Endothelial Cells Modulate Immune-Brain Signaling After Psychosocial Stress

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A. Introduction

Psychosocial stress is a well-known precipitant for mental health disorders such as anxiety and depression[1-3]. Anxiety and depression are among the most common psychiatric disorders, with up to 1 in 3 adults experiencing either Major Depressive Disorder (MDD) or an anxiety disorder within their lifetimes[4]. These conditions negatively impact length and quality of life and contribute to billions of dollars in lost productivity every year[5]. Despite the prevalence and severity of anxiety and depression, up to 35% of patients are refractory to approved therapies[6,7]. Thus, understanding the biological mechanisms by which stress confers susceptibility to anxiety and depression is essential in developing new interventions to prevent or treat these debilitating conditions.

Growing evidence suggests that bidirectional communication between the central nervous system (CNS) and peripheral immune system plays a role in stress-related psychiatric disease. Chronic stress, such as caring for a loved one with dementia or low socioeconomic status, is linked to higher plasma levels of the pro-inflammatory cytokine IL-6, and increased susceptibility to mental health disorders[8,9]. Additionally, subsets of patients with MDD or Generalized Anxiety Disorder (GAD) also have higher peripheral markers of inflammation such as CRP and IL-6 and an altered, pro-inflammatory transcriptional profile of blood leukocytes[10-13]. Finally, depressed patients who died by suicide had evidence of increased reactivity of microglia, the innate immune cells of the brain, suggesting that the CNS is also in an inflammatory state during depression[14]. Thus, neuroinflammation may be the mechanism linking chronic stress to the development of mental health disorders.

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To study how the CNS and immune system are impacted by chronic psychosocial stress, our lab has developed a mouse model of stress termed Repeated Social Defeat (RSD)[15]. In this model, three male mice are co-housed in a cage, where they naturally develop a dominance hierarchy. An aggressive intruder mouse is introduced to the cage to disrupt that hierarchy and establish dominance over the resident animals. The resident mice experience a loss of social status, which is an ethologically relevant psychosocial stressor in mice[16]. We have used RSD to extensively study how neuroinflammation contributes to behavioral changes after chronic psychosocial stress.

We have shown that RSD causes anxiety-like behavior and increased peripheral and central inflammation in mice, recapitulating the behavior and inflammation seen in humans with anxiety or depression[17,18]. RSD also causes proliferation of myeloid cells in the bone marrow and release of pro-inflammatory Ly6C^{hi} monocytes into circulation[17,19]. Importantly, these Ly6C^{hi} monocytes are the functional analog of the CD14+/CD16- monocytes observed in humans with mental health disorders[13]. Ly6C^{hi} monocytes are recruited to the brain, where they interact with the endothelial cells of the brain vasculature[20]. As brain endothelial cells reside at the interface between the periphery and the CNS, they are uniquely positioned to transduce signals from peripheral circulating cells to cells within the brain. We have shown that this monocyte-to-endothelial interaction is essential for the development of anxiety-like behavior after stress, suggesting that endothelial cells play a key role in modulating behavioral responses to stress[20,21]. We have previously shown that RSD causes neurovascular endothelial cells to express ICAM-1 and VCAM-1, which are key molecules involved in leukocyte adhesion and migration into the brain[22]. Additionally, neurovascular endothelial cells also express the receptor for the pro-inflammatory cytokine IL-1β, IL-1R1, indicating that they are responding to pro-inflammatory signals[20].

However, it is still unclear what endothelial cells are producing in response to stress that can impact cells of the CNS.

