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Effects of Retinoic Acid on Beta-Catenin Transcriptional Activity in Melanoma Cells

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EFFECTS OF RETINOIC ACID ON BETA-CATENIN TRANSCRIPTIONAL ACTIVITY IN MELANOMA CELLS

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by

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LIST OF SYMBOLS / NOMENCLATURE

ATRA ……..……All *trans* Retinoic acid

ABC……Active beta-catenin

c-Myc……v-myc myelocytomatosis viral oncogene homolog (MYC)

MITF…….……..…Microphthalmia-associated transcription factor

PBS …….…….. Phosphate Buffered Saline

LEF1……. lymphoid enhancer-binding factor 1

APC ……..……...…… adenomatous polyposis coli

GSK …….. Glycogen Synthase Kinase

TCF ……..…… T-cell factor

MAP Kinase……....Mitogen Activated Protein Kinase

TBE …… Tris-Borate-EDTA

DNA …… Deoxyribonucleic acid

RPMI……..……..…… Roswell Park Memorial Institute

Shh ……...…….…….sonic hedgehog homolog (*Drosophila*)

Wnt……...……..……..…Wingless (*Drosophila*)

EMSA…….……Electrophoretic Mobility Shift Assay

EGF…….……epidermal growth factor

Abstract

Melanoma is the most dangerous form of skin cancer and its incidence has been increasing in the United States. Most melanomas are resistant to current chemotherapies; therefore, understanding the mechanism of melanomagenesis is beneficial to treatment of the diseases. Accumulation of β-catenin has been shown in colon and other cancers, including melanomas, but the transcriptional role of β-catenin in melanomas is still unclear. All-*trans* retinoic acid (ATRA) is an anti-tumor agent that has an inhibitory effect on β-catenin, but this effect has not been studied in melanomas. It has been shown that Microphthalmia-associated transcription factor (MITF), a melanocyte specific protein, can redirect the function of β-catenin from cell proliferation to melanocyte-specific gene expression. In addition, ATRA induced *Mitf* mRNA expression was observed in mouse melanocyte. My objective is to understand the effects of ATRA on the β-catenin signal pathway in ATRA-sensitive melanoma cells. I hypothesize that ATRA will decrease β-catenin transcriptional activity. Western blotting was used to determine the effects of ATRA on β-catenin target genes. Gel Shift assays were used to investigate DNA/ protein interactions. Reporter gene assays were used to examine the effects of ATRA on the transcriptional activity of β-catenin. Results showed a 20% and 40% reduction in c-Myc protein expression after 2 days and 4 days ATRA treatment, respectively. Cyclin D-1 protein expression was reduced by 40% after 4 days treatment compared to the control. ATRA increased the protein levels of MITF after 2 days treatment. Reporter gene assay showed that ATRA reduced transcriptional activity of exogenous active β-catenin in human melanoma cells. Together, ATRA inhibits

melanoma progression by reducing β-catenin transcriptional activity, and in part through inducing MITF expression to alter the function of β-catenin away from growth regulatory pathway.

Chapter One

Introduction

According to estimates of the American Cancer Society, there will be over 60,000 new cases of melanoma in the United States each year. Melanoma is the most dangerous form of skin cancer. Although melanoma only represents 5% of all skin cancers, it causes 71% of all skin cancer deaths (2). Melanoma is a malignant tumor that originates from the pigment-producing skin cell, melanocytes. Melanoma, like some other cancers, has a good prognosis if recognized and treated early. However, if it is not, melanoma can metastasize to lymph nodes and other organs beyond the region of the original tumor. The initial treatment of melanoma is usually by surgical excision, generally followed by chemotherapy when treating later stage melanoma. However, the prognosis for survival of the late stage melanoma patient is poor.

In order to find an effective treatment against melanoma or any other cancers, we have to understand the molecular signaling pathways that govern tumor development and survival. Therefore, we utilize all trans-retinoic acid (ATRA) as our inhibitor of melanoma growth. Many studies have shown that ATRA can delay the progression of some tumor cells, including melanoma, by inducing cell cycle arrest (32, 42). Thus, we used the retinoid-sensitive B16 mouse melanoma cell line - F1 (low metastatic ability) and F10 (high metastatic ability). Recent studies suggest that ATRA is capable of inhibiting activation of β-catenin reporter constructs in vitro (56). Many studies have shown that Wnt/ β-catenin pathway is activated in different types of cancer. In addition,

accumulation of β-catenin in the nucleus of colon cancer cells contributes to tumorigenesis. However, ATRA inhibition of β-catenin activity has not been studied in melanoma. Therefore, β-catenin was selected as the molecule of interest, and investigated the interaction of ATRA and β-catenin signaling in melanoma.

The role of β-Catenin was first recognized as a membrane-associated protein, involved in cell–cell adhesion (21). Cytoplasmic β-catenin binds to the carboxyl terminus of E-cadherin at the plasma membrane, and this complex recruits α-catenin, and then further recruits other structural proteins to form the cell-cell junctions (17). In addition to its role as an adhesion protein, β-catenin can also be a transcription coactivator. β-catenin is a key component of downstream signaling in the Wnt/ Wingless pathway, which is extremely important to embryonic development. This pathway is also involved in disease development if mis-regulation of β-catenin occurs (34). In development, Wnt protein binds to the Frizzled receptor and inhibited the adenomatous polyposis coli (APC)/ Glycogen Synthase Kinase (GSK-3 β) complex, which phosphorylates β-catenin, targeting it to proteasomal degradation. When β-catenin is protected from degradation, it enters the nucleus and associates with the T-cell factor and lymphoid enhancer factor (TCF/LEF-1) family of transcription factors. This association activates the transcription of β-catenin target genes, including regulators of cell growth and proliferation, modulators of cell death pathways and cell–cell communication (22). If Wnt/ β-catenin pathway is activated inappropriately, then it might cause tumorigenesis. Thus, this study is to focus on the regulation of the activity of β-catenin and its partner TCF/ LEF-1 DNA binding proteins, by ATRA.

Objective

Unlike the mouse B16 cells, many human melanomas are resistant to ATRA, therefore we hope to use the mouse B16 cells to understand how the ATRA affects the β-catenin signal transduction pathway in a responsive melanoma model. I will focus on the transcriptional activity of β-catenin and its partner signaling molecules in mouse B16 melanoma cells.

Hypothesis

Previously, we observed that ATRA reduces mouse B16 melanoma cell growth; therefore, my hypothesis is the ATRA treated B16 mouse melanoma cells will have a lower β-catenin transcriptional activity compared to the untreated B16 cells.

Chapter Two
Review of Literature

Introduction

This chapter presents a review of the literature relevant to the present study. The Chapter consists of three parts:

1.) The role of β-catenin and partner protein TCF/LEF in tumorigenesis;

2.) The β-catenin target genes related to cell proliferation, cell cycle regulation, and melanocyte survival;

3.) The use of the anti-tumor agent, all trans-retinoic acid (ATRA).

The role of β-catenin and partner protein TCF/LEF protein in tumorigenesis

Activation of Wnt pathways can modulate cell proliferation, cell survival, cell behavior, and cell fate. The core molecule of this pathway is β-catenin/ armadillo. A series of signaling events occurs before β-catenin can function as a co-activator of gene transcription. Generally, the Wnt pathway begins when secreted Wnt glycoproteins bind to cell-surface receptors of the Frizzled family. This activates Dishevelled (DSH), a key component of a membrane-associated Wnt receptor complex, which inhibits a second complex of proteins that includes axin, GSK-3, and the protein APC. In absence of Wnt signaling, APC-axin-GSK-3β complex phosphorylates β-catenin, and then ubiquitination of β-catenin follows, which leads to proteosomal degradation of ubiquitin-tagged β-catenin. However, activated DSH deactivates this "β-catenin degradation complex", which leaves a pool of stabilized and active cytoplasmic β-catenin. High levels of cytoplasmic β-catenin are able to rapidly migrate into the nucleus through gated channels.

In the nucleus, active β-catenin couples with TCF/LEF family transcription factors to promote specific gene expression (22, 34, 57).

In normal differentiated cells, β-catenin either associates with cadherins to form cell-adhesive structures or is phosphorylated by the APC-axin-GSK-3β complex that leads to its degradation by the ubiquitin-proteasome system (1, 40). It is known that the Wnt/ β-catenin signaling pathway can cause tumorigenesis in colon cancer (44). Tumorigenesis is thought to be initiated by mis-regulation or by mutations of β-catenin, which disable regulatory phosphorylation. The ultimate goal of Wnt pathways are to protect free and activate β-catenin and then activate gene transcription. A recent study has shown that in mouse B16 cells, β-catenin mostly accumulates in cytoplasm and some accumulation occurs in the nucleus. In contrast in human melanoma cells (501mel and SK-MEL-5), β-catenin exhibits high levels of nuclear accumulation (49). This suggests that the degree of β-catenin accumulation varies among melanoma cells. Alterations other than dysregulation of Wnt signaling may also result in tumorigenesis. Stabilizing mutations of β-catenin can also promote cancer development. Rimm *et al.* (1999) showed that 6 of 27 melanoma cell lines were found to have β-catenin exon 3 mutations affecting the N-terminal phosphorylation sites (37). In other studies, mutations of β-catenin in melanoma cell lines are rare; therefore, possibly other mechanisms of β-catenin misregulation contribute to melanogensis.

β-catenin acts as a transcriptional coactivator for gene expression by forming a complex with T-Cell Factor (TCF) proteins and regulating gene transcription. The TCF

transcription factor family is a group of high mobility DNA binding proteins. In mammals, there are four proteins in the family referred to as TCF/ LEF transcription factors (LEF-1, TCF-1, TCF-3 and TCF- 4). These factors play a crucial role in WNT/Wingless signaling, a signal transduction cascade that directs cell proliferation, differentiation and survival. Mann et al. (1999) was able to show the direct interaction between β-catenin and TCF/ LEF complex with the promoter region of c-jun and fra-1 in a gel shift assay. Lymphoid Enhancer Factor -1 (LEF-1) is a protein that is expressed during development in many different differentiating tissues and its function is sometime redundant with TCF transcriptional factor. Interestingly, studies in melanoma suggested that LEF-1 and β-catenin synergistically play a role in cancer progression. In the study of Shtutman *et al*. (1999), they had found that in colon cancer cells, β-catenin couples with LEF-1 and binds to the *LEF-1* DNA binding site in the cyclin D1 promoter. In addition, they showed that wild-type APC can prevent the coupling of β-catenin and LEF-1. They concluded that the elevation of cyclin D1 in colorectal cancer promotes uncontrolled proliferation and thus contributes to the neoplastic transformation of cells (38). LEF-1 is thought to be the partner of β-catenin and the transcription factor that is involved in melanoma progression (16, 37). Our previous data also suggest that B16 cells do not express TCF-4.

Cyclin D1

To date, the transcriptional role of β-catenin in melanoma is still unclear, but some studies have identified several genes that are β-catenin target genes. One of which is cyclin D1, a key regulator of progression through the G1 phase during the cell cycle.

