In Vivo Expression of the PTB-deleted Odin Mutant Results in Hydrocephalus

Sunjung Park, Haeryung Lee, and Soochul Park*

Odin has been implicated in the downstream signaling pathway of receptor tyrosine kinases, such as the epidermal growth factor and Eph receptors. However, the physiologically relevant function of Odin needs to be further determined. In this study, we used Odin heterozygous mice to analyze the Odin expression pattern; the targeted allele contained a β-gal gene trap vector inserted into the 14th intron of the Odin gene. Interestingly, we found that Odin was exclusively expressed in ependymal cells along the brain ventricles. In particular, Odin was highly expressed in the subcommissural organ, a small ependymal glandular tissue. However, we did not observe any morphological abnormalities in the brain ventricles or ependymal cells of Odin null-mutant mice. We also generated BAC transgenic mice that expressed the PTB-deleted Odin (dPTB) after a floxed GFP-STOP cassette was excised by tissue-specific Cre expression. Strikingly, Odin-dPTB expression played a causative role in the development of the hydrocephalic phenotype, primarily in the midbrain. In addition, Odin-dPTB expression disrupted proper development of the subcommissural organ and interfered with ependymal cell maturation in the cerebral aqueduct. Taken together, our findings strongly suggest that Odin plays a role in the differentiation of ependymal cells during early postnatal brain development.

INTRODUCTION

Odin is a member of the Anks1 adaptor protein family, which contains six ankyrin repeats (Ank), two sterile alpha motifs (SAM), and a phosphotyrosine binding domain (PTB) (Uhlik et al., 2005). Odin has been identified as one of the tyrosine phosphorylated coproteins such as spondin, which forms the single fibrous region of the third ventricle and is highly conserved throughout the vertebrate phylum (Rodriguez et al., 1998). It secretes glycoproteins into the brain ventricles (Cottrell and Ferguson, 2004). For example, the subcommissural organ (SCO) is a small ependymal gland located in the dorsocaudal region of the third ventricle and is highly conserved throughout the vertebrate phylum (Rodríguez et al., 1998). It secretes glycoproteins such as spondin, which forms the single fibrous

Keywords: ependymal cells, hydrocephalus, Odin, subcommissural organ

Received 30 October, 2014; revised 3 December, 2014; accepted 11 December, 2014; published online 15 May, 2015

Keywords: ependymal cells, hydrocephalus, Odin, subcommissural organ

http://dx.doi.org/10.14348/molcells.2015.2288
Reissner’s fiber (RF), into the CSF (Rodriguez et al., 1998). RF runs along the aqueduct, the fourth ventricle, and the central canal (Picketts, 2006; Rodriguez et al., 1998). Although the role of the SCO has not been clearly defined, its potential function is to regulate CSF flow and maintain brain homeostasis (Hu et al., 2009; Picketts, 2006). Evidence suggests that abnormal development of the SCO leads to congenital hydrocephalus (Hu et al., 2009; Ortloff et al., 2013; Perez-Figares et al., 2001; Picketts, 2006). Interestingly, the Rx or Msx gene is expressed in the SCO during postnatal brain development and their null-mutant mice have a hydrocephalic brain phenotype consistent with a size reduction or agenesia of the SCO (Baas et al., 2006; Hu et al., 2009; Ramos et al., 2004; Zhang et al., 2006).

In this study, we found that Odin is primarily expressed in ependymal cells lining the brain ventricles, such as the third ventricle and cerebral aqueduct. Ectopic expression of the PTB-deleted Odin protein in ependymal tissue was responsible for immature development of ependymal cells in the SCO and midbrain. Accordingly, a severe hydrocephalic phenotype developed in the midbrain of transgenic mice expressing Odin-dPTB. Therefore, we propose that Odin is critically involved in the development of ependymal cells during early postnatal brain development.

