancer cell lines and are believed to assist in the invasive properties involved in metastasis of tumor cells. SSeCKS aids in the suppression of oncogenisis by re-establishing the normal actin-based cytoskeletal architecture (Gelman and Gao, 2006).

My research shows that, not only do mouse and human melanoma cells express AKAP12, its expression is regulated by atRA. During my research on AKAP12, I also made a novel finding that the transcription factor AP-1 is a negative regulator of AKAP12 expression. Functional assays for quantifying the invasive and anchorage-independent growth properties of mouse and human melanoma cells showed that when AKAP12 expression was decreased, invasion and soft agar colony formation was significantly increased. However, knockdown of AKAP12 in melanoma cells treated with atRA only partially antagonized the ability of this retinoid to inhibit Matrigel invasion and soft agar colony formation. The AKAP12 siRNA could not sufficiently decrease the strong atRA induction of AKAP12 enough to establish if there were changes in Matrigel invasion and anchorage-independent growth. Therefore, it is still possible that atRA inhibits these melanoma activities through other mechanisms in addition to stimulating the expression of AKAP12. I hypothesize that AKAP12 may play a tumor

suppressive role in melanoma biology through coordinating signaling pathways that prevent metastasis and anchorage-independent growth.

The ability of AKAP12 to regulate these various pathways stems from its function as a scaffolding protein. This protein works to sequester and inhibit the activity of many different enzymes, including those involved in transducing signals through the cytoplasm of cells, events commonly initiated at the cell membrane. The ability of AKAP12/SSeCKS/Gravin to associate with both the plasma membrane and the actin cytoskeleton places this protein at a position where it can have direct control over regulating the activity of various signaling pathways and the actin based cytoskeletal architecture. Many of these pathways regulate proliferation, differentiation, survival and metastatic properties (Gelman, 2002).

