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Treatment of Sk-Mel-28 Cells With r-Moj-DM Upregulates Pro-Apoptotic Genes P53 and TNF-B While Downregulating Oncogene c-Myc

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TREATMENT OF SK-MEL-28 CELLS WITH r-Moj-DM UPREGULATES
PRO-APOPTOTIC GENES P53 AND TNF- β WHILE DOWNREGULATING
ONCOGENE c-Myc

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Melissa C. Lombardo

May 2016

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The Designated Thesis Committee Approves the Thesis Titled

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by

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ABSTRACT

TREATMENT OF SK-MEL-28 CELLS WITH r-Moj-DM UPREGULATES PRO-APOPTOTIC GENES P53 AND TNF- β WHILE DOWNREGULATING ONCOGENE c-Myc

by Melissa C. Lombardo

Cell death by apoptosis is the result of one or multiple signaling pathways that can be initiated inside or outside the cell for a variety of reasons. Integrins, expressing themselves on the cell surface, play a central role in some apoptotic pathways. Cancer cells have been known to bypass this function and upregulate survival factor genes in order to elude apoptosis and thrive in the body, thus creating an environment in which it is possible for them to grow and metastasize. Previously in Dr. Soto's lab, a recombinant disintegrin mutant, r-Moj-DM, was shown to induce apoptosis in melanoma cell line SK-Mel-28. To test our hypothesis that r-Moj-DM binds to the β_1 integrin subunit, SK-Mel-28 cells were treated with a FITC-conjugated antibody anti- β_1 and analyzed using flow cytometry. In order to ascertain which apoptotic pathways are activated by r-Moj-DM, ten genes, some known pro-apoptotic and some pro-survival, were investigated using RT-qPCR. It was confirmed that r-Moj-DM does bind to the β_1 integrin subunit, and may induce apoptosis through that interaction. It was also found that two known pro-apoptotic genes, p53 and TNF- β , were upregulated in response to treatment with r-Moj-DM while c-Myc, a known survival factor, was downregulated. This shows the possibility that the intrinsic pathway may be activated due to a crossover from other pathways.

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1. Introduction

Disintegrins are small (low molecular weight), non-enzymatic peptides isolated from venom glands in various genera of viper snakes (Nabi et al. 2014). Disintegrins bind specifically to integrins expressed on the surface of platelets and other cells, recognizing the target sequence Arg-Gly-Asp (RGD) binding site as well as other binding motifs (Pasini et al. 2009; McLane et al. 2004). The RGD motif has been found in proteins that bind α_v and $\alpha_5\beta_1$ integrins (Eda et al. 2015). Disintegrins and recombinant disintegrins induce changes in cell behavior when bound to particular integrin receptors (Macêdo et al. 2015; Lucena et al. 2011; Seoane et al. 2010).

Integrins are transmembrane surface receptors that are responsible for cell adhesion to the extracellular matrix (ECM) by binding to fibronectin, vitronectin, laminin, or collagen (Keely 2011). Integrins are heterodimeric molecules composed of one alpha (α) and one beta (β) subunit (Macêdo et al. 2015; Kumar 1998; Hynes 1992). In humans, different α and β subunits combine to form 24 unique integrin heterodimers that perform specialized functions on each cell type (Brakebusch et al. 1997).

One important function of integrins is the ability to induce apoptosis. Integrin-dependent cell adhesion and focal adhesion kinase (FAK) activation can modify the cytoskeleton shape and activate a MAPK signaling cascade, including downstream Erk 1/2, JNK, and p38 (Saldanha-Gama et al. 2010; Giancotti and Ruoslahti 1999). Furthermore, integrins play a major role in cancer growth and progression (Plantefaber

and Hynes 1989). The $\beta 1$ subunit and $\alpha v\beta 3$ integrin are of particular interest (Macêdo et al. 2015; Seoane et al. 2010).

Disintegrins and recombinant disintegrins have been recognized for their anti-tumor abilities such as inhibition of angiogenesis, cell proliferation, and cell migration (Ramos et al. manuscript under journal review; Sánchez et al. 2010; Seoane et al. 2010; McLane et al. 2008). Recombinant disintegrins maintain their biological activity and can be mutated to gain different anti-cancer properties (Lucena et al. 2011; Seoane et al. 2010; McLane et al. 2004). For example, Seoane et al. (2010) demonstrated that one mutant recombinant disintegrin, r-Moj-DM, has remarkable apoptotic action. Recombinant r-Moj-DM was discovered to also have cell proliferation inhibition abilities by antagonizing the αv integrin subunit (Ramos et al. in press).

I hypothesized that r-Moj-DM induces apoptosis by targeting an integrin pathway. To investigate this, ten genes were chosen, in both canonical and newly emerging apoptotic pathways: ACS, APAF1, Bcl-2, c-myc, DAB2IP, Mcl-1, p38, p53, TNF- β /LT- α , and TNXB. Gene expression was measured using qPCR at different time points after r-Moj-DM treatment to determine if treatment resulted in any alteration of expression. Understanding the apoptotic mechanisms used by r-Moj-DM will be an essential tool to further explore ways to induce apoptosis in cancer cells as a possible treatment for many types of cancer.

2. Literature Review

2.1. Apoptosis and survival factors drive cell fate

Apoptosis is physically characterized by chromatin clumping referred to as condensation, DNA fragmentation, mitochondrial rupture, membrane blebbing, and formation of apoptotic bodies (Brill et al. 1999). Cell death by apoptosis is the result of a single or multiple signaling pathways that can be initiated inside or outside the cell. Of interest to this study are two canonical apoptotic signaling pathways: the intrinsic and extrinsic pathways. Under these two umbrella terms, the individual processes are extremely complex. There is much overlap in the genes involved in these pathways, and there are also sub-pathways and signaling cascades.

The Extrinsic Pathway is a receptor dependent pathway. Receptors, namely tumor necrosis factor receptors (TNFR), Fas, or TRAIL, are embedded in the cell surface and when activated, a cytoplasmic team of proteins called the death inducing signaling complex (DISC), assemble. The death domain is responsible for transmitting the signal to the nucleus to elicit a cellular response to the imposing stimulus. Of the first in the signaling cascade is caspase 8, which is activated by the death domain. Caspase 8 then initiates a signaling cascade that results in the activation of caspases 3, 6, 7, and 9. At the end of this chain of signaling and activation, caspase 3 cleaves CAD (caspase-activated DNase) translocates into the nucleus and fragments the DNA, sending the cell into apoptosis (Indran et al. 2011; Pasini et al. 2009; Fulda and Debatin 2006).

The Extrinsic Pathway can also initiate the p38 (also known as the mitogen-activated protein kinase, or MAPK) signal transduction pathway under endoplasmic

reticulum (ER) stress including UV exposure, aging, genetic mutations, or environmental conditions. Fas, a member of the death domain complex clustered with tumor necrosis factor receptors (TNFR), disembarks the complex and initiates p38 (Tobiume et al. 2001). It was also found that Fas forms a trimer with apoptosis signal-regulating kinase 1 (ASK1) and Daxx, thus inducing JNK activation and eventually, the p38 cascade (Chang et al. 1998).

The Intrinsic Pathway is a receptor independent pathway. Radiation, viral infections, or drugs can activate this pathway (Pasini et al. 2009). These damaging stimuli initiate mitochondrial membrane permeabilization (MMP), allowing the release of cytochrome-c (cyt-c) into the cytosol. Cyt-c induces the activation of caspase 9 via APAF1. This activation and oligomerization produces the apoptosome, thus activating caspase 3, resulting in DNA fragmentation and apoptosis (Indran et al. 2011). What begins as the extrinsic pathway can easily cross over to activate the intrinsic pathway, since caspase 8 can also activate tBid, which encourages the release of cyt-c from mitochondria (Indran et al. 2011; Pasini et al. 2009).

