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PRINCIPAL INVESTIGATOR: Nikolaos Kyritsis, Ph.D.

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Nikolaos Kyritsis, Ph.D.

E-Mail: Nikolaos.Kyritsis@ucsf.edu

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14. ABSTRACT

One of the reasons for the lack of successful clinical trials for spinal cord injury (SCI) is the lack of biomarkers for patient stratification. We have recently proposed a novel approach for the discovery of white blood cell (WBC) RNA biomarkers. Our approach yielded transcriptomic signatures in human WBCs that accurately diagnose SCI severity. Our hypothesis is that these gene modules are also functionally involved in the pathophysiology of SCI and could be targeted for the development of therapeutics. We performed Gene Co-Expression Network Analysis on RNAseq data from WBCs in humans and rats after SCI and identified gene modules associated with SCI severity in both species. Using homologenes, we identified conserved WBC gene modules with several of them being highly associated with SCI severity in both species. WBC transcriptomic biomarkers may potentially be used as targets for therapeutic interventions after SCI. Using evolutionarily conserved SCI-induced gene signatures significantly increases the probability for an effective preclinical model to be translatable in human SCI.

15. SUBJECT TERMS
Spinal cord injury, white blood cells, conserved transcriptomic signatures, biomarkers, injury severity.

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INTRODUCTION

Spinal cord injury (SCI) is a devastating condition that affects millions worldwide and adds thousands of newly injured patients to that pool annually. In the US only, 18,000 new SCIs happen annually, altering dramatically the lives of these new patients and their caretakers. Despite the clinical and research efforts over the past four decades, a therapy for SCIs is still lacking. This funded research aims to discover novel therapeutics using a systems biology approach where a preclinical model will be simultaneously validated by clinical research findings in a bedside-to-bench and back approach. We will use acute transcriptomic signatures in the white blood cells (WBCs) of humans and rats significantly associated with the initial SCI severity and/or long-term functional recovery. From this global transcriptomic pattern of both species, we will derive homologenes to be used to discover evolutionarily conserved transcriptomic signatures associated with SCI severity and recovery in both species. These signatures will subsequently be used in the public database CMap to bioinformatically predict compounds from a list of more than 8,000 perturbagens that could reverse the conserved transcriptomic phenotype. The core hypothesis is that the reversal of the expression phenotype of these signatures will lead to improved long-term recovery. The hypothesis will be tested *in vivo* in a preclinical (rat) model of SCI after the top-ranked predicted compounds will be first validated *in vitro* in rat WBC primary leukocyte cultures. A successful *in vivo* drug trial will have a great chance of being clinically important and applicable in human SCI since the discovered drug will be, by definition (conserved signature), targeting a transcriptomic signature behaving similarly in humans after SCI.

KEYWORDS

Spinal cord injury, white blood cells, conserved transcriptomic signatures, biomarkers, drug discovery, injury severity.

ACCOMPLISHMENTS

What were the major goals of the project?

Per the approved Statement of Work (SoW) the goals that would be completed during the first 12 months of the funding period were:

Description of Aim or Task	Timeline (months)	Percent of completion
Major Task 1: Submit regulatory documents and obtain necessary approvals for study initiation	**1-6**	100
Subtask 1.1: Obtain secondary use approval for analysis of human WBC transcript data sets at UCSF IRB.	**1-2**	100
Subtask 1.2: Submit documents for Aims 2 and 3 rat experiment protocol to ACURO	**1-2**	100
Subtask 1.3: Review and make modifications as needed to regulatory document, and obtain ACURO approval	**2-4**	100
Subtask 1.4: Submit documents for this secondary use of the human WBC RNA-seq datasets to HRPO.	**2-3**	100

