Global gene analysis identifying genes commonly regulated by the Ras/Raf/MEK and type I IFN pathways

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A B S T R A C T
Oncolytic viruses exploit alterations in cancer cells to specifically infect cancer cells but not normal healthy cells. Previous work has shown that oncogenic Ras interferes with interferon (IFN) signaling to promote viral replication. Furthermore, inhibition of the Ras/Raf/MEK/ERK pathway at the level of Ras, MEK, or ERK was sufficient to restore IFN signaling. In order to identify genes that were commonly regulated by the inhibition of the Ras pathway and the IFN pathway, we treated NIH/3T3 cells that overexpress oncogenic Ras with the MEK inhibitor, U0126, or IFN-α for 6 h, and performed DNA microarray analysis (Gene Expression Omnibus accession number GSE49469). Here, we also provide additional information on the experimental and functional analysis of the genes responsive to U0126 and IFN.

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http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49469

Experimental design, materials and methods

Cells and reagents

Murine fibroblast cells (NIH/3T3) were obtained from the American Type Culture Collection and maintained in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Burlington, Ontario, Canada) with 10% fetal bovine serum (Cansera, Etobicoke, Ontario, Canada). RasV12 cells were generated as previously described [1]. Recombinant mouse IFN-α was purchased from PBL Interferon Source (Piscataway, NJ) and U0126 from Cell Signaling Technology (Danvers, MA).

RNA isolation

RNA was isolated from RasV12 cells treated with 20 μM U0126 or 500 units/ml IFN-α or treated with vehicle (DMSO) for 6 h. Total RNA was isolated using TRIzol Reagent (Life Technologies, Ontario, Canada), and then treated with DNase using TURBO DNA-free kit (Ambion, Ontario, Canada). PCR analysis verified that the TURBO DNA-free kit removed all detectable contaminating DNA.

DNA microarray analysis

Isolated total RNA was sent to the University Health Network (UHN) microarray facility (Toronto, Canada) for analysis using Affymetrix 430 2.0 mouse DNA microarrays. RNA quality was analyzed with the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) by the UHN. RNA integrity number was determined to be greater than 8.9 for all samples. Data from three biological replicates were analyzed using GeneSpring (v7.3, Agilent) and data was normalized to the median expression level of each gene.

Organism/cell line/tissue
NIH/3T3 mouse embryonic fibroblast cell line that overexpresses human oncogenic H-Ras (RasV12)

Sex
n/a

Sequencer or array type
Affymetrix Mouse Genome 430 2.0 DNA microarray

Data format
Raw

Experimental factors
Cells left treated with vehicle control (DMSO), U0126 (20 μM) or interferon (500 units/ml) for 6 h

Consent
n/a

Sample source location
n/a

Specifications

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Differential expression analysis

Probesets with greater than 2.5 fold change compared to the vehicle treated control were identified as being differentially expressed. A fold-change cut-off strategy was used to reduce the type II (False negative) error rate in order to identify all possible candidate genes. We identified a total of 1883 probesets upregulated with MEK inhibition and 1877 probesets upregulated with IFN-α treatment (Fig. 1). Of these probesets, 619 were commonly upregulated by both MEK inhibition and IFN-α treatment, and termed MEK-downregulated IFN-inducible (MDII) genes [2]. Here we also report that 2184 probesets were downregulated by U0126, 1656 probesets were downregulated by IFN-α, and 424 probesets were downregulated by both (Fig. 1).

