Differential RNA Expression in Human Islet Cells under Polyamine Biosynthesis Inhibition

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BI-GY 7633: Transcriptomics

May 15, 2023

Introduction:

Type 1 diabetes (T1D) is a chronic autoimmune disease that results from the destruction of pancreatic beta cells. This results in the inability of those cells to produce insulin. Insulin is a hormone that canonically regulates glucose metabolism and in patients with T1D, its deficiency leads to hyperglycemia and proceeding diabetes complications. T1D is a very complicated pathogenesis as there are a variety of reasons that one can be diagnosed with it, however, recent studies have shown that cellular stress conditions can play a critical role in its development (Kulkarni *et al*., 2022). Those cellular stress conditions can be ER or oxidative stress. Focusing on ER stress, certain studies have shown that this specific type of stress can come about due to the presence of pro-inflammatory cytokines in and around beta cells. This then leads to the presence of pro-apoptotic factors which subsequently leads to beta cell death (Szegezdi *et al.*, 2006).

This study focuses on this ER stress due to proinflammatory cytokines and how it can possibly be relieved by inhibiting the biosynthesis of polyamines in beta cells. Polyamines are small, positively charged molecules that play an important role in a variety of cellular processes. They are found in all living organisms and are synthesized from numerous amino acids, such as ornithine, arginine, and lysine (Kahana, n.d.). Polyamines also play an important role in regulating gene expression and their expression levels can dictate toxic or safe cell environments. For this study, specifically, it has been shown in various pre-clinical models that polyamine biosynthesis inhibition results in a reduction of beta islet cell stress (Tersey *et al.*, 2014).

In this study, polyamine biosynthesis inhibition was completed by introducing difluoromethylornithine (DFMO) into the human beta cell environments. This chemical is an inhibitor of ornithine decarboxylase, an enzyme important in the creation of polyamines. In mouse models, the introduction of DFMO to beta islet cells has shown to decrease islet cell inflammation and reduce diabetes incidence by 50% (Tersey *et al*., 2014). Thus, using DFMO, we now seek to discover the effect of polyamine biosynthesis inhibition in human beta islet cells under normal and cellular stress conditions. Cellular stress conditions in this experiment were implemented using pro-inflammatory cytokines to simulate ER stress within a cell. It was hypothesized that inhibiting polyamine biosynthesis with DFMO in the presence of proinflammatory cytokines would curtail the stress response and rescue the normal, control islet cell phenotype.

To test this hypothesis, the experimental setup included 5 replicates of control human islet cells, 5 replicates of DFMO-treated human islet cells, 5 replicates of proinflammatory cytokine-treated human islet cells, and 5 replicates of both DFMO and proinflammatory cytokine-treated human islet cells (data was acquired from NCBI GEO database (Edgar *et al.*, 2002), accession GSE226888). From there, a transcriptomic analysis was completed. The analysis pipeline is discussed in the Methods section.

Methods:

The transcriptomics analysis was completed as follows. After loading the data, a counts and experimental design dataframe were made based on the factor type. Following this, the counts

were filtered first based on the median of each experimental design group being greater than 10. This step was completed to make sure that only the differentially expressed genes would be focused on. After the data was filtered, it was then normalized using DESeq 2's Median of Ratios method.

Analysis from DESeq 2 was completed in two ways. The first was by using DESeq 2 two factor analysis which would have involved creating a dataframe of treatment groups, one for DFMO being applied or not and another for proinflammatory cytokines being applied or not. This analysis was completed to find the interaction data between DFMO and proinflammatory cytokines and can be found in the R markdown file named "Final Markdown 2 Factor Test". The data was also analyzed by separating all the data into 3 separate subsets of the initial counts dataframe. This analysis can be found in the R markdown file titled "Final Project Markdown". These would analyze the control vs. DFMO, control vs. proinflammatory cytokines, and control vs. DFMO and proinflammatory cytokine conditions. For each database, the dispersion vs. mean of normalized counts was plotted and all differentially expressed genes between the aforementioned comparisons were found. This was done using DESeq2 and the outputs were three dataframes with p values and log fold changes for each of the genes. The genes with negative log p values greater than 1.13 (which correspond to p values less than 0.05) and log fold changes below -1 and above 1 were found and compiled into a separate dataframe as the differentially expressed genes for each condition. All the genes were also plotted in a volcano plot to visualize the spread of the p values and log fold changes.

