

# Transcriptome Signature of Immune Cells Post Reovirus Treatment in *KRAS* Mutated Colorectal Cancer

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**Purpose:** Reovirus propagates with high efficiency in *KRAS* mutated colorectal cancer (CRC). About 45–50% of CRC patients possess a *KRAS* mutation. Oncolytic reovirus treatment in combination with chemotherapy was tested in patients possessing *KRAS* mutated metastatic CRC. This study evaluates the biological responses to reovirus treatment by determining the gene expression patterns in RAS-related signaling pathways.

**Methods:** Reovirus was administered as a 60-min intravenous infusion for 5 consecutive days every 28 days, at a tissue culture infective dose (TCID<sub>50</sub>) of  $3 \times 10^{10}$ . Peripheral blood mononuclear cells (PBMCs) were isolated from whole-blood pre- and post-reovirus administration at 48 hr, day-8, and day-15. Clariom\_D\_Human\_Assay was used to determine the expression of vital genes compared to pre-reovirus treatment by RNA sequencing. Using exported sample signals,  $\Delta\Delta Ct$  method was used to analyze the fold changes of genes within seven gene pathways. Significance was calculated by students-two-tail-*t*-test. Hierarchical clustering dendrogram was constructed by calculating Pearson's correlation coefficients.

**Results:** As compared to the control, *SOS1* [48 hr; 2.49X], *RRAS* [48 hr; 2.24X], *PIK3CB* [D8, D15; 2.27X, 3.16X], *MIR 16–2* [D15; 1.70X], *CHORDC1* [48 hr, D15; 1.89X, 4.54X], *RTN4* [48 hr; 4.66X], *FAM96A* [48 hr; 4.54X], *NFKB* [D8, D15; 19.0X, 1.42X], *CASP8* [D8, D15; 2.11X, 1.77X], and *CASP9* [D8; 1.45X] are upregulated post-reovirus. *NOS3* [D15; 0.61X], *SYNE1* [D8, D15; 0.78X, 0.71X], *ANGPT1* [D8; 0.62X], *VEGFB* [48 hr, D8, D15; 0.44X, 0.28X, 0.28X], *JUN* [D15; 0.69X], and *IGF2* [D8; 0.73X] are downregulated post-reovirus. Fold change values were significant [ $p < 0.05$ ].

**Conclusion:** This study highlights reovirus as a novel treatment option for *KRAS* mutated CRC and showcases its effect on the expression of crucial genes.

**Keywords:** transcriptome, reovirus, CRC, *KRAS*, *CASP8*, *CHORDC1*, *RTN4*, *VEGFB*

## Introduction

RNA molecules play significant roles in biological functions, including, genetic coding/decoding, gene regulation and gene expressions.<sup>1</sup> The identification of key RNA sequences in proteins and genes, as well as the understanding of the roles they play, provides an overwhelming amount of knowledge on specific biological pathways.<sup>2,3</sup> This can allow for manipulation of the expression and regulation of certain genes in beneficial pharmaceutical and treatment methods. RNA research made a breakthrough in early 2000s with the advent of next-generation RNA-Seq.<sup>1</sup> Two noteworthy studies, which were some of the first to use RNA-Seq, were Nagalakshmi et al<sup>4</sup> on the landscape of the yeast genome and Bainbridge

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et al on prostate cancer cell lines.<sup>5</sup> The two methods by which researchers use to assemble transcriptomes are De novo and Genome guided. The de novo method does not require a known reference genome in order to reconstruct the transcriptome; rather, sequences are assembled as contigs, a set of overlapping DNA segments that, as a collective, embody a consensus region of DNA.<sup>6</sup> In the genome guided method, RNA sequences are aligned within a specific, known, genome, similar to that in the DNA alignment process.<sup>7</sup> In this study RNA-Seq was conducted on peripheral blood mononuclear cells (PBMC) obtained from a set of patients who underwent reovirus treatment for metastatic colorectal cancer.

The use of reovirus as a therapy for cancer is an up-and-coming intervention in the field of cancer biology.<sup>8–10</sup> Reovirus, a proprietary version of unmodified human reovirus, is an oncolytic virus that can lyse cancer cells.<sup>11,12</sup> This virus is being developed as an immune-oncological viral agent to treat hematological malignancies and various forms of neoplasms.<sup>13</sup> Reovirus is a stable form of non-enveloped double-stranded RNA. In humans, infection usually results in an asymptomatic or mild illness, manifested with flu-like symptoms, including mild gastroenteritis.<sup>14</sup> Given the virus' oncolytic properties and its potential to reproduce well in cancer cells, interest in therapeutic reovirus treatment has been proposed since 1970's.<sup>15</sup> Reovirus has become a prolific experimental model for viral pathogenesis.

Reovirus has been shown to replicate remarkably well in cells that have a mutated *KRAS* gene.<sup>12</sup> After infection within *KRAS*-mutated tumor cells, viral replication occurs resulting in cell death, which leads to viral progeny infecting nearby tumor cells. It is hypothesized that upstream activation of *KRAS* proteins may play a role in more than two-thirds of human metastatic cancers.<sup>16</sup> This provides further evidence of the therapeutic potential of reovirus, specifically in *KRAS*-mutated tumors, and its potential as an alternative treatment method for metastatic colorectal cancer. This study utilized the Genome guided method of RNA-Seq in order to analyze seven different biological pathways and how they were affected by reovirus treatment in samples from *KRAS* mutated colorectal cancer.

## Methods

### Patient Selection

Blood samples were obtained from eight patients, all having *KRAS*-mutated mCRC. Five (5) patients had received reovirus as part of a Phase I clinical trial (NCT01274624).

Three (3) patients were not enrolled in the trial but did receive equivalent background chemotherapy (i.e. FOLFIRI and bevacizumab).

## Ethical Considerations

Ethics approval and consent to participate: The entire study was performed in compliance with Institutional and Federal guidelines for clinical research. The study was approved by the ethics committee of Montefiore Medical Center/Albert Einstein School of Medicine. All patient tissue and blood samples were drawn following written informed consent based on a local IRB approved consent form. This trial was conducted in accordance with the Declaration of Helsinki.

## Institutional Review Board/Ethics Committee Approval and Informed Consent

All patient blood samples were drawn with informed consent based on an Albert Einstein College of Medicine IRB committee approved consent form (IRB# 07-10-376). Following the institutional review board's (ethics committee) approval, blood samples were collected from eight patients with *KRAS*-mutated metastatic colorectal cancer at 0 (pre) and 48 hr, and on days 8 and 15. Peripheral blood mononuclear cells (PBMCs) were isolated for transcriptome analysis from eight patients. Five patients had received reovirus (pelareorep) along with FDA approved second-line chemotherapy (i.e. FOLFIRI and bevacizumab) as part of a Phase I clinical trial (NCT01274624), while three patients were not enrolled in this trial but did receive equivalent chemotherapy.

