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# Global gene expression analysis following spinal cord injury in non-human primates

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ABSTRACT

Spinal cord injury (SCI) is a devastating condition with no established treatment. To better understand the pathology and develop a treatment modality for SCI, an understanding of the physiological changes following SCI at the molecular level is essential. However, studies on SCI have primarily used rodent models, and few studies have examined SCI in non-human primates. In this study, we analyzed the temporal changes in gene expression patterns following SCI in common marmosets (*Callithrix jacchus*) using microarray analysis and mRNA deep sequencing. This analysis revealed that, although the sequence of events is comparable between primates and rodents, the inflammatory response following SCI is significantly prolonged and the onset of glial scar formation is temporally delayed in primates compared with rodents. These observations indicate that the optimal time window to treat SCI significantly differs among different species. This study provides the first extensive analysis of gene expression following SCI in non-human primates and will serve as a valuable resource in understanding the pathology of SCI.



## Introduction

Spinal cord injury (SCI) results in a devastating loss of motor and sensory functions due to the poor regenerative capacity of the central nervous system. In the United States alone, approximately 12,000 patients are annually diagnosed with SCI. Despite recent advances in surgical techniques and our understanding of the biology of the spinal cord, few treatment options for SCI are available with marginal benefits for patients. A greater understanding of the pathology and the underlying molecular mechanisms of SCI is, therefore, imperative to develop a better therapeutic modality.

Contusion SCI initially causes the destruction of the blood–spinal cord barrier, and infiltration of inflammatory cells into the spinal cord ensues (Alexander and Popovich, 2009; Beck et al., 2010; Okano, 2002). Proinflammatory cytokines and reactive oxygen species (ROS) are generated by inflammatory cells and lead to secondary damage in the spinal cord (Bains and Hall, 2012; Kubota et al., 2012; Xiong et al., 2007). Thereafter, the acute inflammatory response subsides and is followed by the formation of a cystic cavity and a glial scar. Subsequently, the production of chondroitin sulfate proteoglycans (CSPGs) and extracellular matrix proteins with potent inhibitory activity against axonal growth (Ikegami et al., 2005; Silver and Miller, 2004) increases as the lesion develops into the chronic phase. In due course, changes in the spinal cord microenvironment become irreversible (Afshari et al., 2009; Okano, 2002). We and others have extensively analyzed the temporal

*Abbreviations:* SCI, spinal cord injury; ROS, reactive oxygen species; CSPGs, chondroitin sulfate proteoglycans; mRNA-seq, mRNA sequencing; WPI, week-post-injury; PCA, principal component analysis; GO, gene ontology; HE, hematoxylin–eosin; PECAM-1, platelet endothelial cell adhesion molecule-1; PC, principal component; VEGF, vascular endothelial growth factor; MMP9, matrix metalloproteinase 9.

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changes in gene expression patterns following SCI using rodent models (Beck et al., 2010; Kumamaru et al., 2012; Nakamura et al., 2003). These results not only provide important information to understand the molecular pathology of SCI but also serve as the basis to determine the optimal time window for therapeutic interventions, such as neural stem/progenitor cell transplantation (Abematsu et al., 2010; Cao et al., 2001; Cummings et al., 2005; Nishimura et al., 2013; Nori et al., 2011; Ogawa et al., 2002; Okada et al., 2005; Okano et al., 2013; Tsuji et al., 2010) and administration of anti-inflammatory substances and growth factors (Kitamura et al., 2011; Mukaino et al., 2010; Okada et al., 2004; Tuszynski et al., 1996). As highlighted by the studies in rodent models, it is conceivable that similar analyses in primate models will further deepen our understanding of the pathology of SCI in humans; however, such a study has not yet been performed, and very little is currently known regarding the changes in gene expression following SCI in primates.

To address this issue, we used the common marmoset (*Callithrix jacchus*) as a non-human primate SCI model and performed two different global gene expression analyses: microarray analysis and mRNA sequencing (mRNA-seq) using next-generation sequencing. Additionally, we performed a detailed histological analysis of the injured spinal cord in adult common marmosets to validate the gene expression analysis data. We found that, although the sequence of the gene expression patterns was comparable between common marmosets and rodents, the time course of changes in gene expression was significantly prolonged in common marmosets. This observation indicates that the transition of SCI from the acute to the chronic phase is temporally delayed in common marmosets compared with rodents and that the therapeutic time window following SCI significantly differs among species. This study provides the first extensive gene expression analysis following SCI in non-human primates and may serve as an invaluable resource in understanding the pathology following SCI in primates and in establishing a time frame for the treatment of SCI in humans.

