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SCHOOL OF MEDICINE

Thesis

THE EFFECT OF PROTEIN KINASE C AND BETA-CATENIN INHIBITORS ON UVEAL MELANOMA CELLS

by

ASHA GOWDA

B.S., University of Michigan, 2012

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THE EFFECT OF PROTEIN KINASE C AND BETA-CATENIN INHIBITORS ON UVEAL MELANOMA CELLS

ASHA GOWDA

ABSTRACT

Purpose: Uveal melanoma (UM) is the most common intraocular malignancy in adults with an incidence of six per one million individuals each year. Globe conserving treatments are currently the standard of care, but unfortunately, despite successful local control, a substantial mortality risk exists due to eventual emergence of distant metastasis, which is invariably lethal. There is therefore an unmet need for novel, effective, targeted therapies for metastatic UM. Somatic mutations in the G-protein α subunits, Gαq and Gα11, are present in a mutually exclusive pattern in approximately 80% of UMs, and they abolish the GTPase activity, resulting in a constitutively active protein. We have previously demonstrated that GNAQ-mutant ($GNAQ^{mt}$) UMs are addicted to the oncogenic effect of the mutant GNAQ protein and dissected the GNAQ pathway in an attempt to identify druggable targets. Our findings that the mutant GNAQ protein activates the PKC/PKD axis, which activates beta-catenin (ß-Catenin), prompted us to investigate the role of PKC and ß-Catenin in $GNAQ^{mt}$ UM.

Experimental Design: The $GNAQ^{mt}$ UM cell lines Mel202 and OMM1.3 were treated with either the PKC inhibitor bisindolylmaleimide I (BIM) alone, the Wnt/ß-Catenin inhibitors FH535 or cardamonin alone, the Wnt/ß-Catenin activator Wnt-3a alone, or siRNAs for ß-Catenin in combination with BIM, and their viability was assessed with the MTT assay. Levels of β-Catenin, phosphorylated AKT, ERK1/2, caspase 3 and LC3BII

were assessed with western blotting. β-Catenin mRNA levels were assessed with microarray analysis and RT-PCR.

Results: $GNAQ^{mt}$ UM cells are very sensitive to PKC inhibition and respond with a decrease in cell viability that involves autophagy and cleavage and translocation of LC3BII in autophagosomes, but not caspase activation. PKC inhibition results in the upregulation of ß-Catenin protein, but not mRNA levels, through a post-translational mechanism that involved the phosphorylation and activation of AKT, but not ERK1/2. β-Catenin inhibition by either small molecule inhibitors or siRNA resulted in a dose-dependent increase of cell proliferation, whereas ß-Catenin activation by Wnt-3a had the opposite effects, resulting in a decrease in cell viability.

Conclusions: Our study demonstrates that PKC is a mediator of the oncogenic effect of mutant Gα protein in UM through the Wnt-3/ß-Catenin signaling pathway. These results open exciting opportunities for the development of personalized targeted therapies for UM in a genotype-dependent fashion.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC ..... Adenomatous Polyposis Cell

ß-Catenin ..... Beta-catenin

BIM ..... Bisindolylmaleimide I

CD ..... Cardamonin

CKI ..... Casein Kinase I

DAG ..... Diacylglycerol

ERK ..... Extracellular Signal-Regulated Kinases

FH FH535

GDP ..... Guanosine Diphosphate

$GNAQ^{mt}$ ..... GNAQ-mutant

GPCR ..... G Protein-Coupled Receptor

GRO ..... Groucho

GSK-3 ..... Glycogen Synthase Kinase 3

GTP ..... Guanosine Triphosphate

IP_3 ..... Inositol 1,4,5-Triphosphate

LEF ..... Lymphoid Enhancing Factor

LRP ..... Lipoprotein Receptor-Related Protein

MAPK ..... Mitogen-Activated Protein Kinases

PI3K ..... Phosphatidylinositol 3-Kinase

PIP_2 ..... Phosphatidylinositol 4,5-bisphosphate

PKC ..... Protein Kinase C

PLCβ ..... Phospholipase C-Beta

PTEN ..... Phosphatase and Tensin Homolog

RKIP ..... Raf Kinase Inhibitor Protein

RPPA ..... Reverse Phase Protein Array

RT-PCR ..... Reverse Transcription Polymerase Chain Reaction

TCF ..... T-Cell Factor

TGFα ..... Transforming Growth Factor α

UM ..... Uveal Melanoma

INTRODUCTION

Uveal melanoma (UM) is the most common primary malignancy of the eye that arises from melanocytes in the uveal tract, consisting of the iris, ciliary body, and choroid (Ocular Melanoma 2014). UM represents less than five percent of all melanomas and affects roughly six in every one million people, yearly (Buder et al. 2013). In most cases, UM does not present with any symptoms and requires an eye examination to be detected (Marshall et al. 2013). Although in the past enucleation was the primary treatment, this mode is currently suitable only for large tumors, eyes without useful vision, pain, or patients that are at risk of not being compliant with follow up. In the last several decades, the treatment of UM has shifted to globe conserving therapies that are currently the standard of care; these include radiotherapy with charged particles (proton beam therapy) or radioactive iodine (brachytherapy), photocoagulation, transpupillary thermotherapy, and rarely, local resection (Gragoudas 2006). Unfortunately, despite successful local treatment and control, a substantial mortality risk exists due to eventual emergence of distant metastasis that occur in almost 50% of all cases (Marshall et al. 2013; Spagnolo et al. 2012). This is postulated to be due to early micrometastasis and hematogenous dissemination of tumor cells (Balch et al. 2001).

According to the largest controlled study of UM, the Collaborative Ocular Melanoma Study (COMS), the all-cause mortality for successfully treated large ocular melanomas is close to 50%, whereas for medium-sized ones is 30% (Collaborative Ocular Melanoma Study Group 2001; Collaborative Ocular Melanoma Study Group

2006). Once the disease hematogenously spreads beyond the eye, prognosis is poor, with only 13% of patients surviving more than 1 year, and the median survival time after diagnosis of distant metastasis is only five to seven months despite systemic treatment (Gragoudas et al. 1991; Bedikian 1995; Pons et al. 2011). Survival time for UM patients is dependent on the location of metastasis; about 90% of UM cases metastasize to the liver, after which the median survival time decreases to four to five months (Spagnolo et al. 2012). Even when metastatic disease is discovered early in asymptomatic patients, treatment does not improve overall survival (Rietschel et al. 2005). Therefore, a systemic agent that could be used in conjunction with globe-sparing modalities that eradicate or prevent the micrometastatic disease is an attractive option for the adjuvant treatment of primary UM. Available systemic treatments provide negligible benefit and as we have previously shown, revolutionary treatments that are beneficial for metastatic cutaneous melanomas do not show promising results on metastatic UM (Mitsiades et al. 2011). Therefore, there is an unmet need for novel, effective targeted therapies for metastatic UM. Several pathways have been identified as important players in the pathogenesis of UM that are involved in cell growth, migration survival and apoptosis (Miyamoto et al. 2012). Such pathways include the mitogen-activated protein kinase (MAPK)/ERK, phosphatidylinositol 3-kinase (PI3K)/AKT, Wnt/ß-Catenin, and $GNAQ^{mt}$ pathways, and offer potential candidates for targeted and personalized therapy.

Guanine Nucleotide-Binding Proteins

Guanine nucleotide-binding proteins are a family of heterotrimeric proteins

composed of α, β and γ subunits that couple cell surface seven-transmembrane domain receptors, such as G protein-coupled receptors (GPRCs), to intracellular signaling pathways (Johnston and Siderovski 2007). The GPRCs form the largest known family of cell surface receptors (1% of the human genome) and mediate cellular responses to a diverse array of signaling ligands (Strathmann and Simon 1990; Breitwieser 2004). GPRCs and their ligands are overexpressed in various malignancies and contribute to cancer cell growth and survival via autocrine growth loops (Rozengurt 2002). GPRC activation catalyzes the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) bound to the inactive G protein α subunit, resulting in a conformational change and dissociation of the β-γ subunits from the complex (Van Raamsdonk et al. 2008). The G protein α, β and γ subunits that belong in the Gαq family of G protein α subunits are ubiquitously expressed in tissues, and upon activation they stimulate phospholipase C-beta (PLCβ) (Gaudi and Messina 2011). Gα signaling is normally terminated by a GTPase intrinsic to the Gα subunit (Gaudi and Messina 2011). PLCβ catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) to produce two second messengers: inositol 1,4,5-triphosphate (IP_3), which triggers the release of Ca^{2+} from internal stores, and diacylglycerol (DAG), which elicits cellular responses through a variety of effectors including protein kinase C (PKC) and MAPK (Gaudi and Messina 2011; Patel et al. 2011). The PKC family consists of several isozymes that exhibit differential expression patterns, subcellular localization, and responsiveness to extra-cellular signals that are thought to explain their distinct and unique functions in the cell (Dekker and Parker 1994). Specific isotypes of PKCs are reported to be

overexpressed in various carcinomas, suggesting their importance as players in cancer pathophysiology (Donson et al. 2000; Eder et al. 2005). Through a cascade of events, GTP-bound Gα subunits ultimately influence cellular properties and activities, including cell growth and differentiation (Spagnolo et al. 2012; Van Raamsdonk et al. 2008).

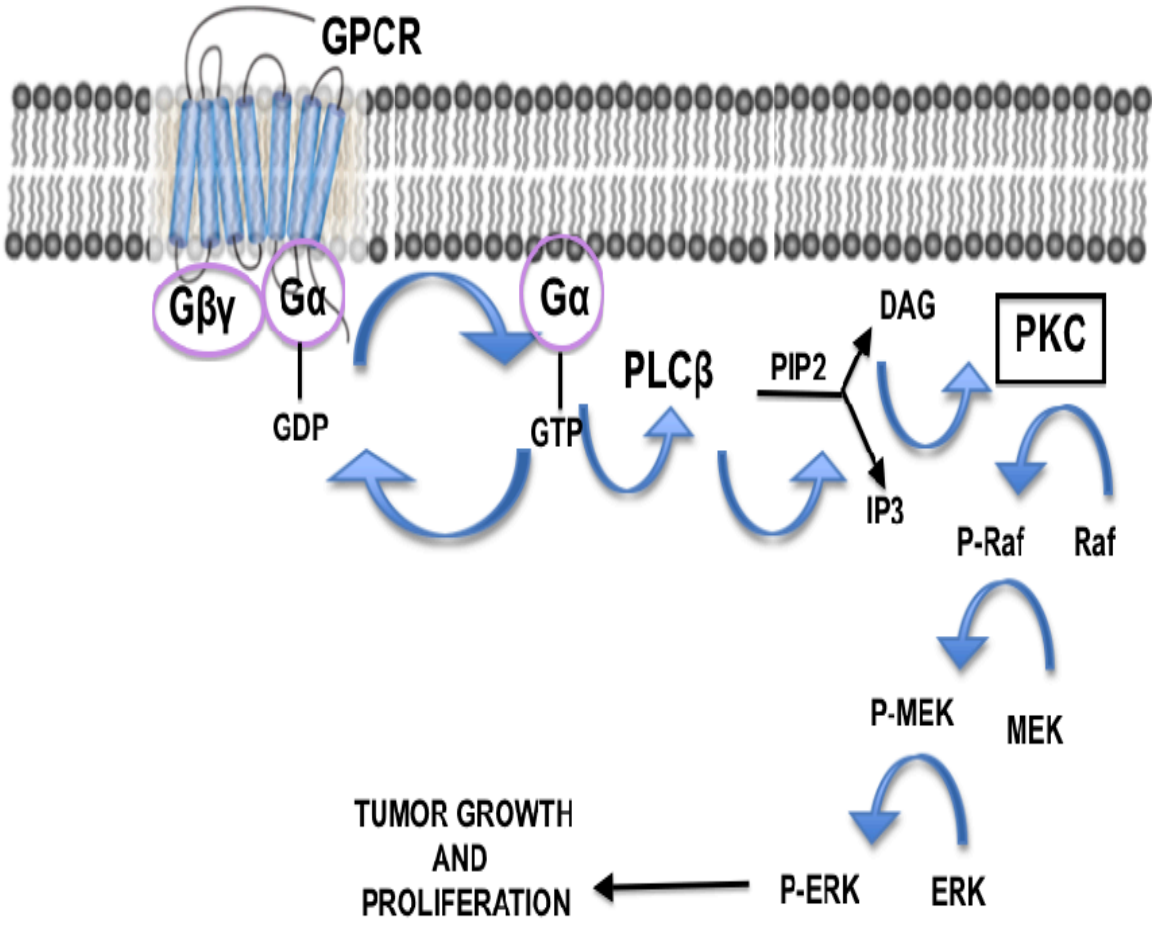


Figure 1: GNAQ/GNA11-Stimulated Activation of PKC and MAPK/ERK Signaling Pathway. GNAQ and GNA11 mutations cause aberrant signaling, initiated by GTP-bound Gα subunit. Upon activation, its downstream target, PKC, can proceed to stimulate the MAPK/ERK pathway, which leads to tumor growth and proliferation.

