

Identification of regulatory mechanisms of the hepatic response to thermal injury

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Received 30 October 2006; received in revised form 11 February 2007; accepted 13 February 2007

Available online 25 February 2007

Abstract

The purpose of this paper is to evaluate the hypothesis that a systems biology approach can be developed such that a set of transcription factors, relevant to burn-induced inflammatory response can be identified and modulated to control the host response. We explore a novel method for identifying coherent and informative expression motifs and we subsequently determine conserved transcription factor binding sites for the sub-sets of co-expressed genes. The responses are rationalized in the context of burn-induced inflammation and the putative transcription factors are rationalized in the context of intervention targets for controlling gene expression.

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Keywords: Gene expression; Burn; Inflammation; Transcription factors

1. Introduction

Deep thermal injury over greater than 20 percent of the total body surface area is one of the most severe forms of trauma. Following the early acute phase response dealing with the initial injury and shock (Carter, Tompkins, Yarmush, Walker, & Burke, 1988), there exists an equally serious secondary response which include changes in metabolism leading to hypermetabolism and catabolism, decreased function of the immune system, and sepsis (Strock, Singh, Abdullah, Miller, & Herndon, 1990). Due to improvements in hospital care, more burn victims survive the acute response to the injury, and are faced with the secondary effects of thermal injury, which have proven to be more difficult to treat and control. In particular, prolonged sepsis and hypermetabolism following severe injury can result in Multiple Organ Dysfunction Syndrome (MODS), currently the most common cause of death in non-coronary intensive care units in the U.S. (Cooper et al., 2006; Tredget & Yu, 1992). A better understanding of the mechanisms by which the early responses

to thermal injury predispose to the later hypermetabolic state would make it possible to define points of intervention by which such outcomes can be avoided.

The response to burn injury and trauma results from a complex interplay between inflammation caused by the initial injury and hypermetabolism. The advent of DNA microarrays enables to systematically examine expression changes of a very large number of genes, which provides an opportunity to identify pathways, which have not been previously known as important. Given the importance to better understand the progression of the response after injury, temporal expression profiling in which the dynamics of mRNA expression are measured over time is more useful than simply the measurement of gene expression pre burn and post burn.

Changes in energy expenditure, increased glucose and lipid turnover are hallmarks of the hypermetabolic state (Wilmore, Long, Mason, Skreen, & Pruitt, 1974). Given that the liver plays a key role in metabolic processes of energy production, gluconeogenesis and lipid synthesis and oxidation, it is likely that the liver is one of the primary organs driving the systemic response to severe thermal injury (Mager, Wykes, Roberts, Ball, & Pencharz, 2006). Furthermore, a better understanding of the liver response to thermal injury may provide mechanistic insights and suggest

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new ways in which the hypermetabolism associated with severe thermal injury could be mitigated or even prevented.

To use temporal expression data from microarrays to formulate possible compensatory strategies the following steps must be accomplished:

1. Selection and classification of relevant genes.
2. Functional characterization of extracted genes.
3. Generation of hypothetical regulatory networks.
4. Associating between functional characterization and regulatory networks.

We will propose an integrative method that combines temporal gene expression profiling, sequence analysis, database mining and network construction in order to formulate the parameters for a future experiment to better understand the response of an organism to severe burn injury. It is our goal to place the bioinformatics approach into an iterative formalism where experimental data guides data analysis which in turn guides the course of future experiments.

2. Methods

Prominent in engineering analysis is the concept of developing appropriate mathematical representations of the response of the system in order to identify critical components and optimal strategies (Aris, 1999; Biegler, Grossmann, & Westerberg, 1997). Biological systems, much like reaction engineering systems are characterized by input, output, control variables and an underlying dynamic model that propagates disturbances due to input variability across the system. These are manifested in the response of the output variables. The goal of an engineering analysis is to identify the control points that modulate the response. An additional complexity of biological systems is that, by and large, fundamental first principles models that describe these dynamic responses are not available. Therefore, data-based methods are needed to develop appropriate models and dependencies. This is a well-accepted paradigm in chemical engineering that has generated significant success. In order to enable the development of such descriptive models a number of critical questions need to be answered. Specifically:

1. We need to identify the inherent dynamic of the system and the informative output variables that best characterize that response.
2. We need to identify the controls that modulate the observed responses, and finally.
3. We need to determine the functional relationships that dictate how inputs propagate across the systems and how outputs are modulated by the controls.

In the sections that follow we identify these steps and demonstrate how such models can be generated and analyzed to identify and propose intervention strategies that modulate the systemic response.

3. Experimental data

In a previously published study, male Sprague–Dawley rats were subjected to a cutaneous third degree burn injury consisting of a full skin thickness scald burn of the dorsum, calculated to be ~20 percent of the rat's total body surface area (Vemula, Berthiaume, Jayaraman, & Yarmush, 2004). Liver samples were obtained at five time points (0, 1, 4, 8, and 24 hr post burn). RNA extracted from the extracted livers was isolated and subsequently hybridized to a U34A GeneChip that had 8799 probes represented on each chip. The control for this experiment was obtained at time 0, which was prior to the injury. It has been previously shown that time had no significant effect upon the response of rats to the sham treatment (Lee, Berthiaume, Stephanopoulos, & Yarmush, 2003).

3.1. Selection and classification of relevant genes

One of the strengths of microarrays is the fact that they are able to measure the levels of gene expression of thousands of genes at once. Therefore, the researcher is able to measure the expression levels of genes whose role in a given biological phenomenon was previously unknown. However, this strength is problematic for researchers because it then becomes difficult to determine which gene expression levels are important to the organism's response to an external stimulus.

Most automated techniques such as dChip, MAS5, PMMM, ANOVA, *t*-tests, and RMA attempt to extract genes from microarrays based upon the notion that genes which show statistically significant changes in gene expression levels ought to be informative (Millenaar et al., 2006). The primary drawback with these methods is that they look for genes whose differential expression is statistically significant rather than evaluating whether or not a given gene is relevant to the experiment at hand. The difference between a statistically significant gene and a gene, which is relevant to the experiment is a subtle but important one. This difference arises due primarily to the dynamic nature of homeostasis. Even in an unperturbed state there is significant transcriptional activity taking place within the organism regulating events such as feeding, resting, or physical activity. Therefore, even in a control state in which the organism has not been experimentally perturbed, there ought to be gene expression profiles, which change at a statistically significant level. However, what researchers should be interested in is the identification of gene expression profiles that are directly responding to the experimental perturbation and not just genes that show a statistically significant dynamic.

Adjunct to this concern is the fact that the currently established filtering techniques are essentially answering the wrong question. All of these techniques were derived in response to two state microarray experiments in which the primary question was, "Does the gene expression of a given gene change significantly between the two states." However, with temporal expression microarray data the relevant question should be, "Is the shape of the temporal expression profile accurate."

SeLection of Informative Genes via Symbolic Hashing of Time Series (SLINGSHOTS) was developed specifically to iso-

late genes that could be hypothesized to be important to the underlying response of an organism. The fundamental hypothesis, which underlies SLINGSHOTS is that in response to an external perturbation, there ought to be a coordinated set of genes whose expression profiles are highly correlated. In addition to this, this set of genes in aggregate ought to illustrate significant deviations from the baseline distribution of expression levels. By identifying these genes, one ought to be able to obtain the genes whose expression profiles are representative of the underlying changes. The prime innovation of this technique is that the selection criteria uses a global metric to assess the “informativeness” of the isolated genes by evaluating the set in aggregate rather than the local metrics used by other selection techniques which checks the informativeness of each genes individually. The details of the method were recently discussed in (Yang, Maguire, Yarmush, Berthiaume, & Androulakis, 2007).

