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Genome Expression Profiling and Network Analysis of Nitrite Therapy during Chronic Ischemia: Possible mechanisms and interesting molecules

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Abstract

Sodium nitrite is widely recognized to be a highly effective NO donor for the treatment of several ischemic tissue disorders. However, mechanisms by which nitrite confers cytoprotection during ischemic disorders remain largely unknown. In this study, we used genome expression profiling approaches to evaluate changes in gene expression in the hind-limb ischemia model using vehicle or sodium nitrite therapy. Sodium nitrite significantly restored ischemic tissue perfusion by day 3 post-ligation which returned to normal by day 7. Genesifter analysis of Affymetrix GeneChip data revealed a significant down-regulation of gene expression profiles at day 3, whereas gene expression profiles were predominantly up-regulated at day 7. Ingenuity network analysis of gene expression profiles at day 3 showed a strong decrease in gene expression from networks associated with immune functions such as acute inflammatory responses, antigen presentation, and humoral immune responses while networks containing increased gene expression profiles were associated with cardiovascular, skeletal, and muscle system development and function. Network analysis of day 7 gene array data revealed predominant up-regulation of genes associated with cell survival, tissue morphology, connective tissue function, skeletal and muscular system development, and lymphoid tissue structure and development. These data suggest that sodium nitrite elicits potent anti-inflammatory and pro-angiogenic gene responses at early time points which is later followed by up-regulation of genes associated with tissue repair and homeostasis.

Keywords

gene array; mRNA; angiogenesis; inflammation; vascular function

Introduction

Nitrite anion is now widely appreciated as a highly useful therapeutic agent for various ischemic disorders due to its one electron reduction to nitric oxide (NO) under such conditions. Nitrite reduction to NO during ischemia can occur in a host of various ways

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including but not limited to deoxyhemoglobin and deoxymyoglobin [1, 2], acidic diproportionation [3], mitochondrial electron transport molecules [4], xanthine oxidoreductase [5], eNOS [6], and other recently identified mechanisms described in this issue. This array of potential reduction mechanisms for nitrite makes it a useful source of nitric oxide during ischemic conditions which could be beneficial in a number of disease states encompassing a role for these responses.

Nitrite therapy has been successful in diverse experimental models of cardiovascular disease involving ischemic and inflammatory mediated tissue injury. Nitrite therapy plays a major cytoprotective role in murine models of myocardial, hepatic, and neurological ischemia/reperfusion injury [7–10]. Moreover, nitrite therapy confers significant protection in a canine model of acute coronary artery occlusion which was also associated with increased cytoprotection and improvement of microvascular perfusion [11]. Stokes et al has recently reported nitrite to have anti-inflammatory properties in a high cholesterol diet induced model of inflammation [12]. We have previously reported that nitrite therapy rapidly restores blood flow to chronically ischemic tissue by stimulating angiogenesis and endothelial cell proliferation as well as augmenting collateral vessel development [13]. Recent work from Isenberg et al also demonstrates that nitrite therapy enhances skin flap wound healing which is coupled with increased angiogenesis and attenuated thrombospondin-1 function [14]. Together, these studies demonstrate that nitrite therapy is a potent mediator of tissue cytoprotection during ischemia which implies a wide impact on several biological functions.

Despite the overwhelming beneficial data of nitrite therapy for ischemic cardiovascular system disorders, there is much less information regarding molecular mechanisms of nitrite mediated tissue protection. Moreover, very little specific information is available regarding what specific genes are affected by nitrite therapy in ischemic tissue pathologies. As nitrite therapy is becoming increasingly attractive for clinical uses, a clear understanding of the genes affected and how they work together is necessary to determine its mechanisms of action and ideal situations for the therapeutic use of nitrite anion. The purpose of this study was to perform genome wide expression profile analysis to identify unique molecular targets of nitrite therapy during chronic hind-limb ischemia. Data gleaned from this study reveals that nitrite anion potently down regulates gene expression of cell mediated and humoral immune responses while simultaneously increasing gene expression of cardiovascular and muscular system development and function at early time points which is followed by a later, more moderate induction of tissue repair related genes. These data provide the first insight into molecular pathways which are involved in the beneficial effects of nitrite therapy for chronic ischemia.

Experimental Procedures

Animals

14 week old male C57BL/6J mice were obtained from Jackson Labs and housed at the Association for Assessment and Accreditation of Laboratory Animal Care, internationally accredited Louisiana State University Health Sciences Center–Shreveport animal resource facility, and maintained according to the National Research Council's Guide for Care and

Use of Laboratory Animals. All experiments were performed with approval of the Institutional Animal Care and Use Committee.

Induction of chronic ischemia and measurement of hind limb blood flow

Induction of chronic hind limb ischemia was performed as we have previously reported [13, 15]. 165 μg/kg sodium nitrite therapy or PBS control treatments were began the afternoon after hind limb ligation via intraperitoneal injection. Two separate 165 μg/kg sodium nitrite doses were given as we have previously published the first in the morning and the second in the afternoon [13]. Gastrocnemius muscle tissue was harvested on the morning of the 4^{th} and 8^{th} days to give an entire 3 and 7 day period for study. Four separate experimental cohorts of animals were used for this study including: day 3 PBS treatment (n=4), day 7 PBS treatment (n=4), day 3 sodium nitrite treatment (n=4), and day 7 sodium nitrite treatment (n=4). Each individual ischemic gastrocnemius muscle specimen was harvested per animal, per cohort with each tissue specimen analyzed on a single gene array for a total of 16 individual arrays (n=4 per experimental cohort) being used to survey changes in genome expression profiles. Harvested tissue was immediately frozen in liquid nitrogen for subsequent total RNA isolation. Laser doppler blood flow measurements were taken from non-ischemic and ischemic hind limbs prior to tissue collection to determine the amount of ischemic tissue perfusion using a Vasamedics Laserflo BPM2 deep tissue penetrating doppler device as we have previously reported [13, 15]. Percent restoration of blood flow was determined by dividing the ischemic hind limb blood flow by the non-ischemic hind limb blood flow and multiplying by 100. Statistical analysis of changes in tissue blood flow between treatment groups at specific time points were performed with an unpaired students t-test using Prism software (Graphpad).

RNA isolation and microarray hybridization

Total RNA was isolated from either PBS or sodium nitrite treated gastrocnemius muscle tissues specimens at day 3 or day 7 post-ischemia using the RNeasy Midi Kit (Qiagen, Chatswort, CA). RNA integrity was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The RNA integrity number (RIN) for all specimens used for gene array analysis was between 8–9. Double stranded cDNA was synthesized from 5ug total RNA using a Superscript cDNA synthesis Kit (Invitrogen, Carlsbad, CA) and purified using the Gene Chip Sample Cleanup Module (Affymetrix, Santa Vlara, CA). After biotin-labeling, the cDNA were fragmented at 94°C and hybridized to the Mouse Genome 430 2.0 Array (Affymetrix), which contains 39,000 fully annotated transcripts of the mouse genome. After washing and staining, the arrays were scanned using GeneChip Scanner 3000 (Affymetrix) and hybridization efficiency determined by housekeeping control and spike control probe sets. Analysis of all control probe sets received a pass call demonstrating consistent hybridization efficiency across all specimens.