Gα Protein Mutations in Uveal Melanoma

The GNAQ and GNA11 genes code for two Gα subunits, Gαq and Gα11, respectively, which are 88% homologous (Strathmann and Simon 1990). Somatic mutations in the heterotrimeric G protein α subunit genes GNAQ (40-50% of primary UMs) or its closely related GNA11 (30% of primary UM) represent an oncogenic effect in UM (Lamba et al. 2009; Onken et al. 2008). The most frequent mutations occur in codon Q209, where a glutamine residue is substituted with a different amino acid residue and affects the Ras-like domain, abolishing the intrinsic GTPase activity in a manner similar to the effect of the NRAS mutations found in cutaneous melanomas (Van Raamsdonk et al. 2008; Van Raamsdonk et al. 2010). These mutations result in a constitutively active protein that functions as a bona fide oncogene (Figure 1) and occur early in UM carcinogenesis, contrary to other genetic events that are associated with increased metastatic potential, such as the recently described BAP1 (BRCA1-associated protein 1) mutations (Van Raamsdonk et al. 2010; Harbour et al. 2010). Primary melanocytes that are transduced with mutant GNAQ or GNA11 form rapidly growing tumors in immunocompromised mice, which provides evidence, together with the high frequency of activating somatic mutations in GNAQ or GNA11 in UMs, for the crucial role of Gαq/Gα11 proteins in the development of UM (Van Raamsdonk et al. 2008).

Irregularity in the functions of these heterotrimeric G-protein α subunits has been considered pivotal in the development of malignant UM because mutant Gα subunits are capable of constitutively activating a number of downstream elements, including the MAPK/ERK pathway that is a dominant route for UM pathogenesis (Jovanovic et al.

2013; Khalili et al. 2012). Activation of this pathway is present in about 86% of primary UM tumors and correlates with mutated GNAQ and GNA11 genes in more than 80% of primary UM cases (Patel et al. 2011). More than half of all metastatic UM have mutations in the GNA11 gene, as opposed to the 22% of metastatic UM having GNAQ mutations; thus, GNAQ is suggested to play a greater role in the early stages of UM and GNA11 in the later stages of malignancy (Jovanovic et al. 2013). Taking into account the predominance of GNAQ mutations during the early development of UM, a better understanding of its behavior and expression may lead the way to successful treatment options for UM prior to its metastasis.

Mutations in the GNAQ gene are associated with nearly half of all UM cases (Jovanovic et al. 2013). The effects of aberrant GNAQ activity are similar to those of growth factors, as this oncogene is believed to contribute to melanocyte growth and transformation in mice (Onken et al. 2008). Our particular study examined the molecular pathways involved in two UM cell lines, MEL202 and OMM1.3. Although the MEL202 line originates from primary UM and OMM1.3 stems from UM liver metastases, both of these cell lines carry mutations in GNAQ (Wu et al. 2012). The missense mutation in GNAQ leads to the substitution of glutamine at codon 209 with leucine in MEL202 cells and with proline in OMM1.3 cells (Wu et al. 2012).

Protein Kinase C

The PKC family of serine/threonine kinases is involved in phosphorylating and regulating of a wide array of substrates that trigger diverse cellular reactions (Newton

1995; Wu et al. 2012). Conventional isoforms of PKC include α, βI, βII, and γ, and novel isoforms include δ, ε, θ, and η; both groups are activated by DAG and phospholipids, but only the former is dependent on intracellular calcium levels to influence their particular downstream targets (Wu et al. 2012). Each of these isoenzymes has disparate regulatory effects on cell behavior (Teicher 2006). For instance, PKCβI partakes in tumor progression, while PKCδ participates in tumor suppression (Teicher 2006). Aberrant activity of PKC can trigger the MAPK/ERK pathway and influence cell death, proliferation, and angiogenesis, to name a few (Figure 1) (Wu et al. 2012). This pathway is triggered when activated PKC signals the kinase MEK to phosphorylate ERK, which in its reactive form can accumulate in the nucleus to control gene transcription and expression (Patel et al. 2011). The phosphorylated form of ERK can have strong effects on cellular activity, as it is capable of upregulating the pathway's constitutive activity by increasing the expression of specific extracellular receptor tyrosine kinase ligands that are responsible for initiating this cascade effect (Roberts & Der 2007).

Protein Kinase C in Uveal Melanoma

Since a large portion of UM cases exhibit GNAQ mutations, PKC involvement in the development of UM tumors can be expected (Van Raamsdonk et al. 2008). Treatment of $GNAQ^{mt}$ UM cell lines with PKC inhibitors prevents cell growth, and confirms that PKC is largely responsible for promoting cell proliferation in $GNAQ^{mt}$ cells (Chen et al. 2013). Although PKC's oncogenic role in UM tumor progression has been established, it can activate a number of downstream targets, and so the exact method through which it

influences proliferation is not completely understood (Koivunen et al. 2006). In addition to PKC's large impact on UM development, its ability to bind oncogenic phorbol esters makes it an attractive target for therapies, and so its behavior must be further investigated in UM progression (Teicher 2006).

Wnt/ß-Catenin Signaling Pathway

The Wnt/ß-Catenin pathway (Figure 2) is a signal transduction pathway that relays messages to the interior of the cell upon binding of a secreted glycoprotein of the Wnt family to a seven-transmembrane domain receptor (MacDonald et al. 2009). Multifarious cellular responses can be triggered due to the many types of Wnt ligands and cell surface receptors that exist within a system (Miller et al. 1999). To date, there are three distinct Wnt signaling pathways: the canonical, or ß-Catenin-dependent, pathway, and the two non-canonical, planar cell polarity and Wnt/Ca^{2+}, pathways (Komiya and Habas 2008). The canonical Wnt/ß-Catenin signal transduction pathway has been observed to be involved in many UM cases, and so it will be highlighted in this paper.

When the Wnt/ß-Catenin pathway is not activated, ß-Catenin is predominantly localized at the cell membrane, where it functions to mediate the adhesion between E-cadherin in the plasma membrane and actin microfilaments of the cytoskeleton (Cadigan 2008). If ß-Catenin is present in the cytoplasm, it is degraded by the APC-Axin-CKI-GSK-3 protein complex (Cadigan 2008). Degradation is initiated when the APC-Axin tumor suppressing complex binds to ß-Catenin to allow for easy and accessible phosphorylation of serine 45 by CKI, and serine 33, serine 37 and threonine 41 by GSK-3

(Cadigan 2008; MacDonald et al. 2009). These modifications of ß-Catenin prompt poly-ubiquitination, which marks the protein for complete degradation and prevents unwanted gene transcription (Cadigan 2008). Within the nucleus, the repressor TLE/Groucho (Gro) interacts with DNA-bound T-cell factor/Lymphoid enhancing factor (TCF/LEF) transcription factors and promotes their activity as transcriptional repressors of Wnt target genes (Logan & Nusse 2004).

When Wnt ligands bind Frizzled, contact between the GPCR and two lipoprotein receptor-related proteins (LRP), LRP5 and LRP6, are stimulated and the downstream cascade effects are initiated (Logan & Nusse 2004; Cadigan 2008). Upon pathway activation, ß-Catenin evades degradation and accumulates in the cytoplasm (Logan & Nusse 2004). The high concentration of ß-Catenin in the cytoplasm enables it to pass into the nucleus, bind the DNA-bound TCF/LEF transcription factors to release the repressors' functions, and direct gene transcription (Logan & Nusse 2004; MacDonald et al. 2009; Miller et al. 1999; Staal and Clevers 2000). Wnt/ß-Catenin-dependent gene expression is capable of affecting cell proliferation and leading to cancer, and so strict regulation of the Wnt/ß-Catenin pathway is imperative (Logan & Nusse 2004). Mutations in molecules involved in major events such as the degradation of ß-Catenin or repression of transcription can lead to aberrant activation of the pathway (Logan & Nusse 2004).

ß-Catenin in Uveal Melanoma

Many cancers, including a large number of human melanoma cases, have shown to involve unregulated activity of ß-Catenin (Widlund et al. 2002). Studies show that ß-

Catenin behavior varies depending on the tissue type (Chien et al. 2008). Accumulation of ß-Catenin in either the cytoplasm or nucleus has been observed during melanoma progression; but, whereas metastatic melanoma cells rely on the transcriptional role of ß-Catenin for survival purposes, primary and benign cells are not as dependent (Sinnberg et al. 2011). An in vitro study by Guo et al. (2012) on mature mouse melanocytes used Wnt-3a, a stimulator of the Wnt/ß-Catenin pathway and thus ß-Catenin accumulation, and found that it reduced the population of cells in the S phase and increased the number of cells in the G1 phase of the cell cycle. This demonstrated that increased Wnt/ß-Catenin signaling prevented cell proliferation by strictly controlling cell division (Guo et al. 2012). Similarly, in vivo studies also show that in a melanoblast population, ß-Catenin stabilization may actually reduce the cell proliferation (Delmas et al. 2007). In contrast, other in vivo studies on melanoma cell lines suggest that ß-Catenin stabilization may lead to malignancy, and thus increased cell proliferation (Delmas 2007).

The effects of ß-Catenin in melanoma appear to vary depending on the cell type considered, which may insinuate patterns between ß-Catenin and survival in UM. Most UM cell lines do not carry genetic mutations of the ß-Catenin protein, which insists investigation on a possible post-translational mechanism through which its expression increases (Edmunds et al. 2002; Zuidervaart et al. 2007). A number of in vitro studies have utilized protein factors to study ß-Catenin expression and observe its upregulation through post-translational means, such as inhibition of its degradation (Zuidervaart et al. 2007). Although in vitro studies on melanoma cells indicate cell proliferation due to ß-Catenin accumulation, Kim et al. (2010) showed that overexpression of ß-Catenin in a

particular UM cell line can prompt apoptosis and restrict their proliferation. Ye et al. (2008) employed hepatocyte growth factor, which promotes UM cell migration, and observed a dose-dependent downregulation of ß-Catenin without nuclear translocation, suggesting that metastasis does not rely heavily on nuclear levels of ß-Catenin; at the same time, however, it has also been suggested that cytoplasmic ß-Catenin may behave as the trigger for cell migration (Zuidervaart et al. 2007). This discrepancy highlights the importance of compartmentalization of ß-Catenin within the cell. A clinical study by Zuidervaart et al. (2007) on UM patients noted a significant increase in Wnt ligand and thus Wnt/ß-Catenin signaling in primary tumors of patients with poor prognosis. Activation of the Wnt/ß-Catenin pathway and subsequent increases in ß-Catenin levels are believed to correlate with shorter survival time for UM patients (Zuidervaart et al. 2007). These dissimilar effects of ß-Catenin expression in melanoma may be due to variations in how ß-Catenin interacts with other proteins in a cell (Zuidervaart et al. 2007). Nonetheless, the distinct functions of ß-Catenin in different cells or tissues suggest that examination of ß-Catenin expression and Wnt/ß-Catenin signaling in individually isolated tumor specimens could help predict survival chances (Zuidervaart et al. 2007).

