A Genome-wide siRNA Screen Identification of Proteins Involved in the Modulation of ER Stress

Jody Groenendyk[1], Marcin J Mizianty[2], Lukasz Kurgan[2] and Marek Michalak[1]

Department of Biochemistry[1] and Electrical and Computer Engineering[2], University of Alberta, Edmonton, Alberta, T6G 2H7

Michalak Lab

Abstract

Endoplasmic reticulum (ER) stress has recently been identified to be significantly involved in numerous disease states and conditions and therefore presents a unique opportunity for therapeutic treatment due to the unique capacity of the ER to control cellular growth and death. Here we used a genome-wide siRNA screen to categorize the role of ER stress, ER signaling and the common functions of the ER. We utilized an Ambion mouse drug-able library composed of over 6000 genes, with determination of the relationship of these three key areas allowing us better understanding of disease state biology leading to the development of therapies to treat and prevent cancer and other diseases. The focus of this project was on ER stress responses dependent on ER calcium emptying and perturbation of oxidative stress. Preliminary results demonstrated the importance of post translational modifications, protein transport and degradation and apoptosis as well as the critical role of metabolism in the maintenance of ER stress. To identify an effect on ER stress, we utilized a luciferase reporter assay based on IRE1α activity, with an increase in endonuclease activity leading to splicing and read-through of the reporter luciferase. siRNAs were used to induce sequence-specific gene silencing in mouse fibroblast cells to study mammalian gene function and its effect on ER stress. We adapted chemical reverse transfection for an adherent cell line in a 96-well format and screened a synthetic siRNA library that targeted over 6000 mouse genes to identify genes that when silenced either increased ER stress or decreased ER stress. This screen yielded over 800 candidate genes that are in the process of validation. Upon specific validation, gene interaction networks were generated and specific biochemical experiments were performed to authenticate the pathway effect on ER stress. Recent evidence suggests the important involvement of ER stress in oncogenesis and due to the fact that calreticulin performs a crucial role in maintaining ER folding and calcium homeostasis, thereby avoiding ER stress, our findings may have a direct effect on the development or diagnosis of cancer.

Supported by CIHR

XBP1 splicing reporter

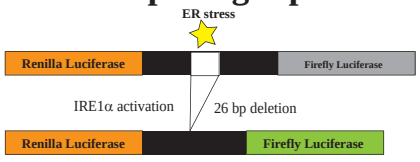
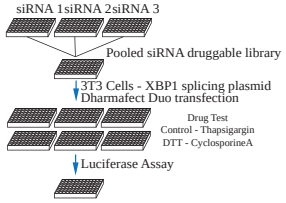


Figure 1 - XBP1 Splicing Reporter.
Upon ER stress, an endoribonuclease, IRE1 cleaves a 26 bp fragment from XBP1 mRNA, generating an active transcription factor. In the case of the reporter plasmid, this cleavage generates a frameshift in the reporter plasmid, bringing Firefly luciferase into frame. Renilla luciferase is used to normalize for tranfection efficiency.

siRNA library screen using XBP1 splicing reporter



Primary Screen

Figure 2 - siRNA Library Screen.
Mouse Druggable Library (~6000 genes) with pooled siRNA composed of three siRNA per gene. 3T3 Fibroblasts were transfected with the XBP1 splicing plasmid to monitor ER stress induced using Thapsigargin, Dithiolthreitol (DTT) or Cyclosporine A.

Normalization

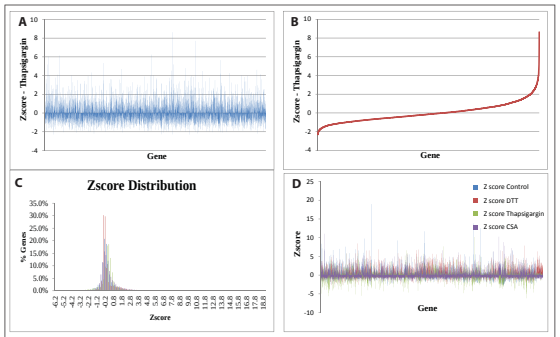
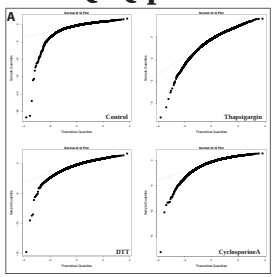


Figure 3 - Normalization of Raw Luciferase Data.
Luciferase light units were normalized to the internal plate standard and the experimental standard deviation. A) Zscore analysis of the thapsigargin treatment. B) Distribution of the Zscore analysis of all the treatments (Control, Thapsigargin, DTT, CSA). C) Zscore distribution of the treatments, Control, Thapsigargin, Cyslosporine A and Dithiolthreitol. D) Zscore analysis of all the treatment conditions. DTT, Dithiolthreitol, CSA, Cyclosporine A.

Plate Summary

Figure 5 - Plate Summary of siRNA Library Screen.
Zscore analysis was mapped to the library, demonstrating the significant hits generated in each plate. Blue indicates genes that when silenced resulted in an increase in ER stress, red indicates genes that when silenced resulted in a decrease in ER stress.