The purpose of the current study was to determine how endothelial cell gene expression changes in the brain after social stress. To do so, this study utilized a RiboTag model expressing Tie2-Cre, in which ribosomes in endothelial cells are tagged with hemagglutinin (HA)[23]. The HA tag allows for isolation of those ribosomes and the messenger RNA that are actively being translated to protein. We exposed male Tie2-Cre::RiboTag mice to six cycles of RSD or left them undisturbed as controls and isolated endothelial-specific mRNA for RNA sequencing. RSD caused increased translation of genes associated with cell adhesion and trafficking (*Sele*, *Icam1*), which corroborated our previous reports[20,22]. Furthermore, we found that RSD induces expression of *Ptgs2*, the gene encoding the cyclooxegenase-2 (COX-2) enzyme, in neurovascular endothelial cells. COX-2 is an essential enzyme for the conversion of arachidonic acid to prostaglandins, suggesting that endothelial production of prostaglandins may be involved in anxiety-like behavior after stress. Targeting endothelial secreted factors or signaling pathways may be a novel axis to prevent or treat stress-related psychiatric disease.

B. Materials and Methods

<u>Mice</u>: Male Tie2-Cre [strain 008863] and female RiboTag [strain 011029] mice were obtained from Jackson Laboratory (Bar Harbor, ME) for breeding. The male offspring with the genotype Tie2-Cre+, $RiboTag^{het}$ were used as experimental animals. Wild type C57BL/6 mice were ordered from Charles River Laboratory (Wilmington, MA). All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Repeated Social Defeat: RSD was conducted as previously described[19]. In brief, three (6-8 week old) male Tie2-Cre::RiboTag or C57BL/6 mice were housed in one cage. An aggressive male CD-1 intruder was introduced to the cage for 2 h a day, 6 consecutive days. Mice were monitored daily and mice that lost >20% of their body weight or appeared lethargic or moribund were removed from the study. No mice in the current study met criteria for early removal.

Immunohistochemistry for CD31 and HA: Tie2-Cre::RiboTag mice were euthanized with CO_2 asphyxiation and transcardially perfused with PBS and 4% paraformaldehyde. Whole brains were dissected and stored in 4% paraformaldehyde for 24 h, followed by 30% sucrose for 72 h. Next, brains were frozen in isopentane on dry ice and stored at -80C until sectioning. Whole brains were sectioned at 30 uM thickness on a Leica CM1950 Cryostat and stored in cryoprotectant (30% ethylene glycol, 30% polyethylene glycol in 0.2M phosphate buffer). Brain sections were rinsed with PBS and then blocked with 10% NDS, 0.3% Triton-X in PBS for 2 h at RT on an orbital shaker. Sections were then incubated overnight with primary antibodies to CD31 (Biolegend, San Diego, CA) and HA (Cell Signaling, Danvers, MA). The following morning, sections were rinsed 3x with PBS and then incubated with Donkey anti-Rat 594 and Donkey anti-Rabbit 488 fluorescent secondary antibodies (Thermo Fisher, Waltham, MA) for 1 h at RT on an orbital shaker. Sections were mounted onto Superfrost Plus slides (Thermo Fisher) and coverslipped using Fluoromount G (Thermo Fisher). Images were taken with an Evos 2 microscope with the 20X objective lens and combined using ImageJ software.

Immunoprecipitation of Ribosome-bound mRNA: 14 h following the final cycle of RSD, Tie2-Cre::RiboTag mice were euthanized by CO2 asphyxiation. Mice were transcardially perfused with ice-cold PBS. Whole brains were dissected and flash-frozen in liquid nitrogen until homogenization. Homogenization and immunoprecipitation of HA-tagged ribosomes was