SLINGSHOTS is broken down into two related steps, a hashing step in which the genes are clustered into a large number of highly correlated clusters, and a selection step in which a set of these clusters is evaluated for their ability to represent the experimental perturbation. The behavior of SLINGSHOTS is defined by two primary parameters, α and w , of which α is the size of the alphabet used and w is the number of time points to average together for longer time series. In this evaluation, α was selected to be 4 and w was selected to be 1. Given that the clustering and selection are combined, the results of the selection are already clustered negating the need for a separate clustering step.

The result of the hashing step is a large set of gene clusters of which all of the gene expressions show a correlation coefficient to the average profile above a certain threshold. Unlike in QT clustering where the threshold is set explicitly, SLINGSHOTS sets this cutoff through a combination of w and alpha. In this case, the minimum correlation coefficient for any of the given clusters is greater than .75.

The identification of clusters that comprise up of the hypothetical primary response of the organism to thermal injury then allows for further analysis in terms of the functional role of the genes and the identification of the regulatory mechanisms which give rise to the observed expression profiles. The following steps will allow for the identification of possible mechanism with which to mediate the undesired responses associated with severe thermal injuries.

3.2. Identification of functional ontologies

The purpose behind the identification of functional ontologies is the determination of the underlying biological processes that are related to the phenomenon being investigated. This essentially allows for the interpretation of the biological significance of isolated genes. This will be conducted by isolating the ontologies, which are related to the selected genes via the database present at <http://www.geneontology.org> (Ashburner et al., 2000). The primary assumption behind this analysis is that co-expressed genes ought to have related functionality (Wolfe, Kohane, & Butte, 2005). Therefore, by looking at an aggregate set of clustered genes, it is expected that there should prefer-

ence of certain biological process ontologies to be localized to a specific cluster.

To determine which of the isolated processes are significant, it is important to determine which of the associated ontologies are enriched at a statistically significant level ($p < .05$). Due to the fact that many genes can participate in different biological processes especially those associated with cellular signaling, it is important to determine which of these biological processes are consistent over all of the selected and clustered genes. This is done utilizing the hypergeometric distribution. The hypergeometric distribution however, is inaccurate if the total number of counts is less than 5, a more accurate assessment of the count is given by the Fisher distribution (Ashburner et al., 2000; Draghici, Khatri, Martins, Ostermeier, & Krawetz, 2003). A secondary benefit of such analysis is that it allows for an ad hoc evaluation as to the correctness of selection and clustering. If the genes that were isolated and classified do not show any notable enrichment, then it would suggest that there was a flaw in the either the data or the methodology.

3.3. Regulatory network construction

The dynamic response of biological organisms is governed by a large interconnected network, which ties the response of each gene to intercellular conditions or the expression levels of other genes. Part of the rationale behind conducting temporal gene expression experiments is that the measured dynamics can help in the construction of a transcriptional network, which gives insights as to how an organism responds to external stimulus. We make the additional assumption that by grouping genes into co-expressed clusters, the network is simplified through the assumption that these co-expressed genes are regulated by the same mechanism. This prunes many of the connections thereby simplifying the overall network. This network can be further simplified by treating it as a bi-partite graph Fig. 1, in which a set of inputs drives a set of output. It does not explicitly model the existence of feedback loops. However, feedback loops can be handled if an input gene is also present in the output. For example, if a transcription factor such as GATA6 were driving the system, it could also be present in the output. Therefore, despite the fact that a bi-partite graph is a simplification of the overall network interactions, it is possible to retrieve the original DAG (Directed Acyclic Graph) from a bipartite network (Liu & Lai, 2000). The primary benefit of treating our gene regulatory network as a bi-partite graph, is that there are robust methods for estimating the connectivity strengths from gene expression data such as Network Component Analysis (NCA) (Boulesteix & Strimmer, 2005; Liao et al., 2003).

Various methods exist for the construction of transcriptional networks from gene expression data. These techniques fall broadly under two primary categories of algorithms, those which seek to identify relationships between the expression level of different genes over multiple conditions (Dojer, Gambin, Mizera, Wilczynski, & Tiuryn, 2006; Kauffman, Peterson, Samuelsson, & Troein, 2003), and those which utilize outside information to generate regulatory network such as using the set of gene regulators which have been previously identified, or analyzing the

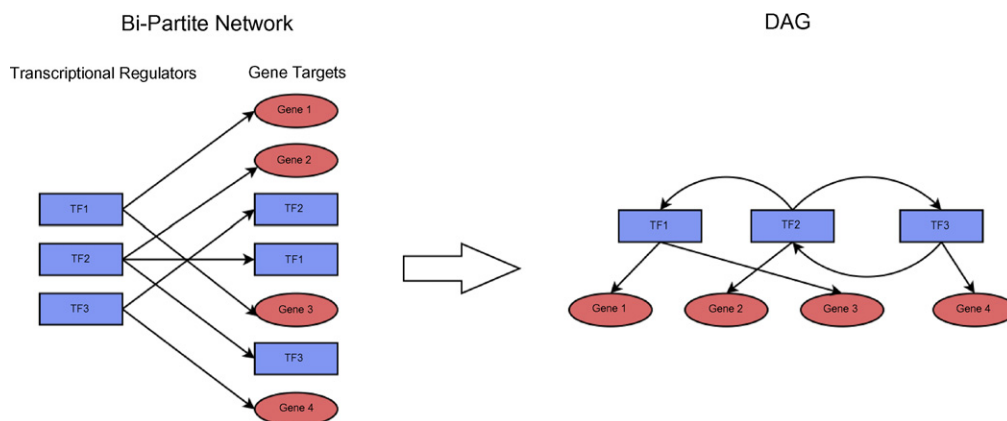


Fig. 1. A bi-partite representation of a transcriptional network and its associated DAG. There is no loss in generality in terms of the possible networks that can be represented. The representation as a bi-partite network however allows for efficient quantification of the network through various algorithms such as NCA and PLS.

promoter region to look for possible links between genes and their regulators (Manson McGuire & Church, 2000; Segal et al., 2003; Tavazoie, Hughes, Campbell, Cho, & Church, 1999). Given the form of our data which consists of a single condition and five time points, the construction of the network requires external data in the form of predicted transcription factor binding sites. Due to the relative dearth of data, it is imperative for us to localize our analysis to a small portion of the regulatory network, namely the genes and regulators that are directly responding to the input perturbation, and hence make the gene selection process discussed previously an integral part of the analysis.

The network associated with temporal gene expression data is the gene regulatory network in which the primary links are the transcription factors whose activity mediates the production of the genes. Transcription factors are proteins that bind to the promoter region of a given gene and through that activity can either up or down-regulate the gene expression of that gene. Given that transcription factors themselves are genes, they are also regulated by other transcription factors and sometimes by a protein product further down in the signaling cascade, which they initiate (Ayte, Schweitzer, Zarzov, Nurse, & DeCaprio, 2001). In yeast, many of the transcription factors have been experimentally identified. However, in more complex mammalian systems such as rat, there is a limited number of experimental information forcing the use of algorithms, which predict transcription factor binding sites. These transcription factors binding site prediction algorithms fall under two general categories, algorithms such as AlignACE (Manson McGuire & Church, 2000) which work by looking for over-expressed motifs (n -mers) within the promoter region of a cluster of genes, and those like CONSITE (Sandelin, Wasserman, & Lenhard, 2004), which base their predictions off transcription factor position weight matrices which are obtained experimentally through procedures such as SELEX (Klug & Church, 1994). The latter method was chosen for this analysis due to the relative difficulty in associating the over-expressed motifs to transcription factors whereas the second method is based off of known transcription factors. The specific tool used for the determination of possible transcription factor binding sites was Comparative Regulatory Genomic (CORG) (Dieterich, Wang, Rateitschak, Luz, & Vingron, 2003), which

is an online tool that is able to extract the promoter sequence from homologous genes between two organisms and obtain the associated transcription factor binding sites. The one piece of information which we utilized was simply whether or not a possible connection existed between a transcription factor and its associated binding site.