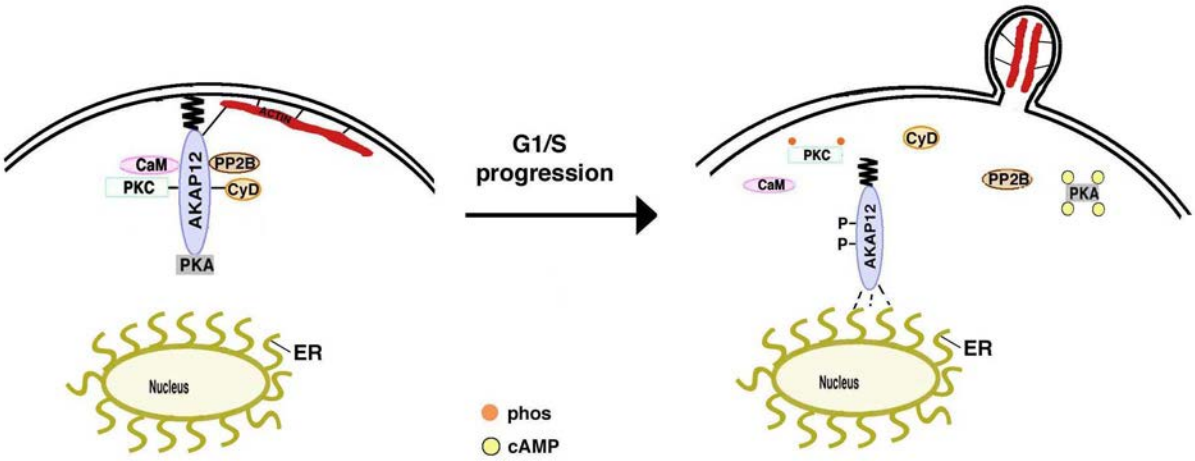
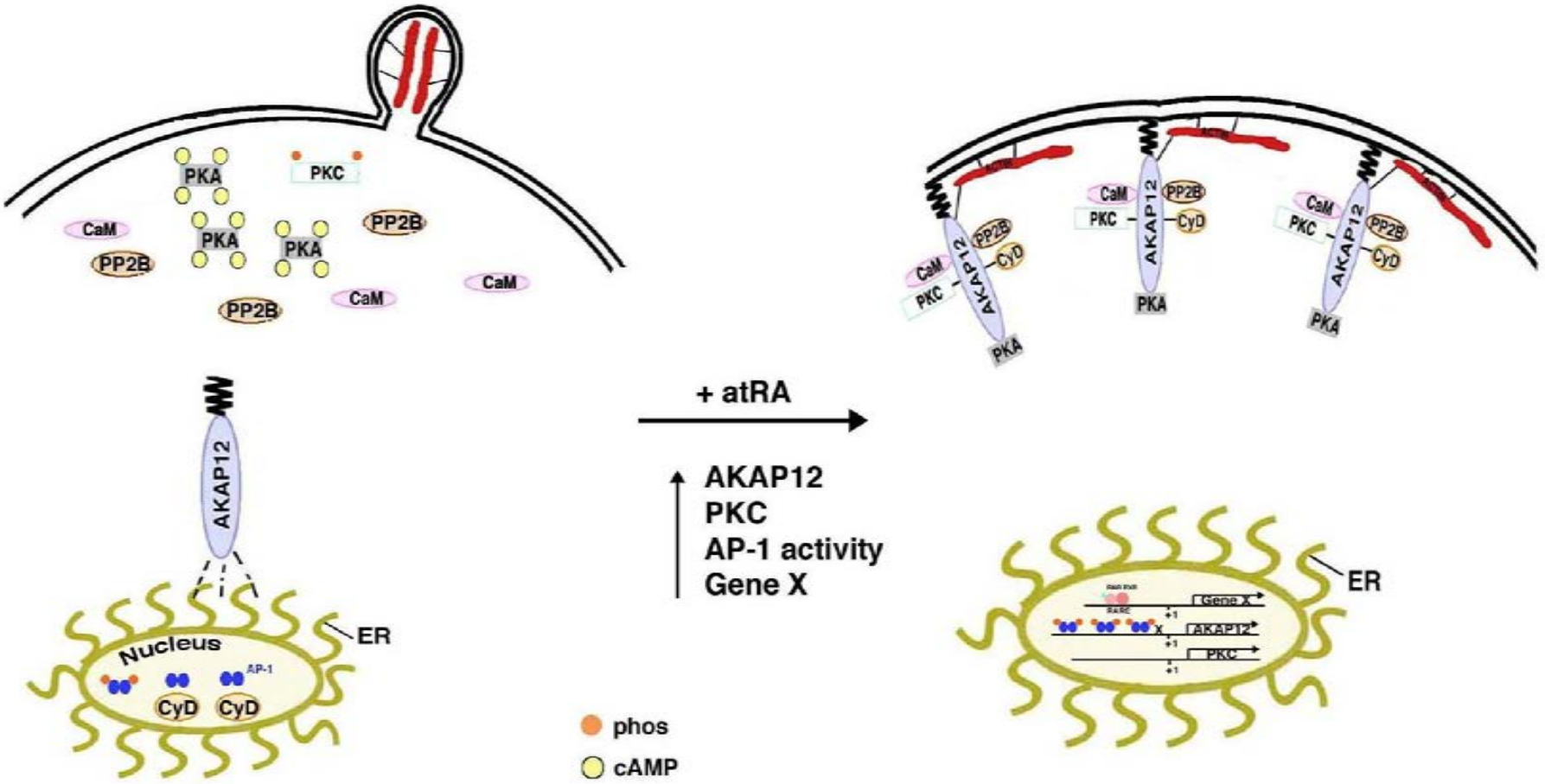


Figure 4.1 Model for AKAP12 scaffolding functions. During G_0 or the early G_1 phase of the cell cycle, the AKAP12 protein sequesters key signaling proteins such as PKA, PKC, calmodulin (CaM), phosphatase 2B (PP2B), and cyclin D

(CyD). After mitogenic stimuli, AKAP12 is rapidly phosphorylated by PKC, inducing a conformational change that leads to the release of the various enzymes and translocation of AKAP12 to the endoplasmic reticulum (ER) in peri-nuclear area. This process leaves all of the enzymes free to become active themselves and to regulate the activities of a variety of their substrates. The change in AKAP12s ability to interact with actin, as well as its various binding proteins, enable the cell to undergo changes in shape more easily (formation of podosome), which is necessary for invasion and metastasis.

