RNA sequence analyses of r-Moj-DM treated cells: TXNIP is required to induce apoptosis of SK-Mel-28

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Abstract

RNA sequencing of untreated and r-Moj-DM treated SK-Mel-28 cells was performed after 6 h, to begin unraveling the apoptotic pathway induced by r-Moj-DM. Bioinformatic analyses of RNA sequencing data yielded 40 genes that were differentially expressed. Nine genes were upregulated and 31 were downregulated. qRT-PCR was used to validate differential expression of 13 genes with known survival or apoptotic-inducing activities. Expression of BNiP3, IGFBP3, PTPSF, Prune 2, TGF-β, and TXNIP were compared from cells treated with r-Moj-DN (a strong apoptotic inducer) or r-Moj-DA (a non-apoptotic inducer) for 1 h, 2 h, 4 h, and 6 h after treatment. Our results demonstrate that significant differences in expression are only detected after 4 h of treatment. In addition, expression of TXNIP (an apoptotic inducer) remains elevated at 4 h and 6 h only in r-Moj-DN treated cells. Based on the consistency of elevated TXNIP expression, we further studied TXNIP as a novel target of disintegrin activation. Confocal microscopy of anti-TXNIP stained SK-Mel-28 cells suggests nuclear localization of TXNIP after r-Moj-DM treatment. A stable TXNIP knockdown SK-Mel-28 cell line was produced to test TXNIP’s role in the apoptotic induction by r-Moj-DM. High cell viability (74.3% ± 9.1) was obtained after r-Moj-DM treatment of TXNIP knocked down SK-Mel-28 cells, compared to 34% ± 0.187 for untransduced cells. These results suggest that TXNIP is required early in the apoptotic-inducing pathway resulting from r-Moj-DM binding to the αv integrin subunit.

1. Introduction

Apoptosis can result from the detachment of cells from the extracellular matrix or adjacent cells in tissues (Gilmore, 2005). Cell shrinkage and blebbing, chromatin condensation, chromosomal fragmentation, and formation of apoptotic bodies are morphological characteristics associated with apoptosis (Lockshin and Zakeri, 2004). When normal cells detach, an extrinsic pathway of apoptosis is initiated (Gilmore, 2005). However, cancer cells can efficiently avoid apoptosis by a variety of mechanisms (Hanahan and Weinberg, 2011). Because of this apoptotic avoidance, apoptotic induction of cancer cells has been a target for the development of anti-cancer treatments (Meng et al., 2006).

Disintegrins induce apoptosis of normal and cancer cell lines by antagonizing integrins (Ramos et al., 2016; Huveneers et al., 2007; Barja-Fidalgo et al., 2005). Examples of apoptotic inducing disintegrins include: r-Moj-DL (Ramos et al., 2016), r-Moj-DM (Ramos et al., 2016), DisBa-01 (Ribeiro et al., 2014), r-Rub (Carey et al., 2012), vicrostatin (Minea et al., 2010), agkistin-s (Ren et al., 2006), rhodostomin (Wu et al., 2003), salmosin (Hong et al., 2003), contortrostatin (Zhou et al., 1999), and accutin (Yeh et al., 1998).

The complete elucidation of signal transduction pathways resulting in apoptotic induction after disintegrin treatment remains an important, mostly uncharted line of research. Moreover, some cytoplasmic and nuclear targets have been identified. FAK, JNK, ERK, and PI-3K are important targets of integrin-induced apoptosis (Chiarugi and Giannoni, 2008) and are also common targets of several disintegrins (Park et al., 2012; Selistre-de-Araujo et al., 2010). For instance, echistatin treatment results in the activation of caspase-3 and the Tyr phosphorylation inhibition of FAK (Alimenti et al., 2004). Rhodostomin inhibits the MAPK pathway by inhibiting the phosphorylation of p38, JNK, and ERK (Hsu et al., 2010, 2016). Other disintegrins inhibit NF-κB (Jain and Kumar, 2012), over-express TGF-β (Ribeiro et al., 2014) or TNF-α (Calderon et al., 2014), or suppress IL-8 expression (Kim et al., 2007).
We have previously demonstrated that r-Moj-DM induces apoptosis of SK-Mel-28 cells by antagonizing the αv integrin subunit (Ramos et al., 2016). There are two distinct recombinant mojastin disintegrins, one that has a 10 amino acid truncation at the amino terminus (r-Moj-WN) and the other one with a full-length sequence (r-mojastin-1). The first recombinant version (r-Moj-WN) was originally derived from a truncated cDNA clone that was synthesized from mRNA isolated from a *Crotalus scutulatus scutulatus* (Mohave) rattlesnake venom gland (Soto et al., 2007). The Mohave venom contains two disintegrin isoforms named mojastin 1 and 2 (Sanchez et al., 2006). Both mojastins inhibit platelet aggregation and T24 cell binding to fibronectin. Recombinant mojastin 1 disintegrin also inhibits metastasis and tumor colonization (Lucena et al., 2011). The recombinant r-Moj-DM contains two mutations in the binding loop (WN to DM) making this disintegrin different than r-mojastin-1 (Seoane et al., 2010).