4. Results

Out of the original 8799 probes, the algorithm has identified 281 probes corresponding to 208 known genes located in four clusters (Appendix B) of which the z -score normalized expression profiles are given in Fig. 2. The transcriptional state of these genes over the experimental time course is given in Fig. 3, and what is clearly evident is that these genes illustrate a two-wave response to the initial burn injury. At hour 1, a large disjoint in the transcriptional levels can be seen in where there is a large amount of activity with all of the genes either significantly up-regulated or down-regulated. At hours 4 and 8, the expression profiles are fairly close to the pre-injury profile, suggesting a return to the initial homeostatic state. Then, at hour 24, an even greater disjoint representing a major shift in the cellular transcriptional state is visible, which is associated with an abundant over-expression of the inflammation marker and acute phase protein Alpha 2 Macroglobulin (A2M). Elucidating the mechanism that gives rise to this more delayed response would have significant implications in the treatment of severe thermal injury.

In the transformation from gene expression profile to hash value by SLINGSHOTS (Yang, Berthiamume, Yarmush, & Androulakis, 2006), the z -score normalization was used. Therefore scale information is discounted when forming the clusters. Commonly used selection metrics such as n -fold changes or t -test require the preservation of scale information within the individual expression profiles. Since this information is eliminated during the transformation, the selection of incorrect genes was a concern. If for the most part, the selected genes are part of co-regulated processes and the clusters that were selected, there ought to be a distinct separation in the functional ontologies between the different clusters. The ontology localization is evident in Fig. 4. It is clearly evident that the

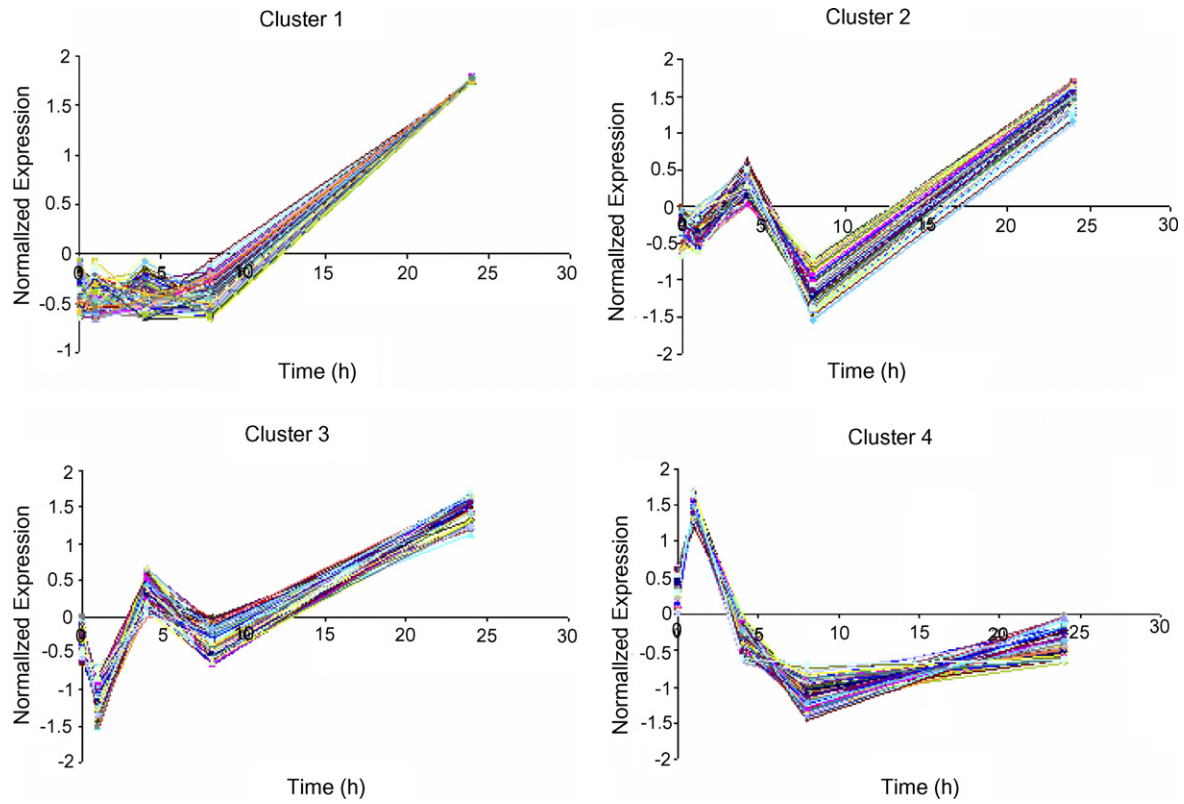


Fig. 2. The expression profiles of the selected genes. Clusters 3 and 4 have an early phase response, while clusters 1 and 2 primarily have a late term response.

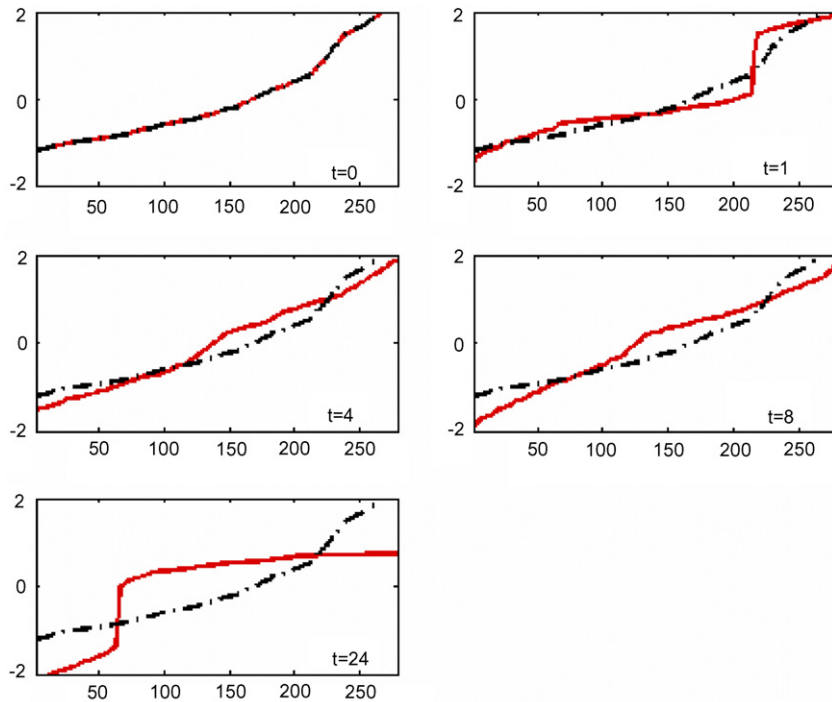


Fig. 3. The transcriptional state of the selected genes. At $t=1$ and 24, we have evidence of a 2 wave effect in which significant transcriptional processes are being altered. The response at $t=1$ is evidence of the short term compensatory mechanism, while the response at $t=24$ represents a potentially irreversible, state change into the chronic inflammation and hypermetabolic state.