## Data Sharing

The authors have shared individual deidentified participant data in the public domain to be available indefinitely. These raw data include peripheral mononuclear cell (PBMC) samples at four timepoints (pre-treatment, 48 hr, day 8 and day 15) for each individual involved in the clinical trial. This data can be accessed through the NCBI GEO Accession Viewer (Series GSE173636) using the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173636>

## Reovirus Administration

Reovirus was supplied by Oncolytics Biotech, Inc. as a translucent to clear, colorless to light blue liquid in vials containing  $7.2 \times 10^{10}$  tissue culture infective dose (TCID<sub>50</sub>) per mL of reovirus in a phosphate-buffered solution and

stored at minus 70°C. Reovirus was administered as a 60-min infusion for 5 consecutive days every 28 days, at a tissue culture infective dose (TCID<sub>50</sub>) of  $3 \times 10^{10}$ /day. Plasma was collected pre-reovirus [0 hr], at 48 hr, Day 8 (D8) and Day 15 (D15) after first dose of reovirus.

## Transcriptome Analysis

Transcriptome Analysis Console (TAC) Software: Thermo Fisher Scientific is a software, which conducts RNA-Seq via the Genome guided method. The TAC 4.0 uses Expression Console™ software (EC), which utilizes cell intensity files (\*.CEL) to create probe-level summarization files. Thus, allowing for initial data quality examination of Affymetrix expression arrays.

Blood was collected into CPT tubes (BD Biosciences, Franklin Lakes, NJ) for isolation of PBMC (Peripheral Blood Mononuclear Cells). Total RNA was isolated from the patient's PBMC and reverse transcribed using a reverse transcription priming method from an engineered set of primers that exclude sequences that match ribosomal RNA (rRNA). These primers specifically prime non-ribosomal RNA from the sample, including both poly(A) and non-poly(A) mRNA and convert it into double-stranded cDNA using first- and second-strand enzyme from Kit. The templates are used for in vitro transcription reaction at 37°C for 16 hr to yield cRNA. The ss-cDNA generated from cRNA was chemically fragmented, biotin-labeled and made into a hybridization cocktail using hybridization kit (Catalog Number 900454) according to the Affymetrix Genechip protocol, which was then hybridized to Clariom D Gene Chip human probe array. The array image is generated by a high-resolution Gene Array Scanner 3000 7G (Thermo Fisher Scientific, Santa Clara, CA) and the Cq value for each gene expression of a patient at a given timepoint was recorded. Individual sample signals for each patient at each timepoint were extracted from the TAC 4.0 software, organized and compiled in Microsoft Office Excel. Gene expression data were analyzed by the  $2^{-\Delta\Delta C_T}$  method<sup>17</sup> and normalized to "Pre" treatment (Day 0) expression. Two tailed *t*-test was used to determine statistical significance ( $p < 0.05$ ). Statistics were calculated using Microsoft Office Excel.

After the results were collected from the patients, the data was uploaded to Transcriptome Analysis Console 4.0.1 (TAC). Seven growth and apoptotic related pathways were observed as detailed in Table 1.

Two thousand eighty-three genes, across all seven pathways, were filtered via the software's filter function and their sample signals were exported to their respective

**Table 1** Pathway Sources

	Pathway	Reference
1	Apoptosis Overview	Zambon A, Pico A, et al: Apoptosis Overview (Homo Sapiens). <a href="https://www.wikipathways.org/index.php/Pathway:WP254">https://www.wikipathways.org/index.php/Pathway:WP254</a>
2	Angiogenesis Overview	Helix, Willighagen E, et al: Angiogenesis Overview (Homo Sapiens). <a href="https://www.wikipathways.org/index.php/Pathway:WP1993">https://www.wikipathways.org/index.php/Pathway:WP1993</a>
3	miR-targeted Genes of Lymphocytes	Sklar S, Hanspers, miR-targeted Genes in Lymphocytes (Homo Sapiens). <a href="https://www.wikipathways.org/index.php/Pathway:WP2004">https://www.wikipathways.org/index.php/Pathway:WP2004</a>
4	Ras Signaling	Hanspers K, Slenker D, et al: Ras Signaling (Homo Sapiens). <a href="https://www.wikipathways.org/index.php/Pathway:WP4223">https://www.wikipathways.org/index.php/Pathway:WP4223</a>
5	MAPK Signaling Pathway	Burel S, Hanspers K, et al: MAPK Signaling Pathway (Homo Sapiens). <a href="https://www.wikipathways.org/index.php/Pathway:WP382">https://www.wikipathways.org/index.php/Pathway:WP382</a>
6	EGF/EGFR Signaling Pathway	Pandey A, Hanspers, K, et al: EGF/EGFR Signaling Pathway (Homo Sapiens). <a href="https://www.wikipathways.org/index.php/Pathway:WP437">https://www.wikipathways.org/index.php/Pathway:WP437</a>
7	PI3K-Akt Signaling Pathway	Hanspers K, Riutta A, et al: PI3K-Akt Signaling Pathway (Homo Sapiens). <a href="https://www.wikipathways.org/index.php/Pathway:WP4172">https://www.wikipathways.org/index.php/Pathway:WP4172</a>

Excel spreadsheets. This was done for each set of genes of the seven biological pathways which were studied. Sample signal data was exported for each patient timepoint (Pre, 48 hr, Day 8 and Day 15). Four Excel sheets, one for each timepoint, were created with the sample signals for all experimental patients. Fold changes were calculated. This was accomplished by comparing the 48 hr, Day 8 and Day 15 timepoints to the Pre timepoint, separately. First, the  $\Delta\Delta C_T$  (Difference in Cq values) were calculated for each timepoint; then, the  $2^{-\Delta\Delta C_T}$  (Actual fold change) was calculated using the data attained from the  $\Delta\Delta C_T$  results. The  $2^{-\Delta\Delta C_T}$  results that were obtained for each patient at that respected timepoint for every gene used to calculate the mean fold change. Upon obtaining the results from these calculations, all 2083 genes (many of which overlapped into different pathways) were filtered by a p-value of 0.05.