Subtask 1.5: Amend IRB protocol to include analysis of human tissue biospecimen	**2-3**	100
Subtask 1.6: Review and make modifications as needed to regulatory documents and obtain HRPO approval.	**4-6**	100
Milestone(s) Achieved: UCSF *IRB approval obtained, UCSF IACUC approval obtained, HRPO approval obtained, and ACURO approval obtained.*		
Specific Aim 1: Identify conserved and distinct transcriptional phenotypes of peripheral WBCs between rats and humans with SCI		
Major Task 2: Identify evolutionary conserved gene modules after SCI between humans and rats utilizing two RNA-seq datasets previously acquired at UCSF BASIC	7-12	95
Subtask 2.1: Identify relevant samples within the rat and human RNA-seq database and map the genes of each dataset in a shared space by converting them to HomoloGene IDs (human n = 90 rat n = 42)	7-10	100
Subtask 2.2: Compute the "consensus matrix" for use as input for detecting coexpression modules by calculating the genome-wide similarity matrices for each species and integrating them.	11-12	100

Per the SoW the goals that would be initiated during the first 12 months of the funding period but would be completed during the 2nd year are:

Specific Aim 2: Discover conserved WBC-derived gene expression patterns that are preserved in the human and rat injured spinal cords		
Major Task 4: Identify gene expression patterns in the rat spinal cord of different severities at two different time points.	9-20	70
Subtask 4.1: Perform SCIs of three injury severities (naïve, sham, 75, 100, and 150 kdynes, n = 12 each), sacrifice half the animals at 48 hours post SCI and the other half at 6 weeks post SCI and extract total RNA from the injured hemicord.	9-15	100

What was accomplished under these goals?

During the first 12 months of the funded research we accomplished the following milestones:

1) Completed all necessary regulatory documents and obtained approvals.

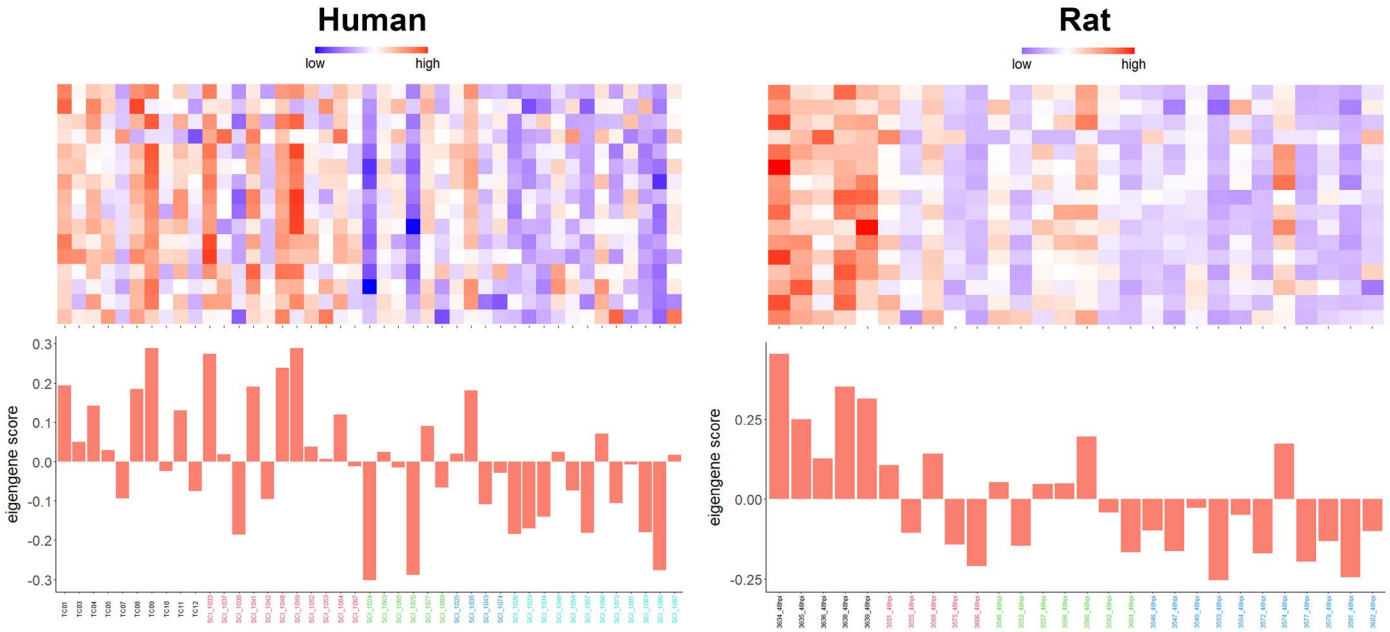
2) Identified preliminary evolutionarily conserved transcriptional signatures in the WBCs of humans and rats that respond similarly after SCI.

