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Expression of Dbn1 during mouse brain development and neural stem cell differentiation

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ABSTRACT

Dbn1 is a newly discovered gene in the drebrin gene family of mice. Previous studies have reported that Dbn1 is specifically expressed in the mouse brain suggesting its potential role in brain development. However, a detailed analysis of Dbn1 expression during mouse brain development has not been demonstrated. Here, we describe the expression pattern of Dbn1 and the coexpression of Dbn1 and actin during the development of the mouse brain from embryonic day 14 (E14) to adulthood and during the differentiation of neural stem cells (NSCs), as determined using immunohistochemistry, double-labeling immunofluorescence, and quantitative real-time polymerase chain reaction. During mouse brain development, Dbn1 expression level was high at E14, attenuated postnatally, reached its highest point at postnatal day 7 (P7), and showed a very low level at adulthood. Imaging data showed that Dbn1 was mainly expressed in the hippocampus, ventricular zone, and cortex, where NSCs are densely distributed, and that the intracellular distribution of Dbn1 was predominantly located in the cytoplasm edges and neurites. Moreover, the signal for colocalization of Dbn1 with actin was intense at E14, P0, and P7, but it was weak at adulthood. During NSC differentiation, Dbn1 mRNA expression increased after the onset of differentiation and reached its highest point at 3 days, followed by a decrease in expression. The imaging data showed that Dbn1 was increasingly expressed in the extending neurites in accordance with the cell morphological changes that occur during differentiation. Furthermore, obvious colocalization signals of Dbn1 with actin were found in the neurites and dendritic spines. Collectively, these results suggest that Dbn1 may play a key role in mouse brain development and may regulate NSC differentiation by filamentous actin.

1. Introduction

Developmentally regulated brain protein (drebrin) is a major filamentous (F)-actin-side-binding protein in the brain and is located in dendritic spines in the central nervous system (CNS) [1–5]. Drebrin forms a complex with F-actin, myosin, and gelsolin in vivo [3]. Previous studies have demonstrated that drebrin not only inhibits the actin-binding activity of F-actin-stabilizing proteins such as α-actinin and tropomyosin, but also suppresses actomyosin interactions [3,6,7]. In addition to F-actin, profilin, an actin monomer-binding protein that stimulates actin polymerization, has been shown to interact directly with drebrin [8]. These results indicate that drebrin is involved in the actin cytoskeleton dynamics in the CNS.

It has been reported that the actin cytoskeleton regulates dendritic spine morphogenesis during neuronal development [9–11]. Moreover, transfection experiments have shown that overexpression of drebrin remodels straight actin bundles into thick and winding bundles in fibroblasts, resulting in elongated dendritic spine lengths in cortical neurons [12–14]. On the contrary, down-regulation of drebrin expression in cultured hippocampal neurons significantly decreases the density and width of filopodia spines [15]. Thus, drebrin is considered to play an important role in the regulation of dendritic spine architecture.

Dbn1 is a novel mouse drebrin gene and is a newly discovered gene in the drebrin gene family, which has been mapped to the central portion of chromosome 13. Dbn1 is specifically expressed in the mouse brain and regulates the actin cytoskeleton [16]. In

Abbreviations: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; Cx, cortex; CNS, central nervous system; DAB, 3,3N-diaminobenzidine tetrahydrochloride; Drebrin, developmentally regulated brain protein; EGF, epidermal growth factor; F-actin, filamentous actin; FITC, fluorescein isothiocyanate; Hi, hippocampus; HRP, horseradish peroxidase; IgG, immunoglobulin G; NSC, neural stem cell; PBS, phosphate buffered saline; PFA, paraformaldehyde; qRT-PCR, quantitative real-time polymerase chain reaction; RT, room temperature; TRITC, tetramethylrhodamine-5-(and 6)-isothiocyanate; VZ, ventricular zone.

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our previous work, we investigated gene expression during CNS development. We found that 1033 genes were hierarchically clustered, and seven genes that are facilitatory to axons were detected in the developmental period, including *Dbn1* [17]. Based on the role drebrin plays in the regulation of development and synaptic plasticity, we hypothesized that Dbn1 contributes to neuronal differentiation and mouse brain development.

In this study, we used Dbn1 immunohistochemistry/immunocytochemistry, double-labeling immunofluorescence, and quantitative real-time polymerase chain reaction (qRT-PCR) to analyze the expression pattern of Dbn1 in mouse brain development and in the differentiation of neural stem cells (NSCs). In order to assess the possibility of Dbn1 binding to F-actin during brain development and NSC differentiation, coexpression of Dbn1 and actin was examined.

