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Single-cell RNA sequencing provides new insights into the interaction between astrocytes and neurons after spinal cord injury in mice

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ABSTRACT

Background: Spinal cord injury (SCI) is a devastating neurological disease in which astrocytes play a central role. Understanding the relationship between different subtypes of astrocytes and neuron subtypes during the progression of SCI is critical to understanding the disease.

Methods and results: In this study, single-cell RNA sequencing (scRNA-seq) was used to analyze the transcriptome data of acute, subacute and intermediate stages of SCI in mice as well as normal tissues. Different subtypes of astrocytes and neuronal cells were identified and their dynamic changes and functionalities during the development of SCI. An intriguing discovery was the identification of a specific subtype of astrocytes characterized by unique expression of Gap43, Vim, Aldoc, and Mt1. This subtype of cells shows similarities in gene expression with neurons and potentially transitioned into neurons during the course of SCI. Furthermore, we have uncovered the important role of the glycolytic pathway in this cellular transformation process. Furthermore, through cellular interaction analysis, we validated pathways (mdk-ptprz1,ptn-ptprz1,ptn-sdc3) associated with the potential conversion of these specific cell subsets into neurons. Finally, these cells were observed by fluorescence microscopy and critical gene expressions were validated by Western blot.

Conclusions: The results of this study not only deepen our understanding of the mechanisms underlying SCI, but also provide new insights and opportunities for the development of novel therapeutic strategies and interventions.

1. Introduction

Spinal cord injury (SCI) is a devastating neurological disorder that usually affects patients' everyday functions for the rest of their lives. Currently, there is still no effective treatment that significantly restores function in SCI patients. SCI can be divided into three stages: acute phase (<48 h), subacute phase (>48 h, <2 weeks), and intermediate to chronic phase (>2 weeks, <6 months) [1]. In the subacute phase, necrosis of neuronal axons and myelin activates macrophages that release proinflammatory, leading to a widespread inflammatory response in the SCI microenvironment. As a result, the blood-spinal cord barrier is disrupted, leading to a dysregulation of the immunological

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microenvironment and impairing the regeneration of the damaged spinal cord [2]. Studies suggest that astrocytes play a crucial role in the dynamic formation of the injured microenvironment after SCI. On the one hand, astrocytes are involved in the proliferation of glial scars after spinal cord injury, which impede axon regeneration. On the other hand, certain astrocytes can promote the survival of neurons by exerting neuroprotective effects, and even participate in neuronal transformation and reprogramming through proliferation [3]. Therefore, a more precise characterization of the heterogeneity of astrocytes during the SCI process and the investigation of their relationship to neurons are of great importance.

The relationship and interaction between astrocytes and neurons have been a recent focus of research. Roberta de Ceglia et al. [4] have identified a "hybrid cell" that belongs to a subgroup of astrocytes and can take over partial functions of neurons. Hong Fan et al. [5] reported that a certain subset of astrocytes can serve as neural stem cells (NSCs) that initiate cell division and differentiation. The question of whether astrocytes can be reprogrammed into neurons and the mechanisms underlying the conversion of astrocytes into neurons has long fascinated many scientists. The discovery of the above-mentioned subset of astrocytes increases the potential to clarify these questions. Identifying the heterogeneity of astrocytes helps to understand their role in the process of spinal cord injury (SCI). However, the heterogeneity and functional differences of astrocytes during SCI have not yet been fully elucidated. Therefore, we performed a detailed analysis of the heterogeneity and dynamic development of astrocytes after SCI.

In this study, we performed single-cell RNA sequencing analysis (scRNA-seq) on tissue data of mice with spinal cord injury (SCI) from the GEO 189070 database. The data included transcriptomes of SCI at acute phase (1 day), subacute phases (3 days, 7 days, 14 days), intermediate phase (60 days), and normal tissue. We identified six distinct subtypes of astrocytes and seven different subtypes of neurons. We then discovered potential transformation processes between specific subpopulations by integrating and performing pseudo-temporal analyzes. Finally, we investigated the interactions and transformation mechanisms between these subpopulations by analyzing the coexpression of genes and cell communication.

To summarise, we have identified a potential intermediate subtype of cells between astrocytes and neurons. This intermediate subtype can transform into neurons after spinal cord injury, and glycolysis was found to promote proliferation and transformation of these cells.

2. Material and methods

2.1. Acquisition and preprocessing of scRNA-seq data from mouse spinal cord injury tissue

Mouse spinal cord injury tissue scRNA-seq data were downloaded from the Gene Expression Omnibus (GEO) database (GSE 189070). The data were subjected to quality control using the R package Seurat v.4.3. Cells with fewer than 400 detected genes or genes expressed in fewer than 10 cells were excluded from the analysis. Cells expressing mitochondrial genes at a high percentage (above 20 %), indicative of dying cells, were also removed. The R package DoubletFinder was employed to detect and remove doublets. Following the removal of all unnecessary cells from the dataset, We ultimately obtained 60,407 cells. data were normalized using the global-scaling normalization method LogNormalize.

2.2. Single-cell clustering analysis

The Seurat package's FindVariableFeatures function was utilized to identify the top 2000 most variable genes. Principal Component Analysis (PCA) was employed to reduce the dimensionality of the data. Cell clustering was performed using the FindClusters and FindNeighbors functions. t-Distributed Stochastic Neighbor Embedding (t-SNE) and Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction techniques were applied to visualize cells and identify distinct cell clusters. To identify significantly differentially expressed genes in each cluster, the FindAllMarkers function, along with the Wilcoxon test option, was employed. Cell type annotation was performed using SingleR [6], and known marker genes were used to verify the classification of each cell cluster. For example, astrocyte marker genes (Gfap, Aldoc, Atp1b2, Aqp4) [7,8], and neuron marker genes (MAP2, CAMK2A, Gpr37) [9,10] were used to ensure the accuracy of the classification.

2.3. Extraction and subgroup classification of astrocytes and neurons

The identified cells from the clusters of astrocytes and neurons were extracted for further investigation. The same clustering methods as in section 2.2 were employed for the classification and visualization of subtypes within astrocytes and neurons.

2.4. PPI network analysis

We first identified the DEGs between astrocytes and neurons in SCI and non-SCI mice. Based on these DEGs, we constructed a PPI network using the STRING database (https://string-db.org/), which provides known and predicted interaction relationships among genes. Next, we visualized the PPI network using Cytoscape software (https://cytoscape.org/). Finally, we analyzed the Hub genes within the PPI network using Cytoscape to identify key genes potentially playing central roles in the transdifferentiation of astrocytes into neurons after spinal cord injury.

2.5. Pseudo-time analysis

Using the R package monocle (version 2.28.0), pseudotime analysis was performed on the astrocyte clusters obtained through the aforementioned steps. Initially, the GetAssayData function was employed to extract raw count data, which underwent data cleaning and normalization. Subsequently, the newCellDataSet function was used to combine the count matrix, feature data, and phenotype data into a CellDataSet object. Following this, the estimateSizeFactors function was applied to estimate size factors for each cell. The estimateDispersions function was then used to estimate the dispersion of each gene. Next, the detectGenes function was utilized to filter a set of genes with an average expression level greater than or equal to 0.1. The setOrderingFilter function was applied to filter and sort genes based on the selected gene order. Subsequently, the reduceDimension function was used for dimension reduction, and the orderCells function was employed to pseudotime-sort the cells.