Microarray data normalization and statistical analysis

Array data were globally scaled to a target intensity value of 500 to compare individual array experiments. To determine differentially expressed genes between PBS and nitrite treatment, data were log transformed and uploaded into Genesifter program (www.genesifter.net). Identification of changes in gene expression were determined using a

pair-wise comparison of PBS versus nitrite therapy gene array data at from an individual tissue specimen hybridized to a single gene chip for each time point per treatment condition (i.e. day 3: PBS n=4 and sodium nitrite n=4, same for day 7). The following analysis criteria was used to identify significant changes in gene expression using Genesifter: a minimal 2-fold difference in expression with no maximal threshold limit, a hybridization quality cutoff of 1, and an unpaired student t-test between treatment groups followed by Benjamini and Hochberg post test to limit false discovery rates.

Ingenuity network analysis

Due to the complexity of reactions between molecules, changes in gene expression are often influenced by other genes, that is to say one gene may be regulated by many different molecules which itself then also regulates many other gene targets, thus classical hierarchical expression analysis cannot reveal complicated relationships between various changes in gene expression. Network analysis using Ingenuity Pathway analysis software can organize gene expression changes into groups of genes which highly influence one another governing specific biological functions. As such, network analysis of large data sets facilitates identification of novel gene expression associations advancing discovery of potential mechanistic pathways which have not been considered. Therefore, genes which were significantly altered as identified by Genesifter analysis were uploaded into Ingenuity software (www.ingenuity.com) to perform network analysis. The software scans the input gene expression data to provide networks by using the Ingenuity Pathway Knowledge Base, which is a data base created from data mining for expression and functional relationships between molecules extracted from previously published peer reviewed papers found in NCBI Pubmed, Medline, and several other databases. The resulting output of network analysis is broken down into genetic composition of networks, biological function of networks, the number of interrelated network relationships, as well as statistical analysis of individual networks.

Results

Relationship between sodium nitrite therapy and global gene expression

We and others have shown that nitrite therapeutic intervention elicits rapid and robust resolution of tissue dysfunction during ischemia [10, 16]. However, no data exists regarding genome expression profile changes at various stages of nitrite therapeutic intervention. Figure 1A illustrates that sodium nitrite therapy progressively restores ischemic hind limb perfusion over the course of seven days which is first observed at day 3 and maximal at day 7. Therefore, we performed genome expression profile analysis from either PBS vehicle or sodium nitrite (165 μg/kg) ischemic gastrocnemius muscle tissue at day 3 and day 7. Figure 1B shows heat map results between PBS control and sodium nitrite gene expression profiles at day 3. 457 distinct gene expression profiles were significantly altered with nitrite therapy eliciting a large down regulation of 346 genes compared to up-regulation of 111 genes. Conversely, figure 1C demonstrates that sodium nitrite therapy differentially impacts the expression profile of 274 genes at day 7 of ischemia but that the majority of these genes are up-regulated (219 genes) compared to fewer that were down-regulated (55 genes). Supplementary Table 1 and 2 list the genes, change ratio (± SEM) of expression levels

compared to PBS control, and p-values which were identified at days 3 and 7, respectively. Together, these data reveal an unexpected yet interesting gene expression profile pattern whereby nitrite therapy mediates a large and preferential down-regulation of gene expression at day 3 which transitions to a smaller yet positively up-regulated group of genes at day 7.

Nitrite therapy regulation of canonical Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

KEGG pathway analysis was performed to determine what biological pathways may be affected by sodium nitrite therapy. Table 1 reports the list of KEGG pathways involved at days 3 and 7. Interestingly, the dominant KEGG pathway identified at day 3 was the cytokine-cytokine receptor interaction pathway with 13 out of a possible 198 genes being significantly altered. Other KEGG pathways which were also identified at day 3 include metabolic and signaling regulatory pathways as well as adhesion molecule dependent responses such as extracellular matrix-receptor interactions, gap junction molecules, focal adhesions, and leukocyte transendothelial migration pathways. The top KEGG pathway identified at day 7 involved the PPAR signaling pathway as well as others involving sphingolipid metabolism and extracellular matrix-receptor interactions. Closer examination of all the KEGG pathways involved at day 7 revealed a wide yet smaller number of molecules associated with established KEGG pathways.

Ingenuity network analysis of nitrite therapy during chronic ischemia

With nitrite therapy exerting a significant change in global gene expression profiles yet showing a modest involvement with classically established KEGG pathways and molecules, we next examined our gene array data using Ingenuity analysis software to identify novel network relationships among differentially expressed genes which may not clearly segregate into classical KEGG pathways. Figure 2 illustrates the number of networks and relationships among them at days 3 and 7. Ingenuity analysis of global expression data from both days revealed 17 distinct networks. However, the number of interactive relationships (found in parentheses) among networks were much greater at day 3 versus 7 suggesting nitrite therapy mediates wide ranging effects intertwining several biological functions. Together, these data suggest that nitrite therapy has a significant impact on biological functions which do not fall under classically defined pathways suggesting novel mechanisms of cytoprotection, tissue preservation, and healing.

Network functions during nitrite therapy

Tables 2 and 3 list all of the identified networks at day 3 and 7 which demonstrate a diversity of differential gene expression profiles. Network analysis of day 3 gene array data shows a general attenuation of early immune response related gene expression yet a significant increase in cardiovascular and skeletal muscle gene expression. Conversely, networks identified at day 7 show a predominant up-regulation of tissue remodeling and repair responses yet the number of affected genes per network is significantly reduced compared to day 3 data. Figure 3 illustrates the strongest network of gene relationships (i.e. network 1) at day 3 and 7 during sodium nitrite therapy. Panel A shows that day 3-network 1 is associated with genetic disorder, immunological disease, and organismal survival. A

striking feature of this and other networks at day 3 is the number of genes which are significantly down regulated. The nature of the relationships within this network is largely indirect (i.e. the presence of hashed lines) reinforcing the notion that network analysis cannot completely reveal the effect of nitrite on ischemic tissue gene expression profiles but do identify key gene targets within a discrete biological function. This issue aside, it is clear that nitrite therapy blunts expression of numerous immunological associated genes including chemokine/cytokine ligands and receptors (e.g. CCL2, CXCL6, CCL9, IL1R1, and CSFR3), the innate immune receptor TLR-1 and the TLR homologue CD180, interferon-inducible proteins IFITM1 and IFI16, the innate immune DNA sensing protein ZBP-1, and the Fc receptor Fcgr1a. Similarly, nitrite therapy also attenuated expression of genes which influence extracellular matrix composition and function, namely MMP9 and collagen 1, alpha 2. Nitrite therapy differentially altered coagulation regulatory gene expression which contributes to both inflammation and angiogenesis responses as complement 3A receptor 1 was down-regulated while proteinase activated receptor 2 was up-regulated [17, 18]. Lastly, day 3 nitrite treated ischemic tissue showed a robust increase in metallothionine 3 gene expression.

