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A mechanism of impaired mobility of oligodendrocyte progenitor cells by tenascin C through modification of wnt signaling ☆

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Abstract In demyelinating diseases, the mechanisms of how oligodendrocyte (OLG) progenitor cells are affected in the demyelinated area remain to be elucidated. To investigate one aspect of the mechanisms, we focused on the role of tenascin C in regulating the migratory mobility of the progenitor cells via β-catenin. By cDNA subtraction screening, we found tenascin C expression to be increased in OLG progenitors (rat primary O2A cells). Tenascin C inhibited the migration of OLG progenitors and CG-4 cells, and β-catenin accumulated at focal adhesions in these cells. These changes were associated with the inactivation of canonical wnt signaling. Overexpression of the wnt-signaling antagonist Dapper prevented the migration of CG-4 cells. This suggests that inactivation of the wnt signal is responsible for impaired migration of OLG caused by tenascin C. Our results suggest that tenascin C is involved in the impaired mobility of OLG progenitor cells through increased amounts of adhesion complex as well as the prevention of wnt signaling.

Keywords: Oligodendrocyte progenitor cell; Tennascin C; wnt; β-Catenin

1. Introduction

Autoimmune-mediated demyelination is a major aspect of the pathogenesis of demyelinating diseases, including multiple sclerosis (MS). A well-known animal model of human MS is experimental autoimmune encephalomyelitis (EAE), which is induced in rodents by the injection of myelin components [1]. In the complicated pathogenesis of EAE or MS, oligodendrocytes (OLGs) are believed to be an initial target for the immune cells. We previously demonstrated that the OLG-specific expression of p35, a baculovirus-derived anti-apoptotic factor, remarkably inhibits the progression of EAE [2,3]. These findings suggest that OLG plays some role in the chronic phase of pathophysiology of EAE as well as the injury of neuronal axons does.

During development, OLG progenitor cells initially proliferate specifically in the ventral neuroepithelial portion of the neural tube, and from there they migrate and differentiate into mature cells that produce myelination-related proteins [4,5]. Therefore, the process of OLG progenitor cells migration might be crucial, not only for development but also for pathophysiological situations. Tenascins are produced by OLG progenitor cells and also by astrocytes, specifically as extracellular matrix proteins with roles in adhesion [6], differentiation [7], and migration [8,9]. However, it remains to be investigated whether and how tenascin C is involved in pathological conditions from the view point of OLG progenitor migration.

In this study, cDNA subtraction screening showed increased gene expression of tenascin C in OLG progenitors compared with mature OLGs. We then investigated how tenascin C affects the migration of OLGs, and found that it stimulated the accumulation of β-catenin in the cytoplasmic membrane, but not in the nucleus, of OLG progenitor cells, in the cultured cell line CG-4 and in the spinal cords of mice with EAE lesions. Tenascin C inhibited the migration of OLG progenitors in vitro, suggesting that an accumulation of tenascin C might play a crucial role in preventing migration of OLG progenitor cells.

2. Materials and methods

2.1. Rat primary OLG culture

As previously reported [10], the brains of rat embryos at ED16 were digested in 0.03% tyrosine–0.03 mM EDTA at 37 °C for 5 min, and inoculated into an 80 cm^2 flask containing DME medium with 20% FBS. After OLG progenitor cells (O2A cells) were detected in the intercellular space between astrocytes, they were separated by manually shaking the flask. The detached O2A cells were collected and cultured in DMEM containing 2% FBS and Bottestein and Sato's supplement on 100 μg/ml poly-L-lysine-coated dishes [11]. O2A cells, when inoculated in an aggregated form, started to migrate from the aggregate leading to a remarkable distribution throughout the dish. The purity of the primary cultured OLGs was more than 92%, as estimated by immunohistochemistry using mouse monoclonal antibodies to CNPase (1:500; Sigma, Chemical Co., Saint Louis, MO, USA) and A2B5 (1:200; Chemicon International, Temecula, CA, USA).

