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The Impact of Cytokine Responses in the Intra- and Extracellular Signaling Network of a Traumatic Injury

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Abstract

Investigations of cellular responses involved in injury and repair processes have generated valuable information contributing to the advancement of wound healing and treatments. Intra- and extracellular regulators of healing mechanisms, such as cytokines, signaling proteins, and growth factors, have been described to possess significant roles in facilitating optimal recovery. This study explored a collection of 30 spatiotemporal responses comprised of cytokines (IL-1 α , IL-1 β , IL-2, IL-6, TNF- α , MIP-1 α), intracellular proteins (Akt, c-Jun, CREB, ERK1/2, JNK, MEK1, p38, p53, p90RSK), phosphorylated proteins (p-Akt, p-c-Jun, p-CREB, p-ERK1/2, p-GSK-3 α / β , p-HSP27, p-I κ B α , p-JNK, p-MEK1, p-p38, p-p70S6K, p-p90RSK, p-STAT2, p-STAT3), and a protease (Caspase-3), measured in skeletal muscle tissue following a traumatic injury (rodent Gustilo IIIB fracture). To optimize the analysis of context-specific data sets, a network centrality parameter approach was used to assess the impact of each response in relation to all other measured responses. This approach identified proteins that were substantially amplified and potentially central in the wound healing network by evaluation of their corresponding centrality parameter, radiality. Network analysis allowed us to distinguish the progression of healing that occurred at certain time points and regions of injury. Notably, new tissue formation was proposed to occur by 168 h post-injury in severely injured tissue, while tissue 1-cm away from the site of injury that experienced relatively minor injury appeared to exhibit signs of new tissue formation as early as 24 h post-injury. In particular, hallmarks of inflammation, cytokines IL-1 β , IL-6, and IL-2, appear to have a pronounced impact at earlier time points (0–24 h post-injury), while intracellular proteins involved in cell proliferation, differentiation, or proteolysis (c-Jun, CREB, JNK, p38, p-c-Jun; p-MEK1, p-p38, p-STAT3) are more significant at later times (24–168 h). Overall, this study

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demonstrates the feasibility of a network analysis approach to extract significant information and also offers a spatiotemporal visualization of the intra- and extracellular signaling responses that regulate healing mechanisms.

Keywords

Traumatic injury; spatiotemporal response; cytokine; phosphoprotein; network centrality parameter analysis

1. Introduction

The elucidation of molecular and signaling responses involved in traumatic injuries has gained considerable interest for potential treatments that may enhance the healing process. Previous studies have investigated the roles of cytokines, chemokines, growth factors [1], or signaling proteins [2] involved in the characteristic stages of wound repair: hemostasis, inflammation, tissue formation, and remodeling [3]. However, certain factors can influence the measured responses, such as varying classes and severities of injuries (e.g. fractures, minor cuts) and different tissue types (e.g. cutaneous tissue, bone, or myofibroblasts), which all appear to have distinct repair mechanisms [4]. Additionally, the measured response may be affected by temporal factors (e.g. time post-injury), as well as the spatial distribution of the zones of injury [5]. An increasing collection of intra- and extracellular regulators continues to emerge as prospective contributors of healing, allowing researchers to examine signaling pathways [6–7] or networks [8] involved in repair mechanisms. However, as the number of injury biomarker grows, it can become difficult to interpret large datasets and translate the results into information useful to practitioners and researchers.

Complex datasets, particularly those describing biological responses, may benefit from a data-driven mathematical modelling or a network analysis approach [9]. Leading bioinformatic software programs specializing in network analysis include Ingenuity Pathway Analysis (IPA), Cytoscape, and Pathway Studio, to name a few. These software programs allow for the construction of molecular networks and signaling pathways, or the identification of involved molecular functions and even disease states using experimental data [10]. Network analyses of biochemical responses are best recognized as tools to evaluate protein-protein interactions, construct signal transduction pathways, or elucidate metabolic patterns in order to better understand how entire systems respond when perturbed by detrimental events [11]. In particular, taking a graph theory approach to evaluate mathematical relationships among the components in a network can aid in sorting through and dissecting large networked datasets. Network analytics, such as centrality parameters, assigns a value to each node (measured response) that may describe how central one node is, relative to all other nodes in the system. Examples of centrality parameters include degree (number of nodes directly connected to a given node), diameter (the distance between the two furthest nodes in the network), or radiality (sum of shortest paths between a given node and all other nodes, normalized to the network diameter) [12]. Node centrality has been used to examine biological networks, such as protein-protein interactions resulting from chronic diseases [13] or phosphorylation responses elicited by toxic exposures [14]. In these studies,

centrality parameters were used to identify biological components that were highly impactful, relative to all other measured responses in the network. A node with a high centrality can be interpreted as having a greater degree of likeness to all other nodes, while a node with a low centrality can be construed as having a more peripheral position in the network (behaving differently from all other nodes in the group) [12].