**Table 2** Genes Analyzed

	Gene Name	Gene Symbol
1	Son of Sevenless Homologue 1	<i>SOS1</i>
2	Ras-related Protein	<i>RRAS</i>
3	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform	<i>PIK3CB</i>
4	MiR-16 microRNA precursor	<i>MIR16-2</i>
5	Cysteine and Histidine-rich Domain	<i>CHORDC1</i>
6	Reticulon-4	<i>RTN4</i>
7	Family with Sequence Similarity 96, Member A	<i>FAM96A</i>
8	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells	<i>NFKB1</i> , <i>NFKBIA</i>
9	Caspase-8	<i>CASP8</i>
10	Caspase-9	<i>CASP9</i>
11	Nitric Oxide Synthase 3	<i>NOS3</i>
12	Synaptic Nuclear Envelope Protein 1	<i>SYNE1</i>
13	Angiopoietin 1	<i>ANGPT1</i>
14	Vascular Endothelial Growth Factor	<i>VEGF</i>
15	Jun proto-oncogene	<i>JUN</i>
16	Insulin-like Growth Factor	<i>IGF2</i>

This yielded 155 significant genes, across all pathways and timepoints. Of the 155 genes, 16 (Table 2) of them were chosen to be further analyzed by virtue of their levels of expression and their effects on *KRAS* mutated CRC proliferation.

## Dendrogram Generation

Fold change values at all timepoints for the 16 genes analyzed were fed into XLSTAT as an agglomerative hierarchical clustering. The similarities of expression between the genes were quantified using the Pearson's Correlation Coefficient.

## Results

### *NF-κB*, *SOS1*, and *RRAS* Gene Expression Indicated Successful Immune Response to Reovirus

*SOS1* and *RRAS* were upregulated at 48 hr; *NF-κB* gene expression showed exceptional increase at Day 8 and a significant decrease at Day 15.

In PBMC samples of patients who underwent reovirus treatment, a statistically significant ( $p < 0.05$ ) increase in expression of *SOS1* and *RRAS* was observed at 48 hr with fold changes of 2.49 [ $p = 0.00339$ ;  $SEM = 0.706$ ] and 2.24 [ $p = 0.0186$ ;  $SEM = 0.340$ ], respectively; both of these genes lost significance at D8 and

D15. It was also observed that *NF-κB* had a fold change of 19.0 [ $p = 0.050$ ;  $SEM = 6.34$ ] at D8 and a fold change of 1.42 [ $p = 0.0285$ ;  $SEM = 0.181$ ] at D15. The major increase of *NF-κB*, followed by a drastic decrease at D15, implies that reovirus was effectively administered, thus promoting an immune response in the patients' innate and adaptive immune systems. The loss of significance for *SOS1* and *RRAS* post-48 hr, in conjunction with the major increase of *NF-κB* expression at D8, indicates that the major effects of reovirus treatment took effect at around D8. Consequently, this timepoint is where we find many of our genes starting to respond to reovirus treatment. These data are presented in Figure 1 and Supplementary Table 1.

### Genes Linked to Cell Proliferation Decrease in Transcription

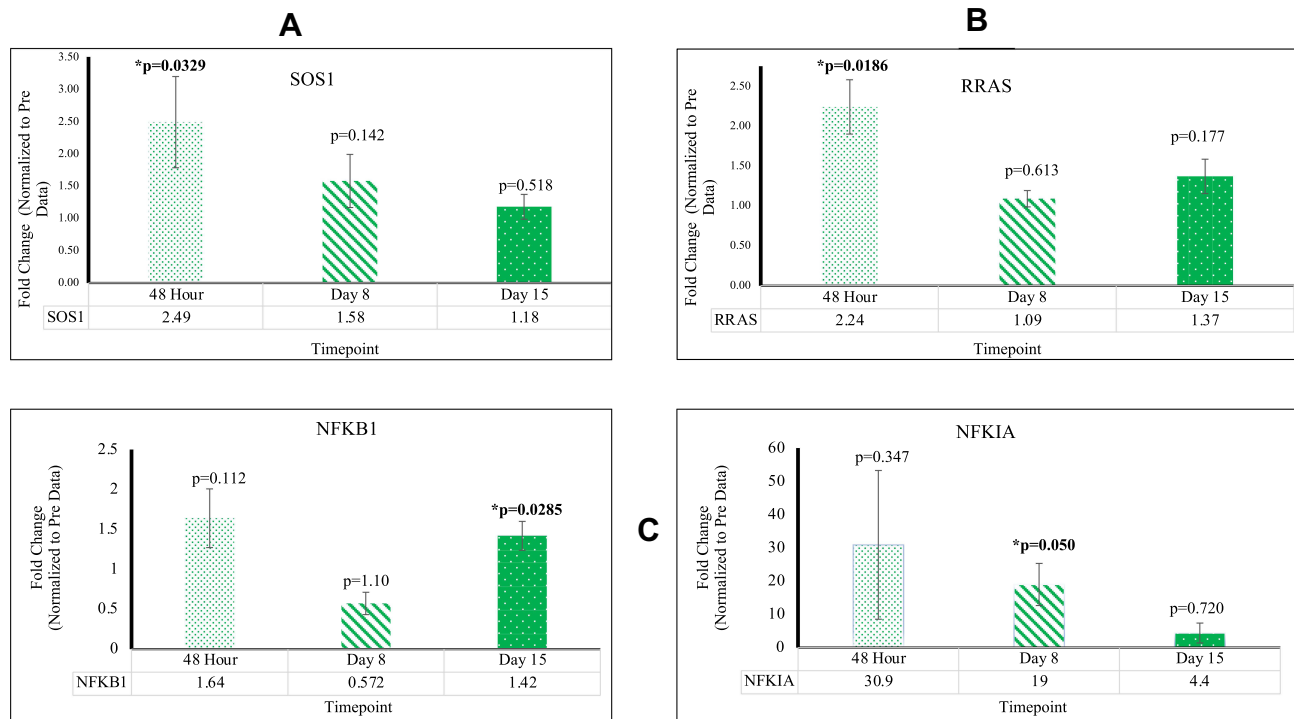
#### Angiogenesis Related Genes, *ANGPT1*, *VEGFB*, and *SYNE1* are Found to Be Significantly Downregulated

We observed a decrease in gene expression of *ANGPT1* and *VEGFB* which were signified by a reduction in mean fold change values relative to Pre. This data is highlighted in Figure 2. *ANGPT1* was found to be downregulated at D8 with a fold change of 0.62 [ $p = 0.0120$ ;  $SEM = 0.113$ ]. *VEGFB* was found to be downregulated at 48 hr with a fold change of 0.44 [ $p = 0.00872$ ;  $SEM = 0.0882$ ], at D8 with a fold change of 0.28 [ $p = 0.00380$ ;  $SEM = 0.0410$ ], and at D15 with a fold change of 0.28 [ $p = 1.31 \times 10^{-5}$ ;  $SEM = 0.0456$ ]. *SYNE1* was found to be downregulated at D8 with a fold change of 0.78 [ $p = 0.0392$ ;  $SEM = 0.0410$ ] and at D15 with a fold change of 0.71 [ $p = 0.0100$ ;  $SEM = 0.0585$ ]. Down regulation indicates reduction in cell proliferation as well as decrease in angiogenesis. This data is highlighted in Figure 2.

