

Genome expression profiling predicts the molecular mechanism of peripheral myelination

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Abstract. The present study aimed to explore the molecular mechanism of myelination in the peripheral nervous system (PNS) based on genome expression profiles. Microarray data (GSE60345) was acquired from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) were integrated and subsequently subjected to pathway and term enrichment analysis. A protein-protein interaction network was constructed and the top 200 DEGs according to their degree value were further subjected to pathway enrichment analysis. A microRNA (miR)-target gene regulatory network was constructed to explore the role of miRs associated with PNS myelination. A total of 783 upregulated genes and 307 downregulated genes were identified. The upregulated DEGs were significantly enriched in the biological function of complement and coagulation cascades, cytokine-cytokine receptor interactions and cell adhesion molecules. Pathways significantly enriched by the downregulated DEGs included the cell cycle, oocyte meiosis and the p53 signaling pathway. In addition, the upregulated DEGs among the top 200 DEGs were significantly enriched in natural killer (NK) cell mediated cytotoxicity and the B cell receptor (BCR) signaling pathway, in which Fc γ receptor (FCGR), ras-related C3 botulinum toxin substrate 2 (RAC2) and 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase γ -2 (PLCG2) were involved. miR-339-5p, miR-10a-5p and miR-10b-5p were identified as having a high degree value and may regulate the target genes TOX high mobility group box family member 4 (Tox4), DNA repair protein XRCC2 (Xrcc2) and C5a anaphylatoxin chemotactic receptor C5a2 (C5ar2). NK cell mediated cytotoxicity and the BCR pathway may be involved in peripheral myelination by targeting FCGR, RAC2 and PLCG2. The downregulation of oocyte meiosis, the cell cycle and the cellular tumor antigen p53 signaling pathway suggests decreasing schwann cell proliferation following the initiation

of myelination. miR-339-5p, miR-10a-5p and miR-10b-5p may play important roles in PNS myelination by regulating Tox4, Xrcc2 and C5ar2.

Introduction

Myelination is the production of the myelin sheath that surrounds the axons of nerve cells; it occurs through reciprocal interactions between the glial cells and the axons that they ensheath (1). Schwann cells (SCs) in the peripheral nervous system (PNS) wrap their membranes spirally around long segments of axons. Subsequently, myelinated fibers with multilayered sheaths are developed, something that allows the propagation of rapid impulses (2,3). Demyelination is a hallmark of numerous neurological diseases, such as Charcot-Marie-Tooth disease, which is a PNS hereditary disorder affecting 1/2,500 individuals (1,4). The mechanism of remyelination in neurological diseases is similar to developmental myelination (2), therefore it is possible to identify potential therapeutic treatments for neurological diseases by studying the process of normal myelination in the PNS.

Several previous studies have investigated the genes and pathways associated with peripheral myelination focusing on SC plasticity, polarization, function of the neuregulin (NRG), and extracellular matrix and cytoskeletal signals (2,3,5). These reviews revealed that NRG1 served a key role in myelination by regulating the majority of SC biological processes (6). NRG1 bounded to the epidermal growth factor receptor and subsequently activated several secondary-messenger cascades, including the phosphoinositide 3-kinase/phosphatidylinositol 3,4,5 trisphosphate/protein kinase B (AKT) (7), intracellular calcium (8) and mitogen-activated protein kinase signaling pathways (9). In addition, the proto-oncogene Wnt/ β -catenin (10), endocytic (11) and hedgehog signaling pathways (12) were also reported to be associated with peripheral myelination. However, further investigation is required to clarify the mechanisms of peripheral myelination.

MicroRNAs (miRs) serve a post-transcriptional role in PNS myelination (13). The majority of previous studies in this area have focused on the function of miRs in peripheral remyelination following nerve injury (14-18). However, the functional roles of miRs are rarely studied in developmental peripheral myelination. miR-29a has been identified to prevent the expression of peripheral myelin protein 22, which is a disease-associated protein in myelinating SCs (19). miR-318

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has been identified as a suppressor of certain myelination inhibitors, including sex determining region Y-box 2, Jun proto-oncogene and cyclin D1 (20). Let-7 miR promotes the expression of positive transcriptional factor early growth response protein 2 through suppression of the myelination inhibitor neurogenic locus notch homolog protein 1 (21). These findings reveal only a small part of the mechanisms of miR in PNS myelination.