2. Materials and methods

2.1. Animals and tissue treatment

C57BL/6 mice were obtained from the Laboratory Animal Center of the Third Military Medical University (Chongqing, China) and were bred in standard conditions. Pups, timed-pregnant mice, and adult mice were killed via decapitation or cervical dislocation. The midpoint of the day a vaginal plug was discovered was designated embryonic day 0.5 (E0.5), the day of birth was designated postnatal day 0 (P0), and the sixtieth day after birth was designated adulthood. The mouse brains were treated for immunostaining as described previously [18].

2.2. NSC culture

NSCs were isolated from E14 mouse telencephalon and cultured in Dulbecco's modified Eagle medium/F12 medium with stemPro Neural Supplement (2%), epidermal growth factor (EGF; 20 ng/mL), basic fibroblast growth factor (bFGF)-2 (20 ng/mL), and GlutaMax-I Supplement (2 mM) for proliferation. For differentiation, NSCs were exposed to Neurobasal media with B27 Serum-Free Supplement (2%) and GlutaMax-I Supplement (2 mM). All of these reagents were purchased from GIBCO (Invitrogen, Carlsbad, CA, USA).

2.3. Antibodies

The following antibodies, all which were obtained from commercial sources, were used in this study: mouse anti-Dbn1 (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-actin (Santa Cruz Biotechnology, Dallas, TX, USA), fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG; Jackson Immunoresearch, West Grove, PA, USA), tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC)-conjugated goat anti-mouse lgG (Jackson Immunoresearch, West Grove, PA, USA), and horseradish peroxidase (HRP)-conjugated goat anti-mouse lgG (SinoBio, Shanghai, China) antibody.

2.4. Immunocytochemistry

Immunocytochemistry was performed as described previously [18]. Briefly, mouse NSCs were seeded at 1×10^5 cells/mL in 4-well chamber slides. At each test time point after the onset of differentiation, NSCs were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT), washed in phosphate buffered saline (PBS; pH 7.4), and permeabilized and blocked in normal blocking buffer (PBS, 0.2% bovine serum albumin [BSA] and 0.1% Triton X-100) for 30 min at RT. For staining Dbn1, NSCs were stained with the primary antibody mouse anti-Dbn1 1:500 in normal blocking buffer for 30 min at 37 °C and then overnight at 4 °C. Cells were washed in PBS and incubated with the secondary antibody TRITC-conjugated goat anti-mouse lgG 1:50 in normal blocking buffer for 30 min at 37 °C and then at RT. After 2 h, slides were washed in PBS and embedded with 75% glycerin. Slides were photographed using an Olympus BX51 microscope equipped for detecting immunofluorescence (Olympus, Tokyo, Japan).

2.5. Immunohistochemistry

Immunohistochemistry was performed as described previously [18,19]. In the immunofluorescence assays, sections were washed in PBS (pH 7.4) and then incubated in normal blocking buffer (PBS, 0.2% BSA, and 0.1% Triton X-100) for 30 min at RT. For staining, the sections were incubated with mouse anti-Dbn1 1:500 in normal blocking buffer for 30 min at 37 °C and then overnight at 4 °C. The next day, sections were washed in PBS and incubated with the secondary antibody TRITC-conjugated goat anti-mouse lgG 1:50 in normal blocking buffer for 30 min at 37 °C and then at RT. After 2 h, sections were washed in PBS and embedded with 75% glycerin. In the immunoperoxidase assays, sections were washed in PBS (pH 7.4) and then incubated with 3% hydrogen peroxide at RT for 15 min to inactivate the endogenous peroxidases, and then washed again in PBS. The subsequent steps were the same as the procedures for the immunofluorescence assays, but the 1:50 diluted HRP-conjugated goat anti-mouse lgG was applied as the secondary antibody for 2 h at 37 °C. After washing in PBS, immunoreactivity on the sections was visualized using a DAB HRP color development kit (Beyotime, Beijing, China) according to the manufacturer's instructions. All of the slides were photographed using an Olympus BX51 microscope equipped for detecting immunofluorescence.