Network 1 result from day 7 of nitrite therapy shows that these genes are associated with regulation of post-translational modlificantion, tissue morphology, and connective tissue disorders. Both direct and indirect relationships (solid versus dashed lines) are observed by day 7 of nitrite therapy which implicates a diverse array of tissue restitution responses. Several growth factor related proteins are up-regulated including connective tissue growth factor (CTGF), granulin (GRN), integrin beta 3 (ITGB3), insulin growth factor binding protein 4 (IGFBP4), and adiponectin (ADIPOQ). Several extracellular matrix associated proteins are also up-regulated including biglycan (BGN), procollagen C-endopeptidase enhancer (PCOLCE), matrix metalloproteinases 2, 3, 13, and 14, and immunoglobulin superfamily member 8 (IGSF8). This combined expression profile suggests that nitrite therapy attenuates tissue injury and damage by altering immune responses while augmenting tissue healing programs over time.

Interrelated network relationships of nitrite therapy

It is difficult to ascribe biological importance of one network over another for the effects of nitrite therapy; however, it is possible to infer potential significance of certain networks by the number of relationships or ‘connections’ between them. Figure 4 illustrates day 3-network 6 and 13 which have at least four connections with other networks. Network 6 functions involve antigen presentation and cell/humoral-mediated immune responses while network 13 functions encompass inflammatory responses and mediators of neurological disease. As mentioned earlier, figure 2 illustrates the number of interactions between individual networks; therefore we chose to examine networks with multiple connections which are indicative of a high degree of relationships. Figure 4 illustrates network 6 and 13 from day 3 nitrite therapy. Day 3 network 6 top functions relate to antigen presentation and cell mediated and humoral immune responses. Numerous immune regulatory genes are significantly down regulated including C-type lectin domain family 4, members A and E, allograft inflammatory factor 1, formyl peptide receptor 2, cysteinyl leukotriene receptor 1, the cysteine proteinase inhibitor Cathepsin C, and CD163 macrophage scavenger receptor.

Similarly, genes associated with day 3 network 13 are involved in inflammatory responses as well as neurological diseases, and cellular development. Genes that influence immune function include C-type lectin domain family 5 member A which influences immune cell adhesion and signal regulatory protein beta 1 that modulates tyrosine kinase signaling involving immunotyrosine activating motifs. Interestingly, several solute carrier family members involved in cell development and homeostasis are differentially regulated as solute carrier family 16 member 3 and 8 member 3 are up-regulated versus solute carrier family 15 member 3 which is down-regulated. Lastly, uridine-cytidine kinase 2 is significantly up-regulated suggesting increased pyrimidine nucleoside triphosphate production which is necessary for DNA/RNA synthesis.

Figure 5 illustrates gene expression profiles of day 3 network 3 and day 3 network 10 which are associated with cardiovascular system development and function, skeletal muscle system development and function, and changes in tissue morphology, respectively. Suprisingly, day 3 network 3 is the only network in which there was a preferential up-regulation of gene expression versus other networks which showed predominant down-regulation of gene expression. Genes within day 3 network 3 are associated with cardiovascular system development and function which include up regulation of the potential pro-angiogenic mediators vascular endothelial cell cadherin (VE-cadherin), purinergic receptor P2Y, and vitronectin, as well as down regulation of anti-angiogenic and vasoregulatory molecules thrombospondin-2 and the serotonin receptor 2B [19–27]. Numerous myocyte specific genes are also significantly up-regulated including myosin heavy and light chain 2, myosin light chain 4 and 9, troponin I, and troponin T type 2 indicating alteration of muscle cell function and possibly development. Lastly, several dual purpose angiogenic and inflammatory mediators are also significantly down-regulated including CXCL7, CXCR6, and stromal derived factor-1/CXCL12 suggesting that nitrite therapy augments physiological versus pathological angiogenesis responses [28]. Genes contained within day 3 network 10 included several inflammatory associated genes which were down-regulated including macrophage scavenger receptor-1 (MSR-1), C-type lectin domain family 4 member D, immunoglobulin superfamily member 6, and Wnt inducible signaling protein 2 (WISP2) [29–33]. Conversely, genes associated with myocyte function were up-regulated including myosin heavy chain 7 and smoothelin-like 1 which modulates myosin phosphatase activity governing contractile responses [34, 35]. Together, these two networks illustrate that nitrite therapy distinctly augments gene expression profiles associated with vascular system development (i.e. angiogenesis) while decreasing inflammatory molecule gene expression.

Figure 6 demonstrates network 3 and 4 associations of nitrite induced changes in gene expression at day 7. Top cellular functions of networks 3 and 4 relate to cancer, cell death, nervous system development, and endocrine system disorders representing a seemingly odd array of responses. However, closer examination of the identified genes reveals interesting and logical associations such as increased expression of transcription factors involved in cell fate determination and healing responses such as FoxP1, KLF16, and TWIST1 suggesting nitrite therapy could act to augment repair responses and possibly augment cell fate determination of resident stem cell populations [36–39]. Nitrite therapy also augments expression of proteins involved in attenuating cell activation responses such as protein tyrosine phosphatase receptor type O and leukocyte associated immunoglobulin-like

receptor 1 (LAIR1) which both negatively regulate cellular activation responses [40–42]. Genes found in day 7 network 4 also influence tissue remodeling as regulators of proteolysis including MMP13 and SERPINB1 are elevated. The anti-angiogenic molecules semaphroin 3B (sema3B) and thrombospondin-1 are up-regulated at this time point suggesting induction of negative feedback loops governing vascular growth [43, 44]. Lastly, genes governing cellular metabolism are also significantly elevated including 3-phosphoglycerate dehydrogenase which is essential for de novo serine biosynthesis and stearoyl-CoA desaturase that catalyzes the formation of oleic acid via desaturation of stearic acid [45]. These molecules play important roles in modulating cellular metabolism of neurological and muscle function which could also influence the formation of other nitric oxide related species such as nitro-fatty acids [46].

Discussion

The utility of nitrite anion therapy for attenuating numerous ischemic tissue disorders is now well appreciated. However, molecular mechanisms mediating nitrite dependent effects on tissue preservation and restoration of homeostasis still remain largely unknown. Here we employed genome wide expression profiling techniques using the Affymetrrix 430 2.0 mouse GeneChip which contains a highly comprehensive and annotated representation of the whole mouse genome with slightly greater than 39,000 transcripts evaluated per array. With such comprehensive coverage of genome expression profiles, we were able to broadly survey for nitrite induced alterations of genome wide changes in expression patterns. To our knowledge, this is the first report of such wide scale genome analysis of nitrite therapy under chronic ischemic settings. A recent report by Raat et al used a similar approach with the same Affymetrix GeneChip 430 2.0 array to determine genome expression changes in response to dietary nitrite supplementation during acute liver ischemia-reperfusion injury [47]. Data generated from the Raat et al study identified 144 different genes which were significantly altered with 83 being up-regulated and 61 down-regulated using a fold change cut off value of 1.5 compared to 457 different genes at day 3 post-ischemia and 274 genes at day 7post-ischemia using a fold change cut off value of 2 in our current study. The differences between our current study and that of Raat et al likely involves altered delivery routes (i.e. intraperitoneal injection versus oral administration in the drinking water) as well as tissue specific differences (i.e. muscle versus liver).