Given the parameters for selection referenced above, these genes were significant and important. A hypergeometric test and GO term analysis was then conducted on these genes for each dataframe to see what type of genes were being differentially expressed. Pie charts of the GO terms were also created based on the processes that the GO terms best aligned with. The specific key words can be found in the Appendix. The categories created were "immune response associated", "stress response associated", and "other processes" to judge the change in number of genes with immune and stress response associations before and after DFMO inclusion.

After GO term analysis, clustering was also completed to see which genes had similar levels of expression. Using a silhouette plot, the number of clusters was determined. A dendrogram and heatmap were completed to visualize the differentially expressed genes based on factor type.

Results:

The results from the analysis pipeline can be seen below. Figures 1, 2, and 3 show the dispersion graphs used to check the integrity and reliability of the data. We see that in all three figures, the lines begin by descending and the mean of normalized counts increases, which leads to a flattened curve. Thus for all comparisons, the averages for all datasets must be relatively reliable.

Figures 4, 5, and 6 show the volcano plots of the control vs. DFMO, control vs. proinflammatory cytokines, and control vs. DFMO and proinflammatory treatments, respectively. The control vs. DFMO plot shows all genes are gray indicating that no genes had a log fold change greater than 1 or less than -1 and there were no genes with a significant p value. Figures 5 and 6, however show that when comparing control vs. proinflammatory cytokine treatment, there were a variety

of genes that were differentially expressed. In both Figures 5 and 6, the x axis was limited to bounds of -2 and 2 and the y axis was limited to bounds of 0 and 4. This was done to show the shape of the plot. This caused the exclusion of certain genes that had very high log fold changes and very low p values such as TAPSAR, NUMA1, and WARS. These genes were still included in subsequent analyses, but left out only for the presentation of the data.

Figures 7 and 8 show the corresponding heatmaps of control vs. proinflammatory cytokines and control vs. DFMO and proinflammatory cytokine treatments, respectively. With these graphs, a clear distinction can be seen in expression between the treatments and the controls for both factors.

Finally, Figures 9 and 10 show the hypergeometric test results as a GO term analysis to see the type of genes that were differentially expressed between the different treatment groups. Because control vs. DFMO had no differentially expressed genes, the GO term analysis could not be completed for that treatment. In Figure 9, we see that a large portion of the genes that are expressed are immune response associated genes and stress response associated genes. In Figure 10, we see this section increase in size based on the same search terms. Figures 9 and 10 also feature p values for the top 10 most significant terms of each comparison. As can be seen from the tables, most, if not all of the terms, are immune and stress response related, which show that immune and stress response-related genes are prevalent and significant.