In this study, 30 intra- and extracellular responses were measured in skeletal muscle tissue following a traumatic injury, in an effort to expand on our understanding of the spatial and temporal signaling changes that occur during wound healing. Cytokine, total intracellular protein, phosphorylated intracellular protein, and Caspase-3 levels were observed at varying time points (0, 6, 24, 168 h post-injury) and locations of injury (at the site of injury, 1-cm away). This complete dataset was examined with IPA to provide a comparison between a recognized software application, often used for analyzing and integrating large biological datasets, and the proposed method. Eight different networks of all responses were analyzed for each spatiotemporal condition (e.g. At the site of injury, 0 h) in order to obtain the centrality parameter, radiality, for each node. This approach was used to assess the value of each node, relative to all other nodes, under various temporal and spatial conditions. Radiality values were then used to identify nodes that had particularly distinctive responses, potentially holding a prominent role in driving the mechanisms of wound healing. This method of analysis allows for an unbiased comparison of all observations regardless of biological hierarchical assignments (e.g. extracellular cytokines and intracellular phosphorylated proteins).

2. Materials and Methods

2.1 Animals

Adult male Sprague–Dawley rats were housed individually with a 12:12 light/dark cycle with ad libitum access to standard rat chow and water. Three sampling (injury) locations were studied at four time points, with 3 replicates each ($N = 3$) for a total of 12 rats for the study. All procedures approved by the West Virginia University Animal Care and Use Committee.

2.2 Sample Analysis

Intra- and extracellular biomolecule responses were examined after animals were subjected to a standardized femur fracture (Gustilo IIIB), as described in Currie, et al. [5], and Han, et al. [15]. Briefly, muscle tissue samples were collected at the respective time points (0 h, 6 h, 24 h, and 168 h post-injury) and injury locations (at the site of the injury, 1-cm away, and in the uninjured leg), rinsed with ice cold phosphate buffered saline, snap frozen, and stored at -80°C . Homogenized tissues were analyzed for 30 targets, including nine intracellular proteins, 14 phosphoproteins, six cytokines, and one protease (listed in Table 1). These targets were chosen based on previously reported molecular responses of injuries in literature, as well as known responses involved cellular processes vital to the healing mechanism (e.g. cell death, cell proliferation, inflammation). The relative abundance of total intracellular protein and phosphoprotein were measured with a Bio-plex kit containing polystyrene, non-magnetic antibody coated beads. Cytokine responses were measured with a

Bio-Plex Pro multiplexed magnetic bead-based immunoassay reagent kit and Caspase-3 activity was measured with a fluorescence assay kit from Cayman Chemical (Item No. 10009135). All beads were analyzed using the Bio-Plex 200 suspension array system, along with the Pro II Wash Station (Bio-Rad, Hercules, CA). Fluorescence readings were taken with the Infinite M1000 microplate reader (Tecan US, Raleigh NC, USA).

2.3 Data processing and statistical analysis

Data were analyzed using Prism 5 (GraphPad, San Diego, CA), and SAS JMP Pro 12.0.1 (Carey, NC). Total intracellular protein, phosphoprotein, cytokine, and Caspase-3 abundances measured in muscle tissue at the site of fracture and 1-cm away from the site were normalized to levels found in tissue collected from the uninjured leg. Normalizing to the uninjured leg served as a systemic control, accounting for any responses that were not directly involved in the response to the local injury. Additionally, normalization accounted for any discrepancies brought on by the differences in measurement units (e.g. large RFU signals of phosphorylation levels vs. ng/g of cytokine response) in the calculation of radiality. Statistically significant ($p < 0.05$) differences were determined by 2-way ANOVA with a Bonferroni post-test.

2.4 Network evaluation with Ingenuity Pathway Analysis

The relative responses were also investigated with QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City). The 30 nodes were split into two groups to allow for analysis: one group consisted of total intracellular protein, cytokine, and Caspase-3 responses, and the second group included only relative phosphorylated protein responses. Proposed signaling networks for all time-points (0, 6, 24, 168 h) and locations (at fracture, 1-cm away) of injury were generated. All networks are comprised of nodes from the experimental dataset, as well as other projected nodes likely to be involved in the network, based on the Ingenuity Knowledge Base. Up- and downregulated responses are represented by red and green color coding, respectively. It must be noted that IPA constructs networks by building on the "Focus Genes" or the nodes that are the most interconnected. Experimental expression values can influence which nodes are designated as "Focus Genes" and can alter the structure of the networks as well as the ranking of top networks and associated biological functions. IPA additionally reported the top ranked molecular and cellular functions describing the network, along with the corresponding p-scores (negative $\log_{10}$ (p-value of Fisher's exact test)). The Fisher's exact test (p-value) describes the probability of finding the Focus Genes in randomly selected genes from the Global Molecular Network constructed from the Ingenuity Knowledge Base. In other words, the network score reports the probability that the associations between the experimental nodes and molecular networks are due to random chance.

