Coordination analysis of gene expression points to the relative impact of different regulators during endoplasmic reticulum stress

Youwen Zhang[1], Matthew D. Lucius[1], Diego Altomare[1], Amanda Havighorst[1], Elena Farmaki[1], Ioulia Chatzistamou[2], Michael Shtutman[1], and Hippokratis Kiaris[1,3]

[1]Department of Drug Discovery and Biomedical Sciences, College of Pharmacy, University of South Carolina, SC, USA.
[2]Department of Pathology, Microbiology and Immunology, School of Medicine, University of South Carolina, SC, USA.
[3]Peromyscus Genetic Stock Center, University of South Carolina, SC, USA

Analysis of gene expression can be challenging, especially if it involves genetically diverse populations that exhibit high variation in their individual expression profile. Despite this variation though, it is conceivable that in the same individuals a high degree of coordination is maintained between transcripts that belong to the same signaling modules and are associated with related biological functions. To explore this further, we calculated the correlation in the expression levels between each of ATF4, CHOP (DDIT3), GRP94, DNAJB9 (ERdj4), DNAJ3C (p58IPK) and HSPA5 (BiP/GRP78) with the whole transcriptome in primary fibroblasts from deer mice following induction of endoplasmic reticulum (ER) stress. Since these genes are associated with different transducers of the unfolded protein response (UPR) we postulated that their profile, in terms of correlation of transcripts, reflects distinct UPR branches engaged and therefore different biological processes. Standard gene ontology analysis was able to predict major functions associated with the corresponding transcript, and of the UPR arm related to that, namely regulation of the apoptotic response by ATF4 (PERK arm) and the ER stress associated degradation for GRP94 (IRE1). BiP, being a global regulator of the UPR, was associated with activation of ER stress in a rather global manner. Pairwise comparison in the correlation coefficients for these genes' associated transcriptome showed the relevance of selected genes in terms of expression profiles. Conventional assessment of differential gene expression was incapable of providing meaningful information and pointed only to a generic association with stress. Collectively this approach suggests that by evaluating the degree of coordination in gene expression in genetically diverse biological specimens may be useful in assigning genes in transcriptome networks and more importantly in linking signaling nodules to specific biological functions and processes.

Introduction

Signaling networks respond to stimuli by modulating the expression of a multitude of genes. This activation proceeds by high quantitative variation and for certain transcripts it can be high while for others it can be minimal, yet both highly and marginally regulated transcripts are equally important for the

production of the desired cellular response. This variation in the magnitude of the response increases if biological samples from genetically diverse populations are analyzed. Correlation clustering had been widely used for the discovery of the group of genes show similar expression pattern under different conditions (Ben-Dor et al, 1999; Ng et al, 2006). Such approaches have been used to define population structures and to identify variation in expression profiles between different groups (Brown et al, 2018; Wei et al, 2011) or to reveal disease-related genes (Cai et al, 2017; Tai et al, 2017).

The utilization of genetically diverse organisms allows applying such analysis to experiments performed on the same conditions where the diversity of the animals is projected to the diversity of the output evaluated. Despite the variation in the intensity of the response among individuals, it is conceivable that a high degree of coordination is maintained between targets that belong to the same signaling cascades (Komili & Silver, 2008). To that end it is conceivable though that co-regulated transcripts, due to their participation in the same networks will exhibit higher coordination than with those associated with other signaling modules and that identification and analysis of coregulated transcripts may convey information regarding the function regulated by the corresponding signaling module. Furthermore, by focusing on the degree of coordination as opposed to fold induction, even minimal albeit impactful differences in gene expression, should be unveiled and appreciated. In the present study we sought to exploit these hypotheses by testing if the degree of coordination in gene expression, rather than the magnitude of overexpression, bears information more valuable in assessing signal integration and biological function.

As a model for the transcriptional analyses we used the induction of endoplasmic reticulum (ER) stress following exposure of fibroblasts to tunicamycin. ER stress is defined as the state of the cells at which protein production exceeds the capacity for protein folding and therefore results in the accumulation of misfolded and unfolded proteins (Walter & Ron, 2011; Almanza et al, 2018). ER stress, for its resolution, inflicts the unfolded protein responses (UPR) that is mediated by 3 major transducers, IRE1, ATF6 and PERK, each of which results in the activation of well-defined downstream targets (Fu & Gao, 2017; Hetz et al, 2017; Lemus & Goder, 2014; Szegezdi et al, 2006). Despite certain redundancies in the regulation of the downstream mediators, the activities and the molecular determinants of the corresponding 3 branches of UPR remain well defined, representing an appropriate system to study the integration of signals associated with different transcriptional nodules, into an overall cascade of a well-orchestrated response.