MATERIALS AND METHODS

Generation of BAC transgenic mice

Odin knockout mice and Odin-dPTB cDNA have been described previously (Kim et al., 2010; Shin et al., 2007). RP24-258K7, which includes homology arms A (824 bp) and B (530 bp) flanking the mouse Odin translation start site (ATG), were synthesized by polymerase chain reaction (PCR) using the following primers sets: 5'-TGCTCTTTAACCAGGAAACCATC-3' (forward primer for A arm), 5'-CCACACCCACCCCCCTCCG-GG-3' (reverse primer for A arm), 5'-GACCAGATTGCGGAATCCTC-3' (forward primer for B arm), 5'-ATGCACTCTGACAGACAT-3' (reverse primer for B arm) to generate a targeting vector for inserting floxed green fluorescent protein (GFP) plus Odin-dPTB into Odin BAC. Next, homology arms A and B were inserted into a plasmid vector described above. The resulting insert containing the A arm, floxed GFP expression cassette (with three SV40 polyadenylation sites) and the Odin-dPTB expression cassette were inserted into the vector described above. The resulting insert containing the A arm, floxed GFP, Odin-dPTB, frt-Kana-frt, and the B arm was digested out with SfII/NsiI and then recombined with Odin BAC genomic DNA using a bacterial homologous recombination method, as described previously (Kim et al., 2007). The recombinant Odin BAC was injected into fertilized C57BL/6 mouse eggs as described previously (Kim et al., 2007). Each BAC transgenic line was identified by PCR analysis of DNA extracted from tail biopsy specimens: a 200 bp PCR product was synthesized with primers 5'-GGACCATGCGGGCCTCTGCAAG-3' and 5'-AGCGTAGATCCTGGAACATCG-3'. Wnt1-Cre mice were purchased from The Jackson Laboratory (USA). All mice were generated and maintained in accordance with the institutional guidelines approved by the Sookmyung Women’s University Animal Care and Use Committee.

Histology, X-gal staining, and immunohistochemistry

Mouse brains at embryonic (E) day 18.5 or postnatal (P) day P5 were collected and fixed overnight in 4% paraformaldehyde (PFA), dehydrated in ethanol, cleared in HistoClear, and embedded in paraffin as described previously (Park et al., 2013). Sections were cut to 10 μm and collected on slides. The slides were stained in a Histoclear series in ethanol and then in an ethanol series in PBS to rehydrate the sections. Eosin staining and cresyl violet staining were performed as described previously (Park et al., 2013). Mouse brains were fixed in 4% PFA and permeated with detergent buffer containing 2% NP-40 and 1% deoxycholate acid sodium salt at room temperature with shaking, stained with X-gal for 3 days at 4°C, and then the X-gal-stained tissues were washed in 1× PBS, dehydrated in ethanol, cleared in HistoClear, and embedded in paraaffin. Sections were cut at 10 μm and collected on slides as described previously (Park et al., 2013).

Sections were rinsed in PBS, immersed in antigen unmasking solution (Vector Laboratories, USA), and boiled in a pressure cooker. After washing in cooled-PBS, the sections were blocked in 0.3% Triton X-100/2% horse serum in PBS for 1 h at room temperature (RT) and incubated with primary antibodies overnight at 4°C. The sections were washed and incubated with secondary antibodies for 2 h at RT. Then, the sections were washed in PBS and mounted in VECTASHIELD (Vector Laboratories) with DAPI (Molecular Probes, USA).

Antibodies

A monoclonal mouse anti-acetylated tubulin was purchased from Sigma-Aldrich (USA). A polyclonal rabbit antibody specific for Odin was generated in our lab. Goat anti-mouse IgG conjugated with FITC and goat anti-rabbit with TRITC were purchased from Life Technologies (USA).