Kangas *et al* (22) presented the genome-wide RNA expression microarray data at different stages of PNS myelination using a dorsal root ganglion (DRG) explant culture. Amongst the differentially expressed genes (DEGs), enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms associated with PNS differentiation were identified. Several previous studies have focused on the potential role of miRs in peripheral myelination (23,24). However, studies on the interactions between DEGs and miRs based on genome expression profiles in regards to peripheral myelination are rare. In the present study, the microarray dataset GSE60345 (21) was downloaded, and DEGs were analyzed and subjected to KEGG pathway and GO term enrichment analyses. Several miRs were identified as being associated with peripheral myelination based on the miR-target gene regulatory network analysis.

Materials and methods

Data acquisition. The GSE60345 microarray dataset (21) gene expression profiles were obtained from the National Center for Biotechnology Information Gene Expression Omnibus database (ncbi.nlm.nih.gov/geo/) (25). The microarray dataset GSE60345 was originally obtained using a SurePrint G3 Mouse GE 8x60K Microarray kit (cat. no. 028005; Agilent Technologies, Inc., Santa Clara, CA, USA). The organism studied was *Mus musculus*. A total of 18 samples of DRG explant cultures were collected at 0 (n=6), 10 (n=6) and 22 days (n=6) following switching to myelination medium from embryonic day 13.5 C57BL/6J mouse embryos. The different time-points that the samples were collected at represented the different stages of the myelination process; i) premyelination (0 days), ii) early myelination (10 days); and iii) advanced myelination (22 days).

Data preprocessing and DEG statistical analysis. The raw data was normalized using the limma package (version 3.10.3) of R software (26). The gene expression matrix was divided into two groups of 10 day vs. 0 day and 22 day vs. 0 day. The P-value of each gene was calculated using the non-paired t-test in the limma package software and the P-value was adjusted using the Benjamini-Hochberg method. According to the cut-off value of adjacent $P < 0.01$ and \log_2 fold change ≥ 2 , the DEGs of each group were identified. The upregulated and downregulated genes were obtained by pooling the DEGs of the two groups.

GO term and KEGG pathway enrichment analyses. The upregulated and downregulated genes were subjected to GO term (27) and KEGG pathway enrichment analysis. The analyses were conducted with Fisher's exact test using the 'mRNA enrichment' module in the Multifaceted Analysis

Tool for Human Transcriptome (biocloudservice.com). The cut-off value was set as $P < 0.05$.

Protein-protein interactions (PPI) network. The interaction of proteins encoded by the DEGs was predicted using the Search Tool for the Retrieval of Interacting Genes version 10.0 (28). The PPI score (medium confidence) was set at 0.4. Cytoscape version 3.2.0 software (29) was used to visualize the PPI network. The modules consisted of the 200 nodes with the highest degree values.

miR-target gene regulatory network analysis. The validated miRs that were identified to regulate DEGs from the two groups were downloaded from a validated gene-miR interaction information retrieval system, miRWalk version 2.0 (30). Cytoscape software was used to construct the miR-target gene regulatory network.

Results

Identification of DEGs. The gene expression data was normalized following preprocessing. A total of 276 DEGs, including 248 upregulated and 28 downregulated genes were obtained in the 10 day vs. 0 day group (Fig. 1). By contrast, 1,045 DEGs were identified in the 22 day vs. 0 day group comprised of 747 upregulated and 298 downregulated genes (Fig. 1). There were a higher number of upregulated genes compared with downregulated genes, and the 22 day vs. 0 day group contained more genes than the 10 day vs. 0 day group. The Venn diagram revealed that the overlapping upregulated and downregulated genes between the two groups were 212 and 18, respectively (Fig. 1). A total of 783 upregulated genes and 307 downregulated genes were identified by pooling the DEGs of the two groups.