conducted as previously described[23]. In brief, homogenization buffer (HB) was prepared as follows: 50 mM Tris, pH 7.5, 100 mM KCl, 12 mM $MgCl_2$, 1% Nonidet P-40 in RNase-free H_2O and supplemented with 1 mM DTT, 200 U/mL Promega RNasin, 100 ug/mL cycloheximide, Sigma protease inhibitor. Whole brains were homogenized on ice in 3 mL of supplemented HB with Dounce homogenizers and centrifuged at 10,000 x g for 10 mins. Supernatants were incubated with anti-HA antibody (BioLegend) for 4 hours with rotation and then added to Protein A/G magnetic beads (Thermo Fisher) for 6 hours with rotation. Samples were then placed on a magnet on ice and supernatants were removed before washing 3x with a high salt buffer (50 mM Tris, 300 mM KCl, 12 mM $MgCl_2$, 1% Nonidet P-40, 1 mM DTT, 100 ug/mL cycloheximide). Finally, 350 uL of Qiagen RLT buffer was added to the beads and vortexed vigorously for 1 min to dissociate bound RNA from the beads. RNA was isolated according to the manufacturer's instructions using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA quality and concentration were analyzed using the Agilent Bioanalyzer system High-Sensitivity TapeStation assay.

Quantitative Real-Time PCR: Tie2-Cre::RiboTag mice were euthanized and whole brains were flash frozen for immunoprecipitation as described above. Samples were taken after the initial homogenization step (Input, before IP) and the final lysis step (Bound, after IP) for analysis. RNA was isolated using the Qiagen RNeasy Micro Kit per manufacturer's instructions. cDNA was synthesized using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. For qPCR, the following probes were used: *Mfsd2a, Cdh5, Cx3cr1, Gfap, Npy* (Thermo Fisher). qPCR was conducted using the Taqman Fast Master Mix and analyzed using the QuantStudio 3 system (Thermo Fisher).

RNA Sequencing: RNA samples were sent to the University of Miami genomics core for library preparation and sequencing. cDNA libraries were synthesized using the NuGen SoLo prep kit and sequenced on the Illumina HiSeq with 25 million single-end reads per sample.

Data Analysis: RNA sequencing files were aligned to the mouse mm10 reference genome using STAR Aligner (refs). Differential expression was analyzed using the DESeq2 package in R. The threshold of significance was set to padj<0.05.

C. Results

Tie2-Cre::RiboTag model allows for enrichment of endothelial genes.

First, the specificity of the Tie2-Cre::RiboTag model was determined. Naïve male RiboTag^{het} mice that either expressed Cre recombinase (Cre+) or did not (Cre-) were sacrificed for immunohistochemistry to localize expression of hemagluttinin (HA)-tagged ribosomes. In Cre- mice, there was no apparent HA signal (Figure 1A). In Cre+ mice, most of the HA signal co-localized with CD31, an endothelial intercellular protein, indicating that HA was expressed in endothelial cells (Figure 1A). However, they was non-endothelial HA reactivity, which suggests that a small population of non-endothelial cells also expressed HA. qPCR was performed on cell signature genes to determine whether the immunoprecipitation protocol could specifically isolate RNA from endothelial cells. The signature genes assessed were endothelial (*Mfsd2a*, *Cdh5*), microglial (*Cx3cr1*), astrocytic (*Gfap*), and neuronal (*Npy*). Before the immunoprecipitation step, samples from both Cre- and Cre+ mice contained similar amounts of all signature genes (Figure 1B). After immunoprecipitation, samples from Cre+ mice were enriched for endothelial cells and microglia (Figure 1B). Overall, the Tie2-Cre::RiboTag mouse model allows for enrichment of endothelial transcripts with off-target effects in microglia.

RSD alters the gene expression profile of neurovascular endothelial cells.

Male Tie2-Cre::RiboTag mice were exposed to 6 cycles of RSD or left undisturbed as controls. 14 h following the final cycle of defeat, mice were euthanized and whole brains were collected for immunoprecipitation and RNA sequencing. RSD caused a shift in endothelial gene expression (Figure 2A). Over 300 genes were statistically different between CON and RSD samples (Figure 2B). As reported previously, stress induces endothelial expression of cell adhesion molecules such as *Icam1* (Figure 2C; padj < 0.05) and *Sele* ($p < 0.05$). Interestingly, several genes associated with prostaglandin synthesis, processing, and transport were increased by stress. These included *Ptgs2*, which is the gene encoding the enzyme cyclooxegenase-2 (COX-2); and *Slco2a1*, which encodes Prostaglandin Transporter. Importantly, the non-inducible form of cyclooxygenase, *Ptgs1*, was not increased by stress. COX-2 is induced by a variety of physical and psychological stressors, including trauma, infection, and social stress[24,25]. This data indicates that endothelial cells may be an important source of prostaglandins after social stress.