There are a few interesting similarities as well as distinct differences between our current data and that of Raat et al [47]. Firstly, dietary nitrite therapy altered carbohydrate metabolism during liver acute I/R as did i.p. nitrite therapy at day 3 of chronic ischemia. Nitrite therapy also similarly affected solute carrier family proteins in acute I/R and day 3 chronic ischemia with different solute carrier family molecules involved in either response. Secondly, nitrite therapy appears to preferentially augment overall gene expression during acute I/R as Raat et al reported more up-regulated genes than down; whereas, our findings suggest that nitrite therapy during chronic ischemia results in an initial down-regulation of the majority of genes identified at day 3 followed by up-regulation of fewer genes at day 7. Differences between ours and the previous study could be due to the duration of ischemic event (acute with reperfusion versus permanent with no reperfusion). These discrepancies aside, our data are consistent with a previous report by Braam et al demonstrating that nitric

oxide donor treatment of endothelial cells results in a temporal and dose-dependent decrease in gene expression which is consistent with our current observations and that of our previous work demonstrating that nitrite restores ischemic tissue perfusion in a NO dependent manner [13, 48]. Our data together with Raat et al suggest that nitrite therapy exerts significant effects on genome expression profiles which are undoubtedly influenced by the duration of ischemia and tissues examined.

The combined use of traditional heat map gene array analysis and KEGG pathway analysis coupled with Ingenuity network analysis provided a comprehensive and illuminating picture of what biological processes may be targeted by nitrite anion therapy during chronic hind limb ischemia. So what then can we conclude regarding the biological effects of nitrite therapy for restoration of chronically ischemic tissue perfusion? Our data unexpectedly revealed that nitrite therapy inhibits gene expression in a dominant fashion at day 3 of ischemia which involved several important biological networks such as immunological disease, cell and humoral mediated immunity, antigen presentation, cancer, cell signaling and cycle, lipid metabolism, and endocrine system disorders. However, of the minority of genes which were up-regulated, they centered squarely on cardiovascular and muscular system development and function with clear up-regulation of pro-angiogenic genes (e.g. VE-cadherin, vitronectin, purinergic receptor PY2) and down-regulation of anti-angiogenic and vasoregulatory genes (e.g. thrombospondin 2 and serotonin receptor 2B) along with induction of myocyte functional responses (e.g. myosin heavy and light chains and troponin T and I). While it is impossible to truly infer precise mechanisms of nitrite action from these data, it does appear that nitrite therapy may serve to blunt inflammatory responses while enhancing vascular and muscle growth or repair.

Consistent with the idea that nitrite could be facilitating tissue healing and repair, network data from day 7 revealed that a fewer number of genes were affected and they were all predominantly up-regulated. Restoration of tissue metabolism and homeostasis is likely the key goal at this time point as several anti-angiogenic mediators were significantly up-regulated (e.g. semaphorin 3B and thrombospondin-1) as well as transcriptional regulators of tissue differentiation (e.g. Twist-1 and FoxP1). These expression patterns and networks are consistent with the observation that ischemic tissue perfusion is restored back to pre-ischemic levels at that time and may represent a shift toward a more homeostatic form of gene expression. However, additional studies are needed to better understand the prolonged effects of nitrite therapy after resolution of tissue ischemia.

In summary, we have presented data showing that sodium nitrite therapy elicits a robust change in gene expression profiles at both early and later time points of chronic ischemia which appear to be directly related with restoring tissue reperfusion and stimulating repair responses. These data reveal novel and interesting networks of biological function which have not been previously considered for correcting chronic ischemic tissue dysfunction. Future studies directly probing the biological roles of these mediators during chronic ischemic injury will likely reveal unique molecular mechanisms necessary for establishing reperfusion of tissues experiencing permanent or intermittent ischemic stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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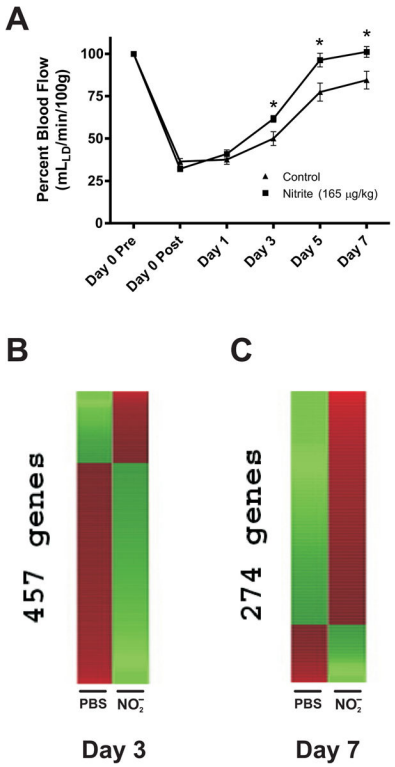

Figure 1. Nitrite therapy restores ischemic hind limb blood flow and stimulates changes in gene expression

Panel A shows the effect of 165 μg/kg sodium nitrite therapy on ischemic hind limb blood flow compared to PBS control treatment. n=4 mice per cohort, *p<0.05 nitrite versus PBS per respective time point. Panel B shows nitrite dependent heat map changes in ischemic tissue gene expression compared to PBS control treatment at day 3 post ischemia. The expression of 457 genes was significantly altered with 111 genes up-regulated and 346 genes down-regulated. Panel C illustrates nitrite dependent heat map changes in ischemic tissue gene expression compared to PBS control at day 7 post ischemia. The expression of 274 genes was significantly changed with 219 up-regulated and 55 down-regulated. Red indicates up-regulation whereas green indicates down-regulation.

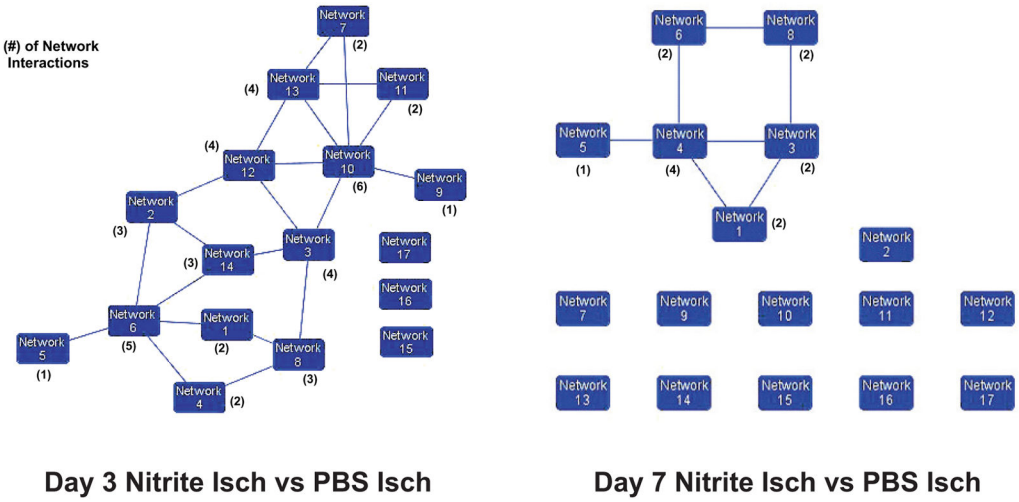

Figure 2. Internetwork relationships during nitrite therapy

Ingenuity network analysis of day 3 and day 7 gene array data identified 17 discreet networks of genes which displayed various degrees of interrelationships (i.e. genes which influenced others in a network or that were found in more than one network). Network relationships for day 3 revealed that 14 out of 17 networks had some degree of interaction with another network with others showing a larger number of interactions. Network relationships for day 7 showed only 6 out of 17 networks had some degree of interaction with other networks. Moreover, the number of network interactions was much smaller compared to day 3.