## Materials and methods

### *Animals*

All interventions and animal care procedures were performed in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA), the Guidelines and Policies for Animal Surgery provided by the Animal Study Committee of the Central Institute for Experimental Animals and Keio University, and the guidelines outlined by the Weatherall Report and were approved by the Animal Study Committee of Keio University (numbers: 10017 and 11014). Female common marmosets (*C. jacchus*; 2-years-old) were purchased from CLEA Japan Inc. (Tokyo, Japan). The animals were housed at 26 °C with 65% humidity and illumination for 12 h/day. All of the animals had free access to food and water in the cage.

### *Contusive SCI in common marmosets*

The subjects were anesthetized with an intramuscular injection of ketamine (50 mg/kg; Sankyo Co., Ltd., Tokyo, Japan) and xylazine (5 mg/kg; Bayer AG, Leverkusen, Germany), followed by inhalation of isoflurane (Fluren; Abbott Japan Co., Ltd., Tokyo, Japan). Contusive SCI was induced using a modified New York University weight-drop device, as previously described (Iwanami et al., 2005; Kobayashi et al., 2012). Briefly, a 17-g weight, 3.5 mm in diameter, was dropped from a height of 50 mm onto the exposed dura mater at the C5 level following laminectomy. For the sham control, laminectomy of the C5 vertebra was performed without any manipulation of the spinal cord. During the surgical procedures, the physiological condition of the animals was continuously monitored by electrocardiography, transcutaneous pulse oximetry (which estimates $O_2$ saturation), and skin and rectal temperature readings. After the procedure, the animals were placed in a temperature-controlled chamber until thermoregulation was reestablished. Manual bladder expression was performed twice per day until voiding reflexes were reestablished. Paralyzed animals were provided adequate amounts of food and water until they recovered their ability to ingest food and water without assistance. Thereafter, animals had free access to food and water in the cage.

### *RNA isolation*

The animals were anesthetized and transcardially perfused with heparinized saline (5 U/ml) at 1-, 2-, 4-, and 6-weeks-post-injury (WPI). Dissected segments (6 mm) of the spinal cord at the C5 level were placed in TRIzol (Invitrogen, Carlsbad, CA, USA) and immediately frozen. Total RNA was isolated using a Qiagen RNeasy Kit (Qiagen Inc., Hilden, Germany). Samples were collected from two different animals with SCI at each time point following injury and one sham control animal at 2-WPI.

### *Microarray analysis*

Microarray analysis was performed using Affymetrix GeneChip technology as previously described (Heishi et al., 2006; Matsui et al., 2012). Sham control samples were independently analyzed three times. Total RNA (100 ng) was reverse transcribed, biotin labeled, and hybridized for 16 h to the Marmoset Genome oligonucleotide custom array Marmo2 (Shimada et al., 2012; Tomioka et al., 2010). The arrays were subsequently washed and stained in a Fluidics Station 450 (Affymetrix Japan, Tokyo, Japan) according to the manufacturer's instructions. The arrays were scanned using a GeneChip Scanner 3000 7G (Affymetrix Japan). Data analysis was performed using Expression Console 1.1 (Affymetrix Japan). Signal detection and quantification were performed using the MAS5 algorithm with the default settings. Principal component analysis (PCA) was performed using Spotfire DecisionSite 9.1.2 (TIBCO Spotfire, Somerville, MA, USA). Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer test ($P < 0.001$) using GeneSpring GX software 11.5.1 (Agilent Technologies Inc., Santa Clara, CA, USA). The heat map was visualized using Java TreeView (Saldanha, 2004). A total of 708 genes were grouped into eight clusters by K-means clustering using Gene Spring GX software 11.5.1 (Agilent Technologies Inc.). Gene ontology (GO) analysis was performed using the gene lists of each cluster. Enriched GO terms were extracted using corrected *P* values with a cut-off at 0.05. *P* values were calculated using Fisher's exact test. To obtain the corrected *P* values, the false discovery rate was controlled using the Benjamini–Yekutieli method.

### *Library preparation and mRNA-seq*

Samples for mRNA-seq were prepared using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, 100 ng of each total RNA sample was used for poly-A mRNA selection using poly-T oligonucleotides attached to magnetic beads. The purified mRNA was then fragmented and subjected to cDNA synthesis using random primers. Following conversion of the fragmented mRNA to double-stranded cDNA, a single adenosine nucleotide was added to the 3′ ends of the blunted cDNA fragments and then ligated to the Y-shape adapter. These cDNAs were purified using AMPure beads (Beckman Coulter, Brea, CA, USA) to remove excess adapter. Finally, the cDNA libraries were amplified by PCR and purified using AMPure beads. Each library was adjusted to a concentration of 11–12 pM and analyzed using a Genome Analyzer IIx (Illumina) in 75-bp single-read mode. The marmoset genomic DNA sequence including scaffold sequences was obtained from the Ensembl FTP site (ftp://ftp.ensembl.org/); concatemerized scaffold sequences were treated as single chromosomes and indexed using bowtie-build v.0.12.7



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