Our present work aims to unravel the signal transduction pathway that is activated upon r-Moj-DM treatment. Instead of examining the expression of known pro-apoptotic or survival factors, we identified the expression of gene targets using RNA sequencing after 6 h of r-Moj-DM treatment of SK-Mel-28 cells. Several insufficiently studied gene targets were identified. In the present work, we demonstrate that TXNIP, an apoptotic inducer protein linked to endoplasmic reticulum (ER) damage (Lerner et al., 2012; Osłowski et al., 2012) and sugar metabolism (Ji et al., 2016; Shen et al., 2015), is required for the induction of apoptosis of SK-Mel-28 after r-Moj-DM treatment.

2. Materials and methods

2.1. Expression and purification of r-Moj-DA, r-Moj-DM, r-Moj-DN, and GST-Moj-DM peptides

Expression and purification of r-Moj-DA, r-Moj-DM, and r-Moj-DN peptides were performed with a method described earlier (Ramos et al., 2016). GST-Moj-DM peptides used in confocal microscopy experiments were expressed and purified with the method described by Seoane et al. (2010).

2.2. Cell culture conditions

SK-Mel-28 cells were grown in Eagle’s minimum essential medium (ATCC). Media was supplemented with 10% fetal bovine serum (FBS) and penicillin-100 (IU/mL), streptomycin (0.1 mg/mL), and amphotericin B (0.25 μg/mL). Cells were grown at 37°C in a humidified incubator with 5% CO₂.

2.3. RNA isolation and RNA sequencing

One million SK-Mel 28 cells were treated with r-Moj-DA or r-Moj-DN for 1 h, 2 h, 4 h, and 6 h. Total RNA was extracted from each sample and purified using the method described in section 2.1. The isolated RNA was reverse transcribed at 42°C for 15 min using Quantscript Reverse Transcriptase (Qiagen). Reactions were prepared for each sample cDNA with correlating primers. Sample reactions containing 2.5 μL of cDNA and 1.0 μM of forward and reverse primers (Supplemental Table 1) were prepared in triplicate wells with the use of Quantitect SYBR Green PCR kit (Qiagen). The PCR conditions were 1 cycle of 95°C for 5 min, followed by 40 cycles of amplification (95°C for 10 s and 60°C for 30 s), and a final extension cycle at 95°C for 15 s and 60°C for 1 min. A melting curve was used to verify the presence of one specific peak for the gene. Average Ct was used to calculate relative fold change. Normalization of the data was performed by the use an endogenous control, HPRT primers (forward: ATGACCAGTCAACAGGGAC, and reverse: GGTCTTTTCACCCAGGAAC), and an untreated control sample.

2.4. Bioinformatics analyses

Sequencing results (reads) were analyzed using the software tools tophat (Trapnell et al., 2009), bowtie (Langmead et al., 2009), and cufflinks (Roberts et al., 2011). All mapped reads were analyzed using cufflinks to identify transcripts and the FPKM (Fragments Per Kilobase of transcript per Million) was then calculated. Differential expression levels between treated and control samples were analyzed using the software tool cuffdiff (Roberts et al., 2011).