3) Initiated the experiments for the discovery of conserved gene expression patterns in the human and rat spinal cords.

Specifically, we used our previously acquired WBC RNAseq datasets from human SCI patients and from a rat preclinical SCI model of various severities and identified homologenes across both of them. 17,500 genes from the human dataset and 11,178 from the rat dataset resulted in 9,688 common genes (homologenes) for which we have expression levels across all samples on both species. We used these genes to identify conserved gene modules. The gene co-expression networks are constructed from the gene cross-correlation matrix (the correlation of each gene with all the rest in the data). We created cross-correlation matrices for both humans and rats and then collapsed them into a consensus matrix using the lower correlation value for each gene pair. Using that consensus matrix to discover gene modules by definition yields modules that exist in both species.

We identified many conserved WBC gene modules and subsequently, we used linear modeling against the outcome measures in humans and rats.

In the figure below, we present our current top module. This module meets our previously set criteria of conservation between the two species and high correlation with outcome measures on both species separately. In the bar plots, one can appreciate the reversal of the gene module expression from high to low as the injury severity increases. On the left side of each bar plot there are the Trauma Controls samples in the humans and the sham animals in the rat followed by the AIS D, AIS C, AIS B, and AIS A in humans and mild (75 kdynes), moderate (100 kdynes), and severe (150 kdynes) in the rat (each group is color coded). Specifically, this gene module is significantly associated with injury severity in humans (AIS grade at 3-10 days post-SCI; adjusted p-value = 0.00249), injury severity in rats (impact force; adjusted p-value = 0.00049), early recovery at 2 days post-SCI in rats (PC1 score at day 2; adjusted p-value = 0.00002), and early recovery at 7 days post-SCI in rats (PC1 score at day 7; adjusted p-value = 0.00005).



Conserved gene coexpression module in human and rat WBCs highly associated with injury severity in both species. The gene module shown here is derived after analyzing the human RNAseq data together with the 48 hours post-injury rat RNAseq data. This module consists of 16 genes (*CTSC*, *CXCL10*, *DAPP1*, *EPSTI1*, *GBP4*, *GBP5*, *HERC6*, *IFI44*, *IFI44L*, *IFIH1*, *IFIT2*, *IFIT3*, *MANEA*, *MX2*, *SSR3*, and *STAT1*) and it reduces its overall expression as the injury severity increases. In both graphs, the severity of the injury increases from left to right and the gradual shift from positive to negative eigengene scores is observed.

Intriguingly, we also discovered conserved gene modules in humans and rats with opposite responses to SCI. Conserved modules are upregulated as the severity increases in one species and downregulated as the severity increases in the other species. Besides the great biological interest this discovery poses, it may also explain and justify the translation failure of many successful preclinical drug tests. Some of these drugs may have been destined to fail if the downstream molecular mechanisms respond differently after SCI across species. Therefore, our finding has the potential to serve as proof of principle and reestablish the criteria based on which preclinical trials are designed.

Furthermore, during the first 12 months, we began the first experiment of Specific Aim 2 (Major Task 4 of the SoW). We performed a C5 SCI on 48 adult Long Evans Rats (males and females) using the same three injury severities as before (75, 100, and 150 kdynes). We also used 12 adult Long Evans rats on which we only performed a laminectomy surgery at the C5 level to use as trauma controls (sham). All animals were tested on three behavioral tests (paw placement, grooming, and the Irvine-Beattie-Bresnahan forelimb digit test) before

the injury, and at 2, 7, 14, 21, 28, 35, and 42 days post-SCI. Half of the animals were sacrificed at 2 days post-SCI (right after their respective behavioral tests) whereas the other half were terminated at 42 days post-SCI. One animal developed severe skin lesions that were not improving even after the veterinarian's instructions, and we were asked by them to euthanize it early at 4 weeks post-SCI. That animal is removed entirely from the study. During termination, all animals were transcardially perfused with ice-cold PBS, and 1 cm around the SCI epicenter was dissected. Subsequently, the two halves of the spinal cord segment were split and we dissociated mechanically the ipsilateral half in TRIZOL solution. Next, total RNA was isolated. All 59 RNA sample concentrations were within the expected range. Currently, we are measuring the RNA integrity using the Agilent 2000 Bioanalyzer before submitting these samples to the RNAseq facility of UC Davis.