RESULTS

Odin is primarily expressed in the brain ventricle-lining cells during early postnatal development

In our previous study, we reported on Odin null-mutant mice carrying a β-geo gene trap inserted into the 14th intron of the Odin gene (Kim et al., 2010). We further used X-gal staining of brain sections from heterozygous mice to analyze Odin gene expression during early postnatal brain development. LacZ activity of the β-geo gene trapped in the intron was expected to recapitulate endogenous Odin expression. Interestingly, we found that Odin was prominently expressed along the cells lining the brain ventricles, such as the third ventricle, cerebral aqueduct, and fourth ventricle (Fig. 1A). We also observed that Odin was expressed from E16.5 to P10, whereas it was barely expressed after P10 (data not shown). Importantly, the SCO, a small ependymal gland of the circumventricular system, was strongly positively stained with X-gal, suggesting that it is the main brain tissue expressing high levels of Odin. The choroid plexus, where CSF is produced, is also positively stained with Odin (Fig. 1B). Taken together, our Odin expression analysis suggests its potential role in the development of brain ependymal tissue. We further investigated whether the SCO was morphologically abnormal in Odin null-mutant mice. Immunohistochemical staining analysis revealed that the SCO of wild-type mice displayed positive staining for anti- Odin antibodies, whereas that of the null-mutant mice did not (Figs. 1C and 1D). However, we did not observe any significant morphological differences in the SCO in between wild-type and null-mutant mice. These results suggest that Odin knockout mice may be incompatible to analyze the biological function of Odin possibly because the Odin-related PTB adaptors provide a redundant function in the SCO and other ependymal tissue.

Identification of the BAC clone recapitulating endogenous Odin gene expression

The Odin gene contains 25 exons and is about 153 kb in length.
STOP cassette would be specifically excised primarily in the dPTB transgenic line for tissue-specific expression of Odin-(Dietrich et al., 2009) transgenic line to cross with the Odin-compete for signaling proteins. We selected the wnt1-Cre negative mutant against endogenous Odin because it may elucidated that the Odin-dPTB mutant may function as a dominant-negative mutant against wild-type Odin. These results support our hypothesis that Odin-dPTB functions as a dominant-negative mutant against wild-type Odin in vivo. In addition, these findings suggest a potential role for Odin in the proper development of ependymal cells in the SCO.

Ectopic expression of the PTB-deleted Odin mutant disrupts SCO development

The Odin PTB domain is critically involved in the signaling pathway downstream of EphA8 (Shin et al., 2007). We postulated that the Odin-dPTB mutant may function as a dominant-negative mutant against endogenous Odin because it may compete for signaling proteins. We selected the wnt1-Cre (Dietrich et al., 2009) transgenic line to cross with the Odin-dPTB transgenic line for tissue-specific expression of Odin-dPTB (Fig. 3). We predicted that a floxed GFP-expression-STOP cassette would be specifically excised primarily in the diencephalon and mesencephalon in the wnt1-Cre Odin-dPTB double transgenic line and that Odin-dPTB would be expressed subsequently instead of GFP in the corresponding brain tissues. Strikingly, all double transgenic lines (100% penetrance) displayed enlarged brains and a hydrocephalic phenotype, primarily in the midbrain (Figs. 3A and 3F). Cresyl violet staining analysis using coronal sections revealed that the SCO of double transgenic mice was much smaller than that of the Odin-dPTB transgenic line and lacked Cre expression (Figs. 3B, 3C, 3G, and 3H). We used Odin-dPTB mice without the Cre transgene as a control throughout all experiments as a comparison with the double transgenic mice. To further investigate whether ependymal cells in the SCO are immature, immunohistochemical staining analysis using anti-acetylated tubulin antibody was performed (Lang et al., 2006; Qin et al., 2011). As a result, ependymal cells in the SCO of the double transgenic lines were mostly negative for acetylated tubulin, a marker for ependymal cell multicilia (Figs. 3D, 3E, 3I, and 3J). Ependymal tissues near the SCO also weakly stained for the acetylated tubulin antibody. Taken together, these results support our hypothesis that Odin-dPTB functions as a dominant-negative mutant against wild-type Odin in vivo. In addition, these findings suggest a potential role for Odin in the proper development of ependymal cells in the SCO.