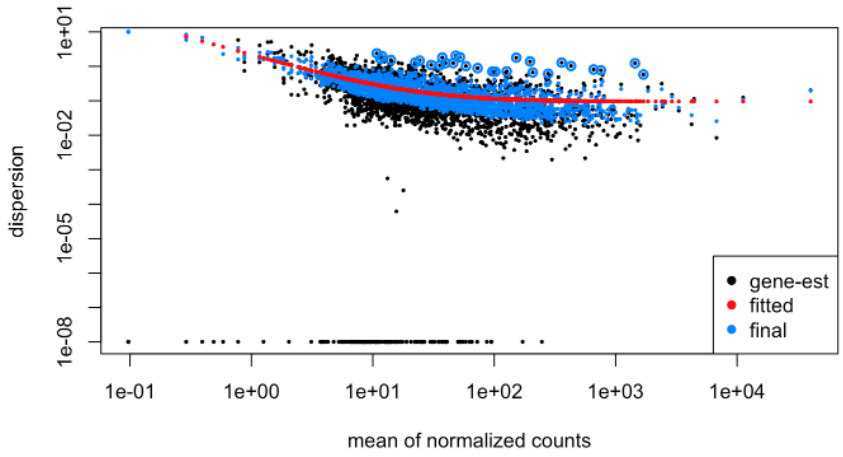


Figure 1. Dispersion plot of control vs. DFMO treatment

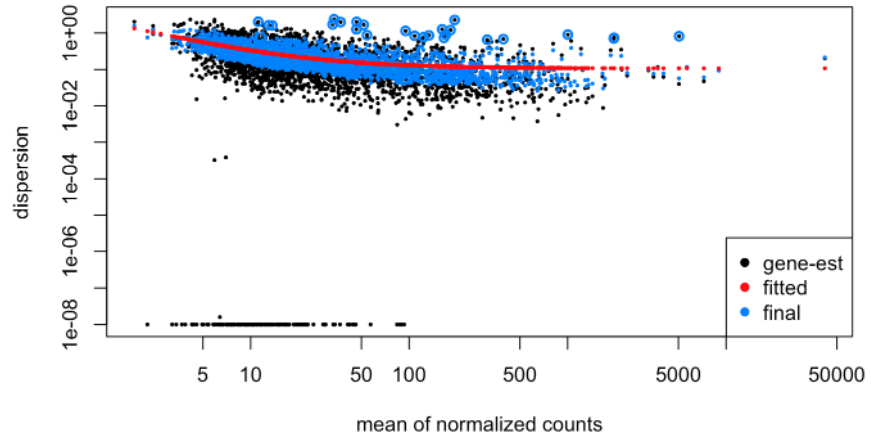


Figure 2. Dispersion plot of control vs. proinflammatory cytokine treatment

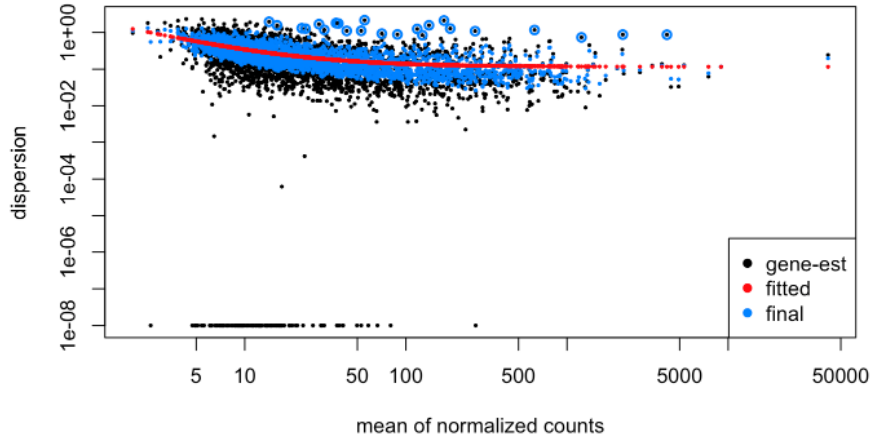


Figure 3. Dispersion plot of control vs. both treatments

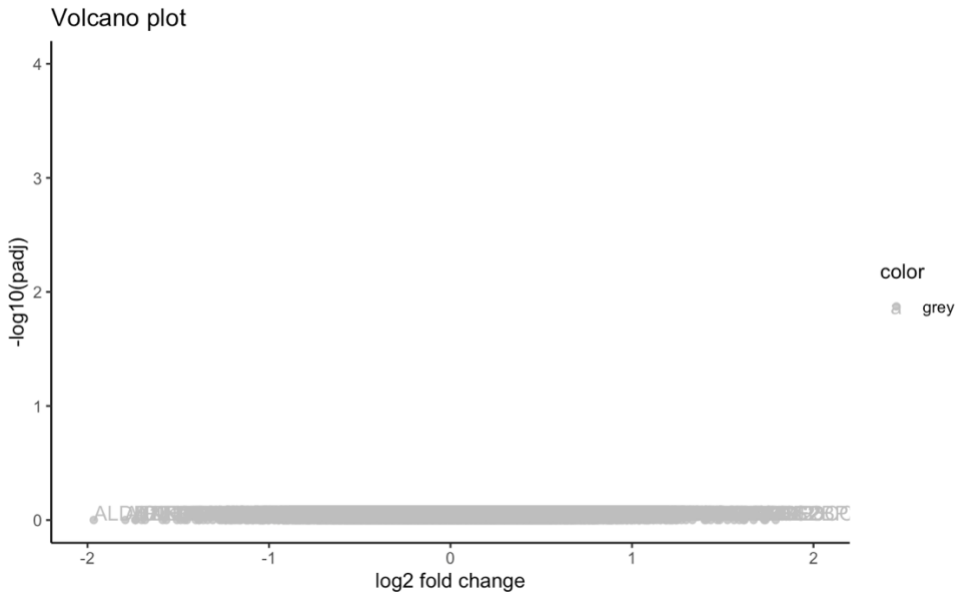


Figure 4. Volcano plot of Control vs. DFMO treatment. The grey represents all insignificant and unimportant genes.