Most melanoma cells harbor mutated proteins, such as NRAS and BRAF, that constitutively activate the MAPK pathway. Evidence suggests a link between the MAPK pathway, the ability of AKAP12/SSeCKS to bind and regulate the activity of protein kinase C (PKC), and cancer cell invasiveness (Su, *et. al.,* 2010). PKC is a serine/threonine protein kinase that plays a role in regulating many cellular functions including cell growth and differentiation. AKAP12 suppresses metastatic motility in MAT-LyLu (MLL) prostate cancer cells by inhibition of the RAS/RAF/MEK/ERK1/2 pathway through direct binding of PKC (Su, *et. al.*, 2010). Re-expression of SSeCKS in MLL cells causes a 50-65% decrease in total PKCα activity, yet there is no change in total PKCα expression. This is interesting, because our laboratory has previously shown that RA induces a 6-8 fold induction of PKCα mRNA and protein, and a 4-fold induction of AP-1 activity (Desai and Niles, 1997; Huang, *et. al.,* 2002). In addition, only the PKCα protein, but not enzyme activity, was required for the RA-induced AP-1 activity

seen in live melanoma cells. The atRA stimulation of AP-1 activity contributes to growth arrest and differentiation. Treatment of B16 mouse melanoma cells with the PKC enzyme inhibitor bisindoylmaleimide did not block the RA response (Desai and Niles, 1997). Also, over-expression of PKCα in these cells resulted in a more differentiated phenotype (Gruber, *et. al.,* 1992). From this information and my investigation of AKAP12 expression and function, I suggest that the increase in AKAP12 expression seen in RA treated melanoma cells is coupled to an increase in PKCα expression. This increase in PKCα protein expression seen in melanoma cells may be necessary for AKAP12's normal functioning as a scaffolding protein. PKCα activity is not needed because AKAP12 is able to bind and block its ability to function as a kinase protein.


PKA
PKC
CaM
PKA
PP2B
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PP2B
CaM
CaM
PP2B
AKAP12
Nucleus
AP-1
CyD
CyD
ER
+ atRA
AKAP12
PKC
AP-1 activity
Gene X
phos
cAMP
CaM
PP2B
PKC
CyD
AKAP12
PKA
Gene X
AKAP12
PKC
ER

Figure 4.2. Model of AKAP12 scaffolding function in melanoma cells before and after treatment with atRA. In melanoma cancer cells, there are decreased levels of AKAP12 protein. Also, these cells are highly proliferative, with a higher fraction of cells in the G2/S phase of the cell cycle relative to normal melanocytes. The AKAP12 protein becomes positioned at the (endoplasmic reticulum) ER near the nucleus of the cell, not at the cell membrane where it would normally be located. The protein Cyclin D (CyD) has also translocated to the nucleus, because of its involvement in cell cycle progression. The enzymes PKA, PKC, CaM, and PP2B, are free to become active, regulating the activity of their substrate proteins, and in turn the activity of various signaling pathways. Retinoic acid works to inhibit melanoma cell growth by first halting proliferation at the G1 phase of the cell cycle. It is also responsible for increased expression levels of the proteins PKC and AKAP12 (as an indirect target gene), and for increased activity of the transcription factor AP-1. As the cells halt their proliferation at G1, cyclin D translocates from the nucleus to the cell membrane to become bound to AKAP12. PKC protein levels also increase, similar to those of AKAP12. The docking of PKC to the AKAP12 scaffolding protein is essential for the ability of AKAP12 to bind all of the appropriate proteins in the correct conformation. The re-localization of cyclin D and increased PKC protein levels, combined with increased AKAP12 protein, allow AKAP12 to function as a negative mitogenic regulator, sequestering and inhibiting the enzymatic properties of the various proteins.