2.5. Validation of RNA sequencing results using quantitative RT-PCR

Three independent cell cultures were treated with r-Moj-DM for 6 h and the total RNA isolated as described in the methods section 2.3. The isolated RNA was reverse transcribed at 42°C for 15 min using Quantscript Reverse Transcriptase (Qiagen). Reactions were prepared for each sample cDNA with correlating primers. Sample reactions containing 2.5 μL of cDNA and 1.0 μM of forward and reverse primers (Supplemental Table 1) were prepared in triplicate wells with the use of Quantitect SYBR Green PCR kit (Qiagen). The PCR conditions were 1 cycle of 95°C for 5 min, followed by 40 cycles of amplification (95°C for 10 s and 60°C for 30 s), and a final extension cycle at 95°C for 15 s and 60°C for 1 min. A melting curve was used to verify the presence of one specific peak for the gene. Average Ct was used to calculate relative fold change. Normalization of the data was performed by the use an endogenous control, HPRT primers (forward: ATGACCAGTCAACAGGGAC, and reverse: GGTCTTTTCACCCAGGAAC), and an untreated control sample.

2.6. Detection of six apoptotic gene expression by qPCR after r-Moj-DA or r-Moj-DN peptide treatment

One million SK-Mel 28 cells were treated with r-Moj-DA or r-Moj-DN for 6 h and the total RNA isolated as described in the methods section 2.3. The isolated RNA was reverse transcribed at 42°C for 15 min using Quantscript Reverse Transcriptase (Qiagen). Reactions were prepared for each sample cDNA with correlating primers as described in the methods section 2.5.

2.7. TXNIP cellular localization using confocal microscopy

Two hundred thousand SK-Mel-28 cells were seeded onto tissue culture microscope slides containing 2 mL of media. Cells were incubated with 3 μM of GST-Moj-DM or with 1X PBS for 24 h. Then cells were washed with warm 1X PBS and fixed using 3% EM grade formaldehyde for 1 h. This was followed by a 10 min incubation with 1 mL of 0.2% triton x-100, and then a 20 min incubation with 1 mL of goat serum. Three slides were incubated with anti-GST IgG goat antibody conjugated to DyLight 650 (ab117497) at a 1:200 dilution, and three slides were incubated with anti-VDUP1 (TXNIP) (H-2) mouse IgG (sc-271328), at a 1:1000 dilution, overnight at 4°C with constant shaking. After this incubation, the slides were rinsed with warm 1X PBS. Rabbit anti-mouse IgG conjugated to FITC (diluted at 1:1000, sc-358946) was added to the slides that were previously incubated with the anti-VDUP (TXNIP) antibody. Cells then were incubated for 45 min (in the dark) at room temperature with constant shaking. The secondary antibody excess was removed with three washes of 1X PBS. Cells were mounted and their nuclei stained with Vectashield mounting medium with DAPI. Slides were viewed at 100X, oil immersion with a confocal Zeiss LSM 700 microscope.

2.8. TXNIP gene expression knockdown

Inhibition of TXNIP expression was performed in SK-Mel-28 cells using VDUP (TXNIP) shRNA (h) lentiviral particles (Santa Cruz Biotech, sc-44943-V). Control shRNA lentiviral particles (sc-108080) were used as a negative scrambled shRNA sequence.
control. Cells were plated in 12-well plates, 24 h prior to viral infection with 1 mL of complete EMEM media. Then, the media was changed with complete media containing 5 μg/mL of Polybrene (sc-134220). TXNIP shRNA (h) lentiviral particles were thawed and added to the cells, mixed gently, and incubated overnight in a humidified 5% CO₂ air incubator at 37 °C. After 24 h, the media was changed with 1 mL of complete media without Polybrene and incubated overnight. To select stable clones, cells were split in the ratio of 1:5 and incubated for 24 h in complete media. After 48 h, stable clones expressing TXNIP shRNA were selected using Puromycin dihydrochloride (sc-108071) at 5 μg/mL. The cells were expanded and grown in puromycin containing complete media until ready for use.

2.9. Detecting a reduction of TXNIP transcript and protein on TXNIP-knocked downed SK-Mel-28 cells

The success of the stable TXNIP stable shRNA knockdown was determined by QPCR and densitometry analysis of western blots. Three types of cells were used in these experiments: stable TXNIP knocked down, scrambled shRNA control, and untransduced cells. Both types of Westerns were performed in triplicates. First, QPCR was used to detect TXNIP transcript levels after 24 h of r-Moj-DM treatment of 1 × 10⁶ cells. Total RNA of treated cells was performed as described in section 2.3. QPCR was performed as described in section 2.5. Second, whole cell lysates were obtained from cells after 24 h of r-Moj-DM treatment and separated in a 4–12% NuPAGE Bis–Tris gel under reducing conditions. Proteins were then transferred to a nitrocellulose membrane. Membranes were blocked in 5% skim milk containing 5% BSA (bovine serum albumin) overnight at 4 °C with constant shaking. After this incubation, the slides were rinsed with warm 1X PBS, followed by rabbit anti-mouse IgG conjugated to FITC (sc-358946). Cells were mounted and their nuclei stained with Vector shield mounting medium with DAPI. Slides were viewed at 100× oil immersion with a confocal Zeiss LSM 700 microscope.