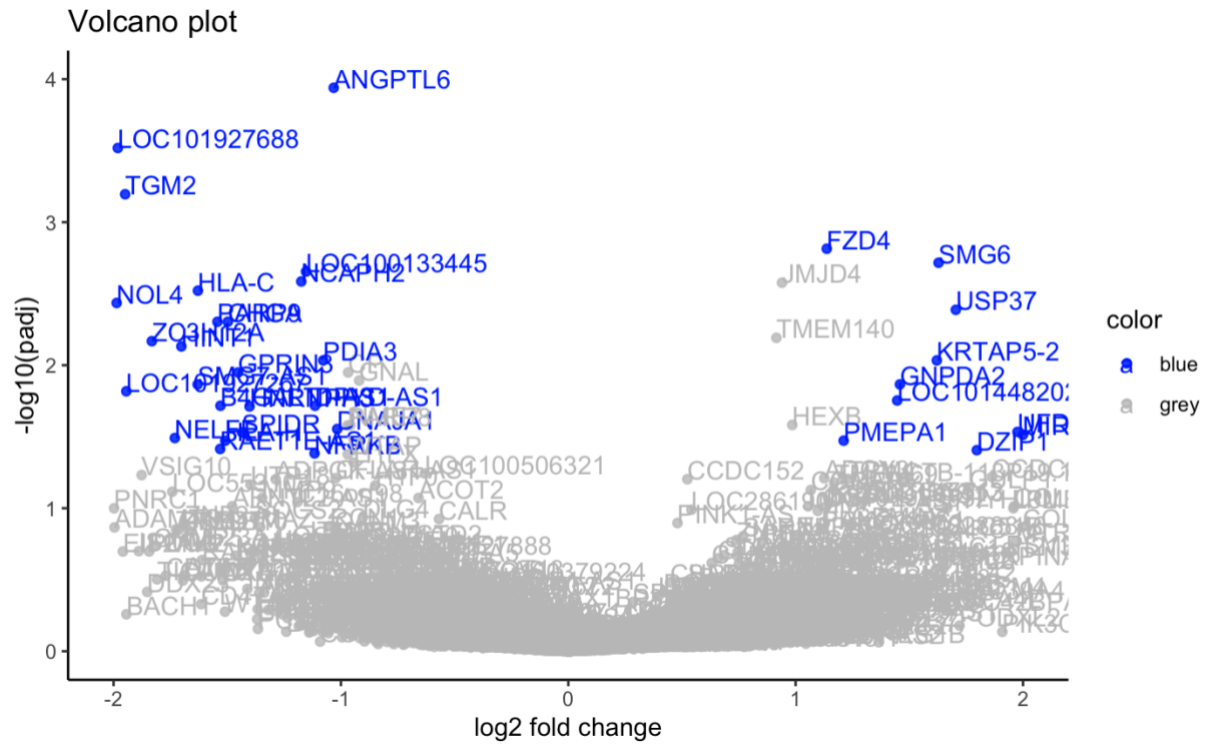


Figure 5. Volcano Plot of Control vs. Proinflammatory cytokines treatment. The blue represents all significant and important genes while the grey represents all insignificant and unimportant genes.

