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14. ABSTRACT

The overall goal of this grant is to establish the role of non-agonist PPAR-gamma ligands as potential therapeutic candidates for lung cancer. In this grant period, we have been able to identify a core gene set that is indicative of the inhibition of the phosphorylation of PPAR gamma in the setting of carboplatin treatment. Using gene set enrichment analysis, we have shown that p53 signaling and the DNA damage response is a key transcriptional target of inhibiting S273 phosphorylation. We have further shown genetically using lung cancer cell lines lacking p53, that p53 is an important mediator of ability of non-agonist PPAR-gamma ligands to sensitize lung cancer to DNA damaging agents. We have demonstrated a biochemical interaction between p53 and PPAR-gamma, which provides insight into the groups of patients for whom this combination therapy may benefit. We continue to make progress on the other aims of this grant, which aim to test this hypothesis in genetic animal models of lung cancer and to identify new partners for PPAR-gamma that may play a role in DNA repair.

15. SUBJECT TERMS

PPAR-gamma, lung cancer, gene expression, p53, thiazolidinedione

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INTRODUCTION

In the veteran population, lung cancer is the number one cause of cancer death. Our previous work demonstrated that ligands for the transcription factor PPARγ, the thiazolidinediones (TZDs), synergized with carboplatin treatment of lung cancer in vitro and in vivo. Unfortunately, the use of TZDs has declined as the adverse effects of these drugs have come to light. Recently, we have demonstrated that novel PPARγ ligands that lack any agonist activity, but inhibit phosphorylation of pS273 are effective anti-diabetic agents that lack many side effects caused by TZD treatment. To generate novel therapeutics with potential in lung cancer, we have explored the role of these non-agonist PPARγ ligands in cancer treatment. We have demonstrated that there is robust phosphorylation of PPARγ after carboplatin treatment in A549 cells, which can be inhibited by non-agonist ligands (NALs). These drugs are active in vitro and in vivo in genetic mouse models and xenografts. In this work, we are exploring the role of PPARγ ligands in lung cancer treatment by identifying biomarkers of activity, mechanisms of action and validating their role in genetic models of lung cancer. We have generated a gene set representative of the activity of these agonist ligands in combination with carboplatin. We have identified p53 signaling as a key mediator of the ability of these non-agonist ligands ability to sensitize cells to carboplatin. Given the broad importance of this pathway in the DNA damage response, we have also shown that non-agonist PPARγ ligands are able to sensitize lung cancer to a wide variety of DNA damaging agents, which further broadens the potential clinical applications of this line of inquiry. We anticipate this will develop a new avenue to combine anti-diabetic drugs and cancer therapy and will lead to a significant improvement in overcoming treatment resistance or chemoprevention of lung cancer death for veterans

KEYWORDS

PPAR-gamma; Lung cancer; DNA damage response; Thiazolidinediones; p53

ACCOMPLISHMENTS

Aim 1 / Major Task 1: Generate gene set altered by carboplatin treatment with and without inhibition of pS273 phosphorylation.

One of the goals of the project was to generate a gene set representative of the inhibition of pS273 phosphorylation after carboplatin treatment. To accomplish this task, we turned to Affymetrix gene expression profiling. To get the purest representation of this gene set, we generated fibroblasts from the brown adipose tissue of mice with the S273→A mutation or wild type *Pparg* (*Pparg*$^{KI/KI}$ or *Pparg*$^{+/+}$). We immortalized these cells using SV40 retrovirus. We treated these cells with increasing doses of carboplatin and demonstrated that this model recapitulates the effects of non-agonist ligand treatment. As seen in Appendix Figure 1A, there is a significant increase in markers of cell death including cleaved PARP1 and cleaved Caspase 3 in cells with the S273A mutation.