2.5 Network centrality parameter analysis

Using SAS JMP (Cary, NC), Euclidean distances between each pair of normalized observations (total intracellular protein, phosphoprotein, cytokine, Caspase-3) were determined for all time points and locations of injury (e. g. Time = 0 h/At the site of fracture). Euclidean distance is defined as:

$$E(v, \omega) = \sqrt{\sum_{i=1}^n ((\text{normalized response } v_i) - (\text{normalized response } \omega_i))^2} \quad (1)$$

where v and ω represent the 2 responses for which the distance between is being calculated, and n signifies the replicate number.

To construct a spatiotemporal network of the measured responses to the injury, Euclidean distances between each pair of normalized observation (node) were used to calculate the node centrality parameter, radiality:

$$Radiality-C_{rad(v)} = \frac{\sum_{\omega \in N} (\Delta G + 1 - \text{dist}(v, \omega))}{n - 1} \quad (2)$$

where G represents the network (N) diameter (longest path length connecting any 2 nodes), $\text{dist}(v, \omega)$ is defined as the shortest path between two nodes (v, ω), and n is the number of nodes in the network. Briefly, radiality describes how close (high values) or distant (low values) in proximity one node is from all of the other nodes in the network. High radiality values will result if the distances are short, while low values will be produced by long distances. Radiality values were normalized to the average radiality for all proteins in the network, indicating that proteins with relative values higher than 1 are more central than average, and proteins with relative values lower than 1 are less central than average. A threshold of the average radiality $\pm$ the standard deviation was set to identify nodes with significant radiality outcomes.

3. Results

3.1 Relative spatial and temporal cytokine response

Responses of the following six cytokines were normalized to levels detected in the uninjured leg: IL-6, IL-1 β , IL-2, TNF- α , IL-1 α , and MIP-1 α (Figure 1). IL-6 and IL-1 β levels were highest at 6 h in tissue from the site of fracture, with a significantly higher ($p < 0.05$) response at the site of fracture compared to samples acquired from 1-cm away. Tissue located 1-cm away experienced a considerable increase in IL-2 response at 24 h post fracture, while MIP-1 α levels at the site of fracture slightly decreased 24 h following the injury. IL-1 α and TNF- α did not exhibit any statistically significant spatial nor temporal differences.

3.2 Relative spatial and temporal intracellular protein response

Total protein responses were normalized to levels measured in the uninjured tissue for the following 9 targets: c-Jun, CREB, p38, ERK1/2, JNK, Akt, MEK1, p53, and p90RSK (Figure 2). The relative protein levels of c-Jun, CREB, p38, ERK1/2, JNK, and Akt at the site of the fracture - 168 h post fracture were significantly ($p < 0.05$) increased, compared to responses measured at 0, 6 and 24 h. These protein levels were also significantly higher at the site of injury than in the uninjured leg (>1) at 168 h. There were no significant temporal

or spatial changes for those 6 proteins at the three earlier times (0, 6, and 24 h). In tissue 1-cm away from the fracture, only JNK and p38 responses were statistically increased at 168 h, in comparison to levels found at 0, 6, and 24 h. Furthermore, JNK and p38 levels at time point 168 h were significantly higher in tissue 1-cm away from the fractured location when compared to tissue from the uninjured leg. Notable spatial differences of c-Jun and CREB responses were also observed between the two regions of injury, with significantly higher levels measured at the site of fracture for both proteins. Additionally, MEK1 protein response at the site of fracture decreased considerably at 24 h (statistically different from MEK1 response in uninjured tissue). There were no spatial nor temporal differences for p53 and p90RSK and all measurements were statistically comparable to levels measured in the uninjured tissue.

3.3 Relative spatial and temporal response of phosphorylated proteins

To further examine the impact of the observed proteins (c-Jun, CREB, p38, ERK1/2, JNK, Akt, MEK1, p53, and p90RSK), associated phosphorylation levels were measured. Phosphoprotein responses of 6 additional targets were added: STAT3, p70S6K, STAT2, HSP27, I κ B α , GSK-3 α / β (Figures 3 and 4). At the site of fracture, spatiotemporal variations in the phosphorylation of STAT3, c-Jun, p70S6K, and Akt were observed: significantly ($p < 0.05$) increased responses at 24 h for STAT3, increased response at 168 h for c-Jun and p70S6K, and decreased response at 6 h and 24 h for Akt. In tissue 1-cm away from the fracture, phosphorylation on MEK1, STAT3, ERK1/2, p38, p70S6K, CREB and GSK-3 α / β proteins displayed significant changes over time. Significantly elevated responses at 24 h were measured for MEK1, STAT3, ERK1/2, p70S6K, CREB, and at 168 h for p38. A reduction in GSK-3 α / β phosphorylation occurred at 0 h and increased to comparable levels found in the uninjured leg at the later time points for 1-cm away.