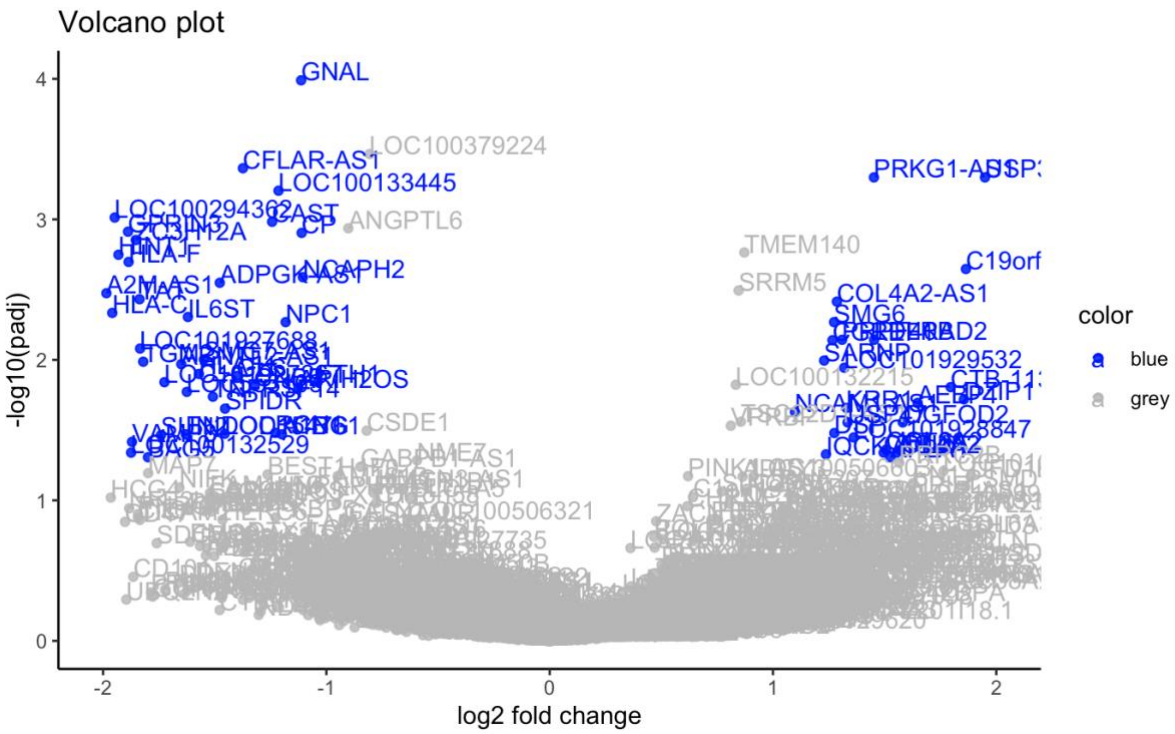


Figure 6. Volcano Plot of Control vs. DFMO and Proinflammatory cytokine treatment. The blue represents all significant and important genes while the grey represents all insignificant and unimportant genes.