3. Results

3.1. RNA sequencing and bioinformatics analyses of r-Moj-DM treated SK-Mel-28 cells

FASTQ analysis was done to examine the quality and filtering of the raw sequencing reads. RNA sequencing quality control data show that sequencing quality was good with all mean Phred scores at base positions 1–100 > 30. Both samples mean per sequence quality scores were 38. Sequence comparisons between untreated and treated cells were done in order to identify genes that were differentially expressed in r-Moj-DM treated cells. Density and volcano plots were obtained to identify those sequences. The sequencing coverage was calculated to be 8.33. RNA sequencing was done at single reads of 100 bp with approximately 250 million read pairs per sample.

3.2. Differential expression after r-Moj-DM treatment

Forty genes were differentially expressed after 6 h of r-Moj-DM treatment (Table 1). Nine genes were upregulated and 31 were downregulated. The expression of 13 of those genes was validated by qRT-PCR (Fig. 1). CXCL1, IL-8, TXNIP, BirC3, and Hist1-H2B were upregulated in both the RNA sequencing data and qRT-PCR. IGFBP3, Prune2, BNP3, HLA-B, RGS1, PTPRF, TGFβ-1, and PTPRD were downregulated in both methods.

3.3. Apoptotic genes are upregulated as a result of r-Moj-DN but not r-Moj-DA treatment

R-Moj-DN is a strong apoptotic inducer of SK-Mel-28 cells, while r-Moj-DA is not (Ramos et al., 2016). Differential expression was examined by qRT-PCR of the genes BNP3, IGFBP3, PTPRF, Prune2, TGFβ-1, and TXNIP (Fig. 2). There were no differences in expression in between cells treated with r-Moj-DN or r-Moj-DA early after treatment (1 h–2 h). However, higher expression was detected for all genes examined in r-Moj-DN treated cells from 4 h to 6 h after treatment.