To assess the genetic changes induced by inhibiting the phosphorylation of PPARγ, we used unbiased gene expression profiling using Affymetrix arrays. RNA from *Pparg*$^{+/+}$ and *Pparg*$^{KI/KI}$ fibroblasts treated with 25μM carboplatin or saline were

harvested and hybridized to arrays. The resulting gene expression data was analyzed and unbiased hierarchical clustering revealed that the samples segregated according to genotype and then by treatment with carboplatin (Appendix Figure 1B). Examination of volcano plots revealed that at baseline, 187 genes were significantly downregulated more than 2 fold in the mutant cells, and 67 genes were upregulated. Upon treatment with carboplatin, the differences between the genotypes was much more prominent, with 395 genes downregulated in mutant cells and 215 genes upregulated. The greater difference in gene expression between these two genotypes upon treatment with carboplatin is consistent with the idea that S273 phophorylation is a critical event in response to carboplatin treatment, and inhibition of this phosphorylation by changing a single amino acid results in a profound change in the transcriptome of the cells.

We subsequently validated these gene expression changes using QPCR from cDNA prepared from wild type and mutant fibroblasts cultures in the presence or absence of carboplatin. We selected a group of genes that were at least 3-fold upregulated with an ANOVA p value <0.5. A total of 59 genes (excluding predicted genes and uncharacterized cDNAs) met these criteria and were analyzed (Appendix Figure 1C). Forty of these were significantly ($p<0.05$) regulated in separate experiments (chi-square $p<0.0063$) and multiple others trended towards significance.

To examine whether similar changes were seen in the lung cancer cell lines in which PPARγ phosphorylation has been inhibited, we examined the expression of these genes in other cell types to generate a core signature of PPARγ phosphorylation inhibition after carboplatin treatment. A core set of genes was generated based on their expression in multiple cell types with and without carboplatin treatment. A set of 12 genes that were upregulated in the S273A mutant and 11 genes that were downregulated in the S273A mutant were assessed in A549 cells treated with the non-agonist ligand SR1664 in combination with carboplatin (Appendix Figure 1D). Ten of the 12 upregulated genes were coordinately upregulated in A549 cells treated with SR1664 and carboplatin. Seven of the 11 genes were appropriately downregulated with SR1664 treatment with carboplatin, for a total of 17/23 genes appropriately regulated (chi square $p=0.0218$.) This core gene set represents gene expression based readout of the inhibition of PPARγ phosphorylation in response to carboplatin.

To assess for potential mechanisms of the increased sensitivity to genotoxic drugs, we performed Gene Set Enrichment Analysis using the microarray data generated from wild type and S273A mutant fibroblasts treated with carboplatin with the Hallmark gene sets (Appendix Figure 2A.) The most enriched gene set associated with S273A mutation was the p53 pathway (Appendix Figure 2B). Interestingly, several other pathways involving the DNA damage response were upregulated, including the UV response, and DNA repair pathways, although the FDR q-value was >0.05 for these sets. This analysis raises the intriguing possibility that the single amino acid change in the S273A knock in mutants that eliminates phosphorylation results in alteration of certain aspects of the DNA damage response.

Given the critical role that p53 plays in the response both to DNA damage and the initiation of apoptosis, we hypothesized that the interaction of p53 and PPARγ may play an important role in the ability of non-agonist PPARγ ligands to sensitize cancer cells to the cytotoxic effects of carboplatin. We are examining the effect of the rescue of p53 in p53 null cell lines as well as the effect of CRISPR mediated deletion of *TP53* from the A549 cell lines that are responsive to non-agonist ligand induced sensitization to carboplatin.

For this aim, we are still planning to analyze human samples for the gene set changes as described above, and are in the process of obtaining IRB approval to do so.

Aim 2 / Major task 1: Immunoprecipitation of PPARγ from cells treated with and without carboplatinum and non-agonist PPARγ ligands.

One of the goals of this project was to use an unbiased approach to purify a complex of phosphorylated and non-phosphorylated PPARγ in the presence and absence of carboplatin. We have made some strides in developing a good general immunoprecipitation condition to obtain PPARγ from these wild type and mutant fibroblasts as described above. However, we have been limited by the total amount of PPARγ obtainable from the cells, so we are in the process of scaling up our cell culture preparations to start from a larger pool of total cellular protein.

Aim2/ Major task 2: Assess protein complex members for function in response to carboplatin.

