

Enhanced migration of engrafted retinal progenitor cells into the host retina via disruption of glial barriers

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Purpose: Migration and integration remain critical challenges for stem cell replacement therapy. Glial barriers play an important role in preventing cell migration and integration. The purpose of this study was to investigate the effect and mechanisms of chondroitinase ABC on the migration of murine retinal progenitor cells (mRPCs) transplanted into the subretinal space of B6 mice.

Methods: mRPCs were harvested from the neural retinas of P1 enhanced green fluorescent protein (GFP) B6 mice. Two μ l containing 2×10^5 expanded RPCs alone or combined with chondroitinase ABC in suspension were injected into the subretinal space of the recipient B6 mice. Immunohistochemistry was performed on the recipient B6 retinas to evaluate the glial barrier formation and migration of the mRPCs. Western blotting was also used to check the expression of the glial barriers.

Results: Glial fibrillary acidic protein (GFAP) and vimentin could be seen around the transplanted mRPCs in the B6 mice. Formation of glial barriers prevented the migration of donor cells into the retinal layers. Chondroitinase ABC promoted the migration and survival rates of the engrafted retinal progenitor cells in the retinal layers of recipient B6 mice. Injection induced upregulation of GFAP, chondroitin, and CD44 expression. Chondroitinase ABC disrupted the glial barriers. The CD44 around the mRPCs was much lower in the chondroitinase group. However, the CD44 in the retinal layers was considerably higher in the chondroitinase group. With the employment of chondroitinase ABC, more cells migrated into the outer nuclear layer or inner nuclear layer. The chondroitin and CD44 expression decreased 3 weeks after transplantation in the chondroitinase ABC group.

Conclusions: Chondroitinase ABC degraded glial barriers and enhanced the migration of transplanted mouse retinal progenitor cells. Chondroitinase ABC may also have induced activation of the CD44 signaling pathway to exert the effect.

Age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are the most common retinal degeneration diseases in the world, and they are characterized by the loss of photoreceptor cells [1]. Since photoreceptor cells cannot be regenerated, stem cell replacement therapy is the most promising method to cure these diseases. Previously, most studies focused on the transplantation of retinal sheets [2], neural progenitor cells [3], retinal progenitor cells (RPCs) [4,5], postmitotic photoreceptor precursors [6], human embryonic stem (ES) cells [7], and induced pluripotent stem cells (iPSCs) [8] in the retinal degeneration mouse model. New agents for transplantation, such as three-dimensional retinal tissues with functional photoreceptors, have also been generated from iPSCs [9]. Recent studies have shown that stem cell transplantation can restore some visual function [5-7,10]. However, such low integration remains a big challenge for

stem cell-based therapy [6]. Injection can induce glial activation, which causes donor cell death and prevents grafted cells from migrating into the target position. In addition, activated microglia and Müller cells would produce extracellular matrix (ECM) components, such as chondroitin sulfate proteoglycans (CSPGs), which have been shown to prevent axon extension in the brain [11]. In addition, CSPGs may limit the migration and integration of Müller stem cells [11]. Chondroitinase ABC has been reported to improve axonal regeneration or functional recovery in spinal cord injury by degradation of the CSPGs [12-15] and enhance synaptogenesis between transplanted stem cells and host neurons in the retinal degeneration mouse model [16]. Our previous study showed that CSPGs could be activated and upregulated in the outer nuclear layer and in the vicinity of the donor cells after subretinal injection, which played a vital role in hindering the migration and integration of donor progenitor cells. Comparably, the CSPG expression around mRPCs was much lower in the chondroitinase ABC group. This suggests that chondroitinase ABC has a potential effect on degrading CSPGs [17]. However, the mechanisms of chondroitinase

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TABLE 1. THE mRPCs TRANSPLANTED WITH CHONDROITINASE HAD HIGHER POWER TO MIGRATE.

Migration and survival rates	Control	Chabc	P value (n=9)
Migration (um)	620.56±159.49um	1795.56±414.76um	<0.05
survival	25.56±15.63	99.22±23.51	<0.05

More mRPCs survived in the retinal layers.

ABC on transplanted RPCs were not fully investigated. As reported, CD44 can regulate its downstream effectors and coordinate downstream intracellular signaling pathways that exert multiple cellular functions, such as cell migration and differentiation [18,19]. Here, we transplanted mRPCs with chondroitinase ABC into B6 mice to check the migration of the donor cells. At the same time, we explored the expression of glial barriers and CD44 to evaluate possible mechanisms.