What opportunities for training and professional development has the project provided?

This funded research is the first independent project of the PI in his newly established laboratory at UCSF. It allowed the PI to establish his own laboratory culture and take some different paths from how he was previously trained. A good example is the record-keeping of the lab. While all regulatory agencies do not demand it, the PI establishes a fully electronically record-keeping platform for animal surgical records and daily animal caretaking. All involved lab members have access to the platform and the data are entered there "live" as they are gathered. That step promotes an environmentally "greener" laboratory with much less paper used. Paper that according to several previous fire marshal inspections can be a serious fire hazard. More importantly, though, it promotes transparency between the PI and his lab members as well as with IACUC. IACUC inspectors and the vet staff can very easily scan the QR code placed in the animal rooms and momentarily gain access to all necessary animal records of the lab and monitor them as they are being collected. Because of that, our lab received a special appraisal from the IACUC.

The same system is being currently developed for all biospecimen collected. An online inventory is currently being created that will include all sample-specific info, it will link to the animal the sample derived from and its surgical record, and it will also include storage location in our freezers. Lastly, we are developing an R script that will allow us to automatically generate animal recovery graphs as new data are being collected and entered "live" in the system. The idea is that a researcher will be behaviorally examining animals in one room and adding the data to the platform and the PI and other senior lab members will be able to see the behavioral curves live from their office.

Another opportunity this project has provided is for the PI to present it in front of an international audience of experts. The results of this work were presented by the PI with an oral presentation at the International Neurotrauma Symposium in Berlin and will be presented again at the upcoming ISCoS meeting in Vancouver.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

As mentioned before, we are finalizing the analysis of the second cohort of human SCI patients' RNAseq data. That analysis is almost completed and subsequently, the data will be entered into our analytical pipeline to refine the evolutionarily conserved gene modules discovered. In addition, we will complete the rat spinal cord RNAseq experiment we have already initiated. Within the next month, the rat spinal cords will be sequenced and will begin the bioinformatic analysis of the data.

Furthermore, we are ready to begin the isolation of RNA from the human postmortem spinal cord sections. We have been practicing on “junk” FFPE tissue donated to our lab by other UCSF labs (placenta and brain tissues) on how to isolate good quality RNA from FFPE samples. The results of our training were very promising and we are ready to proceed with the precious human spinal cord samples. After that, the RNA samples will be sequenced and along with the rat spinal cord data will be used to prioritize the WBC gene signatures based on whether traces of them can be found in both human and rat spinal cords.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The major finding of this reporting period is the discovery of evolutionarily conserved WBC transcriptomic signatures between humans and rats whose expression after SCI behaves similarly on both species and is strongly associated with injury severity. This is an exciting finding and basically the entire premise of the project is based on the existence of these conserved signatures. The potential non-existence of such signatures was mentioned as a caveat in the proposal of this project and if that was true the project would take a very different direction. With excitement, we found that our core hypothesis is true and conserved WBC gene expression patterns do exist and their expression levels are strongly associated with SCI severity. That finding alone has the potential to transform the way we utilize preclinical research to develop therapeutics not only for neurotrauma but for more diseases and syndromes that show a considerable discord between preclinical findings and clinical transferability. As mentioned previously we identified conserved gene expression signatures that behave in an opposite manner in humans and rats in response to SCI. That would mean that targeting successfully that signature in rats will very likely not yield a similar result in humans. Identifying a common target or research space that functions similarly in humans and the preclinical model of choice promises to provide a smoother transition to a clinical application and will increase tremendously the chances for a successful clinical trial.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

CHANGES/PROBLEMS

Nothing to report.