Further complicating the apoptotic processes are survival factors that neutralize the apoptotic signaling proteins. There are many factors that work to prevent apoptosis once signaling begins in either the extrinsic or intrinsic pathways. Mcl-1, and the Bcl-2 family are excellent examples of these proteins. Bcl-2 has been shown to physically block apoptotic signaling proteins by directly interacting with and sequestering them (Indran et al. 2011; Reed 1998). The overexpression of Bcl-2 has been shown to halt apoptosis in certain cancer cells. This interesting family, consisting of both survival

factors and pro-apoptotic signalers, has three main functions. First, they dimerize with other family members to inhibit each other's activity. For example, Bax and Bcl-2 commonly pair, though Bcl-2 is a known survival factor while Bax is pro-apoptotic (Reed 1998). The proteins will also dimerize with other proteins associated with cell survival or apoptosis, such as APAF1, protein kinase Raf-1, and the GTPase R-Ras (Reed 1998). It is speculated that Bcl-2 family proteins can actually regulate APAF1 and that Bcl-2 blocks apoptotic signaling downstream of APAF1 activation in some cell types (Reed 1998; Hengartner 1998). Third, it appears that Bax, Bcl-2, and Bcl-X_L are able to form ion channels. Bax and Bak dimerize, creating mitochondrial pores to release cytochrome c into the cytosol (Reed 1998).

Mcl-1 is a peptide of interest due to its unique biology and therapeutic opportunities. Mcl-1 is a highly regulated gene and is regularly expressed during certain stages of cell differentiation and in response to a number of cell signals (Bingle et al. 2000). A member of the Bcl-2 family, Mcl-1 also acts as a survival factor. Structurally quite similar to Bcl-2, Mcl-1 has been shown to be extremely important during human fetal development and essential for the maturation and maintenance of B and T lymphocytes (Warr and Shore 2008). In cancerous cells, Mcl-1 is primarily localized to the outer membrane of the mitochondria, where it inhibits the release of cytochrome-C in the intrinsic pathway of apoptosis (Warr and Shore 2008). It has been demonstrated that Mcl-1 is upregulated not only in blood cancers such as chronic lymphocytic lymphoma, but also in solid tumors, particularly in pancreatic cancer, testicular cancer, ovarian cancer, and melanoma (Warr and Shore 2008). Mcl-1 is required for melanoma cell

resistance to anoikis, a form of cell death induced when a cell unbinds the extracellular matrix (Boisvert-Adamo et al. 2009). In 50%-70% of melanoma cells, Sk-Mel-28 cells included, the serine threonine kinase B-RAF is mutated. This protects malignant melanoma cells from anoikis. Boisvert-Adamo et al. (2009) found that malignant cells with the B-RAF mutation and Mcl-1 knocked down had high levels of caspase-3 cleavage. Thus, Mcl-1 mediates the resistance to anoikis in mutant B-RAF melanoma cells. Further, it has been found by Song et al. (2005) that human non-small cell lung cancer (NSCLC) solid tumors have elevated Mcl-1 expression when compared with normal surrounding lung tissue. Mcl-1 was knocked down in NSCLC cells and an increase in apoptosis was observed, suggesting the gene has a central role in supporting the survival of these cancer cells. Cells expressing relatively high levels of Mcl-1 were also found to have increased viability after exposure to cytotoxic agents. Thus, having emerged as an obvious therapeutic target, chemotherapy drugs such as Sorafenib, Seliciclib, and Obatoclax have been developed to specifically target Mcl-1 (Warr and Shore 2008).

Despite these bold developments, Mcl-1 is not as simple as one would think. The gene also has the ability to undergo differential splicing. Normally transcribed, Mcl-1 has three coding exons; however, there is a variation where first and third exons are joined, excluding the central one, creating Mcl-1_{s/ΔTM} (Bingle et al. 2000). This variant actually acts as a promoter of cell death.

The fate of each cell is regulated and fought for by the interactions of apoptotic and survival proteins. In cancer cells, it is seen that survival factors are highly over-

expressed in order to overcome apoptotic signaling. This contributes to cancer's ability to overcome apoptotic signals and achieve a sort of cell immortality (Hanahan and Weinberg 2011).

2.2. Integrins can induce both anoikis and apoptosis

Integrins are transmembrane surface receptors that are responsible for endothelial cell adhesion to the extracellular matrix (ECM) by binding to fibronectin, vitronectin, laminin, and collagen (Keely 2011). If a normal cell becomes detached from the ECM, the cell undergoes apoptosis due to integrin intracellular signals (Frisch and Francis 1994) and all adherent cells require adhesion to the ECM to advance through the next cell cycle (Streuli 2009). The structure of tissues is in part due to integrin mediated cell-cell adhesion with E-cadherin (Keely 2011). The integrin adhesion sites are also the mounting points for cytoskeletal assembly, and thus control the architecture of the cell (Streuli 2009). In healthy tissue, when a cell loses its adhesion to E-cadherin and the cytoskeleton is compromised, the cell undergoes a cell death known as anoikis (Keely 2011). Jun N-terminal kinase (JNK) is increasingly phosphorylated, which activates pro-apoptotic p53 (Keely 2011; Lane 1992). Cancerous cells are able to metastasize, which requires breaking away from E-cadherins and the ECM, circumventing the normal anoikis process. Without this regulation, tumor cells have also been known to degrade the basal lamina, allowing themselves to invade connective tissue (Keely 2011).

Integrins have two non-covalent components, alpha (α) and beta (β) subunits, and are therefore called heterodimeric (Macêdo et al. 2015; Kumar 1998; Hynes 1992). Different α and β units that combine to form unique integrin units that perform specified

functions on each cell type (Brakebusch et al. 1997). Endothelial cells normally express combinations of α_1 , α_2 , α_3 , α_5 , α_6 , α_8 , α_9 , and α_v with β_1 . There are a few other known α combinations with other β subunits, namely $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Muñoz-Chápuli et al. 2004). All the various combinations of the subunits compose 24 different integrins (Macêdo et al. 2015; Pasini et al. 2009). Integrin expression is also cell type dependent, for example, $\alpha_6\beta_4$ is found on epithelial cell surfaces, but not most other cell membranes (Kumar 1998). These α and β subunits work in tandem and closely associate with growth factor receptors, among other signaling molecules (Muñoz-Chápuli et al. 2004). Integrin expression can vary greatly when comparing normal cells to cancer cells (Macêdo et al. 2015).

Integrins also carry out intracellular signal transduction (Muñoz-Chápuli et al. 2004). Intracellular signals alter the affinity of integrins to their ligands (Dedhar and Hannigan 1996). There is transfer of information into the cell from the outside, and vice versa (Brakebusch et al. 1997). Thus, integrins are an extremely important avenue for a cell to communicate with its environment and trigger signaling pathways that control a cell's actions such as mobility, proliferation, death, differentiation, and even morphology (Streuli 2009; Brakebusch et al. 1997). It makes sense, then, that different integrins are expressed on the cell membrane at specific points in the cell life cycle and development (Brakebusch et al. 1997). For example, β_1 has been found to play an instrumental role in myogenesis as well as assist cardiac muscle cell differentiation during fetal development (Brakebusch et al. 1997). Additionally, the absence of α_4 subunits has been shown to

result in the production of defective white blood cells, namely T-cells and B-cells (Hirsch et al. 1996).

Yet another important function of integrins is the ability to induce apoptosis. Integrin-dependent cell adhesion and Focal adhesion kinase (FAK) activation can modify the cytoskeleton shape and activate MAPK signaling cascade, including downstream Erk 1/2, JNK, and p38 (Saldanha-Gama et al. 2010; Giancotti and Ruoslahti 1999). Upon binding, FAK autophosphorylates Tyr³⁹⁷, which creates a binding site for Src or Fyn (Giancotti and Ruoslahti 1999).

Integrin activation also leads to nuclear translocation of NF- κ B, a survival factor also responsible for immune response (Saldanha-Gama et al. 2010). There is also another opportunity for integrin signaling to contribute to cell survival: FAK-Src complex can activate PI-3K pathway, which triggers Akt activation. Akt is a survival factor that inhibits the actions of Bad and Caspase-9 (Giancotti and Ruoslahti 1999). It seems FAK plays a role in linking connection to the ECM and survival signals. When FAK is deficient, p53 mediates anoikis signaling (Giancotti and Ruoslahti 1999).