3.4. TXNIP protein localization dynamics after GST-Moj-DM treatment

Confocal microscopy results from immunostaining of anti-GST antibody staining of GST-Moj-DM treated cells suggest that GST-Moj-DM results in integrin clustering. In addition, anti-TXNIP antibodies suggest that some TXNIP protein is localized to the nuclei of treated cells (Fig. 3).
Table 1
Differentially expressed genes between r-Moj- DM treated and control SK-Mel-28 cells at 6 h and identified with RNA-SEQ (n = 40).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Fold change</th>
<th>Gene</th>
<th>Function</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL8</td>
<td>Proinflammatory heparin-binding protein, negative regulation cell proliferation, locomotion</td>
<td>5.52</td>
<td>STC1</td>
<td>Glycoprotein, angiogenesis, bone and muscle development, cellular metabolism</td>
<td>−3.70</td>
</tr>
<tr>
<td>BIRC3</td>
<td>Negative regulation of apoptosis</td>
<td>5.44</td>
<td>RGS1</td>
<td>Attenuates g-protein activity</td>
<td>−3.72</td>
</tr>
<tr>
<td>TXNIP</td>
<td>Positive regulation of apoptosis, negative regulation cell proliferation, redox regulation</td>
<td>4.98</td>
<td>BNIP3</td>
<td>Positive regulation of apoptosis, cell projection</td>
<td>−3.72</td>
</tr>
<tr>
<td>CXCL1</td>
<td>Melanoma Growth factor, negative regulation cell proliferation</td>
<td>4.93</td>
<td>C2orf72</td>
<td>Uncharacterized protein</td>
<td>−3.83</td>
</tr>
<tr>
<td>HIST1H2AG</td>
<td>Chromatin dynamics</td>
<td>3.37</td>
<td>FLNB</td>
<td>Vascular injury repair</td>
<td>−3.98</td>
</tr>
<tr>
<td>HIST1H2BB</td>
<td>Chromatin dynamics</td>
<td>3.34</td>
<td>FGFBP2</td>
<td>Plasma protein, cytotoxic lymphocyte-mediated immunity</td>
<td>−4.15</td>
</tr>
<tr>
<td>HIST1H2BJ</td>
<td>Chromatin dynamics</td>
<td>3.08</td>
<td>DDX41</td>
<td>DEAD box protein family member, function not determined</td>
<td>−4.41</td>
</tr>
<tr>
<td>HIST1H2AH</td>
<td>Chromatin dynamics</td>
<td>3.00</td>
<td>NRN1</td>
<td>Neuritogenesis, glutamate target gene</td>
<td>−4.51</td>
</tr>
<tr>
<td>HIST1H1B</td>
<td>Chromatin dynamics</td>
<td>2.85</td>
<td>TGFB1</td>
<td>Positive regulation of apoptosis, growth factor, cell projection, negative regulation cell proliferation, locomotion</td>
<td>−5.01</td>
</tr>
<tr>
<td>A2M</td>
<td>Extracellular glycoprotein, plasma protein, protease inhibitor</td>
<td>−2.78</td>
<td>PPFIA4</td>
<td>Tyrosine phosphatase, hypoxia-induced gene, cell-cell adhesion</td>
<td>−5.25</td>
</tr>
<tr>
<td>ANKGF1</td>
<td>Ankyrin repeat and zinc finger domain-containing protein 1, protein-protein interactions</td>
<td>−2.81</td>
<td>LOXL2</td>
<td>Activation of TGFB1 signaling, disease-associated fibroblasts, and growth factors</td>
<td>−5.61</td>
</tr>
<tr>
<td>TNS1</td>
<td>Focal adhesions, crosslinks actin filaments, Src homology 2 (SH2) domain</td>
<td>−2.84</td>
<td>DOK3</td>
<td>Adaptor protein, TLR signaling, inhibits Ras-Erk pathway</td>
<td>−5.66</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>Positive regulation of apoptosis, negative regulation cell proliferation, locomotion</td>
<td>−2.94</td>
<td>PTPRD</td>
<td>Tyrosine phosphatase</td>
<td>−5.71</td>
</tr>
<tr>
<td>VGF</td>
<td>Growth factor, cell projection</td>
<td>−3.02</td>
<td>CACNA1H</td>
<td>Voltage-gated calcium channel, cell projection</td>
<td>−5.92</td>
</tr>
<tr>
<td>PRUNE2</td>
<td>Positive regulation of apoptosis</td>
<td>−3.07</td>
<td>C2orf46</td>
<td>Transmembrane protein</td>
<td>−6.27</td>
</tr>
<tr>
<td>NDRG1</td>
<td>Growth arrest, cell differentiation</td>
<td>−3.25</td>
<td>ISM2</td>
<td>ECM protein</td>
<td>−6.29</td>
</tr>
<tr>
<td>HLA-B</td>
<td>Immune system, membrane macroglobulin, antigen presenting</td>
<td>−3.43</td>
<td>SEC14L5</td>
<td>Secretory protein</td>
<td>−6.33</td>
</tr>
<tr>
<td>RORA</td>
<td>Nuclear hormone receptors</td>
<td>−3.54</td>
<td>SLC8A2</td>
<td>Sodium-calcium exchanger</td>
<td>−6.70</td>
</tr>
<tr>
<td>PTPRF</td>
<td>Positive regulation of apoptosis, negative regulation cell proliferation</td>
<td>−3.58</td>
<td>BEND5</td>
<td>Uncharacterized protein, BEN domain</td>
<td>−7.08</td>
</tr>
<tr>
<td>EN02</td>
<td>Glycolysis, enolase</td>
<td>−3.63</td>
<td>CA9</td>
<td>Carbonic anhydrase, cell projection</td>
<td>−7.40</td>
</tr>
</tbody>
</table>

Fig. 1. Thirteen genes that were significantly differentially expressed in RNA sequencing experiment were validated using qRT-PCR. Cells for the qRT-PCR analysis were treated for 6 h.
3.5. TXNIP knockdown

A stable knockdown of TXNIP resulted in a significant ($p < 0.001$) reduction of TXNIP transcript (Fig. 4A) and protein (Fig. 4B) after treatment with r-Moj-DM for 24 h.