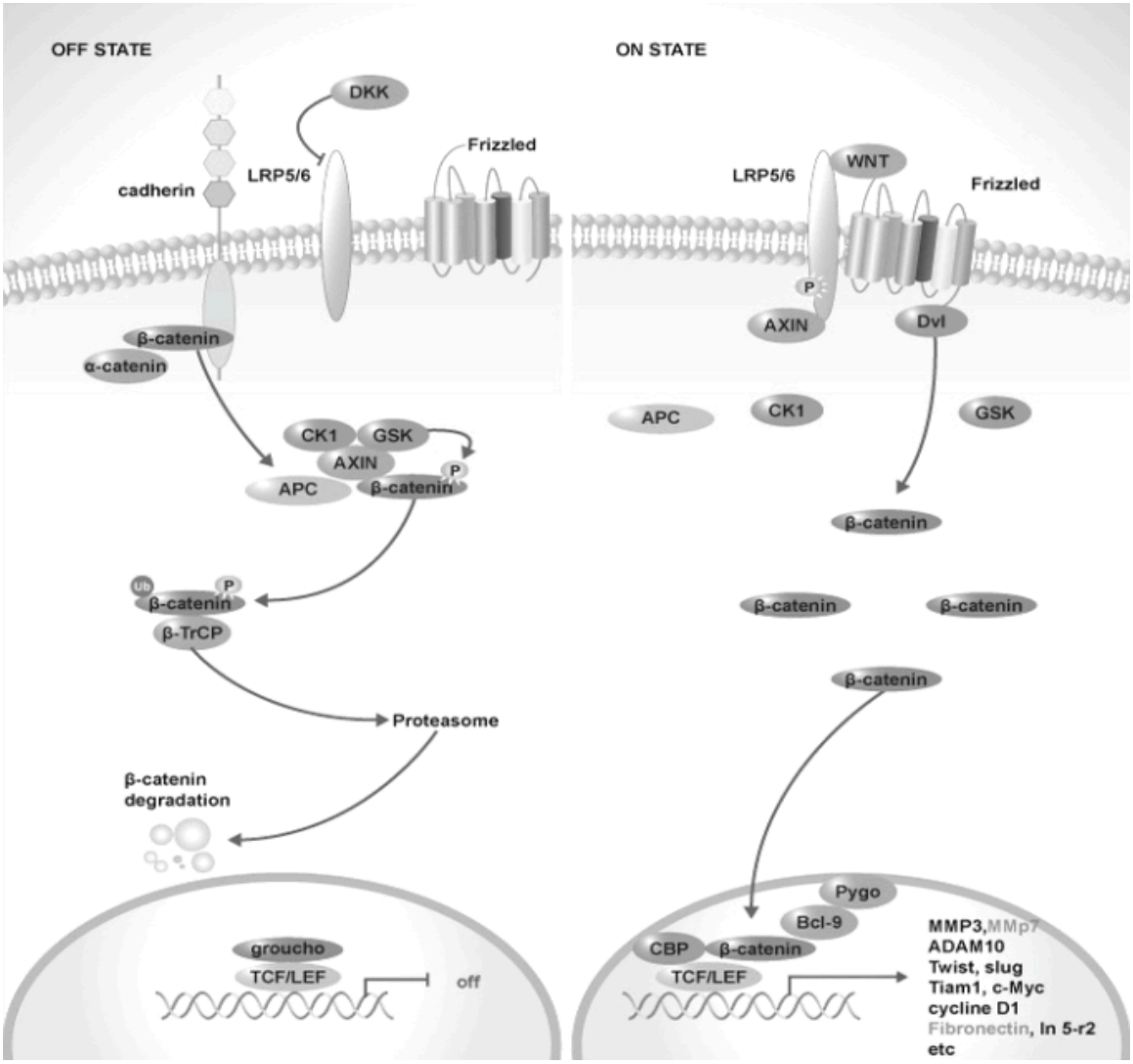


Figure 2: Wnt/ß-Catenin Signaling Pathway (Canonical (beta-Catenin-Dependent) Wnt Signaling Image). This figure provides a simple representation of the currently accepted model of the Wnt/ß-Catenin signaling pathway. In the "off " state, there is no Wnt ligand bound to the frizzled receptor, and so any cytoplasmic ß-Catenin is degraded by the APC-Axin-CKI-GSK-3 complex. The TLE/Groucho co-repressors bind TCF/LEF and trigger their functions as repressors of gene transcription. When Wnt ligands bind frizzled, the signaling cascade is activated and in the "on" state. Consequently, ß-Catenin accumulates in the cytoplasm, translocates to the nucleus, and binds TCF/LEF to trigger their functions as promoters of gene transcription. This can lead to aberrant cell growth and cancer.
This image is downloaded and adapted from Sino Biological Inc. (www.sinobiological .com).

Specific Aims

Although UM represents a small percentage of all melanomas, UM has a high risk of liver metastasis and subsequently, death (Bedikian 1995; Buder et al. 2013). Development of UM may be due to a variety of genetic mutations, constitutive activation of signaling pathways, or irregular associations among molecular factors responsible for regulating cellular functions. This complicates the search for successful therapies because each melanocyte cell can transform into a distinct malignant cell line through unique modifications and interactions. Further, findings suggest that UM tumors can be composed of more than one type of malignant UM cell line (Patel et al. 2011). Thus, depending on the root source of the malignancy and specific cell lines involved, treatment options may vary.

Our study focuses on investigating the activity of PKC and its influence on the Wnt/ß-Catenin pathway. PKC and ß-Catenin were selected as targets to inhibit in two $GNAQ^{mt}$ UM cell lines, MEL202 and OMM1.3. Inhibition of these pathway points was accomplished using cardamonin (CD), a Wnt/ß-Catenin inhibitor, FH535 (FH), a ß-Catenin/TCF inhibitor, and bisindolylmaleimide I (BIM), a highly specific PKC inhibitor, in a dose-dependent manner. Activity of ß-Catenin was investigated further in depth with Wnt-3a ligand to comprehend the effects of stimulating the Wnt/ß-Catenin pathway. In addition, western blotting, siRNA transfection, reverse phase protein array (RPPA), and reverse transcription polymerase chain reaction (RT-PCR) were used to justify our claims. Previous studies have shown that combination therapies aiming to inhibit activity of more than one pathway or their respective molecular components are more effective in

controlling cell growth and development of cancer, and so relationships between these distinct protein molecules were sought (Khalili et al. 2012).

METHODS

Cell Culture

The genotype of MEL202 and OMM1.3 ($GNAQ^{mt}$, $GNA11^{wt}$, and $BRAF^{wt}$) UM cell lines has been reported (Van Raamsdonk et al. 2008; Mitsiades et al. 2011; Griewank et al. 2012). All cell lines used were confirmed by Sanger sequencing to harbor the genotypes described above and were passaged for fewer than 6 months afterwards. All cells were grown in filtered DMEM/F12 (Invitrogen, Carlsbad, CA) with 100 units/ml penicillin, 100 µg/ml streptomycin, 10% FBS (Invitrogen), 1% MEM vitamin solution (Gibco, Gaithersburg, MD), and 1% MEM non-essential amino acids (Gibco).

Reagents

The Wnt/ß-Catenin inhibitor, CD, the ß-Catenin/TCF inhibitor, FH, PKC inhibitor, BIM, and human recombinant Wnt-3a were purchased from R&D Systems, (Minneapolis, MN), and reconstituted in DMSO (Sigma-Aldrich, St. Louis, MO) according to the manufacturers instructions. ß-Catenin antisense oligonucleotides were purchased from Invitrogen. Antibodies for ß-Catenin, ß-actin, the phosphorylated form of AKT, Erk1/2, and LC3BII were purchased from Cell Signaling (Danvers, MA).

Treatment with Inhibitors

Dose response curves were created for each cell line with each inhibitor under sterile conditions. Prior to treatment with the various reagents, cells were plated in 24-well cell culture plates with 5,000 cells per well in a volume of 500 µL media. The plated

cells were incubated for a minimum of 24 hours to ensure adherence. All treatments, including controls, were performed using a total volume of 400 μL of media. Cells were treated with CD alone at concentrations of 0 μM, .5 μM, 3 μM, 10 μM, and 30 μM. Treatment with FH alone was conducted at concentrations of 0 μM, .05 μM, .1 μM, .3 μM, 1 μM, and 10 μM. Each treatment condition for CD and FH was repeated in four separate wells. After treatment with the inhibitors, cells were incubated for 72 hours, after which MTT assays were performed.

Treatment with Wnt-3a

Dose response curves were created for each cell line with different concentrations of Wnt-3a under sterile conditions. Five thousand cells were plated 24 hours prior to treatment to ensure adherence. Treatments with Wnt-3a alone were conducted at concentrations of 0 ng/mL, 100 ng/mL, 200 ng/mL, and 300 ng/mL. Following the addition of Wnt-3a, the treatment conditions were replenished after 96 hours. MTT assays were done 168 hours after the initial treatment.

MTT Assay

MTT assays were performed to determine the effect of the inhibitors and activators on cell viability. MTT solution was made using 1 mg of Molecular Probes® MTT reagent per 2 mL of sterile phosphate buffered saline. After ensuring the solution was completely dissolved, the cells were incubated with the MTT solution at 37°C with 5% CO_2 for four hours. The formazan crystals were dissolved with a 1:1 ratio mixture of

DMSO and isopropanol (Sigma-Aldrich) that was added to each well. Complete dissolution and homogeneity were ensured by keeping the plates on a vigorous shaker for about 20 minutes. Softmax® Pro Data Acquisition and Analysis Software was used to read the absorbance of the samples at a wavelength of 570 nm.

Western Blotting

$1x10^6$ cells from both cell lines, MEL202 and OMM1.3, were plated and treated with BIM at concentrations of 0 μM, 5 μM, 10 μM, and 20 μM. After 48 hours of incubation under these treatment conditions, western blots were performed after collecting, centrifuging, and lysing the cells in a solution of 50 mM Tris-HCl with 120 mM NaCl, 1% Igepal, and proteinase inhibitors from Complete™ (Indianapolis, IN). After collecting the protein lysates, 30 μg of protein was loaded into each well in a 12% sodium dodecyl sulfate-polyacrylamide membrane to perform gel electrophoresis. The proteins were then electroblotted onto nitrocellulose sheets and incubated in blocking buffer (20% IgG-free horse serum and PBS) for one hour. The respective primary antibodies (1:1000) were added to the membrane-bound proteins and incubated overnight at 4°C. Dilute (1:10,000) respective secondary antibodies were added to the membrane after washing with PBS. All the membranes were subsequently stripped of the primary and secondary antibodies and incubated with the anti-ß-actin Clone AC-15 mouse monoclonal antibody (Sigma-Aldrich) for normalization. Enhanced chemiluminescence (ECL kit, Pierce Chemical, Rockford, IL) was used to detect the proteins.

siRNA Transfection

siRNA silencing of ß-Catenin (siCTNNB1) was used to interpret the influence of ß-Catenin gene knockdown alone and in combination with BIM on MEL202 cell viability. Cells were plated in two 24-well plates and incubated for 24 hours to ensure adherence. After, a mixture of siRNA and Lipofectamine™ RNAiMAX (Invitrogen) was made in OptiMEM I Reduced Serum Medium (Invitrogen). The Lipofectamine was used to transfect the cells with 30 nM Stealth RNAi™ siRNA Duplex Oligoribonucleotides (Invitrogen) as directed by the manufacturing company. Cells were treated with either control or siCTNNB1 oligonucleotides alone, 1 μM or 2 μM BIM alone, or their combinations. MTT cell viability assay was performed 6 days after the initial treatment to assess the effects of the treatment conditions and statistical analyses of data were done after subtracting absorbance values obtained at 630 nm from values obtained at 570 nm.