It is no surprise, then, that integrins have been found to play a major role in cancer formation and progression (Plantefaber and Hynes 1989). The β_1 subunit and $\alpha v \beta_3$ integrin are of particular interest. The β_1 subunit has been shown to play an integral role in tumorigenesis due to its function in angiogenesis, new blood vessel growth. Bloch et al. (1997) demonstrated that VEGF (vascular endothelial growth factor) action depends on functional β_1 integrins in teratomas grown on embryonic stem cells. In the absence of β_1 , however, teratoma growth is dramatically weakened, suggesting a role in tumor

proliferation, as well (Brakebusch et al. 1997). Teratoma modifications due to the β_1 deficient cells range from increased apoptosis to insufficient angiogenesis. However, it could be that the increased level of apoptosis is simply due to damaged interaction of the cell with the ECM due to the subunit deficiency (Brakebusch et al. 1997). Still, the variation of effects on β_1 deficient teratomas suggests that the integrin subunit regulates different functions in different cell types, perhaps depending upon the α subunit interaction. This data coincides nicely with Weaver et al.'s study (1997) which found that breast tumor cells treated with β_1 antibodies and injected into mice had significantly reduced the number and size of successful tumor growth, implicating the integrin in tumor formation. This has been shown in various additional studies, including one by Zhang et al. (1995). Chinese hamster ovary (CHO) cells were transfected with a truncated version of the $\alpha_5\beta_1$ integrin, which lacked the cytoplasmic domain. This cell line, as well as CHO cells with fully functional $\alpha_5\beta_1$ integrins and CHO cells with $\alpha\nu\beta_1$ integrins were subjected to serum-free fibronectin plate attachment assays. Once the serum was removed, almost 100% of the CHO cells with truncated $\alpha_5\beta_1$ and $\alpha\nu\beta_1$ integrins showed signs of apoptosis. Less than 25% of the functional $\alpha_5\beta_1$ cells had become apoptotic within the same time period. The detection of Bcl-2, a well-known anti-apoptotic agent, was carried out via RT-qPCR. This showed that the mRNA *bcl-2* levels were elevated in the functional $\alpha_5\beta_1$ cells, indicating that $\alpha_5\beta_1$ integrin interaction with fibronectin actually suppresses apoptosis by inducing Bcl-2 upregulation.

The molecular pathways and mechanisms of integrin-triggered cell survival and apoptosis in cancer cells are still not clear, however, it is known that integrins are

involved in both tumor progression and restraint. For example, in healthy cells, adhesion to the ECM activates FAK, which translocates into the nucleus and binds directly to p53 to inhibit p53 function (Keely 2011). In normal cells, p53 is a proapoptotic cell cycle regulator. However, in many cancers, p53 is mutated and becomes a contributor to tumor survival (Keely 2011).

During melanoma development, a subset of integrins, including $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_6\beta_4$, directly activates the p38/MAPK pathway (Pasini et al. 2009). Further, $\alpha_v\beta_3$ has been thoroughly implicated in survival signaling in M21 cells, a melanoma cell line. Montgomery et al. (1994) concluded that the α_v subunit is required in dermal collagen to avoid apoptosis when the cells are deprived of any external growth factors. Physical symptoms of apoptosis were observed after only a few days α_v (-) M21 cells were placed in dermal collagen growth environment void of any external growth factors, mimicking the inner tumor mass environment. This implies that the α_v subunit is involved with survival signaling.

There is currently extensive research being done on the clinical implications of integrin antagonists as treatments for cancer. One example is Vitaxin, a monoclonal antibody that interacts with integrin $\alpha_v\beta_3$. Vitaxin is currently in Phase II clinical trials for treatment in colorectal cancers due to its ability to inhibit angiogenesis (Eda et al. 2015).

2.3. Disintegrins bind to integrins and initiate a cellular response

Disintegrins are small (low molecular weight) peptides isolated from snake venom glands in various genera of venomous snakes (Nabi et al. 2014). Disintegrins are non-enzymatic peptides that bind specifically to integrins expressed on the surface of platelets and other cells using the target sequence Arg-Gly-Asp (RGD) binding site as well as other binding motifs (Pasini et al. 2009; McLane et al. 2004). The RGD site specifically binds α_v and $\alpha_5\beta_1$ integrins, among others (Eda et al. 2015). The binding of disintegrins to integrins can potentially initiate one or multiple signal transduction pathways in order to elicit a response from the cell (Keely 2011; Pasini et al. 2009; McLane et al. 2004), possibly leading to inhibition of platelet aggregation, inhibition of cell adhesion, angiogenesis, and migration (Lucena et al. 2015).

The functionality of disintegrins depends on the motif in the hairpin loop. There are three motifs which separate the three functional classes: RGD, MLD (Met-Leu-Asp), and R/KLS (Arg/Lys-Leu-Ser). Disintegrins with the RGD motif are most common. Induced or natural variations of the arginine in the RGD motif results in a change or elimination of the biological effect the disintegrin possesses (Macêdo et al. 2015).

In addition to their functional classes mentioned above, disintegrins are also separated into structural classes. Commonly, disintegrins are derived from proteolytically processed precursors called metalloproteases (MPs), or more specifically, snake venom metalloproteases (SVMPs) (Macêdo et al. 2015). However, some may result from short coding of mRNAs, which does not have a MP domain. SVMPs are also sub-categorized into classes based on their domains. The P-I class possesses a MP

domain only. P-II disintegrins have a MP domain as well as a disintegrin-like domain. Lastly, the P-III class consists of disintegrins with a MP domain and a cysteine-rich domain following the disintegrin region (Macêdo et al. 2015).

There is excellent potential for therapeutic uses of disintegrins. *Crotalus durissus terrificus* isolated venom disintegrin caused cell death in sarcoma and leukemia (Nabi et al. 2014). Cytotoxin IIa, a disintegrin isolated from Indian cobra (*Naja naja*) venom, exhibited cytotoxicity toward Yoshida sarcoma cells as well as human breast cancer cells (MCF-7), liver cancer cells (HepG2) and lung carcinoma cells (A549). It is also a potent *in vitro* growth inhibitor of MCF-7, HepG2 and A549 cells (Macêdo et al. 2015). Yeh et al. (1998) found that accutin, another RGD-motif disintegrin, bind to $\alpha v \beta_3$ and induce apoptosis as well as reduce angiogenesis. Teklemariam et al. (2011) have shown that recombinant acocostatin induces apoptosis in HUVEC and HeLa cells while preventing cell migration of Sk-Mel-28 cells. Many more disintegrins with useful functional properties are currently being investigated.

2.4. *r-Mojastin Disintegrin*

Until recently, only a few disintegrins had been cloned and expressed while preserving biological activity. Sánchez et al. (2010) exemplified that disintegrins can, in fact, be cloned and purified utilizing chromatographic techniques to produce functional biological assays. *Crotalus scutulatus scutulatus*, the Mohave rattlesnake, contains venom that is proteolytic, hemorrhagic, and possesses disintegrin activity. Two RGD disintegrins, mojastin 1 and mojastin 2, were isolated from Mohave rattlesnake venom. Recombinant mojastin 1 (r-mojastin 1) was produced and compared with the functional

abilities of the natural mojastin 1 disintegrin. It was found that not only was the expressed r-mojastin 1 identical in amino acid sequence, but that the natural effects on adhesion, activation, and platelet aggregation was preserved, albeit less aggressively (Sánchez et al. 2010).

Shortly after, the anti-tumor properties of r-mojastin 1 were studied extensively. Human bladder carcinoma cells (T24), fibrosarcoma cells (HT-1080), and melanoma cells (SK-Mel-28) were used. It was found that r-mojastin 1 inhibited the migration of all three cell types and inhibited SK-Mel-28 cell adhesion to fibronectin. Further, r-mojastin 1 inhibited invasion of T24 and SK-Mel-28 cells *in vitro* (Lucena et al. 2011). Later, R-mojastin 1 was also found to inhibit proliferation and cell migration, and induce apoptosis in human pancreatic adenocarcinoma cells (BXP-3) (Lucena et al. 2015). Again, this demonstrates that recombinant mojastin is an extremely useful tool to assist in the study and development of anti-tumor drugs and agents.