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Abbreviations: EAE, experimental autoimmune encephalomyelitis; OLG, oligodendrocyte

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2.2. cDNA subtraction assay for detecting genes that are more highly expressed in O2A cells than mature differentiated oligodendrocytes

The cDNA subtraction assay was performed using the CLONTEC SMART™ PCR cDNA Synthesis Kit and PCR-Select™cDNA Subtraction Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) [12]. One μg of the total RNA from O2A cells or mature differentiated OLGs was reverse-transcribed using an oligo dT primer (cDNA synthesis primer) possessing a specific DNA element that is suitable for further PCR amplification. The amplified PCR products were divided into three groups. Two samples of O2A cDNA (testers) were ligated with different adapters such that each contains a specific PCR primer annealing site. A sample of reference (or driver) cDNA was then hybridized with the testers to subtract the many genes expressed at a similar level between the tester and driver to obtain cDNAs rich in the tester. PCR using primers possessing the specific DNA elements that were found in the adapters ligated to the testers specifically amplified these cDNAs. These PCR products were subcloned and sequenced. The subtraction was then evaluated again by RT-PCR using primers targeting the sequenced genes.

2.3. The CG-4 cell line of rat OLG progenitors

As previously reported, CG-4 cells were cultured on poly-L-ornithine coated dishes in growth medium containing 70% DMEM, 30% conditioned medium from B104 cells, 2% FBS, N1 supplement including insulin, transferrin, selenite, and progesterone, and biotin [13]. For differentiation, CG-4 cells were cultured in DMEM containing N1 supplement but neither FBS nor B104-conditioned medium. After inducing the differentiation of CG-4 cells, each differentiated CG-4 efficiently loses the cell–cell contact, and E-cadherin in the adherent junction between each cell was hardly detected.

2.4. Transfection of CG-4 cells and luciferase assay

To introduce genes into CG-4 cells, we used a cationic reagent, Effectene™ (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The plasmids transfected in this study contained a wnt signal antagonist Xenopus Dapper (kindly given by Dr. R.T. Moon, University of Washington, Seattle, USA) [14], a luciferase reporter gene for wnt signaling, i.e., TOPFLASH (Upstate Biotechnology, Lake Placid, NY, USA), and a GFP gene. The cells which were cultured on tenascin C-coated or non-coated dishes were harvested 48 h after transfection for Western blot analysis or luciferase activity assay. One hundred μL of the cell lysates was combined with 20 μL of luciferase assay buffer (Promega, Madison, WI, USA). Luciferase activity was measured by luminometer with correction of transfection efficiency among the wells.

2.5. Immunocytochemical study using the CG-4 cell line

CG-4 cells were fixed and treated with 0.1% Triton X. After blocking, the cells were incubated with a polyclonal antibody against phosphorylated FAK (1:100; Upstate Biotechnology, Lake Placid, NY, USA), a rabbit anti-GFP antibody (1:500; MBL, Nagoya, Japan) or a monoclonal antibody against β-catenin (1:300; BD PharMingen, San Diego, CA, USA). Then, they were followed by reaction with a FITC-conjugated anti-rabbit IgG antibody or a Cy 3-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA).

2.6. Migration assay for primary cultured oligodendrocytes or CG-4 cells

After co-transfection of CG-4 cells with the genes for Dapper and GFP by Effectene™, they were collected and the aggregates were prepared by gentle suspension. They were transferred to the center of culture dishes coated by BSA (100 μg/ml) or tenascin C (100 μg/ml), on which the scale bar was present so that the distance of migration of CG-4 from the center could be easily calculated. Within 24 h since the transfer, the migratory distance of at least 10 GFP-positive cells from the center of the aggregate was evaluated and compared with each other. At least four aggregates from each were used for the quantification.

2.7. Western blot analysis

The samples from rat primary OLG progenitor cells or CG-4 cells were fractionated by SDS–PAGE and transferred onto membranes (Millipore Corp., Bedford, MA, USA). The membranes were incubated with a monoclonal β-catenin antibody (1:500), or a monoclonal β-tubulin antibody (1:2000). After the reaction with HRP-conjugated secondary antibodies, the signal was detected using an enhanced chemiluminescence system (ECL Plus, Amersham, Buckinghamshire, UK).

3. Results

3.1. Identification of the tenascin C gene by its enhanced expression in OLG progenitor cells

To identify genes that are enriched in OLG progenitors, we performed subtractive cDNA screening using rat OLG progenitor cells, i.e., O2A. The cDNAs of rat O2A cells were subtracted by those of mature OLGs. Tenascin C was found to be among the genes that were more highly expressed in rat O2A. The level of gene expression of tenascin C was more elevated in rat O2A than that in mature OLG, evaluated by RT-PCR (Fig. 1).