Considerable spatial differences were also observed between the site of fracture and 1-cm away for MEK1, c-Jun, and p70S6K: phosphorylation responses in tissue at the site of fracture were significantly higher for c-Jun and p70S6K at 168 h, while the response was higher in 1-cm away tissue at 24 h for MEK1. Although there were no significant differences over time or between the two sampling locations for the phosphorylation of JNK and STAT2, there was a slight increase in response at 6 h at the site of fracture, where the response was higher than levels detected in the uninjured leg (relative response is >1). Phosphorylation of HSP27, I κ B α , and p90RSK remained constant over time for the two regions associated with the injury, and were not significantly different than responses observed in the uninjured leg.

3.4 Relative Spatial and Temporal Caspase-3 Response

The activity of Caspase-3 at the site of the fracture and 1-cm away was measured and normalized to levels found in the uninjured leg (Figure 5). At the site of the fracture, Caspase-3 activity was initially reduced (0 and 24 h) and then increased at 168 h, in relation to the uninjured leg. In tissue 1-cm away from the fracture, relative responses observed at 24 and 168 h were significantly different from each other. Additionally, at 0 h, activity was substantially decreased at the fracture compared to the region 1-cm away.

3.5 IPA generated networks

Since all networks were constructed from the same collection of nodes, the overall components of the networks and the general connectivity of the nodes were similar across all time points and locations of injury. The only major difference shown among the networks are the upregulated and downregulated responses that did vary temporally and spatially. Two examples of IPA generated networks using the intracellular protein, cytokine, and protease or the phosphoprotein dataset are shown (Figures 6 and 7). Temporal changes between 0 and 168 h post-injury were demonstrated with the intracellular protein, cytokine, and protease network, while spatial differences between the two injury regions, at 6 h post-injury, were observed with the phosphoprotein network. The generated networks using intracellular protein, cytokine, and protease responses were postulated to encompass cellular processes including free radical scavenging, cell death and survival, skeletal and muscular disorders, cell signaling, small molecule biochemistry, drug metabolism, and cardiac necrosis/cell death. Cellular processes suggested to be involved in the phosphoprotein networks include cell death and survival, cell morphology, cellular development, lipid metabolism, small molecule biochemistry, organismal injury and abnormalities, cellular movement, skeletal and muscular disorders, and free radical scavenging (Table 2). The probability that the uploaded experimental data are associated with the reported networks are expressed by the p-scores. All spatiotemporal networks were given high probability scores of 22. Again, these scores are based on the p-value that describe the probability of finding the Focus Genes in randomly selected genes from the Global Molecular Network constructed from the Ingenuity Knowledge Base.

3.6 Normalized radiality of all 30 nodes

Radiality values that were determined for each node (response) were normalized to the average radiality value for each dataset (e.g. At Fracture, 0 h), as presented in Tables 3 and 4. Nodes with significant radiality outcomes (significance threshold: average radiality $\pm$ standard deviation) are bolded. Normalization to the average was performed to allow for cross comparison across time and location. Monitoring the changes in radiality for each node over time, or identifying when the value significantly rises or falls, can identify critical times when a certain node behaves similarly to or deviates from the rest of the nodes in the network. At the site of the fracture, normalized radiality values of most nodes were comparable to the average ($= 1$) and remained unchanged over all 4 time points: Akt, ERK1/2, JNK, MEK1, p38, p53, p90RSK, p-Akt, p-CREB, p-ERK1/2, p-GSK-3 α/β , p-HSP27, p-I κ B α , p-JNK, p-MEK1, p-p38, p-p70S6K, p-p90RSK, p-STAT2, p-STAT3, IL-1 α , IL-2, MIP-1 α , TNF- α , Caspase-3. Figure 8 shows normalized radiality scores for certain nodes (c-Jun, CREB, p-c-Jun) decreased considerably only at 168 h, while IL-1 β and IL-6 responses contributed to significantly low radiality scores at 0, 6, 168 h, and 0, 6, 24 h, respectively.

In tissue 1-cm away from the fracture, the associated normalized radiality of most nodes were also comparable to the average ($= 1$) for all 4 time points: Akt, c-Jun, CREB, ERK1/2, MEK1, p53, p90RSK, p-Akt, p-c-Jun, p-CREB, p-ERK1/2, p-GSK-3 α/β , p-HSP27, p-I κ B α , p-JNK, p-p70S6K, p-p90RSK, p-STAT2, IL-1 α , MIP-1 α , and Caspase-3. Figure 9 shows radiality for TNF- α was notably reduced at 0 h, but increased to a value ~ 1 at all

later time points. Only at 24 h, radiality scores of p-MEK1, p-STAT3, and IL-2 were significantly decreased. In addition, radiality scores for JNK, p38, and p-p38 were ~ 1 at the earlier time points (0–24 h), but dropped to lower scores at 168 h. Radiality values for IL-1 β and IL-6 were significantly low at 0, 6 h, and 0, 6, 24 h, respectively, which is similar to radiality determined for responses measured at the site of fracture.