Q-Q plot



Distribution

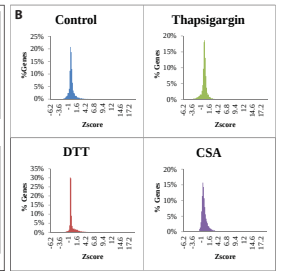


Figure 4 - Zscore Analysis with Q-Q plot and Distribution Graphs.
A) Zscores were displayed using Q-Q plot, demonstrating the relative fitness of the experimental conditions. Sample quantiles were plotted against theoretical quantiles. Deviations from the line indicate non-normally distributed values. B) Distribution graphs show the Zscore centering around 0 with long tails. The density of the data centering around 0 demonstrates the majority of the genes were not statistically significant. Density was plotted versus the Zscore.

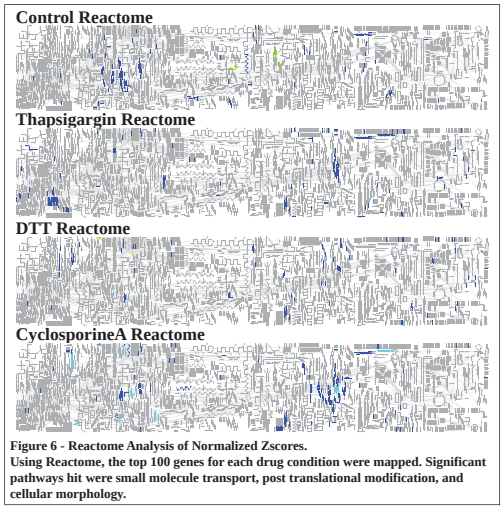


Figure 6 - Reactome Analysis of Normalized Zscores.
Using Reactome, the top 100 genes for each drug condition were mapped. Significant pathways hit were small molecule transport, post translational modification, and cellular morphology.

Statistical Analysis

The raw data were subjected to rudimenary statistical analysis using cellHTS, including:
- plate specific normalization

Followed by byzantine analysis:
- Zscore calculations:
  Z = (x - mean)/SD
- two sided significance:

pvalue<0.001 with selection of the top 100 hits for each drug condition analyzed for validation

Putative Novel Hits

Thapsigargin Treatment
-ACAA1 - acetyol-CoA acyltransferase 1
- ADCY8 - adenylate cyclase 8 (brain)
- CSTB - cystatin B
- MMP7 - matrix metallopeptidase 7
- SLC7A3 - solute carrier family 7, member 3

Dithiolthreitol Treatment
- BEST2 - bestrophin 2

Cyclosporine A Treatment
- CD6 - CD6 molecule
- PTGS2 - prostaglandin synthase 2

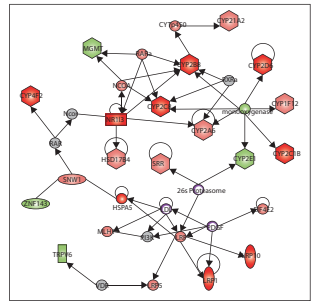
Validation

- Repetition of experiment using Dual Signal Luciferase assay with top 400 genes.
- siRNA followed by qPCR and Western Blot of downstream genes.
- identify an inhibitor for the selected hits and quantitate the effect on ER stress.
- follow-up experiments to elucidate the role of the proteins in the ER stress pathway.

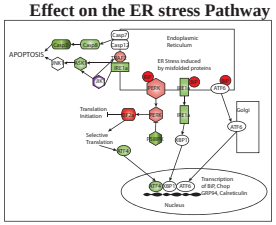
Informatics Analysis:

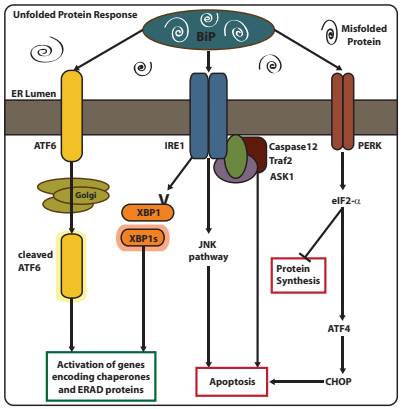
CellHTS, IPA, InGenuity, David Database, Reactome, GeneMania, Cluster

Thapsigargin Treatment - Molecular Transport



Thapsigargin Treatment - Effect on the ER stress Pathway





Conclusions

- **ER stress is a unique phenomenon where a cell utilizes this pathway to recover homeostasis as well as signal apoptosis**
- **Using an XBP1 splicing reporter that monitors ER stress, we have identified new participants in the ER stress pathway.**
- **ER stress presents a novel area of research in the treatment of numerous diseases including cardiovascular disease, cancer and diabetes.**

Funded By:

AstraZeneca · Heart & Stroke Foundation of Canada · Alberta Innovates Health Solutions · CIHR IRSC