3.2. Inhibitory effect of tenascin C on the migration of rat primary oligodendrocytes and rat OLG progenitor cell line CG-4

Considering the molecular mechanisms of how tenascin C affects the function of OLG progenitor cells, we focussed on migration of the cells and evaluated if there was a direct effect of tenascin C on the migration of OLG progenitors (rat O2A) by using tenascin C-coated dishes. O2A showed an initial adhesion in an aggregated form, followed by remarkable migration away from aggregates after inoculation. The presence of 100 μg/ml of tenascin C impaired the migration of O2A cells from aggregates in this system (Fig. 2A), compared to BSA; however, adhesion to a tenascin C-coated dish was conserved. The O2A cells cultured on BSA, which was surrounded by tenascin C, could not move across a margin of tenascin C (dotted line). On the other hand, O2A cells on the non-tenascin C-coated part of the dish migrated normally from the aggregates.

To further evaluate the inhibitory effect of tenascin C on migration, the cell migration assay was also performed using the rat OLG cell line, CG-4. Tenascin C had the same effect on CG-4 cell migration as on primary cultured O2A cells (Fig. 2B). Compared with the control, the migration of CG-4 cells from aggregates was inhibited by tenascin C. However, CG-4 cells formed aggregates on a tenascin C-coated dish and further dispersion of CG-4 was inhibited by 100 μg/ml tenascin C, indicating impaired migration.

3.3. Mechanism responsible for tenascin C-mediated inhibition of cell migration

To further investigate the molecular mechanisms underlying the inhibition of OLG migration, we tested whether the wnt signal is affected by tenascin C. Western blot analysis dem-

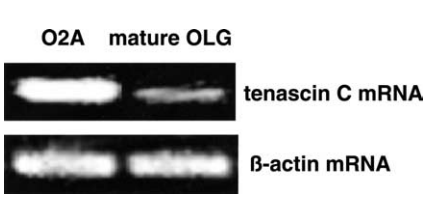

Fig. 1. Gene expression of tenascin C in rat O2A cells. Expression of tenascin C mRNA in rat O2A was more increased than that in mature OLG.

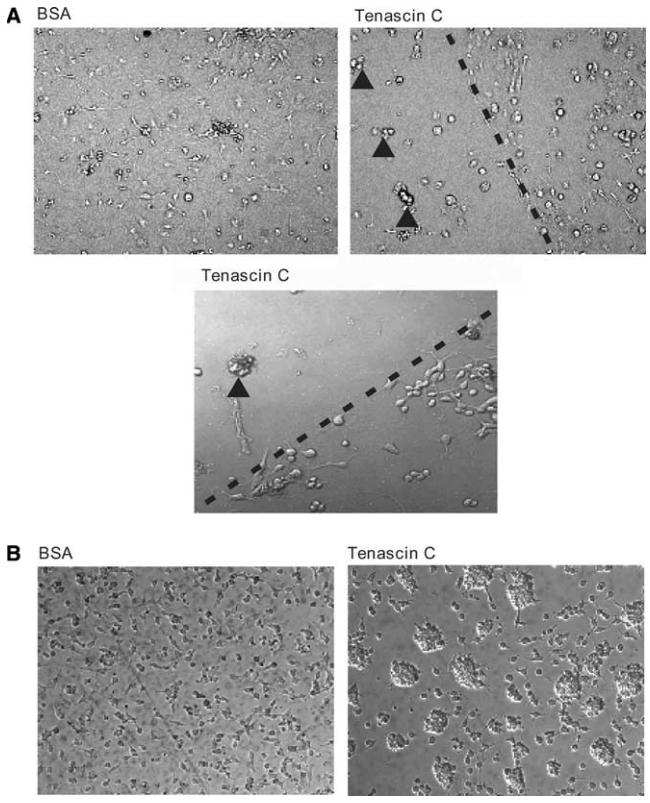

Fig. 2. Effect of tenascin C on cell migration of primary cultured rat OLGs. Primary OLGs did not migrate well on tenascin C (100 μg/ml)-coated dishes; they tended to form aggregates in comparison with OLGs plated on dishes coated with 100 μg/ml of BSA (non-tenascin C)-coated dish (A). The dotted line shows the margin of tenascin C. Left of the dotted line (tenascin C-coated area), OLGs did not migrate from aggregates (arrowhead). In contrast, OLGs on the right side (non-tenascin C but BSA coated area) did not migrate across the dotted line. Likewise, CG-4 cells did not migrate efficiently on tenascin C (100 μg/ml)-coated dishes and formed aggregates (B). Bar, 20 μm.