METHODS

Animals: All experiments were treated in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and all experimental protocols were approved by the Animal Care and Use Committee of the Schepens Eye Research Institute and Zhongshan Ophthalmic Center. Green fluorescent protein positive (GFP+) C57BL/6J (B6) mice and C57BL/6J mice (Jackson Laboratory) were housed under a 12h:12h light-dark cycle with water and food provided ad libitum.

Cell isolation and culture: mRPCs harvested from the neural retinas of P1 GFP+ B6 mice were isolated and maintained in neurobasal medium, as previously described [5]. Briefly, mouse neural retina layers were dissected free from the retinal pigment epithelium and vitreous body. Then, the tissue was minced and dissociated enzymatically (0.1% collagenase in Hanks' Balanced Salt solution-HBSS, Sigma, St. Louis, MO) at room temperature. Isolated cells were collected through a 100 um mesh strainer, centrifuged, and then resuspended in the neurobasal culture medium. The medium was supplemented with epidermal growth factor (EGF, 20 ng/ml), L-glutamine (2 mM, Sigma), nystatin (2,000 U, Sigma), penicillin/streptomycin (100 ug/ml, Sigma), and 2% B-27 supplement and N-2 supplement (Invitrogen, Carlsbad, CA). Subsequently, RPCs were fed by 50% medium exchange every 3 days and passaged at confluence.

Subretinal transplantation: B6 mice (between 4 and 8 weeks of age) were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (20 mg/kg), followed by the dilation of pupils with topical tropicamide (all from Phoenix Pharmaceutical Inc., St. Joseph, MO) to

view the fundus. By transpupillary direct observation using a binocular surgical microscope, we injected 2 ul of HBSS containing RPCs (approximately 200,000 cells) transsclerally into the subretinal space of the B6 mouse eye, as has been described [5]. All injections were performed using a beveled glass micropipette (internal diameter, 150 um) connected to a 20 ul syringe (Hamilton, Reno, NV) by polyethylene tubing. mRPCs were transplanted alone or together with chondroitinase ABC (0.01 U/ul, Sigma) into the subretinal space of the recipient B6 mice.

Tissue preparation and histology: The B6 mice were euthanized at 1 or 3 weeks after mRPC transplantation. The eyeballs were fixed in 4% paraformaldehyde, cryoprotected in 10% and 30% sucrose in 0.1% phosphate buffer, and sectioned at 8 um on a cryostat. Tissue sections were immunostained for GFAP (1:250, Chemicon, Temecula, CA), vimentin (1:250, Abcam, Cambridge, MA), chondroitin (1:100, Chemicon), and CD44 (1:500, Abcam), followed by reaction with Cy3-conjugated or Cy5-conjugated secondary antibody. Sections were examined with either conventional immunofluorescence microscopy or confocal microscopy.

Migration and survival evaluation: To assess the migration of the engrafted cells, we measured the farthest horizontal distance from which the mRPCs migrated from the injection site. To quantify the live RPCs, we counted the mRPCs in the retinal layers with normal form, excluding the donor cells in the subretinal space and other cell debris. GFP-positive cells were calculated within one random microscopy field at 40× (n = 9).

Immunoblotting: For western blot analyses, retinas from the control eyes or transplanted eyes were homogenized in lysis buffer (50 mM Tris HCl, pH 7.6, 150 mM NaCl, 10 mM CaCl₂, 1% Triton X-100, 0.02% NaN₃) and centrifuged; the supernatants were isolated, and protein concentrations determined using a bicinchoninic acid (BCA) protein assay (Pierce Chemicals, Rockford, IL). Equivalent amounts of protein (30 mg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 7% acrylamide), transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with the following antibodies: chondroitin (1:1,000, Chemicon), GFAP (1:1,000, Chemicon),