2.6. Double-labeling immunofluorescence

For immunostaining, the mouse brain sections were washed in PBS (pH 7.4) and then incubated in normal blocking buffer (PBS, 0.2% BSA, and 0.1% Triton X-100) for 30 min at RT. Then, the sections were incubated with the primary antibodies mouse anti-Dbn1 1:500 and rabbit anti-actin 1:500 in normal blocking buffer for 30 min at 37 °C and then overnight at 4 °C. The next day, sections were washed in PBS and incubated with the secondary antibody TRITC-conjugated goat anti-mouse lgG 1:50 in normal blocking buffer for 30 min at 37 °C and then for 2 h at RT. Subsequently, sections were washed in PBS and incubated with another secondary antibody FITC-conjugated goat anti-rabbit IgG 1:50 in normal blocking buffer for 30 min at 37 °C and then at RT. After 2 h, sections were washed in PBS and embedded with 75% glycerin. Slides were photographed using an Olympus BX51 microscope equipped for detecting immunofluorescence.

For immunostaining, mouse NSCs were seeded at 1×10^5 cells/mL in 4-well chamber slides. At each test time point after the onset of differentiation, NSCs were fixed with 4% PFA for 15 min at RT, washed in PBS (pH 7.4), and permeabilized and blocked in normal blocking buffer (PBS, 0.2% BSA, and 0.1% Triton X-100) for 30 min at RT. Then, cells were incubated with the primary antibodies mouse anti-Dbn1 1:500 and rabbit anti-actin 1:500 in normal blocking buffer for 30 min at 37 °C and then overnight at 4 °C. The next day, slides were washed in PBS and incubated with the secondary antibody TRITC-conjugated goat anti-mouse lgG 1:50 in normal blocking buffer for 30 min at 37 °C and then for 2 h at RT. Subsequently, slides were washed in PBS and incubated with another secondary antibody FITC-conjugated goat anti-rabbit IgG 1:50 in normal blocking buffer for 30 min at 37 °C and then at RT. After 2 h, slides were washed in PBS and embedded with 75% glycerin. Slides were photographed using an Olympus BX51 microscope equipped for detecting immunofluorescence.