2.6. Cell interaction analysis

We created a cell-cell communication object using the CellChat package. By integrating data and loading information from databases, we identified and characterized overexpressed genes and their interactions. Subsequently, we computed the communication probability between cells. We filtered out relationships with communication occurrences fewer than 10 times, constructing a network of pathways and communication relationships. We visualized the communication intensity and specific communication patterns between pathways using the netVisual_circle function and netVisual_bubble function.

2.7. Spinal cord injury surgery in mice

This study protocol was approved by the Animal Ethics Committee of Wuxi City Hospital of Traditional Chinese Medicine (Ethics No. SZYYKJFZJH2020111810). Twelve 6-week-old female C57BL/6 mice were purchased from the Qinglongshan Animal Farm and housed at the Jiangsu Institute of Parasitic Diseases. The mice were randomly divided

into three groups: the Sham group (Naive), the 7-day post-spinal cord injury group (SCI 7), and the 14-day post-spinal cord injury group (SCI 14), with four mice in each group. All mice were anesthetized by intraperitoneal injection of 1 % sodium pentobarbital (50 mg/kg). The location of T13 (attached to the 13th rib) was identified by palpating along the ribs towards the spine, followed by identifying the position of T10 by tracing the spinous process anteriorly. The T9-11 segments of the spinal cord were exposed during surgery, and the T10 segment was impacted using a spinal cord impactor (RWD company). Successful model establishment was indicated by the immediate loss of hind limb tension and the absence of observable hind limb movement. In the Sham group, the spinal cord was exposed without injury. After muscle and skin suturing, erythromycin was applied to prevent infection. Mice in the SCI 7 group were euthanized on day 7 post-injury, those in the SCI 14 group were euthanized on day 14 post-injury, and Naïve mice were euthanized on days 7 and 14, with two mice each. The spinal cord tissue from the injury site was collected, mechanically dissociated, and digested with 0.25 % trypsin for 30 min. Fetal bovine serum was added to terminate the digestion, followed by centrifugation at room temperature at 1200 r/min for 5 min. The supernatant was discarded, and the cell pellet was resuspended in high-glucose DMEM containing 10 % fetal bovine serum and 1 % penicillin-streptomycin. The suspension was filtered through a 200-mesh sieve, and differential adhesion was used to remove non-target cells. The cells were seeded in T25 culture flasks and observed under a microscope. Glial fibrillary acidic protein (GFAP) immunofluorescence staining was performed to identify spinal cord astrocytes. Primary cells were passaged when 90 % confluent, and cells in the logarithmic growth phase at passage 3 were used for subsequent experiments.

2.8. Cell Immunofluorescence

Astrocytes obtained through the above procedure were fixed with 4 % pre-cooled paraformaldehyde at room temperature for 20 min, followed by permeabilization with 0.2 % Triton X-100 (Sigma) for 10 min. The cells were then blocked with 1 % BSA at room temperature for 30 min. Next, primary antibodies GAP43 (DF7766, Affinity) and ALDOC (PA5-12317, Invitrogen) (1:50 dilution) were diluted in 1 % BSA and incubated in a humidified chamber at 37 °C for 2 h. Secondary antibodies, diluted in 1 % BSA (1:100 dilution), were incubated in the dark at 37 °C for 1 h. Finally, nuclei were stained with DAPI for 10–20 min. Each step was followed by three PBS washes, each lasting 5 min. After processing, images of the cells were captured using a fluorescence microscope.

2.9. Western Blot

Proteins were extracted from astrocytes obtained the above procedure using RIPA lysis and extraction buffer. Protein concentrations were determined by the BCA method. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were then blocked with bovine serum albumin. The membranes were incubated overnight at 4 °C with primary antibodies diluted 1:1000 (GAP43, ALDOC, VIM), followed by a 1-h incubation at room temperature with secondary antibodies diluted 1:5000 (goat anti-rabbit IgG). ECL chemiluminescence detection was used for exposure and imaging. Finally, ImageJ was used for semi-quantitative analysis of protein bands, quantifying protein expression levels.

3. Results

3.1. Single-cell clustering of spinal cord injury tissue

We performed single-cell clustering analysis on spinal cord injury (SCI) tissue at different time points and identified 17 cell types. We evaluated the proportions and trends of cell populations at each time point and found that neurons and astrocytes exhibited similar expression patterns. Notably, the proliferation of astrocytes was correlated with that of neurons, suggesting the potential for astrocytes to trans-differentiate into neurons.

We utilized the FindIntegrationAnchors function to identify anchors and the IntegrateData function to integrate all cells from spinal cord tissues at different time points (uninjured, 0d, 1d, 3d, 7d, 14d, 60d) from the GEO database (GSE 189070). After quality control, we obtained a total of 60,407 transcriptome data points. The data were standardized, clustered, and visualized using t-distributed stochastic neighbor embedding (tSNE). Through SingleR analysis, we identified 17 cell types, including microglia, neurons, fibroblasts, macrophages, oligodendrocytes, monocytes, endothelial cells, NK cells, astrocytes, B cells, epithelial cells, erythrocytes, granulocytes, T cells, adipocytes, dendritic cells and cardiomyocytes (Fig. 1A). Validation by differential gene expression within each cell cluster showed significant differences and good separation between the clusters (Fig. 1B).

In addition, we calculated the proportions of each cell cluster at different injury time points as identified by SingleR, and presented the results in a stacked bar chart (Fig. 1A). At the same time, we counted the number of cells, selected neurally related cells and visualized the trends using a line graph (Fig. 1C). We observed a similar trend in the expression patterns and cell numbers of neurons and astrocytes, with both reaching their lowest numbers on the third day after spinal cord injury and increasing thereafter. Astrocyte proliferation appears to be correlated with neuron proliferation, consistent with previous reports suggesting potential transformation of astrocytes to neurons [11]. Therefore, we have focused on investigating the subtypes of astrocytes and neurons, their cell communication and interaction, and the potential of astrocytes for neuronal reprogramming.

3.2. Extraction and subtyping of astrocytes

We extracted and analyzed subtypes of astrocytes, identifying six subtypes. Through cell count trends and differential gene analysis, we found that subtypes 1 and 3 may play a significant role in the astrocyte transformation process after spinal cord injury. GO analysis revealed that genes enriched in subtypes 1 and 3 are involved in regulating neurodevelopment and oxygen level changes, which may be associated with the transformation of astrocytes after spinal cord injury.

We isolated Astrocytes annotated through SingleR for further analysis, and conducted expression pattern analysis of well-known Astrocyte marker genes ("Gfap", "Aldoc", "Atp1b2″, "Aqp4″) (Fig. 1B), confirming a robust clustering of Astrocyte cell clusters. These cells were then re-clustered into 6 distinct clusters (Fig. 2A). Using the same approach, cell counts within each subtype cluster at different injury time points were calculated and presented in line graphs (Fig. 2B), while proportions were visualized through stacked bar charts (Fig. 1C). We observed that the expression pattern of the cells in cluster 1 corresponded to the previous trends we had seen in the cell numbers of neurons and astrocytes. It showed an increase from the third day after spinal cord injury, peaking around day 7, and then declining (Fig. 2B). AC3, on the other hand, rapidly increased after injury, reaching its peak on the third day, followed by a decline, and experiencing another peak on the 14th day. We suspect that these two subtype clusters may be associated with neuronal proliferation.