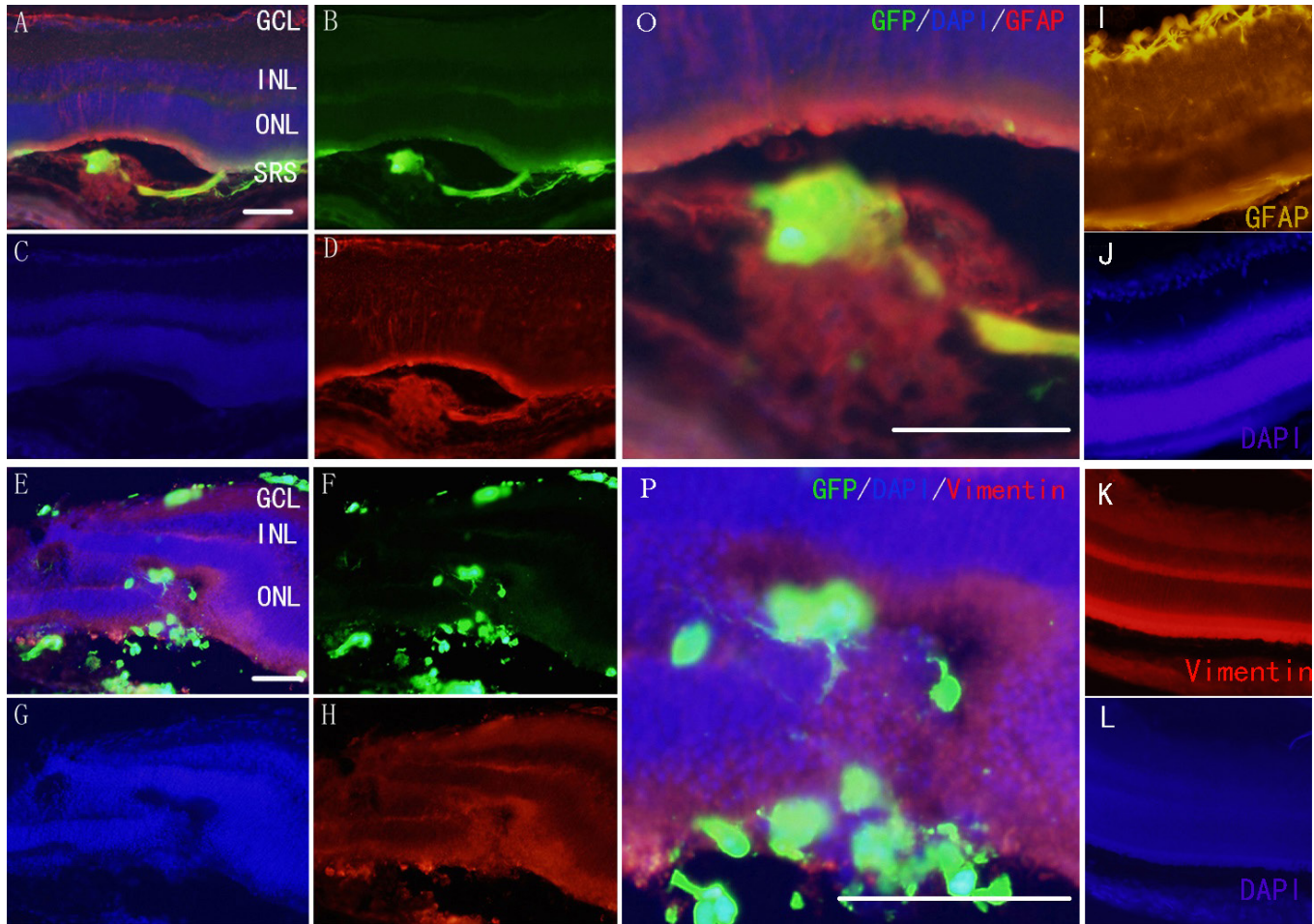


Figure 1. Injection induces glial barriers in the subretinal space 1 week after transplantation of mRPCs into the B6 mice. GFAP and vimentin expressions were seen around the donor cells and the outer nuclear layer. **A**: Overlay, **B**: GFP, **C**: 4,6-diamino-2-phenyl indole (DAPI), **D**: GFAP, **O**: high magnification of **A**. **E**: Overlay, **F**: GFP, **G**: DAPI, **H**: vimentin, **P**: high magnification of **E**. Control image: **I**: GFAP, **J**: DAPI, **K**: vimentin, **L**: DAPI. Scale bar: 50 μ m (**D**, **H**). Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; SRS, subretinal space.

CD44 (1:500, Millipore), and b-actin (1:5,000, Abcam). Blots were cut and reprobbed sequentially, visualized with electrochemiluminescence (ECL) reagents (NEN, Boston, MA), and exposed to X-ray film (Kodak/Carestream Health, Bio Max Light Film, Rochester, NY). Developed films were subsequently digitized and metrically analyzed for density using ImageJ software (NIH image; each substrate was normalized against b-actin). Digital images of the western blots were used to make composite figures with Adobe Photoshop graphics software (Adobe Corp., Mountain View, CA).