Although we are still working on our unbiased mass spectrometry approach to yield results, I had also proposed targeted immunoprecipitation experiments to explore potential candidates that may play a functional role in the sensitization of lung cancer cells to non-agonist ligands. Given the data presented above that the p53 pathway was the leading gene set that was changed in the S273A knock in fibroblasts treated with carboplatin, we examined whether there was a biochemical interaction between p53 and PPARγ. We performed immunoprecipitation of PPARγ from nuclear extracts of WT or mutant fibroblasts in the presence and absence of carboplatin. Immunoblotting for p53 demonstrates that the wild type PPARγ associates with p53 more closely than the S273A mutant (Appendix Figure 2C). This is true both in the presence and the absence of carboplatin. This suggests that phosphorylation of PPARγ stabilizes the interaction of PPARγ and p53, and that mutant PPARγ that cannot be phosphorylated is not able to associate with p53 as efficiently. We are verifying this finding in the setting of the A549 cells in combination with non-agonist ligands as well. These data represent a novel interaction between p53 and PPARγ, and have implications that PPARγ may modify p53 function in a variety of settings.

AIM 3 / Major task 1 and 2: Mouse studies with KRAS mouse and mouse studies with KRAS/LKB1 mice.

We are assembling our cohorts of mice with a large enough number to perform our experiments for both of these genotypes. We plan to induce tumors, treat mice and analyze the results in the upcoming year.

Training and Professional Development Opportunities

This project has provided a number of opportunities for training. I have had the opportunity to attend weekly clinical conferences as well as monthly research conferences. I was also able to present my work at the Spiegelman Lab group meeting, and get feedback from a group of scientists with expertise in a diverse group of fields. I met with Dr. Spiegelman, my mentor, at least twice a month to review data and discuss scientific issues and directions. An abstract based on this work was accepted as a poster presentation at the Keystone Symposium Conference: New Frontiers in Understanding Tumor Metabolism.

Dissemination of Results

At this point, the major dissemination has been the abstract presentation for the Keystone Symposium conference as discussed above. I also gave a seminar at the Obesity and Cancer Working group at Memorial Sloan Kettering Cancer Center.

Plans for Next Year

In the next year, I am planning to analyze the clinical samples after obtaining IRB approval. I will also scale up our protein production to isolate PPARγ complexes for analysis by mass spectrometry. Finally, I am planning to induce lung tumors and treat animals with carboplatin and assay the effects of inhibition on the phosphorylation of PPARγ on lung tumor sensitivity in vivo. These goals should complete the experiments outlined in the grant.

IMPACT

We have not yet published our findings related to this research, and thus the impact has been limited to date. However, we anticipate that there will be interest in the lung cancer community. Currently, there are limited options for patients with locally advanced or metastatic lung cancer. Although advances in immunotherapy have been made, only about 20% of lung cancer patients will respond to these types of treatments. Thus, there is an urgent need for ways to make conventional chemotherapy more effective. We anticipate that the ability to add an agent that works in a pathway that has been traditionally well tolerated will be well received. Furthermore, we are hopeful that by identifying biomarkers of efficacy (e.g. *PPARG* expression and wild type *TP53)*, we can specifically identify a group of patients who are most likely to benefit from combination therapy and thus maximize the likelihood of success in early phase studies.

This work may have implications for other disciplines as well. By showing the ability of these drugs to modulate PPARγ activity in lung cancer, we are hopeful that other cancer types where PPARγ is expressed (e.g. breast cancer, endometrial cancer, ovarian cancer,) may also be potential candidates for combination therapy. Furthermore, we hope that demonstration of the relative safety of these non-agonist ligands in cancer may propel them to be explored as anti-diabetic agents for metabolic disease as well. Our data describing the physical interaction between p53 and PPARγ is novel, and may suggest that PPARγ has a role in modifying p53 function in other tissues. It has been shown that p53 plays an important role in metabolism and adipose tissue function, and this may be modulated by PPARγ expression as well.

At this point, I do not have anything to report regarding potential impacts on technology transfer or on society beyond science and technology as a whole.

CHANGES/PROBLEMS

Changes in approach: Nothing to report

Actual or anticipated problems or delays: We have had some delays in a few areas which have pushed back our timeline from the SOW. For Aim 1, I am still in the process of obtaining IRB approval for the clinical samples, which has delayed completion. However, given that we have the gene set information already identified, analysis of these samples will still be able to be completed within the timeline of this grant.