Inflammatory cytokines are predicted to regulate endothelial protein translation after RSD.

Differentially expressed genes between RSD and CON animals were analyzed using Ingenuity Pathway Analysis to determine which pathways are activated in endothelial cells after stress and which molecules are predicted to regulate these changes. The most prominent pathway involved is Chemokine Signaling (Figure 3A), which is involved in recruiting cells during cell migration. Other activated pathways included Oxidative Phosphorylation and Endothelin-1 Signaling, which suggest that metabolic activities and vascular structure in endothelial cells may be impacted by stress. This pattern of changes is predicted to be caused by inflammatory mediators

such as IL-6 and NF-κB (Figure 3B). IL-1β produced by monocytes that are recruited to the brain with stress is an activator of the NF-κB pathway, indicating that monocyte signaling may be driving endothelial gene expression changes.

Prostaglandin synthesis is an enriched pathway in endothelial cells after RSD.

Endothelial genes that were increased by stress were further analyzed using Cytoscape to visualize relationships between genes (Figure 4). Changes in endothelial gene expression were predominantly categorized into vascular structure or cell adhesion categories. However, stress induced the "Lipid Biosynthetic Process" pathway, which was not related to any structural pathways. This provides further evidence that prostaglandins, which are lipid-based signaling molecules, play an important role in biological responses to social stress.

D. Conclusions and Future Directions

We have shown previously that RSD activates endothelial cells specifically in regions of the brain associated with fear, anxiety, and threat appraisal. These reactive endothelial cells increase expression of cell adhesion molecules and IL-1R1, and interact with IL-1β-producing monocytes[20,22]. This study corroborated our previous findings that activated endothelial cells express leukocyte adhesion molecules such as *Icam1* (Figure 2B). The unique gene expression profile of brain endothelial cells after stress is likely mediated by inflammatory cytokine signaling including IL-6 and the NF-κB pathway (Figure 3). This is an intriguing finding, as activation of IL-1R1 is one pathway that can activate NF-κB[26]. This suggests that monocyte-to-endothelial signaling via IL-1β/IL-1R1 is mediating the gene expression changes in endothelial cells after stress. We have shown that monocyte production of IL-1β is crucial for the development anxiety-

like behavior after stress[20], which indicates that endothelial changes may be a critical component of behavioral adaptations to stress.

Additionally, we found that RSD also induces COX-2 expression in endothelial cells (Figure 2B&C). COX-2 is the inducible isoform of the rate-limiting enzyme in prostaglandin production[24], suggesting that endothelial cells may be producing prostaglandins in response to stress. Neurons in stress-responsive brain regions such as the hypothalamus and amygdala express prostaglandin receptors[27]. Thus, prostaglandins may be the mediator that endothelial cells produce to signal back to cells of the CNS to affect behavior.

The present study profiled endothelial cells after RSD in the whole brain. Because neuroinflammatory changes are thought to be region-specific, future studies will characterize the gene expression profile of endothelial cells only in regions involved with threat appraisal and stress responses, including the hypothalamus, hippocampus, amygdala, and prefrontal cortex. Additionally, future studies will determine whether prostaglandin concentrations are increased in the brain after stress, and whether prostaglandins are necessary for the development of anxiety-like behavior after stress.

Overall, this study shows a unique profile of endothelial cells after stress that is characterized by expression of leukocyte adhesion molecules and proteins involved in prostaglandin synthesis and transport. These findings suggest that neurovascular endothelial cells play an important role in mediating neuroinflammatory responses to stress.