4. Discussion

Biomarkers involved in wound healing processes have been examined for many types of injuries, ranging from muscle strains/contusions [16] to severe femur fractures [17]. Studies have investigated wound healing from intracellular signaling perspectives [18], spatiotemporal aspects [19], or even a genetic approach [20]. In our own previous work, we addressed the spatial and temporal distribution of cytokines, intracellular protein expression, and phosphorylation levels [5, 15], but in this study, we expanded on the research by investigating intra- and extracellular responses of an injury model with a network analysis approach in an effort to better define the significant spatiotemporal contributors of wound repair.

The cytokine, total intracellular protein, phosphorylated protein, and Caspase-3 targets are known to be significant contributors to wound repair [21–27]. The responses measured in this study exhibited spatiotemporal distribution patterns that are in agreement with the current knowledge of the stages of wound healing. Normalized levels of cytokines IL-1 β , IL-6, and IL-2 appeared to possess a prominent impact (significantly higher than uninjured leg) at 6 h post injury through 24 h (Figure 1), indicating the early repair stages of hemostasis and inflammation [28]. Additionally, most total intracellular protein (Akt, c-Jun, CREB, ERK, JNK, and p38), select phosphorylation responses (c-Jun, ERK1/2, MEK1, p38, p70S6K, and STAT3), and Caspase-3 activity (as shown in Figures 2–5) were significantly higher at the site of fracture during the later time points (24–168 h). Overall, this dataset suggests cytokines are most essential in the intermediate stages (6–24 h), while increased intracellular protein expression, phosphorylation, and caspase-3 activity are more prominent later when new tissue formation is essential.

IPA is a valuable tool that can be used to explore signaling networks, predict which pathways and cellular processes may be involved, and visualize the connectivity within the network. Using literature references, other peripheral nodes and proteins that are likely to be involved are inserted in the network, providing researchers more potential targets to investigate. Figure 6 shows two example IPA generated networks that depict the temporal differences (0 h vs 168 h) in intracellular protein, cytokine, and protease response. Figure 7 demonstrates the spatial difference in phosphorylation response at a single time point (6 h post-injury). While IPA proved to be a useful approach to visualize the changes in response and connectivity, as well as extrapolate prospective cellular functions and mechanisms associated with the observed responses, there were a few limitations due to the nature of the experimental dataset. The complete dataset included protein expression, as well as phosphorylation of the proteins (e.g. total protein MEK1 expression and phosphorylated MEK1). To evaluate experimental responses with IPA, the data must be uploaded using identifiers supported by IPA (e.g. Genbank, UniProt/Swiss-Prot, RefSeq), meaning both

protein expression and phosphorylation levels on the same protein cannot be analyzed at the same time. When only phosphoproteins were analyzed with IPA (Figure 7) there appears to be spatial differences/significance at 6 h post-injury, as phosphorylation signaling overall increased in tissue that suffered severe damage, compared to tissue 1-cm away. However, when processed through network parameter analysis – where all phosphoproteins, cytokines, intracellular proteins, caspase-3 were analyzed together, it appears phosphoproteins may not be significant at that time point, relative to all the other responses (Figures 8–9). IPA also proposed general functions and mechanisms (e.g. cell death and survival, cellular growth and proliferation, organismal injury and abnormalities), expected for an injury model, but there were no distinct patterns that may have corresponded to specific stages of wound healing. There were also proposed networks that were not relevant to an injury model (e.g. drug metabolism), but may have shared similar molecular responses as the ones observed in this study (Table 2).

In an effort to better understand the action/reaction of the elements involved in wound healing, we applied a network analysis based on radiality, as described in the Materials and Methods section. The intent in using this analysis was not to identify direct signaling relationships, but to assess each node in comparison to all other nodes instead of evaluating one isolated response at a time. Briefly, nodes with high radiality were interpreted to overall behave similarly to all the other nodes in the network, whereas a node with low radiality was interpreted to behave much differently than all the other nodes in the network, potentially driving the network. Our results found that for most nodes normalized radiality was ~ 1, indicating similar response elicited by most nodes in the network (Tables 3 and 4). While analysis of relative responses determined that there were no significant cytokine responses at 0 h (Figure 1), network analysis recognized certain cytokines to have a prominent impact in the network at the earliest time point. For instance, at time point 0 h, radiality scores identified IL-1 β and IL-6 as significant nodes at the site of injury, and IL-1 β , IL-6, and TNF- α as significant nodes in tissue 1-cm away (bolded in Tables 3 and 4). However, the relative levels of these cytokines were not statistically different than levels found in the uninjured leg at 0 h (Figure 1), de-emphasizing their key role in the network immediately following the injury.