Statistical analysis: Each experiment was repeated three times. Data were represented as mean \pm standard deviation (SD) if appropriate. Statistical analysis of data was performed using SPSS 15.0 software; significance of difference was examined with the Mann–Whitney U test, one-way

analysis of variance (ANOVA) with Tukey testing for post hoc comparisons, or a standard Student *t* test. A *p*-value < 0.05 was considered significant.

RESULTS

To confirm the role of chondroitinase ABC in the migration and survival of donor cells, we transplanted the mRPCs alone or combined with chondroitinase ABC into the B6 mice. The concentrations of the cells and chondroitinase ABC were used as reported above. As shown in Table 1, the cells had a higher ability to migrate and survive in the retinal layers in the chondroitinase ABC group. More mRPCs survived in the outer or inner retinal layers. Chondroitinase ABC greatly enhanced the migration and survival rates of transplanted mRPCs.

Glial scarring is known to be an obstacle to the migration of donor cells. To elucidate whether transplantation caused the formation of glial barriers, we checked the GFAP and vimentin expression around the subretinal area. GFAP and vimentin are normally expressed in the retinal layers, which can be demonstrated by our control group (Figure 1I,K). One week after transplantation, the cells resided in the subretinal space, which was surrounded by GFAP+ glial barriers (Figure 1A,O). Similarly, vimentin expression was seen around the donor cells near the injection site area (Figure 1E,P). Surrounded by glial barriers, the transplanted cells could hardly migrate into the retinal layers. Most were located in the subretinal space or vitreous cavity. In contrast, GFAP expression around the donor cells was incomplete in the chondroitinase ABC group (Figure 2B). Vimentin expression could hardly be seen in the whole retinal layers and subretinal space (Figure 2F). Without intact glial barrier interference, more mRPCs migrated into the retinal layers (Figure 2A,E). The donor cells, which migrated into the inner nuclear layer, appeared like bipolar cells (Figure 2J,L).

In our previous study, CSPGs could be upregulated in the outer nuclear layer and in the vicinity of the donor cells after subretinal transplantation, which restrained the donor cells from migrating to the retinal layers. Chondroitinase ABC could digest the CSPGs and induce migration [17]. CD44 is a type I transmembrane CSPG. To confirm the effect of chondroitinase ABC on CD44, we also examined the CD44 expression around the transplanted cells. As shown in Figure 3B, expression of CD44 was much higher in the mRPC transplantation alone group. This means that subretinal transplantation induced the upregulation of CSPGs. Thus, the cells lost the ability to migrate. In the chondroitinase ABC-treated group, CD44 in the subretinal area was not visible (Figure 3F). More cells migrated into the retinal layers when CD44 was degraded by chondroitinase ABC (Figure 3H). However, the expression of CD44 in the retinal layers seemed to be upregulated by chondroitinase ABC treatment (Figure 3H). This may suggest the activation of the hyaluronan (HA)/CD44 signaling pathway.

We also checked GFAP, chondroitin, and CD44 expression of the recipient mouse retina after transplantation. As