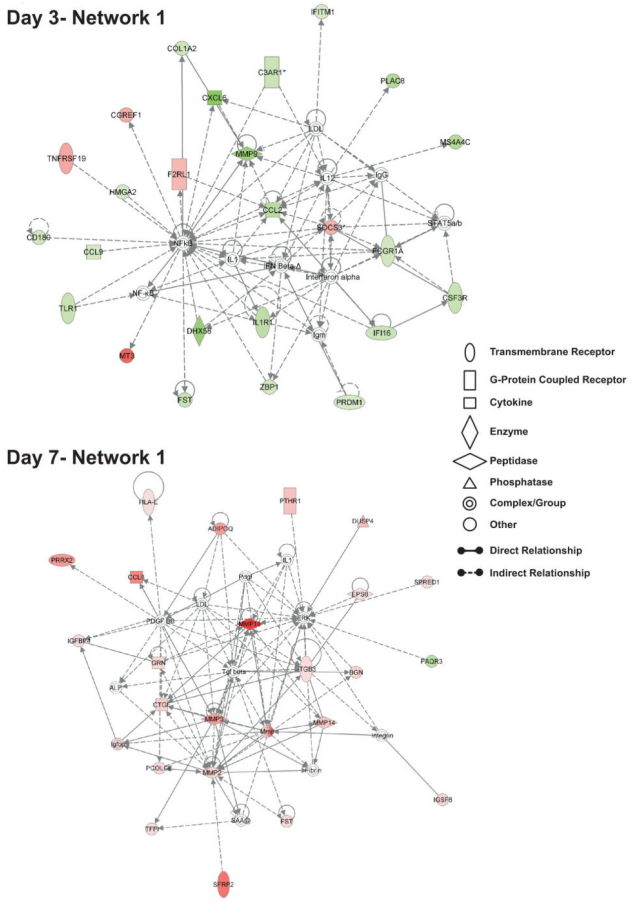

Figure 3. Top ranking networks identified during nitrite therapy at day 3 and day 7

The top network illustrates day 3 network 1 which has biological functions associated with genetic disorder, immunological disease, and organismal survival. The bottom network shows day 7 network 1 that has biological associations with post-translational modification, tissue morphology, and connective tissue disorders. Red shading indicates up-regulation whereas green shading shows down-regulation. A molecule classification legend is provided to clarify the classification of molecules involved and molecular relationships.

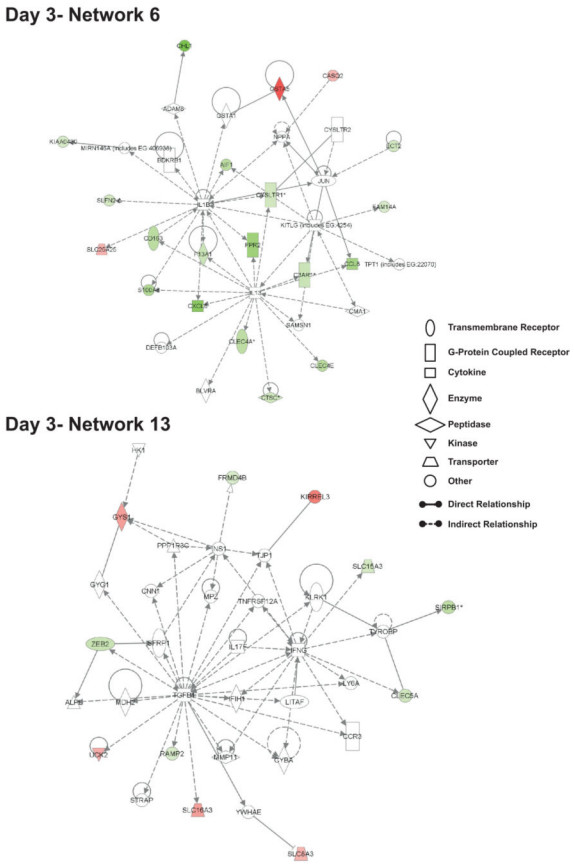

Figure 4. Highly interrelated networks affecting immune responses at day 3

The top network illustrates day 3 network 6 which has 5 interactions with other networks and has biological functions associated with antigen presentation, cell-mediated immune response, and humoral mediated immune response. The bottom network shows day 3 network 13 that has 4 interactions with other networks and its biological functions are associated with inflammatory responses, neurological disease, and cellular development. Red shading indicates up-regulation whereas green shading illustrates down-regulation. A molecule classification legend is provided to clarify the classification of molecules involved and molecular relationships.

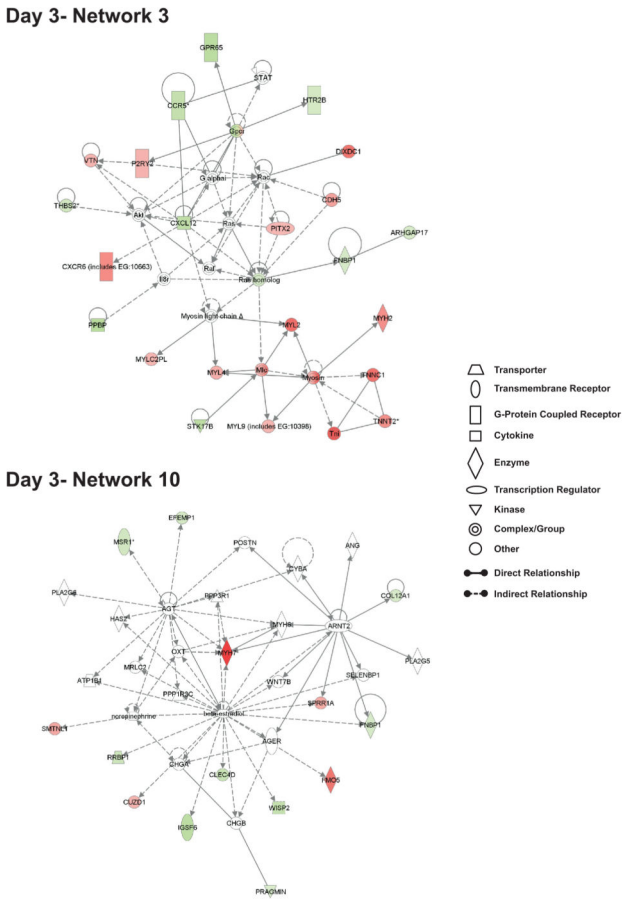

Figure 5. Highly interrelated networks affecting cardiovascular and muscle system functions at day 3

The top network shows day 3 network 3 which has 4 interactions with other networks and its biological functions are associated with cardiovascular system development and function, skeletal and muscular system development and function, and tissue development. The bottom network illustrates day 3 network 10 that has 6 interactions with other networks and has biological functions associated with cardiovascular system development and function, tissue morphology, and carbohydrate metabolism. Red shading indicates up-regulation whereas green shading illustrates down-regulation. A molecule classification legend is provided to clarify the classification of molecules involved and molecular relationships.