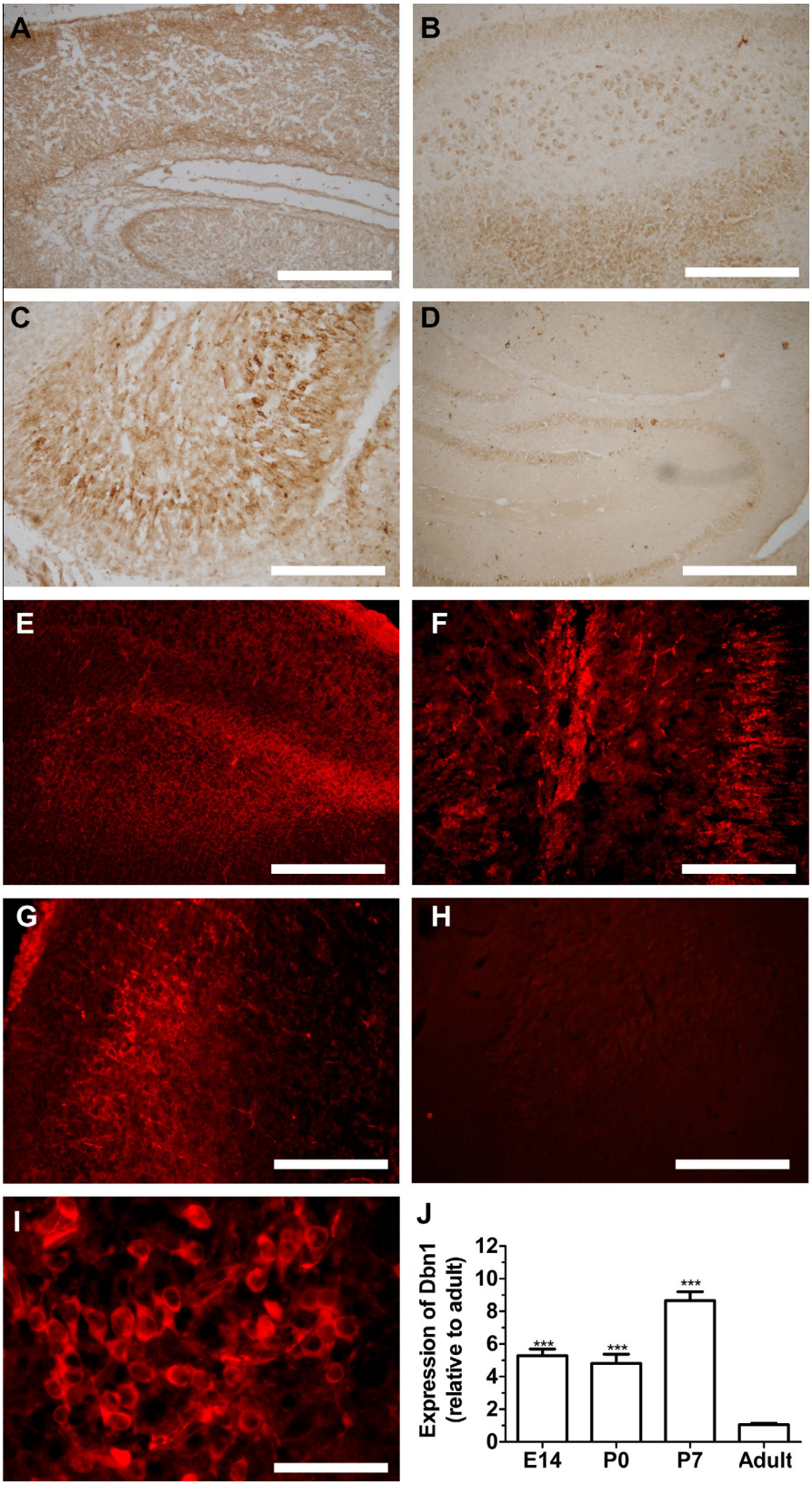

Fig. 1. Immunohistochemistry and quantitative real-time polymerase chain reaction (qRT-PCR) assays of Dbn1 during mouse brain development. (A)–(D) Immunoperoxidase assay of Dbn1. (A) Embryonic day 14 (E14). (B) Postnatal day 0 (P0). (C) P7. (D) Adulthood. (E)–(I) Immunofluorescence assay of Dbn1. (E) E14. (F) P0. (G) P7. (H) Adulthood. (I) P7. (J) qRT-PCR assay of Dbn1. Scale bars indicate 250 μm. Error bars denote ± the standard deviation of the mean; $^{***}p < 0.001$.

2.7. qRT-PCR

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) following the manufacturer's instructions. Real-time PCR was performed with appropriate mRNA primers purchased from Roche (Basel, Switzerland). Conditions for real-time PCR were 94 °C for 10 min, 45 cycles of 94 °C for 10 s, 55 °C for 30 s, 72 °C for 1 s, and 40 °C for 30 s. The results were analyzed using the comparative threshold cycle method. Melt curve analyses were performed to check for specific primer sets. All of the data were normalized against the level of actin mRNA.

2.8. Statistical analysis

All values are expressed as the mean ± the standard deviation. Means were compared with an unpaired Student's two-tailed *t*-test and values were considered significant if $^{**}p < 0.01$, or $^{***}p < 0.001$.

3. Results

3.1. The expression pattern of Dbn1 during mouse brain development

To examine the expression pattern of Dbn1 during mouse brain development, immunohistochemistry, immunoperoxidase, and immunofluorescence assays were performed. Dbn1 expression was initially detected at embryonic day E14, and the images showed that the expression of Dbn1 was prominently found in the cortex (Cx), ventricular zone (VZ), and hippocampus (Hi) (Fig. 1A and E). At P0, marked Dbn1 expression was observed in the VZ (Fig. 1B and F). At P7, strong immunoreactive cells were observed in the Cx (Fig. 1C and G). At adulthood, the Hi regions were only slightly stained by the immunoperoxidase assay (Fig. 1D). However, a few immunoreactive cells were detected by immunofluorescence (Fig. 1H). At high magnification, Dbn1-positive signals were mainly found in the cytoplasm edges and neurites (Fig. 1I).

To demonstrate the expression levels of Dbn1 during mouse brain development, we used qRT-PCR. Dbn1 was sequentially