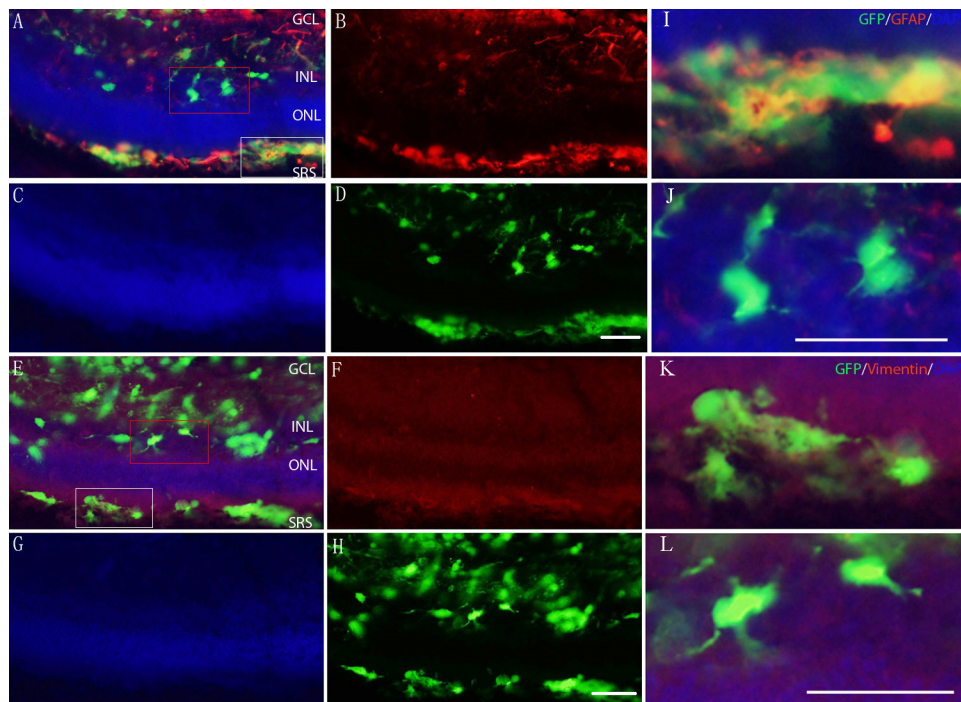


Figure 2. Chondroitinase ABC disrupted the glia barrier 1 week after transplantation of mRPCs combined with chondroitinase ABC into the B6 mice. GFAP and vimentin expression in the subretinal area was degraded by chondroitinase. No GFAP or vimentin expression was seen around the cells that migrated into the retinal layers. **A**: Overlay, **B**: GFAP, **C**: DAPI, **D**: GFP, **E**: high magnification of the white boxed area in **A** in the subretinal area, **F**: high magnification of the red boxed area in **A** in the inner nuclear area. **E**: Overlay, **F**: vimentin, **G**: DAPI, **H**: GFP, **I**: high magnification of the white boxed area in **E** in the subretinal area, **L**: High magnification of the red boxed area in **E** in the inner nuclear area. Scale bar: 50 μ m (**A**, **E**). Abbreviations: GCL, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; SRS, subretinal space.

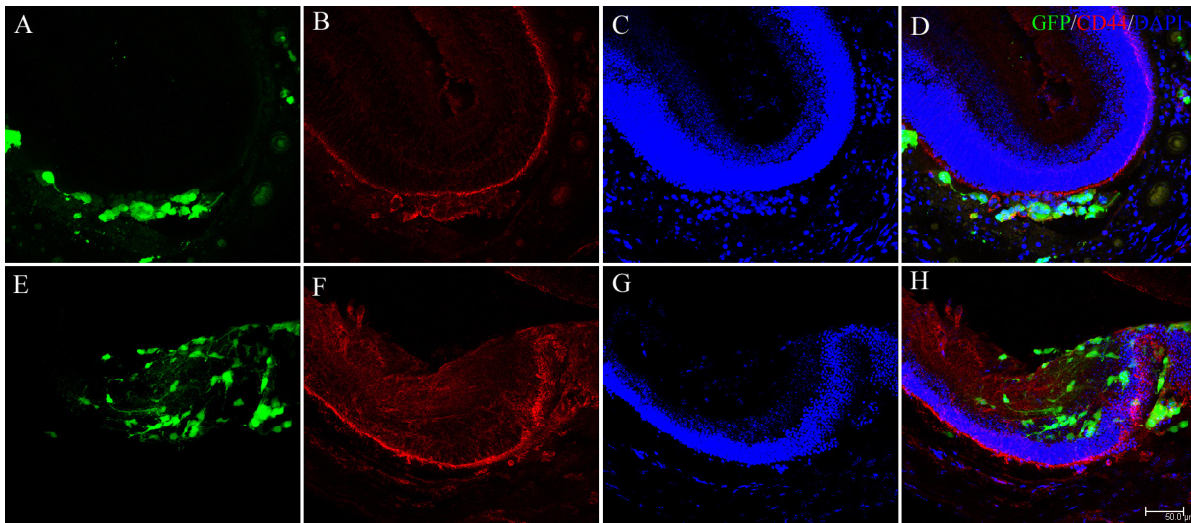


Figure 3. Expression of CD44 at 3 weeks after transplantation. CD44 expression was seen around the transplanted cells in the subretinal space. Higher expression of CD44 was seen in the retinal layers in the group of murine retinal progenitor cells (mRPCs) transplanted with chondroitinase ABC than in the mRPC group. **A:** GFP, **B:** CD44, **C:** DAPI, **D:** Overlay. A-D are from the group transplanted with mRPCs only. **E:** GFP, **F:** CD44, **G:** DAPI, **H:** Overlay. E-H are from the group transplanted with mRPCs combined with chondroitinase ABC. Scale bar: 50 μ m (D, H). Abbreviations: GCL, retinal ganglion cell layer; ONL, outer nuclear layer; SRS, subretinal space.