Seoane et al. (2010) compared the functions of three short, recombinant mojastin disintegrin mutants, r-Moj-WN, r-Moj-NN, and r-Moj-DM, to find a variant that would successfully induce apoptosis in melanoma cells. It was found that r-Moj-WN and r-Moj-NN inhibit platelet aggregation and inhibit SK-Mel-28 cell adhesion to fibronectin. Recombinant r-Moj-DM was found to induce apoptosis in approximately 15% of melanoma cells. Gross morphological changes and nuclear fragmentation were observed by Hoechst staining and differential interference contrast (DIC) microscopy.

Ramos et al. (in press) produced six recombinant r-Moj-D_ mutants containing a second aspartate carboxyl immediately following the RGD binding motif. Interestingly,

it appears that each disintegrin mutant possesses different strengths of various biological activities. For example, all r-Moj-D₁ peptides inhibited platelet aggregation, but at varying potencies. Only three of the six mutants induced apoptosis of SK-Mel-28 cells: r-Moj-DL, r-Moj-DM, and r-Moj-DN. All three mutants were shown to bind the α v integrin subunit.

2.5. Emerging apoptotic and survival signals in cancer cells

Hanahan and Weinberg's (2011) last hallmark, resisting cell death, occurs because of cancer cell expression of survival factors, also known as anti-apoptotic signals. Survival factors manage to allow the cell to survive in spite of the numerous apoptotic signals that may be working to kill the cell. These signaling cascades can be triggered in multiple ways (Indran et al. 2011).

2.5.1. Bcl-2

Bcl-2 has been found at elevated levels in many types of human cancers, including blood-based (Fulda and Debatin 2006). Even when cancer cells are exposed to chemotherapy drugs, Bcl-2 can reduce or even prevent cell death in many cancer types (Reed 1998). More specifically, Bcl-2 inhibits the release of cytochrome c from the mitochondria (Green and Reed 1998). Of the Bcl-2 family of proteins, which has at least 16 members, Bcl-2 is arguably the most well known and researched (Reed 1998). For this reason, it seems necessary to include Bcl-2 in the RT-qPCR studies carried out here.

2.5.2. p53

p53 is a transcriptional regulator that translocates into the nucleus and binds to its response element (Beckerman and Prives 2010). The main role of p53 is a sequence-

specific DNA binding protein (Beckerman and Prives 2010). Over 125 protein-coding genes and noncoding RNAs are direct transcriptional targets of p53, speaking to the complexity of the p53 signaling network (Beckerman and Prives 2010). During non-stressful times, p53 is maintained at low concentrations and has very little effect on cell fate (Oren 2003). However, in response to stressors such as replication, DNA damage, and oncogene activation, p53 determines the appropriate cellular process (Beckerman and Prives 2010). Thus, p53 can literally determine whether a cell will survive or die, given the context of p53 activation, by activating other sets of either proapoptotic or prosurvival genes, depending highly on the cell type and stimulus (Soengas et al. 2001). p53 was the first tumor suppressor gene linked to apoptosis. Interestingly, p53 mutations occur in the majority of cancers, augmenting aggressiveness and chemoresistant abilities. However, these mutations are rarely seen in melanoma cases (Soengas et al. 2001).

Survival signals present in the cell may encourage p53 to lead the cell in the direction of DNA repair or cell-cycle arrest (Oren 2003). When these signals are absent or insufficient, it is likely the cell will undergo apoptosis. The molecular pathway for the activation of the two completely different sets of genes is not well known, though. It is most likely that multiple mechanisms are involved in p53 target gene selection (Oren 2003). In its pro-apoptotic state, p53 transactivates death receptor genes such as FAS and DR5 as well as a myriad of apoptotic mitochondrial proteins such as Bax (part of the Bcl protein family) (Oren 2003).

Inactivation of p53 is extremely important in the formation of cancers due to its strong tumor suppressor nature (Beckerman and Prives 2010). The simplest way this can

occur is via a mutation in p53, rendering the protein permanently active. This will also eradicate all wild-type p53 from the affected cells (Oren 2003). Otherwise, there are ample opportunities for other proteins in the p53 signaling chain to become mutated. Just one example is the *mdm2* gene, which encodes for a protein that is tightly bound to p53 in non-stressful times, rendering it inactive (Oren 2003). With the p53 signaling cascade malfunctioning, the entire Intrinsic Pathway is at risk, and apoptosis signals could be easily eluded.

Brash et al. (1991) found that 58% of a collection of samples of squamous cell carcinoma patients from New England, USA and Uppsala, Sweden had a particular type of p53 mutation. The mutation was base changes CC to TT, which is a distinctive mutation caused only by UV light. These base pair changes in the p53 gene altered the amino acid sequence, causing the invasive cancer. The sunlight-driven mutation was probably not enough to alone induce squamous cell carcinoma, but it certainly played a major role.

Mcl-1 is upregulated in many melanoma cells, probably as an adaptation to increased stress on the endoplasmic reticulum (ER) (Jin et al. 2011, Jiang et al. 2008). Jin et al. propose a novel pathway connecting p53 and Mcl-1 up-regulation. Jin and his team have found that p53 over expression leads to up-regulation of miRNA-149*, which plays a central role in targeting Mcl-1 in Mel-RM cells. The over expression of Mcl-1 stabilizes melanoma cells under ER stress. This pathway has yet to be tested in various other melanoma cell types, such as Sk-Mel- 28, Sk-Mel-2, or HT-144 cells.

2.5.3. p38 (MAPK)

p38 (or MAPK) is a highly conserved protein. Responding to a multitude of stimuli, such as growth factors, inflammatory cytokines, heat, osmotic shock, and UV light, p38 plays a central role in mediating cell stress, much like p53. It is important to note that p38 activation and action is not only dependent on stimuli, but on cell type, as well. There are many different upstream activators of p38 kinases, but they are chiefly activated by partnered kinases called MAP kinases, or MKKs.

Further, two main MKKs are known to directly activate p38: MKK3 and MKK6. Multiple transcription factors are phosphorylated by p38, notably GADD153 (growth arrest and DNA damage inducible gene 153), c-Myc, Bcl-2, and p53 (Zarubin and Han 2005; Ivanov and Ronai 2000), all of which are thought to play important roles in cancer cell survival or apoptosis. p38 has been shown to have a role in post-transcriptional regulation of inflammatory gene expression, including IL-1 β and TNF α . It is no surprise then, that p38 has been implicated in Rheumatoid arthritis, inflammatory bowel disease, and Alzheimer's disease (Zarubin and Han 2005).

In cancer, p38 functionality is complicated and not yet well understood. In some cell lines, p38 activation seems to promote cell death while in other cell lines, p38 has been shown to assist in cell survival (Zarubin and Han 2005; Ivanov and Ronai, 2000). It has been reported that p38 activation may be reduced in tumors, further indicating a possible role in apoptosis (Zarubin and Han 2005). According to Ivanov and Ronai (2000) however, in melanoma cell lines LU1205 and WM1552, p38 promotes survival. They have shown that MKK6 and p38 protect these cell lines from UV-induced apoptosis

and actually decrease death domain protein expression. Further, the inhibition of p38 lead to a marked increase (up to fourfold) of transcriptional activity by NF- κ B and approximately 3.5-fold increase in the activation of Fas (death domain) promoters. Because the p38/MAPK signaling process is necessary for the relay of extracellular signals to the nucleus, some believe that p38 and downstream proteins are overexpressed in all metastatic melanomas (Pasini et al. 2009).

A novel pathway for p38 apoptotic signaling has been proposed by Zhang et al. (2007), implicating a new gene holding a vital role in the TNF complex activation. In human umbilical vein endothelial cells (HUVEC), AIP1 or DAB2IP peptide is essential for TNF-induced formation of complex 1 (TRAF2-RIP1-DAB2IP-ASK1), ultimately leading to the activation of p38 apoptotic signaling. Mutation of the DAB2IP binding site for TNF blocks the complex 1 formation, thus halting p38 MAPK activation. In short, DAB2IP is a novel protein that seemingly contributes greatly to cell survival signaling.