GO term and KEGG pathway enrichment analyses. A total of 26 pathways were significantly enriched in the upregulated DEGs and 5 pathways were significantly enriched in the downregulated DEGs. The top 10 enriched pathways according to their P-value are listed in Table I. The complement and coagulation cascades, cytokine-cytokine receptor interaction, cell adhesion molecules, natural killer (NK) cell mediated cytotoxicity and B cell receptor (BCR) signaling pathways were significantly enriched by the upregulated DEGs. Pathways significantly enriched by the downregulated DEGs included the cell cycle, oocyte meiosis and the cellular tumor antigen p53 (p53) signaling pathway.

The top 5 GO terms in the biological process, molecular function and cellular component categories according to their P-values are illustrated in Fig. 2. The upregulated DEGs were mainly associated with immune response processes and extracellular region components. The cell cycle process was significantly enriched in the downregulated DEGs.

PPI network. A PPI network with 812 nodes and 13,141 edges was constructed using all of the DEGs (Fig. 3A). The network revealed that the majority of the nodes gathered together with a complex connectivity, indicating that the proteins were closely associated with each other. A PPI sub-network was subsequently constructed from the 200 DEGs with

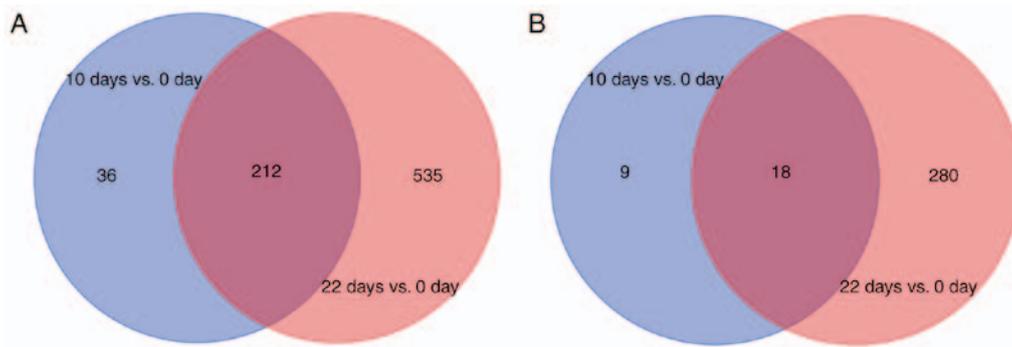


Figure 1. Venn diagram of differentially expressed genes. Following normalization of the gene expression data the (A) upregulated and (B) downregulated genes in each group were identified.

Table I. Kyoto Encyclopedia of Genes and Genomes pathways that the DEGs were significantly enriched in.

A, Upregulated genes

Pathway ID no.	Pathway name	No. of DEGs enriched in pathway	P-value
mmu04610	Complement and coagulation cascades	31	4.43×10^{-22}
mmu04060	Cytokine-cytokine receptor interaction	32	5.84×10^{-8}
mmu05330	Allograft rejection	15	1.24×10^{-7}
mmu05332	Graft-vs.-host disease	15	1.24×10^{-7}
mmu04940	Type I diabetes mellitus	15	3.76×10^{-7}
mmu04662	B cell receptor signaling pathway	16	1.47×10^{-6}
mmu04514	Cell adhesion molecules	22	2.92×10^{-6}
mmu04650	Natural killer cell mediated cytotoxicity	19	4.96×10^{-6}
mmu05322	Systemic lupus erythematosus	17	8.62×10^{-6}
mmu05320	Autoimmune thyroid disease	14	1.16×10^{-5}

B, Downregulated genes

Pathway ID no.	Pathway name	No. of DEGs enriched in pathway	P-value
mmu04110	Cell cycle	13	2.73×10^{-9}
mmu04114	Oocyte meiosis	11	1.38×10^{-7}
mmu04914	Progesterone-mediated oocyte maturation	8	1.77×10^{-5}
mmu04115	p53 signaling pathway	5	4.68×10^{-3}
mmu04020	Calcium signaling pathway	6	4.02×10^{-2}

DEG, differentially expressed gene.