shown in Figure 4, 1 week after transplantation, the expressions of chondroitin, GFAP, and CD44 were much higher in the transplantation groups compared with the control group ($*p < 0.05$). There was no statistically significant difference in the expression of chondroitin, GFAP, and CD44 between the mRPC group and chondroitinase ABC-treated group 1 week after injection. However, 3 weeks after transplantation, expressions of chondroitin and CD44 were lower in the chondroitinase ABC group ($**p < 0.05$).

DISCUSSION

RP and AMD are the leading causes of retinal degeneration blindness. Almost 100,000 persons suffer from RP in the United States, and they usually lose vision in childhood or young adulthood [20]. Among older people, AMD is the main cause of blindness in the United States and Europe. It will affect 288 million people worldwide by 2040 [21]. Although RP and AMD have different causes and demographics, in both diseases, the patient will eventually experience central visual loss in the end stage because of photoreceptor cell death. Cell replacement therapy can be used for sight restoration by transplanting stem cells into the subretinal space. Ideally, stem cell-based replacement therapy would be promising even in the late stages of retinal degeneration. Recent studies have shown that photoreceptors or human ES cell-derived retinal tissue engrafted into mouse or primate models of degenerative retinas could survive, construct host-graft synaptic connections, and even restore some

visual function [10,22]. However, only 3% of the injected cells survived 12 weeks after transplantation if more than 200,000 cells were transplanted into the subretinal space; even fewer cells migrated into the ideal location and formed synaptic connections with host neurons.

There are several limitations of stem cells transplanted into the degenerated retina to migrate and integrate with the host. CSPGs and microglia constitute glial barriers that restrict stem cell migration following transplantation [11]. Brain injury would cause a reactive response to induce the formation of scar tissue and upregulation of vimentin. The effect of GFAP in central nervous system (CNS) injury involves suppressing neuronal proliferation and neurite extension in the mature brain, producing a physical barrier to isolate damaged tissue [23]. It is almost the same in the retina. Our study showed that subretinal injection caused glial barriers at the injection site, which was created by the host Müller cells. GFAP and vimentin expression was seen clearly around the mRPCs in the subretinal space. Most mRPCs were scattered near the injection area and could not migrate into the retinal layers. When chondroitinase ABC was injected with mRPCs, the glial barriers caused by Müller cells vanished. More cells migrated into the inner or outer retinal layers and extended their axons. This finding is in accordance with a previous study [24].

CD44 is a type I transmembrane CSPG. It is composed of a large extracellular domain containing a HA-binding region and four consensus sites for attachment of chondroitin