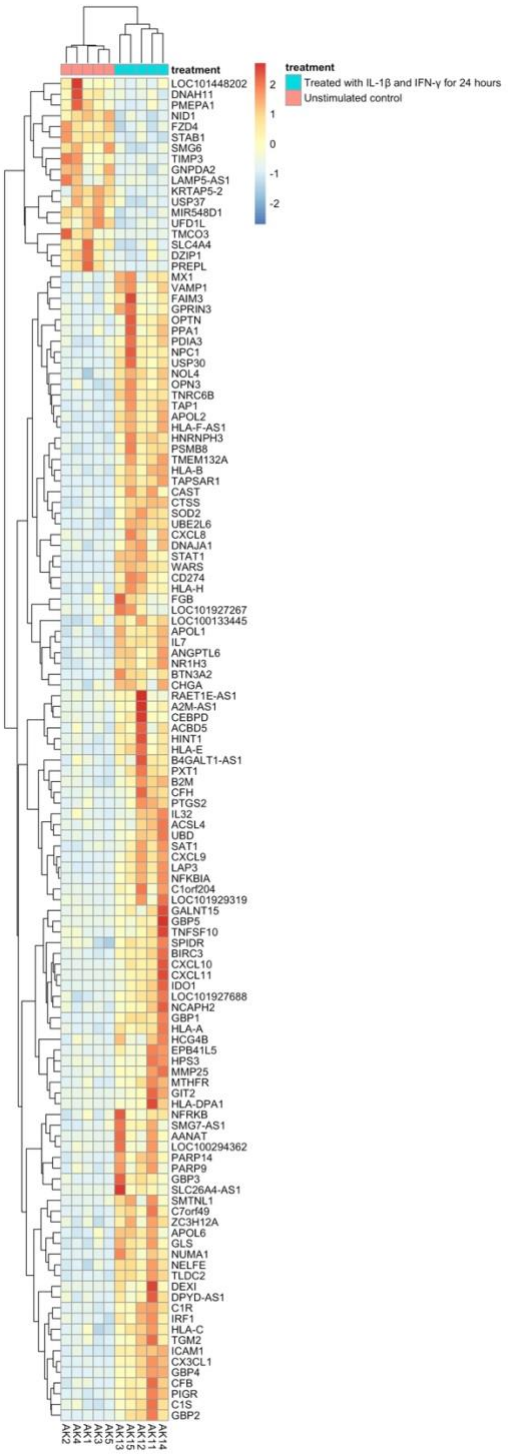


Figure 7. Heatmap of control and proinflammatory cytokine treatments.