While the damage was not as severe in tissue located 1-cm away from the fracture, a repair response was nonetheless exhibited. Figures 8 and 9 show the similarities and differences of networks defined by the two regions of injury, as well as a proposed progression of wound healing for these two disparate zones of injury. Time points 0–24 h post-injury capture inflammation in both regions of injury, primarily regulated by cytokines. Although markers of hemostasis were not measured in this study, it is known to be a conserved process of early wound healing, and is assumed to have occurred, considering the nature of the injury. Additionally, cytokines are prominent mediators of hemostasis, known to regulate procoagulant and anticoagulant mechanisms [29,30]. The end of the inflammatory stage and the beginning phases of tissue formation appear to occur by 168 h at the site of injury, marked by the emergence of significant transcription factors (CREB, c-Jun, p-c-Jun) associated with wound healing, as well as the concurrent loss of cytokine significance. CREB is shown to promote satellite cell proliferation [31], while c-Jun is associated with both cell proliferation and pro-apoptotic roles [32]. In tissue 1-cm away, intracellular

proteins associated with cell death/survival, proliferation, differentiation, and proteolysis (p-MEK1, p-STAT3, p38, p-p38, JNK) [33–37] were more relevant to the network of responses, suggesting the beginning of new tissue formation starting as early as 24 h and continuing through 168 h.

Overall, centrality parameter analysis appeared to successfully identify relative responses that may hold more relevance than others, allowing us to match distinct stages of wound healing to the time points selected in this study. Many of the same conclusions, such as the importance of cytokines at early time points, were made with IPA as well. It may also be advantageous to analyze the data with *both* centrality parameter analysis and IPA, as there are strengths and limitations with each approach. With centrality parameter analysis, there is more control over data inclusion, but lacks the vast database integrated into IPA. In addition, the node inputs for centrality parameter analysis can be spatially disparate and thus there may, or may not, be a relationship between each pair of nodes in the network. However, in this study, we were able to treat the dataset as a single network because all of the responses were measured in the same tissues and collected from the same animals, so there is certainty that the measurements are defined by a specific time and location of injury. Nonetheless, caution must still be taken in using this approach and interpreting the results. It is also necessary to note that with many animal and time course studies, the measurements are confined to the time points selected for tissue collection and, consequently, responses that were not significant spatiotemporally or in the network analysis of this study may still be important to the injury response, but at different time points post-injury.

In conclusion, we examined relative intra- and extracellular responses of a traumatic injury and observed spatiotemporal dependent patterns of cytokine, intracellular protein, phosphoprotein, and Caspase-3 activity. Network centrality parameter analysis identified outcomes that were not readily apparent with traditional data analysis approaches and allowed us to discern the possible stages of wound healing associated with the different regions of injury. Molecular indications of inflammation and new tissue formation were observed within 168 h at the site of injury, while muscle tissue further from the site of injury (that experienced less damage) may have progressed to new tissue formation as early as 24 h. This study demonstrated the potential in using a network centrality analysis approach to investigate relative cellular responses in different stages of wound healing, and to monitor changes under varying spatial and temporal conditions, thereby allowing for the identification of new targets timed for specific injury patterns.

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Figure 1. Relative cytokine levels measured across time and injury location in muscle tissue resulting from a femur fracture

Relative cytokine response (normalized to levels measured in uninjured leg) were observed across four time points and different regions of injury following the femur fracture: IL-1 α , IL-1 β , IL-2, IL-6, TNF- α , and MIP-1 α . Statistically significant ($p < 0.05$) differences were determined by 2-way ANOVA with a Bonferroni post-test. Temporal differences are notated with brackets pairing certain time points when substantial alterations in protein response were observed. Statistically significant spatial differences are marked with matching striped bars. Responses at the different regions of injury (at the site of fracture and 1-cm away) that

were significantly higher (>1) or lower (<1) than levels found in the uninjured leg are indicated the symbol (*). Error bars reflect $\pm$ standard error of the mean.

Figure 2. Relative intracellular protein response measured across time and injury location in muscle tissue resulting from a femur fracture

Relative response (normalized to levels measured in uninjured leg) associated with total protein responses of the following proteins were observed across four time points and different regions of injury following the femur fracture: Akt, c-Jun, CREB, ERK1/2, JNK, MEK1, p38, p53, and p90RSK. Statistically significant ($p < 0.05$) differences were determined by 2-way ANOVA with a Bonferroni post-test. Temporal differences are notated with brackets pairing certain time points when substantial alterations in protein response were observed. Statistically significant spatial differences are marked with matching striped bars. Responses at the different regions of injury (at the site of fracture and 1-cm away) that were significantly higher (>1) or lower (<1) than levels found in the uninjured leg are indicated with the symbol (*). Error bars reflect $\pm$ standard error of the mean.

Figure 3. Relative phosphorylation response measured across time and injury location in muscle tissue resulting from a femur fracture

Relative phosphorylation response (normalized to levels measured in uninjured leg) of the following proteins were observed across four time points and different regions of injury following the femur fracture: Akt, c-Jun, CREB, ERK1/2, GSK-3 α / β , HSP27, I κ B α . Statistically significant ($p < 0.05$) differences were determined by 2-way ANOVA with a Bonferroni post-test. Temporal differences are notated with brackets pairing certain time points when substantial alterations in protein response were observed. Statistically significant spatial differences are marked with matching striped bars. Responses at the different regions of injury (at the site of fracture and 1-cm away) that were significantly

higher (>1) or lower (<1) than levels found in the uninjured leg are indicated with the symbol (*). Error bars reflect $\pm$ standard error of the mean.