PTB-deleted Odin mutant expression interferes with proper development of ependymal cells in the cerebral aqueduct

The SCO is located in a dorsocaudal region of the third ventricle, at the entrance of the cerebral aqueduct (Rodriguez et al., 1998). Therefore, malformation of the SCO may have a causative role in the severe midbrain hydrocephalic phenotype (Picketts, 2006). We further examined the anatomical structure of the midbrain in the double transgenic lines. Consistent with the hydrocephalic morphology of the whole brain, the midbrain...
Expression of Odin-dPTB in Ependymal Cells
Sunjung Park et al.

**Fig. 3.** Subcommissural organ (SCO) development is defective in mice ectopically expressing Odin-dPTB. Littermate mice at P5 were obtained from a cross between the Odin-dPTB BAC transgenic line and wnt1-Cre mice to analyze their morphological abnormalities. (A and F) Whole brains were dissected out of the indicated mice for morphological comparison. Note that the brain of the double transgenic mice reveals a severe hydrocephalic phenotype with prominent enlargement in the midbrain. (B and G) Cresyl violet staining analysis of the coronal section. Note that the SCO is much smaller in the brain of the double transgenic line. PC, posterior commissure. (C and H) Enlarged view of the SCO regions in B and G, respectively. (D and I) The mid-sagittal section was subjected to immunohistochemical staining using anti-acetylated tubulin antibody, a marker for multicilia of ependymal cells. (E and J) Enlarged view of the SCO regions in D and I, respectively. Note that the ependymal cells in the SCO display much weaker staining with anti-acetylated tubulin antibody.

**Fig. 4.** The midbrain of Odin-dPTB mice becomes severely expanded upon Cre expression. (A and D) The coronal sections of the indicated brains were analyzed by cresyl violet staining. Note that both the cerebral aqueduct and inferior colliculus (ic) are severely expanded. ME, medulla. (B and E) Enlarged view of the cerebral aqueduct regions in A and D, respectively. (C and F) High magnification of the ependymal cells lining the cerebral aqueduct in B and E. Note that the ependymal cells of control mice have a regular and well-organized morphology, whereas those of the double transgenic mice do not.

and cerebral aqueduct were severely enlarged in double transgenic mice (Figs. 4A, 4B, 4D, and 4E). In contrast, the sizes of the cerebellum and medulla were not significantly altered in double transgenic mice. In addition, neither apoptotic cells nor proliferative (pH3-positive) cells increased significantly in the midbrain region (data not shown). Importantly, ependymal cells of control mice appeared to be well organized with epithelial-like morphology, whereas those of double transgenic mice looked irregular and disorganized (Figs. 4C and 4F). To further investigate whether ependymal cells lining the cerebral aqueduct were properly differentiated in double transgenic mice, we performed acetylated tubulin antibody staining on midbrain sagittal sections. As we predicted, the ependymal cells lining the cerebral aqueduct of double transgenic mice were mostly negative for acetylated tubulin antibody staining (Figs. 5A-5J). This result strongly suggests that ependymal cells in the midbrain are defective for proper development of multi-cilia. Taken together, these findings support our hypothesis that Odin plays a role in the development of ependymal cells in the diencephalon and mesencephalon during early postnatal brain development.