Reverse Phase Protein Array (RPPA)

After treatment with 20 μM BIM, MEL202 cellular protein expression was analyzed. Cells were incubated for 6, 24, or 48 hours after initial treatment. Each treatment condition had its particular set of controls with DMSO. Nitrocellulose-coated slides were used to arrange and probe the cell lysates with antibodies. Diaminobenzidamine (DAB) colorimetric reaction was used to visualize the proteins, upon which the proteins were scanned and their image density was evaluated numerically.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

After treatment with 20 μM BIM, MEL202 ß-Catenin mRNA production was analyzed. Cells were incubated for 6 or 24 hours, after which absolute quantities of mRNA transcripts were retrieved. Primers were purchased from Sigma-Aldrich and probes were purchased from Roche Universal Probe Library (Indianapolis, IN). Cellular content of mRNA was separated using a Qiagen RNeasy kit (Qiagen, Valencia, CA) and the company's recommended instructions. TaqMan® RNA-to-CT™ 1-Step Kit (Foster City, CA) and a StepOne™ PLUS Real-Time polymerase chain reaction (PCR) from Applied Biosystems were used to perform reverse transcription and PCR.

Statistical Analysis

Since each well in the treated 24-well plates was represented by two separate wells in the 96-well plates, two absorbance values were obtained for each trial of different treatment conditions. To assess cell viability after treatment with any one reagent, the mean of the two measurements were calculated and used to compute the standard deviations. The effects of each treatment were interpreted by performing independent (unpaired) samples t-test analyses. $P < 0.05$ was considered to be statistically significant.

RESULTS

PKC Activity in GNAQ-Mutant Uveal Melanoma Cells

We have previously mentioned that Gαq-induced transformation in UM involves the activation of PKC. To investigate the role of PKC in UM, we utilized BIM, a highly specific inhibitor of PKC activity. MEL202 and OMM1.3 cells were treated with 20 μM BIM and cell viability was assessed with MTT assay after a 96-hour incubation (Figure 4). These data show that BIM decreased more than 80% of the cell viability in both cell lines.

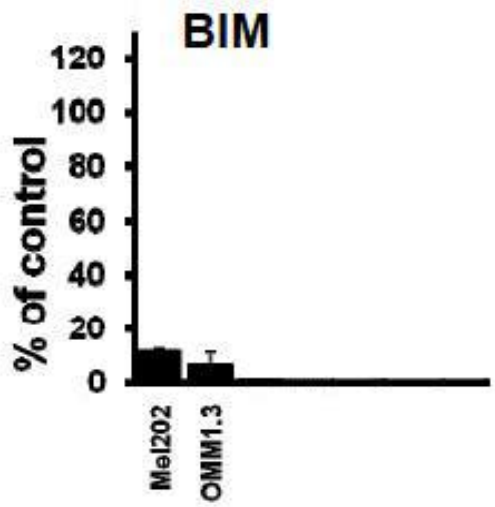


Figure 3: PKC Inhibition Decreases Cell Viability of GNAQ-Mutant Uveal Melanoma Cells. Treatment with the PKC inhibitor BIM decreased the proliferation of UM cells by more than 80% over 96 hours. Results are presented as mean absorbance of the respective control ± SD.

We then proceeded to investigate the mechanism of this effect by assessing whether it is an apoptotic death that involves the activation of caspases (Figure 4).

MEL202 cells were treated with 20 μM BIM and levels of cleaved (and therefore activated) caspase 3 and LC3BII were assessed with western blotting. We saw that caspase 3 is not activated during the BIM-induced decrease in cell viability, but the intracellular concentration of an autophagy marker, LC3BII, was increased, which is the hallmark of autophagy.

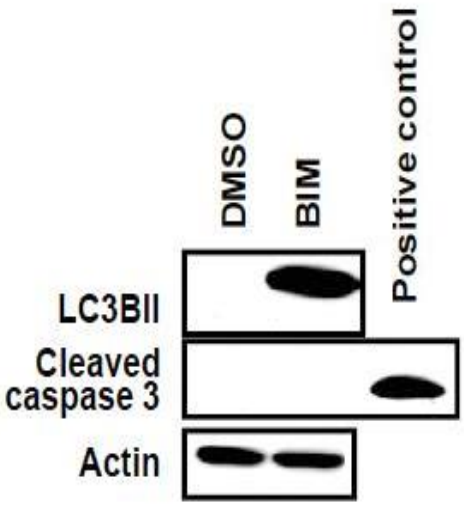


Figure 4: PKC Inhibition Results in Uveal Melanoma Cell Death that is Mediated Through Autophagy and Does Not Involve the Activation of Caspases. MEL202 cells were treated with 20 μM of BIM for 72 hours or DMSO. BIM-treated cells accumulate LC3BII, an autophagy marker, but do not cleave and therefore activate caspase 3, a marker of apoptosis, confirming that cell death is caspase-independent in this model.

Regulation of ß-Catenin Expression Through PKC

Although it is known that the Wnt/ß-Catenin pathway plays a crucial role in melanoma pathogenesis, the precise nature of its contribution is still unclear. We investigated the role of the Wnt/ß-Catenin pathway in PKC-induced UM cell death by visualizing the levels of ß-Catenin after PKC inhibition (Figure 5). After 48 hours of

incubation with BIM at concentrations of 0 µM, 5 µM, 10 µM, and 20 µM, western blots were performed with ß-actin as the loading control. Expression of ß-Catenin was found to increase, more significantly in the OMM1.3 cell line, as the concentration of BIM was increased. RPPA technology was also used to evaluate the increase in ß-Catenin protein expression in MEL202 cells after PKC inhibition (Figure 6). RPPA results show that compared to the control treatment with DMSO, treatment of MEL202 cells with 20 µM of BIM significantly increased ß-Catenin protein expression by 374.28% after 6 hours, 454.5% after 24 hours, and 404.12% after 48 hours of incubation.

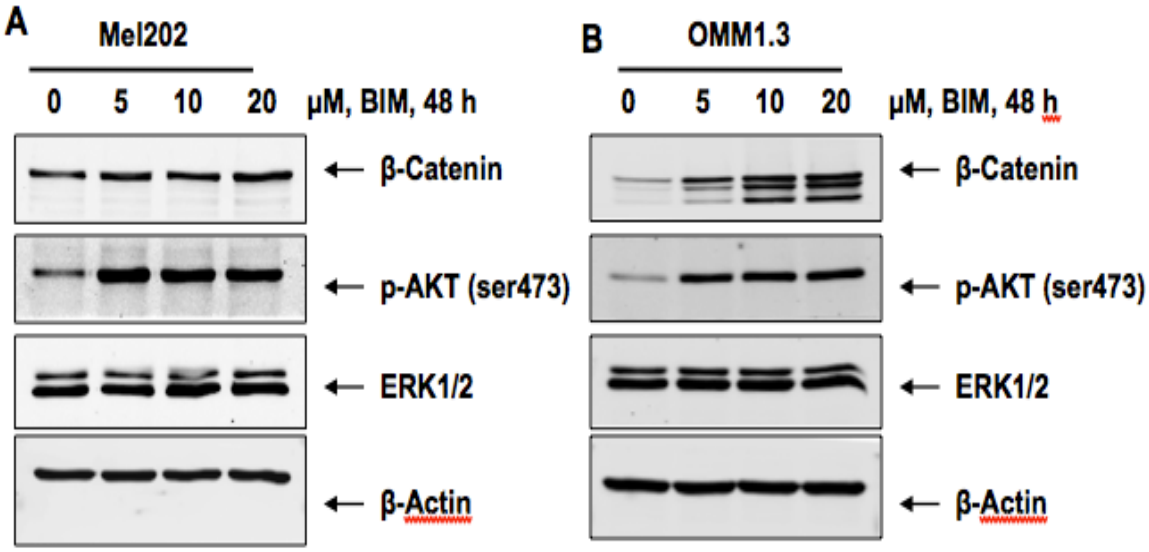


Figure 5: Effect of BIM on the Protein Expression Levels of ß-Catenin, p-AKT, and ERK1/2. Western blots of A) MEL202 and B) OMM1.3 after 48 hours of treatment with BIM show an increase in ß-Catenin and p-AKT expression in a concentration-dependent manner. Protein expression levels of ERK1/2 did not change in response to treatment with BIM.

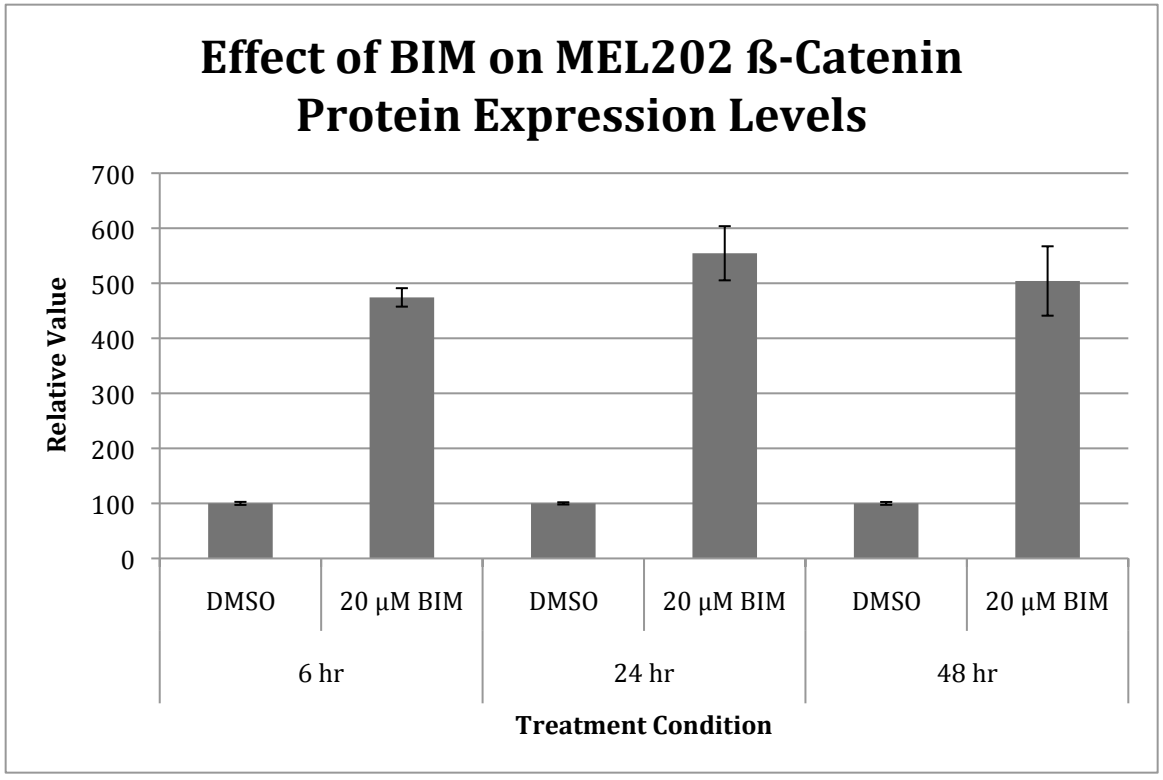


Figure 6: Effect of BIM on MEL202 ß-Catenin Protein Expression Levels. RPPA analysis of ß-Catenin protein levels after treatment with 20 µM of BIM in MEL202 cells was done after 6 hours, 24 hours, and 48 hours. Results show that treatment with BIM, regardless of incubation times, leads to an increase in the expression of ß-Catenin. Results are presented as mean absorbance of the respective control ± SE.