Figure 4. Relative phosphorylation response measured across time and injury location in muscle tissue resulting from a femur fracture

Relative phosphorylation response (normalized to levels measured in uninjured leg) of the following proteins were observed across four time points and different regions of injury following the femur fracture: JNK, MEK1, p38, p70S6K, p90RSK, STAT2, and STAT3. Statistically significant ($p < 0.05$) differences were determined by 2-way ANOVA with a Bonferroni post-test. Temporal differences are notated with brackets pairing certain time points when substantial alterations in protein response were observed. Statistically significant spatial differences are marked with matching striped bars. Responses at the different regions of injury (at the site of fracture and 1-cm away) that were significantly

higher (>1) or lower (<1) than levels found in the uninjured leg are indicated with the symbol (*). Error bars reflect $\pm$ standard error of the mean.

Figure 5. Relative Caspase-3 activity measured across time and injury location in muscle tissue resulting from a femur fracture

Relative Caspase-3 response (normalized to levels measured in uninjured leg) were observed across four time points and different regions of injury following the femur fracture.

Statistically significant ($p < 0.05$) differences were determined by 2-way ANOVA with a Bonferroni post-test. Temporal differences are notated with brackets pairing certain time points when substantial alterations in protein response were observed. Statistically significant spatial differences are marked with matching striped bars. Responses at the different regions of injury (at the site of fracture and 1-cm away) that were significantly higher (>1) or lower (<1) than levels found in the uninjured leg are indicated with the symbol (*). Error bars reflect $\pm$ standard error of the mean.

Figure 6. IPA generated networks constructed from intracellular protein, cytokine, phosphoprotein, and protease dataset

Proposed signaling networks generated by IPA used experimental relative intracellular protein, cytokine and protease responses at the site of injury for 0 h and 168 h post-injury, depicting the similarities and differences between disparate temporal networks (top).

Networks portraying phosphoprotein responses at 6 h for both injury locations (at the site and 1-cm away) are shown, illustrating the similarities and differences between disparate spatial networks (bottom). All networks were supplemented with other projected nodes likely to be involved in the network, based on the Ingenuity Knowledge Base. Up- and downregulated responses are represented by red and green color coding, respectively.

Figure 7. IPA generated networks constructed from intracellular protein, cytokine, phosphoprotein, and protease dataset

Proposed signaling networks generated by IPA used experimental relative intracellular protein, cytokine and protease responses at the site of injury for 0 h and 168 h post-injury, depicting the similarities and differences between disparate temporal networks (top). Networks portraying phosphoprotein responses at 6 h for both injury locations (at the site and 1-cm away) are shown, illustrating the similarities and differences between disparate spatial networks (bottom). All networks were supplemented with other projected nodes likely to be involved in the network, based on the Ingenuity Knowledge Base. Up- and downregulated responses are represented by red and green color coding, respectively.

Figure 8. Significant radiality outcomes associated with responses at the site of fracture
 Nodes that possessed significant radiality outcomes (significance threshold: average radiality $\pm$ standard deviation) were plotted over time. Five nodes (IL-6, IL-1 β , CREB, c-Jun, p-c-Jun) from networks describe responses at the site of fracture that were significant (in boxes below graph) at varying times.

Figure 9. Significant radiality outcomes associated with responses 1-cm away from the site of fracture

Nodes that possessed significant radiality outcomes (significance threshold: average radiality $\pm$ standard deviation) were plotted over time. Nine nodes (IL-6, IL-1 β , IL-2, TNF- α , JNK, p38, p-p38, p-MEK1, p-STAT3) from networks associated with injury 1-cm away from the fracture were significant (in boxes below graph) at varying times.

Table 1

Intra- and Extracellular biomolecules measured after traumatic injury

	Target
Total intracellular protein	Akt
	c-Jun
	CREB
	ERK1/2
	JNK
	MEK1
	p38
	p53
	p90RSK
Phosphorylated protein (site)	p-Akt (Ser473)
	p-c-Jun (Ser63)
	p-CREB (Ser133)
	p-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187)
	p-GSK-3 α/β (Ser21/Ser9)
	p-HSP27 (Ser78)
	p-I κ B α (Ser536)
	p-JNK (Thr183/Tyr185)
	p-MEK1 (Ser217/Ser221)
	p-p38 (Thr180/Tyr182)
	p-p70S6K (Thr421/Ser424)
	p-p90RSK (Ser380)
	p-STAT2 (Tyr689)
	p-STAT3 (Tyr705)
Cytokine	IL-1 α
	IL-1 β
	IL-2
	IL-6
	TNF- α
	MIP-1 α
Protease	Caspase-3