2.5.4. *DAB2IP*

DAB2IP emerged in 2003 as a novel peptide with a central role in TNF- α signaling (Zhang et al. 2003; Guicciardi and Gores 2003). Also referred to as ASK1-interacting protein-1 (AIP1), it is believed that DAB2IP aids in the dissociation of ASK1 from its inhibitor, 14-3-3 in the TNF- α signaling pathway. DAB2IP possibly recruits an unknown phosphatase to ASK1, which disrupts the ASK1/14-3-3 complex (Zhang et al. 2003). In turn, the JNK or p38 MAPK pathways are activated, which leads to apoptosis or cell survival. Zhang et al. (2003) have shown that TNF- α activation induces DAB2IP

interaction with ASK1 in HUVECs. Further, DAB2IP/ASK1 complex formation was found to increase significantly when TRAF2 expression increased, but not when ASK1 concentrations increased, further supporting their proposed pathway. When DAB2IP was knocked down in HeLa cells, ASK1 and JNK activation by TNF- α was reduced five- to seven-fold. This shows the potential importance of DAB2IP to ASK1/JNK activation and pathway. Later, Zhang and researchers (2004) revised the pathway. They showed that DAB2IP remains on the cell membrane complexed with TNFR1 in endothelial cells at rest. Binding of a substrate with TNFR1 releases DAB2IP, and a signaling complex forms comprised of TRADD, RIP1, TRAF2, and DAB2IP. This pathway functions to specifically activate ASK1 and, in turn, JNK. Intriguingly, this same complex without the addition of DAB2IP leads to NF- κ B activation instead of JNK. This lead to the conclusion by Zhang et al. (2004) that DAB2IP is actually responsible for mediating a balance between JNK activation and NF- κ B.

In endothelial cells, the activation of ASK1/JNK induces intrinsic pathway apoptotic signaling (Zhang et al. 2003). Because DAB2IP enhances JNK activation, eventually leading to apoptosis, it is believed that DAB2IP acts as an apoptotic signaler in endothelial cells (Zhang et al. 2004). This is only supported by the findings that DAB2IP suppressed the expression of NF- κ B, a long established survival peptide (Zhang et al. 2004).

DAB2IP has been found to have a significant impact on prostate cancer cells, as well. For example, Duggan et al. (2007) may have found a link between a DAB2IP allele and a more aggressive prostate cancer. In another study, Xie and researchers (2009)

found that decreased expression of DAB2IP is found in prostate cancer cell lines C4-2 and PC-3. Further, Xie et al. (2009) uncovered that DAB2IP is not only pro-apoptotic under cell stress, but is also a potent cell growth inhibitor. Later, Xie and colleagues found that down regulation of DAB2IP promotes tumor proliferation and metastasis (2010). In fact, a knockdown of the gene in mice initiated the epithelial-to-mesenchymal transition (EMT), a necessary landmark for prostate tumor cells to metastasize (Xie et al. 2010). DAB2IP has even recently been recognized as a factor in chemotherapy resistance. DAB2IP knockdown prostate cancer cells showed strong resilience against several types of drugs (Wu et al. 2013). Interestingly, the opposite was found in C4-2 prostate cancer cells. Increased levels of DAB2IP restored drug sensitivity in that cell line (Wu et al. 2013).

Since DAB2IP has only begun to be researched, the status of this peptide in many other cancers, melanoma included, is unknown. Clearly this gene is a promising therapeutic target in prostate cancer, thus it is reasonable to consider that it may be a potential target in other cancers, as well.

2.5.5. *Cytosolic acetyl-CoA synthetase (ACS)*

Cytosolic acetyl-CoA synthetase (ACS) converts long-chain fatty acids into acyl-CoA during lipid metabolism (Mashima et al. 2009). This enzyme is overexpressed in many different types of cancers including glioma, colon cancer, and hepatocellular cancer. The specific role of ACS in cancer cell growth and malignancy is unknown, however, it is clear that ACS is necessary for glioma cell survival. Even more interestingly, Mashima and colleagues found that when a glioma cell line was treated

with Triacsin c, a specific ACS inhibitor, apoptosis was induced. While cytochrome c was activated, ACS inhibition did not affect levels of Bcl-2, Bcl-XL, and Bax, suggesting yet another possible non-canonical pathway of apoptosis.

Yoshii et al. (2009) also found that ACS affected tumor cell survival. Four mouse carcinoma cell lines (Lewis lung, melanoma, colon, and mammary) had higher expression levels of ACS in hypoxic conditions compared to normoxic. A knockdown of ACS showed increased tumor cell death when under hypoxia. Further, it was found that a knockdown of ACS *in vivo* slowed tumor growth significantly. ACS expression in human melanoma cells is unknown at this point.

2.5.6. Tenascin-X

In 1993, Bristow et al. described a novel extracellular matrix protein, Tenascin-X. The peptide is broken up into two segments: TNX-A and TNX-B. TNXB encodes for an extracellular matrix protein that may be capable of forming multimers. TNXB is highly expressed in fetal tissues, especially in skeletal, cardiac, and smooth muscle. Though TNXB does not contain an RGD sequence and hence cannot bind to RGD-dependent receptors (such as integrins), it may still play an important role in metastasis.

In a study by Matsumoto et al. (2001), TNX^{-/-} mice had normal development and life spans. Upon injection of B16-BL6, a melanoma cell line, TNX deficient mice experienced a much more aggressive response. The tumor grew much larger, by approximately 1.2 fold, than in normal mice. It was also found that TNX deficiency led to an up-regulation of matrix metalloproteinases (MMPs), which degrade the macromolecules of the extracellular matrix. The increased expression of MMPs

encourages rounding and metastasis of the B16-BL6 cells. In fact, pulmonary metastasis increased by 6.8-fold in TNX^{-/-} mice. This supports previous findings that as malignancy progresses, TNXB expression decreases. Fujie et al. (2009) had similar findings. In order to investigate the adhesive properties of TNX, a TNX substrate was treated with mouse fibroblast L cells. Compared to the PBS control substrate, more than half the L cells were not adhered at all and of the adhered cells, half of those were rounded and did not spread. Again, this indicates that TNX promotes detachment and rounding of cells, leading to metastasis in cancerous cells. Fujie et al. (2009) then found that TNX is a driving force of the activation of p38 MAP kinase. No activation of the kinase was found in the PBS substrate cells.

TNXB may also have important diagnostic implications. In 2009, Yuan et al. found that TNXB is expressed significantly more in malignant mesotheliomas than in ovarian and breast carcinomas. TNXB levels were even further elevated in mesotheliomal effusions, again suggesting a role in the inhibition of cellular adhesion to the extracellular matrix. Yuan et al. (2009) are even calling TNXB a “novel diagnostic marker” of malignant mesothelioma.

2.5.7. *APAF1*

In healthy cells, APAF1 is a pro-apoptotic peptide that interacts with cytochrome *c* to cleave procaspase 9 into the active form. The active caspase then activates downstream apoptotic signaling (Lowe and Lin 2000). In 1999, Soengas et al. showed that APAF1 and Caspase-9, but not p53, are necessary in controlling tumor growth. When tumor growth was induced in APAF1^{-/-} and Casp9^{-/-} mice, they had appalling

tumor volume growth when compared with both wild type and p53^{-/-} mice. According to Soengas et al. (2001), no mutations in *APAF1* have been described as of yet, but in metastatic melanoma, a highly aggressive and chemoresistant cancer, *APAF1* is often inactivated due to loss of heterozygosity and gene silencing by methylation. This loss of APAF1 leads to a disabling of the p53 apoptotic signaling cascade, thus giving the cells power to survive even when treated with doxorubicin, a very common chemotherapy drug. In Sk-Mel-28 cells, APAF1 was expressed in intermediate amounts (15-30%) relative to the melanocyte control. In many SK-Mel cell lines, there was zero expression of APAF1. This contributes immensely to the destructive nature of melanoma and the failure of treatment with traditional chemotherapy drugs. Soengas' (2001) conclusions correspond nicely with Robles' et al. (2001) findings that p53 directly induces APAF1 gene expression in lymphoblastoid cells responding to stress and, eventually, undergoing apoptosis. Interestingly, Robles also found that functional p53 is necessary for DNA damage-induced expression of APAF1 and that p53 can actually transcriptionally activate *APAF1*. They have shown that APAF1 is an essential player in the p53 apoptotic signaling system.