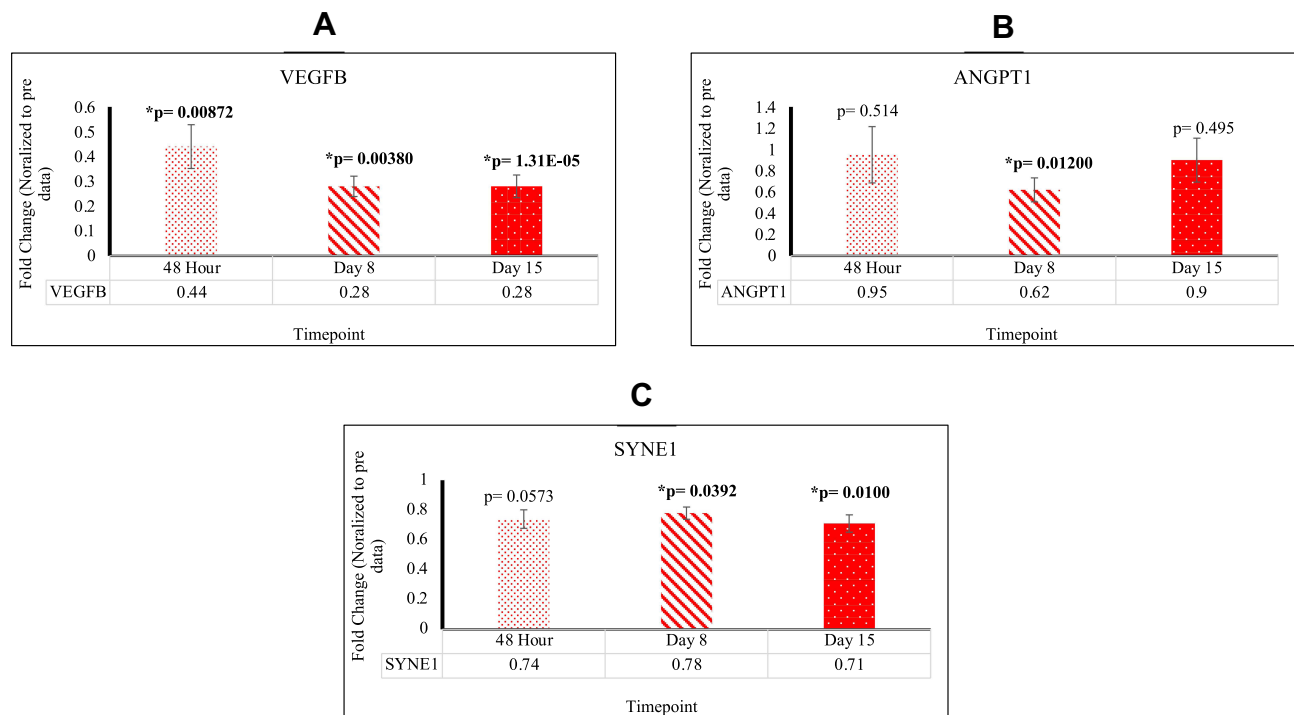
### Apoptotic Related Genes Increase in Reovirus Treated *KRAS* Mutated CRC

#### Key Apoptotic Genes *CASP8* and *CASP9* are Found to Be Upregulated; *JUN* and *IGF2* are Found to Be Downregulated

*CASP8* and *CASP9* were upregulated at D8 with fold changes of 2.11 [ $p = 0.0410$ ;  $SEM = 0.487$ ] and 1.45 [ $p = 0.00299$ ;  $SEM = 0.105$ ], respectively. In addition, *CASP8* was upregulated at D15 with a fold change of 1.77 [ $p = 0.0425$ ;  $SEM = 0.344$ ]. These findings indicate a significant increase in apoptotic activity in *KRAS* mutated CRC post-reovirus treatment. *JUN* was found to



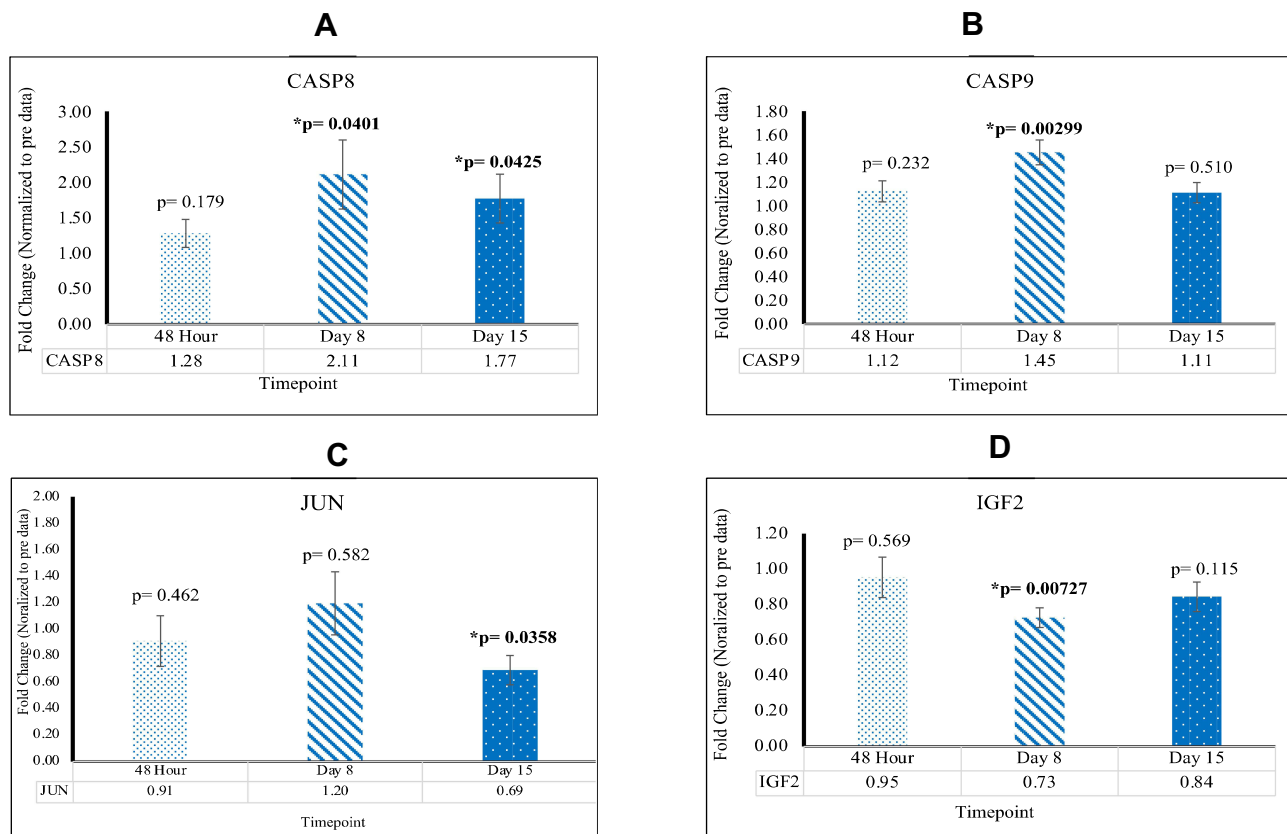
**Figure 1** (A) The expression of *SOS1* significant at 48 hr (B) The expression of *RRAS* significant at 48 hr (C) The expression of *NFKB* significant at D8 and D15.