onstrated that 3 h after the inoculation of CG-4 cells, β-catenin expression was increased by tenascin C (Fig. 3A). To determine whether the increased β-catenin was responsible for the activation of the wnt signal and whether the localization of β-catenin was altered, we performed immunocytochemistry. Interestingly, as shown in Fig. 3B, the β-catenin in CG-4 cells that were cultured on a tenascin C-coated dish was markedly localized to focal adhesions which were identified by immunostaining of phosphorylated FAK, compared with CG-4 cells cultured on a non-tenascin C-coated dish. Furthermore, the β-catenin in the cytoplasmic membrane fraction of CG-4 lysates was greatly increased by tenascin C, whereas in contrast, its nuclear localization was attenuated by tenascin C (Fig. 3C). These results suggest that tenascin C facilitates the accumulation of β-catenin at the focal adhesions causing inactivation of the wnt signal.

These findings prompted us to speculate that the accumulation of β-catenin at the focal adhesions was responsible for the inactivation of the wnt signal. To test this possibility, we performed a luciferase activity assay using TOPFLASH, which is transactivated by β-catenin located in the nucleus (Fig. 3D). The luciferase activity was decreased by tenascin C-coating, consistent with our finding that more β-catenin was located at the focal adhesions. This suggested that tenascin C decreased the wnt signal in CG-4 cells. Next, to study the involvement of the tenascin C-induced inhibition of wnt signaling in decreasing the CG-4 cell migration, we expressed a wnt signaling antagonist, Dapper, which inhibits the β-catenin-mediated transcriptional activation of wnt in CG-4 cells (Fig. 3E). The cells transfected with GFP alone migrated efficiently away from the aggregates within 30 h after inoculation. In contrast, CG-4 cells co-transfected with GFP and Dapper did not migrate markedly from the aggregate. Furthermore, immunocytochemistry demonstrated that Dapper caused them to make more contact with the dish, compared with control CG-4 cells, which made few contacts at focal adhesions (Fig. 3F). In