2.5.8. *c-myc*

c-myc is an oncogene that can potentially induce apoptosis in human cancers. It is estimated that 70,000 cancer deaths per year in the US are associated with *c-myc* mutations (Dang 1999). In healthy cells, c-Myc expression encourages proliferation, but can also induce apoptosis with help from Bcl-2 (Lowe and Lin 2000). Bcl-2 inhibits c-Myc mediated apoptosis in some cells, but this has no effect on proliferative ability of c-

Myc (Hoffman and Liebermann 1998). Oddly though, overexpression of c-Myc induces pro-apoptotic Bcl-2 (plus Bcl-2 family members Bax and Bak) independent of p53 activation (Hoffman and Liebermann 2008). Because Bax or Bak is required for apoptosis signaling via the intrinsic pathway, this means that c-Myc expression helps govern both intrinsic and extrinsic apoptotic pathways. Essentially, it seems that if apoptosis is occurring using any canonical pathway, one could expect c-Myc to be expressed.

Many types of cancers show amplification in c-myc expression, including lung, breast, and rare cases of colon (Dang and Boxer 2001). c-Myc is a long standing cell growth mediator, but when interacting with p53 or ARF, becomes a pro-apoptotic signal. It was shown that ARF and p53 were triggered by ectopic c-Myc expression in murine embryonic fibroblasts. It does seem that p53 is a necessary component to the apoptotic pathway, and that c-Myc affects the transcription of other genes that participate in the apoptotic signaling pathway, such as ornithine decarboxylase, ODC.

In most colorectal cancers, the tumor suppressor gene adenomatous polyposis coli gene (APC) is rendered inactive. He et al. (1998) propose that c-Myc is highly overexpressed due to this inactivation, leading to increased β -catenin/Tcf-4 activity. In turn, this *c-myc* expression elevation enhances tumor growth.

2.5.9. Lymphotoxin α (LT α , originally named tumor necrosis factor- β , TNF- β)

Lymphotoxin α (LT α , originally named tumor necrosis factor- β , or TNF- β) is a cytokine that binds and signals through receptors in the tumor necrosis factor family receptors, TNFR1 and TNFR2 (Etemadi et al. 2013). The TNF “superfamily” aids in

regulating many cellular processes, including cell death. Historically, it was thought that LT α was a weaker cell death inducer when bound to TNFR1 when compared to its pro-apoptotic brother, TNF. However, Etemadi et al. (2013) have concluded that both cytokines have equal potency to induce cell death and MAPK/p38 pathway activation.

TNFR1 and TNFR2 receptors have been found on most normal and transformed cells, including endothelial cells and immune cells. LT α seems to be most active with natural killer cells, B cells, and T cells (Aggarwal 2003). Similarly to most of the signaling proteins discussed thus far, the TNF family has multiple capabilities, and can initiate survival and death, depending on a myriad of conditions (Aggarwal 2003).

3. Methods

3.1. Recombinant r-Moj-DM expression

Seoane et al. first produced the recombinant (r) Moj-DM mutant in 2010. To grow cells containing the r-Moj-DM mutant, a 1.5 mL tube of the Moj-DM plasmid containing BL21 cells was thawed and two LB agar plates with ampicillin were streaked with the cell solution. The plates were incubated for approximately 24 h at 37° C. A single colony from each plate was chosen, and two tubes of 5 mL of LB broth with 50 mg/mL ampicillin were inoculated with one colony each. The tubes were incubated for approximately 18 h at 37° C with gentle agitation. Once cell growth was established, 2.5 mL was transferred to 500 mL of prepared, pre-warmed 2xYT broth with 50 mg/mL ampicillin. To induce expression of the mutant r-Moj-DM, 1.0 mL of 1M IPTG was added to each flask. After adequate growth was verified by OD₆₀₀ reading, cells were pelleted by centrifugation.

3.2. Recombinant r-Moj-DM Isolation

E. coli cell pellets were resuspended in PBS buffer and lysed using sonication. Separation of r-Moj-DM was performed using glutathione sepharose (GST) bead pull-down technique. The lysate was incubated with GST beads for approximately 16 hours at 4° C without agitation. The peptide was cleaved from the beads using a thrombin enzyme capture kit (Novagen). The volume of biotinylated thrombin used was determined using the formula listed in the manufacturer user guide, which is based on the estimated protein concentration ascertained by Bradford assay. The presence of purified

peptide was confirmed using NuPAGE[®] Novex[®] SDS-PAGE Bis-Tris Mini Gel (Invitrogen). Concentration of r-Moj-DM was determined using a Bradford assay.

3.3. SK-Mel-28 Cell Culture

SK-Mel-28 cells were ordered from ATCC (HTB-72). Cells were cultured in T75 flasks in Eagle's Minimum Essential Media, 10% fetal bovine serum and 5% HyClone antibacterial and antimicrobial, at 37° C, and 5% CO₂.

3.4. Live SK-Mel-28 β_1 Integrin Surface Staining

Cells were cultured to confluency as described above, then trypsinized and transferred to 6-well plates. Five hundred thousand cells were seeded into each well. The cells were immediately treated with the following r-Moj-DM concentrations: 20 nM, 40 nM, 60 nM, 80 nM, 100 nM, and 120 nM. These wells were seeded and treated in triplicate.

After a 5 min incubation period at 37° C, cells were incubated for 10 min with 1% rabbit blocking serum in PBS solution at room temperature. FITC-labeled antibodies for integrin β_1 were added and incubated for 5 min at 37° C, then in the dark for 30 additional min. Cells were then fixed with 1% formaldehyde and detected using a Becton Dickinson FACScan flow cytometer. This protocol was previously described in Juliano et al. (1996).

3.5. RNA Isolation

Approximately 500,000 SK-Mel-28 cells were seeded into each well of an eight-well plate and incubated for approximately 24 h. They were then treated with 5 μ M r-Moj-DM and incubated for 8-16 h, in 2 h intervals. Two wells were left untreated and

used as controls. Cells were harvested using cell scrapers and cell pellets were stored at -80° C until RNA isolation. RNA was isolated using Qiagen® RNeasy® Mini kit.

Immediately following isolation procedure, the concentration was estimated using a NanoDrop. To remove any genomic DNA contamination, all RNA was treated with Ambion® DNA-free™, exactly as described in the kit user guide.

3.6. Reverse Transcription (RT)

To perform reverse transcription (RT), approximately 50 ng of RNA was incubated for 10 min at 70°C and immediately put on pre-refrigerated cold block for 5 min. Table 1 shows master mix components used to perform RT.

Table 1. Components and volumes used in RT master mix.

Reagent (Starting Concentration)	Volume/sample (µL)	Final Concentration
First-Strand Buffer, 5x	4.0	1x
MgCl ₂ (50 mM)	2.0	5 mM
dNTPs (10 mM)	2.0	1 mM
DTT (100 mM)	2.0	10 mM
RNase inhibitor (40 U/µL)	1.0	2 U/µL
Random nonamer (100 µM)	5.0	25 µM
Superscript® II RT (200 U/µL)	0.5	5 U/µL
FINAL VOLUME	16.5	

The total volume per sample was brought to 20.0 µL with nuclease-free water and/or RNA, as shown in Table 2.

Table 2. RT reaction RNA concentrations and volumes.

Sample ID	Original RNA Concentration (ng)	Vol. RNA Added to Reaction (μL)	Total RNA in RT Reaction (ng)	Vol. H ₂ O Added to RT Reaction (μL)
Untreated 1	18.4	3.0	55.2	0.5
Untreated 2	41.4	1.5	62.1	2.0
8 Hours	32.6	1.7	55.4	1.8
10 Hours	37.7	1.5	56.6	2.0
12 Hours	22.7	2.5	56.8	1.0
14 Hours	30.0	1.7	51.0	1.8
16 Hours	23.2	2.5	58.0	1.0
18 Hours	13.6	3.5	47.6	0.0

Thermocycling was completed using Applied Biosystems™ Veriti Thermal Cycler (Table 3).

Table 3. Thermocycling parameters used for RT reaction.