**Figure 2** (A) The expression of *VEGFB* significant at all timepoints (B) The expression of *ANGPT1* significant at D8 (C) The expression of *SYNE1* significant at D8 and D15.

be downregulated at D15 with a fold change of 0.69 [p=0.0358; SEM=0.110]. *IGF2* was downregulated at D8 with a fold change of 0.73 [p=0.00727; SEM=0.0560].

Down regulation indicates a reduction in cell proliferation and an increase in cellular apoptosis. This data is highlighted in Figure 3.



**Figure 3** (A) The expression of *CASP8* significant at D8 and D15 (B) The expression of *CASP9* significant at D8 (C) The expression of *JUN* significant at D15 (D) The expression of *IGF2* significant at D8.

### Genes Pertaining to miR in Lymphocytes Exhibit Significantly Positive Alteration Post Reovirus Treatment

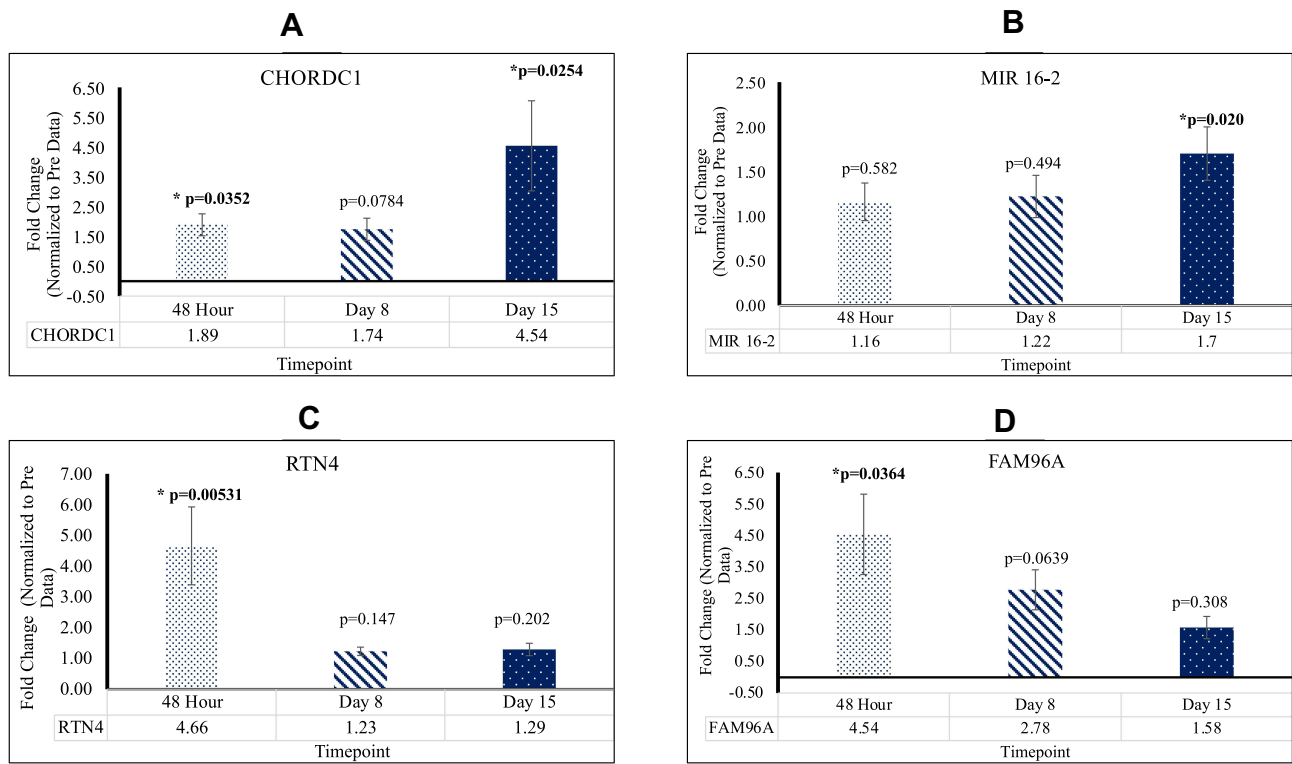
*RTN4* and *FAM96A* Were Upregulated at 48hr; *CHORDC1* Was Upregulated at 48hr and D15; *MIR16-2* Was Upregulated at D15

When compared to Pre mean Cq values, patients who underwent reovirus treatment exhibited higher levels of *RTN4* and *CHORDC1* gene expression. At 48 hr, *RTN4*, *FAM96A*, and *CHORDC1* were upregulated with fold changes of 4.66 [p=0.00531; SEM=1.27], 4.54 [p=0.0364; SEM=1.27], and 1.89 [p=0.0352; SEM=0.369], respectively. Additionally, *CHORDC1* and *MIR16-2* were upregulated at D15 with fold changes of 4.54 [p=0.0254; SEM=1.51] and 1.70 [p=0.0204; SEM=0.301], respectively. These results showcase that in *KRAS* mutated CRC, post-reovirus treatment, patients possessed a significant increase in lymphocytic activity and strengthening of their adaptive immune systems. This data is highlighted in Figure 4.