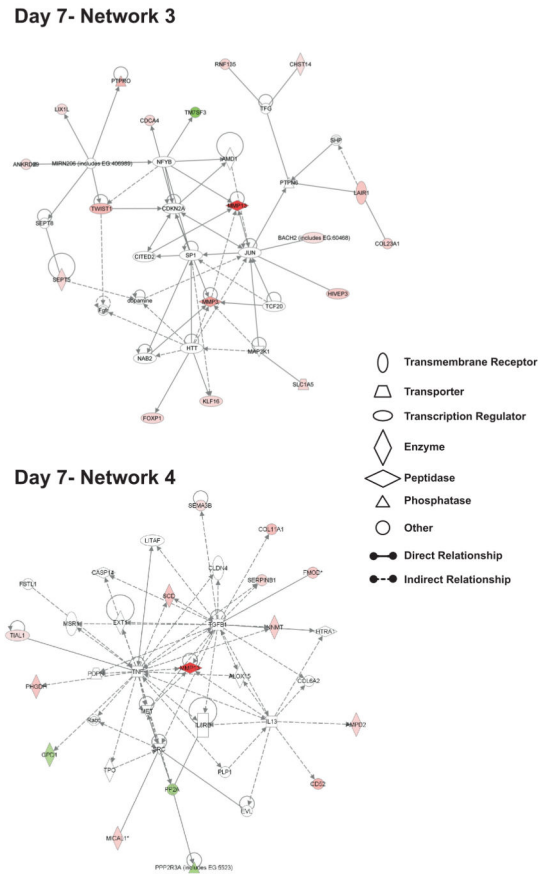

Figure 6. Highly related networks affecting multiple cell responses and tissue morphology at day 7

The top network illustrates day 7 network 3 which has 2 interactions with other networks and is associated with biological functions involving cancer, cell death, and endocrine system disorders. The bottom network shows day 7 network 4 that has 4 interactions with other networks and has biological functions associated with cancer, nervous system development and function, and tissue morphology. Red shading indicates up-regulation whereas green shading illustrates down-regulation. A molecule classification legend is provided to clarify the classification of molecules involved and molecular relationships.

Table 1

Identified KEGG Pathways

Day 3	**List**	**Gene Set**	**z-score**
Cytokine-cytokine receptor interaction	13	198	4.42
ECM-receptor interaction	4	50	2.86
Starch and sucrose metabolism	3	35	2.62
Porphyrin and chlorophyll metabolism	2	20	2.42
Calcium signaling pathway	6	115	2.3
Focal adhesion	6	119	2.21
Pentose and glucuronate interconversions	1	7	2.21
Bile acid biosynthesis	2	24	2.09
Metabolism of xenobiotics by cytochrome P450	3	49	1.92
Leukocyte transendothelial migration	4	79	1.8
Gap junction	3	55	1.7
Glycosphingolipid biosynthesis - neo-lactoseries	1	11	1.58
Melanoma	2	35	1.45
Cysteine metabolism	1	14	1.29
Keratan sulfate biosynthesis	1	14	1.29
Parkinson’s disease	1	14	1.29
Cell Communication	3	70	1.24
B cell receptor signaling pathway	2	41	1.21
Glycan structures - biosynthesis 2	2	43	1.13
Day 7			
PPAR signaling pathway	5	50	4.21
Sphingolipid metabolism	3	23	3.91
ECM-receptor interaction	4	50	3.17
Complement and coagulation cascades	3	41	2.54
Alanine and aspartate metabolism	2	23	2.38
Cell Communication	4	70	2.35
Wnt signaling pathway	5	102	2.24
Nicotinate and nicotinamide metabolism	2	28	2.03
Jak-STAT signaling pathway	5	114	1.97
Gap junction	3	55	1.94
GnRH signaling pathway	3	57	1.87
Focal adhesion	5	119	1.86
Glycine, serine and threonine metabolism	2	33	1.75
Chondroitin sulfate biosynthesis	1	11	1.74
Carbon fixation	1	14	1.43
Glioma	2	44	1.28
Insulin signaling pathway	3	79	1.24
Adipocytokine signaling pathway	2	48	1.15
Citrate cycle (TCA cycle)	1	20	1.01

Day 3	List	Gene Set	z-score
Small cell lung cancer	2	53	1

Table 2

Ingenuity network list of Day3

ID	Molecules in Network	Score	Focus Molecules	U/D ratio	Top Functions
1	C3AR1, CCL2, CCL9, CD180, CGREF1, COL1A2, CSF3R, CXCL6, DHX58, F2RL1, FCGR1A, FST, HMGA2, IFI16, IFITM1, IFN Beta, IgG, Igm, IL1, IL12, IL1R1, Interferon alpha, LDL, MMP9, MS4A4C, MT3, NF-κB, NFkB, PLAC8, PRDM1, SOCS3, STAT5a/b, TLR1, TNFRSF19, ZBP1	44	25	5/20	Genetic Disorder, Immunological Disease, Organismal Survival
2	ALCAM, ALP, ANXA6, CCL8, CD3, CD52, CD244, DOCK2, ERK, FCER1G, Fgf, FGF13, FHL1, FPR2, HMMR, HMOX1, IGFBP5, ITGA7, KIT, LILRA6, LTBP2, Mmp, MYBPC1, NCAM1, Pdgf, PDGF BB, PDGFC, PLC gamma, Pld, PTPRO, TEAD4, Tgf beta, VAV, VAV1, VCAN	41	24	6/18	Cellular Movement, Cellular Growth and Proliferation, Cell-To-Cell Signaling and Interaction
3	Akt, ARHGAP17, CCR5, CDH5, CXCL12, CXCR6 (includes EG:10663), DIXDC1, FNBP1, G alphai, Gpcr, GPR65, HTR2B, Il8r, Mlc, MYH2, MYL2, MYL4, MYL9 (includes EG:10398), MYLC2PL, Myosin, Myosin light chain, P2RY2, PITX2, PPBP, Rac, Raf, Ras, Ras homolog, STAT, STK17B, THBS2, Tni, TNNC1, TNNT2, VTN	36	22	13/9	Cardiovascular System Development and Function, Skeletal and Muscular System Development and Function, Tissue Development
4	Actin, ADAM12, Alpha Actinin, Ap1, Calmodulin, CAP1, CORO1A, CSRP3, EGLN3, EPS8, F Actin, FYB, Hsp90, KCNN4, MAP4K1, MARCKSL1, Mek, MYO5A, MYOG, MYOZ2, Nfat, P38 MAPK, PACRG, Pkc(s), PTPN3, Rb, S100A8, S100A9, SFPQ, TCR, TNNI1, TNNT1, TOP2A, TPM3, UBE2G2	35	23	10/13	Cellular Development, Inflammatory Response, Lipid Metabolism