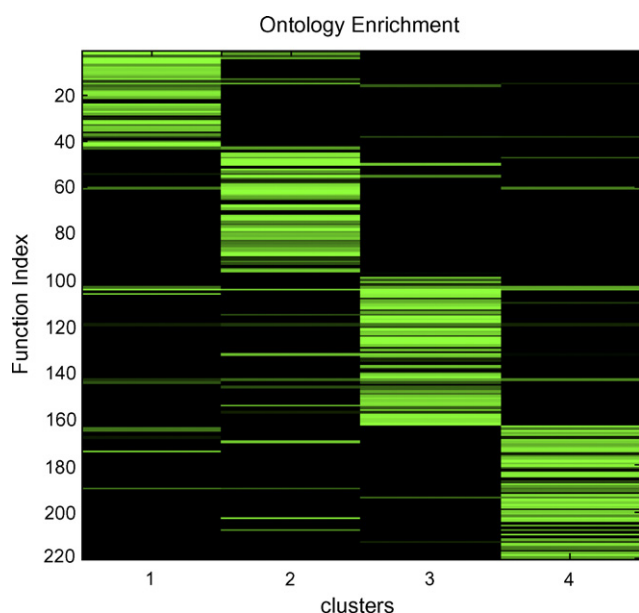


Fig. 4. The enrichment of ontologies associated with the clustered and selected genes. The diagonally dominant nature of the graph suggests that our clustering has indeed separated out genes with related functionalities despite superficial similarities in their overall shape.

ontologies are indeed localized to the cluster, and despite the fact that clusters 2 and 3 seem to differ by only one time point after normalization, they do encapsulate two different sets of functions.

Taking the set of genes as a whole instead of as four clusters, we find that the most significant biological processes revolve around metabolism, inflammation, protein production, and signaling which is in agreement with the macroscopic observations of pathophysiology after severe thermal injury. The localization of functional ontologies to each cluster suggests to us that our clustering is indeed correct and the selection of significant ontologies related to metabolism, inflammation and protein catabolism and synthesis suggests that the selection process was likewise successful. However, while significant processes and their underlying dynamics have been identified, there still remains an open question as to what the best way to mediate the response of the system.

The most prevalent transcription factors amongst all of the clusters are STAT5/STAT6 associated with the JAK-STAT pathway, and Translation Elongation Factor (TEF1). While there are genes, which are part of the JAK-STAT pathway expressed in cluster 4 such as erythropoietin, the JAK-STAT transcription regulation pathway is not particularly informative due to its widespread use in cellular signaling. In fact, looking at a random selection of genes, the JAK-STAT pathway is found to also be highly prevalent.

The only clusters with a consistent set of transcription factors binding sites that were not STAT5, STAT6, and TEF1 amongst the genes of that cluster (greater than 95 percent of identified genes containing a transcription factor binding site) were clusters 2 and 4. It was somewhat surprising that we were not able to find a small set of transcription factors that regulated all

Table 1
Associated transcription factors

Cluster	Associated transcription factors
1	STAT5, STAT6, TEF1
2	AP2 alpha
3	STAT5, STAT6
4	TEF1, STAT5, STAT6, CIZ, CDXA, GATA6, AP2 alpha

Those highlighted in orange are transcription factors that are highly conserved, but not found.

of the clusters. However, it was noticed that the clusters that contained a significant set of genes that coded for transcription factors had a consistent set of activators, while the clusters of genes that did not contain transcription factors (clusters 1 and 3) were not regulated by transcription factors other than STAT5 and STAT6. The associated transcription factors are given in Table 1.

The primary activators that we found were AP2 alpha, GAT A 6, and CIZ. AP2 alpha was localized to cluster 2, while GATA 6 and CIZ were co-localized to cluster 4. While cluster 1 and cluster 3 did not have a set of consistent transcriptional regulators, they did however, have a large fraction of genes that were regulated by the Octamer Binding Family (OCT) of transcription factors and genes that respond to myogenin (MYOD) both of which are transcription factors that were present in clusters 2 and 4. This suggests that there are some elements of a signaling cascade within the transcriptional regulatory network. A gross overview of the transcriptional network is given in Fig. 5.

5. Discussion

From the results of the clustering, we find that the two wave phenomenon can be rationalized by the expression dynamics of cluster 1 and cluster 4, in which cluster 1 remains constant up until hour 24 in which there is a large increase in the level of gene expression. Cluster 4 is indicative of the early response showing significant up-regulation during this period, and a relaxation afterwards with significant overshoot. Clusters 2 and 3 are similar in terms of their response except at hour 1 with cluster 1 remaining constant up until that time and cluster 3 being down regulated at hour 1.

Characteristic of the genes that show activity early on such as clusters 3 and 4, we find that there is a significant over-representation of genes that code for transcription factors, regulate metabolism, and protein production within these clusters (Appendix B). We hypothesize that the initial thermal injury causes the change in the expression levels of clusters 3 and 4, which affect the dynamics of clusters 1 and 2, either directly through the production of transcription factors or indirectly by altering the levels of circulating metabolites through changes in metabolism of macromolecules such as proteins, carbohydrates and fatty acids.

Coupled with the transcriptional driving forces found within these genes, are associated metabolic processes associated with catecholamines, which are important in the hyperme-