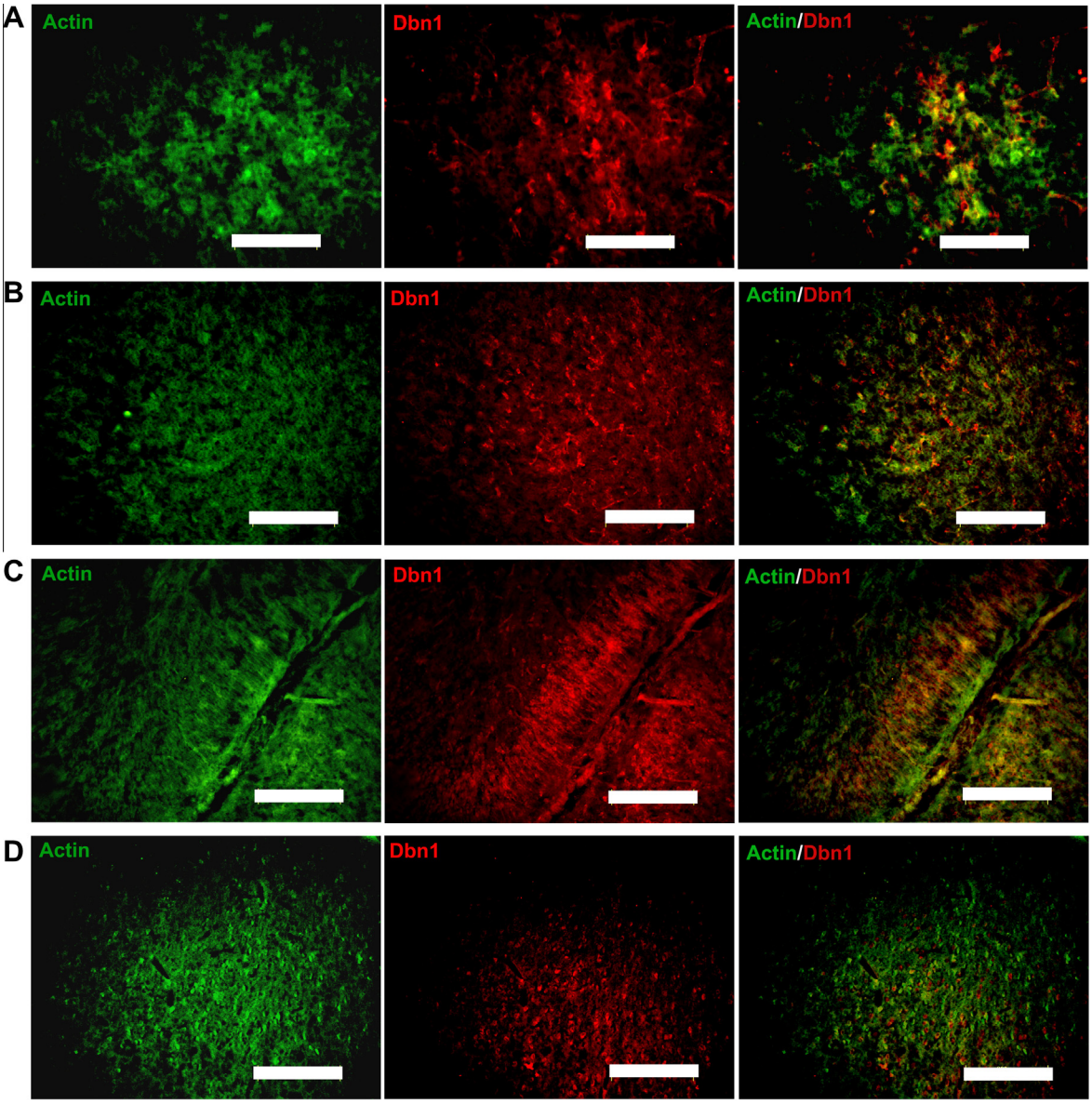

Fig. 2. Double-labeling immunofluorescence assay of Dbn1 in mouse brain development. (A) Embryonic day 14 (E14). (B) Postnatal day 0 (P0). (C) P7. (D) Adulthood. Scale bars indicate 200 μm.

expressed in the four stages of mouse brain development. However, its expression level was significantly different depending on the developmental stage. The expression level was high at E14, was attenuated at P0, reached its highest point at P7, and finally showed a very low level at adulthood (Fig. 1J). Generally, the expression levels at E14, P0, and P7 were higher than the levels at adulthood. These results are consistent with the image data.

The expression pattern of Dbn1 during the different mouse brain developmental stages was scored. The results revealed that Dbn1 was mainly expressed in the Cx, VZ, or Hi regions of the embryonic and neonatal mouse brain, while the intracellular distribution of Dbn1 was predominantly located in the cytoplasm edges and neurites, suggesting that Dbn1 was involved in mouse brain development.

3.2. The coexpression of Dbn1 and actin during mouse brain development

Previous studies have demonstrated that drebrin could change neuronal morphology by regulating the polymerization of cytoskeletal proteins, F-actin [13,20,21]. Therefore, we speculate that Dbn1 may also regulate F-actin in mouse brain development.

To verify this speculation, we analyzed the coexpression of Dbn1 and actin in mouse brain development by double-labeling immunofluorescence. Actin (green fluorescent) was widely expressed in various cerebral regions, and the expression of Dbn1 (red fluorescent) was similar to the results shown in Fig. 1. Furthermore, at E14, P0, and P7, the colocalization signal (yellow fluorescent) was intense (Fig. 2A–C), but was weak at adulthood (Fig. 2D). These results suggest that Dbn1 colocalizes with actin during mouse brain development, indicating that Dbn1 may participate in mouse brain development by regulating F-actin.