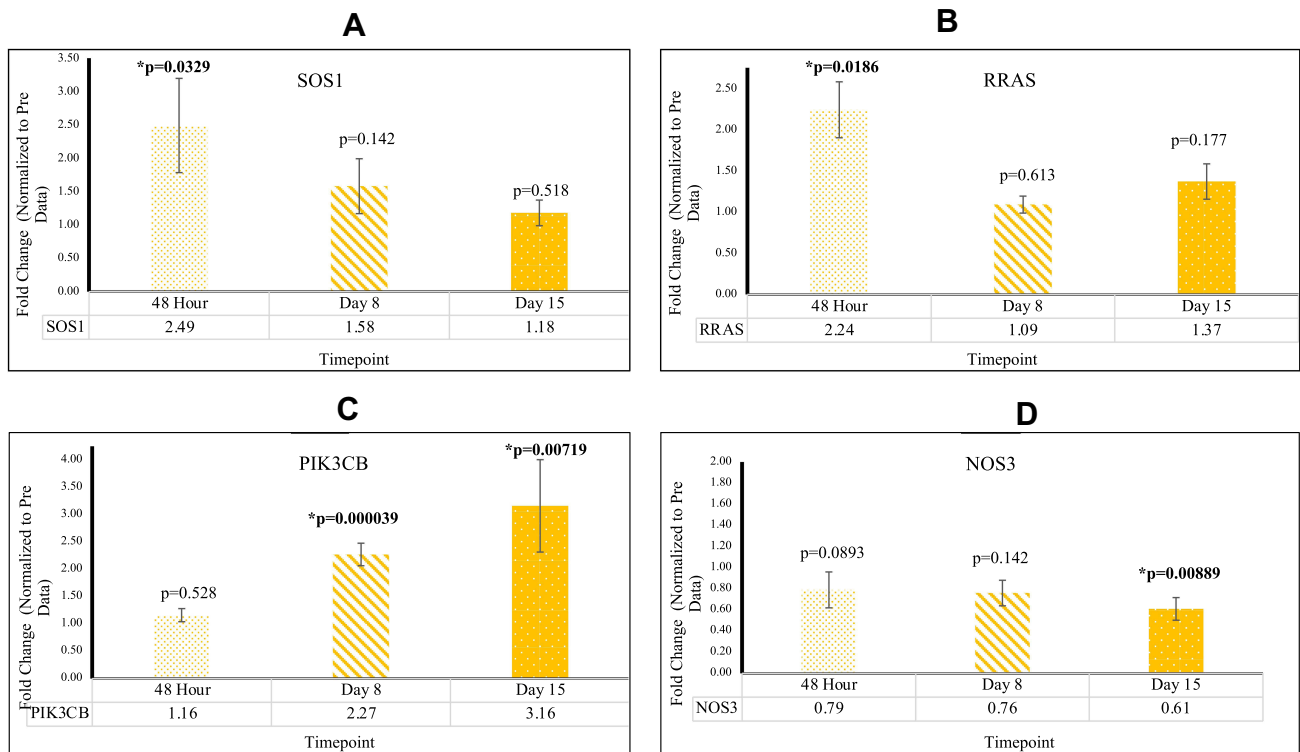
### Post Reovirus Treatment, Genes Within the RAS-Signaling Pathways of RAS, MAPK, EGFR, and PI3K-Akt, Showcased Gene Alteration

*PIK3CB* Was Upregulated at D8 and D15; *NOS3* Was Downregulated at D15

Two significant RAS-signaling genes were identified across the four RAS-related gene signaling pathways. Upon comparison with mean Pre Cq values, CRC patients exhibited higher levels of *PIK3CB* gene expression, as well as lower levels of *NOS3* gene expression post-reovirus treatment. At D8 *PIK3CB* was upregulated with fold a change of 2.27 [p=0.0000390; SEM=0.204]. At D15 *PIK3CB* was upregulated and *NOS3* was downregulated with fold changes of 3.16 [p=0.00719; SEM= 0.846] and 0.61 [p=0.00889; SEM=0.107], respectively. These results confirmed that patients who underwent reovirus treatment saw tumor-suppressor effects of *PIK3CB* and *NOS3*. This data is highlighted in Figure 5 and [Supplementary Table 1](#).



**Figure 4 (A)** The expression of *CHORDC1* significant at 48hr and D15 **(B)** The expression of *MIR16-2* significant at D15 **(C)** The expression of *RTN4* significant at 48hr **(D)** The expression of *FAM96A* significant at 48hr.



**Figure 5 (A)** The expression of *SOS1* significant at 48 hr **(B)** The expression of *RRAS* significant at 48 hr **(C)** The expression of *PIK3CB* significant at D8 and D15 **(D)** The expression of *NOS3* significant at D15.

## Pathway Correlations

Wanting to identify statistical correlations, four types of graphical interpretation of the data were created to interconnect the genes which were focused on. First, a Venn diagram was generated, showing common genes among the different pathways. A STRING interaction network was created, depicting the relationship between the 16 main genes. The third statistical representation was a heat map, which was generated using the mean fold changes from each timepoint (significant and not significant). Finally, a dendrogram-statistical analysis for the similarity of genes was created, with assessment based on the Pearson's Correlation Coefficient. This data is displayed in Figure 6.