the highest PPI degree value (Fig. 3B). The upregulated and downregulated DEGs were gathered together. The 200 DEGs with the highest PPI degree values were subsequently subjected to KEGG pathway analysis. A total of 28 pathways were significantly enriched in the upregulated DEGs and 5 pathways were significantly enriched in the downregulated DEGs. Table II lists the top 10 pathways enriched by the DEGs according to their P-value. The leishmaniasis, osteoclast differentiation, NK cell mediated cytotoxicity and BCR signaling pathways were significantly enriched by the upregulated DEGs, including the Fc γ receptor (FCGR), ras-related C3 botulinum toxin substrate 2 (RAC2) and 1-phosphatidylinositol-4,5-bisphosphate phosphodi-

esterase γ -2 (PLCG2). The downregulated DEGs were significantly enriched in the cell cycle, oocyte meiosis and the p53 signaling pathway.

miR-target gene regulatory network. The miR-target gene regulatory network comprised of 135 nodes and 144 edges, as shown in Fig. 4. The association between 109 miRs and 26 DEGs (15 upregulated and 11 downregulated) was clearly illustrated. According to their connectivity degree value, the top 10 genes and miRs are listed in Table III. miR-10b-5p, miR-10a-5p and miR-339-5p were identified as having a high connectivity degree value. The validated target genes of these miRs were TOX high mobility group box family

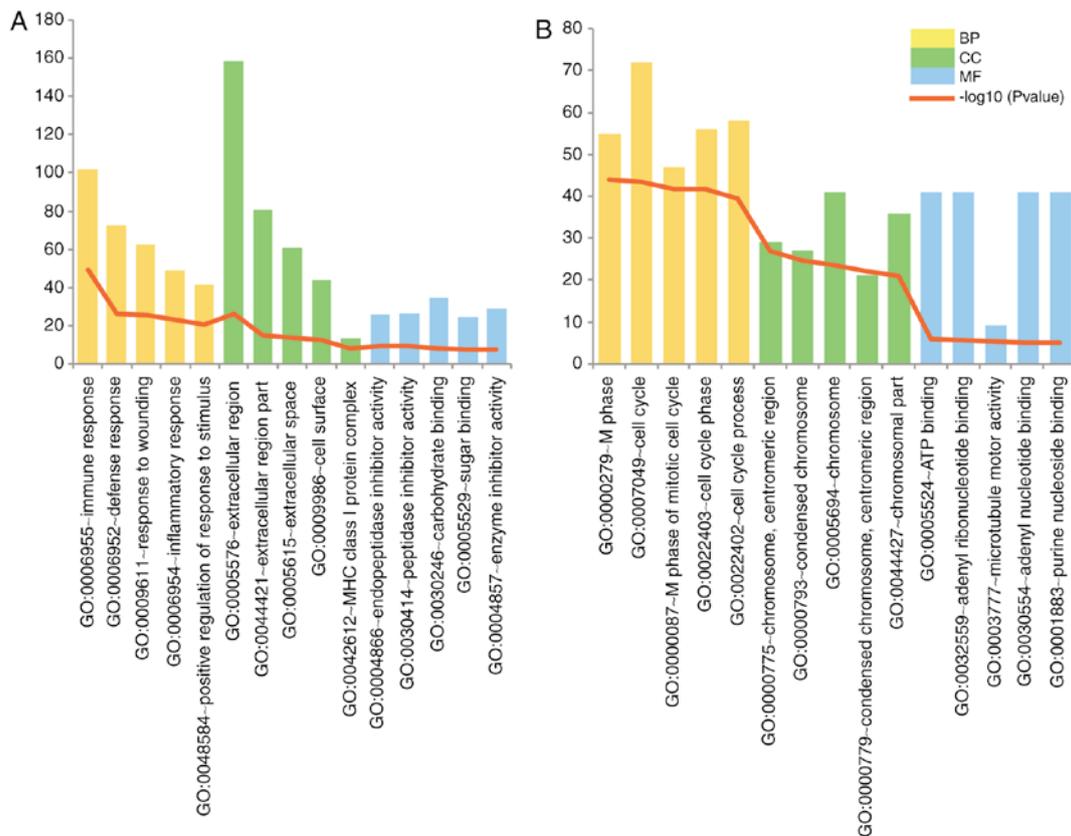


Figure 2. GO terms enrichment analysis of the differentially expressed genes. According to their P-value, the top 5 GO terms in the BP, CC and MF categories were listed for the (A) upregulated and (B) downregulated genes. BP, biological process; CC, cellular component; MF, molecular function; GO, gene ontology.

member 4 (Tox4), DNA repair protein XRCC2 (Xrcc2) and C5a anaphylatoxin chemotactic receptor C5a2 (C5ar2).