We analyzed the differentially expressed genes in subtypes of astrocytes, identifying the top ten significantly different genes in each subtype and visualizing them using volcano plots. Additionally, volcano plots were generated for the marker genes "Gfap," "Atp1b2," "Vim," "Aldoc," "Mt1," "Gap43," "Gpr37l1," "Gpr37," and "Sox2" (markers for AC and NC) (Fig. 1D), demonstrating robust differential expression of marker genes across astrocyte subtypes. Furthermore, expression bubble plots were created for these genes (Fig. 2D). We observed that clusters 1 and 3 not only differentially expressed astrocyte marker genes but also cluster 1 exhibited differential expression of Mt1, Gfap, Vim, and Gap43.

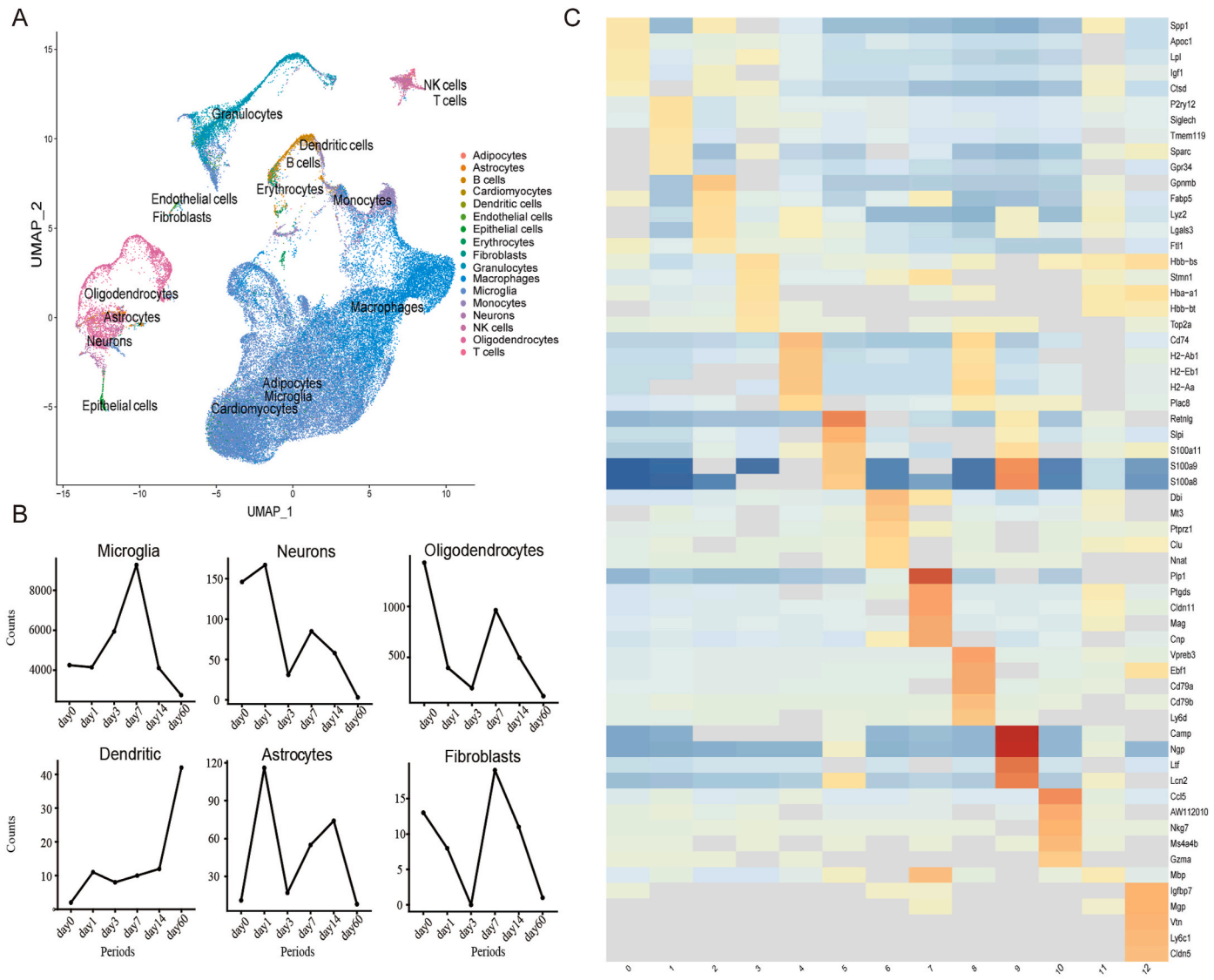

Fig. 1. (A) Clustering of mouse spinal cord injury tissue using U-MAP for dimensionality reduction and clustering, annotated with SingleR using known marker genes for 18 clusters. (B) Expression heatmap of the top five differentially expressed genes in each cluster after U-MAP clustering. (C) Line chart depicting the cell count changes over time for Microglia, Neurons, Fibroblasts, Oligodendrocytes, Astrocytes, and Dendritic cells.

The Mt1 gene has been shown to play a role in regulating cell proliferation [12], while GFAP + cells may act as neural stem cells (NSCs) initiating cell differentiation during development [13]. The Vim gene is believed to be involved in spinal cord regeneration after injury [14], and cluster 1 also differentially expressed the Gap43 gene (Fig. 2D), which is considered a neural marker gene and a key gene in neuronal growth [15].

Cluster 3 also differentially expressed Gfap, Gap43, Vim, and Mt1. Additionally, we conducted Gene Ontology (GO) functional enrichment analysis for each subtype of Astrocytes. The top 10 functional annotation terms of differentially expressed genes in cluster 1 included positive regulation of developmental growth and cellular response to oxygen levels. In cluster 3, the labels included regulation of neuroblast proliferation (Fig. 2C). This suggests that clusters 1 and 3 (differentially expressing Gap43, Vim, Aldoc, Mt1) may play crucial roles in the developmental transformation of astrocytes after spinal cord injury. Furthermore, this process appears to depend on the regulation of cellular responses to changes in oxygen levels, which may impact the cell's glycolytic pathway to meet its energy requirements.

3.3. Extraction and subtyping of neuronal cells

We performed extraction and subtyping analysis of neurons after spinal cord injury, identifying 7 neuronal subtypes. Through cell count trends, differential gene analysis, and GO enrichment analysis, we identified the cell types of several subtypes. By combining the expression patterns of AC and NC marker genes across these subgroups, along with PPI analysis, we revealed interactions between astrocytes and neurons following spinal cord injury. Notably, we found intermediate cells with the potential to transdifferentiate into neurons in AC1, AC3, and NC1. Additionally, we identified several key genes involved in this process.