The analyses were performed in primary cultures of fibroblasts from genetically diverse (outbred) *Peromyscus* (deer mice) (Havighorst et al, 2017). Recently we reported that major UPR targets such as chaperones BiP, GRP94 and calnexin are highly coordinated in fibroblasts from outbred deer mice (P. maniculatus) (Havighorst et al, 2019). The utilization of an outbred species for this analysis allows assessment of the variation in gene expression in the context of the naturally existing diversity and within what should be considered as physiological range.

Materials and Methods

Animals. Deer mouse, *Peromyscus maniculatus bairdii* (BW Stock) was closed colony bred in capitivity since 1948 and decended from 40 ancestors wild-caught near Ann Arbor, Michigan. Sonoran deer mouse, *Peromyscus maniculatus sonoriensis* (SM2 Stock) was derived from about 50 animals wild-caught by Jack Hayes in 1995 near White Mountain Research Station, CA (Havighorst et al, 2017). In this study, we picked 3 outbred BWs, including 2 males and 1 female. They were 4-week old at weaning. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and the Department of Health and Human Services, Office of Laboratory Animal Welfare, University of South Carolina (Approval No. 2349-101211-041917).

Cell culture. Fresh ear punches were collected from deer mice during routine weaning and marking procedures. Ear punches were washed for 2 minutes in 70% EtOH and moved to RPMI-1640 medium (HyClone) supplemented with 10% FBS (Gibco), 500 u/mL Penicillin and 500 uL/mL L-Glutamine (complete RPMI). Ear punches were minced into small pieces and then digested by collagenase I (5 mg/ml in RPMI-1640) for 1 hour. Tissue debris from digested ear punches was removed once cells were visible. Cells were cultivated in complete RPMI at 37°C in a humidified incubator containing 5% CO_2, and passaged when cells were at 90% confluency or above to 45% confluency or above. Cells were passaged no more than 7 times before tunicamycin treatment. For tunicamycin treatment, cells were split into 6-well plates, 300,000 cells/well, and cultivated for 24 hours. Then cells were treated with tunicamycin (5 μg/mL) for 5 hours, immediately followed by RNA extraction. The analysis was performed in 3 pairs of fibroblasts, all obtained from different animals that resulted in 6 datasets (3 treated and 3 untreated).

RNA sequencing. RNA and library preparation, sequencing, and post-processing of the raw data and data analysis were performed by the USC CTT COBRE Functional genomics Core. RNAs were extracted with Qiagen RNeasy Plus Mini kit as per manufacturer recommendations (Qiagen, Valencia, CA, USA). RNA integrity was assessed using the Agilent Bioanalyzer and samples had a quality score >8.0. RNA libraries were prepared using established protocol with NEBNExt Ultra II Directional Library Prep Kit for Illumina, (NEB, Lynn, MA). Each library was made with one of the TruSeq barcode index sequences and samples were sequenced across three lanes. The pools were clustered at 6.5 pM on a pair end read flow cell and sequenced for 300 cycles on an Illumina NextSeq. Sequences were aligned to the Peromyscus maniculatus genome using STAR v2.6.1 (Dobin et al., 2013). Reads were counted using the featureCounts function of the Subreads package (Liao et al., 2013) in R (https://www.R-project.org/) using Gencode M6 GTF (http://www.gencodegenes.org/mouse_stats/archive.html) and summarized at exon, transcript, or gene level. Only reads that were mapped uniquely to the genome were used. Mapping quality (MAPQ) minimum threshold was set at 10. Differential expression analysis was performed in R using the edgeR package (Robinson et al., 2010). The average read depth for the samples was 48 million reads and only genes with at least one count per million average depth were considered for differential expression analysis. Raw counts were normalized using the trimmed mean of mvalues (TMM)

method. Dispersion estimates were then calculated using the estimateGLMRobustDisp function (Zhou et al., 2014). The normalized read counts were then fitted to a generalized linear model using the function glmFit (McCarthy et al., 2012). Genewise tests for significant differential expression were performed using the function glmLRT. The P-value was then corrected for multiple testing using Benjamini-Hochberg's FDR (Benjamini and Hochberg, 1995). The results have been deposited in GEO (GSE131429).