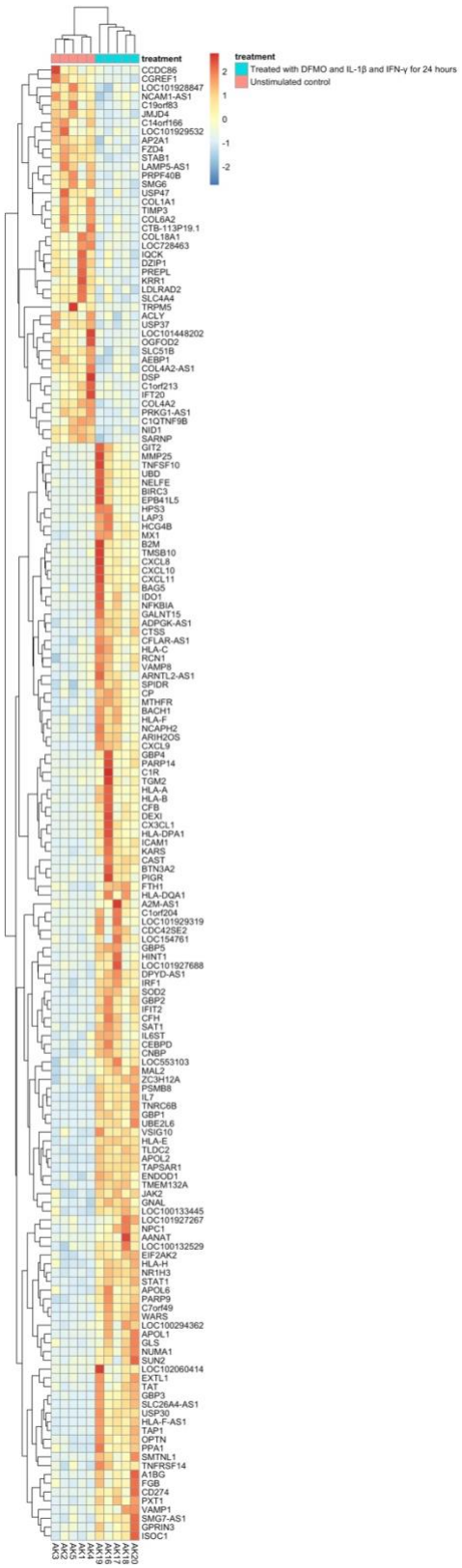
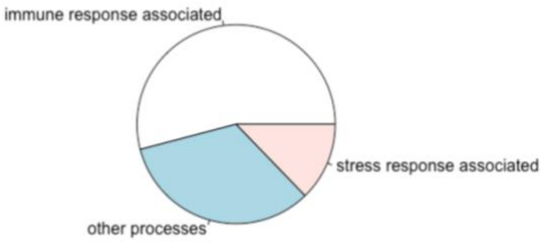
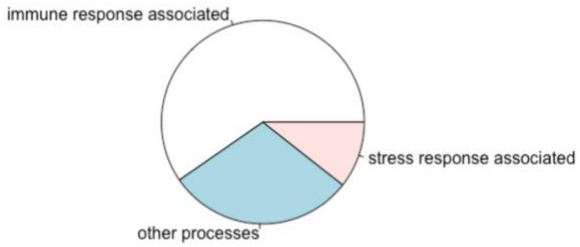


Figure 8. Heatmap of control and both treatments.



	GOBPID	Pvalue	Term
1	GO:0006955	1.114637e-20	immune response
2	GO:0051707	1.269427e-17	response to other organism
3	GO:0043207	1.367462e-17	response to external biotic stimulus
4	GO:0044419	1.458706e-17	biological process involved in interspecies interaction...
5	GO:0009607	3.452271e-17	response to biotic stimulus
6	GO:0002376	5.360037e-17	immune system process
7	GO:0034097	4.393935e-16	response to cytokine
8	GO:0098542	4.507235e-16	defense response to other organism
9	GO:0006952	1.311726e-15	defense response
10	GO:0045087	1.096300e-14	innate immune response

Figure 9. Pie chart of GO terms for differentially expressed genes of control vs. proinflammatory cytokines treatment. The table shows the most significant terms represented amongst the genes.



	GOBPID	Pvalue	Term
1	GO:0006955	8.182186e-22	immune response
2	GO:0098542	5.646930e-17	defense response to other organism
3	GO:0051707	6.067290e-17	response to other organism
4	GO:0043207	6.568828e-17	response to external biotic stimulus
5	GO:0044419	1.339839e-16	biological process involved in interspecies interaction...
6	GO:0009607	1.764416e-16	response to biotic stimulus
7	GO:0034097	1.826949e-16	response to cytokine
8	GO:0002376	3.487899e-16	immune system process
9	GO:0045087	2.168770e-15	innate immune response
10	GO:0006952	1.141696e-14	defense response

Figure 10. Pie chart of GO terms for differentially expressed genes of control vs. both treatments. The table shows the most significant terms represented amongst the genes.

Table 1. DESeq2 Two factored data analysis significant and important genes.