PRODUCTS

Publications, conference papers, and presentations

1. Oral presentation at the International Neurotrauma Symposium in Berlin, Germany, 18-20 July 2022. Kenneth Fond, Megan McCune, Michelle Por, Patrick Schupp, Abel Torres-Espin, Amity Lin, Cleopa Omondi, Adam Ferguson, Jacqueline Bresnahan, Michael Oldham, Michael Beattie, and Nikos Kyritsis. Evolutionarily conserved blood transcriptomic signatures as diagnostic biomarkers and pharmacological targets for spinal cord injury.

2. Oral presentation at the 61st ISCoS Annual Scientific Meeting in Vancouver, Canada, 15-18 September 2022. Megan McCune, Kenneth Fond, Michelle Por, Patrick Schupp, Abel Torres-Espin, Amity Lin, Cleopa Omondi, Adam Ferguson, Jacqueline Bresnahan, Michael Oldham, Michael Beattie, and Nikos Kyritsis. Evolutionarily conserved blood transcriptomic signatures as diagnostic biomarkers and pharmacological targets for spinal cord injury.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Nikolaos Kyritsis*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-7801-5796*
Nearest person month worked:	4
Contribution to Project:	*Dr. Kyritsis has been involved in every experiment and oversees the entire project.*

Funding Support:	

Name:	*Kenneth Fond*
Project Role:	*Staff Research Associate*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-9154-6599*
Nearest person month worked:	5
Contribution to Project:	*Mr. Fond was involved in animal caretaking, behavioral testing, RNA isolation, and data organization in this project.*
Funding Support:	

Name:	*Amity Lin*
Project Role:	*Staff Research Associate*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-5433-3623*
Nearest person month worked:	1
Contribution to Project:	*Ms. Lin performed all animal spinal cord injuries and supervised the animal caretaking*
Funding Support:	

Name:	*Michelle Por*
Project Role:	*Student volunteer/Intern*
Researcher Identifier (e.g. ORCID ID):	-
Nearest person month worked:	1

Contribution to Project:	*Ms. Por was involved in scoring the behavioral tests from the videos.*
Funding Support:	Interns are working 20 hours per week for 14 weeks and are not compensated financially.

Name:	*Megan McCune*
Project Role:	*Graduate student rotation*
Researcher Identifier (e.g. ORCID ID):	-
Nearest person month worked:	1
Contribution to Project:	*Ms. McCune was involved in the data analysis of the rat data and the generation of the consensus matrix for the identification of conserved gene modules.*
Funding Support:	Graduate students are sponsored by their program during the rotations.

Name:	*Michael Oldham*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-7633-6932*
Nearest person month worked:	1
Contribution to Project:	*Dr. Oldham shared code and supervised closely the identification of the conserved gene modules.*
Funding Support:	

Name:	*Adam Ferguson*
Project Role:	*Co-Investigator*

Researcher Identifier (e.g. ORCID ID):	*0000-0001-7102-1608*
Nearest person month worked:	1
Contribution to Project:	*Dr. Ferguson assisted in developing the experimental design of the rat study to ensure proper statistical analysis and he reviewed all statistical analysis.*
Funding Support:	

Name:	*Michael Beattie*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-9463-3631*
Nearest person month worked:	1
Contribution to Project:	*Dr. Beattie provided his expertise and thoughts on the progress of the project through weekly 1-on-1 hourly meetings specifically dedicated to this project.*
Funding Support:	

Name:	*Jacqueline Bresnahan*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-2243-7054*
Nearest person month worked:	1
Contribution to Project:	*Dr. Bresnahan was closely participating in the rat study design and was offering her expertise and knowledge on all aspects of animal surgery and caretaking.*
Funding Support:	

Name:	*Abel Torres-Espin*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-9787-8738*
Nearest person month worked:	1
Contribution to Project:	*Dr. Torres-Espin reviewed and corrected when necessary all R scripts generated for this project. He also supervised the linear modeling approach to associate the conserved gene modules with SCI severity.*
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.