**DISCUSSION**

Here, we report that Odin, a PTB-domain containing adaptor...
protein, may play a role in the development of ependymal cells in the SCO and midbrain during early postnatal brain development. Odin-dPTB was ectopically expressed in ependymal cells by wnt1-Cre, and we subsequently showed that Odin-dPTB plays a causative role in the severe midbrain hydrocephalic phenotype. This hydrocephalic phenotype was likely due to agenesis of the SCO and/or immature development of ependymal cells lining the cerebral aqueduct (Picketts, 2006). As the hydrocephalic phenotype was rarely found in the midbrain of Odin null-mutant mice, we postulate that Odin-related PTB adaptors may supplement the deficient Odin function to develop ependymal cells in these mice. For example, AIDA-1b is highly homologous to Odin, and these two members constitute the Anks family proteins containing six ankyrin repeats at their NH2-terminus, two SAM domains, and a PTB domain at their COOH-terminus (Ghersi et al., 2004). Further experiments will be needed to determine the role of AIDA-1b in the development of ependymal cells during early postnatal brain development. In particular, it would be interesting to generate double knockout mice lacking both Odin and AIDA-1b to examine whether these mice display the hydrocephalic phenotype. However, it would be far more complex and time-consuming to generate AIDA-1b null-mutant mice because they have various splicing variants and an enormous genome size of > 1000 kb.

The SCO is one of the secretory organs in the circumventricular system (Cottrell and Ferguson, 2004). Although the main function of the SCO is not clearly defined, some evidence suggests that it plays a role as a neuroendocrine organ circulating CSF. In particular, the ependymal cells of the SCO are specialized to secrete glycoproteins, such as spondin, a giant glycoprotein, and they play a role in homeostasis (Huh et al., 2009; Picketts, 2006; Rodriguez et al., 1998). It has been proposed that malfunction of the SCO in various genetically mutant mice is related to the development of congenital hydrocephalus (Picketts, 2006). Our transgenic mice ectopically expressing Odin-dPTB also had an immaturely developed SCO with a severe midbrain hydrocephalic phenotype. Thus, Odin may have an essential role in SCO cell secretory function during early postnatal brain development. For example, the lack of secretory function in SCO cells may result from ectopic expression of the Odin-dPTB protein, and this defect may play a causative role in immature development or malformation of the SCO in Odin-dPTB mice. The secretory function of SCO cells will need to be examined to determine if they are partially ab-

normal in Odin null-mutant mice.

Our results strongly suggest that the PTB-deleted Odin protein may be effective as a dominant-negative mutant against the endogenous Odin protein. However, it is unclear how Odin-dPTB disturbs the wild-type Odin-mediated signaling pathway. Odin-dPTB contains six Ank repeats and two SAM domains, so it may interact with other proteins critically involved in the Odin-mediated signaling cascade. These large and stable protein complexes involving Odin-dPTB may prevent the endogenous Odin protein from binding to those signaling proteins in the pathway. However, we cannot rule out the possibility that Odin-dPTB may not be restricted to Odin but may also disturb other PTB-domain containing adaptors in various ways. A recent ependymal cell culture technique (Guirao et al., 2010; Paez-Gonzalez et al., 2011) will be an essential tool to analyze the mechanism by which Odin-dPTB disrupts endogenous Odin in cultured cells. Although the mechanism underlying Odin function remains to be analyzed in the future, our findings highlight the potential role of Odin in the proper development of the SCO in the third ventricle and in ependymal cells in the cerebral aqueduct.

**Note:** Supplementary information is available on the Molecules and Cells website (www.molcells.org).

**ACKNOWLEDGMENTS**

This study was supported by a grant from the Korean Health Technology R&D Project, Ministry of Health & Welfare grant (A111706).

**REFERENCES**

Baas, D., Meineli, A., Benadiba, C., Bonnaffe, E., Meiniel, O., Reith, W., and Durand, B. (2006). A deficiency in RFX3 causes hydrocephalus associated with abnormal differentiation of ependymal cells. Eur. J. Neurosci. 24, 1020-1030.

Blatt, E.N., Yan, X.H., Wuerffel, M.K., Hamilos, D.L., and Brody, S.L. (1999). Forkhead transcription factor HFH4 expression is temporally related to ciliogenesis. Am. J. Respir. Cell Mol. Biol. 21, 168-176.