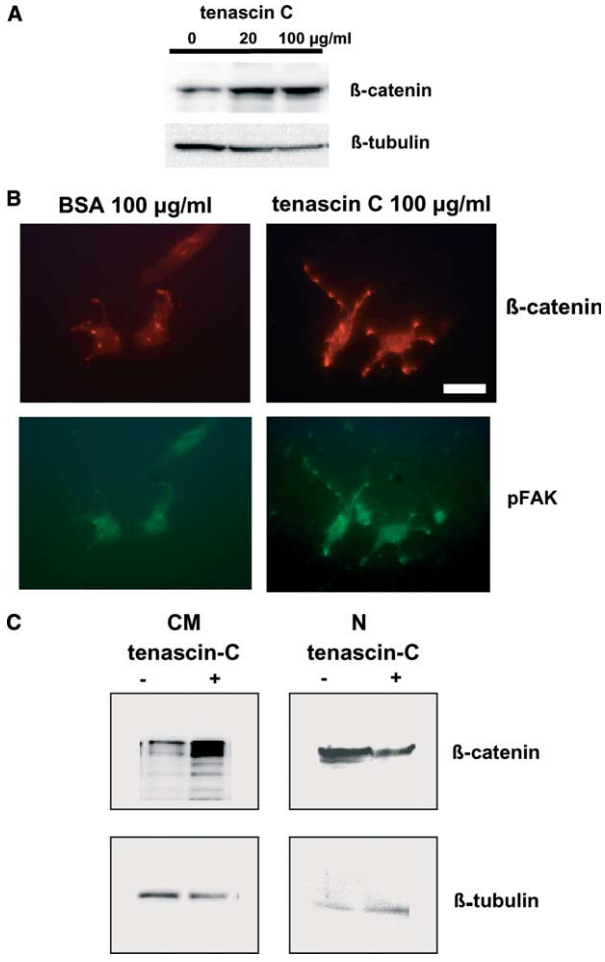

Fig. 3. Tenascin C-induced translocation of β-catenin to focal adhesions. β-Catenin (upper bands) expression was greatly increased by tenascin C as compared with β-tubulin (lower bands) (A). By tenascin C, more β-catenin was localized to focal adhesions demonstrated by immunostaining of phosphorylated FAK (pFAK). Bar, 10 μm (B). Tenascin C (100 μg/ml) increased the amount of membrane-localized β-catenin with a decrease in the accumulation of nuclear β-catenin (C). CM: cytoplasmic membrane fraction; N: nuclear fraction. Tenascin C (100 μg/ml), coated on culture dishes, attenuated luciferase activity that was transactivated by the nuclear translocation of β-catenin, as evaluated by TOPFLASH (mean ± S.E.) ($P < 0.01$ vs control) (D). An antagonist of wnt signaling, Dapper, affected the migration of CG-4 cells. CG-4 cells transfected with GFP alone showed efficient migration compared with cells transfected with GFP and Dapper 30 h after inoculation (arrowhead), as also shown by quantitative analysis of the fold increase in migration distance (mean ± S.E.) ($P < 0.01$ vs control) (E). Dapper also modulated the translocation of β-catenin more to the focal adhesions, compared with the β-catenin in cells transfected with GFP alone. Bar, 20 μm (F).