3.3. The expression of Dbn1 during NSC differentiation

The previous results showed that Dbn1 is prominently expressed in cerebral regions where NSCs are densely distributed [22,23]. Thus, we surmise that Dbn1 may be involved in the differentiation of NSCs.

The expression of Dbn1 at five time points during NSC differentiation, including 0 h, 12 h, 24 h, 3 days, and 7 days, were detected by immunocytochemistry. At 0 h, Dbn1 expression was weak (Fig. 3A). At 12 h and 24 h, NSCs demonstrated extended neurites where some positive signals appeared, while other positive signals were distributed around the cell (Fig. 3B and C). At 3 days, the neurites became longer where Dbn1 expression was obvious (Fig. 3D). At 7 days, when NSC differentiation was almost complete, the cells exhibited long and serried neurites where Dbn1 was mainly expressed (Fig. 3E). These results suggest that Dbn1 may be involved in the formation of neurites. Furthermore, the expression of Dbn1 mRNA at 0 h, 12 h, 24 h, 3 days, and 7 days during NSC differentiation was investigated by qRT-PCR. At 0 h, low expression of Dbn1 mRNA was detected. Dbn1 expression increased after the onset of differentiation and reached its highest point at 3 days, followed by decreased expression at 7 days (Fig. 3F). The changes in Dbn1 mRNA expression were consistent with the morphological changes in NSCs during differentiation, which demonstrates that Dbn1 may be involved in NSC differentiation.

3.4. The coexpression of Dbn1 and actin during NSC differentiation

Dbn1 colocalizes with actin during mouse brain development. Here, we analyzed the colocalization of Dbn1 with actin during NSC differentiation. Through double-labeling immunofluorescence, colocalized signals were found in the differentiated NSCs at 7 days (Fig. 4A), especially in the cytoplasm edges and neurites of