To determine if PKC inhibition amplifies ß-Catenin protein expression through increased transcription and subsequently translation, expression of ß-Catenin mRNA transcripts were determined. MEL202 and OMM1.3 cells were treated with 0 µM, 5 µM, 10 µM, and 20 µM of BIM and incubated for 48 hours prior to RT-PCR experiments (Figure 7). The RT-PCR failed to show an upregulation of ß-Catenin mRNA transcription, but instead showed a decrease in ß-Catenin mRNA levels. Collectively, this data suggests showing that the mechanism of PKC-induced upregulation of ß-Catenin levels was post-translational.

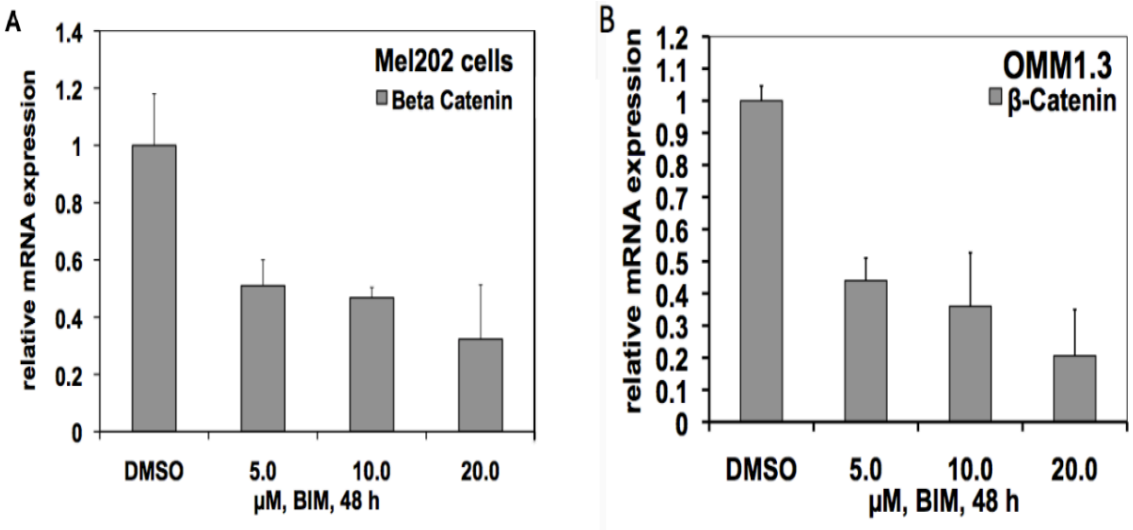


Figure 7: Effect of BIM on mRNA Expression Levels of ß-Catenin. MEL202 and OMM1.3 cells were treated with the indicated concentrations of BIM for 48 hours, mRNA was isolated, and RT-PCR was performed. Treatment with BIM decreased mRNA levels in a dose-dependent fashion in A) MEL202 and B) OMM1.3. Results are presented as mean ± SD.

PKC Inhibition Increases the Phosphorylation of AKT

In an effort to investigate the mechanism of BIM-induced increases in ß-Catenin levels, we studied the involvement of kinases, such as AKT and ERK1/2, that are known to modify ß-Catenin post-translationally (Figure 5). Cells were incubated with 0 μM, 5 μM, 10 μM, and 20 μM of BIM for 48 hours and immunoblotted for the phosphorylated (and therefore activated) form of AKT and ERK1/2. Treatment with BIM increased the phosphorylated form of AKT (p-AKT) but did not affect ERK1/2, pointing to the role of AKT in the increase of ß-Catenin levels.

β-Catenin Inhibition Has Dose-Dependent Effects on GNAQ-Mutant Uveal Melanoma Cells

To assess the effects of inhibiting components of the Wnt/β-Catenin signaling pathway on cell proliferation, cells were treated with CD, a Wnt/ß-Catenin inhibitor, and

FH, a ß-Catenin/TCF inhibitor, separately at various concentrations. After 36 hours, MTT cell viability assays showed that low doses of CD (.5 μM and 3 μM) increased cell proliferation while higher concentrations of CD (10 μM and 30 μM) inhibited cell proliferation in both, MEL202 and OMM1.3 (Figure 9). In comparison to the control cells, average growth of MEL202 cells after treatment with .5 μM and 3 μM of CD increased by 15.84% and 10.99%, respectively. At 10 μM and 30 μM of CD cell growth was reduced by 32.57% and 57.23%, respectively. Similarly, treatment with .5 μM and 3 μM of CD led to an increase in OMM1.3 cell proliferation of 71.8% and 12.73%. After treatment with 10 μM and 30 μM of CD, cell proliferation was reduced by 73.73% and 78.05%, respectively.

Results were similar with the inhibitor FH that increased cell proliferation at low levels but inhibited cell proliferation at higher levels (10μM) in both, MEL202 and OMM1.3 (Figure 10). In comparison to the control cells, average growth of MEL202 cells after treatment with .05 μM, .1 μM, .3 μM, and 1 μM of FH was increased by 10.45%, 10.94%, 14.22%, and 12.88%, respectively. Treatment with 10 μM of FH significantly reduced cell growth by 53.22%. Likewise, after treatment with .05 μM, .1 μM, .3 μM, and 1 μM of FH, OMM1.3 cells further proliferated by 24.53%, 57.63%, 122.68%, and 6.35%, respectively. Contrastingly, 10 μM of FH reduced cell proliferation by 75.59%.

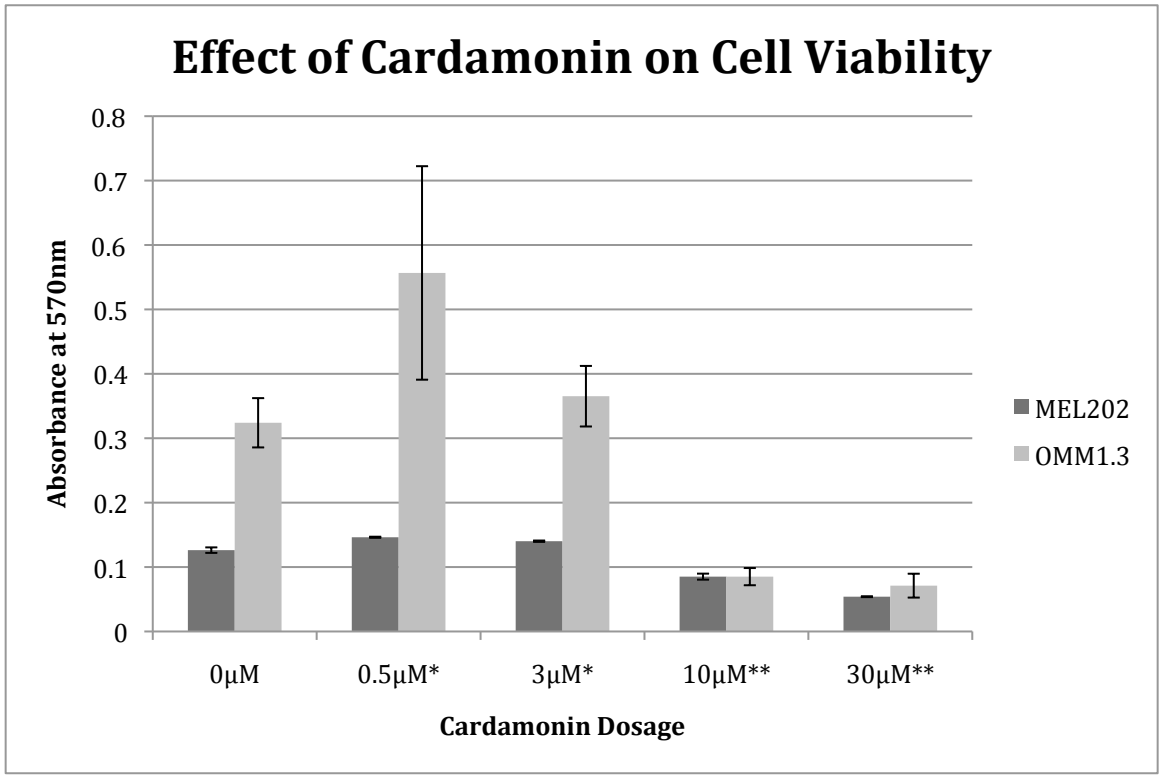


Figure 8: Treatment with Cardamonin Increased Proliferation at Low Levels and Decreased It at High Levels. The effect of cardamonin on the proliferation of MEL202 and OMM1.3 cells is concentration-dependent, with $P < 0.05$ considered significant. After 36 hours of incubation with CD at various concentrations, MTT results indicate that at .5 μM and 3 μM, cell proliferation increases while at 10 μM and 30 μM, CD causes a significant decrease in cell growth compared to the control cells. Results are presented as mean ± SE of four trials. *Significant values for only MEL202. **Significant values for MEL202 and OMM1.3.

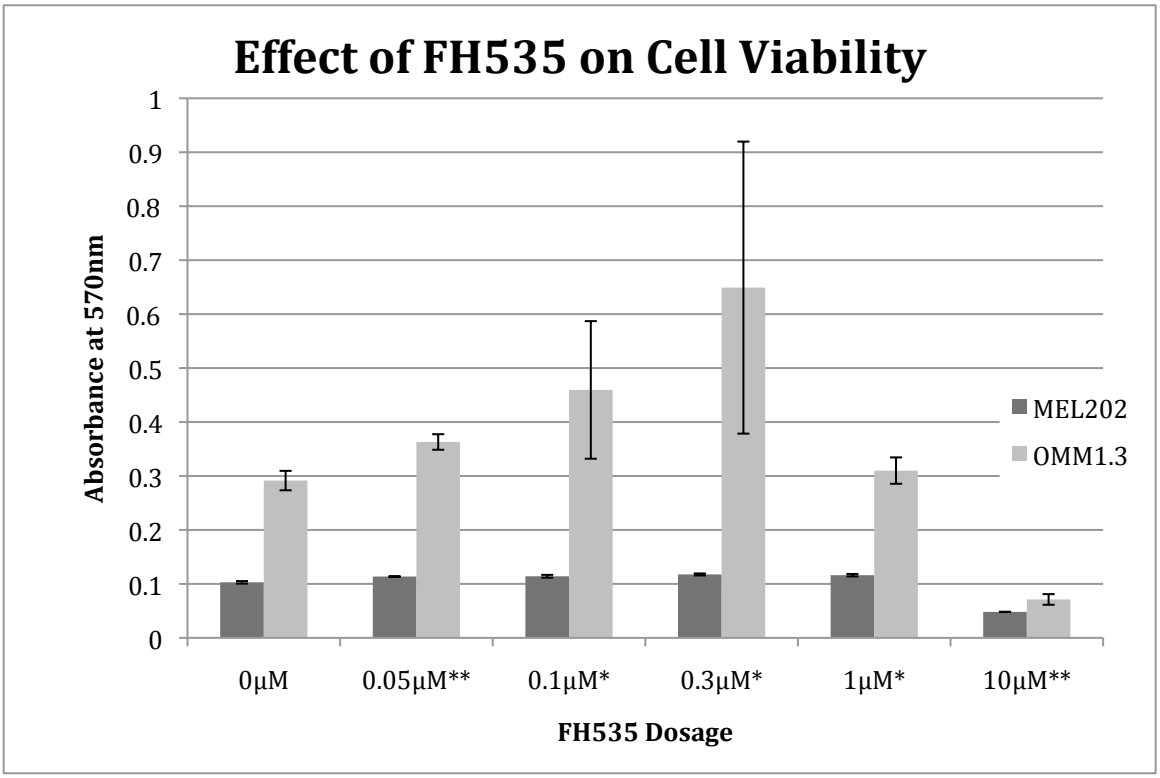


Figure 9: Treatment with FH535 Increased Proliferation at Low Levels and Decreased It at High Levels. The effect of FH535 on the proliferation of MEL202 and OMM1.3 cells is concentration-dependent, with $P < 0.05$ considered significant. After 36 hours of incubation with FH at various concentrations, MTT results indicate that FH increases cell growth, but a higher dose of FH (10 μM) significantly decreases cell growth in comparison to the control cells. Results are presented as mean ± SE of four trials. *Significant values for only MEL202. **Significant values for MEL202 and OMM1.3.