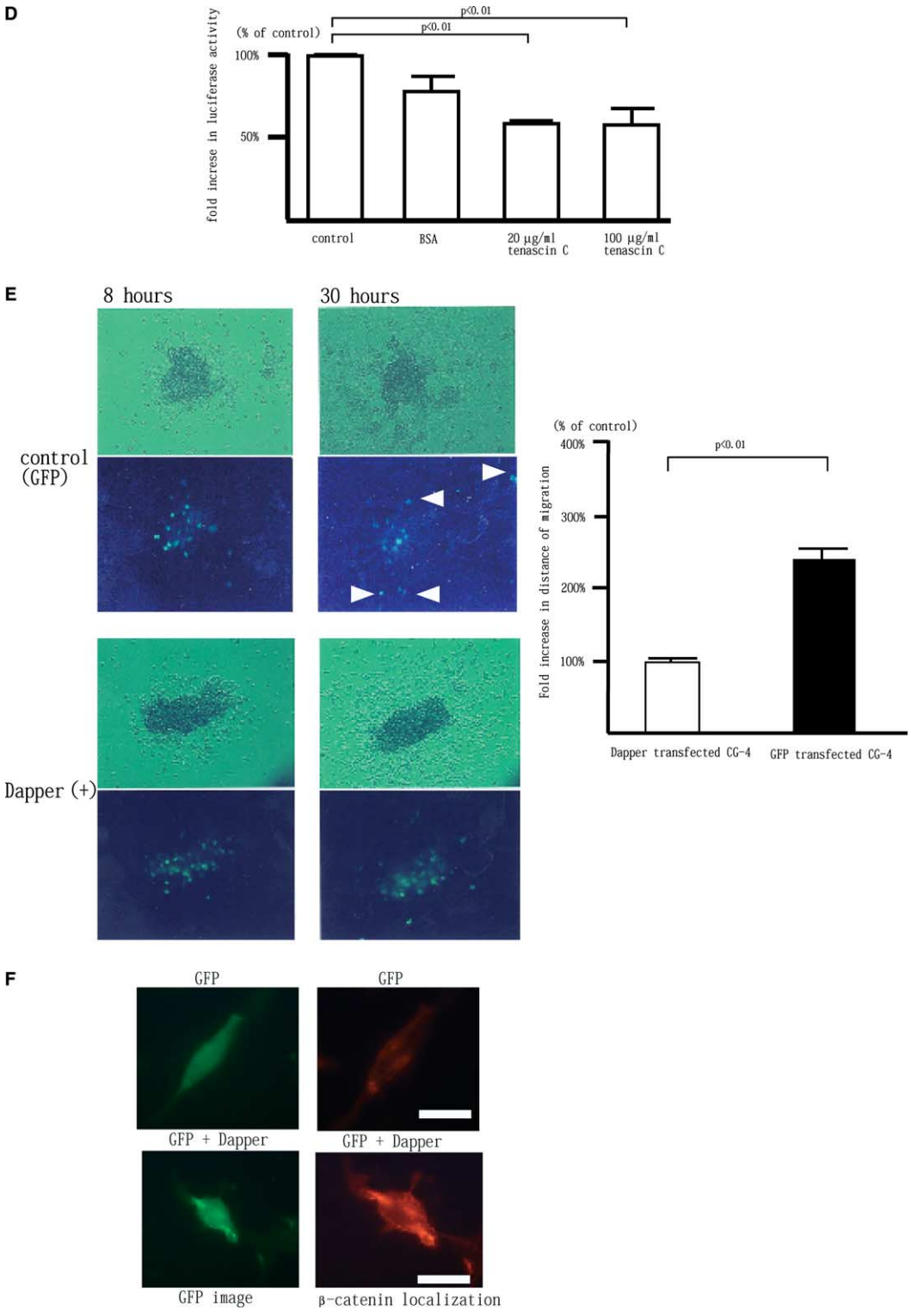

Fig. 3 (*continued*)

Dapper-transfected CG-4 cells, β-catenin was expressed exclusively in the focal adhesions, in comparison with control CG-4 cells with less β-catenin localized to the focal adhesions. These results suggest that tenascin C inhibits CG-4 cell migration by causing β-catenin to accumulate at the focal adhesions, rather than the nucleus, resulting in the inhibition of wnt signaling.

4. Discussion

In the present study, we found that one of the key molecules involved in the poor mobility of OLG progenitor cells is tenascin C. This molecule inhibits the migration of OLG progenitor cells by causing β-catenin to accumulate at the focal adhesions, thus inhibiting wnt signaling. Thus, our study elu-

cidates a novel aspect of tenascin C responsible for activation of wnt signaling.

To elucidate the effect of tenascin C on OLG progenitor cells, we performed migration assays using CG-4 and O2A cells. In both these cell types, tenascin C interfered with cell migration, suggesting that it can inhibit OLG progenitor migration. This inhibitory effect of tenascin C on the migration of OLG progenitor cells was also verified by tenascin C-knockout mice; our in vitro data are consistent with the previous in vivo data evaluating the migratory activity of OLG precursors in tenascin C-knockout mice [15]. These tenascin C-deficient mice conclusively demonstrated that tenascin C has an inhibitory effect on the migration of OLG progenitor cells.

As demonstrated in this study, this inhibition of the migration by tenascin C was accompanied by a translocation of β-catenin. To further investigate the molecular mechanisms by which tenascin C transduces an inhibitory effect on migration, we focused on the wnt signal in OLGs [16]. It is well accepted that during development, the wnt signal is involved in cell mobility; however, the role of wnt signaling in pathological situations remains to be studied [17–19]. In the present study, tenascin C is known to enhance FAK phosphorylation, which is accompanied by an elevation of β-catenin expression. β-Catenin is known to co-localize with cadherin in cell-cell adhesion. A very restricted localization of β-catenin to the cytoplasmic membrane with cadherins, and not to the nucleus, is known to make cells less mobile [20–24]. On the other hand, a more nuclear than cytoplasmic membrane localization of β-catenin, a condition associated with more disrupted focal adhesions, tends to make cells migrate [25–27]. Our preliminary study demonstrated that differentiated CG-4 cells lose their contact between each cell and E-cadherin immunoreactivity is not clearly detected. The present study demonstrated that tenascin C recruits the β-catenin to the focal adhesion resulting in the impairment of translocation of β-catenin to the nucleus, because the transcriptional activity of β-catenin was shown to be decreased by tenascin C, which was consistent with the translocation of β-catenin to focal adhesions. This finding suggests that blockage of the wnt signal by tenascin C is responsible for the poor migration. Furthermore, in some tumor cell lines including melanocytes, elevated β-catenin signaling in the nucleus causes extensive migration and is responsible for increased metastasis [27] To investigate whether the wnt signal is directly involved in OLG progenitor migration, we used Dapper, an antagonist to wnt signaling [14]. Dapper binds to wnt signaling molecules including Dishevelled, Axillin, and GSK-β leading to the antagonization of β-catenin-mediated nuclear transcriptional activity [14]. In our study, Dapper clearly enhanced the translocation of β-catenin to the focal adhesions and inhibited cell migration, suggesting that tenascin C inhibits cell migration by blocking wnt signaling.

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