Cyclin D1 is one of the three D-type cyclins, which are essential for progression from G1 to S-phase. These D cyclins bind to and activate both cyclin-dependent kinases (CDK4 and CDK6). The CDK are then activated due to phosphorylation by CDK-activating kinase (CAK). The activated CDKs phosphorylate the retinoblastoma (Rb) protein, which leads to release of the E2F (family of transcription factors (TF) in higher eukaryotes) transcription factors and cells can then proceed to S phase (52). Due to abnormal regulation of β-catenin in cancer cells, β-catenin accumulates inside the nucleus, which results in the binding of β-catenin and LEF-1. This complex then binds to the *Lef-1* DNA binding site in the target genes' promoter and initiates transcription. Shtutman *et al*. (1999)have shown that β-catenin couples with LEF-1 protein and binds to a LEF-1 binding site in the cyclin D1 promoter; thereby the cyclin D1 gene is a direct gene expression target for β-catenin/LEF-1 in human colon cancer cells (43, 44, and 48). Studies have also indicated that the cyclin D1-gene (CCND1) acts as an oncogene in human hepatocellular carcinoma and esophageal cancer (20, 53). In addition, aberrant expression of cyclin D1 is commonly displayed in human cancers (5, 45, and 47); therefore, cyclin D1 would be a marker for cell proliferation.

c-Myc

c-Myc is a transcription factor in the basic Helix Loop Helix-Leucine Zipper (bHLH-LZ) family. There are three members in Myc family: n-Myc, L-Myc and c-Myc. They all possess the ability to induce cell proliferation, differentiation and tumorigensis. Aberrant expression of the c-Myc gene has been found in several types of human tumors, and the n-Myc gene is expressed/ overexpressed in tumors or cells that derived from

neuroblastomas (31). Abnormal expression of the L-Myc gene has been found in human small cell lung carcinomas (30). C-Myc plays a key role in up-regulation of the transcription of growth-related genes. The mechanism of Myc transcriptional regulation is thought to be by coupling with the Max protein to form a heterodimeric complex and binding to target DNA binding sites to enhance transcription. Phosphorylation of c-Myc by the p44/42 MAP kinase at Ser62 and Thr58 and Ser62 can resulted in activation (12). Activation of c-Myc requires mitogenic signals such as Wnt, Sonic hedgehog homolog (Shh), and epidermal growth factor (EGF). Therefore, Wnt/ β-catenin signaling can induce c-Myc expression level. In addition, c- Myc is similar to cyclin-D1, in what it is involved in cell proliferation, differentiation and apoptosis. It also has been shown that c-Myc is one of the target genes of Wnt/ β-catenin in colorectal cancers (13). Therefore, we chose c-Myc and cyclin D1 as molecules of interest.

MITF

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix-leucine zipper (bHLH-LZ) protein encoded by the mouse microphthalmia locus. It plays a vital role in differentiation and survival of melanocytes (14, 15). MITF is a down stream target of β-catenin (8). Surprisingly, the bHLH-LZ region of MITF is shown to bind to LEF-1 as a coactivator in its own transcriptional activation (41). In addition, the interaction between LEF-1, not TCF-1, and MITF is detected in melanocytes. MITF was also shown to up-regulate BCL2 (anti-BCL2 apoptotic protein) expression in pigment cells (26), which could also be happening in melanoma cells. It is noteworthy that GSK3-β, an inhibitor of β-catenin, can be activated by cAMP and then phosphorylates

MITF, and therefore activates MITF (27). One hypothesis is that GSK3-β may play a role to maintain functional MITF-M levels in melanocytes (41). It would be interesting to know whether ATRA affects the level of GSK3-β, because ATRA can induce melanin production *in vitro* (33). MITF then cooperates with LEF-1 and β-catenin to activate gene transcription by binding to the dopachrome tautomerase (DCT) gene promoter, an early melanoblast marker (51). Watabe *et al*. showed that *Mitf* mRNA levels increased after 72 hrs ATRA treatment. (66) In a recent study, MITF have been shown to interact with β-catenin to modulate melanocyte-specific gene transcription (67).

Anti-tumor agent – all trans-retinoic acid (ATRA)

ATRA is a derivative form of Vitamin A (retinol). The major source of ATRA in the body is conversion of dietary β-carotene to retinal, and then it further oxidizes to retinoic acid (RA), also called vitamin A acid (3, 55). It is known to be important to embryonic inner ear development (36). In addition, it is involved in formation of visual purple in the rod cells, which function to allow sight in dim light. It is also involved in the formation of the three color pigments (red, green, and violet) in the cone cells, which function in bright light to provide color vision (28). There are isoforms of retinoid receptors, which belong to the family of nuclear receptors mediating the activity of steroid and thyroid hormones vitamin D, prostaglandins, and certain drugs. Retinoids have also been shown to have a role in prevention of cancer (32). Recent study has shown that all trans-retinoic acid (ATRA) mediates the G1/ S arrest, and inhibition of retinoic acid receptor (RAR) results in high percent S phase in normal human mammary epithelial cells and lower G1 phase cells (42). This suggests that ATRA and RAR are

vital regulators of the G1 to S phase cell cycle transition. After ATRA translocates by cellular retinoic acid binding protein II (CRABP-II), a carrier protein (6), to the nucleus, it binds to retinoid receptors and activates its target genes, including CRABP-II gene expression (18). Some of the ATRA/RAR target genes that have been identified to be up-regulated by RA/RAR pathway are activator protein-1 (AP-1), protein kinase calpha (PKC), and T-box binding protein type 2 (Tbx-2) (33). ATRA promotes melanoma cell differentiation by inducing PKCα and *MITF* mRNA level (66). Many studies have shown that ATRA synergistically suppresses tumor development by induction of tumor suppressor gene proteins, such as p53 and p105 (Rb or retinoblastoma protein), which are strong inhibitors of cell proliferation (24, 29, 50). An interesting finding showed that RAR, but not RXR (another isoform of retinoic acid receptor), is a competitor of TCF for binding to β-catenin (56). This interaction was accelerated under the treatment of retinoic acid. This study also showed that retinoic acid inhibits activation of β-catenin reporter constructs (56). Interestingly, retinoic acid seems to have no effect on the cytoplasmic regulation of β-catenin, suggesting it does not influence β-catenin degradation.

Retinoids have also been shown to regulate other genes that are involved in normal cellular architecture, cellular adhesion, and cell-cell communication (11, 23). ATRA also increased ICAM-1, a type of intracellular adhesion molecule, which may involved in interaction between immune cells and melanoma (60). Adhesion modulated by ATRA can be seen in our previous immunohistochemistry data (Fig. 1), which showed that ATRA promotes localization of active β-catenin at the membrane for cellular adhesion, as opposed to intracellular sites and restoring a more normal cellular distribution of β-catenin. Therefore, RA may indirectly prevent β-catenin from inducing

gene expression in the nucleus that leads to cell proliferation. In fact, our previous Western Blot data showed that ATRA did not reduce the level of β-catenin but instead it relocated and converted the active form of β-catenin to cell adhesion. (Henry & Delidow, personal communication)

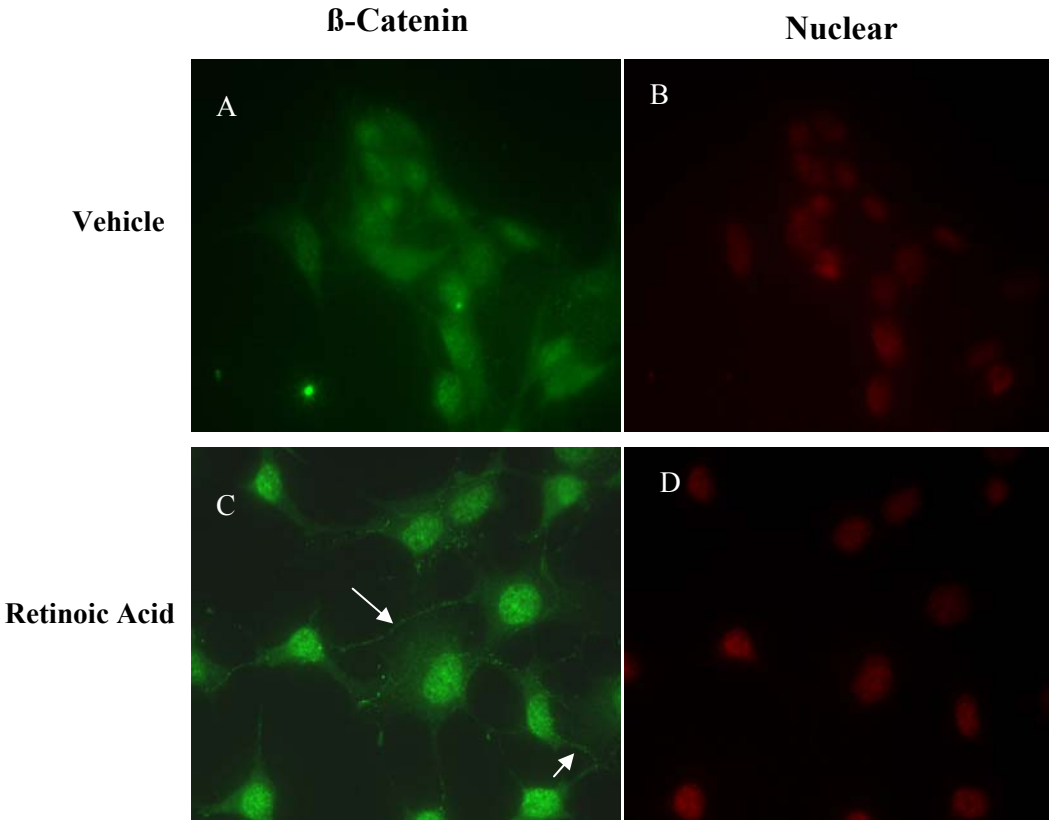


Figure 1. Control and ATRA-treated B-16 mouse melanoma cells treated with anti-active-β-catenin (A-D) primary antibodies. [A & C] Vehicle (DMSO) and RA-treated B-16 cells were stained with anti-active β-catenin and detected with Alexa-488 anti-mouse secondary antibody (green). Nuclei of vehicle and RA-treated B-16 cells were counter-stained with Propidium Iodide nuclear stain (red). Treatment with RA reduced the number of cells. In vehicle-treated cells, active β-catenin is more evenly distributed throughout the whole cell and especially in the nucleus of the vehicle-treated cells. In the RA-treated cells Active β-catenin is more clearly relocated to the membrane (see arrows, panel C). – Cara Henry

The effect of ATRA on β-catenin transcriptional activity in melanoma is still not well understood. C-Myc and cyclin D1 protein expression are excellent markers for cell proliferation; therefore, I will examine these β-catenin target genes proteins expression under the treatment of ATRA in B16 cells. It is noteworthy that neither c-Myc nor cyclin

D1 is a direct ATRA target, thus, regulation of these genes in the presence of ATRA suggests ATRA is able to interfere with a direct regulator, such as β-catenin. Previously, we observed that ATRA reduces mouse B16 melanoma cell growth. Thus, we expected the ATRA treated B16 mouse melanoma cells will have a lower c-Myc and cyclin D1 protein expression. We also need to investigate the transcriptional activity of β-catenin under the treatment of ATRA by using a reporter gene assay. The reporter gene assay can provide us direct evidence of transcriptional activity of β-catenin. Therefore, we expected to see the β-catenin transcriptional activity is reduced by the ATRA treatment.

Chapter Three
Materials and methods

Cell line

B16 mouse melanoma cells (F1 and F10) were obtained from American Type Culture Collection (ATCC). Cells were grown in RPMI Medium 1640 (Gibco) This medium contained 10% fetal bovine serum (Gibco Co.), and 1% penicillin-streptomycin (Gibco). Cells were cultured in a humidified incubator with 5% CO2 at 37 °C.

Retinoic Acid

Vitamin-A-acid (all-trans-Retinoic acid, or ATRA) (Fluka) was purchased from Sigma-Aldrich Co. A concentrated stock solution of ATRA (10 mM) was prepared in dimethyl sulfoxide (DMSO) under subdued light environment to prevent photo-oxidation of the retinoid. Fresh stock was prepared every 2 weeks. The final working concentration (10-20 μM) was obtained by diluting the stock solution in tissue culture medium.