3.6. TXNIP is required for the apoptotic induction initiated by r-Moj-DM/integrin signaling

Cell viability experiments were performed to determine the role of TXNIP in the apoptotic inducing activity originated from the r-Moj-DM/integrin pathway. High cell viability (74.3% ± 9.1) was obtained after r-Moj-DM treatment of TXNIP knocked down SK-Mel-28 cells, compared to 49.8% ± 0.58 for control shRNA cells and 34% ± 0.187 for untransduced cells (Fig. 5A).

Chromatin condensation, an apoptotic event, was observed using confocal microscopy (Fig. 5B). After r-Moj-DM peptide treatment, chromatin condensation was observed in the scrambled shRNA control cells, but not in the TXNIP stabled knocked down SK-Mel-28 cells.

![Fig. 2.](image1.png) (A) Gene expression of pro-apoptotic genes after r-Moj-DN treatment of SK-Mel-28 cells. (B) Gene expression of pro-apoptotic genes after r-Moj-DA treatment of SK-Mel-28 cells.

![Fig. 3.](image2.png) Confocal Microscopy of GST-Moj-DM treated (top left and right panels) and untreated (bottom left and right panels) SK-Mel-28 cells. Nuclei were stained with DAPI. Arrowheads in the top anti-GST panel indicate integrin crosslinking. Arrowheads on the anti-TXNIP panel indicate chromatin condensation. The white arrow in the anti-TXNIP panel suggest increased nuclear and cytoplasmic localization. Similar results were obtained when cells were treated with r-Moj-DM and stained with anti-TXNIP antibodies.
4. Discussion

4.1. Gene expression changes as a result of r-Moj-DM treatment of SK-Mel-28 cells

Microarrays were used to examine gene expression changes in HUVECs after *Crotalus atrox* or *Bothrops jararaca* venom treatment (Gallagher et al., 2003). However, RNA sequencing demonstrating changes in gene expression after cancer cell line treatment with snake venom or purified toxins have not been reported. RNA sequencing has been used to determine snake venom transcriptome profiles of several species (Rokyta et al., 2013; Rokyta et al., 2012; Durban et al., 2011) and to examine molecular evolution (Reeks et al., 2016; Aird et al., 2015; Junqueira-de-Azevedo et al., 2015; McGivern et al., 2014; Hargreaves et al., 2014; Tanaka et al., 2013; Moura-da-Silva et al., 2011).

Our gene expression analysis yielded several unexpected results. First, genes associated to the TNFR (tumor necrosis factor receptor) involved in the activation of extrinsic apoptosis or integrin-induced apoptosis were not differentially expressed after r-Moj-DM treatment. These targets include FAK, ERK, NF-κB, and JNK (Sharma et al., 2014; Ashkenazi and Dixit, 1998). Second, most of the identified genes have not been studied extensively. Differentially expressed genes after r-Moj-DM treatment are grouped into seven categories: pro-apoptotic (TXNIP, CXCL1, IGFBP3, Prune2, PTPRF, TGF-β, BNIP3, and DOK3), survival (BIRC3), inflammation (IL-8), chromatin dynamics (HIST1H2AG, HIST1H2BB, HIST1H2BJ, HIST1H2AH, and HIST1H1B), tumor metastasis inhibition (NDRG1), focal adhesion (TNS1), and hypoxia-induced (PPIA4). There were 22 genes with no known connection to apoptosis or survival.

Gene expression changes of pro-apoptotic and anti-apoptotic genes after treatment can be confusing and sometimes contradictory. However, it has been proposed that the expression ratio between pro-apoptotic and anti-apoptotic proteins may be more important than the overexpression of particular pro-apoptotic proteins in the cellular decision to undergo apoptosis (Zhivotovsky and Orrenius, 2006). Two of the pro-apoptotic genes identified in the RNA sequencing of r-Moj-DM treated SK-Mel-28 cells were upregulated (TXNIP and CXCL1), while the rest in this category were downregulated. In contrast, qRT-PCR experiments...

![Fig. 4.](image-url)
indicated that six pro-apoptotic genes (BNIP3, IGFBP3, PTPRF, Prune2, TGFß-1, and TXNIP) were upregulated after 4 h of r-Moj-DN treatment. Similar results were not obtained with r-Moj-DA, as upregulation of those genes was not significantly high after 4 h. Ramos et al. (2016) demonstrated that in SK-Mel-28 cells, r-Moj-DN is a stronger apoptotic inducer than r-Moj-DM. Recombinant r-Moj-DA does not induce apoptosis in SK-Mel-28 cells (Ramos et al., 2016).