For Aim 2, it has taken longer than anticipated to find appropriate conditions to immunoprecipitate an amount of PPARγ protein from these cells sufficient for mass spectrometric analysis. However, I am confident that with a large enough pool of starting material we will be able to achieve this goal. In the meanwhile, I have used a candidate approach informed by the gene expression analysis to identify p53 as a novel interacting factor which seems to play an important mechanistic role in sensitization of these cells to cytotoxic DNA damaging agents.

For Aim 3, we are still in the process of assembling our cohorts, which has taken a bit longer than outlined in the SOW. However, I believe we will still be able to complete analysis of these results within the timeline specified in the grant.

Changes that had impact on expenditures: We have come in under budget as most of the experiments outlined above involve overhead and supplies that are paid via my mentor's grant and institutional funding.

Changes in use of human/animals: Nothing to report

PRODUCTS

Publications, conference papers, and presentations.

Poster Presentation: Melin J. Khandekar, Alex S. Banks, Dina Laznik-Bogoslavski, James P. White, Jang H. Choi, Kwok-kin Wong, Ted Kamenecka, Patrick R. Griffin, Bruce M. Spiegelman. "Non-agonist PPAR-gamma ligands sensitize cancer cells to the effects of cytotoxic chemotherapy by inhibiting phosphorylation of Serine 273 and modulating the response to DNA damage." New Frontiers in Understanding Tumor Metabolism, Banff, Canada. February 2016

Invited Oral Presentation: Selective modulation of PPAR-gamma influences the response of cells to DNA damage and potentiates cytotoxic therapy. Obesity and Cancer Working Group Meeting. Memorial Sloan Kettering Cancer Center. New York, NY. March 21, 2016.

PARTICIPANTS

Name:	Melin Khandekar
Role:	PI
Nearest Person Month Worked	6 months
Contribution:	Performed all work described above
Funding Support	No change

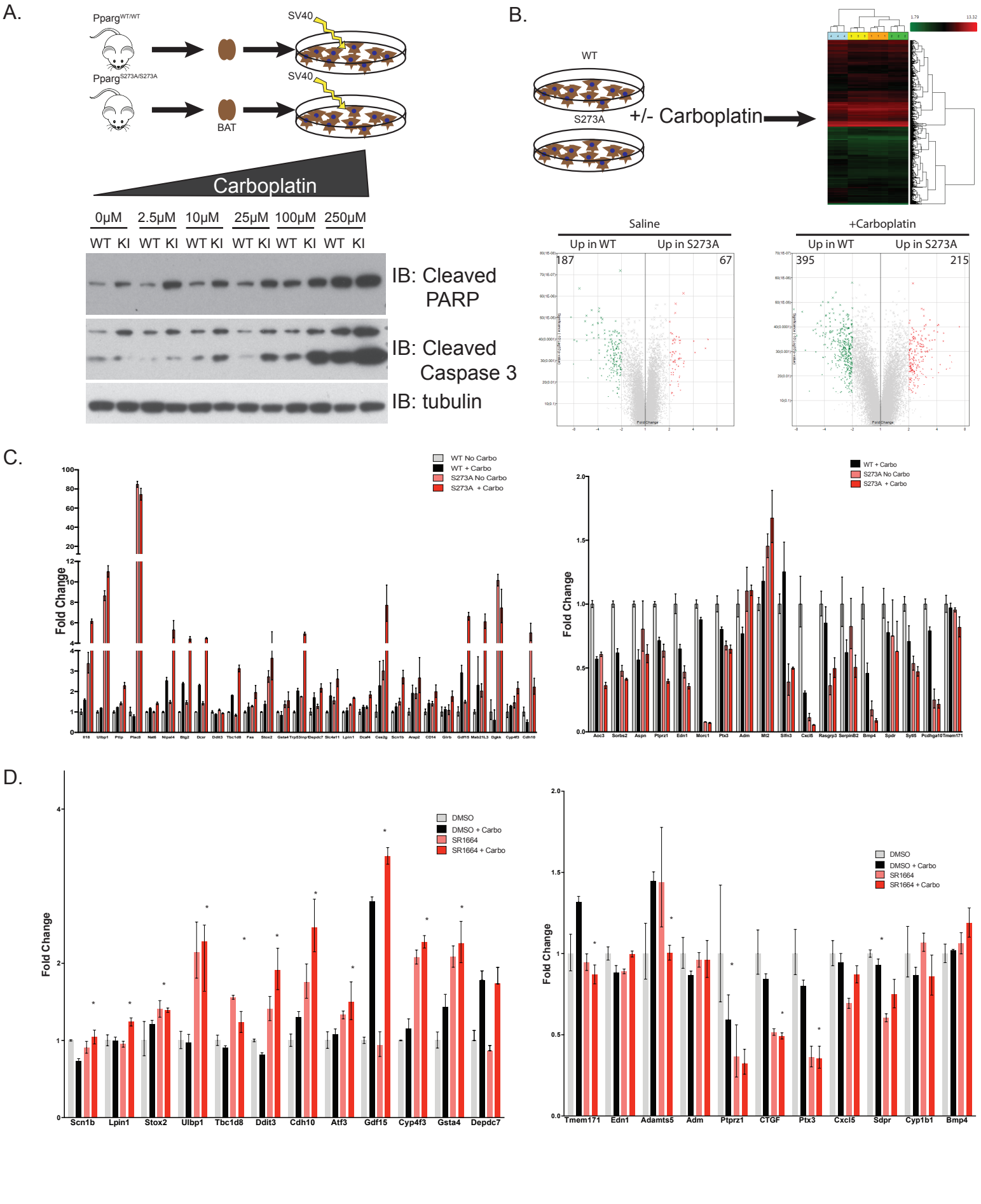
Name:	Bruce Spiegelman
Role:	Mentor
Nearest Person Month Worked	NA
Contribution:	Advised PI regarding experimental design, and analysis of experiments
Funding Support	No change