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ID	Molecules in Network	Score	Focus Molecules	U/D ratio	Top Functions
5	ARHGAP9, ASPM, BCL2L11, BIRC5, BIRC6, BTG1, Caspase, CCNA2, CD163, Ck2, DAB2, DBF4, FBXO5, FSH, GCNT1, hCG, Histone h3, Insulin, Jnk, KRT18, MAP2K1/2, Mapk, PEG3, PI3K, PIP4K2A, PLK1, Proteasome, PTGS2, RNA polymerase II, SCN5A, SMC2, SMC6, SRRM2, Ubiquitin, Vegf	34	21	3/18	Cancer, Cell Cycle, Reproductive System Disease
6	ADAM8, AIF1, BDKRB1, BLVRA, C3AR1, CASQ2, CCL8, CD163, CHL1, CLEC4A, CLEC4E, CMA1, CTSC, CXCL6, CYSLTR1, CYSLTR2, DEFB103A, ECT2, F13A1, FAM14A, FPR2, GSTA1, GSTA5, IL13, IL1B, JUN, KIAA0430, KITLG (includes EG:4254), MIRN146A (includes EG:406938), NPPA, S100A8, SAMSN1, SLC25A25, SLFN2, TPT1 (includes EG:22070)	32	20	3/17	Antigen Presentation, Cell-mediated Immune Response, Humoral Immune Response
7	ABCC9, AHNAK, ATP10A, BAT2, BRF2, C14ORF1, C6ORF211, DLG4, EXOD1, FOXA2, GCG, GSTK1, HNF4A, IMMT, JARID1C, KCNJ11, KIF22, LDHA, LDHB, LEF1, LIF, NOS2, PDZK1, PPP1R3C, PYGL, SAP18, SCARB1, SETDB1, SLC22A4 (includes EG:6583), SLC38A1, SOX17, SSR3, STAT5B, TMEM176A, ZFP64	22	15	7/8	Endocrine System Disorders, Genetic Disorder, Metabolic Disease
8	1600029D21RIK, amino acids, BTC, CDH5, CDKN2A, CRCT1 (includes EG:54544), DCK, DDR1, ERBB2, GLYCTK, GMFG, HMGA2, KIAA1598, LNX1, LTK, MATK, MELK, MERTK, MRC1, NCAPD2, PLAC8, PLK3, PTPN3, PTPN18, PTPRB, SHC1, SHCBP1, STRADA, TAOK1, TRIP13, TXK, UGT1A6, VIM, YWHAG, ZNF655	22	15	4/11	Amino Acid Metabolism, Post-Translational Modification, Small Molecule Biochemistry

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ID	Molecules in Network	Score	Focus Molecules	U/D ratio	Top Functions
9	ARPP-21, B4GALT4, DNAJA, DNAJA1, DNAJA4, DNAJB, DNAJB1, DNAJB6, DNAJB9, DNAJB11, DNAJB14, DNM1L, FABP7, Galactosyltransferase beta 1,4, HECTD2, Hsp22/Hsp40/Hsp90, HTT, LMCD1, MCOLN2, MIRN122 (includes EG:406906), MIRN17 (includes EG:406952), NPL, PACSIN1, progesterone, RHBDL1, SFRP, SFRP2, SFRP4, SHH, STIP1, TBX1, TBX18, TMCC1, WNT4, WNT7B	22	15	7/8	Drug Metabolism, Endocrine System Development and Function, Lipid Metabolism
10	AGER, AGT, ANG, ARNT2, ATP1B1, beta-estradiol, CHGA, CHGB, CLEC4D, COL12A1, CUZD1, CYBA, EFEMP1, FMO5, FNBP1, HAS2, IGSF6, MRLC2, MSR1, MYH6, MYH7, norepinephrine, OXT, PLA2G5, PLA2G6, POSTN, PPP1R3C, PPP3R1, PRAGMIN, RRBP1, SELENBP1, SMTNL1, SPRR1A, WISP2, WNT7B	20	14	5/9	Cardiovascular System Development and Function, Tissue Morphology, Carbohydrate Metabolism
11	AFP, AKAP12, ANAPC7, ANKRD2, APOBEC1, APOBEC2, AURKB, BUB1B, C14ORF106, CAST, CRIP2, DDIT4, EGR3, EP400, FIGNL1, FXYD6, GSR, H2AFZ, HAS2, HUWE1, KNTC1, LRRN1, LY6A, MIRN101-1, MIRN101-2, MTA1, MYC, RAD54B, SPN, TGFBI, TMEM97, Top2, TP53, ZW10, ZWILCH	19	14	5/9	Cell Cycle, DNA Replication, Recombination, and Repair, Cellular Assembly and Organization

ID	Molecules in Network	Score	Focus Molecules	U/D ratio	Top Functions
12	ABHD5, ADFP, ARFIP1, ATP1B1, ATPase, CEBPA, CHI3L3 (includes EG:12655), CNNM4, CTSK, DDX19B, EHD2, glucosamine, KIF20B, LITAF, LST1, MIRN124-1 (includes EG:406907), MIRN194-2 (includes EG:406970), MYH1, MYH6, MYH7, MYH9, MYL9 (includes EG:10398), POPDC2, PSMC2, PSMC5, RAB4A, RAD51, RBM47, S100A4, SLC2A4, SNX6, TEAD1, TEAD4, Tni	16	12	6/6	Cellular Assembly and Organization, Carbohydrate Metabolism, Molecular Transport
13	ALPL, CCR3, CLEC5A, CNN1, CYBA, FRMD4B, GYG1, GYS1, HK1, IFIH1, IFNG, IL17F, INS1, KIRREL3, KLRK1, LITAF, LY6A, MDH2, MMP11, MPZ, PPP1R3C, RAMP2, SFRP1, SIRPB1, SLC15A3, SLC16A3, SLC8A3, STRAP, TGFB1, TJP1, TNFRSF12A, TYROBP, UCK2, YWHAE, ZEB2	14	11	5/6	Inflammatory Response, Neurological Disease, Cellular Development
14	ASCL2, BMX, CCR1, CCR5, CCR7, CD69, CD40LG, CD5L, CXCL5, DIAPH3, FCER1G, GP1BA, HLA-DQA1, HLA-DQB2, IL6, IL1RAP, IL7R, KIAA0101, KIF2C, MLL2, MX1, NAMPT, NCKIPSD, PAXIP1, PLEK, POLD1, PVR, RBBP5, SRC, TBC1D9, TNFRSF11A, TPT1 (includes EG:22070), TUBB, UTX, VAV1	12	10	0/10	Antigen Presentation, Cell-mediated Immune Response, Humoral Immune Response
15	ATP1B4, Na-k-atpase	2	1	1/0	
16	Biotin carboxylase, MCCC2, Methylcrotonoyl-CoA carboxylase	2	1	1/0	Amino Acid Metabolism, Small Molecule Biochemistry
17	4-phenylbutyric acid, CDV3, MIRN361 (includes EG:494323), YBX2	1	1	0/1	Inflammatory Response, Neurological Disease, Lipid Metabolism