Collection of cells

Cells were seeded at 2×10^5/100-mm tissue culture dish. The following day the medium was replaced and cells were treated with 10-20 μM RA or an equal volume of vehicle (DMSO). Cells were treated every 2 days and collected after 2 or 4 days of RA treatment. Cells were collected by scraping and transferring into a 15-ml centrifuge tube.

Cells were then spun down and the medium was decanted. The cell pellet was washed with phosphate-buffered saline (PBS). The cells and the PBS were then transferred to a micro-centrifuge tube and spun down. Cells were incubated in lysis buffer - (10 mM of HEPES pH 7.5, 150 mM NaCl, 1.5 mM EDTA, 1% NP-40, and 2% (v/v) protease inhibitor) for 5 min. After 5 min incubation, samples were spun down and the supernatant (protein) were transferred to a fresh micro-centrifuge tube. The protein concentration of each sample was determined by using the BCA protein assay kit from Pierce. Protein samples (20 µg) were mixed with equal volume of gel loading dye and denatured by boiling for 10 min.

Cell fractionation

Cells were collected as mentioned above. The cell pellet was re-suspended in an appropriate amount of cell fractionation buffer (CFB) (20 mM of HEPES – pH 7.5, 10 mM KOAc pH 7.5, 1.5 mM MgCl2, and 2 % (v/v) the protease inhibitor) and incubated on ice for 15 min. The cells were transferred to small dounce homogenizer and homogenized using approximately 10 strokes. The cells were transferred back to the original microtube and spun down at 5000 rpm at 4°C for 2 min. The supernatant (cytoplasmic and membrane protein) was transferred to a new tube. The cell pellet was washed 2 times in the CFB. The cell pellet containing nuclear protein was re-suspended in PBS-NP40 (1X PBS, 1% NP-40, and 2% (v/v) protease inhibitor) and incubated on ice for 15 min. The sample was spun down at 13,200 rpm for 1 min at 4°C and the supernatant was collected into a new tube as nuclear protein.

Plasmids, Transient transfection, and Reporter Gene Assay

Transient transfection was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were seeded the day before the transfection at a density of 1×10^5 cells per well in 6 well plates. The following plasmids were used in this assay: luciferase reporter constructs TOP flash (TCF reporter plasmid), FOP flash (mutant TCF binding sites) (Upstate Biotechnology, Lake Placid, NY), point mutated β catenin gene (ABC) (generous gift from Dr. Mayion Park) and thymidine kinase (TK) renilla luciferase reporter vector (Promega) as a transfection control. The following day, cells were transfected according to the manufacturer's protocol: 10 ng of TOP flash, FOP flash or ABC and 2 ng of control vectors were diluted in Opti-MEM I medium (without serum and penicillin) (Invitrogen) in a 2 ml eppendorf tube. In another 2 ml eppendorf tube, 5 µl of Lipofectamine was diluted in Opti-MEM I medium (without serum and penicillin) and incubated at room temperature for 5 min. After 5 min incubation, the DNA-medium was mixed into the Lipofectamine 2000 (Promega) – medium tube, and incubated for 20 min at room temperature. The growth medium was replaced with Opti-MEM I medium (without serum and penicillin) and the DNA-Lipofectamine 2000 complex was then add into the medium (1.5 ml). The cells were transfected at 37°C in a CO_2 incubator for 24 hrs. The growth medium was replaced and the cells were grown for 24 more hrs or until they were ready to assay for luciferase expression. After the cells were transfected, growth media was removed from the cultured cells. Cultured cells were rinsed with in PBS, and then disrupted in 250 µl (for 6 well plate) of passive lysis buffer (provide by the Promega Dual-Luciferase Reporter gene assay kits) by gentle rocking for 15 min at room temperature. To measure luciferase

enzyme activity, 20 µl of lysates were transferred to a siliconized polypropylene tube. One hundred µl of Luciferase assay reagent was added and mixed with the lysates, and then the tube was placed in the luminometer and the flash signal was measured; after that 100 µl of Stop and Glow reagent was added and the glow signal (Renilla control) was measured and recorded.

Western blotting

Cellular proteins denatured in SDS-loading buffer were loaded into wells of a SDS gel (10% separating and 3% stacking) for electrophoresis. A BioRad Criterion cell was used to run the gel electrophoresis at 100 V for 1 hr 45 min. Proteins were then transferred at 100 V for 1 hr to nitrocellulose membranes (Osmonics Inc.) by using a BioRad transfer cell. The membrane was incubated in blocking solution (Tris-buffered saline containing 0.2% Tween 20 and 3% nonfat dry milk, TBST) for 1 hr. Blots were then incubated with 1:1000 dilution of either rabbit polyclonal anti-c-Myc antibody (Cell Signaling Technology Inc.) overnight at 4°C, or with 1 µg/ml mouse monoclonal anti-Cyclin D1 antibody (Upstate Inc.) for 1 hr at room temperature. They were then washed two times in TBST for 5 min and incubated with 1:3000 dilution of secondary antibody (horseradish peroxidase-conjugated anti-rabbit/ mouse IgG, Amersham Corp., according to the source of the primary antibody) for 1 hr. The membranes were then washed three times in 1X TBST, and signals were detected by use of the ECL kit from Amersham Corp.

Densitometry

Signals from the ECL and horseradish peroxidase were captured with Fuji X-ray films. A Bio-Rad ChemiDOC was used to scan the proteins bands and the image was then analyzed using Quantity One software.

Electrophoretic Mobility Shift Assay (EMSA)

The cells were collected using the same method as mentioned in "Collection of cells". The cells were then incubated in cell fractionation buffer (10 mM NaOH-HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5% NP-40) for 15 min on ice. The fractionated cells were then spun down at 800 x g (3000 rpm) for 5 min at 4°C. The supernatants were discarded by aspiration. The nuclear pellets were then washed once with washing buffer (10 mM NaOH-HEPES pH 7.9, 1.5 mM MgCl2, and 10 mM KCl) and centrifuged, as above. The nuclear pellets were incubated in hypotonic salt buffer (20 mM NaOH-HEPES pH 7.9, 420 mM NaCl, 1.5 mM EDTA, and 10% glycerol) for 30 min on ice and spun down at 10,000 x g (10,300 rpm) for 5 min at 4°C. The supernatants were collected as nuclear proteins. Biotin labeled oligonucleotides containing the following consensus sequences were purchased from Panomics, Inc.:

TCF/LEF sequence (5-CCTTTGATCTTCCTTTGATCTT-3),

LEF1 sequence (5-CCCATTTCCATGACGTCATGGTTA-3),

RAR (DR5) sequence (5-TCGAGGGTAGGGTTCACCGAAAGTTCACTCG-3).

Nuclear extracts from control and ATRA treated B16 cells (5 µg of protein) were incubated with binding reagents from electrophoretic mobility shift assay (EMSA) kits (Panomics, Inc.): 2.0 µl 5X binding buffer, 1.0 µg poly d (I-dC), 10 ng transcription

factor (TF) probe, in a total reaction volume of 10 µl. The nuclear extracts were incubated with poly d (I-C) for 5 min at room temperature before adding the transcription factor probe. The binding reaction was allowed to incubate for 30 min at room temperature. To detect preformed complexes, the samples were run on 6% non-denaturing polyacrylamide gels in 0.5×Tris/borate/EDTA pH 8.0 buffer at 120 V for 1 hr 30 min 4°C by using a BioRad Criterion Cell. Protein and DNA were then transferred to a nylon membrane (Osmonics Inc.) at 300 mA for 30-45 min by using a BioRad transfer cell. Oligonucleotides were covalently attached to the membrane by UV-crosslinking. The DNA–protein complexes were detected on the membranes by the chemiluminescence detection method (EMSA Kits User Manual, Panomics, Inc.) followed by autography using a Fuji X-ray film for 2-10 min.

Data Analysis

Graphs are presented as mean ± standard error. Multiple group comparisons were performed by one way ANOVA. Significance of correlation, p-Value, was analyzed by one way ANOVA.

Results

ATRA effects on β-catenin target gene expression: c-Myc and cyclin D1

The transcriptional function of the β-catenin in melanoma cells is still unclear. It is known that cyclin D1 and c-Myc are target genes in the canonical Wnt pathway, because of their role in regulation of the cell cycle and cell proliferation. Cyclin D1 is a key regulator of progression through G1 phase during the cell cycle (52). C-Myc is a transcription factor, which possess the ability to induce cell proliferation, differentiation and tumorigenesis (12, 30, 31). Wnt signaling free and protect β-catenin and causes accumulation of β-catenin inside the nucleus. The ultimate task of intracellular β-catenin is to regulate the expression of genes, such as cyclin D1 and c-Myc. Therefore, we expected there will be a high level of cyclin D1 and c-Myc protein expression in melanoma cells. In contrast, reduction of these protein expressions is expected in ATRA-treated cells.

Western Blot analysis was used to examine the effect of ATRA on the protein expression of the c-Myc and cyclin D1 in B16 F1 and F10 mouse melanoma cells. Results showed that c-Myc protein levels were reduced by 15.5 % (p-value = 0.022) after 2 days treatment, and by 58.0% (p-value = 0.00006) after 4 days ATRA treatment in F1 cells compared to control. In F10 cells, c-Myc protein levels were reduced by 18.4% (p-value = 0.0001) after 2 days treatment, and by 35.8% (p-value = 0.0049) after 4 days ATRA treatment compared to control (Figure 2-1). Similarly, cyclin-D1 protein expression also decreased after ATRA treatment. There was only a slight decreased in cyclin D1 protein levels after 2 days ATRA treatments and it was reduced by 17.1% (p-value = 0.004) in F1 cells and 5% (p-value = 0.79) in F10 cells. After 4 days of ATRA

treatment, cyclin D1 protein levels were reduced by 31.1 % (p-value = 0.32) in F1 cells and by 79.3% (p-value = 0.0037) in F10 cells (Figure 2-2).