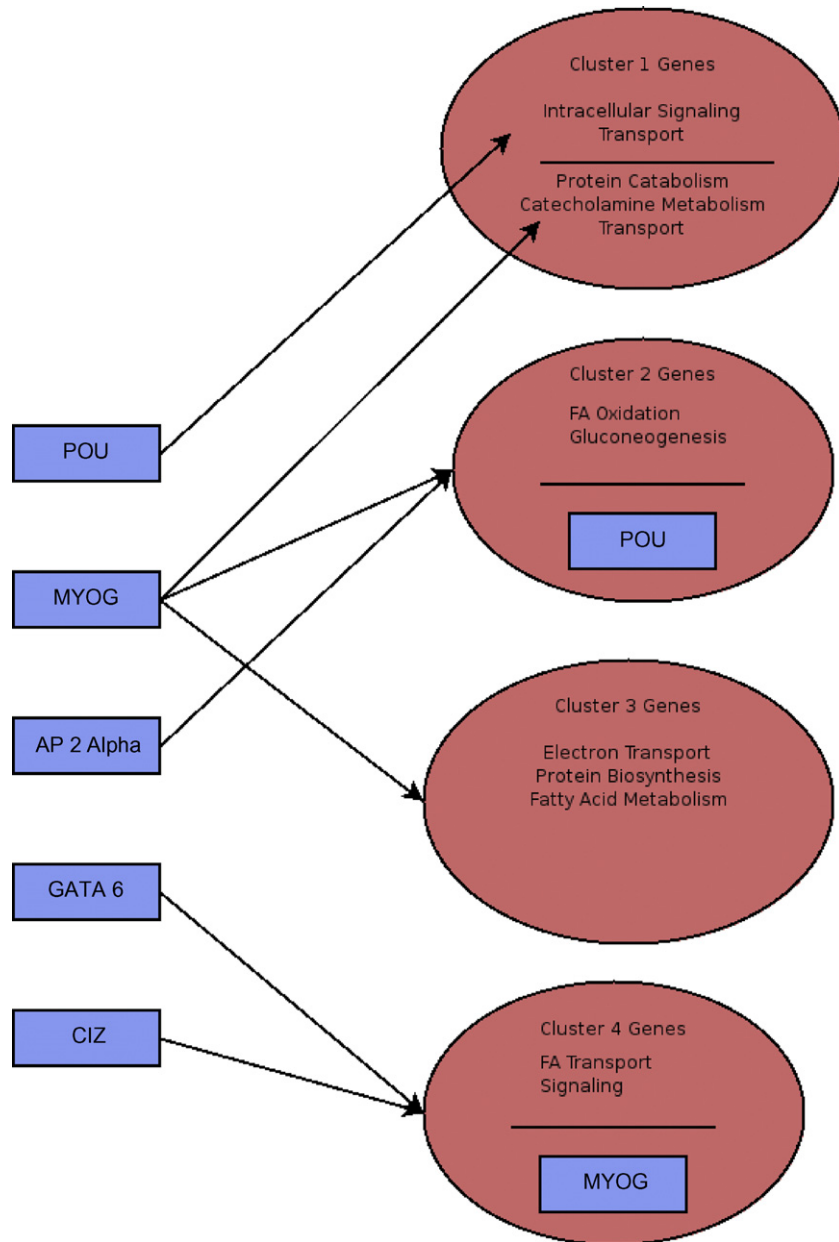


Fig. 5. A gross schematic of the predicted response mechanisms. The notable thing is that MYOG which is up-regulated early is a regulator in a significant number of genes in clusters 1 and 2, and may drive the secondary response.

tabolic response seen after thermal injury (Herndon, Hart, Wolf, Chinkes, & Wolfe, 2001; Wilmore et al., 1974). Although the hypermetabolic response is known to occur 3 days or more after injury, the presence of these metabolic genes in Appendix B suggests that there is a significant metabolic component early on in the response of the burn injury. It is currently unclear as to whether these early changes in liver metabolism and corresponding changes in levels of metabolites in the circulation play a role in the subsequent more systemic and chronic changes in metabolism and the inflammatory response, events that lie outside of the experimental time frame analyzed herein. There is evidence that the sustained inflammation leads to inhibition of transporters leading to abnormal levels of circulating

metabolites such as lipids and glucose (Khovidhunkit, Moser, Shigenaga, Grunfeld, & Feingold, 2003; Savage, Petersen, & Shulman, 2005), which may affect the other organs in the organism.

As part of the early response to thermal injury, a large portion of the genes appears to be either metabolic in nature or involved in transcriptional signaling. Cluster 4, which has an immediate response at hour 1, contains genes that are responsible for the metabolism of fatty acids. This is paired with the activity in cluster 2 in which a major component of its activity is a corresponding down-regulation in fatty acid transport. It has been noted in previous work that gene expression levels often do not correlate well with the levels of their cor-

responding metabolites in circulation (Vemula et al., 2004). This opposing dynamics of the fatty acid transporters and the fatty acid enzymes suggests that the levels of fatty acids are controlled by competing transport and metabolic processes. Coupled with the changes in metabolism are a series of transcriptional changes. What we find is that within these early response clusters, there are changes in expression of murinoglobulin 1, and complement component 5. These regulate key inflammatory and acute phase responses and work to dampen the long term inflammatory response of the genes found in clusters 1 and 2 (Arumugam et al., 2004; Northemann, Shiels, Braciak, & Fey, 1989).

The longer term responses shown by clusters 1 and 2 seem to revolve around a transcriptional signaling component and a protein catabolism component. Given the localization of protein catabolism along with inflammation in cluster 1, we believe that the increased protein turnover rate may be one of the primary driving forces leading to the hypermetabolic state. So, while there are undoubtedly changes in the energetics of an organism after thermal injury, we believe that long-term changes in energetics manifest themselves primarily in the levels of protein turnover rate. Given the severe fall in ATP levels (Vemula et al., 2004) post burn, we believe the organism is making up for a significant shortfall in available energy through the catabolism of protein. This counter-productive process may be one of the significant barriers to recovery from thermal injury.

While there is a significant metabolic component to the burn response, there is also a significant role played by transcriptional signaling pathways. The regulatory network which we were able to infer from a combination of gene expression data and promoter region analysis has suggested three possible initiators of the burn response GATA 6, AP2 alpha, and CIZ. We believe that the identification of these initiatory transcription factors are not wholly unreasonable. These three transcription factors have been cited in functions related to inflammation (Gille, Swerlick, & Caughman, 1997; Lin, Spoor, Gerth, Brody, & Peng, 2004; Torrungruang et al., 2002). Unlike other factors such as the ubiquitous JAK-STAT pathway, these transcription factors offer the best hope of altering individual burn injury-induced responses independently.

While clusters 2 and 4 have a possible set of regulators with which their responses can be perturbed, clusters 1 and 3 do not have a readily apparent set of regulators. It is possible that the co-expression of the genes within clusters 1 and 3 is due primarily to the co-expression of their regulators, meaning that the genes in clusters 1 and 3 may be co-expressed not because they have precisely the same regulators, but because the activity of their individual regulators is similar. Looking at clusters 2 and 4, we find a set of regulators, which may not bind to the same recognition sequence but may have very similar responses, of which the most notable ones are MYOG and the POU family of transcription factors which are known to be important regulators in liver. MYOG is present in more than 35 percent of the genes found in clusters 1–3. This is notable given the relative long length of the MYOG recognition sequence, which is 29 base pairs long. Finding it in such a large number of pro-

motor regions is highly statistically significant given the fact that the expected hit rate of MYOG is 1 out of 5.21×10^{12} bases. What is even more notable is that MYOG is found in many of the genes, which are responsible for metabolism, including catecholamine metabolism. The transcription factor family POU seems to be more biased towards the genes, which code for calcium and potassium ion channels. Given that the transcription factor MYOG is highly significant in terms of the number of matches, and the fact that it appears to regulate genes related to metabolism rather than the genes associated with inflammation, we hypothesize that by altering the activity of MYOG, we may be able to affect the severity of the hypermetabolism while having a minimal impact upon the inflammatory response.

6. Conclusions

The role of bioinformatics is not to look for answers independently of experimentation, but rather to look for the basis of new experiments. By integrating the experimental gene expression data with genomic data and the results of SELEX experiments, we were able to construct a rough network, which gave hints as to possible points of intervention. Seeking to prevent the induction of the system into hypermetabolism, we have identified the myogenin transcription factor as perhaps one of the critical signals, which drives the system from its acute response to burn injury to the longer term systemic hypermetabolic state.

Identifying a regulatory layer and the core nodes of that layer provides a mechanism to elucidate intervention points to attenuate the inflammatory process. Intervention utilizing these TF proteins could theoretically take one of three forms: (1) inhibition of TF production using knockout or silencing techniques; (2) blocking TF activity through competitive inhibition; (3) blocking TF activity through suicide inhibition. Promising approaches for silencing focus on the use of siRNA techniques (Lee & Church, 2003). In this approach double-stranded RNA (dsRNA) is digested by the dsRNA-specific RNase III enzyme dicer into small interfering RNAs (siRNAs). The siRNAs then assemble with a multiprotein nuclease complex, RNA-induced silencing complex (Wu, Kwon, Driscoll, & Faeth, 1991), which unwinds the dsRNAs and degrades target mRNAs homologous to the single stranded siRNA in a sequence-specific manner. The result of this process is the degradation of mRNA needed as a template for protein production, thereby inhibiting the production process, and depleting pools of proteins needed for specific enzymatic reactions. One specific example of siRNA utilized for intervention in inflammatory response is the application of siRNA techniques to inhibition the production of STAT-3 in order to elucidate key signaling molecules in the inflammatory response pathway (Verga Falzacappa et al., 2006). Therefore, a key advantage of the methodology discussed in this work is the systematic identification of several putative regulatory proteins of the inflammatory response, thus enabling the rational selection of multiple targets and design of combination therapies for the modulation of the inflammatory response.