We isolated Neurons annotated through SingleR for further analysis, and these extracted cells were re-clustered into 7 different clusters (Fig. 3A). Similarly, using the same approach, we calculated cell counts and proportions within each subtype cluster at different injury time points and presented the results (Figs. 1E and .3B). Clusters NC1, NC3, NC4, NC5, and NC6 were detectable before injury, and NC1, NC4, NC6 significantly decreased after injury, with NC1 and NC4 reaching their lowest points on the third day, then starting to rise and peaking around the seventh day before declining—a trend consistent with the expression patterns of certain subtypes of Astrocytes. In contrast, NC5 and NC7

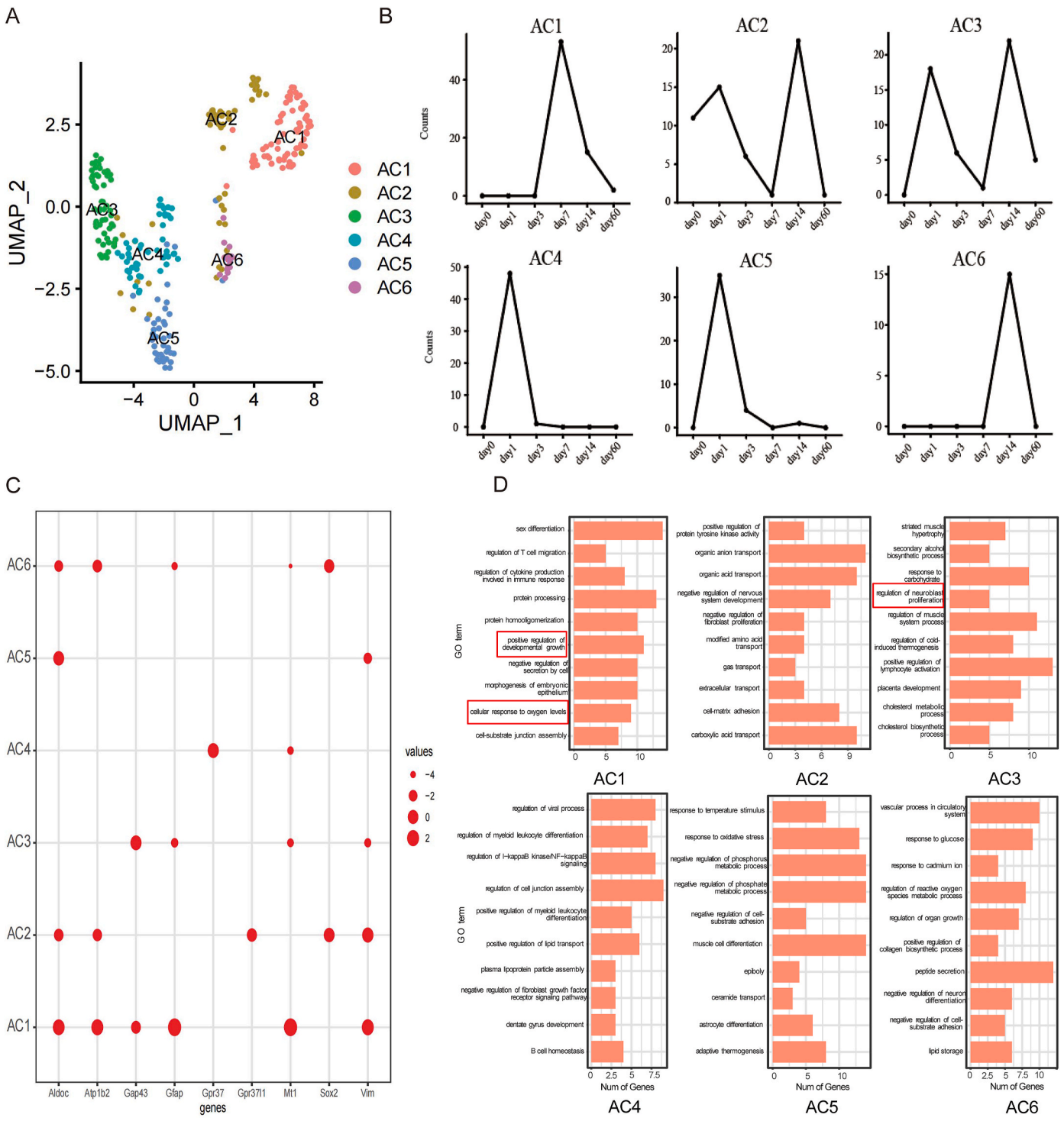

Fig. 2. (A) U-MAP clustering of Astrocytes. (B) Line chart depicting the cell count changes over time for each subtype cluster. (C) GO functional enrichment of differentially expressed genes in each subtype cluster, displaying the top ten terms. (D) Bubble plot illustrating the expression patterns of selected marker genes for Neurons and Astrocytes in each subtype.

were undetectable before injury but continuously increased after injury (Fig. 3B), exhibiting characteristics of neurons or glial scar cells undergoing changes. At the same time, volcano plots of differential genes among neuronal subgroups also indicate distinct separation of neuronal subgroups (Fig. 1F).

We conducted Gene Ontology (GO) functional enrichment analysis for each subtype of Neurons (Fig. 3C). Cluster NC1 included labels such as plasminogen activation and Neuron remodeling, NC2 included Response to monosaccharide, Cellular response to hydrogen peroxide, and presynapse organization labels, NC4 included cell fate commitment and regulation of glial cell proliferation labels. Combining the GO functional enrichment results, post-injury expression trends, and the expression of differentially expressed genes (Fig. 1F), these clusters may represent neuronal precursor cells and immature neurons. In contrast, NC5 and NC7 enriched GO functional annotations include negative regulation of cytoskeleton organization, negative regulation of nervous