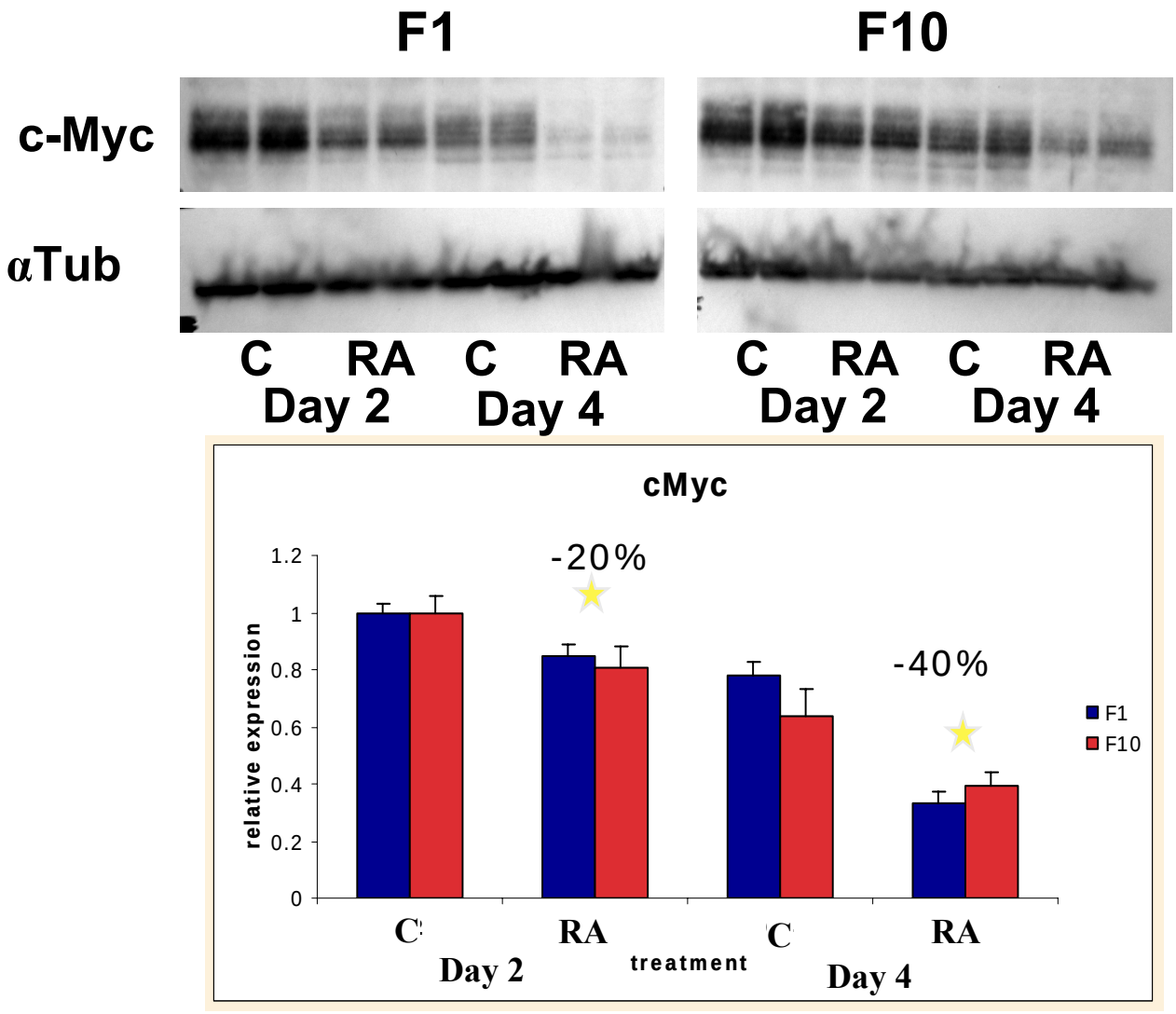


Figure 2-1. **ATRA effects on β-catenin target genes protein expression** Three independent duplicate experiments were performed for statistical analysis, n=6. We found that c-Myc protein levels were reduced by 20 % after day 2 and by 40% in both cell lines after day 4 of RA treatment compare to control. An asterisk (*) indicates significant difference from the control, $p < 0.05$ or less. Bars represented mean ± standard error. C= control, DMSO; RA= 20 μM ATRA.

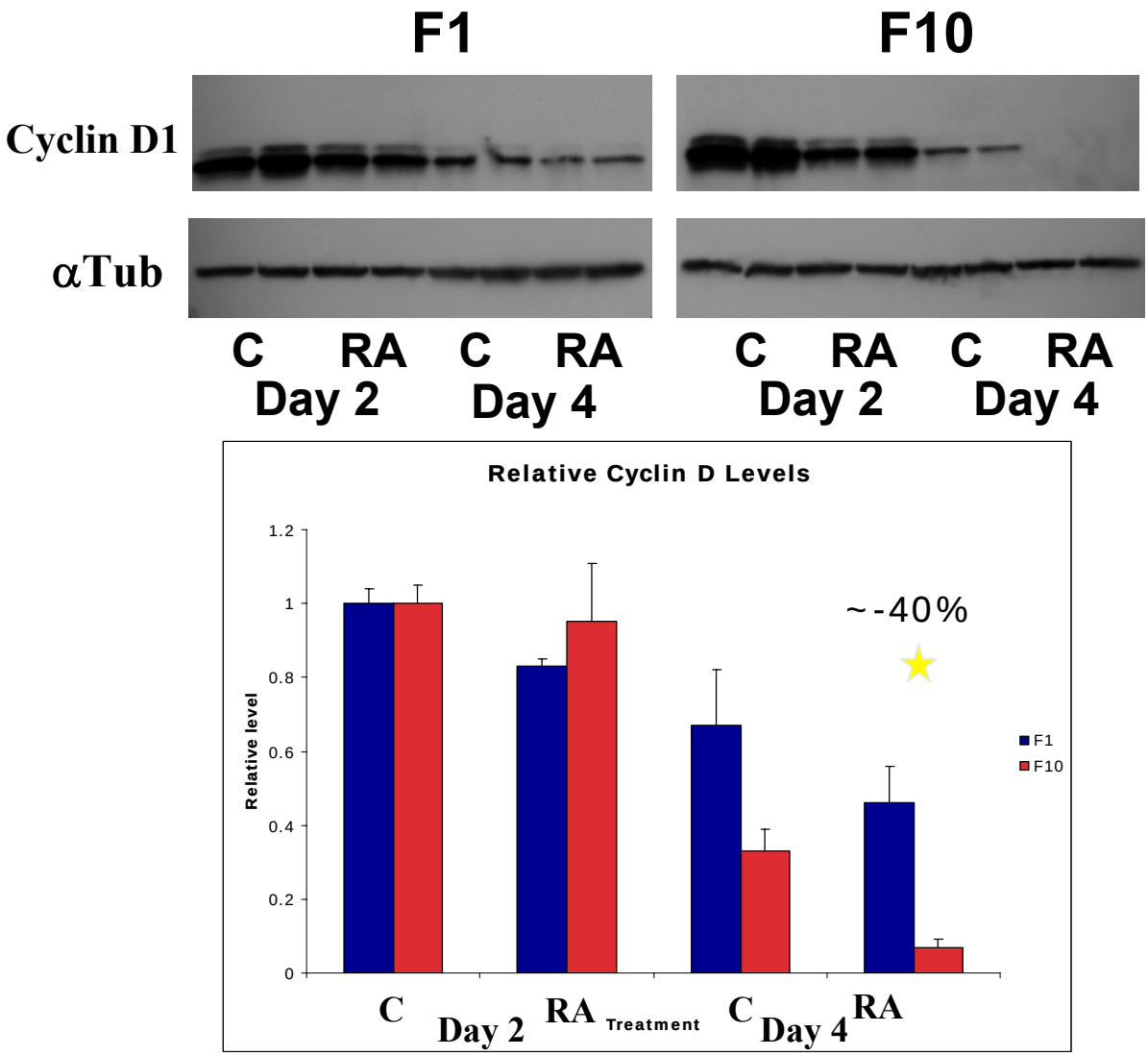
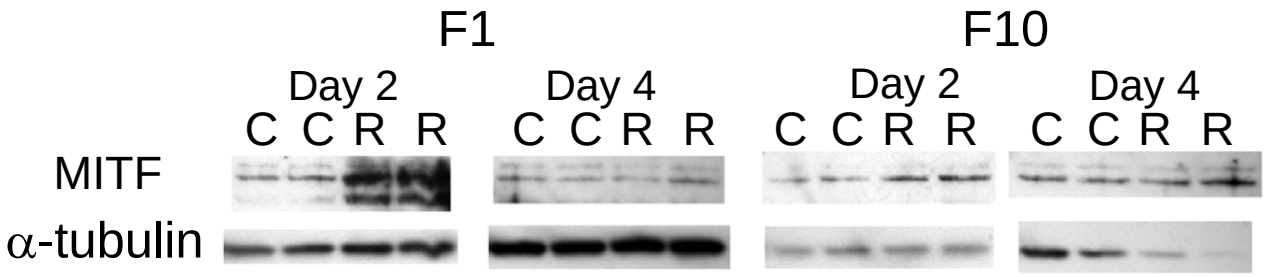


Figure 2-2. **ATRA effects on β-catenin target genes protein expression.** Three independent duplicate experiments were performed for statistical analysis, n=6. We found that cyclin D1 protein levels were reduced by 40% in both cell lines after day 4 of RA treatment compare to control. An asterisk (*) indicates significant difference from the control, $p < 0.05$ or less. Bars represented mean ± standard error. C= control, DMSO; RA= 20 μM ATRA.

MITF protein expression after treatment of ATRA

MITF is vital for the differentiation and survival of melanocytes. (14, 15) Watabe *et al*. (2002) showed that 72 hrs of ATRA treatment increased the mRNA levels of PKCα and MITF (66). Therefore, we investigated whether ATRA treatment could induce MITF protein levels in B16 F1 and F10 mouse melanoma cells. Western Blot data showed that

MITF protein level was increased 4 folds after ATRA treatment compared to the control in B16 F1 cells, but not after 4 days. In B16 F10 cells, the MITF protein level was increased 2 fold after 2 days ATRA treatment, and increased 4 fold after 4 days. (Figure 2-3)



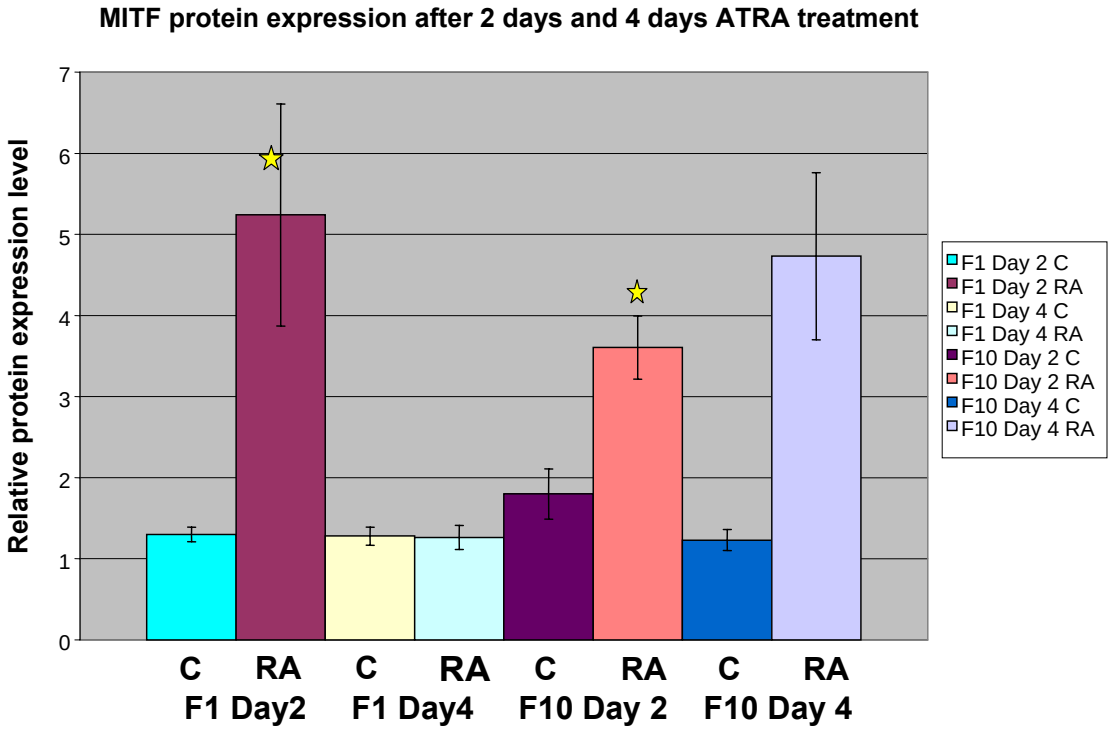


Figure 2-3. **ATRA effects on β-catenin target genes protein expression- MITF.** Two independent duplicate experiments were performed for statistical analysis, n=4 for day 2 and n=2 for day 4. We found that MITF protein level was increased 4 fold in F1cells after day 2 of ATRA treatment compare to control. In F10 cells, MITF protein level was increased 2 fold. An asterisk (*) indicates significant difference from the control. Bars represented mean ± standard error.