Specific Knockdown of ß-Catenin Increases UM Cell Proliferation

Our results from the Wnt/ß-Catenin inhibitors effect on UM cell proliferation and viability were further solidified with treatment with ß-Catenin antisense oligonucleotides that target only ß-Catenin and do not have the non-specific side effects of small molecule inhibitors (Figure 10).

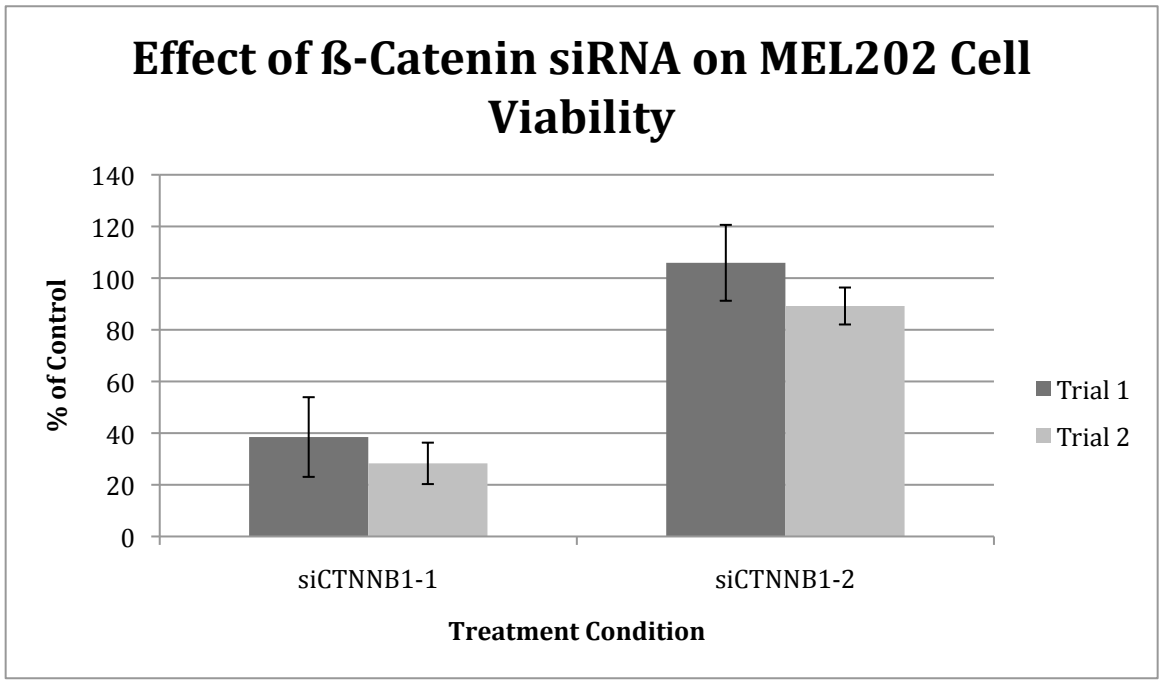


Figure 10: Treatment with ß-Catenin Antisense Oligonucleotides Increased Proliferation in MEL202 Cells. ß-Catenin knockdown in MEL202 increases cell growth in two distinct trials. Results are presented as % increase in proliferation in relation to the respective control ± SD.

Activation of Wnt/ß-Catenin Pathway with Wnt Treatment Decreases Uveal Melanoma Cell Proliferation

To further confirm the pro-survival effect of ß-Catenin inhibition on UM cells, we studied the effect of Wnt-3a, a natural activator of the pathway. Stimulation of the Wnt/ß-Catenin with recombinant human Wnt-3a was found to have a dose-dependent effect on UM cell proliferation by significantly inhibiting cell proliferation at high concentrations in both, MEL202 and OMM1.3 lines (Figure 11). The effect of Wnt-3a was dependent on the cell line with mild proliferating effect on OMM1.3 in lower doses (33.11% for the

200 ng/mL dose). Wnt-3a treatment resulted in significant decrease in cell proliferation that was reduced 37.78% after treatment with 300 ng/mL of Wnt-3a in MEL202 and 55.27% in OMM1.3.

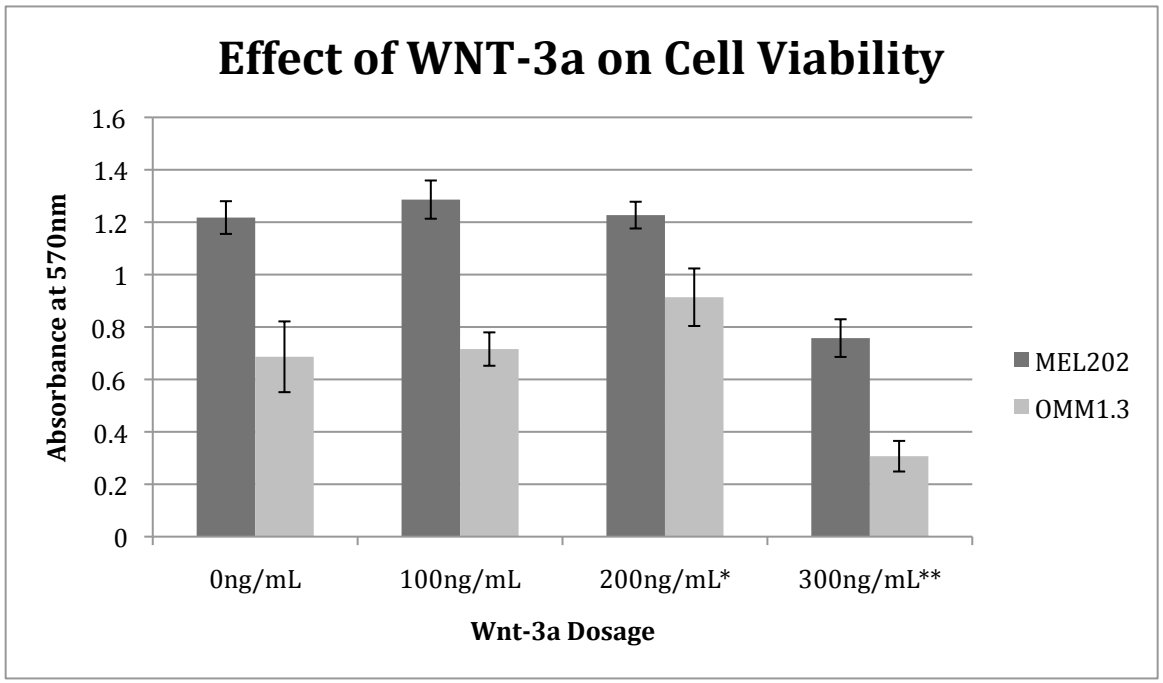


Figure 11: Wnt/ß-Catenin Decreases Uveal Melanoma Cell Proliferation. After 168 hours of incubation with Wnt-3a at various concentrations, MTT results indicate that Wnt-3a decreases cell viability at 300 ng/mL in both cell lines compared to the control cells. Results are presented as mean ± SE. *Significant values for only OMM1.3. **Significant values for MEL202 and OMM1.3.

ß-Catenin Mediates BIM-Induced Cell Death

Based on the data that ß-Catenin downregulation has a prosurvival effect on UM cells, we investigated whether ß-Catenin mediates the cell death induced upon PKC inhibition (Figure 12 and 13). The results show that compared to the controls, treatment with only 1 μM BIM decreased proliferation by 35.36%. In comparison to

treatment with only 1 μM BIM, combination treatment with siCTNNB1 and BIM increased cell viability by 69.41% and 132.89%.

MTT assays from another experiment showed similar results. Compared to the controls, treatment with only 2 μM BIM led to a 58.86% decrease in cell viability. Compared to treatment with 2 μM BIM alone, combination treatment with siCTNNB1 and BIM increased cell proliferation by 23.86% and 126.14%.

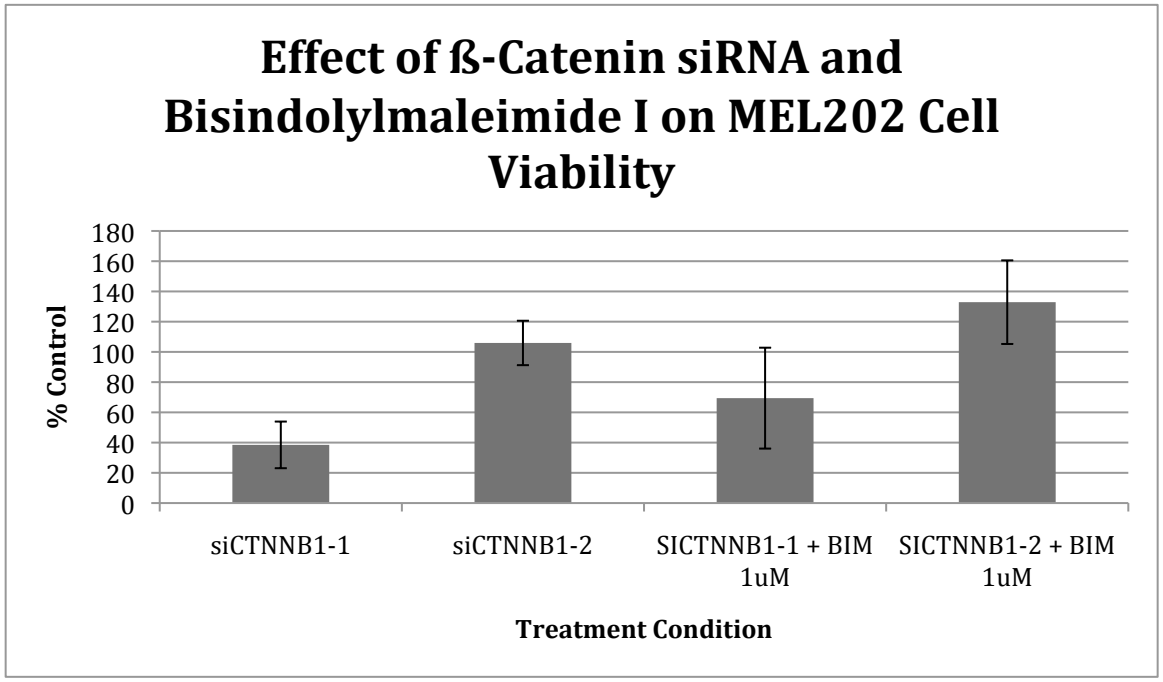


Figure 12: Effect of ß-Catenin siRNA and BIM on MEL202 Cell Viability. MTT cell viability assays show that treatment of siCTNNB1 increases cell growth and combination treatment with siCTNNB1 and 1 μM BIM increases cell growth when compared to treatment with BIM alone. Results are presented as mean % increase in cell proliferation in relation to the respective control ± SD.

