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Transcriptional analysis of glial cell differentiation in the postnatal murine spinal cord

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ABSTRACT

Postnatal murine spinal cord represents a good model system to study mammalian central nervous system myelination *in vivo* as a basis for further studies in demyelinating diseases.

Transcriptional changes were analyzed in SJL/J mice on postnatal day 0, 14, 49 and 231 (P0, P14, P49, P231) employing Affymetrix GeneChip Mouse Genome 430 2.0 Arrays. Additionally, marker gene signatures for astrocyte and oligodendrocyte lineage-stages were defined to study their gene expression in more detail. In addition, immunohistochemistry was used to quantify the abundance of commonly used glial cell markers.

6092 differentially regulated genes (DEGs) were identified. The up-regulated DEGs at P14, P49 and P231 compared to P0 exhibited significantly enriched associations to gene ontology terms such as myelination and lipid metabolic transport and down-regulated DEGs to neurogenesis and axonogenesis. Expression values of marker gene signatures for neural stem cells, oligodendrocyte precursor cells, and developing astrocytes were constantly decreasing, whereas myelinating oligodendrocyte and mature astrocyte markers showed a steady increase. Molecular findings were substantiated by immunohistochemical observations.

The transcriptional changes observed are an important reference for future analysis of degenerative and inflammatory conditions in the spinal cord.

1. Introduction

In general, the spinal cord and the brain vary widely from the development of most other tissues or organs in the body. A considerable amount of cell differentiation or functional and morphological development takes place during postnatal development (Clinton et al., 2000). Neurogenesis and migration of neuronal cell populations take place rather early at embryonic day 11 in mice, followed by neuronal differentiation and gliogenesis. Synaptogenesis, oligodendrocyte and astrocyte development are late embryonic or postnatal events (Brumwell and Curran, 2006; Petrik et al., 2013; Ulrich et al., 2005). Myelination starts at birth in the murine spinal cord and is considered to be completed around postnatal day 45–60 (Baumann and Pham-Dinh, 2001). Apart from the timing, the processes in central nervous system (CNS) maturation are similar between humans and rodents (Baumann and Pham-Dinh, 2001; Jakovcevski et al., 2009; Semple et al., 2013).

The above-mentioned postnatal processes suggest extremely dynamic changes in the gene expression pattern during postnatal development until the adult nervous system is functionally mature. As shown by multiple studies in different species focusing on various regions of the brain and developmental stages, gene expression profiles are crucial for future molecular and genetic studies of neuropathies and diseases by providing a robust framework (Dorrell, 2004; Johnson et al., 2009; Kagami and Furuichi, 2001; Kang et al., 2011; LeDoux et al., 2006; Lee et al., 2000; Matsuoka et al., 2010; Oldham et al., 2008; Zahn et al., 2005). The gene expression of hindbrain regions and the spinal cord displays a distinct profile in comparison to other CNS regions (Zapala et al., 2005). Therefore, results from other CNS regions cannot be directly transferred to the spinal cord. Thus, gene expression studies from the developing spinal cord are needed in order to gain in depth insights in processes

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and networks participating during its physiologic development and to use this knowledge as a basis for further studies of pathologic conditions. Moreover, considerable similarities are shown between developmental myelination and remyelination in certain disorders (Chung et al., 2011; Fancy et al., 2009), therefore, the focus of the present analysis was particularly set on the delineation of oligodendrocytic maturation and the myelination process as a basis for further studies in demyelinating diseases, including multiple sclerosis. Expression values indicated that the spinal cord exhibited a higher rate of myelin production and metabolism compared to other CNS areas (Yan et al., 2009). For that reason we believe that the postnatal murine spinal cord is a good model to study particularly processes involved in oligodendrocyte maturation and myelination *in vivo*. Furthermore, with the results of microarray analysis of *ex vivo* isolated and purified neural cell types (Cahoy et al., 2008) or lists summarizing the results from *in vitro* generated marker genes (Kuegler et al., 2010) we were provided with a profound transcriptome database for cell type specific marker gene signatures. This allows a better discrimination of the single cell types of interest.

Therefore, the aims of the study were (i) to provide a detailed exploration of the transcriptome on a gene and functional level with specific focus on the myelination process, (ii) to transfer the *in vitro* generated marker gene signatures for different glial cell lines in an *in vivo* model and (iii) to identify genes involved in oligodendrocyte lineage proliferation and differentiation *in vivo*.