Electrophoretic Mobility-Shift Assay

EMSA were performed to determine whether B16 nuclear extracts contain protein complexes capable of binding DNA consensus elements for LEF-1 or TCF. Comparison was made using equal amounts of nuclear extracts harvested from B16 cells treated with or without ATRA (Figure 3). The binding of the proteins and biotinylated *TCF/ LEF* DNA binding site is indicating by the arrow (Figure 3-1) (64). Unlabeled DNA probe (cold probe) was added to validate specific binding. There is no specific pattern of the band intensity. Anti-active β-catenin antibody was added in Figure 3-1 (lane 12), but there was no super shift occurred. In Figure 3-2, specific bands indicated the binding of B16 nuclear proteins to the LEF-1 probe. In Figure 3-3, RAR protein binding to the *RAR (D5)* binding site occurred. The intensity of the bands was reduced after ATRA treatment (lane 5&6), suggesting that RAR protein might be used and degraded after the binding of ATRA. Together, these studies suggest B16 cells do have proteins that bind to *TCF/ LEF* and the *LEF-1* DNA binding site and there is regulation of binding by ATRA.

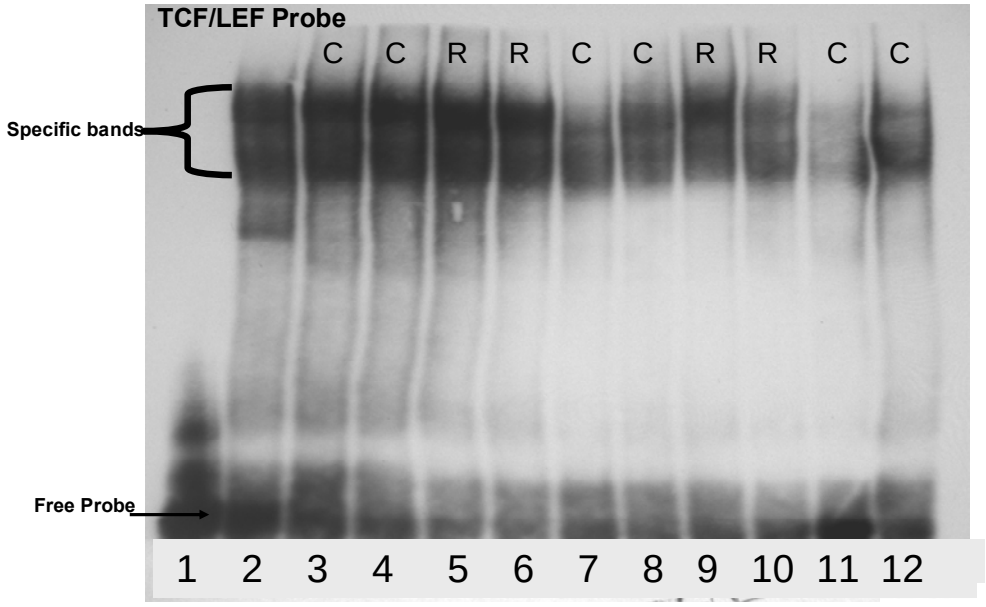


Figure 3-1. EMSA. Nuclear extracts of the Hela cells (lane 2), the B16 F1 cells (lanes 3-6) and B16 F10 cells (lanes 7-11) were incubated with a biotinylated probe (lanes 1-12). C= control and R= ATRA treated. Unlabeled Cold probe was added as a specific competitor (lanes 11). Supershift was performed using anti-active β-catenin antibody (upstate) (lane 12).

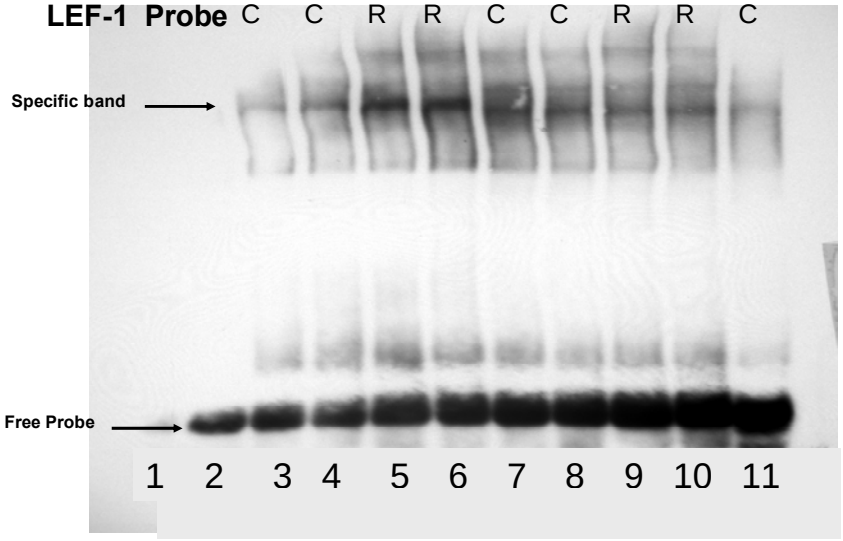


Figure 3-2. EMSA. Nuclear extracts of the Hela cells (lanes 2)), the B16 F1 cells (lanes 3-6) and B16 F10 cells (lanes 7-11) were incubated with a biotinylated probe (lanes 1-11). C= control and R= ATRA treated Unlabeled Cold probe was added as a specific competitor (lanes 11).

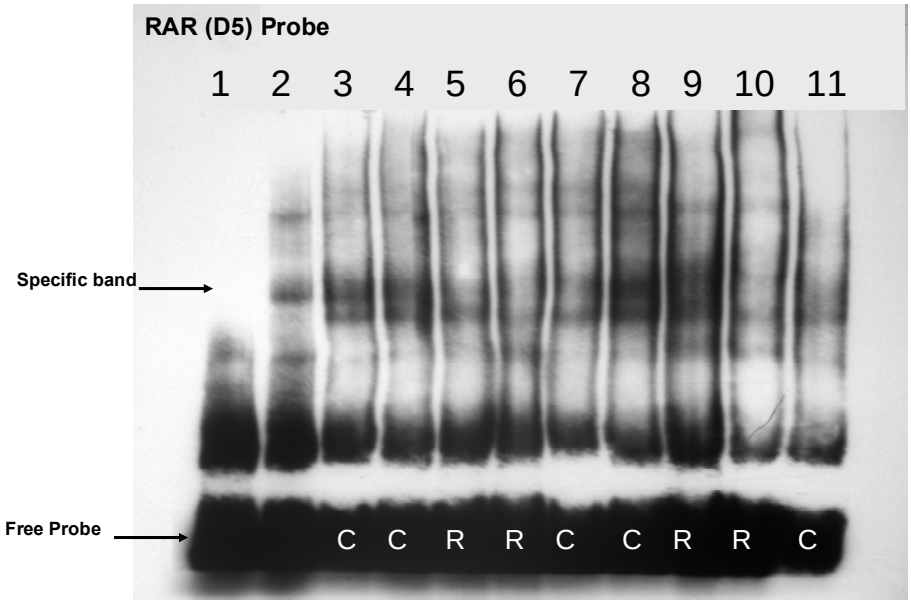


Figure 3-3. EMSA. Nuclear extracts of the Hela cells (lanes 2), the B16 F1 cells (lanes 3-6) and B16 F10 cells (lanes 7-11) were incubated with a biotinylated probe (lanes 1-11). C= control and R= ATRA treatedUnlabeled Cold probe was added as a specific competitor (lanes 11).

Luciferase reporter gene assay

In order to investigate whether the β-catenin signaling pathway is active in tumor cell lines, we chose to use a reporter gene assay, one of the methods to determine transcriptional activity of specific transcription factors *in vitro*. The Western Blot analysis can only show the presence of signaling pathway components, which cannot be used as direct evidence for the transcriptional activity. We investigated the Wnt/ β-catenin pathway in B16 mouse melanoma cells by using reporter genes, TOP-flash (wild type *TCF/ LEF* binding sites) and FOP-flash (mutated *TCF/ LEF* binding sites). In transcriptional activation, active β-catenin binds to TCF/ LEF protein, and then this protein complex binds to a *TCF/ LEF* DNA binding site, enhancing transcription of the

luciferase gene. TOP-flash and FOP-flash were first used to identify activation of the Wnt/ β-catenin signaling pathway in human colon cancer cell lines (62). Therefore, we employed Caco cells (human colon cancer cell line) to determine the optimal amount of the reporter plasmids that we need to use and the efficacy of our transfection. First, we investigated whether we could successfully transfect Caco cells using Lipofectamine ™ 2000 reagent; the plasmids that we we used for transfection were TOP- or FOP-flash (negative control) and pRL-TK renilla (a transfection control driving constitutive expression of renilla luciferase). We found that the Caco cells express considerable luciferase 48 hr after transfection, regardless of the amount of reporter plasmids that we used (Figure 4-1). Having established successful transfection in Caco cells, we used the same technique in B16 mouse melanoma cells.

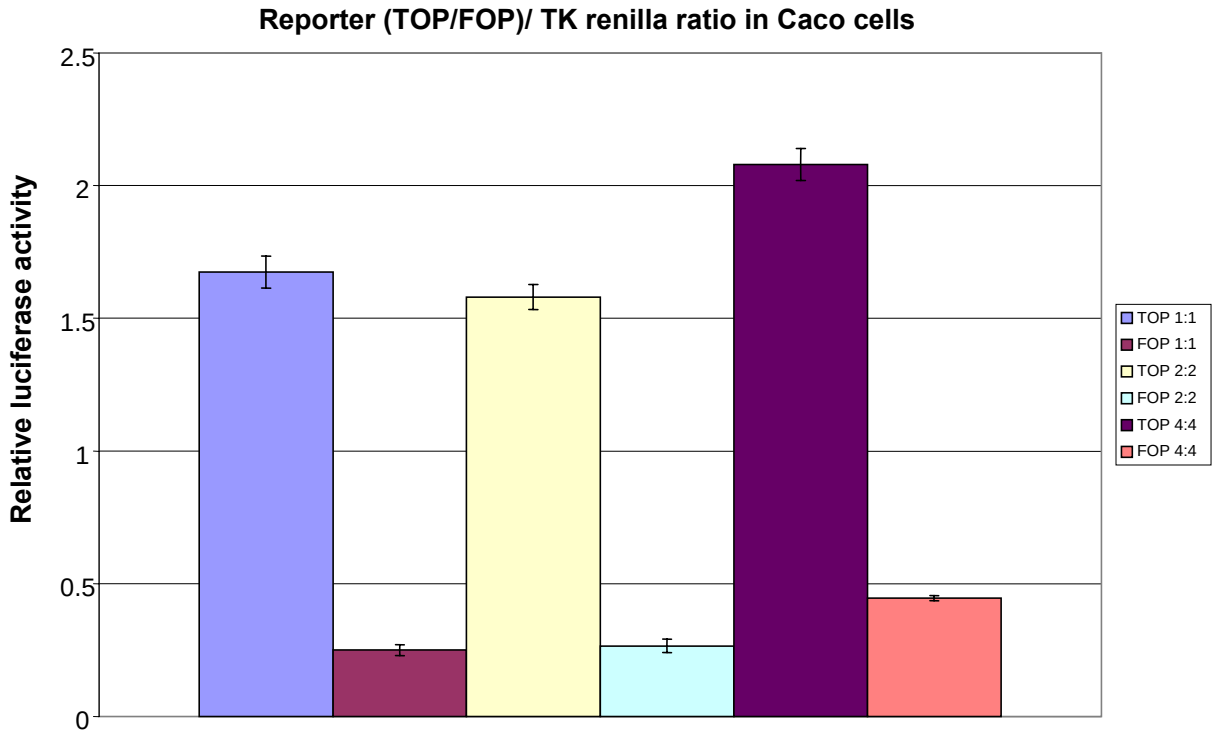


Figure 4-1. **The reporter/ TK renilla ratio in Caco cells** Caco cells were transfected with TOP-flash or FOP-flash plasmid (control) and pRL-TK vector (transfection control) with the amount of plasmids consisting at 1 μg, 2 μg, or 4 μg. Luciferase expression was assessed by a luminometer after 48 hrs of transfection; n=4. Bar represented standard error.