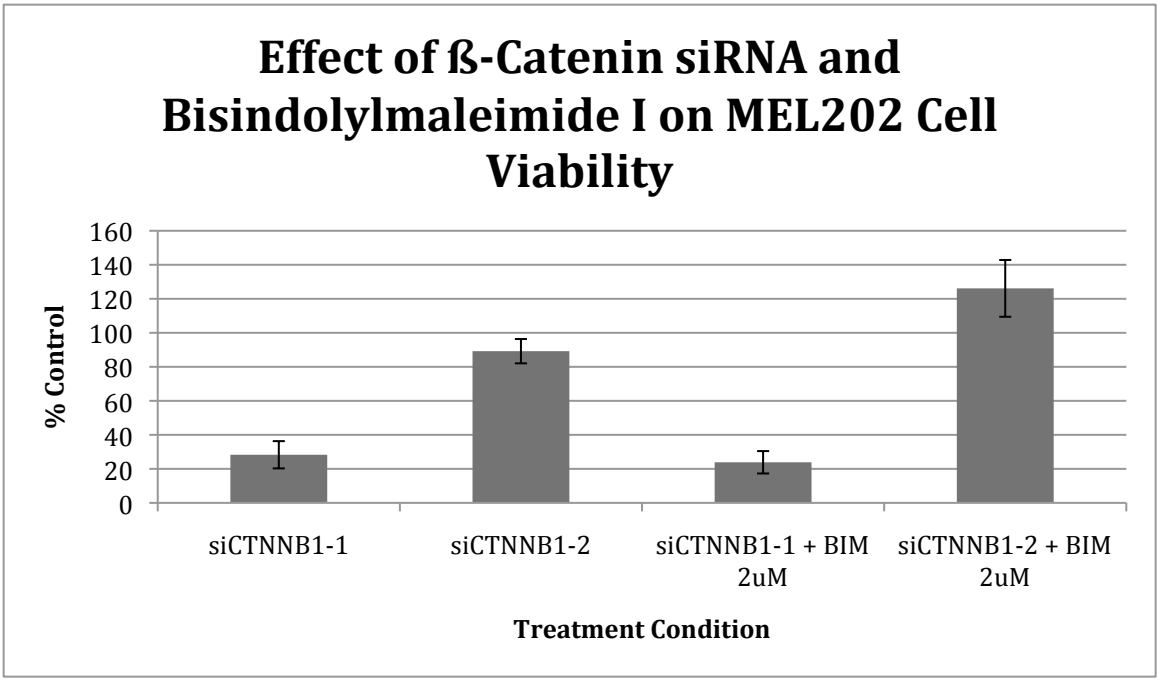


Figure 13: Effect of ß-Catenin siRNA and BIM on MEL202 Cell Viability. MTT cell viability assays show that treatment of siCTNNB1 increases cell growth and combination treatment with siCTNNB1 and 2 μM BIM increases cell growth when compared to treatment with BIM alone. Results are presented as mean % increase in cell proliferation in relation to the respective control ± SD.

DISCUSSION

Uveal melanoma (UM) is the most common intraocular malignancy in adults and despite successful local control, leads to substantial mortality due to early metastasis. Thus, there is an unmet need for novel anticancer agents with activity against UM. The discovery that 50-70% of cutaneous melanomas carry mutations in the BRAF gene that cause constitutive activation of the MEK/ERK pathway and increased cell proliferation led to the development of specific B-Raf kinase inhibitors, such as PLX4032, that revolutionized the clinical treatment of metastatic cutaneous melanoma (Colombino et al. 2012; Heakal et al. 2011). However, BRAF mutations are quite rare in UM. Instead, an equivalent oncogenic effect present in 80% of UMs is in the form of mutually exclusive somatic mutations in GNAQ or GNA11, which abolish GTPase activity, resulting in a constitutively active Gα protein (Onken et al. 2008; Van Raamsdonk et al. 2008; Van Raamsdonk et al. 2010; Lamba et al. 2009; Dratviman-Storobinsky et al. 2010; Wu et al. 2012). Mutated Gα subunits lead to constitutive activity of the MAPK/ERK signal transduction pathway and subsequently, melanocyte transformation, and so have been considered an early event in the progression of UM (Wu et al. 2012; Jovanovic et al. 2013). Distinct sensitivity patterns of UMs to targeted therapies have been identified and Gαq-induced signaling has been established as the oncogenic driver in Gαq-mutant UM cells via a pathway involving PKC (Mitsiades et al. 2011). Dissection of the signaling pathway downstream of $GNAQ^{mt}$ highlighted the role of PKC signaling in UM. We have found that pharmacological inhibition of Gαq or PKC exerts anticancer activity in

$GNAQ^{mt}$ UM that involves autophagy and represents a novel treatment paradigm for cancer. We proceeded to investigate the role of β-Catenin in UM and its crosstalk with the GNAQ/PKC pathway.

PKC Role in GNAQ-Mutant Cells

We have found that the PKC inhibitor BIM has profound effects on several $GNAQ^{mt}$ UM cell lines. Our data highlight the role of the PKC pathway in $GNAQ^{mt}$ UM cell proliferation and survival. It is known that transfection of $GNAQ^{mt}$ into NIH-3T3 cells activates PLCβ that catalyzes the hydrolysis PIP_2 into inositol and triggers Ca^{2+} release and DAG which activates PKC (Wu et al. 1992). We have previously found that RNAi-mediated silencing of GNAQ suppresses PKC activity via depletion of DAG. In detail, we demonstrated that when $GNAQ^{mt}$ UM cells were transfected with siRNAs for GNAQ or various forms of PKC in the presence of increasing concentrations of the phorbol ester PMA that mimicks the effects of DAG, PMA could rescue the viability of cells transfected with GNAQ siRNA but not of cells transfected with PKC siRNA (data not shown). Therefore, Gαq operated upstream while PKC operated downstream of DAG in $GNAQ^{mt}$ UM. By utilizing RNAi screening experiments, we previously identified PKCα as the key mediator of growth signaling and MEK/ERK activation downstream in the signaling cascade of the mutated GNAQ protein in Mel202 cells (data not shown). These data provide proof of concept that $GNAQ^{mt}$ induced signaling can be selectively targeted in melanoma and support a role for PKC in this oncogenic pathway. Nearly half of all UM cases carry somatic mutations in the GNAQ gene, which can lead to aberrant

signaling of the MAPK/ERK pathway and cell proliferation (Jovanovic et al. 2013). A potent upstream regulator of the MAPK/ERK pathway is PKC, which has been shown to be upregulated in many malignancies (Wu et al. 2012). RNAi-mediated silencing of GNAQ suppressed phosphorylation of the MEK and ERK1/2 kinases according to our previous data. In an effort to identify potential links between the PKC and MEK/ERK pathway, we investigated the phosphorylation of Raf kinase inhibitor protein (RKIP). RKIP in its dephosphorylated state is an inhibitor of the Raf/ERK pathway, and upon PKC-dependent phosphorylation can be inactivated, thus releasing the activity of the Raf/MEK/ERK pathway. Therefore, PKC is not only a mediator of the Gαq pathway but can also, through phosphorylation and inactivation of RKIP, activate the Raf/MEK/ERK pathway. This could be a link between the Gαq/PKC pathway and ERK activation.

The PKC inhibition-mediated cell death in our experiments does not appear to involve the activation of caspases (in particular caspase 3), which is the hallmark of apoptosis, but it involves the translocation of LC3BII into the phagosomes that characterizes autophagy, a form of programmed cell death involving the collaboration of autophagosomes and lysosomes (Wolf et al. 1999; Mizushima et al. 2010). Autophagosomes incorporate a protein complex that requires LC3BII, and so the amount of this protein observed within a cell is proportional to the number of functional autophagosomes (Mizushima et al. 2010). We have also previously found that PKC inhibition-mediated cell death – similar to GNAQ downregulation-mediated cell death- is preceded by an early suppression in mitochondrial respiration, as evidenced by decreased oxygen consumption rate, which occurred while the mitochondrial membrane potential

was still intact (data not shown). Therefore, the decrease in mitochondrial respiration was not a non-specific sequel of the cell's demise but an early result of the PKC inhibition. Several published articles show that PKC isoforms such as PKCα regulate mitochondrial function. Translocation of PKCα to the mitochondria occurs with several toxic stimuli such as ischemia following reperfusion, heat shock, and chemotherapeutic agents (Fernández et al.; Wang et al. 2007). This translocation involves targeting PKCα to the inner mitochondrial membrane by PICK1 (protein interacting with protein kinase C) and confers resistance to cell death from drug-induced apoptosis (Wang et al. 2003). This effect involves the PKCα-mediated phosphorylation of BCl2 (B-cell lymphoma 2) on Ser70 and decreased dimerization of the pro-apoptotic protein BAX that promotes cell survival (Wu et al. 2002; Ruvolo et al. 1998). As seen in our model, PKCα inhibition in glioma cells collapses mitochondrial membrane potential, decreases complex I and pyruvate dehydrogenase activities, and increases mitochondrial ROS (reactive oxygen species) production (Wang et al. 2006). Singh et al. (2012) have also found that PKC inhibition induces autophagy and induction of cytoplasmic vacuolations with conversion of microtubule associated protein LC3-I to LC3-II as observed in our model that involves the inhibition of PI3K/Akt/mTOR pathway in human pancreatic cancer stem cells.

PKC-Mediated Increase in ß-Catenin Protein Levels

Our data show that PKC inhibition results in increased ß-Catenin protein but not mRNA levels, pointing to the fact that this regulation is post-translational. Intracellular ß-Catenin levels in the cytoplasm are tightly regulated by multiple pathways. In the GSK-3-

dependent pathway, ß-Catenin is phosphorylated at serine residues and associates with a destruction complex containing GSK-3/APC/Axin that targets it for proteasomal degradation (Cadigan 2008; MacDonald et al. 2009). In the Siah-1 pathway, Siah interacts with APC and recruits the ubiquitinilation of ß-Catenin, independent of GSK-3 (Liu et al. 2001). Upregulation and stabilization of the protein ß-Catenin in a number of cancers has been validated (Logan & Nusse 2004). Mutations in the ß-Catenin, APC or Axin genes that promote the stabilization of ß-Catenin are found in several cancers (Giles et al. 2003). β-Catenin stability is also regulated by protein phosphatases like PP2A that can bind Axin and dephosphorylate GSK-3 substrates, promote the dissociation of GSK-3 from the destruction complex and prevent the phosphorylation of ß-Catenin (Jonkers et al. 1997; Li et al. 1999). Multiple studies have investigated the role of PKC on ß-Catenin regulation. Orford et al. (1997) showed that PKC inhibition causes ß-Catenin accumulation in breast cell lines without Wnt activation, whereas Gwak et al. (2006) showed that PKC directly phosphorylates important residues in ß-Catenin that targets it for the proteasome in the presence of Ca^{2+} and a lipid activator. Several of the ß-Catenin residues that PKC is essential for GSK-3-mediated phosphorylation of its N-terminal in the destruction complex can bypass the need for "priming kinases" (Liu et al. 2002). PKC was also found to phosphorylate the ubiquitination targeting sequence on ß-Catenin to target it for the proteasome (Orford et al). Therefore, our findings that PKC inhibition results in ß-Catenin accumulation could be due to a direct effect.

On the other hand, BIM-induced ß-Catenin upregulation could be indirect and through the activity of other kinases. We found that BIM treatment results in

phosphorylation and therefore activation of AKT, and that treatment with AKT inhibitors significantly decreased the BIM-induced upregulation of ß-Catenin levels (data not shown). Therefore, AKT can partially be responsible for the ß-Catenin stabilization. It was shown that AKT-induced ß-Catenin phosphorylation dissociates it from the cell-cell contacts and accumulates it in the cytoplasm and the nucleus enhancing in parallel its transcriptional activity (Fang et al. 2007). Alternatively, p-AKT was shown to phosphorylate GSK-3, inhibit its activity, and disturb the degradative role of the APC-Axin-CKI-GSK-3 protein complex (Kitagishi et al. 2012). This enables large amounts of ß-Catenin to accumulate in the cytoplasm and translocate into the nucleus (Cadigan 2008). Therefore, our observed increase in ß-Catenin protein expression levels after PKC inhibition with BIM could be due to an increase in p-AKT expression, which in turn inactivates GSK-3 and hinders the breakdown of cytoplasmic ß-Catenin through indirect means.

