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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 down-regulated. 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-ß*, 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' 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 (Hanahhan 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-DN (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), echistatin (Alimenti et al., 2004; Brassard et al., 1999), 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).

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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; Oslowski 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% CO_2.

2.3. RNA isolation and RNA sequencing

One million SK-Mel 28 cells were treated with r-Moj-DM for 6 h, and total RNA extracted using the RNeasy Mini Kit (Qiagen). RNA was quantified using a NanoDrop spectrophotometer. One thousand nanograms of total RNA were isolated from any contaminating genomic DNA with the use of gDNA Wipeout Buffer from the QuantiTect Reverse Transcription Kit (Qiagen). The RNA was checked for quality on the Agilent Bioanalyzer. Libraries were made with Illumina TruSeq according to the manufacturer's protocol. Two RNA samples per sequenced, one from treated cells and one from untreated cells. Sequencing was performed 2 × 100 PE on the HiSeq2000 by Centrillion Technologies.

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 Quantiscript 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: ATGACCAGTCAACAGGGGAC, and reverse: GGTCCTTTTCACCAGCAAGC), 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 peptides 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 Quantiscript 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