Acknowledgements

IPA and EY acknowledge financial support from NSF-BES 0519563 and EPA GAD R 832721-010 is gratefully acknowledged. FB and MLY supported by grants from the Shriners Hospitals for Children.

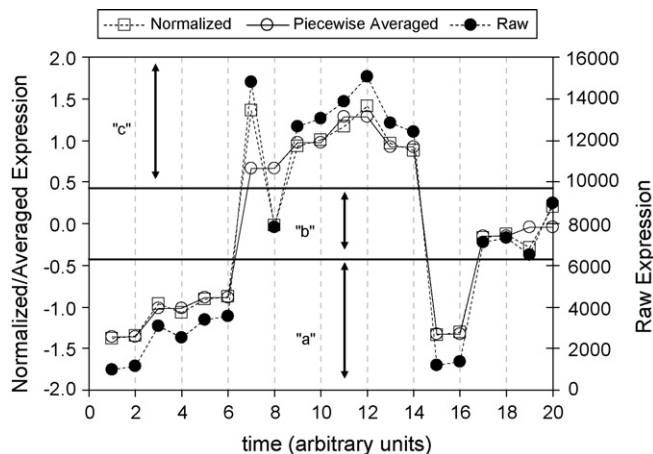
Appendix A

1. Normalization of the gene expression profile to $N(0,1)$ via the z -score transform.
2. If the sequences are longer than 10 time points, piecewise averaging is conducted, that is averaging together sets of n time points to reduce the exponential expansion of the search space. In the case of our data, the 17 time points are interpolated to 18 time points, and the time series are broken down into sets of 2 to be piecewise averaged.
3. These piecewise averaged points are then converted into symbols through the use of Gaussian breakpoints. Gaussian breakpoints are divisions in the Gaussian distribution such that the cumulative probability of each section are equivalent. These can be obtained through the use of CDF tables found in statistics text books or by solving the following equation for b :

$$\frac{i}{k-1} = \frac{1}{2} \left[1 + \operatorname{erf} \left(\frac{b}{\sqrt{2}} \right) \right]$$

$i = 1, \dots, k$; k = number of breakpoints; b = breakpoint value.

The overall process of assigning a letter to each piecewise averaged point is illustrated in below:



4. After the symbolic transformation, the series of symbols is converted into a single integer via the formula:

$$\text{hash}(c, w, a) = 1 + \sum_{j=1}^w [\text{ord}(c_j) - 1] \times a^{j-1}$$

where c is the letter assigned to each piecewise averaged point, a is the size of the alphabet (3), and w is the total length of the expression profile divided by the number of points per piecewise average (2). The parameters of the alphabet were selected to so that the population distribution of motifs is non-exponential, to reflect the non-random distribution of expression profiles present in the data. w was chosen to preserve as much of the high frequency component of the signal as possible.

Appendix B. Informative genes, class membership and ontology annotation

Gene ID	Gene	Cluster	Function
AI030286	Brain derived neurotrophic factor	1	Apoptosis
AA859878	ret proto-oncogene	1	Cell cycle regulation
AI234604	Heat shock protein 8	1	Cell cycle regulation cytoskeleton
U60416	Myosin 5B	1	Organization
AI171243	Replication protein A3 (predicted)	1	DNA repair
Z50084	Ameloblastin	1	ECM organization
AA874941	Adipose differentiation-related protein	1	Fatty acid transport
AA819776	Heat shock 90 kDa protein 1, alpha-like 3 (predicted)	1	Heat shock
M23566	Alpha-2-macroglobulin	1	Inflammation
AA900582	Alpha-2-macroglobulin	1	Inflammation
X13983	Alpha-2-macroglobulin	1	Inflammation
M22670	Alpha-2-macroglobulin	1	Inflammation
M22670	Alpha-2-macroglobulin	1	Inflammation
M83209	Parotid secretory protein	1	L-Serine biosynthesis
D10233	Renin binding protein	1	Metabolism
L12407	Dopamine beta hydroxylase	1	Metabolism
L26043	Perilipin	1	Metabolism neurotransmitter
U24071	unc-13 homolog B (<i>C. elegans</i>)	1	Secretion
D64061	Eukaryotic translation initiation factor 5B	1	Protein biosynthesis
AA892680	Peptidylprolyl isomerase (cyclophilin)-like 3	1	Protein biosynthesis
D50696	Peptidase (prosome, macropain) 26S subunit, ATPase 1	1	Protein catabolism
U50194	Tripeptidyl peptidase II	1	Protein catabolism
D10699	Ubiquitin carboxy-terminal hydrolase L1	1	Protein catabolism

Appendix B (Continued)

D45247	Proteasome (prosome, macropain) subunit, beta type 5	1	Protein catabolism
D21799	Proteasome (prosome, macropain) subunit, beta type 2	1	Protein catabolism
D90265	Proteasome (prosome, macropain) subunit, alpha type 1	1	Protein catabolism
AF054270	Prolactin induced protein	1	Protein processing
X93352	Ribosomal protein L10A	1	Protein synthesis
AA891713	Ribosomal protein L13A	1	Protein synthesis
M17419	Ribosomal protein L5	1	Protein synthesis
AI170685	DnaJ (Hsp40) homolog, subfamily A, member 2	1	Protein synthesis
AI103238	Regulatory subunit B (PR 52), beta isoform	1	Reproduction
AB011068	Deiodinase, iodothyronine, type II	1	Response to cold
X68400	Protein kinase C, eta	1	Signaling
D45412	Protein tyrosine phosphatase, receptor type, O	1	Signaling
M17526	Guanine nucleotide binding protein, alpha o	1	Signaling
AF064706	G protein-coupled receptor 6	1	Signaling
U66274	Neuropeptide Y receptor Y5	1	Signaling
D15069	Adrenomedullin	1	Signaling
Z35654	mcf.2 transforming sequence-like	1	Signaling
AA859752	Noggin	1	Skeletal development
AF053101	Paired box gene 4	1	Transcription
AI639353	Pleiotropic regulator 1 homolog (<i>Arabidopsis</i>)	1	Transcription
AF062594	Nucleosome assembly protein 1-like 1	1	Transcription
AA866472	Nucleosome assembly protein 1-like 1	1	Transcription
M99221	Calcium channel, voltage-dependent, Ltype, alpha 1D subunit	1	Transport
X78997	Cadherin 17	1	Transport
AF019043	Dynamin 1-like	1	Transport
X81448	Similar to cytokeratin	1	No identified ontologies
AI104388	Aurora-A kinase interacting protein	1	No identified ontologies
AA891829	WD40 protein Ciao1 (predicted)	1	No identified ontologies
X99338	Stromal cell derived factor receptor 1	1	No identified ontologies
X77815	Variable coding sequence A2	1	No identified ontologies
AI072634	–	1	No identified ontologies
AA859804	–	1	No identified ontologies
AA892310	–	1	No identified ontologies
AA800017	Similar to hypothetical protein BC011833 (predicted)	1	No identified ontologies
AA893307	Nuclear cap binding protein subunit 2 (predicted)	1	No identified ontologies
H31648	Transcribed locus	1	No identified ontologies
AA874849	–	1	No identified ontologies
AA892369	–	1	No identified ontologies
AA894054	CDNA clone IMAGE:7326015	1	No identified ontologies
AA956941	–	1	No identified ontologies
AA892818	Transcribed locus	1	No identified ontologies
M13949	–	1	No identified ontologies
AA800275	Transcribed locus	1	No identified ontologies
AA799865	Transcribed locus	1	No identified ontologies
AI639039	–	1	No identified ontologies
AA875554	Transcribed locus	1	No identified ontologies
H33467	Transcribed locus	1	No identified ontologies
H31753	Transcribed locus	1	No identified ontologies
AI230789	–	1	No identified ontologies
AI639464	–	1	No identified ontologies
AI639289	–	1	No identified ontologies
AI639459	–	1	No identified ontologies
AI639033	–	1	No identified ontologies
AA800948	Similar to tubulin alpha-4 chain (alpha-tubulin 4)	1	No identified ontologies
L03386	Olf-1/EBF associated Zn finger protein Roaz	1	No identified ontologies
U66707	Densin-180	1	No identified ontologies
AF053987	Putative pheromone receptor V2R1	1	No identified ontologies
AF079873	Zinc finger protein 162	2	Apoptosis
AI070295	–	2	Apoptosis
AA945608	Serum amyloid P-component	2	Cell Adhesion
AA859869	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1	2	Cell cycle regulation
X67805	Synaptonemal complex protein 1	2	Cytokinesis
AF017757	Axin2	2	Development
AA893280	Adipose differentiation-related protein	2	Fatty acid transport
AA858520	Follistatin	2	Gametogenesis
AB014722	Squamous cell carcinoma antigen recognized by T-cells 1	2	Immune response