Discussion

Multiple peripheral nerve diseases have been revealed to be closely associated with dysregulated myelination (31). However, the mechanism of dysregulated myelination has not been fully identified. In the present study, an integrated bioinformatic analysis was performed on the differential gene expression patterns throughout different stages of myelination. A total of 783 upregulated and 307 downregulated genes were identified throughout the different stages of peripheral myelination. The NK cell mediated cytotoxicity and BCR signaling pathways were significantly enriched by the upregulated DEGs, including FCGR, RAC2 and PLCG2. Pathways significantly enriched by the downregulated DEGs included oocyte meiosis, the cell cycle and the p53 signaling pathway. In addition, miR-339-5p, miR-10a-5p and miR-10b-5p demonstrated a high degree value in targeting Tox4, Xrcc2, C5ar2.

The NK cell mediated cytotoxicity and BCR signaling pathways were significantly enriched in the upregulated DEGs among the top 200 DEGs. NK cells are an essential type of cytotoxic lymphocyte in the innate immune system, which have been revealed to be associated with myelin loss (32). NK cells also serve a regulatory role in multiple sclerosis, a disease characterized by chronic demyelination (33). BCRs are also a crucial component of the immune system and the BCR signaling pathway has been implicated in the activation of nuclear factor

(NF)- κ B, which serves an indispensable role in peripheral myelination (34). The present study revealed that these two pathways were significantly enriched in the upregulated DEGs. The upregulation of genes in the NK cell mediated cytotoxicity and the BCR signaling pathways suggested that an improved immune response was closely associated with peripheral myelination. In addition, several genes overlapped between these two pathways, including FCGR, RAC2 and PLCG2. FCGR has previously been identified in SCs, which may aid in the regulation of the immune response (35). A recent study reported that FCGR had a potential role in axonal injury through the mediation of inflammation (36). RAC2 has been previously identified as stimulating AKT activation in primary mast cells (37). AKT silencing was previously reported to be associated with hypomyelination (38). The expression of PLCG2 was revealed to be sensitive to ciliary neurotrophic factor (39), a cytokine which may promote peripheral nerve regeneration (40). Consequently, the NK cell mediated cytotoxicity and BCR signaling pathways may be associated with PNS myelination through FCGR, RAC2 and PLCG2.

KEGG pathways significantly enriched by downregulated DEGs among the top 200 DEGs were identified, such as oocyte meiosis, the p53 signaling pathway and the cell cycle. Oocyte meiosis is linked to the cell cycle and the p53/p21 axis is associated with the cell cycle by regulating G₁/S transition (41). The cell cycle is indispensable for glial cell proliferation, not only in PNS myelination but in cell dedifferentiation in response to nerve injury (42). The results of the present study demonstrated that these three pathways were downregulated, suggesting that