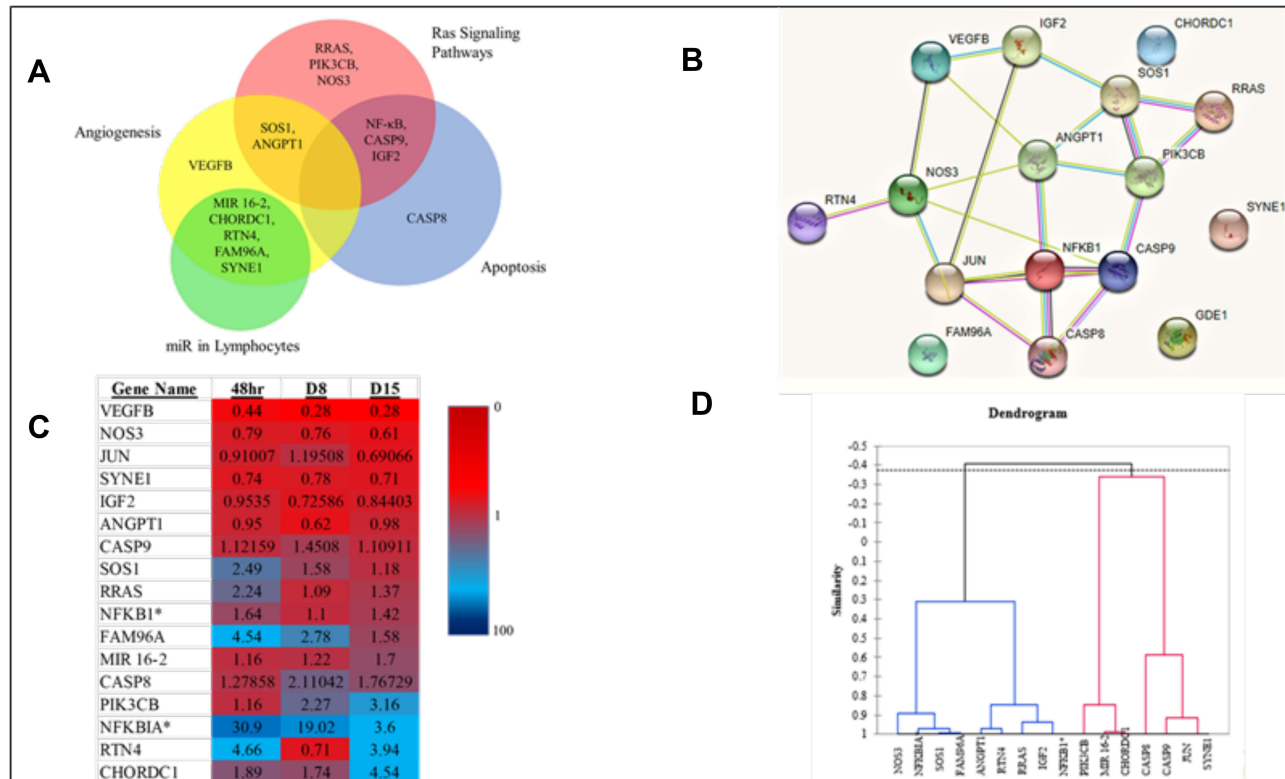
## Discussion

Our research focused on analyzing the affects reovirus treatment had on *KRAS* mutated CRC on seven crucial biological pathways. We have gathered that across these seven pathways, apoptosis was induced, angiogenesis was inhibited, lymphocytic maturation and activity increased, and several RAS-related signaling genes were altered to aid the immune response to CRC. Reovirus induces alterations in several gene transcripts that can be correlated with

oncolysis. Upregulation of apoptotic genes and downregulation of angiogenesis-related genes indicate that reovirus can be an effective CRC treatment in patients possessing *KRAS* mutated cells. The results clearly show the effect of reovirus. Patients receiving background chemotherapy but not the reovirus do not have any significant alterations in these genes at 48 hr, D8, or D15 [Supplementary Tables 1 and 2]. In this study, seven biological pathways were completely analyzed and assessed based on the efficacy of reovirus-altered gene expression on genes within these pathways.

The *NF-κB* family deals with controlling genes that regulate cellular differentiation and the transcription of cytokines and antimicrobial effectors.<sup>18</sup> However, an additional characteristic of *NF-κB* is its contribution to development of immune cells and their survivability and its use as a transcription factor that promotes transcription of many genes including *MAPK*. Our data showed a drastic increase in gene expression of *NF-κB* at the D8 timepoint, suggesting a trigger in immune response due to the administration of reovirus.

This observed increase was enabled by the activity of *SOS1*, which encodes a protein that is a guanine nucleotide



**Figure 6** (A) Venn Diagram exhibiting pathway relationships between the primary 16 genes (B) STRING interaction network (*GDE1* is an alias for *MIR16-2*). \*Indicates a subunit of *NF-κB*. (C) Heat Map with fold changes from all 16 genes (D) Dendrogram showing statistical similarities between the 16 genes.



exchange factor for RAS proteins.<sup>19</sup> By participating in RAS-related signal transduction pathways, *SOS1* has been shown to increase NFκB activation by constitutive stimulation of the EGF receptor and phosphorylation of *MAPK3*.<sup>20</sup> Our data show that *SOS1* was overexpressed at 48 hr, a requirement for the EGF-dependent activation of *NFκB* that was witnessed significantly at D8. Additionally, this increase was followed by a fold change decrease to basal levels at the D15 timepoint, which signified a complete cycle of immune response to the treatment.

Angiogenesis is a biological process, which promotes the creation of blood vessels; observations of genes in the angiogenesis pathway can reveal the state of cancer cell progression or regression.<sup>21</sup> *ANGPT1* directly plays a role in the creation of blood vessels and their maturation as well as vascular development.<sup>22</sup> In metastatic CRC, this gene is generally upregulated, pointing cellular proliferation of cancer cells; however, in our data we see a decrease of *ANGPT1* at the D8 timepoint. While this gene loses significance at D15, another key angiogenesis-related gene, *VEGFB*, was observed to be significant and was down regulated at all timepoints. *SYNE1*, a gene that helps encode a spectrin repeat containing protein, which is expressed in peripheral blood lymphocytes,<sup>23</sup> was found to be downregulated at both D8 and D15. The downregulation of *VEGFB* at every timepoint, in conjunction with the downregulation of *ANGPT1* and *SYNE1*, proves the decrease in cancer cell proliferation and angiogenesis in CRC.

Apoptosis, a form of programmed cell death, is controlled by aspartate-specific cysteine proteases (caspases) and functions in maintaining homeostasis by the removal of damaged cells. We have identified multiple genes, which function in the apoptotic pathway, that have been affected via reovirus treatment. The *CASP8* and *CASP9* are two important members of the pathway that are significantly upregulated favoring apoptosis. *CASP8* not only regulates apoptosis but has also been found to stimulate cell proliferation, malignant transformation and tumor progression when it is dysfunctional or has significantly low gene expression.<sup>24</sup> Thus, these genes play a crucial role in tumor suppression.

*JUN* is an intron-less proto-oncogene, which has a key role in cellular proliferation and apoptosis.<sup>25</sup> When overexpressed, *JUN* has been seen to suppress cellular apoptosis. This function has shown to be highly exacerbated by exposure to UV radiation.<sup>26</sup> A study from 2003 by Eferl

et al<sup>27</sup> revealed that the c-JUN protein is required during early stages of tumor development, and in our study, *JUN* was downregulated at D15.

*IGF2* is involved in tissue differentiation. By facilitating the recruitment of transcriptional coactivators, *IGF2* positively regulates myogenic transcription factor *MYOD1*, thus controlling muscle terminal differentiation.<sup>28</sup> It has been shown that elevated levels of serum IGF2, which is encoded by the *IGF2* gene, have been proven to have an increased risk of developing certain cancers, including breast, lung and colorectal cancers.<sup>29</sup> Given that *IGF2* was downregulated at D8, this indicates that the proliferation of CRC cells, regarding *IGF2* expressivity, decreased. Successive activation of *CASP8* and *CASP9* play a crucial role in the execution-phase of cell apoptosis.<sup>29</sup> It was observed that both genes were not significant in our control data at any time. In our experimental data, the upregulation of *CASP8* and *CASP9*, as well as the downregulation of *JUN* and *IGF2*, provide strong evidence that apoptosis increased in *KRAS* mutated CRC post-reovirus treatment.