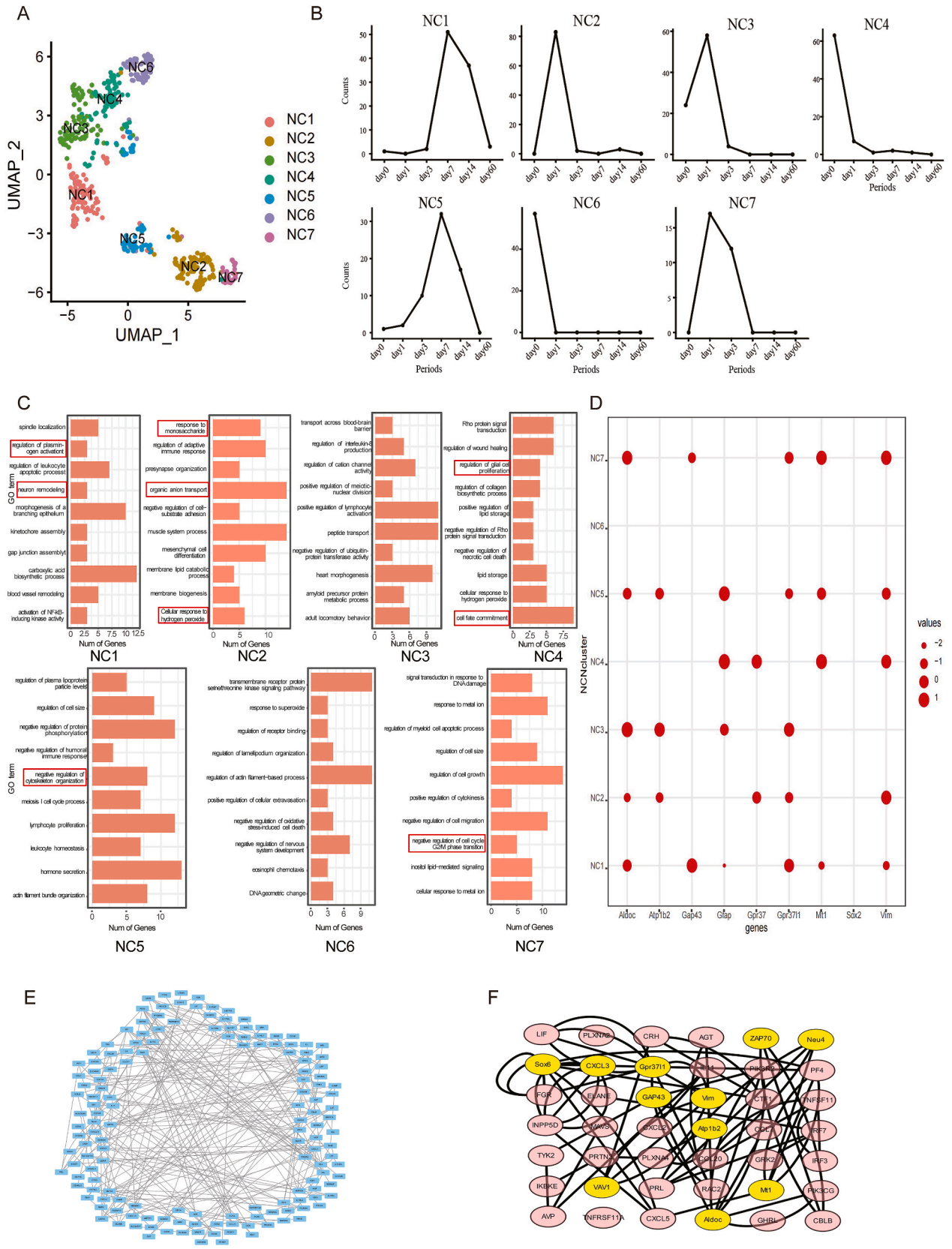

Fig. 3. (A) U-MAP clustering of Neurons. (B) Line chart depicting the cell count changes over time for each subtype cluster. (C) GO functional enrichment of differentially expressed genes in each subtype cluster, displaying the top ten terms. (D) Bubble plot illustrating the expression patterns of selected marker genes for Neurons and Astrocytes in each subtype. (E)The PPI network of DEGs between astrocytes and neurons in SCI and non-SCI mice. (F)Hub gene analysis based on the PPI network, illustrating key genes with potential roles in the transdifferentiation process.

system development, and negative regulation of oxidative stress-induced cell death, indicating these cells may be damaged neurons and scar cells.

Simultaneously, we observed the expression of "Gfap", "Atp1b2″, "Vim", "Aldoc", "Mt1″, "Gap43″, "Gpr37l1″, "Gpr37″, and "Sox2" (markers for AC and NC) in various NC subtypes (Figs. . 2D and .3D). Some subtypes expressed both AC and NC marker genes, and NC1 shared a similar differential expression pattern with AC1 and AC3, all including Gfap, Gap43, Vim, and Mt1. This suggests active interaction between AC and NC after spinal cord injury and implies the presence of intermediate cells with the potential for reprogramming into neurons in AC1, AC3, and NC1. Additionally, through PPI network analysis, we uncovered the interactions between the differential genes of astrocytes and neurons in SCI and non-SCI mice. Fig. 3E illustrates the PPI network, highlighting how these differential genes interact with one another. Furthermore, the Hub gene analysis (Fig. 3F) identifies key genes with central roles in the network(Gap43, Vim, Aldoc, Mt1, Atp1b2, Gpr37l1, etc.), which may play critical regulatory functions in the transdifferentiation of astrocytes into neurons following spinal cord injury. To clarify this question, we conducted pseudo-temporal analysis by integrating AC and NC.

3.4. Integration and pseudo-temporal analysis

We performed pseudo-temporal analysis on astrocytes and neurons following spinal cord injury, revealing that the process of astrocyte-to-neuron conversion occurs in distinct stages. Additionally, we validated the previously defined subgroups and, through gene expression analysis, found that glycolysis may play a crucial role in the reprogramming of astrocytes into neurons.

Previous studies have suggested that Astrocytes may act as neural stem cells (NSCs) initiating cell division and differentiation [16]. To further explore the relationship between Astrocytes and Neurons, we simultaneously extracted and conducted pseudo-temporal analysis on both cell types (Fig. 4A). We retained the original clustering, resulting in