Wnt/ß-Catenin Pathway Role in Uveal Melanoma Survival and BIM-Induced Cell Death

The collective data from the Wnt pathway inhibitors and the ß-Catenin antisense experiments showed that ß-Catenin inhibition increased proliferation in $GNAQ^{mt}$ UM cells. The concept that ß-Catenin inhibition is a pro-survival mechanism in UM was corroborated with utilization of activators of the pathway, such as Wnt-3a, that showed a dose-dependent decrease in cell viability. Activation of Wnt/ß-Catenin signaling typically leads to aberrant cell proliferation due to increased gene transcription, but our results

shed light on new possibilities. Experiments by Guo et al. (2012) also revealed concentration-dependent inhibition of cell proliferation in melan-a cells, or mouse melanocytes, after treatment with Wnt-3a-expressing adenovirus. In agreement, research by Chien et al. (2009) indicates that activation of the Wnt/ß-Catenin pathway in mouse melanoma B16-F1 cells using Wnt-3a leads to nuclear accumulation of ß-Catenin, which corresponded to a decrease in proliferation. In spite of the accepted theory that increased Wnt signaling can lead to cancer, we considered this conflicting phenomena to be a result of ß-Catenin's involvement in some form of programmed cell death (MacDonald et al. 2009). Interestingly, Biechele et al. (2012) have found that endogenous ß-Catenin is required to induce apoptosis in melanoma cells and that activation of the Wnt pathway strongly synergizes with $BRAF^{mt}$ inhibition to decrease tumor growth in vivo and increase cell death in vitro. In particular, they found that administration of PLX4720, an inhibitor of the mutated BRAF protein, synergizes with the activated Wnt pathway in decreasing AXIN1, the limiting component of the destruction complex, and therefore stabilizes ß-Catenin and induces melanoma cell death (Biechele et al. 2012). Although UM cells rarely carry mutations on the BRAF gene, the oncogenic driver in UM is the mutated Gαq protein that has a similar behavior to the mutated BRAF protein. Therefore, it is worthy to speculate that PKC inhibition, which behaves similar to GNAQ inhibition, synergizes with the activated ß-Catenin to promote cell death in UM.

Our data also show that BIM-induced cell death in UM is attenuated significantly with ß-Catenin downregulation with siRNA, further solidifying the pro-apoptotic role of ß-Catenin in UM and its significance in the mutant GNAQ pathway. Compelling

evidence for the validity of our speculation comes from Zimmerman et al. (2013), whose work examined different mouse and human melanoma cell lines. These melanoma cells generally evade cell death mediated by TNF Receptor Death-Inducing Ligand (TRAIL), but when they were treated with recombinant human TRAIL in conjunction with Wnt-3a to increase Wnt/ß-Catenin signaling, there was a significant increase in cell death (Zimmerman et al. 2013). Since activation of the Wnt/ß-Catenin pathway elicits ß-Catenin accumulation in the cytoplasm, its influence on TRAIL-mediated cell death was studied by inhibiting its degradation, which also enhanced cell death (Zimmerman et al. 2013).

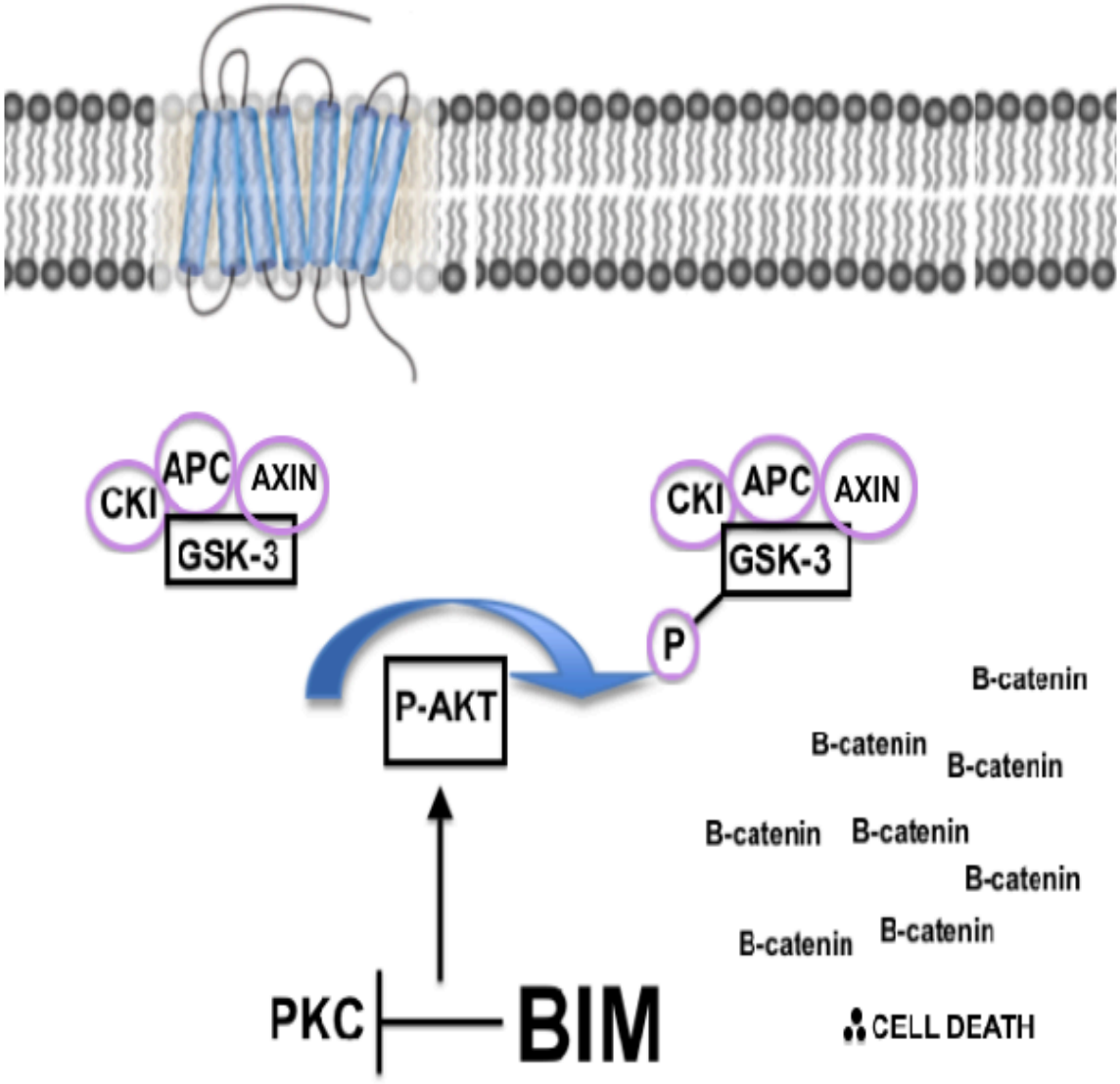
Our data that ß-Catenin plays a pro-apoptotic role in UM can be corroborated from observations from animal models and clinical samples. Murine models for melanoma have demonstrated the anti-proliferative and tumor-suppressing role of Wnt/β-Catenin signaling, which entails nuclear accumulation of β-Catenin (Chien et al. 2008). Recent findings that large amounts of nuclear β-Catenin are common in primary melanoma tumors and suggestive of improved prognosis highlight this protein's capacity to help better understand the etiology of malignant melanoma (Chien et al. 2008). Large amounts of ß-Catenin have been linked to primary tumors compared to metastases, insisting that a decrease in Wnt/ß-Catenin signaling represents melanoma development (Chien et al. 2009). Moreover, in human melanoma cells, high levels of nuclear ß-Catenin correspond with better prognosis and reduced tumor development (Chien et al. 2009). Maelandsmo et al. (2003) reveal that many superficial spreading melanomas have high levels of cytoplasmic ß-Catenin in the preliminary stages, while those undergoing

metastases have lower levels. A decrease in ß-Catenin expression strongly correlates with malignant growth, and so this protein may be central to preventing melanoma progression. Analyses of tumors from melanoma patients with better prognosis also revealed high levels of nuclear β-Catenin, which is suspected to affect tumor development by communicating with the MAPK/ERK and PI3K/AKT pathways, further diversifying and increasing the number of its targets (Chien et al. 2008; Damsky et al. 2011). Wnt/β-Catenin signaling may be a key regulator of melanoma metastasis that influences proliferation and invasiveness in a cell-dependent manner (Damsky et al. 2011). Clinical trials persuasively demonstrate that nuclear accumulation of β-Catenin can be used as a prognostic tool, but predictions regarding survival depend on the context of the melanoma (Maelandsmo et al. 2003).

Conclusion

In summary, we have established the role of PKC and ß-Catenin in $GNAQ^{mt}$ UM. We found that PKC inhibition results in autophagy-mediated cell death that involves the stabilization of ß-Catenin by p-Akt in the cytoplasm. Results from other studies support our claims that increased activation of the Wnt/ß-Catenin pathway and hence, intracellular levels of ß-Catenin promote cell death. Examination of β-Catenin localization can potentially be advantageous in clinic to determine a patient's survival probability, but it is crucial first to fully comprehend how this protein functions in different cell types. Further research on regulating β-Catenin accumulation in the nucleus may also pave the way for targeted therapy.

APPENDIX



Proposed Mechanism for BIM-Mediated Upregulation of β-Catenin and Cell Death.

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CURRICULUM VITAE

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Education

09/2012—present **M.S. in Medical Sciences**
Boston University: Boston, MA
Expected to graduate in May 2014
Academic GPA: 3.8

09/2008—04/2012 **B.S. in Brain Behavior & Cognitive Sciences, Spanish**
University of Michigan: Ann Arbor, MI
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Research Experience

06/2012—07/2012 **Research Assistant in Padmanabhan Lab: Ann Arbor, MI**

- Assisted senior research investigators by capturing images of collected specimen slides of tissue samples
- Used statistical programs to analyze various staining of tissue samples, record data, and analyze the samples to determine whether they were appropriate for study

05/2009—12/2009 **Research Assistant in Wellik Lab: Ann Arbor, MI**

- Gained exposure to the laboratory environment and frequently used research methods
- Aided senior researchers by creating a database and analyzing mice genotypes to confirm their use in the experimental studies
- Collaborated with senior researchers and performed isolated specimen experiments as directed and headed solution making for the research

Volunteer Experience

10/2013—11/2013 **Massachusetts General Hospital: Boston, MA**

- Shadowed pediatric orthopedics clinicians in the clinic and operating room to directly witnessed the importance the doctor-patient bond and critical concerns and proceedings during surgery
- Observed orthopedic nurses and learned proper documentation of patient records

01/2013—12/2013 **Boston Cares: Boston, MA**

- Worked with the American Red Cross to provide groceries to low income families
- Taught athletic skills to mentally disabled children
- Assisted at community events by welcoming guests and cleaning venues afterwards

03/2013—9/2013 **Rosie's Place: Boston, MA**

- Worked with other volunteers to cook and serve meals to women and their children
- Helped women select food items, such as fresh produce, from Rosie's Place Groceries
- Translated English to the women who were fluent in only Spanish

05/2010—06/2011 **St. Andrews Episcopal Church: Ann Arbor, MI**

- Worked with 7-10 other volunteers to prepare and serve early-morning breakfast to approximately 100-175 homeless, underprivileged, and fellow community residents
- Oversaw that all residents and their needs were attended to
- Represented the church when answering questions or providing information to guests

10/2010—04/2011 **University of Michigan Hospital: Ann Arbor, MI**

- Provided entertainment gadgets to patients in the cancer center and presented art for patients desiring a change in environment
- Gained exposure to the medical environment and basic medical care of patients
- Addressed any of the patients' needs or requests and relayed messages to department representatives
- Assisted and comforted patients when requested to spend time with them

August 2009 **Sri Jayadeva Institute of Cardiovascular Sciences and Research: Bangalore, India**

- Shadowed various doctors and observed treatment of adults and children with cardiovascular health issues
- Examined the process of conducting and analyzing cardiac tests such as echocardiograms
- Direct observation of treatment of patients in the intensive care unit and surgical ward
- Spent time and communicated with patients in their native language

Additional Experience

01/2010—04/2012 **F.O.K.U.S (Fighting Obstacles Knowing Ultimate Success): University of Michigan**

- Used art as a platform to unite community members and educate them by bringing awareness to real life issues, such as regional hostility
- Communicated and worked with peers for long hours during creative brainstorming and evaluations of events
- Collaborated with on- and off-campus organizations to promote and present events, lead discussions about topics affecting society, and showcasing unrecognized artists
- Learned the procedures of generating proposals, gathering funding, and supervising events