MicroRNAs (miRNAs) consisting of small non-coding RNAs are utilized in post-transcriptional gene regulation, many of which are involved in T lymphocyte development, differentiation, and function.<sup>30</sup> T cell activation and differentiation are dependent on the expression of multiple miRNAs with many mRNA targets.<sup>31</sup> Our study has highlighted several genes within the miR in Lymphocytes gene pathway, which have shown significant alteration post-reovirus treatment.

The *CHORDC1* gene regulates the centrosome cycle via *ROCKII* Kinase to control genomic stability.<sup>32</sup> *CHORDC1* acts as an onco-suppressor in certain capacities. When under-expressed, *CHORDC1* has been shown to induce centrosome amplification and cytogenic abnormalities.<sup>33</sup> This amplification is largely caused by increased interactions between *ROCKII* and *NPM*, leading to a promotion of *ROCK II* kinase activity.<sup>34</sup> As such, upregulation of *CHORDC1* prevents centrosome amplification and tumorigenesis. Our data indicate that *CHORDC1* was upregulated 48 hr, and increased approximately three-fold by D15.

*RTN4* plays an important role in the formation and stabilization of endoplasmic reticulum tubules.<sup>35</sup> The family of proteins encoded by this gene influences nuclear envelope expansion, nuclear pore complex formation, and proper localization of inner nuclear membrane proteins.<sup>36</sup> *RTN4B* expression in various tissues also regulates angiogenesis, macrophage movement, vascular modeling,

sphingolipid homeostasis, inflammation, and other immune responses.<sup>37</sup> There are data to indicate that the RTN gene family reduces the anti-apoptotic activity of *Bcl-xl* and *Bcl-2*.<sup>38</sup> Another isoform of this gene, *RTN4-C*, is known to be essential in hepatocellular carcinoma regulation, inhibiting cell growth and promoting apoptosis. Our data showed *RTN4* to be significantly upregulated at 48 hr indicating its role as an antitumor effector.

*FAM96A* a component of the cytosolic iron-sulfur protein assembly (CIA) complex has been identified as a novel pro-apoptotic tumor suppressor in gastrointestinal stromal tumors.<sup>39</sup> Post-reovirus treatment, our data exhibits significant upregulation of *FAM96A* at 48 hr.

*MIR16-2* is classified within a group of small RNA genes that regulate gene expression.<sup>40</sup> The *MIR-16* gene acts as a tumor suppressor in cancer cells by inhibiting FEAT translation.<sup>41</sup> Decreased *MIR-16* expression upregulates expression of *MEK1*, which in turn promotes cancer cell invasion and proliferation.<sup>42</sup> Overexpression of *MIR-16* downregulates the ERK/MAPK pathway by inhibiting *MEK1* expression. Consequently, this reduces clone formation and inhibits cell proliferation. As our data shows, *MIR 16-2* was upregulated at D15. All in all, based on the results from the PBMC samples, lymphocytic maturation and activity increased significantly.

The mitogen-activated protein kinase pathway (MAPK/ERK) and PI3K-Akt pathway play an integral role in CRC, dealing with regulation of gene expression, cellular maturation, and survival.<sup>43</sup> Given that reovirus replicates remarkably well within the RAS signaling pathways, our data indicate that after initial reovirus treatment, expression of RAS-specific genes such as *RRAS* immediately increase in gene expression. This effect occurs because of virus propagation within *KRAS* mutated cells. However, these genes become downregulated or lose significance as the immune response begins to take effect at D8. The expression of the *RRAS* gene in our data is a premier example of this phenomenon. At 48 hr, *RRAS* is upregulated, but loses significance at subsequent timepoints.

*PIK3CB* is a gene known to be important in signaling pathways that involve receptors on the outer membrane of eukaryotic cells. Ablation of *PIK3CB* has been shown to impede tumorigenesis with a concomitant diminution of Akt phosphorylation.<sup>44</sup> In our study, *PIK3CB* was shown to be upregulated both at D8 and D15.

*NOS3* is a gene that is implicated in vascular smooth muscle relaxation through its production of nitric oxide

(NO) via a cGMP-mediated signal transduction pathway.<sup>45</sup> Through the activation of platelets, NO mediates VEGF-induced angiogenesis in coronary vessels and promotes blood clotting. Tumor-derived *NOS3* has been shown to promote tumor growth and metastasis by stimulation of tumor cell migration, invasiveness, and angiogenesis. As our data shows, *NOS3* was downregulated at D15.

## Conclusion

This is a valuable and informative study, with CRC patients receiving reovirus therapy. This study confirms that reovirus (commercially known as pelareorep) has a profound effect on gene expression in patients with *KRAS* mutated CRC. We have previously shown using the same transcriptome data set that immune related genes<sup>8,46</sup> and autophagy-related genes<sup>47</sup> are significantly upregulated upon reovirus treatment. Herein, we have further analyzed and reported the genes that are altered in seven important cellular pathways, including the RAS signaling pathway. With deference to genes that displayed significant alteration at multiple timepoints, it was observed that six genes were significant at the 48 hr, 8 were significant at D8, and 10 were significant at D15. Additionally, with respect to genes which were altered in multiple pathways, four genes were significant in the RAS Signaling pathway, four in the MAPK Signaling pathway, three in the EGFR Signaling pathway, six in the PI3K-Akt Signaling pathway, eight in the Angiogenesis pathway, five in the miR-targeted genes in lymphocytes pathway, and five in the Apoptosis pathway. Our transcriptome assay has shown that reovirus treatment causes meaningful alterations in gene expression which indicates that reovirus can be a successful therapy for *KRAS* mutated CRC. When studied at the transcript level, we are confident, upon comparison with the control data, that administering reovirus in combination with chemotherapy leads to the regression of tumor mediators. Our findings strongly indicate that reovirus treatment leads to immune responses, which include increased lymphocytic maturation and activity, increased levels of apoptosis, and a decrease in angiogenesis. Finally, our data show that while reovirus is propagating, *NF-κB*, *SOS1* and *RRAS* promote this immune response.

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## Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval for the version to be published, and agree to be accountable for all aspects of the work. Conception and design: SG, RM. Analysis and interpretation of data: EF, AS, RM. Writing, review, and/or revision of the manuscript: EF, AS, SG, RM. Administrative, technical, or material support: SG, RM. Study supervision: SG, RM.

## Disclosure

The authors declare that there is no conflict of interest.

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