Activity of the Wnt/ β-catenin pathway in B16 mouse melanoma cells

We utilized the luciferase reporter plasmid TOP-flash to examine if the Wnt/ β-catenin signaling pathway is active in B16 cells. First, we transfected B16 F1 and F10 cells using the same method with two different ratios of TOP-flash or FOP-flash vs. pRL-TK vector (2 or 4 μg of reporter plasmid: 2 μg of pRL-TK) (Figure 4-2). After choosing the ratio (4 μg of reporter plasmid: 2 μg of pRL-TK), we transfected B16 cells along with Caco cells as a positive control (Figure 4-3). We found that the TOP-flash luciferase activities of both B16 cell levels were lower than their FOP-flash activity, while Caco cells have much higher TOP-flash activity compared to their FOP-flash activity. These results were unexpected; therefore, we tried to induce the level of β-catenin by inhibiting its inhibitor, GSK3-β, using lithium chloride (LiCl) (63). We treated B16 cells with 10 μM LiCl after 24 hrs of transfection. Previously, we did not detect TCF-4 protein in B16 cells (unpublished observation), which suggested that B16 cells may not contain complexes binding to a TCF consensus element. Therefore, we used a plasmid that contains the LEF-1 binding site in the MITF promoter, Pmic (generous gift from Dr. L. Larue) in B16 cells (Figure 4-4). Although treatment with LiCl increased the TOP-flash and Pmic activity, FOP-flash activity was also increased by LiCl even more than the wild type reporter genes. Because FOP-flash activity was a paradox, we transfected the B16 cells using the same method, and treating the B16 cells with 10 μM of ATRA or an equal amount of DMSO after 24 hrs of transfection (Figure 4-5). We observed that the TOP-flash activity in both B16 cell lines was slightly decreased by ATRA, but FOP-flash activity in F10 cells was decreased after 24 hrs of ATRA treatment, and increased in F1 cells.

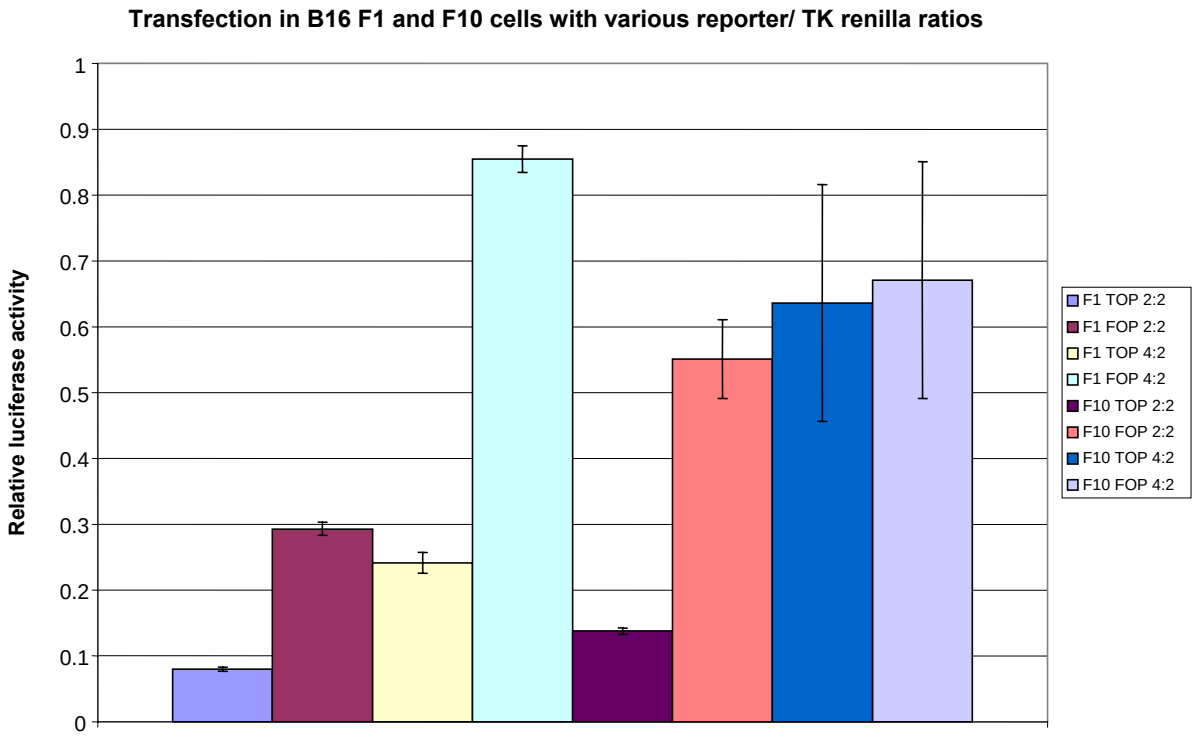


Figure 4-2. **Transfection of B16 cells by Lipofectamine ™ 2000 reagent.** B16 cells were transfected with TOP-flash or FOP-flash plasmid (control) and pRL-TK vector (transfection control), with the plasmid ratios of 2 or 4 µg of reporter plasmid and 2 µg of pRL-TK. Luciferase expression was assessed by a luminometer after 48 hrs of transfection; n=4. Bar represented standard error.

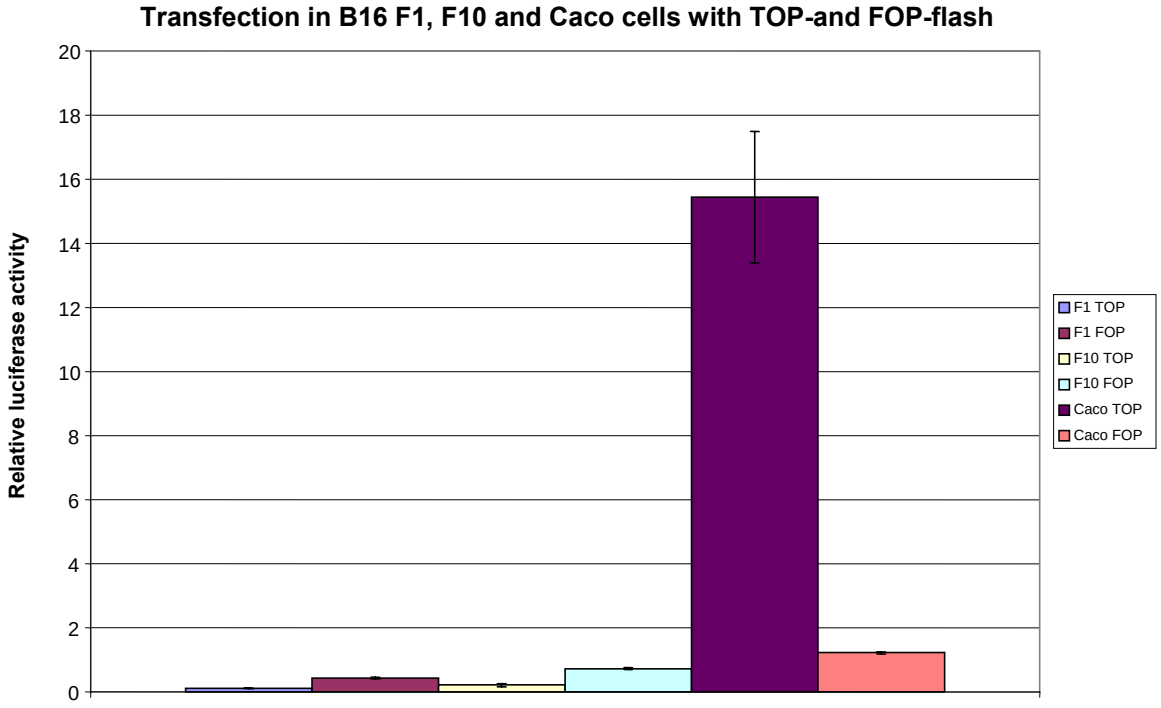


Figure 4-3. **Transfection of B16 cells and Caco cells by Lipofectamine ™ 2000 reagent.** B16 and Caco cells were transfected with TOP-flash or FOP-flash plasmid (control) and pRL-TK vector (transfection control), with the plasmid ratios of 4 µg of reporter plasmid and 2 µg of pRL-TK. Luciferase expression was assessed by luminometer after 48 hrs of transfection; n=4. Bar represented standard error.

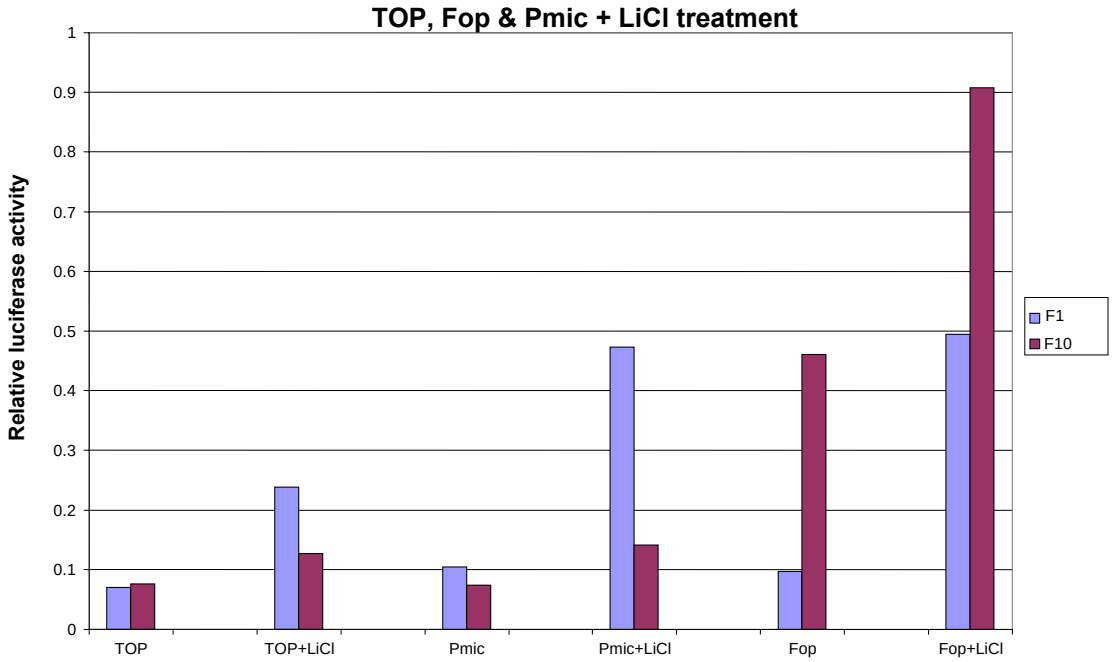


Figure 4-4. **LiCl treatment after 24 hrs of transfection in B16 cells.** B16 cells were transfected in 6-well plates with TOP-flash or FOP-flash plasmids (control) or Pmic and pRL-TK vector (transfection control), with the plasmid ratios of 0.5 μg of reporter plasmid and 0.2 μg of pRL-TK. Luciferase expression was assessed by luminometer after 48 hrs of transfection and LiCl treatment; n=1.