Correlation clustering. The Pearson's correlation was calculated between the whole transcriptome as obtained by the RNAseq analysis, and the UPR-associated transcripts indicated, by using Excel. Subsequently, all transcripts were sorted according to their R value with the given UPR genes and all exhibiting $P<0.05$ (Pearson's) were introduced in the Gene Ontology Enrichment analysis (http://cbl-gorilla.cs.technion.ac.il/). Both transcripts showing positive correlation only (Table 1) or positive and negative correlation (Suppl. Table 1) were analyzed. The process and analysis followed is shown in Figure 1.

Results and Discussion

By using 3 pairs of fibroblasts from different *P. maniculatus* animals that were treated with tunicamycin we performed RNA sequencing that revealed expression of 14,159 transcripts. The upregulation of six selected UPR targets, as revealed by the RNAseq analysis, indicates the canonical activation of the UPR under these conditions (Suppl. Fig. 1). Subsequently, we determined the correlation of the 14,158 genes with each of ATF4, CHOP (DDIT3), GRP94, DNAJB9 (ERdj4), DNAJ3C (p58IPK) and HSPA5 (BiP/GRP78) and then calculated the correlation of the corresponding R (Pearson's) values for all pairwise comparisons. These genes represent targets of different branches of the unfolded protein response (UPR): ATF4 is regulated by PERK (Harding et al, 2003) while CHOP by both PERK (in a manner that is ATF4-dependent) and by ATF6 (Zinszner et al, 1998; Ye et al, 2000). GRP94 is regulated by IRE1 (Yoshida et al, 2001; Merzec et al, 2012) but also by ATF6 (Yamamoto et al, 2007; Shoulders et al, 2013). DNAJB9 and DNAJ3C have been recognized as XBP-1 specific targets (Lee et al, 2003) while BiP (HSPA5) is induced by ATF6 but then it plays a nodal role in orchestrating globally the UPR .

Calculation of the correlation of the transcriptome coordinated with each of the UPR-associated genes analyzed, in all pairwise comparisons, showed similar –yet distinct– profiles. BiP, being a global transducer of the UPR was highly coordinated with all UPR targets while CHOP exhibited the most deviant profile (Figure 2). This is not surprising considering that CHOP is activated by several stress inducing signals, beyond UPR (Jauhiainen et al, 2012). CHOP and ATF4 that are co-regulated following stimulation of the PERK branch of the UPR showed tight coordination with each other exceeding that obtained by CHOP and the other UPR targets analyzed. DNAJB9 and DNAJC3 that are XBP1 targets showed high coordination with all UPR targets and especially with BiP.

These observations imply that interpretation of the degree of co-ordination between specific transcripts and the whole transriptome may be useful in assigning genes into networks, signaling nodules and biological

processes. It is noted though that UPR is a highly integrated response at which different branches of the UPR crosstalk and mask linear and causative associations between specific targets. For example, BiP that is an ATF6 target globally activates all branches of the UPR while XBP1 is regulated by both IRE1 (at the level of splicing) and by ATF6 (at the level of transcription). Yet, we hypothesized that the impact of particular targets and their association with specific biological processes may be revealed.

Based on these notions we explored if the identification of the array of genes that are mostly correlated with each of these UPR-associated genes can predict biological processes that are known to reflect activation of corresponding UPR branches and reveal their corresponding impact in specific processes. The identification of signaling networks and associated biological processes was performed by using the gene ontology online platform (Eden et al, 2007; Eden et al, 2009) at which the list of genes that showed significant ($P<0.05$, Pearson's) with either of GRP94, ATF4, CHOP, DNAJB9 (ERdj4), DNAJ3C (p58IPK) or HSPA5 (BiP/GRP78) were considered hierarchically, in a progressively decreasing manner. For this analysis we considered both genes exhibiting positive correlation only (Table 1 and suppl. Fig. 2 and 3) and genes exhibiting positive and negative correlation (suppl. Table 1 and suppl. Fig. 4 and 5). With GRP94, 1178 genes exhibited significant positive correlation, 1010 with CHOP (DDIT3), 1198 with ATF4, 1182 with BiP, 1250 with ERdj4 and 1202 with p58IPK (for all $P<0.05$, Pearson's). Relevant biological processes are shown in Table 1 at which all functions unveiled, along with the corresponding statistical significance is indicated. Similar results were obtained when both positively and negatively transcripts were analyzed (Suppl. Table 1).