E. Figures

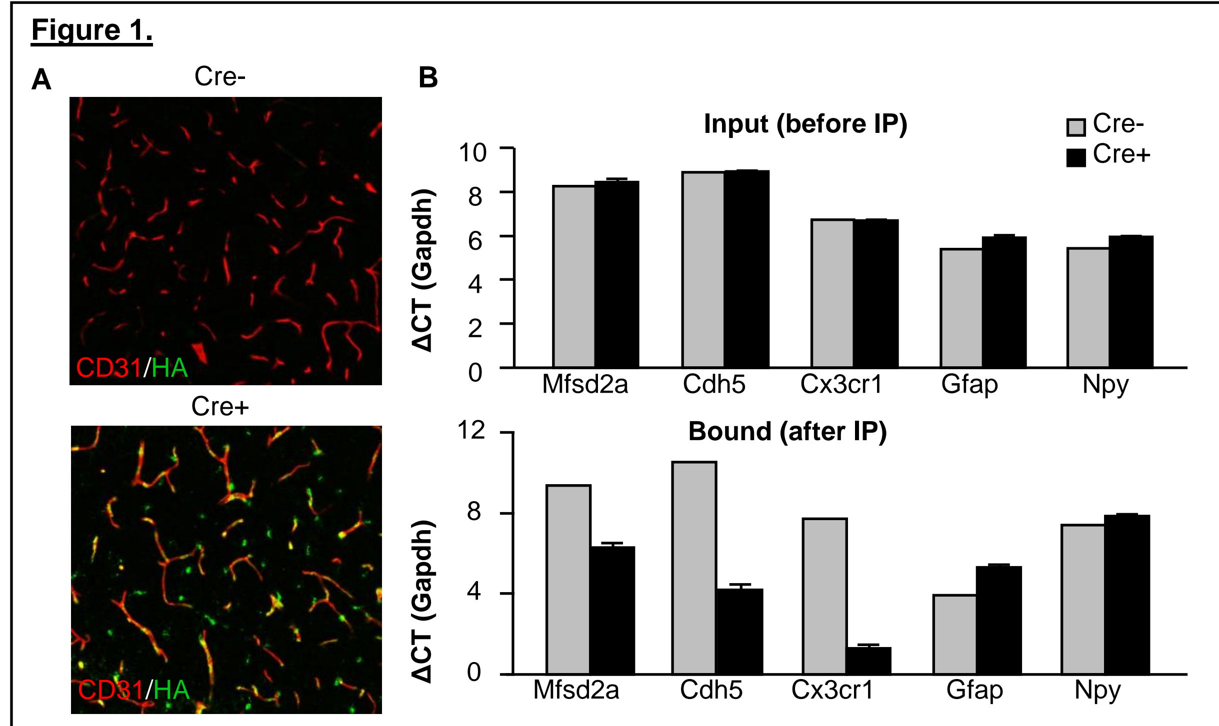

Figure 1. Tie2-Cre and RiboTag mice were crossed to generate Tie2-Cre::RiboTag mice in which endothelial cells expressing HA-tagged RpL22 **(A)** Cre+ mice express HA in endothelial cells while Cre- littermates do not. **(B)** Mfsd2a and Cadherin-5 (endothelial) and CX3CR1 (microglial) mRNA are enriched after immunoprecipitation. n=1-2 per group