Table 2

Top IPA Networks

Time post-injury (h)	At the site of injury			1-cm away from injury		
	IPA Network (<i>Intracellular Protein Cytokine, Caspase-3</i>)	p-score	IPA Network (<i>Phosphorylated Protein</i>)	p-score	IPA Network (<i>Phosphorylated Protein</i>)	p-score
0	Free radical scavenging, Cell death and survival, Skeletal and muscular disorders	22	Cell death and survival, Cell morphology, Cellular development	22	Cell death and survival, Cell morphology, Cellular development	22
6	Cell signaling, Small molecule biochemistry, drug metabolism	22	Cell death and survival, Lipid metabolism, Small molecule biochemistry	22	Cell death and survival, Skeletal and muscular disorders, Cardiac hypertrophy	22
24	Cell death and survival, Skeletal and muscular disorders, Cardiac necrosis/cell death	22	Cell death and survival, Organismal injury and abnormalities, Cellular movement	22	Cell death and survival, Cardiac hypertrophy, Cardiovascular disease	22
168	Free radical scavenging, Cell death and survival, Skeletal and muscular disorders	22	Cell death and survival, Skeletal and muscular disorders, Free radical scavenging	22	Cell death and survival, Skeletal and muscular disorders, Cell morphology	22

Network p-score: negative log10 (p-value) of Fisher's exact test. The p-value describes probability of finding Focus Genes in randomly selected genes from the Global Molecular Network.

Table 3Normalized radiality of nodes **at the site** of injury

Node	0 h	6 h	24 h	168 h
Akt Total	1.057	1.049	1.052	1.046
c-Jun Total	1.074	1.048	1.046	0.601
CREB Total	1.060	1.038	1.053	0.775
ERK1/2 Total	1.079	1.048	1.052	0.862
JNK Total	1.069	1.048	1.046	1.010
MEK1 Total	0.999	1.050	1.052	1.120
p38 Total	0.977	1.041	1.046	0.944
p53 Total	1.061	1.044	1.050	1.083
p90RSK				
Total	1.067	1.049	1.047	1.096
p-Akt	1.060	1.035	1.050	1.084
p-c-Jun	1.005	1.036	1.049	0.528
p-CREB	1.066	1.047	1.042	1.101
p-ERK1/2	1.019	1.046	1.029	1.002
p-GSK-3 α/β	1.038	1.048	1.051	1.076
p-HSP27	1.062	1.051	1.048	1.089
p-I κ B α	1.084	1.042	1.045	1.102
p-JNK	1.060	1.030	1.053	1.033
p-MEK1	0.997	1.046	1.005	1.020
p-p38	1.040	1.042	1.046	1.028
p-p70SK6	1.048	1.046	1.040	0.944
p-p90RSK	0.936	1.036	1.049	1.048
p-STAT2	1.056	1.042	1.044	1.101
p-STAT3	0.915	1.042	0.880	1.062
IL-1 α	0.952	1.044	1.047	1.113
IL-1 β	0.717	0.664	0.829	0.838
IL-2	1.072	1.044	1.037	0.997
IL-6	0.538	0.109	0.120	1.034
MIP-1 α	1.015	1.048	1.051	1.123
TNF- α	0.940	1.040	1.002	1.029
Caspase 3	0.937	1.047	1.040	1.111

Table 4Normalized radiality of nodes **1-cm away** from injury

Node	0 h	6 h	24 h	168 h
Akt Total	1.098	1.045	1.084	1.050
c-Jun Total	1.072	1.037	1.058	1.094
CREB Total	1.063	1.043	1.084	1.099
ERK1/2				
Total	1.095	1.056	1.081	1.017
JNK Total	1.050	1.051	1.077	0.767
MEK1 Total	0.971	1.055	1.081	1.090
p38 Total	0.936	1.053	1.075	0.515
p53 Total	0.950	1.034	1.079	0.937
p90RSK				
Total	0.923	1.043	1.063	1.000
p-Akt	1.060	1.047	1.077	0.992
p-c-Jun	1.065	1.046	1.071	1.037
p-CREB	1.042	1.055	1.064	1.083
p-ERK1/2	1.068	1.045	0.948	1.006
p-GSK-3 α / β	1.059	1.055	1.087	1.092
p-HSP27	1.078	1.052	1.077	1.073
p-I κ B α	1.091	1.052	1.087	1.066
p-JNK	1.092	1.045	1.084	1.046
p-MEK1	1.003	1.042	0.723	1.057
p-p38	0.997	1.056	1.086	0.718
p-p70S6	1.065	1.043	1.050	1.054
p-p90RSK	1.085	1.052	1.087	1.079
p-STAT2	1.052	1.041	1.069	1.087
p-STAT3	1.044	1.045	0.751	1.079
IL-1 α	0.992	0.994	1.054	0.962
IL-1 β	0.735	0.681	0.890	0.914
IL-2	1.087	1.015	0.549	0.895
IL-6	0.653	0.102	0.326	1.014
MIP-1 α	0.988	1.040	1.088	1.090
TNF- α	0.589	1.028	1.076	0.988
Caspase 3	0.996	1.047	1.072	1.097