Step Type (Hold or Cycle)	Temperature (° C)	Time (minutes)
Hold	20	10
Hold	42	45
Hold	99	3
Hold	0	∞

3.7. Real Time PCR Primer Design

cDNA primers were designed using Primer Express software by ABI, with the exception of Mcl-1 primers, which were taken from Desjobert et al. (2011). cDNA sequences were obtained on the Ensembl website (www.ensembl.org). Primer pairs were checked for specificity using NCBI BLAST® tool before ordering from Integrated DNA Technologies (IDT) and validated by conventional PCR and gel electrophoresis on 4% agarose gel to ascertain optimal annealing temperatures (T_m) for each primer pair.

Table 4. Both primer sequences for each gene investigated (with the exception of Bcl-2, explained below).

Gene of Interest	Forward Primer	Reverse Primer	Product Size
β -actin (reference gene)	GATGAGATTGGCATGGCTTT	CACCTTCACCGTTCCAGTTT	100bp
DAB2IP	GGCCAGGACTACTGCTTCGA	GGAGGTTCTCCATCCACTTATCC	97bp
ACS	TCAAAGGCAGTCGGCTCAAC	CTTTGCCTTCCCTTGCAATG	90bp
c-Myc	CTGCTGGGAGGAGACATGGT	CCACATACAGTCCTGGATGATGA	93bp
APAF1	GGCATTCTGTTGTCTCTTCTTC	TACTCCACCTTCACACAGGACTGT	90bp
Mcl-1	AGCTGCATCGAACCATTAGC	TCCTGATGCCACCTTCTAGG	136bp
TNF- β	GTTCCCCAGGGACCTCTCTCT	ACATGGGCTACAGGCTTGTC	90bp
TNX-B	GGAAGCCCCTCTTCTCAGCTT	AGGTTAATGCGGTGGGTGAA	80bp
p53	CCCCTCCTCAGCATCTTATCC	GCACCACCACACTATGTCGAA	90bp
p38	TGGCTCTGTGTGTGCTGCTT	TTCGCATGAATGATGGACTGA	93bp

To analyze Bcl-2, a different method was used due to difficulty designing acceptable primers. A TaqMan[®] Gene Expression Assay (assay ID Hs00608023_m1) from Life Technologies was purchased. The corresponding β -actin assay was purchased (assay ID Hs99999903_m1) and used as a control or reference gene. Bcl-2, along with Mcl-1, ACS, c-myc, and TNX-B are survival factors while the others (DAB2IP, APAF1, TNF- β , p53, and p38) are known to be pro-apoptotic. β -actin was used as a reference gene for each gene tested.

3.8. Real Time (Quantitative) PCR

A master mix of the following components was made, as shown in the table below.

Table 5. Components and volumes of SYBR Green master mix used for real time PCR

Reagent Added	Volume Added (μL) per Reaction
SYBR Green iQ SuperMix	7.5
Nuclease free H ₂ O	4.5
Forward primer	1.0
Reverse primer	1.0

Fourteen microliters of master mix was pipetted into each well of a 96 well plate, following the layout in Figure 4 below. One microliter of cDNA from section 3.1.1 was added to the appropriate wells, following the layout in Figure 4 below.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	UT	UT	UT	no RT UT	GOI NTC		UT	UT	UT	no RT UT	b-actin NTC		Gene of Interest
B	8	8	8	no RT 8			8	8	8	no RT 8			
C	10	10	10	no RT 10			10	10	10	no RT 10			
D	12	12	12	no RT 12			12	12	12	no RT 12			
E	14	14	14	no RT 14			14	14	14	no RT 14			b-actin (+ control)
F	16	16	16	no RT 16			16	16	16	no RT 16			
G	18	18	18	no RT 18			18	18	18	no RT 18			
H													

Figure 1. Layout example of 96-well plate for each gene of interest (GOI) tested.

An ABI 7500 Quantitative PCR System was used for qPCR analysis. The thermocycling program is shown below. Data was taken for 40 PCR cycles.

Table 6. Real Time (quantitative) Thermocycling Parameters

Step Type (Hold or Cycle)	Temperature (°C)	Time seconds)
Hold	95	180
Cycle, Step 1	95	10
Cycle, Step 2	60	30
Cycle, Step 3	72	30

Following the above program, the melt curve analysis program was run. This was comprised of 80 cycles of $55 + 0.5^0$ C per cycle.

3.9. Real Time PCR Data Analysis

Threshold cycle number (Ct) was automatically determined by Applied Biosystem software, but was checked for accuracy. qPCR data was analyzed using the comparative Ct method as described by Schmittgen and Livak (2008). The exact formula to ascertain the $\Delta\Delta C_t$ is below.

$$\Delta\Delta C_t = (C_{t(\text{target, untreated})} - C_{t(\text{reference, untreated})}) - (C_{t(\text{target, treated})} - C_{t(\text{reference, treated})})$$

Fold difference was calculated as $2^{-\Delta\Delta C_t}$.

4. Results

4.1. Expression of mutant *r-Moj-DM*

r-Moj-DM was successfully isolated and purified, then visualized by SDS-PAGE gel (Fig. 2). A single band of ~8.2 kDa was observed, as shown in Figure 2.

Figure 2. Purified *r-Moj-DM*. Lanes 1-3: *r-Moj-DM* (~8.2 kDa). Lane 5: Invitrogen Protein Molecular Weight Standard.

4.2. Primer Design and *T_m* Optimization

All the primers for the genes of interest, with the exception of *Bcl-2*, were successfully designed and the annealing temperatures (*T_m*s) were determined. Primers were designed and tested using conventional, gradient PCR to ascertain the optimal *T_m* for each primer pair. PCR products were separated on a 4% agarose gel (Fig. 3). Primer

pairs with optimal Tms showed a single band, signifying that no nonspecific products were amplified.

Figure 3. An example photo of a gradient gel run with 25bp DNA marker. Gel shows PCR products of the following: Lane M: 25bp ladder; Lane 1: p38 at 62° C Tm; Lane 2: TNF-B at 62° C Tm; Lane 3: TNX-B at 62° C Tm; Lane 4: ACS at 63° C Tm; Lane 5: Mcl-1 at 63° C Tm, Lane 6: p38 at 63° C Tm, Lane 7: p53 at 63° C Tm, Lane 8: TNF-B at 63° C Tm, Lane 9: TNX-B at 63° C Tm, Lane 10: Blank. Lanes 6 and 7 show single bands.

4.3. Binding Analysis of SK-Mel-28 Staining of β_1 Integrins

In order to confirm r-Moj-DM binds to the β_1 integrin, SK-Mel-28 cells were treated with varying concentrations of r-Moj-DM and live stained. Cells were analyzed using a Becton Dickinson FACSscan flow cytometer and analyzed using Cell QuestPro and FloJo software. The presence of β_1 integrins on SK-Mel-28's surface was previously confirmed by Seoane et al. (2010). β_1 integrin staining revealed FITC decreases in relation to the untreated cells, as shown in Figure 4 below.

Figure 4. r-Moj-DM binds to the β_1 integrin subunit of SK-Mel-28 cells. Flow cytometry and FITC-conjugated anti- β_1 antibodies were used to detect β_1 expression on untreated SK-Mel-28 cells, and cells treated with r-Moj-DM at 20 nM, 40 nM, or 60 nM. β_1 integrin detection on untreated cells (*).

4.4. Real Time qPCR Gene Expression Analysis

A total of ten genes, a combination of survival factors and pro-apoptotic factors, were tested using SYBR Green or TaqMan RT-qPCR (Figure 5).

Figure 5. (A) Amplification plot of c-myc and β -actin (control) expression exported from data. All other gene amplification plots looked similar. (B) Melt curve exported from data showing melting temperature of c-myc and β -actin (control). Nonspecific noise is from wells containing no-RT controls and NTCs. All other melt curve plots looked similar.

For each time point, the $\Delta\Delta C_t$ and fold difference was calculated as described in Methods (3.9) to determine any differences in expression of any gene over treatment time. The findings were then separated according to pro-apoptotic or survival factor family.

A total of ten genes were tested. Of the five survival factors, Bcl-2, TNX-B, Mcl-1, and ACS showed no significant changes in expression (under 1.9-fold change) when compared with the untreated cells (Figure 6). c-Myc, however, was found to be downregulated by approximately 13-fold after 8 hours of treatment (Figure 7).