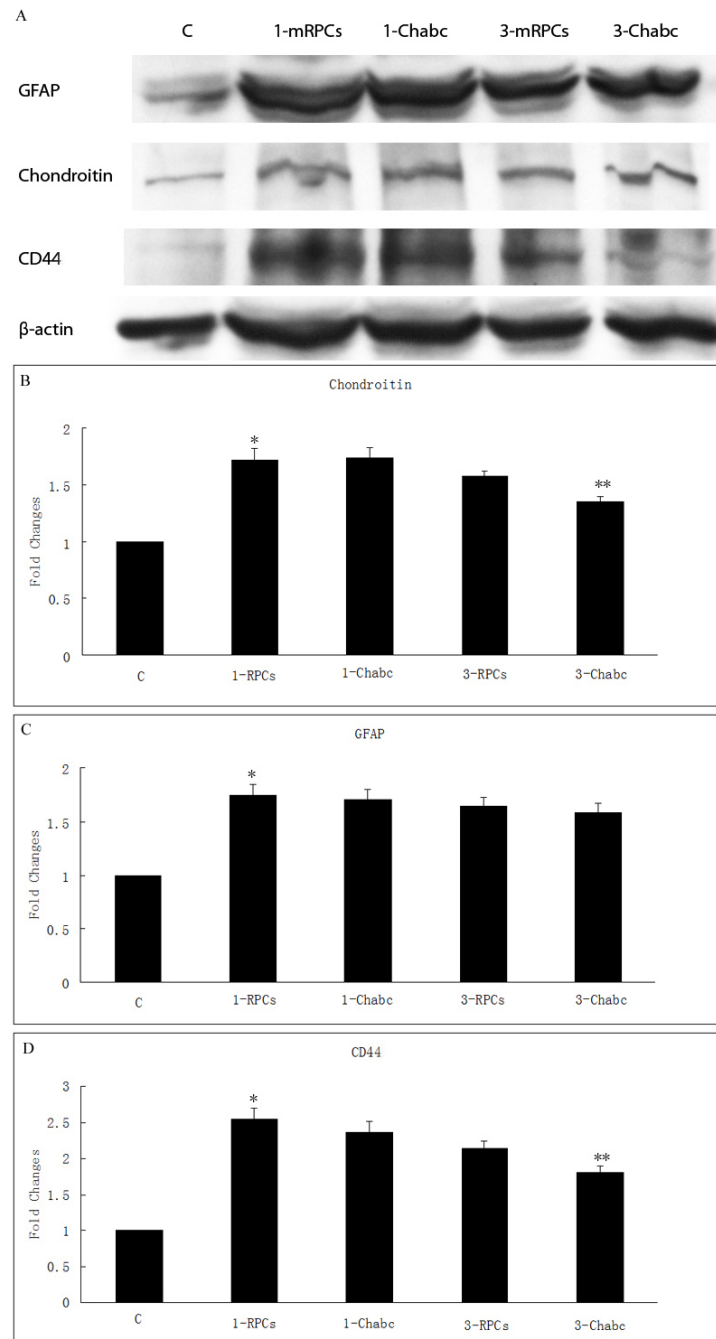


Figure 4. Western blot analyses for glial barriers protein expression after transplantation. Representative western blots and corresponding densitometric analyses of GFAP, chondroitin and CD44 (n=5). Injection induced chondroitin, CD44 and GFAP up-regulation (*P<0.05). The expression of chondroitin and CD44 was lower in the chondroitinase-treated group compared to mRPCs alone transplanted group 3 weeks after transplantation (**P<0.05). Data were represented as mean ± SD. Error bars: SD. P<0.05 was considered significant. Abbreviations: C: control, 1-RPCs: 1w post-transplantation of mRPCs, 1-Chabc: 1w posttransplantation of mRPCs and chondroitinase ABC, 3-RPCs: 3w post-transplantation of mRPCs, 3-Chabc: 3w post-transplantation of mRPCs and chondroitinase ABC.

sulfate and other domains [25]. CD44 can be altered by differential splicing of at least 10 variable exons encoding a segment of the extracellular domain and by cell type-specific glycosylation [25]. CD44 has been shown to mediate cell–cell and cell–ECM interactions [26]. It has also been reported to promote tumor cell motility on HA-coated substrates [27,28], costimulate lymphocyte activation and tissue infiltration [29], and enhance the growth and metastasis of some tumor types [30,31]. HA-CD44 interaction was correlated to tumor cell migration and invasion via microRNA signaling and Rho GTPase activation [18]. However, the interaction is quite complicated because CD44 plays a vital role in cell migration. On the one hand, CD44 prevents the movement of the transplanted cells as one kind of CSPG. On the other, HA/CD44 promotes the migration of the injected cells by activating several signaling pathways. Our results showed that mRPC transplantation induced glial barriers, such as GFAP, chondroitin, and CD44, by western blot. There was not much difference between the mRPCs and chondroitinase ABC groups 1 week after transplantation. Three weeks after transplantation, CD44 and chondroitin expression was lower in the chondroitinase ABC group. This signified that the chondroitinase degraded the chondroitin and CD44 to promote migration. However, it was determined by immunohistochemistry that chondroitinase ABC upregulated CD44 expression in the retinal layers of recipient mice. In contrast, the expression of CD44 in the mRPC transplantation group alone was considerably lower in the mRPC and retinal layers. This result implies that chondroitinase induced the HA/CD44 signal pathway to enhance the migration. Chondroitinase ABC definitely reinforced the migration of mRPCs in the B6 mice. It may activate the HA/CD44 signal pathway via microRNA signaling and RhoGTPase activation. These mechanisms warrant further investigation.

In conclusion, chondroitinase ABC facilitated the migration of mRPCs engrafted into B6 mice via disruption of the glial barriers. Chondroitinase ABC may also induce activation of the HA/CD44 signaling pathway to exert the effect. CD44 may play a dual role in this process.

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