Result Type	**Number of Significant and Important Genes**
DFMO results	0
Pro-inflammatory cytokine results	119
DFMO and Pro-inflammatory cytokine results	0

Discussion:

This analysis has shown that, as per this experiment, DFMO presence in human beta islet cells does not have a similar effect as that seen in mouse models. This conclusion was reached by examining the figures and their relationship with one another. Figure 1, showing any possible differentially expressed genes between the control and DFMO treated cells, displayed no blue, therefore no differentially expressed, genes. Thus, DFMO had little to no effect in changing the phenotype of human beta islet cells. This is juxtaposed by the control vs. proinflammatory cytokine treatment which showed that introduction of proinflammatory cytokines changed the phenotype of the cell enough to show differentially expressed genes (as can be seen in Figure 2) as well as a majority of those differentially expressed genes being related to immune and stress responses (as seen in Figures 7 and 9).

After looking at the DFMO and proinflammatory cytokine factors, it raises the question of whether DFMO causes a different phenotype in cells depending on the initial condition of the cells. This was seen in the last experimental setup, control vs. DFMO and proinflammatory cytokines, to see if cells that were already in a stressed state responded to DFMO. As can be seen in Figure 6, there are a large amount of significant and important genes, which shows that unlike in the control vs. DFMO treatment group, there are some upregulated and downregulated genes present. From there, in Figures 8 and 10, we see a large portion of these genes are still associated with immune and stress response. In fact, the pie chart shows that an even larger portion of the genes are immune and stress response-associated. This shows that DFMO did not decrease the stress response phenotype and instead may have exacerbated it.

In Table 1, we can see that in the two factor analysis conducted, the only DFMO condition had no significant and important genes, the only cytokines condition had a showing of significant and important genes that were differentially expressed, and the DFMO and proinflammatory cytokines interaction showed no significant and important genes expressed. Thus, it can be seen that, as aforementioned, DFMO has no substantial effect on control cells or stressed cells. Therefore, the state of the cells did not affect the cells response to DFMO.

Based on these analyses, it can be concluded that the hypothesis is rejected. Inhibiting polyamine biosynthesis with DFMO in the presence of proinflammatory cytokines did not curtail stress response or rescue normal, control islet cell phenotype. The best course of action to confirm this is to repeat the experiment. This is because of three main reasons. First, because there is such strong of evidence of DFMO's influence in mouse model eukaryotic cells, there is a strong possibility that there could have been errors in the experiment conducted which then necessitates a repetition of the experiment. Second, Figures 7 and 8 are heatmaps of good quality, however, when looking at the numbers that the colors correspond to, most replicates of an experimental condition setup do not have consistent across their corresponding replicates. For example, in Figure 7, the OPTN has a very high value for its second proinflammatory cytokine treatment, however, in the other replicates of the same factor, it has middle level values, often at 0. This is simply one example of a trend that occurs overall in both Figure 7 and 8 heatmaps. Third, when the data filtering was completed, it was found that the amount of genes used for the analysis went from 25,000 to 3,000. In any data filtering process, this is quite a large number of genes to remove and therefore suggests that there must have been an error in the alignment process. Thus, it is imperative to repeat this experiment to get more accurate and precise count data.

We can also look at more questions to understand the role of cellular stress and its involvement in the incidence of diabetes. While this experiment focused on ER stress, oxidative stress can also be examined. Using different chemicals to inhibit polyamine biosynthesis can also be examined, such as guanylhydrazone, which is also a single enzyme inhibitor of polyamine biosynthesis (Wallace & Fraser, 2004).

Sources:

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Tersey, S. A., Colvin, S. C., Maier, B., & Mirmira, R. G. (2014). Protective effects of polyamine depletion in mouse models of type 1 diabetes: implications for therapy. *Amino acids*, *46*(3), 633–642. https://doi.org/10.1007/s00726-013-1560-7

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Appendix:

The search terms used to filter for "immune response" in Figures 9 and 10 were as follows: immune, cytokine, MHC, antigen, defense, T cell, immunity, interleukin, lymphocyte, neutrophil, leukocyte, virus, interferon-gamma, and chemokine.

The search terms used to filter for "stress response" in Figures 9 and 10 were as follows: apoptosis, cell death, stress, cytotoxicity, external stimulus, cell killing, and apoptotic.