PKC can also influence RA signaling by altering the stability of the RARα protein. Activation of PKC in mouse melanoma cells leads to an increase RARα protein levels, while downregulation of PKC diminishes RARα protein half-life and markedly inhibits ligand-independent transcriptional activity (Boskovic, *et. al.,* 2002). Regulation of RARα expression and activity may be one possible way of influencing the RA-induced expression of AKAP12.

Another possible mechanism for AKAP12 regulation of the melanoma phenotype is though its ability to regulate G-protein coupled receptor (GPCR) activity (Shih, *et. al.,* 1999). The melanocortin-1 receptor (MC1R) is a G-protein coupled receptor that is involved in melanoma function and development. Individuals with mutations in this receptor produce more of the red/yellow pigment pheomelanin, than the black photo-protective pigment eumelanin. Because of the decreased eumelanin expression, these individuals usually have fair skin and light colored hair, and are more sensitive to UV induced DNA damage. Stimulation of GPCRs involves phosphorylation of the receptor, which is a critical element of agonist-induced desensitization, leading to internalization of the receptor for recycling. This process includes the involvement of the enzymes PKA, G protein-coupled receptor kinases (GRKs) and the adaptor molecule β-arrestin. The activity of MC1R is regulated by the agonists ACTH (adrenocorticotropic hormone) and αMSH (melanocyte stimulating hormone), while ASIP (agouti signaling peptide) is an antagonist. Receptor activation induces the activity of adenylate cyclase, resulting in increased production of cAMP. This cyclic nucleotide is necessary for PKA activation. AKAP12 not

only binds the kinases PKA and PKC, as well as phophatase 2B (PP2B), but can also directly associate with the GPCR. The physical association of AKAP12 to the β_2-GPCR affects signaling through regulation of agonist-induced desensitization of the receptor. The protein phosphatase PP2B plays a critical role in resensitization due to its ability to dephosphorylate the receptor (Shih, *et. al.*, 1999). Once desensitization and internalization of the MC1R is complete, AKAP12 plays a crucial role in resensitization (dephosphorylation) and recycling of the receptors back to the cell membrane. Therefore, the reduced AKAP12 expression in melanoma cancer cells may influence and downregulate MC1R activity, by impeding recovery from agonist-induced desensitization (Shih, *et. al.*, 1999; Tao, *et. al.*, 2007; Tao and Malbon, 2008).