Appendix B (Continued)

J05446	Glycogen synthase 2	2	Metabolism
AA891286	Thioredoxin reductase 1	2	Metabolism
M27440	Apolipoprotein B	2	Metabolism
S78217	Protein phosphatase 1, catalytic subunit, gamma isoform	2	Metabolism
S78218	Protein phosphatase 1, catalytic subunit, beta isoform	2	Metabolism
J02810	Glutathione <i>S</i> -transferase, mu 1	2	Metabolism
U91847	Mitogen activated protein kinase 14	2	Metabolism
AA859920	Nucleosome assembly protein 1-like 1	2	Nucleosome assembly
U95052	Eukaryotic translation initiation factor 4 gamma, 2	2	Protein biosynthesis
AA875205	Eukaryotic translation initiation factor 3, subunit 9 (eta) (predicted)	2	Protein biosynthesis
U13176	Ubiquitin-conjugating enzyme E2D 2	2	Protein catabolism
L38615	Glutathione synthetase	2	Protein catabolism
U48592	Interleukin 1 receptor accessory protein	2	Signaling
M23591	Protein phosphatase 2a, catalytic subunit, beta isoform	2	Signaling
X16044	Protein phosphatase 2a, catalytic subunit, beta isoform	2	Signaling
X89704	Olfactory receptor 1283	2	Signaling
AF055291	Signal transducer and activator of transcription 4	2	Signaling
AF037199	RE1-silencing transcription factor	2	Transcription
AB017044	Forkhead box A3	2	Transcription
AB017044	forkhead box A3	2	Transcription
AI177751	Transcription elongation factor B (SIII), polypeptide 1	2	Transcription
AI104524	Heterogeneous nuclear ribonucleoprotein A/B	2	Transcription
X66022	Neuronal d4 domain family member	2	Transcription
AJ001641	POU domain, class 3, transcription factor 3	2	Transcription
M18416	Early growth response 1	2	Transcription
D17711	Heterogeneous nuclear ribonucleoprotein K	2	Transcription
S59893	Sjogren syndrome antigen B	2	Transcription
AF044910	Survival of motor neuron 1, telomeric	2	Transcription
X62145	Ribosomal protein L8	2	Transcription
AF083341	Potassium large conductance calcium-activated channel	2	Transport
J05510	Inositol 1,4,5-triphosphate receptor 1	2	Transport
AA799276	ATPase, Ca ²⁺ transporting, cardiac muscle, slow twitch 2	2	Transport
J03969	Nucleophosmin 1	2	Transport
U12402	ADP-ribosylation factor-like 1	2	Transport
AA875099	Nuclear pore associated protein	2	Transport
H31747	–	2	Transport
	Actin related protein 2/3 complex, subunit 5-like (predicted)	2	
H32977	Beta-1,3-glucuronyltransferase 1	2	No identified ontologies
D88035	(Glucuronosyltransferase P)	2	No identified ontologies
AF016702	Glycoprotein hormones, alpha subunit	2	No identified ontologies
AF090692	Cystatin 8	2	No identified ontologies
AFFX-TrpnX-M	–	2	No identified ontologies
AI178828	–	2	No identified ontologies
AB013454	Similar to Ac2-210	2	No identified ontologies
AA893603	Solute carrier family 35, member E1 (predicted)	2	No identified ontologies
AA891842	Tumor necrosis factor receptor superfamily, member 21 (predicted)	2	No identified ontologies
AI639257	–	2	No identified ontologies
AI639476	–	2	No identified ontologies
AI639474	–	2	No identified ontologies
AA892010	Similar to hypothetical protein CGI-128 homolog (predicted)	2	No identified ontologies
AA893422	Transcribed locus	2	No identified ontologies
AI071399	–	2	No identified ontologies
AI639486	–	2	No identified ontologies
AA893180	Transcribed locus	2	No identified ontologies
AA900850	–	2	No identified ontologies
AI639120	Similar to RIKEN cDNA 1700088E04	2	No identified ontologies
S68589	Protein kinase N3 (predicted)	2	No identified ontologies
AA891838	Similar to ribosomal protein P0-like protein	2	No identified ontologies
D10854	Aldo-keto reductase family 1, member A1	3	Aldehyde catabolism
Z75029	Heat shock 70 kDa protein 1A3A	3	Apoptosis
X53428	Glycogen synthase kinase 3 beta	3	Cell cycle
X59859	Decorin	3	ECM organization
AF054618	Cortactin isoform B	3	Endocytosis
S45392	Heat shock 90 kDa protein 1, beta	3	Heat shock
M81225	Farnesyltransferase, CAAX box, alpha	3	Metabolism
AA799466	Adenylate kinase 2	3	Metabolism

Appendix B (Continued)