4.2. TXNIP localizes to the nucleus after r-Moj-DM treatment

For this study we focused on TXNIP, since it is a protein with different cellular roles that intersect different aspects of cell death initiation (Zhou and Chng, 2013; Goldberg et al., 2003). TXNIP (also known as VDUP and thioredoxin binding protein 2, or TBP-2) was first identified as a vitamin D binding protein and can act as a transcription factor (Masutani et al., 2012; Han et al., 2003). Therefore, it is not surprising that TXNIP translocates to the nucleus after pathway activation (Fig. 3). Our confocal experiments with GST-Moj-DM peptides suggest that Moj-DM binding to the integrin results in integrin clustering, increase in TXNIP mRNA expression, and nuclear translocation of TXNIP (Fig. 3).

It is surprising that TXNIP expression was upregulated in r-Moj-DM treated cells as a result of an integrin-induced pathway. TXNIP upregulation has been detected after ER damage (Lerner et al., 2012; Oslowski et al., 2012), hypoxic conditions (Li et al., 2015), glucocorticoid incubation (Wang et al., 2006), ROS production (Gao et al., 2015; Zhou and Chng, 2013), or inhibition of glucose uptake and aerobic respiration (Shen et al., 2015).

4.3. Knockdown of TXNIP revealed its significance in the apoptotic induction by r-Moj-DM mutant peptides

Stable knockdowns of TXNIP and scrambled shRNA controls were used to examine the role of TXNIP in the apoptotic SK-Mel-28 cells. In TXNIP knocked down SK-Mel-28 cells, cell survival increased significantly and chromatin condensation (a morphological characteristic of apoptosis) was inhibited, suggesting that TXNIP is involved in the apoptotic pathway triggered by r-Moj-DM upon antagonizing the αv integrin subunit.

Increased survival of cancer cells has been associated with reduced levels of TXNIP (Zhou et al., 2011). In triple-negative breast cancers, c-myc inhibits TXNIP expression resulting in the increase of cell survival and poor tumor prognosis (Shen et al., 2015). Conversely, high levels of TXNIP correlate to increased apoptosis. Apoptosis of neuroblastomas (Su et al., 2015) and the lung cancer cell line A549 (Liu et al., 2015) was activated by ROS increase and upregulation of TXNIP expression.

4.4. Potential relationship between cell proliferation inhibition and TXNIP

TXNIP gene upregulated expression was also detected in r-Moj-DA treated cells. Recombinant r-Moj-DA is not an apoptotic inducer,
but a strong cell proliferation inhibitor of SK-Mel-28 cells (Ramos et al., 2016). This finding is not surprising since TXNIP has been shown to be upregulated after signals that inhibit cell proliferation (Han et al., 2003). Furthermore, it has been proposed that TXNIP inhibits cell proliferation by inhibiting metabolic pathways that are necessary for cell division (Elgort et al., 2010).

4.5. Conclusions

Our study is the first demonstration of TXNIP gene upregulation induced by a disintegrin/integrin pathway. The qRT-PCR results shown to be upregulated after signals that inhibit cell proliferation (Han et al., 2003). Furthermore, it has been proposed that TXNIP identified by an integrin-type signaling pathway, which may explain this disintegrin’s apoptotic potency. It would be of interest to identify the proteins and signal transduction pathway(s) that transmit the signal from the integrin receptor and results in the upregulation of TXNIP, and ultimately apoptosis. Our study was limited to gene expression changes at a short time range (1–6 h). It would be of interest to identify gene targets at later times (8–24 h). Finally, other candidate genes identified from our study are promising. These include CXCL1, BIRC3, and IL-8. Functional analysis of these gene targets and protein expression levels are possibilities for future studies. Finally, our study focused on gene expression changes and perhaps phosphorylation/inhibition of phosphorylation events of known pro-apoptotic or survival genes are as important as TXNIP in inducing SK-Mel-28 apoptosis induction by r-Moj-DM. These possibilities remained to be studied.

Ethical statement

Our manuscript has not been published and is not under consideration for publication in other journals. All co-authors in the manuscript carefully read the manuscript, carried out the experiments, and agreed on the content presented in the manuscript.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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