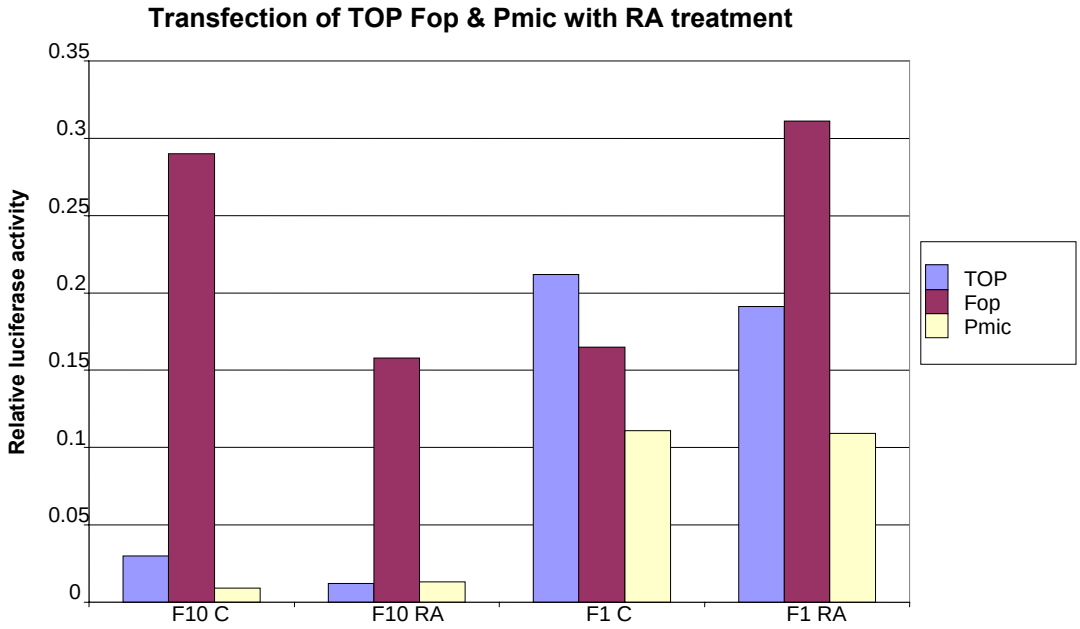


Figure 4-5. **ATRA treatment after 24 hrs of transfection in B16 cells.** B16 cells were transfected in 6-well plates with TOP-flash or FOP-flash plasmids (control) or Pmic and pRL-TK vector (transfection control), with the plasmid ratios of 0.5 μg of reporter plasmid and 0.2 μg of pRL-TK. Luciferase expression was assessed by luminometer after 48 hrs of transfection and ATRA treatment; n=2. Bars represented mean.

These results were unexpected, leading us to test whether the ratio of reporter to control plasmids might be a cause of poor detection of specific transcriptional activity. Therefore, we tested ratios of reporter plasmids to the pRL-TK vector. A broader range of pRL-TK: reporter plasmid ratios (1:2.5, 1:5, 1:10 and 1:100) were examined in B16 F10 cells (Figure 4-6). We noticed that when we transfected at 1:100 ratio of reporter plasmid: pRL-TK renilla, the FOP-flash activity was much higher than the TOP-flash activity. Thus, we selected the 1:10 ratio to be optimal.

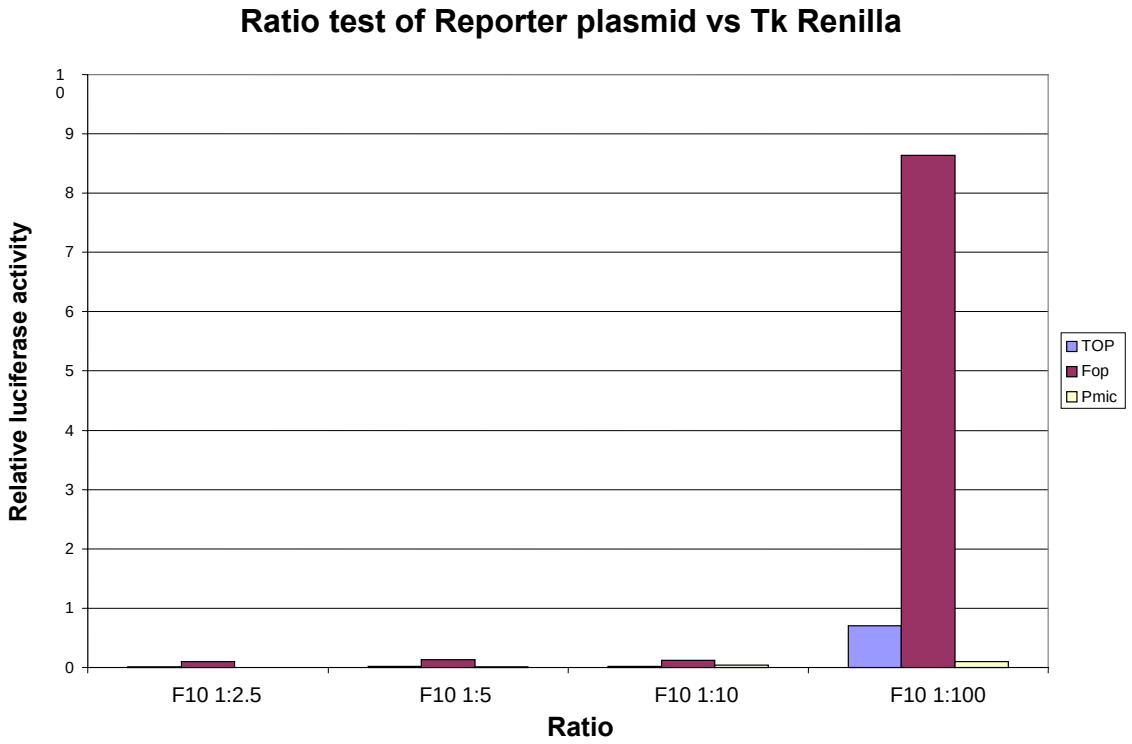


Figure 4-6. **Ratio test of reporter plasmids vs. pRL-TK renilla vector in B16F10 cells.** F10 cells were transfected in 6-well plates with TOP-flash or FOP-flash plasmids (control) or Pmic and pRL-TK vectors (transfection control), with the plasmid ratios of 0.5 μg of reporter plasmid and 0.2 μg, 0.1 μg, 0.05 μg and 0.005 μg of pRL-TK. Luciferase expression was assessed by a luminometer after 48 hrs of transfection; n=2.

As a means of increasing the available proteins to transactivate the reporters, we overexpressed active β-catenin to determine whether the B16 cells utilize the Wnt/ β-catenin signaling pathway. We obtained a point mutated active β-catenin (ABC) gene

and Wnt-8 gene from Dr. Mayion Park. We expected to see TOP-flash activity increase with the overexpression of ABC. We were not certain that Wnt-8 would induce the downstream signaling cascade in B16 melanoma cells because these cells might not express Frizzled 8a receptor (68) that binds to Wnt-8 protein. We received the human melanoma cell line, FO-1, from Dr. L. Larue. We overexpressed ABC or Wnt-8 in F1, F10 and FO-1 cell lines (Figure 4-7). Expression of FOP-flash activity was high in F10 cells, but it became even higher after overexpression of ABC. There was only a slight increase of TOP-flash activity in F10 cells with the overexpressed ABC vector compared to the control. In F1 cells, the pattern is similar to F10 cells, but the activity was not as high. These results are consistent with what we observed above.

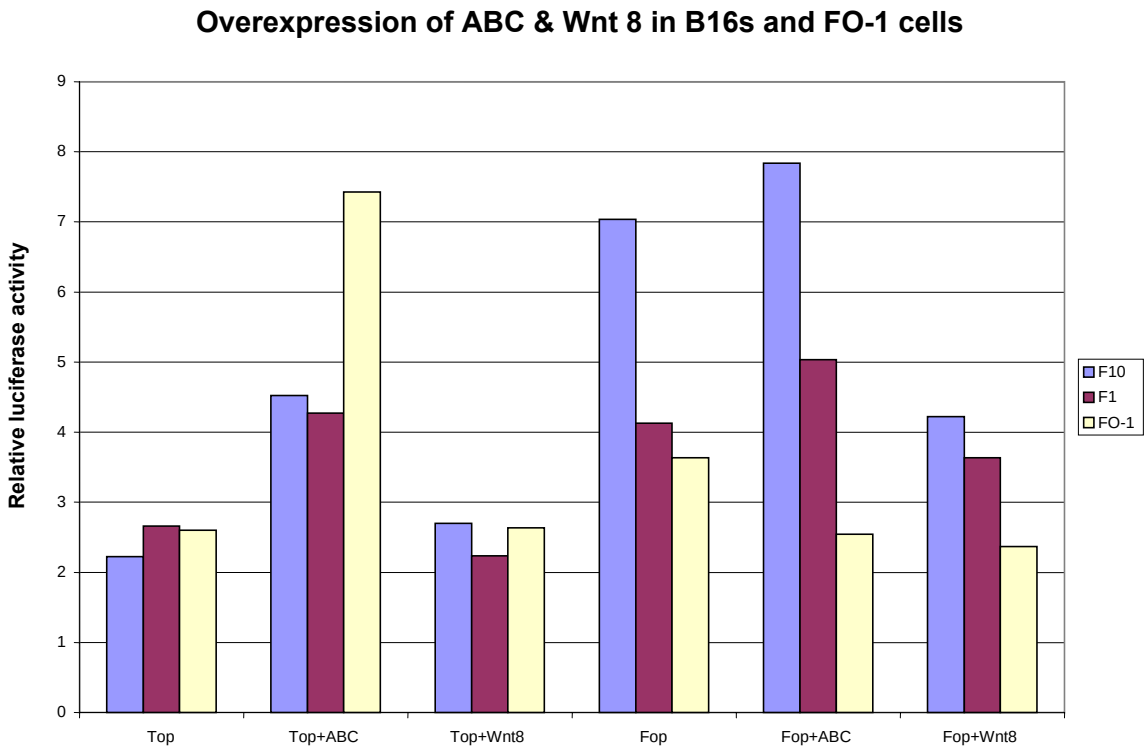


Figure 4-7. **Overexpression of ABC and Wnt 8 in B16 and FO-1 cells.** Experiment was performed in duplicate. B16 cells were transfected in 6-well plates with TOP flash or FOP flash plasmids (control) or Pmic and pRL-TK vectors (transfection control), with the plasmids ratios of 10 ng of reporter plasmid, 10 ng of ABC or 10 ng of Wnt-8 and 2 ng of pRL-TK. Luciferase expression was assessed by a luminometer after 48 hrs of transfection, n=2.

Because FO-1 human melanoma cells exhibited an increased in TOP flash activity, but not in FOP flash activity, when ABC was expressed, FO-1 cells were used as a model to study the ATRA sensitivity of β-catenin transcriptional activity. FO-1 cells have been studied in the field of immunology (61), but ATRA-sensitivity has not been demonstrated. FO-1 cells ($1X10^5$) were plated in 6-well plates the day before transfection. Then, transfected with TOP- or FOP-flash plasmids, in addition we overexpressed ABC in the cells. After 24 hrs of transfection, the cells were treated with 10 μM of ATRA or the same amount of vehicle (Figure 4-8). The luciferase activity in the ATRA treated cells decreased 30 % compared to the DMSO treated cells. In addition, there was no difference when comparing ATRA treated cells to the untreated cells.