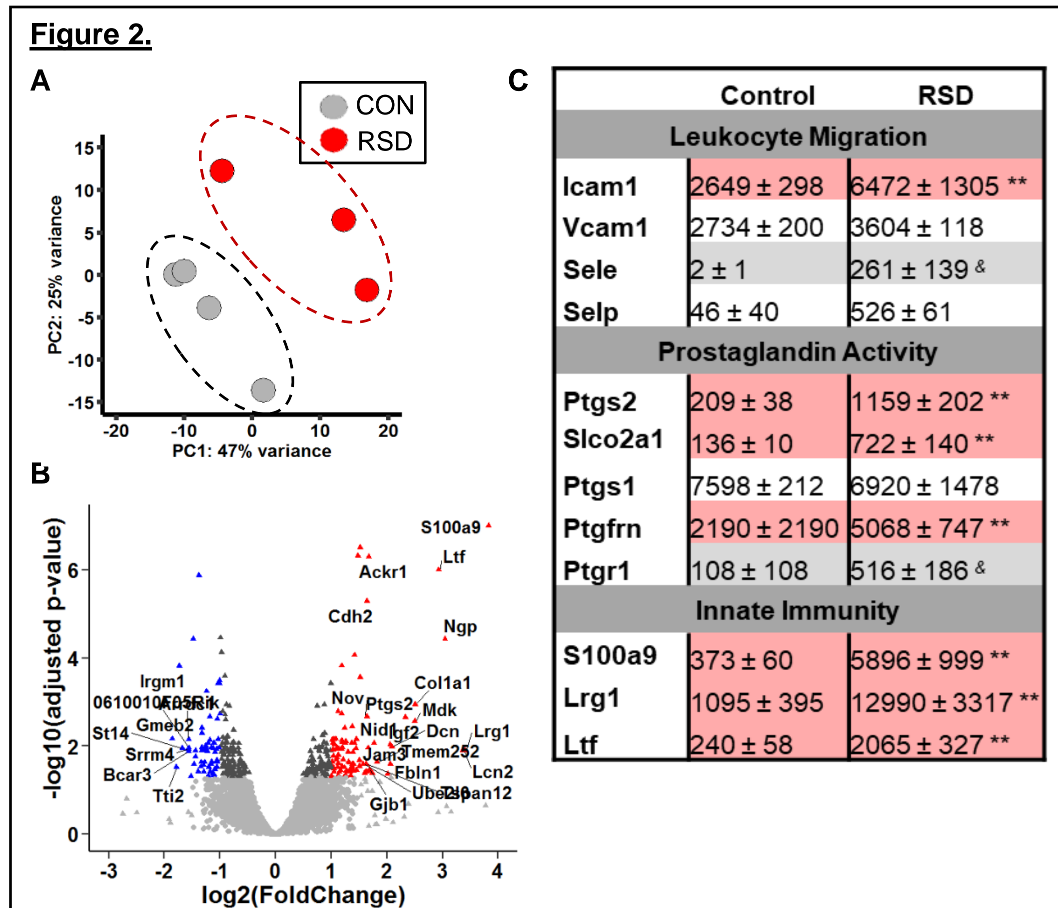

Figure 2. Tie2Cre::RiboTag mice were exposed to 6 cycles of RSD or left undisturbed as controls. Whole brains were collected for RiboTag immunoprecipitation. **(A)** Normalized PCA plot. Control and RSD samples cluster separately. **(B)** Volcano plot; highlighted genes are >1.5 fold change from controls. **(C)** Counts ± SEM for selected genes of interest (** = padj < 0.05; & = unadjusted p-value <0.05). n=3-4 per group