Validation

The induction of nine genes representing genes identified by the microarray as being upregulated by IFN-α alone, U0126 alone, or both IFN-α and U0126 were validated by RT-qPCR, previously [2]. Using RT-qPCR, the expression changes of 5 additional genes were determined (Fig. 2), using the following forward (F) and reverse (R) primers: 

- Pycard F: ACAAAAGCGGCTCAAGTTACTGC, Pycard R: GCCAATCCCTTAAGGAGCA, Cd24a F: ACTGCAAGCAGGAAACTCTCT, Cd24a R: AACACC CAACTCCAGGGTGGAC, Gbp2b F: CCGAGAACCAGAGACATACC, Gbp2b R: GAGGACTGACAAGCAAGAGA, Pycard F: ACCGAGTGCTGATGCTT, Pycard R: CTCGCTCTGCTGCTT, Irf7 F: CCCAAGGAAGACCTGA, and Irf7 R: TAGACAAGCACAGCCAGA. Primers were validated according to previously published strategies [3]. Statistically significant changes in log2-transformed relative expression levels were determined using 1-way ANOVA followed by Tukey HSD post-hoc analysis, if significant, in R v3 [4].

The microarray analysis identified Pycard and Cd24a to be upregulated by U0126 only, BECN1 (Beclin1) by both U0126 and IFN-α, as well as Gbp2b (alias Gbp-1) and Irf7 by IFN-α only. The induction of Cd24a, Gbp2b, and Irf7 was validated by RT-qPCR while changes to Pycard and BECN1 were not (Fig. 2). Of the three validated genes, the expression of Cd24a and Irf7 did not significantly change by the combined U0126 and IFN-α treatment. However, Gbp2b, showed increased induction with the combined treatment. Subsequent analysis of the probesets annotated for BECN1 revealed that these probesets also aligned to the Cntd1 gene. Therefore, this gene was likely erroneously identified due to the cross-reactivity of the microarray probeset.

Gene function analysis

Gene ontology analysis and network analysis of genes that were either upregulated or downregulated by both U0126 and IFN-α treatment were analyzed by GeneMania [5]. To identify potential novel indirect interactions, ten additional associated genes were added to the network by GeneMania. Both upregulated and downregulated lists generated a highly networked set of genes based on co-expression, co-localization, physical interactions, and shared protein domains with only 1 gene not networked in the upregulated list (Fig. 3A) and 6 genes not networked in the downregulated list (Fig. 3B).

Analysis of gene function revealed novel potential alterations to signal transduction pathways and polysaccharide catabolism due to
downregulation of gene expression (Table 1). Additionally, there was enrichment of genes involved in morphogenesis in the upregulated gene list (Table 1). As a validation of this strategy to identify novel functions of IFN-α and U0126 treatments, we also identified “response to IFN” as significantly enriched, which is a function known to be associated with IFN-α treatment and U0126 treatment [1,3,9,10].

Conclusion

The chosen method of analysis by fold-change resulted in the identification of numerous novel genes upregulated and downregulated by both MEK inhibition and IFN-α treatment. As expected, we had a higher type I error rate resulting in two genes that did not show the response that was predicted by the microarray analysis. In addition to the genes that were upregulated by U0126 and IFN-α treatment [2], we have identified additional genes that are commonly downregulated by these treatments. Analysis of gene networks revealed tight networks suggesting that these genes are commonly regulated by other treatments and/or in other cell types.

Functional analysis revealed additional functions of these gene sets that could be analyzed in future investigations. The increase in morphogenic gene changes aligns with recent evidence that infection of the tumor vasculature by oncolytic viruses contributes to anti-tumor effects [6,7]. Specifically, oncolytic Vesicular stomatitis virus (VSV) targets tumor-specific vasculature in preference to normal vasculature [6]. Although the mechanism underlying the tumor-specific targeting of the vasculature by VSV has not yet been elucidated, the list of genes identified with functions relating to morphogenesis includes genes involved in vascular stability and remodeling, such as angiopoietin 1 (Angpt1), and endothelial-specific receptor tyrosine kinase (Tek) [8]. Therefore, it will be of interest to investigate whether destabilization of these genes by oncogenic Ras is exploited by oncolytic viruses for tumorspecific vascular disrupting effects.

Overall, we identified a significant overlap of transcriptional activity induced by oncogenic Ras/Raf/MEK/ERK inhibition and innate immune response stimulated by type 1 IFN.

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