Other Partner Organizations

Name**:** Dana Farber Cancer institute
Location: Boston, MA
Contribution: Facilities and collaboration with mentor's laboratory staff

Name**:** The Scripps Research Institute Florida
Location: Jupiter, FL
Contribution: In kind support (reagents) and collaboration regarding non-agonist ligand dosing

APPENDIX FIGURE 1

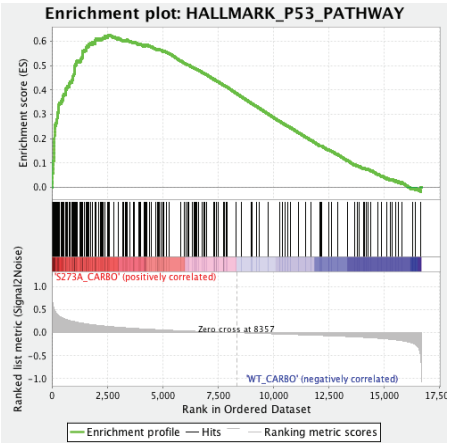


APPENDIX FIGURE 2

A

Hallmark Gene Set	Size	ES	NES	NOM p-val	FDR q-val	FWER p-val	Rank at Max
p53 Pathway	193	0.63	2.4	0	0	0	2603
Xenobiotic Metabolism	197	0.5	1.94	0	0	0.001	3248
Interferon Alpha Response	89	0.54	1.89	0	0	0.001	2533
Bile Acid Metabolism	110	0.47	1.69	0	0.007	0.033	3494
Apoptosis	155	0.42	1.58	0.001	0.026	0.152	1706
Heme Metabolism	182	0.38	1.47	0.006	0.071	0.437	4178
Interferon Gamma Response	195	0.38	1.46	0.008	0.072	0.495	2533
UV Response Up	143	0.38	1.43	0.014	0.08	0.564	3924
Reactive Oxygen Species Pathway	44	0.44	1.39	0.072	0.112	0.717	5660
IL6 Jak Stat Signaling	87	0.39	1.37	0.041	0.131	0.808	1861
Peroxisome	99	0.37	1.33	0.059	0.17	0.9	3355
Hypoxia	191	0.33	1.28	0.06	0.237	0.972	2131
DNA Repair	137	0.34	1.26	0.096	0.262	0.99	4835
Hedgehog Signaling	36	0.42	1.25	0.178	0.259	0.993	1874

B



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