Breunig, J.J., Arellano, J.I., and Rakic, P. (2010). Cilia in the brain: going with the flow. Nat. Neurosci. 13, 654-655.

Carien, M., Meletis, K., Goritz, C., Darsalia, V., Evergren, E., Tanigaki, K., Amendola, M., Barnabe-Heider, F., Yeung, M.S., Naldini, L., et al. (2009). Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke.
Ghersi, E., Noviello, C., and D’Adamio, L. (2004). Amyloid-beta
Dietrich, P., Shanmugasundaram, R., Shuyu, E., and Dragatsis, I.
Cottrell, G.T., and Ferguson, A.V. (2004). Sensory circumventricular
Guirao, B., Meunier, A., Mortaud, S., Aguilar, A., Corsi, J.M., Strehl,
Ihrie, R.A., and Alvarez-Buylla, A. (2011). Lake-front property: a
Kim, Y., Song, E., Choi, S., and Park, S. (2007). Engineering lacZ
Jacquet, B.V., Salinas-Mondragon, R., Liang, H., Therit, B., Buie,
Kristiansen, T.Z., Nielsen, M.M., Blagoev, B., Pandey, A., and Mann,
Lacar, B., Young, S.Z., Platel, J.C., and Bordey, A. (2010). Imaging
Lang, B., Song, B., Davidson, W., MacKenzie, A., Smith, N.,
Ortloff, A.R., Vio, K., Guerra, M., Jaramillo, K., Kaehne, T., Jones,
H., McAllister, J.P., 2nd, and Rodriguez, E. (2013). Role of the
Paez-Gonzalez, P., Abdi, K., Luciano, D., Liu, Y., Soriano-Navarro,
Rawlins, E., Bennett, V., Garcia-Verdugo, J.M., and Kuo, C.T. (2011).
Ank3-dependent SVZ niche assembly is required for the
Park, E., Kim, Y., Noh, H., Lee, H., Yoo, S., and Park, S. (2013).
EphA4+Apelin-A signaling is critically involved in region-specific
apoptosis during early brain development. Cell Death Differ. 20, 169-180.
Perez-Figares, J.M., Jimenez, A.J., and Rodriguez, E.M. (2001).
Subcommissural organ, cerebrospinal fluid circulation, and
hydrocephalus. Microsc. Res. Tech. 52, 591-607.
Picketts, D.J. (2006). Neuropeptide signaling and hydrocephalus: SCO with the flow. J. Clin. Invest. 116, 1826-1832.
Qin, S., Liu, M., Niu, W., and Zhang, C.L. (2011). Dysregulation of
Kruppel-like factor 4 during brain development leads to
hydrocephalus in mice. Proc. Natl. Acad. Sci. USA 108, 21117-
21121.
Ramos, C., Fernandez-Ulbrez, P., Bach, A., Robert, B., and Soriano,
E. (2004). Msx1 disruption leads to diencephalon defects and
devolutys. Dev. Dyn. 230, 446-460.
Rodriguez, E.M., Rodrigo, S., and Hein, S. (1998). The
subcommissural organ. Microsc. Res. Tech. 47, 98-123.
Shin, J., Gu, C., Park, E., and Park, S. (2007). Identification of
phosphotyrosine binding (PTB) domains. J. Mol. Biol.
Uhlik, M.T., Temple, B., Bencharit, S., Kimple, A.J., Siderovski, D.P.,
and Johnson, G.L. (2005). Structural and evolutionary division of
phosphotyrosine binding (PTB) domains. J. Mol. Biol. 345, 1-20.
Whitsett, J.A., and Tichelaar, J.W. (1999). Forkhead transcription
factors are master regulators of the motile ciliogenic
transcription factor critical for brain development. J. Neurochem.
Zhao, C., Suh, H., and Gage, F.H. (2009). Notch keeps ependymal
cells in line. Nat. Neurosci. 12, 243-245.