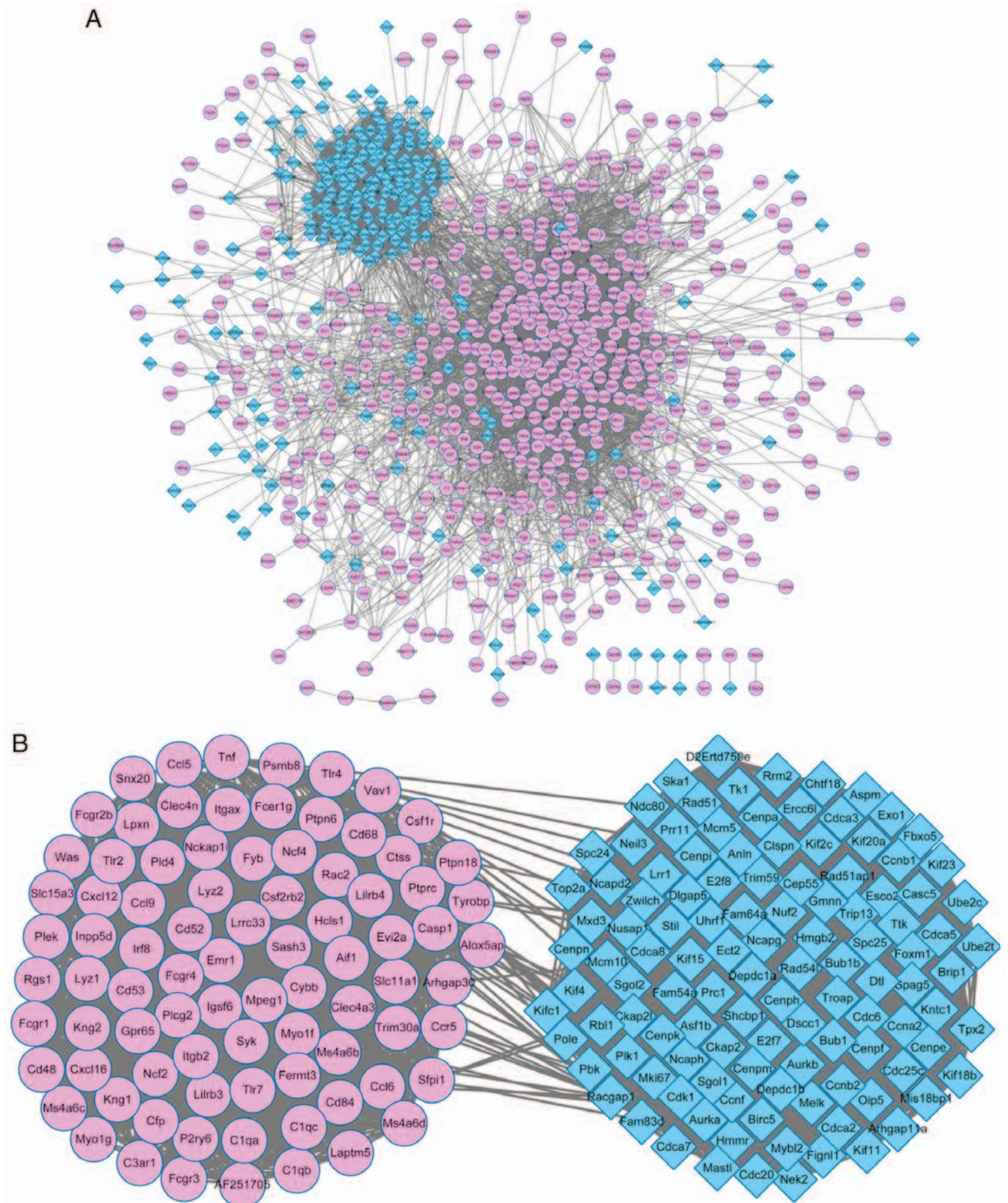


Figure 3. PPI network of the DEGs. (A) A PPI network was constructed using all of the DEGs identified. (B) A secondary PPI was constructed using the 200 DEGs with the highest degree values. The purple circles represent upregulated genes and the blue squares represent downregulated genes. PPI, protein-protein interaction; DEG, differentially expressed gene.

glial cells were prone to differentiation instead of proliferation following the initiation of PNS myelination.

The miR-target gene regulatory network revealed that three miRs (miR-339-5p, miR-10a-5p and miR-10b-5p) possessed a

Table II. Top 10 significantly enriched Kyoto Encyclopedia of Genes and Genomes pathways of the top 200 DEGs.