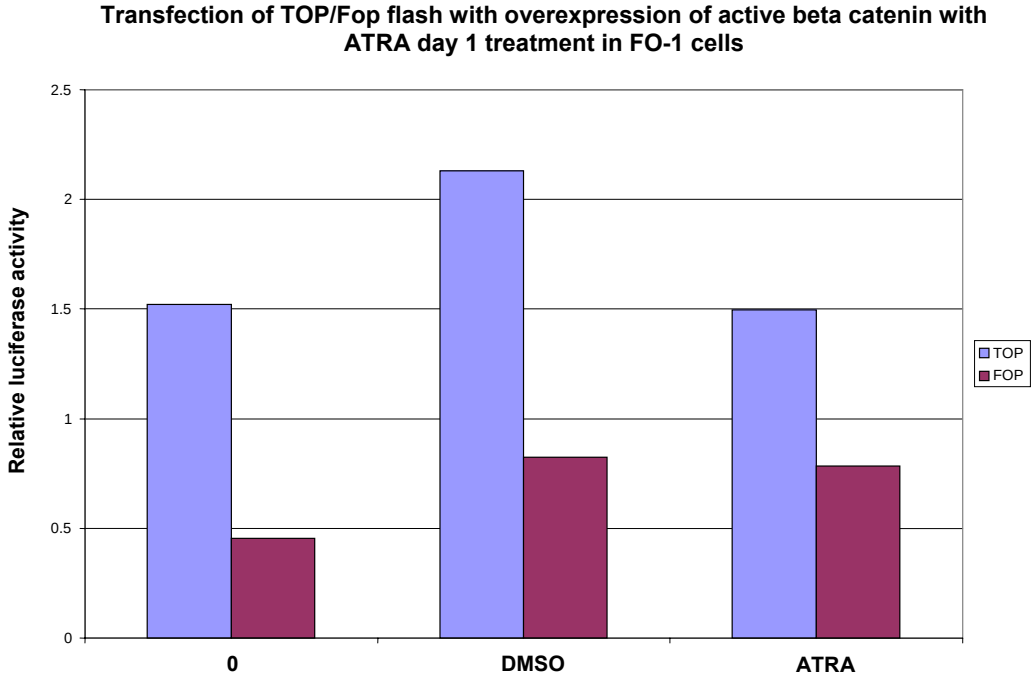


Figure 4-8. ATRA treatment after 24 hrs of transfection in FO-1 cells. FO-1 cells were transfected in 6-well plates with 10ng of TOP flash or FOP flash plasmids (control) and 2 ng of pRL-TK vector (transfection control), and overexpressed with 10 ng of ABC. Luciferase expression was assessed by luminometer after 48 hrs of transfection and 24 hrs of ATRA treatment, n=2.

To validate the data, we repeated the experiment using the same method. We performed the experiment in triplicate with 48 hrs of transfection and after 24 hrs of ATRA treatment (Figure 4-9). These results showed that 10 μM of ATRA decreased the TOP flash activity by 13%. With 20 μM of ATRA, TOP flash activity was reduced by 23%. This is an interesting trend, but the differences between the control and the ATRA cells were not statistically significant.

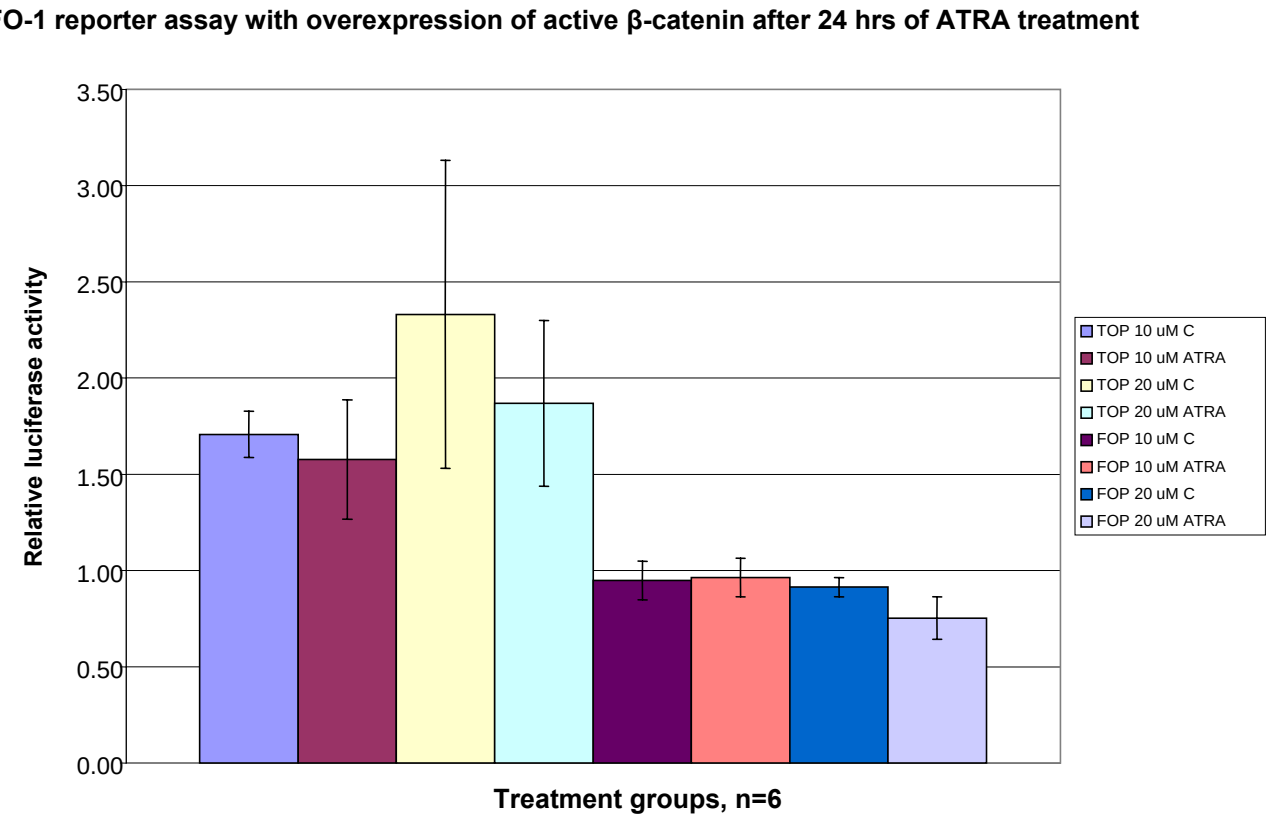


Figure 4-9. **ATRA treatment after 24 hrs of transfection in FO-1 cells.** FO-1 cells were transfected in 6-well plates with 10 ng of TOP-flash or FOP-flash plasmids (control) and 2 ng of pRL-TK vector (transfection control), and overexpressed with 10 ng of ABC. Luciferase expression was assessed by a luminometer after 48 hrs of transfection and 24 hrs of ATRA treatment, n=6. Mean ± SEM.

Discussion

The Wnt/ β-Catenin signaling pathway plays a pivotal role in embryonic development among different species. It can also contribute to cancer formation, which has been extensively studied in human colon cancer (44). The central molecule in the Wnt signaling pathway is β-Catenin. It has been found that 30% of melanomas have β-catenin in their nuclei (65). Although there is evidence that β-Catenin is involved in tumor formation, the transcriptional activity of β-Catenin in melanoma is still unclear.

Our previous results showed that ATRA relocated the β-catenin distribution from nucleus to membrane in B16 cells (Henry and Delidow, personal communication). In addition, other studies showed that disruption of β-catenin adhesion function can lead to nuclear accumulation in melanoma (35). Our previous work showed that ATRA was able to decrease the protein level of active β-catenin and LEF-1 in B16 cells (Kern and Delidow, personal communication). We investigated whether the levels of the c-Myc and cyclin D1 protein were reduced, in response to inhibition of β-catenin. Our Western Blot data showed that the levels of c-Myc and cyclin D1 were reduced after 2 days and 4 days ATRA treatment in B16 cells. Because these genes are not direct targets of ATRA, it suggests they maybe reduced as a result of down regulated active β-catenin. Both c-Myc and cyclin D1 can modulate cell proliferation and cell cycle (5, 13, 45, 47). Thus, the reduction in c-Myc and cyclin D1 expression correlates well with the known effect of ATRA on B16 cell proliferation (33).

Not only were cell proliferation markers levels reduced, our previous result showed that the β-Catenin partner and target protein, LEF-1, was also reduced after ATRA treatment. In order to activate growth regulatory gene, β-Catenin is required to couple with LEF-1 (21, 44, 48, 51). The reduction in LEF-1 expression suggests it is more difficult for β-Catenin to co-activate gene transcription. On the other hand, MITF, an important molecule that modulate melanocyte cell differentiation and survival, also been shown to associate with LEF-1 and β-Catenin to regulate gene transcription (41). MITF can associate with β-catenin in absence of LEF-1 to regulate melanocyte-specific gene transcription that will redirect β-Catenin nuclear function from cell proliferation to melanocyte-specific gene expression (67). In this study, ATRA induced MITF protein level and other have shown that showed ATRA induces *MITF* mRNA expression (66). In addition, ATRA was able to reduce LEF-1 expression and to promote differentiation (33). Thus, ATRA inhibits melanoma progression by redirecting the B16 cells to differentiate, in part through inducing MITF expression. However, these results are only down-stream evidences of effects of ATRA on β-catenin, a direct evidence of β-catenin transcriptional activity will be required.

We examined presence of β-catenin transcriptional complexes in B16 cells by performing a gel shift assay. The gel shift assay results suggested that there are transcription factors in B16 cells that bind to specific DNA elements (TCF/LEF and LEF-1) consistent with transcriptional activity of β-catenin. Reporter gene assays were performed to assess the transcriptional activity of β-catenin and its partner proteins TCF/ LEF. The TOP-flash activity was low compared to the negative control (FOP-flash) in B16 cells. These results may suggest that B16 cells are not compatible with using FOP-

flash as the negative control because of the possibility of binding to the mutant TCF binding sites in the FOP-flash vector. On the other hand, we examined the transcriptional activity of β-catenin in FO-1 cells (human melanoma cell lines). We noticed that this cell line provides us with a consistently low response of the FOP flash activity, which means that there is no non-specific binding of proteins to the mutated *TCF* binding sites in FO-1 cells. The results of reporter gene assays in FO-1 cells showed that TOP-flash does have activity, which is induced in the presence of active β-catenin. In human colon cancer cell, the transcription of TOP-flash construct was reduced by ATRA treatment. (69) Similarly, we observed a reduction in TOP flash activity with presence of exogenous ABC in FO-1 cells after treated with ATRA.

In conclusion, this study shows that ATRA reduces the expression of active β-catenin and its target genes (LEF-1, c-Myc, and cyclin D1) and increases MITF expression in B16 cells. In addition, ATRA reduces the exogenous β-catenin transcriptional activity in FO-1 cells. Thus, ATRA may inhibit melanoma cell growth by decreasing active nuclear β-catenin protein levels and its nuclear activity, which decreases the transcription of cyclin D1 and c-myc and ultimately resulting in slowed cell-cycle progression.

Chapter Four

Future Directions

Future directions for research based on this study should focus on the mechanism by which ATRA affects β-catenin transcriptional activity in melanoma cells. In this study, effects of ATRA on β-catenin transcriptional activity were demonstrated in FO-1 human melanoma cells, but expression of β-catenin target genes and proteins were not examined. Therefore, Western Blot will be required to determine effects of ATRA on protein expression of MITF, c-Myc, cyclin D1, LEF-1, and active β-catenin.

As mentioned in this study, MITF is an important molecule in melanocyte development, survival, and may possess the ability to alter β-catenin nuclear function. Therefore, study of whether MITF can improve or change the behavior of melanoma cells to a more normal phenotype will be required. Alternatively, expression of LEF-1, a partner of β-catenin, can be repressed using small interfering RNA (siRNA) and with/ without ATRA treatment to determine whether melanoma cells will continue to proliferate in the absence of LEF-1 protein. In addition, RAR siRNA can be used to determine whether siRNA reduced the ability of ATRA to decrease the nuclear activity of β-catenin.

Different melanoma cell types may response to ATRA differently because of each cell line does not have exact same signaling cascade that modulate their behavior. Thus, investigation using mouse melanoma cell line may be not accurately depicting the mechanism that occurs in human melanoma cell line. In future research, different human

melanoma cell lines should be used and different pathway that response to ATRA should also be examine.

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