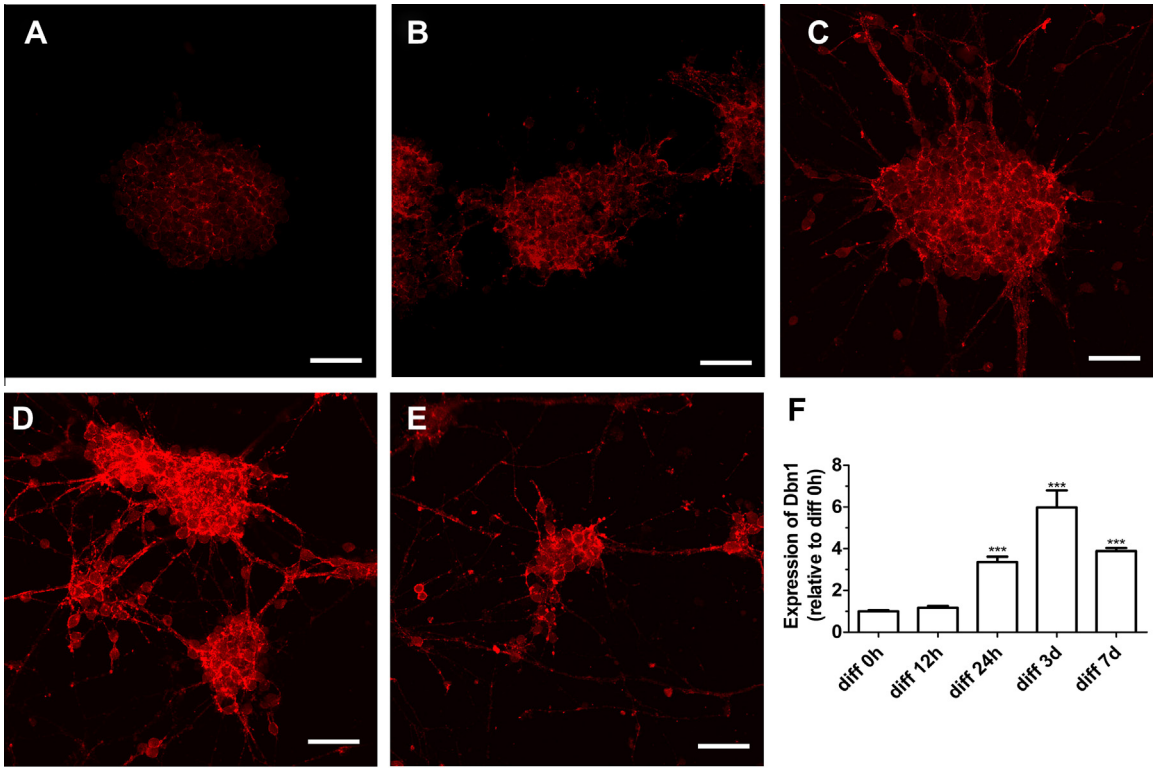

Fig. 3. Immunofluorescence and quantitative real-time polymerase chain reaction (qRT-PCR) assays of Dbn1 during neural stem cell differentiation (diff). (A)–(E) Immunofluorescence assay of Dbn1. (A) Diff at 0 h. (B) Diff at 12 h. (C) Diff at 24 h. (D) Diff at 3 days. (E) Diff at 7 days. (F) qRT-PCR assays of Dbn1. Scale bars indicate 200 μm. Error bars denote ± the standard deviation of the mean; $^{***}p < 0.001$.