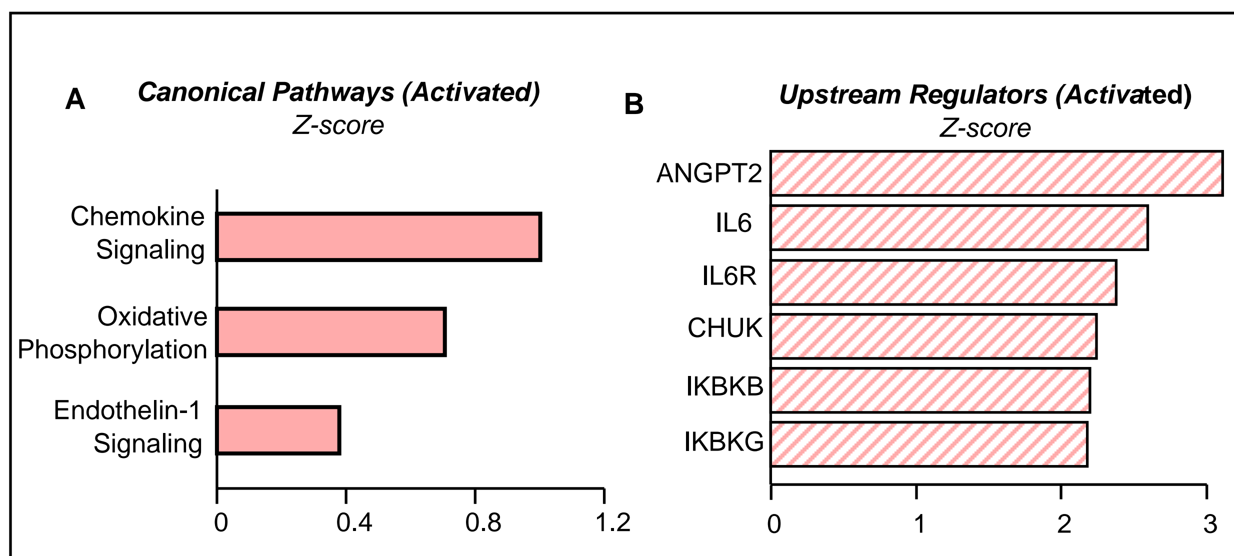

Figure 3. Differentially regulated genes were analyzed using Ingenuity Pathway Anaysis. **(A)** Significantly activated pathways and **(B)** activated upstream regulators in RSD animals compared to controls.

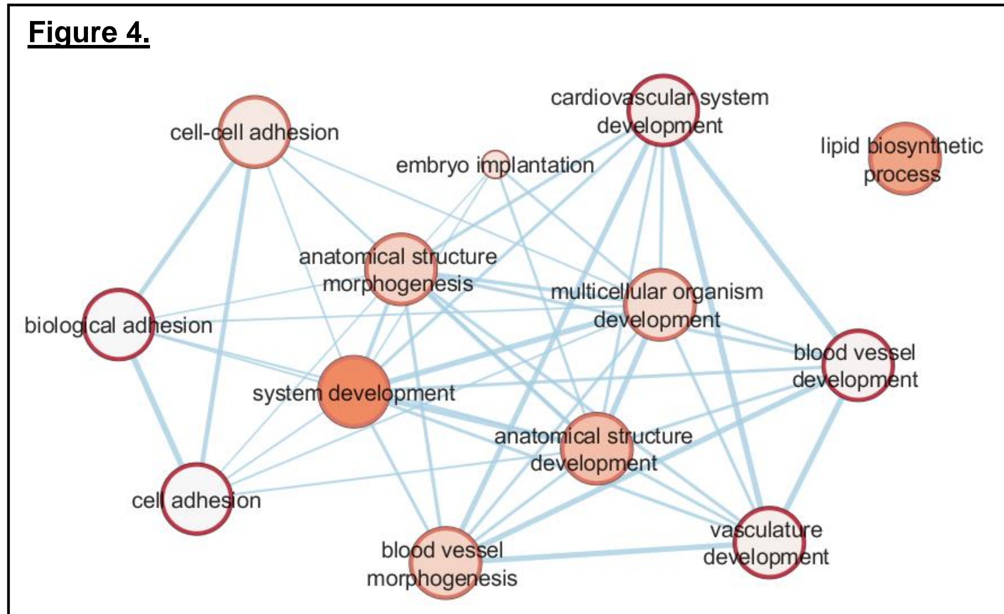

Figure 4. Differentially regulated genes were analyzed using Cytoscape. Biological processes represented by upregulated genes in endothelial cells after stress using Cytoscape EnrichmentMap app. Circle size represents # of genes within the category; circle color represents strength of connections.

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