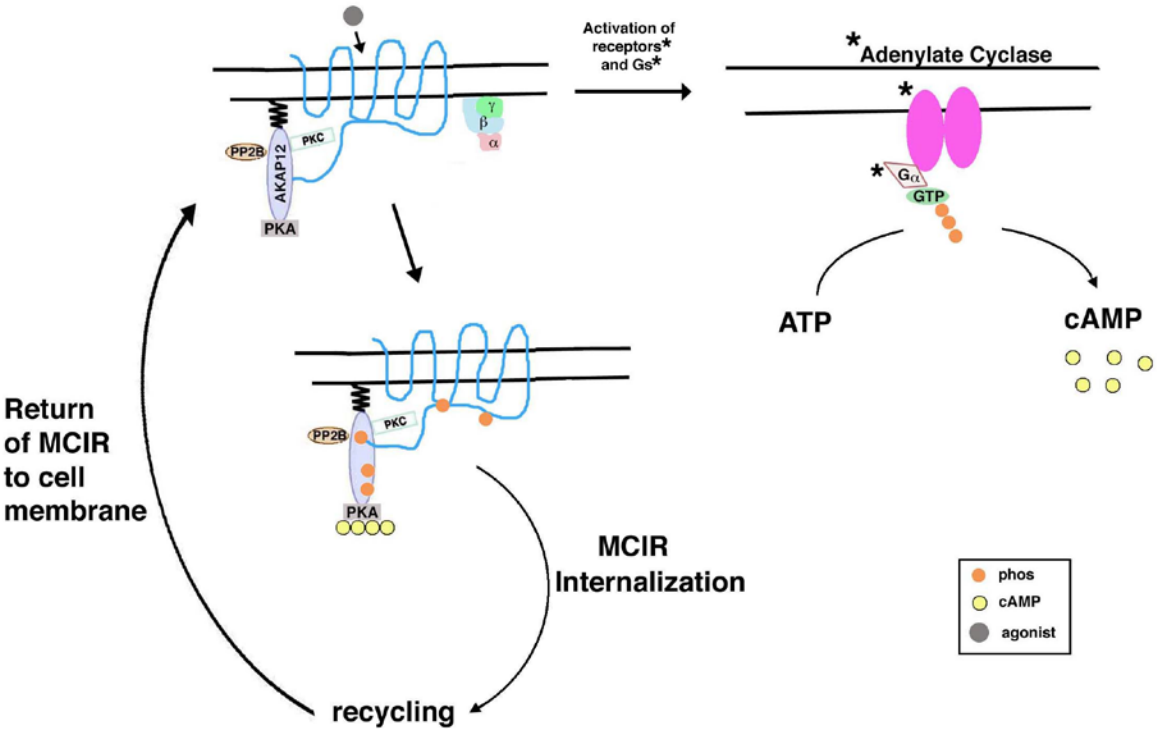

Figure 4.3. Model for the regulation of melanocortin 1 receptor (MC1R) signaling by AKAP12. AKAP12 provides docking sites for protein kinase A (PKA), protein kinase C (PKC), and phosphatase 2B (PP2B) and many other enzymes and adaptor molecules that are not shown. AKAP12 also associates with the MC1R. After the MC1R binds its agonist, this leads to activation of the receptor and the small Gα protein ($G\alpha_S$). $G\alpha_S$ is responsible for the activation of adenyl cyclase, which converts ATP to cAMP. PKA needs to bind cAMP to become active. Once activated, PKA will phosphorylate specific sites on the receptor and the AKAP12 protein. This phosphorylation will markedly enhance the binding of AKAP12 to the receptor. The enzyme PP2B is responsible for dephosphorylation of sites located on the AKAP12 scaffolding protein and the MC1R receptor. This process is critical for AKAP12 ability to mediate the resensitization and recycling of the internalized MC1R back to the cell membrane.

There are numerous avenues of the AKAP12 project that need to be explored. The regulation of AKAP12 gene expression and function in normal melanocytes needs to be determined. Other research questions include: What is the identity of factor "X", i.e. the atRA direct target gene that is responsible for induction of AKAP12 expression after treatment of melanocytes or responsive melanoma cells with atRA? Second, is there a difference in subcellular location of AKAP12 in the melanoma cells compared to the normal human melanocytes? An altered location of AKAP12 in melanoma cells could change the function of this scaffolding protein. Third, how would over-expression of AKAP12 in a cell line

such as WM1366 melanoma cells, a rapidly growing, atRA resistant cell line, change the physiology of these cells?? I was unable to successfully attain overexpression of the AKAP12 gene in these melanoma cells. The cDNA coding for this gene is approximately 5.4kb and is as large as or larger than the expression vectors that were used to express the gene in melanoma cells. The very large size of the plasmid impeded its transfection efficiency. However, an adenoviral delivery system may be more successful in achieving high AKAP12 gene expression. Fourth, the WM3248 cells express very high basal levels of AKAP12 and these levels are further induced by atRA, yet, these cells are only moderately responsive to the growth inhibitory effects of atRA. Why is there a strong induction of AKAP12 in cells that are more resistant to atRA-dependent growth inhibition?? Last, mutation of sites encoding binding domains for important signaling kinases located in the N-terminal region of the protein can give insight into how AKAP12 regulates these pathways. The answers to the questions listed above will provide a much deeper insight into the role of AKAP12 in melanocyte biology and could uncover therapeutic targets to counteract its dysfunction in human melanoma.

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