D86215	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 5	3	Metabolism
J02752	Acyl-coenzyme A oxidase 1, palmitoyl	3	Metabolism
J05470	Carnitine palmitoyltransferase 2	3	Metabolism
U17901	Phospholipase A2, activating protein	3	Metabolism
AA946040	Cytochrome c oxidase, subunit VIb (predicted)	3	Metabolism
AF059530	Heterogeneous nuclear ribonucleoprotein methyltransferase-like 3	3	Methylation
M37568	Homeo boxC8	3	Neuron differentiation
AA875069	H3 histone, family 3B	3	Nucleosome assembly
X62166	Ribosomal protein L3	3	Protein biosynthesis
AA875327	Eukaryotic translation initiation factor 4H	3	Protein biosynthesis
AA859719	–	3	Protein biosynthesis
X62146	Ribosomal protein L11 (predicted)	3	Protein biosynthesis
X62146	Ribosomal protein L11 (predicted)	3	Protein biosynthesis
AA892367	Ribosomal protein L3	3	Protein biosynthesis
D10755	Proteasome (prosome, macropain) subunit, alpha type 6	3	Protein catabolism
X51536	Ribosomal protein S3	3	Protein synthesis
AA800054	Ribosomal protein L19	3	Protein synthesis
X51536	Ribosomal protein S3	3	Protein synthesis
AI178750	Eukaryotic translation elongation factor 2	3	Protein synthesis
X58465	Ribosomal protein S5	3	Protein synthesis
X53378	Ribosomal protein L13	3	Protein synthesis
X78327	Ribosomal protein L13	3	Protein synthesis
M89646	Ribosomal protein S24	3	Protein synthesis
AI176546	Heat shock protein 1, alpha	3	Protein synthesis
AA944397	Heat shock protein 1, alpha	3	Protein synthesis
AA800211	Pyridoxine 5'-phosphate oxidase	3	Pyridoxine biosynthesis
AF036335	NonO/p54nrb homolog	3	RNA splicing
AF036335	NonO/p54nrb homolog	3	RNA splicing
U87960	Protein tyrosine phosphatase, receptor type, F	3	Signaling
U10303	Endothelial differentiation sphingolipid G-protein-coupled receptor 1	3	Signaling
U14409	Melatonin receptor 1A	3	Signaling
U50949	Olfactory receptor 1641	3	Signaling
AB007688	Homer homolog 1 (<i>Drosophila</i>)	3	Signaling
M36317	Thyrotropin releasing hormone	3	Signaling
X01454	Thyroid stimulating hormone, beta subunit	3	Signaling hydroxysteroid dehydrogenase-1, delta(5)-3-beta
S63167	Hydroxysteroid dehydrogenase-1, delta (5)-3-beta (predicted)	3	Biosynthesis
AI009098	Hypoxia up-regulated 1	3	Stress
M96630	SEC61, alpha subunit (<i>S. cerevisiae</i>)	3	Transport
M30312	Potassium voltage-gated channel, shaker-related subfamily, member 3	3	Transport
AI230914	Farnesyltransferase, CAAX box, beta	3	Wound healing
U82591	Chromosome 6 open reading frame 108	3	No identified ontologies
AF051155	G protein beta subunit-like	3	No identified ontologies
X52815	–	3	No identified ontologies
AA891742	Similar to cDNA sequence BC019806 (predicted)	3	No identified ontologies
AA892863	–	3	No identified ontologies
AI104513	–	3	No identified ontologies
S76758	–	3	No identified ontologies
AI012942	–	3	No identified ontologies
AF027188	Similar to RIKEN cDNA 4933424N09 (predicted)	3	No identified ontologies
AI639409	–	3	No identified ontologies
AI233591	–	3	No identified ontologies
AA859835	Transcribed locus	3	No identified ontologies
AF053097	–	3	No identified ontologies
AF034753	Similar to hypothetical protein FLJ22490 (predicted)	3	No identified ontologies
U47311	–	3	No identified ontologies
H33253	Similar to tubulin-specific chaperone d	3	No identified ontologies
AI113046	–	3	No identified ontologies
AA900850	–	3	No identified ontologies
AA875265	–	3	No identified ontologies
D17349	Similar to cytochrome P450 2B15	3	No identified ontologies
AI007820	Heat shock 90 kDa protein 1, beta	3	No identified ontologies
H31907	Embryo-related protein	3	No identified ontologies
H33725	Associated molecule with the SH3 domain of STAM	4	Apoptosis
AA800206	Actin alpha 2 (predicted)	4	Apoptosis
AB010436	Cadherin 8	4	Cell adhesion
X95990	Complement component 5, receptor 1	4	Chemotaxis

Appendix B (Continued)

M33605	Perforin 1 (pore forming protein)	4	Cytolysis
D90219	Natriuretic peptide precursor type C	4	DNA metabolism
X84210	Nuclear factor I/A	4	DNA replication
AA892798	Uterine sensitization-associated gene 1 protein	4	Embryo implantation
M22360	Murinoglobulin 1 homolog (mouse)	4	Inflammation
M22993	Murinoglobulin 1 homolog (mouse)	4	Inflammation
M94548	Cytochrome P450, family 4, subfamily F, polypeptide 2	4	Metabolism
AF008554	Implantation-associated protein	4	Metabolism
D00688	Monoamine oxidase A	4	Metabolism
D00729	Dodecenoyl-coenzyme A delta isomerase	4	Metabolism
AF036761	Stearoyl-coenzyme A desaturase 2	4	Metabolism Microtubule
M83196	Microtubule-associated protein 1A	4	Depolymerization
M91652	Glutamine synthetase 1	4	Nitrogen fixation
AF061726	Calpain 3	4	Protein catabolism
L34262	Palmitoyl-protein thioesterase	4	Protein production
X13905	Similar to Ras-related protein Rab-1B	4	Protein transport
AF073891	Potassium voltage-gated channel, subfamily H (eag-related), member 5	4	Signaling
U57500	Protein tyrosine Phosphatase, receptor type, A	4	Signaling
L19112	Fibroblast growth factor receptor 2	4	Signaling
L35921	Guanine nucleotide binding protein (G protein), gamma 8 subunit	4	Signaling
D10763	Erythropoietin	4	Stress
AB015432	Tumor-associated protein 1	4	Transport
M24393	Myogenin	4	Transcription
L13206	Forkhead box D4	4	Transcription
U01146	Nuclear receptor subfamily 4, group A, member 2	4	Transcription
AI145177	Early growth response 4	4	Transcription
AF059273	Glucocorticoid modulatory element binding protein 2	4	Transcription
AJ006519	Amiloride-sensitive cation channel 2, neuronal	4	Transport
AF104399	Cbp/p300-interacting transactivator	4	Transport
J02844	carnitine <i>O</i> -octanoyltransferase	4	Transport
D12573	Hippocalcin	4	Transport
U02096	Fatty acid binding protein 7, brain	4	Transport
U09211	solute carrier family 18 (vesicular acetylcholine), member 3	4	Transport
AI102031	Bridging integrator 1	4	Transport
X63744	Solute carrier family 1	4	Transport
AF090692	Cystatin 8	4	No identified ontologies
D26492	Dynein, axonemal, heavy polypeptide 1	4	No identified ontologies
AI639159	Solute carrier family 23	4	No identified ontologies
X01115	Seminal vesicle secretion 5	4	No identified ontologies
AA875001	Tripartite motif protein 8 (predicted)	4	No identified ontologies
U77626	WW domain binding protein 4 (formin binding protein 21)	4	No identified ontologies
AI639076	–	4	No identified ontologies
AA892394	CUG triplet repeat, RNA binding protein 1 (predicted)	4	No identified ontologies
AI639179	–	4	No identified ontologies
AI231445	–	4	No identified ontologies
AA866264	–	4	No identified ontologies
AI638972	–	4	No identified ontologies
AA892228	CUG triplet repeat, RNA binding protein 1 (predicted)	4	No identified ontologies
AA866293	Transcribed locus	4	No identified ontologies
AA875089	–	4	No identified ontologies
AI639500	–	4	No identified ontologies
AI009658	–	4	No identified ontologies
H31550	Transcribed locus	4	No identified ontologies
AI137538	–	4	No identified ontologies
AA859992	–	4	No identified ontologies
AI639012	Similar to cDNA sequence BC019776	4	No identified ontologies
AA800803	Similar to RIKEN cDNA6720485C15	4	No identified ontologies
AI169372	RAS-like family 11 member A	4	No identified ontologies
S65091	similar to protein phosphatase 1, regulatory (inhibitory) subunit 1C	4	No identified ontologies
AA858621	CaM-kinase II inhibitor alpha	4	No identified ontologies
AI171848	Apical early endosomal glycoprotein	4	No identified ontologies

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