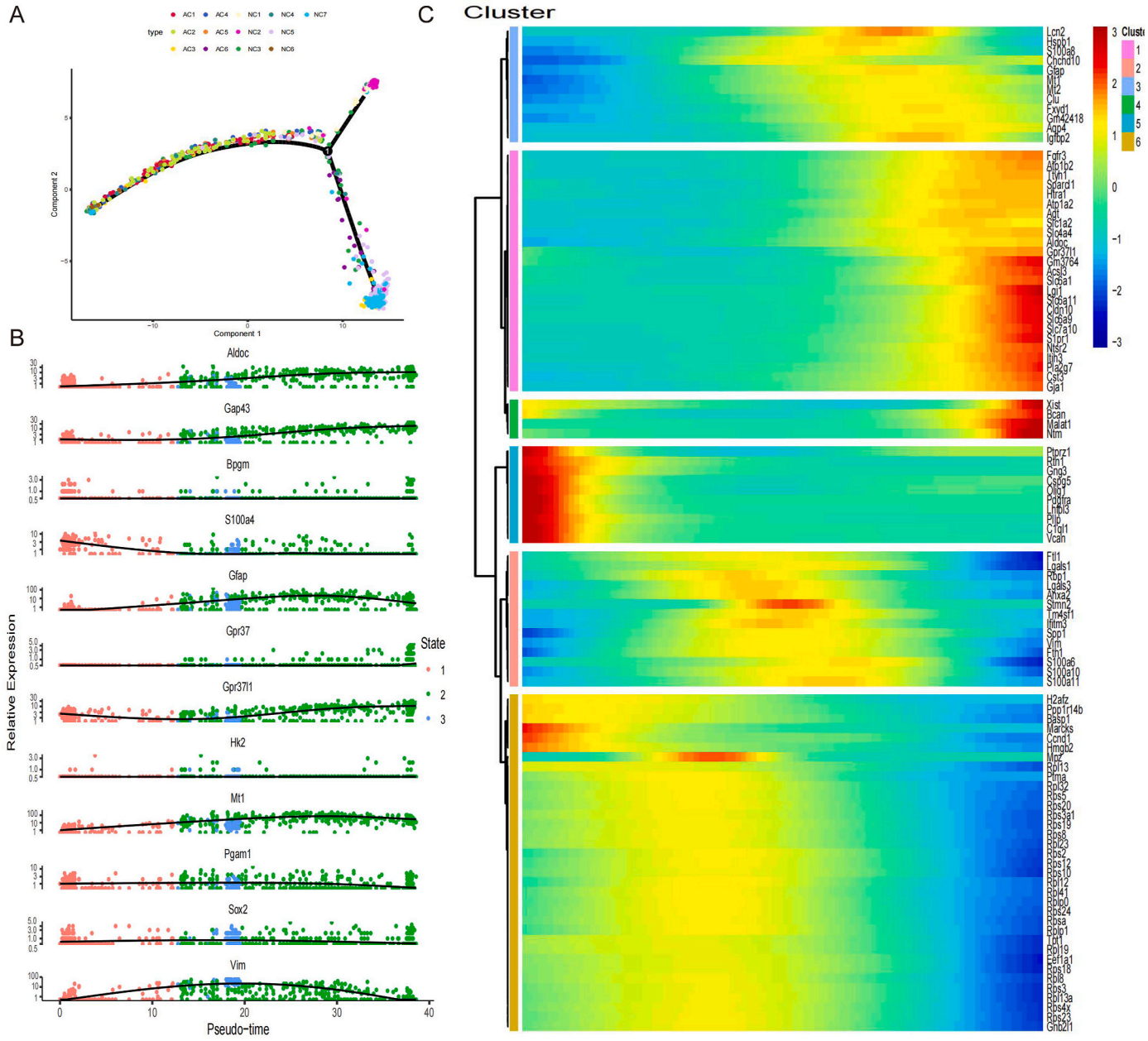

Fig. 4. (A) Visualization of the pseudo-temporal analysis of merged Neurons and Astrocytes, categorized by subtype. (B) Expression patterns of selected glycolysis genes, Astrocyte marker genes, and genes regulating cellular reprogramming. (C) Expression pattern heatmap of differentially expressed genes.

13 groups in total (6 for AC and 7 for NC). Pseudo-temporal analysis of Astrocytes and Neurons cells showed that the initial part of state 2 was mainly composed of Astrocytes, with the AC1 subtype spanning the entire state 2 and scattered distribution near the mutation node. After the mutation node, states 1 and 3 were mainly composed of neuronal cells. State 1 and state 3 represented two outcomes of Neurons after spinal cord injury, with the end segment of state 1 concentrating cells from NC5 and NC7 clusters, previously identified as damaged neurons and scar cells. Cells from AC5 and AC6 clusters were sporadically distributed in the early segment of state 1, and the end segment of state 3 was mainly composed of NC1, NC2, and NC4, which were considered neuronal precursor cells and immature neurons in previous analyses. At 1 and 3 days after injury, there was a significant proliferation in state 2, indicating a proliferative response of both Astrocytes and Neurons. State 1, representing the appearance of damaged neurons and scar cells, was observed. At 7 and 14 days after injury, a large number of cells were detected in both state 2 and state 3, indicating the processes of neural repair, protection, and new neuron generation and transformation (Specific Astrocytic Subpopulation Transforms into Neurons)(Fig. 1H).

Subsequently, we plotted the expression patterns of Aldoc, Pkm2, Hk2, Bpgm, S100a4, Gfap, Atp1b2, Vim, Mt1, Gap43, Gpr37l1, Gpr37, and Sox2 (genes related to glycolysis and cell reprogramming) (Fig. 4B). We found that the Aldoc gene was highly expressed after spinal cord injury with changes during the recovery process. As a key regulatory gene of glycolysis, it regulates energy supply. Importantly, we further analyzed the expression patterns of other genes and discovered that Aldoc had a similar expression pattern to reprogramming-related genes and stem/progenitor cell genes (Gfap, Mt1, Mt2, Clu, Slc1a2) (Fig. 4C). These genes were also highly detected in the AC1 and AC3 subtypes. This indicates that the Aldoc gene may play a crucial role in regulating the repair process after spinal cord injury and is actively involved in the reprogramming of Astrocytes to Neurons.

3.5. Cell interaction analysis

We analyzed the cell-cell interactions between subgroups of Astrocytes and Neurons, finding a strong interaction between AC1 and NC1. Pathway analysis revealed significant interactions between these subtypes through pathways such as mdk-ptprz1, ptn-ptprz1, and ptn-sdc3, which are primarily involved in neural development, synapse formation, and synaptic growth.

To identify and specifically validate the neuronal conversion potential of particular cell subtypes, we extracted Neurons and Astrocytes and performed cell-cell interaction analysis on their subtypes. We observed a strong interaction between subtypes AC1, AC2, AC3 in Astrocytes, and NC1, NC3, NC5 in Neurons, with particularly robust interaction between AC1 and NC1 (Fig. 5A). Further analysis of the interaction pathways between them revealed that the interaction between astrocytes and neurons was only prominent in certain pathways and subtypes. Notably, the interaction pathway between AC1 and NC1 subtypes was particularly strong for the mdk-ptprz1 pathway, which has been shown to play a role in neural development, synapse formation, and neuroprotection [17]. The interaction between AC3 and NC1 subtypes was significant in the ptn-ptprz1 and ptn-sdc3 pathways (Fig. 5B), pathways known to promote neural synapse growth and cell migration in the nervous system [18]. These findings demonstrate ongoing communication between AC1 and NC1 subtypes during spinal cord injury, involving neural development, synapse formation, and synaptic growth. This further supports the potential existence of a reciprocal conversion process between these two cell populations.

3.6. Experimental validation

The bioinformatics findings were further validated through Cell Immunofluorescence and Western Blot analyses, which assessed the expression alterations of hallmark genes and their respective cell subtypes.