A, Upregulated genes				
Pathway ID no.	Pathway name	No. of DEGs enriched in pathway	P-value	Genes
mmu05140	Leishmaniasis	10	3.44x10 ⁻¹⁰	PTPN6, TNF, NCF2, NCF4, FCGR4, TLR2, TLR4, ITGB2, FCGR1, FCGR3
mmu04380	Osteoclast differentiation	12	6.01x10 ⁻¹⁰	CYBB, TNF, FCGR2B, NCF2, NCF4, PLCG2, FCGR4, FCGR1, CSF1R, FCGR3, SYK, TYROBP
mmu05150	<i>Staphylococcus aureus</i> infection	9	1.23x10 ⁻⁹	C1QA, C3AR1, C1QB, FCGR2B, FCGR4, ITGB2, FCGR1, C1QC, FCGR3
mmu04650	Natural killer cell mediated cytotoxicity	11	1.68x10 ⁻⁹	CD48, PTPN6, TNF, RAC2, PLCG2, FCGR4, FCER1G, ITGB2, VAV1, SYK, TYROBP
mmu05152	Tuberculosis	12	2.10x10 ⁻⁸	TNF, ITGAX, FCGR2B, FCGR4, TLR2, FCER1G, TLR4, ITGB2, CTSS, FCGR1, FCGR3, SYK
mmu04666	Fc γ R-mediated phagocytosis	9	8.34x10 ⁻⁸	PTPRC, FCGR2B, RAC2, PLCG2, INPP5D, FCGR1, VAV1, WAS, SYK
mmu04145	Phagosome	11	2.20x10 ⁻⁷	CYBB, FCGR2B, NCF2, NCF4, FCGR4, TLR2, TLR4, ITGB2, CTSS, FCGR1, FCGR3
mmu05133	Pertussis	8	6.04x10 ⁻⁷	C1QA, C1QB, TNF, IRF8, TLR4, ITGB2, CASP1, C1QC
mmu04664	Fc epsilon RI signaling pathway	7	6.19x10 ⁻⁶	TNF, RAC2, PLCG2, FCER1G, INPP5D, VAV1, SYK
mmu04662	B cell receptor signaling pathway	7	7.34x10 ⁻⁶	PTPN6, FCGR2B, RAC2, PLCG2, INPP5D, VAV1, SYK
B, Downregulated genes				
Pathway ID no.	Pathway name	No. of DEGs enriched in pathway	P-value	Genes
mmu04110	Cell cycle	13	1.16x10 ⁻¹⁴	CDK1, CDC6, RBL1, TTK, CDC20, CDC25C, MCM5, CCNB1, CCNB2, PLK1, BUB1, BUB1B, CCNA2
mmu04114	Oocyte meiosis	8	1.53x10 ⁻⁷	CDK1, PLK1, SGOL1, BUB1, FBXO5, CDC20, AURKA, CDC25C
mmu04914	Progesterone-mediated oocyte maturation	7	8.23x10 ⁻⁷	CCNB1, CDK1, CCNB2, PLK1, BUB1, CDC25C, CCNA2
mmu04115	p53 signaling pathway	4	2.15x10 ⁻³	CCNB1, CDK1, CCNB2, RRM2
mmu03460	Fanconi anemia pathway	3	1.65x10 ⁻²	BRIP1, UBE2T, RAD51

DEG, differentially expressed gene.

relatively high degree value. miR-339-5p has been identified to suppress the protein expression of β -secretase 1, which is a positive regulator of myelination (43,44). Additionally, miR-339-5p was reported to be associated with the NF- κ B (45)

and p53 signaling pathways (46) which are crucial to peripheral myelination. miR-10a-5p and miR-10b-5p have been previously reported to regulate the gene encoding brain-derived neurotrophic factor, which may produce a long-term increase in PNS