2. Material and methods

2.1. Animals and tissue preparation

24 female SJL/JHanHsd-mice (6 animals per time-point; Harlan Winkelmann, Borchen, Germany) were euthanized with intraperitoneal injection of medetomidin (2 mg/kg; Domitor®; Pfizer GmbH, Karlsruhe, Germany) and ketamine (400 mg/kg; Ketamin® 10%, WDT, Garbsen, Germany) on postnatal day 0, 14, 49, and 231 (P0, P14, P49, P231), respectively. Immediately after death the spinal cord was removed from the spinal canal. On P0 and P14 the complete, unfixed spinal cord was used for RNA isolation. On P49 and P231 cervical segment 3–7, thoracic segments 5–13 and the lumbar segment 3–6 were immediately snap-frozen and stored at −80 °C for subsequent RNA isolation. For immunohistochemistry spinal cord from 1–3 cervical segment 1–3, thoracic segment 4–7 and lumbar segment 3–7 (with exemption at P0, where the whole vertebral column was sectioned) from age and sex-matched animals of equal genetic background were formalin fixed in 10% formalin for 24 h and embedded in paraffin wax (formalin-fixed and paraffin embedded, FFPE). Animal experiments were authorized by the local authorities (Regierungspräsidium Hannover, Germany, permission numbers: 33-42502-05/963 and 33.9-42502-05-12A278).

2.2. RNA isolation and microarray hybridization

RNA was isolated and hybridized separately from each animal, as described (Ulrich et al., 2010). Shortly, RNA was isolated from spinal cord specimens using the RNeasy Mini Kit (Qiagen, Hilden, Germany). 250 ng total RNA was used as a template for the generation of cRNA using the MessageAmp II-Biotin Enhanced Kit (Ambion, Austin, TX, USA). 10 μg of fragmented and labelled cRNA was hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). After an incubation period of 16 h in a rotating hybridization oven at 45 °C the arrays were washed and stained with Affymetrix GeneChip Fluidics Station 450 (Affymetrix, Santa Clara, CA). Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA) was used for signal detection.

2.3. Low-level analysis

Quality control and low level analysis of raw fluorescence intensities was performed using R (R version 2.14.1) and Bioconductor (Bioconductor version: release 2.11) with the packages "affy" (Gautier et al., 2004) and "simpleaffy" (Miller, 2013). Expression measures were created from raw intensities by quantile normalization and median polish across all microarrays using Gene Chip Robust Multichip Average (GC-RMA) with the R package "GCRMA" (Wu et al., 2013) as previously described (Ulrich et al., 2014b).

2.4. Differential expression

Statistical significance of gene expression differences was determined employing the false discovery rate estimation (FDR) method embedded in the significance analysis of microarray (SAM) using the two class unpaired argument with the R package "samr" (Tusher et al., 2001). The fold change (FC; r) was calculated for each gene (i) of the $\log_2$-transformed expression values from two time-points (a;b) by the formula $r_{(i)} = 2^{\bar{X}a(i)}/2^{\bar{X}b(i)}$. Down-regulations were displayed as negative reciprocal values (Ulrich et al., 2014a; Ulrich et al., 2010). A FDR ≤1% and a FC ≥2.0 or ≤ −2.0 was determined to define differentially expressed probe sets (DEPs; Shi et al., 2008). Lists of differentially expressed genes and expressed sequence tags (DEGs) were generated from DEPs by selecting the probe set with the highest significant absolute fold change in one of the comparisons, when the gene was represented by multiple probe sets. A gene was defined by having a particular UniGeneID and gene symbol. All transcripts with a unique UniGeneID but without assignment to a gene symbol were defined as expressed sequence tags (ESTs). Lists of DEGs (including genes and ESTs) of the different comparisons were spited into up- and down-regulated genes according their FC. Following a broad comparison of all time-points with each another, gene expression values of samples obtained from animals on P0 were defined as reference value in order to focus on oligodendrocyte maturation and myelination. DEGs retrieved in the comparisons of gene expression values at P14, P49 and P231 with P0 were compared with one another using area-proportional venn diagrams employing the R package "VennDiagram" (Chen and Boutros, 2011).

2.5. Principle component analysis and hierarchical clustering

Principle component analysis of the samples and hierarchical clustering of selected gene groups was performed on $\log_2$-transformed expression values employing TM4 Microarray Software Suite, Multi Experiment Viewer (MeV; v 4.8.1; Saeed et al., 2003). Complete linkage method with Euclidian distance as a distance measure was used.

2.6. Functional annotation clustering

Significantly overrepresented functional associations were selected from the biological process category of the gene ontology (GO) database using a modified Fisher's exact test (EASE score) calculated in DAVID 6.7 (Huang et al., 2009). The resulting lists of significantly enriched GO terms were summarized into a manageable number of ≤15 enriched biological modules of functionally related GO terms employing the DAVID functional annotation clustering algorithm with customized settings (Raddatz et al., 2014). Enriched biological modules were ranked based on their respective enrichment score (Huang et al., 2009).