For BiP, as expected, the response to ER stress was predicted to be the major process unveiled, underscoring its role as the nodal activator of the UPR (Table 1). For GRP94, besides the cumulative response to ER stress, major functions that had been identified are also protein catabolism, endoplasmic reticulum-associated (ERAD) degradation, and transcriptional activation, which are all established processes that are linked to the IRE1 arm of the UPR (Chiang et al, 2012). We note that earlier studies utilizing either qualitative changes in gene expression or chemical activators showed that in the absence of IRE1 and its target XBP1, but not in the absence of ATF6, MEFs can induce GRP94 which points to the important role of ATF6 in GRP94 expression (Yamamoto et al, 2007; Shoulders et al, 2013). This association we also noted since GRP94 was identified as an ATF6 target (Table 1). Yet, with such qualitative alterations the relative impact of each of these upstream regulators cannot be evaluated and adequately appreciated. The present experimental setting, at which all branches are physiologically expressed, implies that GRP94 is associated with functions (as opposed to specific genes), previously established as IRE1-regulated functions, such as ERAD. This observation does not contradict the role of ATF6 in the regulation of GRP94 but rather underscore the biological relevance of IRE1 at conditions at which all branches of the UPR are physiologically expressed

With ATF4, major processes revealed were the regulation of translation and regulation of apoptosis in response to stress, which are known processes that are mediated by PERK (Szegezdi et al, 2017).

For ERdj4 and p58IPK, no specific functions were revealed beyond their association with ER stress with the exception of the fact that for both, a negative

function was predicted. This probably implies their function in establishing negative feedback regulatory associations, consistent with the relief of stress following UPR activation.

An unexpected finding was the consistent association of CHOP with the regulation of metabolic processes. While CHOP is known to regulate metabolism, it is believed that this is activity is produced indirectly, by competing with other cEBP family members. Indeed, negative regulation of CREB has been revealed (Rutkowski et al 2008). In addition, a major role of CHOP in regulating metabolism should also be considered.

Although the present analysis possesses limitations in predicting linear association at the level of regulation, it possesses high power in identifying relevant biological processes and underscoring the relative impact of the transcripts of interest when simultaneously several such processes operate. The power of this approach in deciphering biologically relevant processes was reflected to the fact that by using only 3 primary cell lines we were able to distinguish between well established major functions for the different arms of the UPR and accurately predicting processes, such as ERAD and apoptosis for different transcripts. Similar gene ontology analysis by identifying transcripts significantly upregulated during ER stress and subjecting them, by an analogous hierarchical manner, to gene ontology analysis, was powerless to predict specific biological functions and only unveiled a generic and rather wide response to stress (Table 1). Furthermore, while the proposed analysis allows the extraction of information with regards to the function of individual genes, conventional analysis focusing on relative expression can be useful only if genetic manipulation targeting the gene of interest is applied. The latter, beyond its methodological limitation related to that it is hypothesis-driven, it also possesses conceptual limitations since qualitative changes may be distinct from quantitative changes that occur in naturally existing populations. It is plausible that the power of the correlation analysis over the standard overexpression analysis is associated with the fact that different transcription modules exhibit different saturation levels that when reached the coordination in their activation is abolished. This can only be studied by using genetically diverse biological systems at which variation in expression has the capacity to reveal the presence of correlation in expression levels. When, however, only the degree of differential expression is evaluated, co-regulation and deviation from this cannot be assessed.

Collectively, the present study illustrates the power of using outbred species in analyzing gene expression and suggests that evaluation of the degree of coordination as opposed to the magnitude of expression may be particularly valuable in assigning genes into transcriptional networks. It is plausible that by increasing the depth of RNA sequencing and concomitantly analyzing a large number of specimens and transcripts, more precise predictions will be made regarding signal integration. It is conceivable that this strategy can find application to the study of virtually all signaling networks at which variation in the response, accompanied by coordination in the expression of the corresponding transcripts is anticipated. Whether in disease the coordination in particular signaling nodes is abolished, remains to be seen.

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