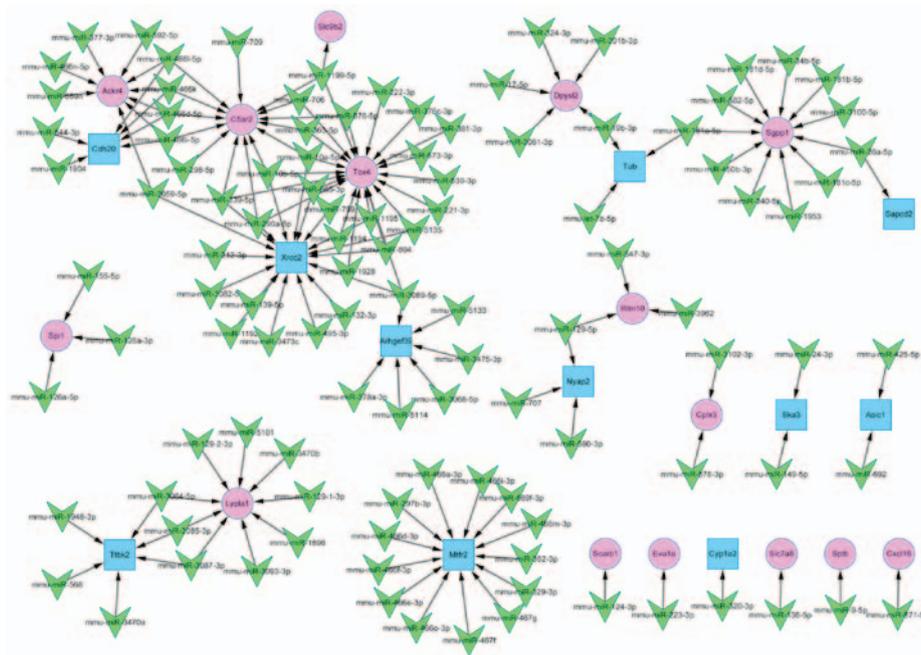


Figure 4. miR-target gene regulatory network. Cytoscape was used to construct a miR-target gene regulatory network. The green arrows represent miRs, the purple circles represent upregulated genes and the blue squares represent downregulated genes. miR, microRNA.

Table III. Top 10 miRs and DEGs according to their degree value.

A, DEGs

Symbol	Regulation	Degree value
Tox4	Up	21
Xrcc2	Down	18
C5ar2	Up	14
Mtrf2	Down	13
Sgpp1	Up	11
Ackr4	Up	9
Lypla1	Up	9
Ttbk2	Down	6
Cdh20	Down	6
Arhgef39	Down	6

B, miRs

Symbol	Regulation	Degree value
mmu-miR-290-5p	-	3
mmu-miR-10a-5p	-	3
mmu-miR-339-5p	-	3
mmu-miR-10b-5p	-	3
mmu-miR-4661-5p	-	3
mmu-miR-466i-5p	-	3
mmu-miR-466k	-	3
mmu-miR-466d-5p	-	3
mmu-miR-1199-5p	-	3
mmu-miR-3089-5p	-	3

miR, microRNA; DEG, differentially expressed gene.

myelin formation (47-49). In the present study, miR-339-5p, miR-10a-5p and miR-10b-5p were identified to regulate Tox4, Xrcc2 and C5ar2, respectively. Tox4, also known as Lcp1, has previously been revealed to bind with calcium and has been implicated in PNS myelination (49). Xrcc2 encodes a member of the RecA/DNA repair protein RAD51 homolog 1-(RAD51L1) related protein family, in which hsRec2/Rad51L1 is included. HsRec2/Rad51L1 may phosphorylate myelin basic protein, p53 and cyclin E (50). C5ar2 encodes C5a anaphylatoxin chemotactic receptor C5a2, which is also known as G protein-coupled receptor 77 (GPR77). The function of GPR77 in peripheral myelination is unclear, whereas another G protein-coupled receptor, GPR126 (encoded by Adgrg6), has been identified to be required for the initiation of SC myelination (51). This suggests that C5ar2 may serve a similar role to Adgrg6. These findings suggest that miR-339-5p, miR-10a-5p and miR-10b-5p may be associated with peripheral myelination by targeting Tox4, Xrcc2, and C5ar2.

In conclusion, NK cell mediated cytotoxicity and the BCR signaling pathway may be associated with peripheral myelination by regulating FCGR, RAC2 and PLCG2. The indicated downregulation of oocyte meiosis, the cell cycle and the p53 signaling pathway suggests that SC proliferation decreases following the initiation of PNS myelination. miR-339-5p, miR-10a-5p and miR-10b-5p serve a potential role in the myelination of the PNS by regulating Tox4, Xrcc2 and C5ar2. The results of the present study may provide information to clarify the mechanisms of PNS myelination; however, experimental validation is required to confirm these results.

Acknowledgements

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