Table 3

Ingenuity network list of Day 7

ID	Molecules in Network	Score	Focus Molecules	U/D Ratio	Top Functions
1	ADIPOQ, ALP, BGN, CCL8, CTGF, DUSP4, EPS8, ERK, Fibrin, FST, GRN, HLA-E, Igfbp, IGFBP4, IGSF8, IL1, Integrin, ITGB3, LDL, Mmp, MMP2, MMP3, MMP13, MMP14, PAQR3, PCOLCE, Pdgf, PDGF BB, PRRX2, PTHR1, SAA, SFRP2, SPRED1, TFPI, Tgf beta	44	23	22/1	Post-Translational Modification, Tissue Morphology, Connective Tissue Disorders
2	Akt, AMPK, AOC3, Ap1, COL1A2, CXCL9, ERK1/2, FAM46A, G alphai, GPSM1, IBTK (includes EG:25998), IFI16, IL12, IL2RG, Insulin, Interferon alpha, IRF7, LCP2, LPAR1, Mek, MYEF2, NFkB, P38 MAPK, PDE3B, PEBP1, PI3K, PLEKHO1, Ras, RNA polymerase II, RRAD, SERPINF1, TCR, TNFAIP8, TYRO3, USP7	36	20	19/1	Skeletal and Muscular System Development and Function, Cellular Development, Lymphoid Tissue Structure and Development
3	AMD1, ANKRD29, BACH2 (includes EG:60468), CDCA4, CDKN2A, CHST14, CITED2, COL23A1, dopamine, Fgfr, FOXP1, HIVEP3, HTT, JUN, KLF16, LAIR1, LIX1L, MAP3K1, MIRN206 (includes EG:406989), MMP3, MMP13, NAB2, NFYB, PTPN6, PTPRO, RNF135, SEPT5, SEPT8, SHP, SLC1A5, SP1, TCF20, TFG, TM7SF3, TWIST1	32	18	17/1	Cancer, Cell Death, Endocrine System Disorders

ID	Molecules in Network	Score	Focus Molecules	U/D Ratio	Top Functions
4	ALOX15, AMPD2, CASP14, CD52, CLDN4, COL11A1, COL6A2, EVL, EXT1, FMOD, FSTL1, GPD1, HTRA1, IL13, IL8RB, LITAF, MET, MICAL1, MMP13, MSR1, NNMT, PDPN, PHGDH, PLP1, PP2A, PPP2R3A (includes EG:5523), Rab5, SCD, SEMA3B, SERPINB1, SRC, TGFB1, TIAL1, TNF, TPO	23	14	12/2	Cancer, Nervous System Development and Function, Tissue Morphology
5	ARHGAP4, ATAD2, BAG3, beta-estradiol, C9ORF61, CCR1, CPT1A, CYB5A, DOCK8, FADS2, FAM14A, FOS, FSTL1, GAMT, GPSM2, HRAS, IFNA2, ITGBL1, KDELR3, LRRK1, LTF, MET, MYC, NRAS, PAK4, PLA2G1B, PNPT1, PTGIS, RAC1, RASSF2, RNF213, RSAD2, SLA, SSBP2, SYNGAP1	23	14	13/1	Connective Tissue Disorders, Cell Cycle, Connective Tissue Development and Function
6	ACD, ATP10A, C2, C1S, C2-C3-C4b, C2-C4b, C4B, CFI, DRAM, ERBB2, FAM115A, FCN3, HNF4A, Hsp70, KLHDC5, LITAF, MFAP2, MIRN31 (includes EG:407035), MYO1E, NNMT, PCSK6, PRG4 (includes EG:10216), SF3B4, SLC38A4, SORBS1, SPOCK1, TERF2IP, TGFB1, TINF2, TNMD, TP53, UBQLN1, XPR1, ZNF644	23	15	12/3	Post-Translational Modification, Infectious Disease, Cancer

ID	Molecules in Network	Score	Focus Molecules	U/D Ratio	Top Functions
7	Actin, ATPase, Calmodulin, CAMK2A, CAMK2N2, CASP4, CCND1, CDK5R2, COL15A1, DAPK2 (includes EG:23604), FBLN1, FSH, GDF10, hCG, Histone h3, ITPKA, Jnk, KLHL2, Mapk, MB, MYH1, MYH9, MYL9 (includes EG:10398), MYO9B, MYOM1, Myosin, NTRK2, PCP4, Pkc(s), PLC gamma, Rac, SLC25A5, TEAD1, TF, TUBB	21	13	10/3	Cell Cycle, Endocrine System Development and Function, Cancer
8	ABI3BP, ACLY, BICD2, CD163, CDH2, CHRDL1, CITED2, DDO, FLJ10357, GNAO1, GRIN1, IQGAP1, MAMDC2, MIRN325, MIRN338, MIRN103-1 (includes EG:406895), MIRN103-2 (includes EG:406896), MIRN134 (includes EG:406924), MIRN18A (includes EG:406953), MIRN199A1, MIRN362 (includes EG:574030), MIRNLET7B (includes EG:406884), MIRNLET7E (includes EG:406887), MTPN, NISCH, NTF3, PEX5, PPM1F, PURB, ROCK1, SEMA6A, TCEB2, TSC22D3, WIF1, ZNF644	13	9	7/2	Genetic Disorder, Skeletal and Muscular Disorders, Cancer
9	TMEM68, ZFP36	2	1	1/0	Nutritional Disease, Organismal Injury and Abnormalities, RNA Damage and Repair
10	GPM6B, IL4	2	1	1/0	Antigen Presentation, Cancer, Carbohydrate Metabolism
11	DCUN1D5, MIRN149 (includes EG:406941)	2	1	1/0	
12	GPI, PARP14	2	1	1/0	Cancer, Cell Morphology, Cell-To-Cell Signaling and Interaction

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ID	Molecules in Network	Score	Focus Molecules	U/D Ratio	Top Functions
13	3-dehydrosphinganine reductase, KDSR	2	1	1/0	
14	MIRN197 (includes EG:406974), RNF26	2	1	1/0	
15	KERA, LUM	2	1	1/0	Skeletal and Muscular System Development and Function, Hair and Skin Development and Function, Tissue Development
16	CYP4X, CYP4X1, Unspecific monooxygenase	2	1	0/1	
17	ARHGAP18, BMP6, MPHOSPH6, MPP6	2	1	1/0	Gene Expression, Endocrine System Development and Function, Lipid Metabolism