Five total pro-apoptotic genes were also tested. Of this group, DAB2IP, c-Myc,

and APAF1 showed no significant changes (under 1.9-fold change) in expression when compared with the untreated cells (Figure 6).

Figure 6. Low expression of survival and pro-apoptotic genes after treatment with r-Moj-DM. Black bars denote pro-apoptotic genes and grey bars show survival genes.

p53 showed an increase in expression ranging from 5-fold to 8-fold over the 8 to 18 hour treatment period. TNF- β expression increased 33-fold after 8 hours and 81-fold after 18 hours of treatment (Figure 8).

Figure 7. Oncogene c-Myc was downregulated after 8 hours of treatment with r-Moj-DM.

Figure 8. Pro-apoptotic genes p53 and TNF-β are overexpressed after 8 hours of treatment with r-Moj-DM.

5. Discussion

This study was completed with two objectives: to determine if r-Moj-DM binds to the β_1 integrin, and to determine which potential gene targets that are down- or upregulated as a result of r-Moj-DM 8-18 hours after treatment.

5.1. *Live SK-Mel-28 β_1 Integrin Surface Staining*

Seoane et al. (2010) determined that SK-Mel-28 cells express $\alpha v \beta_3$ and the integrin subunit β_1 on its cell surface. Ramos et al. (in press) present evidence indicating that r-Moj-DM binds to the $\alpha v \beta_3$ receptor, but not very efficiently. The authors hypothesized that r-Moj-DM could exert some its activity through $\alpha_6 \beta_1$, an integrin expressed on SK-Mel-28. The integrin subunit β_1 has been implicated in tumor invasion and it is expressed on metastatic melanoma cells (Mizejewski 1999). Here, I present findings provide support to the hypothesis presented in Ramos et al. (in press), as r-Moj-DM binds to the β_1 subunit. It remains to be determined if some of the apoptotic activity exerted by r-Moj-DM is activated by its binding to the β_1 or α_6 subunits.

At least 26 lines of melanoma cells exist (ATCC 2016), each potentially having different integrin expression patterns. It is imperative to study integrin expression on all those cell lines and study their role in survival and apoptosis to better develop targeted therapies. Integrins show much promise for alternative treatment options for a wide variety of cancer patients (Eda et al. 2015; Nabi et al. 2014).

5.2. *Real Time (Quantitative) PCR*

In healthy cells, p53 initiates the apoptotic process when environmental stress or DNA abnormalities are present (Beckerman and Prives 2010; Soengas et al. 2001).

Many types of cancer cells have mutations in p53, attesting to the immortal and aggressive nature of these cancers. In melanomas, however, this particular mutation is not common and p53 is wild type in SK-Mel-28 cells (Soengas and Lowe 2003). p53 is upregulated in SK-Mel-28 cells after treatment with the snake venom jararhagin (Klein et al. 2011). Further, it was found that chemotherapy-induced apoptosis is p53-dependent in melanoma cells (Li et al. 2000; Li et al. 1998). This supports our current findings of upregulation of p53 after treatment with r-Moj-DM.

c-Myc is a known survival factor or oncogene; thus it makes sense that while undergoing apoptosis, a downregulation would be seen (Evan and Vousden 2001). Many contradictory results have been found concerning the link of melanoma growth to c-Myc expression (Kraehn et al. 2001). While its expression is tightly controlled in normal cells, it is often deregulated in tumor cells (Evan and Vousden 2001). One study by Kraehn et al. (2001) found that the ratio of expression of c-Myc to the centromere of chromosome 8 was 1.0, indicating no upregulation is present in this cell type. According to Zhuang (2008), c-Myc levels in melanoma cell lines show a 3- to 20-fold increase when compared with normal melanocytes. Further, Zhuang (2008) proposes even $\geq 70\%$ decrease of c-Myc expression could be sufficient to send the cell into senescence. It would be interesting to study the effects of r-Moj-DM on c-Myc directly to determine whether SK-Mel-28 cells are undergoing apoptosis or senescence. A knockdown of c-Myc in SK-Mel-28 cells could also offer some information regarding the exact role in some SK-Mel-28 cell survival after treatment with r-Moj-DM. More testing needs to be completed to determine a more clear role of c-Myc in this interaction.

LT α /TNF- β is a cytokine that binds and signals through receptors in the tumor necrosis factor family receptors, TNFR1 and TNFR2 (Etemadi et al. 2013). The TNF superfamily aids in regulating many cellular processes such as cell death by a variety of pathways. My findings suggest the potential involvement of the p53 (intrinsic) signaling apoptotic process in r-Moj-DM treated SK-Mel-28 cells, possibly crossing over to the p53 intrinsic pathway from the TNF- β /TNFR1 (extrinsic) binding complex. However, more studies need to be completed, such as a knockdown of TNF- β or TNFR1. Also helpful in drawing a full picture of the molecular process induced by r-Moj-DM would be a full apoptotic and survival gene analysis by RNA sequencing after 8 hours of r-Moj-DM treatment. Further, it would be interesting to see if caspases are still activated despite the absence of APAF1 upregulation, as was found by Chen et al. (2001). This would also implicate a crossover to the intrinsic pathway independent of APAF1 activation, possibly via caspase 8.

Previous analysis of SK-Mel-28 cells treated with r-Moj-DM found another set of genes to be affected after a short treatment (1-6 hours) (unpublished data). Here, we propose a new model of apoptosis in SK-Mel-28 cells including one set of genes that are upregulated early after treatment, and another set that is upregulated later (8 hours) after treatment (Fig. 9). TNXIP-1 is a known pro-apoptotic gene that was shown to be upregulated after 6 hours of treatment with r-Moj-DM, while survival factors IGFBP-3, Prune-2, and TGF β -1 were found to be downregulated.

Figure 9. Proposed model of early and late response genes after treatment with disintegrin r-Moj-DM on SK-Mel-28 cells. After 6 hours of treatment, TXNP-1 was highly upregulated while two other known pro-apoptotic genes, BNIP-3 and PTPSF, were downregulated. Survival factors IGFBP-3, Prune-2, and TGFβ-1 were downregulated 6 hours after treatment. After 8 hours of treatment, c-Myc was downregulated.

Bcl-2 is a known potent survival factor, and physically blocks apoptotic signaling proteins by directly interacting with and sequestering them (Indran et al. 2011; Reed 1998). The overexpression of Bcl-2 has been shown to halt apoptosis in certain cancer cells. In my study, Bcl-2 did not show high levels (under 1.4-fold change) of expression in SK-Mel-28 cells. However, these findings are not surprising. Malignant melanoma cells, including the SK-Mel-28 cell line, also showed reduced Bcl-2 expression levels (Placzek et al. 2010; Tang et al. 1998). One study by Chen et al. (2001) revealed Bcl-2

highly upregulated in SK-Mel-28 cells 36 hours after being treated with a polyamine analogue. It is possible that I could have found similar results if the time points of my study been expanded beyond 18 hours. It is important to note that the karyotype of SK-Mel-28 cells is complex: a 4n-hypotetraploid (Reinhold 2012). This should also be considered when comparing to other cell line expression changes as missing chromosomes and rearrangements could effect expression patters.

The expression of apoptotic and survival proteins seem to vary not only with cell types, but also with the stage of progression the cancer cell is in. For example, Mcl-1 expression is found in 70-80% of normal skin cells, but 60% of small (under 1 mm in size) primary melanomas. In larger melanomas (over 1 mm in size), Mcl-1 expression swells to 95%. When these cells metastasize, 100% show Mcl-1 expression (Hartman and Czyz 2013; Placzek et al. 2010). This pattern makes sense as Mcl-1 protects cells from anoikis (Hartman and Czyz 2013).

It is shown here and in previous studies that r-Moj-DM has great potential as a SK-Mel-28 fighting agent. The complexity of the molecular pathways involved in apoptosis and cell survival are complex, sometimes contradictory, and varies with every cell type and stage of cell growth, particularly in cancer. It is imperative that more research be done to elucidate utilized survival and apoptotic pathways in different cancer cells to develop more effective means for patients' treatment.

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