We conducted Western Blot experiments on differentially expressed

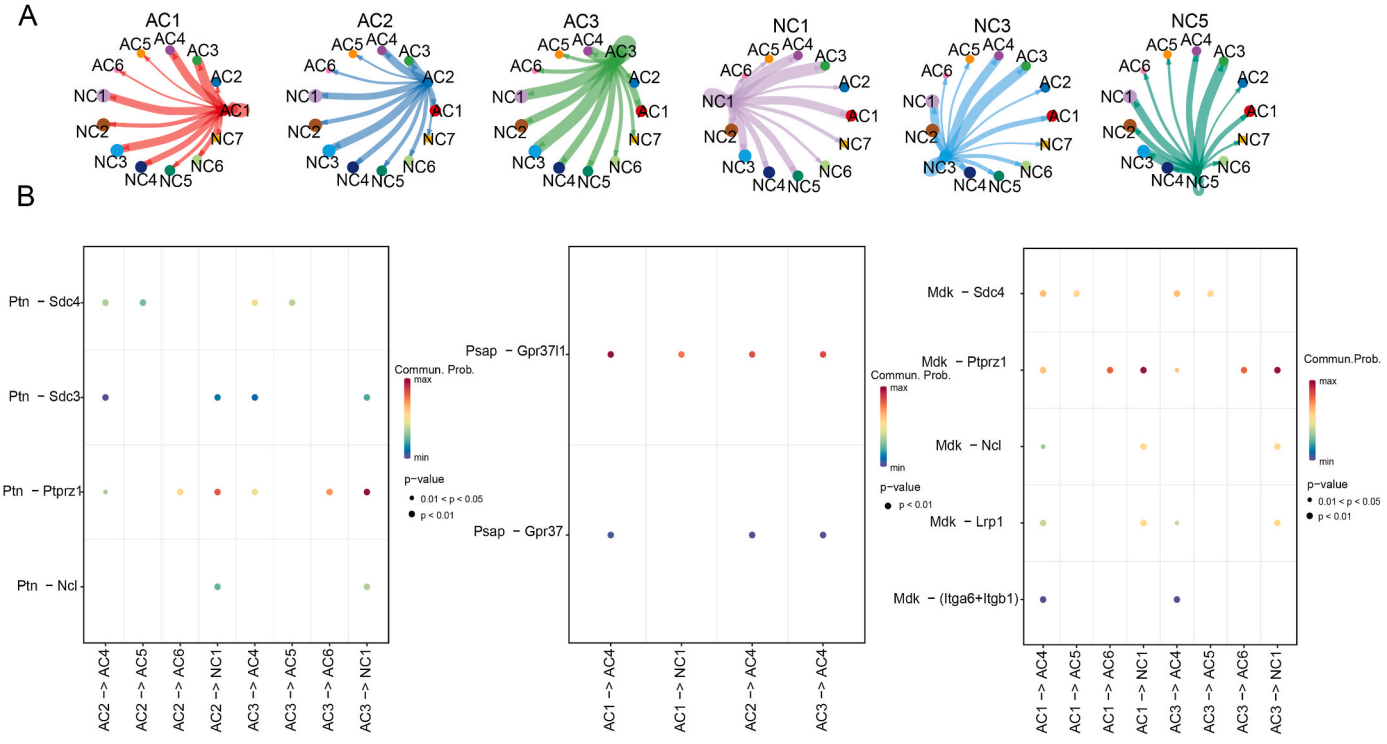

Fig. 5. (A) Visualization of the interaction network between Neurons and Astrocytes, where thicker lines represent more significant interactions. (B) Bubble plot depicting pathway enrichment of interactions between Neurons and Astrocytes.

proteins (Gap43, Vim, Aldoc) in the AC1 and AC3 subtypes to validate their changes at 0, 7, and 14 days post-injury(dpi). The results were consistent with previous analyses, showing a significant increase in their expression levels at 7 and 14 days (Fig. 6A). To further observe and validate this subtype of astrocytes, we performed co-staining for Gap43 and Aldoc on cultured astrocytes at 0 dpi, 7 dpi, and 14 dpi. We found that Gap43 and Aldoc were co-expressed in some astrocytes, clearly identifying this specific subgroup of cells (Fig. 6B). Moreover, the number of these observed cells significantly increased at 7 and 14 days post-injury, aligning with the earlier analyses (Fig. 6A).

4. Discussion

Through pharmacological approaches and regulation by various transcription factors, astrocytes have been successfully reprogrammed into different types of neurons. Recent studies have confirmed that mature astrocytes in the central nervous system (CNS) can be transformed into functional neurons. Building on in vitro work, numerous studies have demonstrated the conversion of astrocytes into neurons in different disease models to replace damaged or lost cells [19]. Puls B and colleagues successfully transformed astrocytes in the spinal cord of mice with spinal cord injury (SCI) into neurons [20]. Non-astrocytic glial cells can also be converted into neurons; endogenous Sox2 can transform NG2 glial cells (not astrocytes) into neuronal cells after SCI, although the levels are insufficient to induce the development of these neurons into mature neurons [21]. Astrocytes are a favorable choice for glial cell reprogramming because astrocytes and neurons both originate from the same precursor cells. When neurons are damaged, astrocytes can be activated and exhibit good plasticity [22].

In this study, we found that subtypes AC1, AC3, and NC1 cells exhibit characteristics of expressing both astrocytic and neuronal marker genes. This indicates overlapping and similar functions, which have been

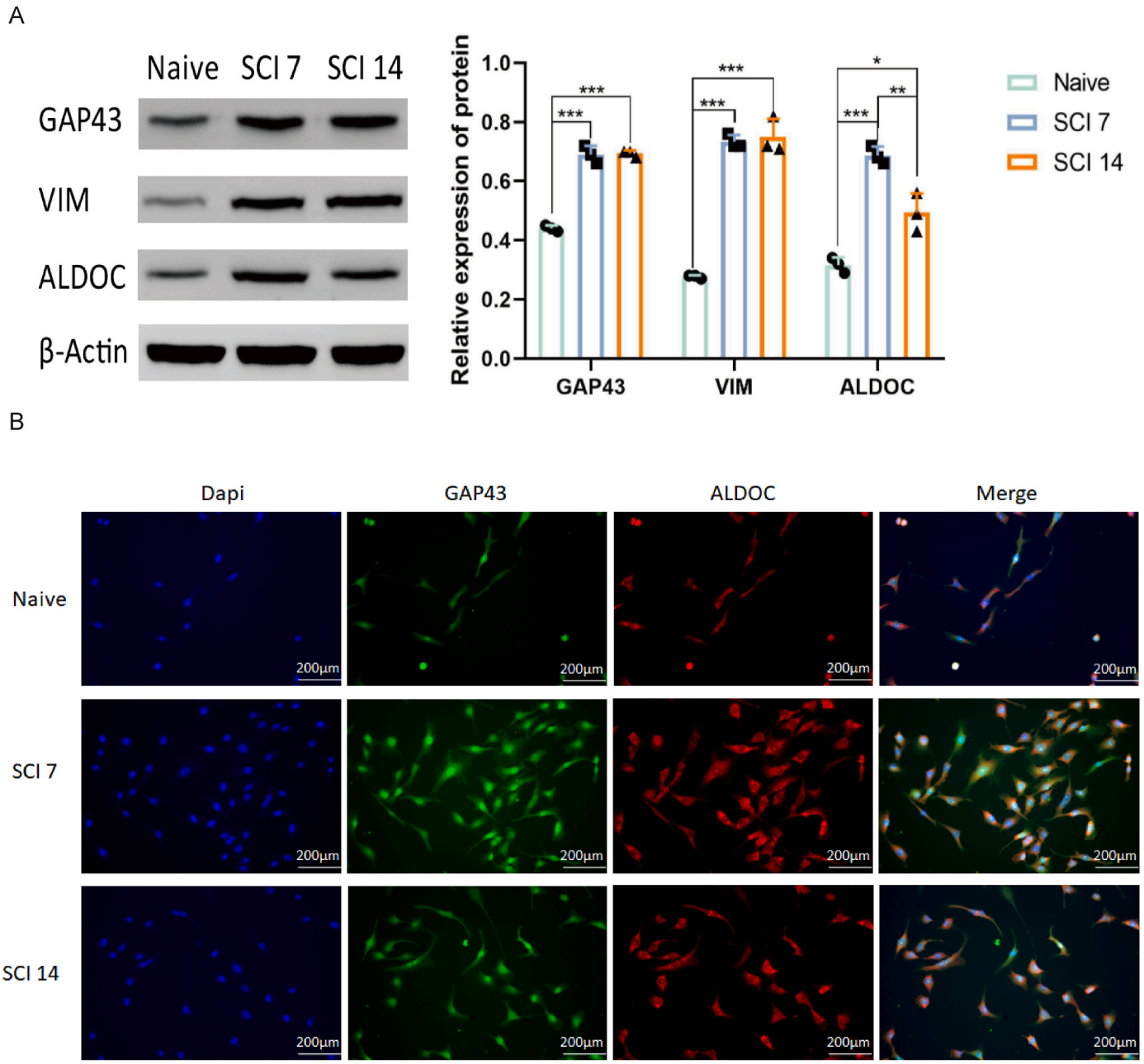

Fig. 6. (A) Quantification of gene expression levels for GAP43, ALDOC, and VIM, validated by Western Blot.(B) Cellular immunofluorescence, utilizing staining for GAP43 and ALDOC, revealing cells with colocalization.

confirmed through the characteristics of marker genes and GO functional enrichment related to the regulation of cell growth and development, neuronal remodeling, and regulation of neuronal proliferation, among other processes relevant to the growth, development, and differentiation of nervous system cells. Additionally, their changes in cell counts and proportions after injury are remarkably similar. These multiple similarities, along with their proactive communication and pathway interactions, lead us to believe that there are intermediate cells between astrocytes and neurons, as corroborated by the analysis. These cells differentially express Gap43, Vim, Aldoc, and Mt1 (with similar expression trends in the pseudo-time analysis), and functional analyses suggest their potential to transform into mature neurons. Furthermore, we identified key protein interactions between astrocytes and neurons through protein-protein interaction (PPI) analysis and selected several hub genes (such as Gap43, Vim, Aldoc, Mt1, Atp1b2, Gpr37l1, etc.). Previous studies have shown (e.g., Li et al., 2024; Xu et al., 2024) [23,24] that these genes play a critical role in the recovery process after injury by regulating the interactions between neurons and astrocytes. Based on the results of this study, we propose that these hub genes are crucial for regulating the transdifferentiation of astrocytes into neurons following spinal cord injury and neuroinflammation.