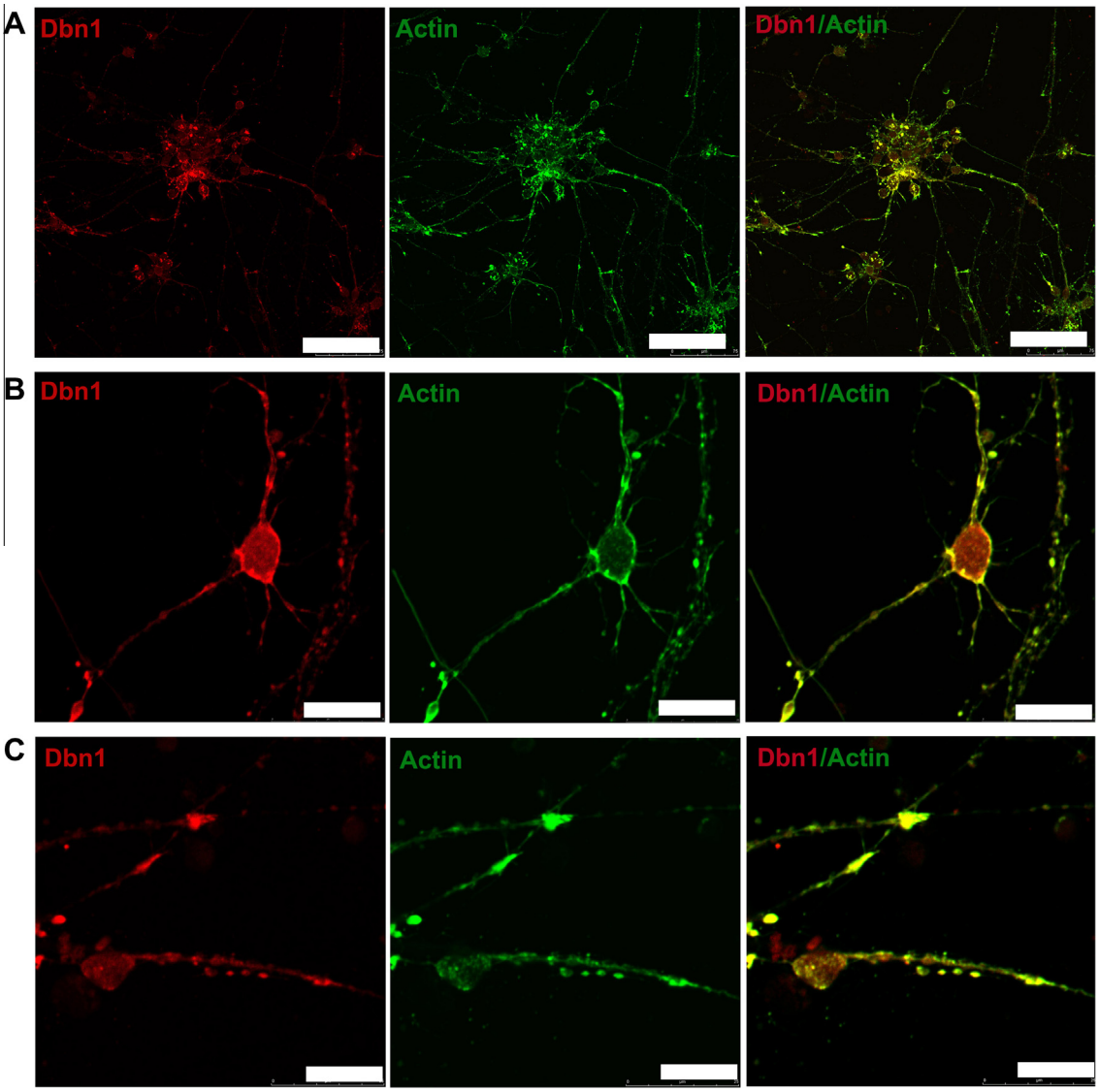

Fig. 4. Double-labeling immunofluorescence assay of Dbn1 during neural stem cell (NSC) differentiation. (A) Colocalization of Dbn1 with actin in the differentiated NSCs at 7 days. Scale bar indicates 100 μm. (B) Colocalization of Dbn1 with actin in the cytoplasm edges and neurites of neuron-like cells. Scale bar indicates 100 μm. (C) Colocalization of Dbn1 with actin in the dendritic spines of neuron-like cells. Scale bar indicates 100 μm.

neuron-like cells (Fig. 4B). Importantly, the colocalization of Dbn1 with actin appeared on the dendritic spine-like structures (Fig. 4C), which was consistent with the results of Takahashi et al. [9] showing the facilitative role of drebrin in dendritic spine morphogenesis by F-actin. These results suggest that Dbn1 may promote the formation of neurites and dendritic spines by regulating F-actin.

4. Discussion

In this study, we investigated the expression pattern of Dbn1 and the colocalization of Dbn1 with actin during mouse brain development and NSC differentiation. Our results showed that during mouse brain development, the expression of Dbn1 is high at E14, and then declines; however, Dbn1 expression increases again and reaches its highest point at P7, and significantly decreases at adulthood. In the period from E14 to P15, most neurons exit the cell cycle and gradually migrate to their appropriate terminal cerebral regions [24]. Meanwhile, a large number of neurons remodel to form a neural network, and various morphological changes occur including cytoskeletal alterations [3,25]. Our results show that Dbn1 is strongly expressed in the embryonic and neonatal brain, but the expression is decreased in adulthood. Moreover, the image data revealed that Dbn1 is mainly expressed in the Cx, VZ, and Hi, and that the intracellular distribution of Dbn1 is mostly located in the cytoplasm edges and neurites. During mouse brain development, around E14, most cells differentiate from NSCs into neuronal precursors; around the time of birth, most neurons have become orientated and are migrating, and the neural network is preliminarily established; after birth, the neural network is gradually established and matured [26–29]. In the present study, the expression pattern of Dbn1 tended to be synchronized with the differentiation of neurons and the establishment of neural networks.

F-actin is a major cytoskeletal protein, whose dynamics are the base of neuronal differentiation and migration, synapse formation, and the establishment of neural networks [30,31]. Previous studies have confirmed that drebrin interacts with F-actin to regulate neuronal differentiation [4,32,33]. Thus, we investigated the coexpression of Dbn1 and actin. The degree of colocalization of Dbn1 and actin is different depending on the developmental stage. The results of the double-labeling immunofluorescence showed that the colocalization of Dbn1 with actin is the most obvious in the

early stages of brain development, suggesting that Dbn1 may participate in the differentiation of neurons by regulating F-actin during mouse brain development.

Dbn1 is prominently expressed in the Cx, VZ, and Hi, where NSCs are densely distributed during mouse brain development [22,23]. Hence, we speculate that Dbn1 may play a role in NSC differentiation. During NSC differentiation, the mRNA expression level of Dbn1 was low at the early stages and increased along with the differentiation process. However, the expression level was reduced in the late stages. In the initial NSC differentiation and migration, both in vivo and in vitro, the main morphological change is the formation of neurites and dendritic spines [24]. In our results, NSCs extended small neurites and Dbn1 was distributed around the cell in the initial differentiation. When NSCs differentiated into long neurites, Dbn1 was expressed in neurites at a high level. These data demonstrate that the expression pattern of Dbn1 is consistent with the morphological changes of NSCs that occur during differentiation, and that Dbn1 may facilitate the formation of neurites. Regarding the coexpression of Dbn1 and actin, the results of the double-labeling immunofluorescence showed that Dbn1 colocalizes with actin, especially in the neurites and dendritic spines. This indicates that, similar to the results of mouse brain development, Dbn1 may also be involved in NSC differentiation by regulating F-actin.

In conclusion, we investigated the expression and localization of Dbn1, as well as the coexpression of Dbn1 with actin during mouse brain development and NSC differentiation. The results suggest that Dbn1 may play a key role in mouse brain development, and may regulate NSC differentiation by F-actin. Although the exact mechanisms are still unclear, our results serve as a valuable framework for further exploration of the role of Dbn1 in mouse neural development.

Acknowledgments

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