It has been established that specific cell types can be operationally defined by their unique metabolic pathway profiles. For instance, astrocytes primarily rely on anaerobic glycolysis to generate ATP, while neurons utilize oxidative phosphorylation to meet their higher metabolic demands [25–27]. Russo et al. reported significant differences in the mitochondrial proteomes between astrocytes and neurons [28,29]. An increase in mitochondrial activity is required before any major metabolic transition occurs and becomes physiologically sustainable [30]. In this study, we observed through GO functional enrichment that labels related to cellular response to oxygen metabolism are enriched in AC1 and NC2. Since astrocytes mainly depend on anaerobic glycolysis for ATP production, engaging in oxygen metabolism and energy supply, the appearance of the AC1 subpopulation after spinal cord injury may suggest a cellular response through enhanced glycolytic pathways for both sugar and oxygen metabolism. This indicates a regulatory role in increasing energy supply, contributing to functions such as cell proliferation and developmental growth regulation, crucial for neuronal reprogramming after spinal cord injury. On the other hand, neurons utilize oxidative phosphorylation for ATP production to meet their higher metabolic demands. Therefore, the enrichment of labels such as "Response to monosaccharide" and "Cellular response to hydrogen peroxide" in NC2 suggests changes in energy supply demands in neurons after spinal cord injury, albeit not through the regulation of glycolytic pathways. Additionally, our pseudo-time analysis in this study focused on the expression changes of glycolysis-related genes and their co-expression with reprogramming-related genes. We found a similar expression pattern between the key glycolytic gene Aldoc and reprogramming-related genes, as well as stem/progenitor cell genes (Gfap, Mt1, Mt2, Clu, Slc1a2), further supporting the crucial role of astrocyte glycolytic pathways in cellular reprogramming.

This study, through cell communication analysis, reveals the interplay among AC1, AC3, and NC1 subtypes through the mdk-ptprz1, ptn-ptprz1, and ptn-sdc3 pathways. Mdk, ptprz1, and Sdc3 are crucial molecules in neurogenesis and neural plasticity. Ptn and mdk are involved in inducing and stimulating neuronal differentiation, and the interaction between ptn and ptprz1 plays a vital role in cell-cell adhesion, cell movement, and migration [31]. Li H et al. experimentally confirmed in vivo and in vitro that the ptn-ptprz1 pathway promotes the proliferation and differentiation of neural stem/progenitor cells (NSPCs) [32]. Between ptn and sdc3, ptn facilitates neurite growth and cell migration by interacting with sdc3 [33]. The binding of mdk to ptprz1 promotes neuronal migration, nuclear localization, and cell survival [34]. Therefore, the interactions among AC1, AC3, and NC1 subtypes still correlate with cell differentiation, proliferation, and neuronal conversion. This further validates the plasticity and functional similarity of these subtypes, supporting their role in the transformation into mature neurons after spinal cord injury.

This study selected the GSE 189070 dataset for analysis primarily because it provides rich and high-quality single-cell data from a mouse model of spinal cord injury, which is highly relevant to our research questions. Although relying on a single dataset may introduce certain limitations, we have performed rigorous preprocessing and standardization of the data to ensure the reliability of our conclusions. Furthermore, we plan to use other publicly available datasets in future studies for cross-validation to enhance the generalizability of our findings.

To extract pure astrocytes as comprehensively as possible, different from using only a few marker genes to define and extract them, this study strictly adheres to SingleR's selection and definitions from relevant literature. This results in a relatively small number of astrocytes extracted from the database, limiting the analyses and calculation methods that can be applied. However, it's crucial to note that this limitation does not impact the results. Additionally, since the study assumes that certain subtypes of astrocytes can undergo transformation into neurons, the distribution of these two cell types was defined as the starting point in the pseudotime analysis. Specifically, the side with a higher distribution of astrocytes and associated GO functions related to neuronal remodeling, developmental growth regulation, and cell proliferation regulation was chosen as the starting point for analysis, aligning with the study's focus.

To explore the role of glycolysis in cell transformation and reprogramming, the analysis of the glycolysis section primarily relies on the definition through differential genes and the functional annotation of co-expressed genes. Further refinement and validation of these findings will be pursued in future research. The experimental part of this study is relatively limited, and in the future, we aim to enhance our understanding of the relationship between astrocytes and neurons during SCI through more refined experimental techniques, such as cell sorting, confocal observations, and gene knockout experiments.

In conclusion, this study investigates the potential of astrocytes to transdifferentiate into neurons after spinal cord injury, with a focus on the possible existence of intermediate transition states between certain subpopulations. Existing studies have primarily concentrated on the transdifferentiation of astrocytes but rarely considered the differing roles of glial subpopulations in this process. By systematically analyzing the mechanisms underlying astrocyte-to-neuron transdifferentiation after spinal cord injury, we reveal dynamic transitions between subpopulations, further elucidating the transdifferentiation potential of astrocytes under specific conditions. Notably, the overlap in marker gene expression between astrocyte subpopulations and neurons in our study provides new cell-level evidence for understanding the mechanisms of transdifferentiation. Additionally, through cell communication and gene co-expression analyses, we explored how these cells interact through various pathways, uncovering the potential of astrocytes in spinal cord injury repair. This extends beyond their fundamental roles in cell proliferation and neural remodeling to investigate their capacity to transform into mature neurons. These findings offer novel insights for future research into neural regeneration and spinal cord injury treatment.

Author contributions

Zhi Tang, Hengyang Lu, Xiao Yang: Methodology, Writing – original draft, Writing – review & editing, Project administration. **Mao Wu, Junfeng Yang**: Investigation, Resources, Writing – original draft. **Shaoshuo Li, Heng Liu, Junkang Zhou, Bin Tang, Xinyao Du**: Visualization. **Fei Xu, Yang Shao, Jianwei Wang**: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Project administration.

Ethics approval

This study protocol was approved by the Animal Ethics Committee of Wuxi City Hospital of Traditional Chinese Medicine (Ethics No. SZYYKJFZJH2020111810).

Animal experimentation compliance

This study adhered to the ARRIVE guidelines and was conducted in accordance with the Animals (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63 for the protection of animals used for scientific purposes, and the NIH (National Research Council) Guide for the Care and Use of Laboratory Animals.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2025.101917.

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