Title: Predominant neuronal differentiation of Olig1+ neural progenitors in forebrain cortex biased by β -catenin over-expression

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ABSTRACT:

Proper neuron-glia ratio is essential for normal brain development and function. Olig1 is a basic helix-loop-helix (bHLH) transcription factor generally used as a lineage tool for oligodendrocyte research in spinal cord. Recent studies have revealed a property of Olig1 as the common progenitor of GABAergic neurons and oligodendrocytes in the forebrain during embryogenesis, and a stage-dependent regulatory role of Wnt/ β -catenin signaling in the differentiation of oligodendrocytes in spinal cord. Given the neurogenic role of Wnt/ β -catenin signaling in neural progenitor cells, it is unclear how β -catenin affects the differentiation of Olig1-positive progenitors in brain. In the present study, we investigated the effects of β -catenin over-expression on the differentiation of Olig1-positive progenitors in the forebrain cortex, by using Olig1-Cre: β -cateninEX3^{loxp/+}:ROSA-YFP (β -cateninEX3 CKO) mice as compared to Olig1-Cre:ROSA-YFP control. The results showed that in the cortex of Olig1-Cre:ROSA-YFP mice, approximately 28.6% of YFP labeled cells are GFAP-positive, 43.7% are NG2-positive, 23.4% are CC1-positive and 3.2% are NeuN-positive, showing that Olig1-positive cells are multi-potential and mainly gliogenic. However, in the cortex of β -cateninEX3 CKO mice, the percentage of astrocytes generated from Olig1-positive cells decreased dramatically to approximately 2%, NG2-positive cells to 0.4%, and CC1-positive cells to 0.5%. In contrast, the percentage of NeuN-positive cells increased to approximately 96% of YFP-labeled cells. Taken together, our data showed that the gliogenic property of Olig1-positive progenitors in forebrain can be efficiently switched to neurogenic by

over-expressing β -catenin, revealing a neurogenic effect of β -catenin in the forebrain
Olig1-positive progenitors.

Key words: Olig1; β -catenin; neurogenesis

1. Introduction

During development, oligodendrocyte progenitor cells (OPCs) share the same precursors with neurons. The mechanisms for the differentiation of oligodendrocyte lineage and the subsequent process of maturation have been extensively studied, particularly in spinal cord [7, 13]. In spinal cord, OPCs and motor neurons are derived from p3 progenitor domain of ventral neural tube [11, 17]. Olig1 is one of the key transcription factors originally demonstrated to be crucial for the separation of oligodendrocyte progenitors from motor neuron progenitors in spinal cord, in together with Olig2 [11, 23]. Later studies have demonstrated that, in contrast to the wide involvement of Olig2 in the reactive astrogliogenesis and neurogenesis [3, 19], the function of Olig1 is restricted to the myelination and re-myelination of oligodendrocytes [2]. Therefore, Olig1 is generally thought to be more specific to oligodendrocyte lineage, and Olig1-Cre has been used by many researchers as the tool of oligodendrocyte research in spinal cord [4, 5, 18].

Wnt/ β -catenin signaling is a conserved intracellular signaling pathway which plays essential roles in the neuronal differentiation and self-renewal of neural stem cells both during development and in the subventricular zone (SVZ) of adult mice [6, 12]. Recently, several studies reported that Wnt/ β -catenin signaling regulates the differentiation of spinal cord oligodendrocytes, either affecting the proliferation of OPCs or the myelination of oligodendrocytes in a stage-dependent manner [5, 8]. In brain, the GABAergic neurons and oligodendrocytes in the cerebral cortex are derived from same multipotent stem cells during embryogenesis [10]. Recent studies [have](#)

revealed that Olig1 is expressed by Nestin-positive ventral neural progenitors in the early stage of brain development, and can produce both GABAergic neurons and oligodendrocytes[16], indicating that Olig1-positive progenitors in the early stage of brain development may act as one group of neuron/oligodendrocyte common progenitors.

Considering that the effects of morphogen signaling during development are usually temporal-spatial specific, and that of Wnt/ β -catenin signaling in neural progenitors are usually neurogenic, it is interesting to test how Wnt/ β -catenin signaling could influence the differentiation of Olig1-positive neuron/oligodendrocyte progenitors in forebrain, which remains unclear yet.

In the present study, we investigated the effects of over-expressing β -catenin on the differentiation of Olig1-positive progenitors in forebrain cortex, and found that stabilized β -catenin can bias Olig1-positive progenitors towards a predominantly neuronal fate.

2. Material and Methods

2.1 Animals

Olig1-Cre mice, in which the Olig1 coding sequence is replaced by Cre [21], ROSA-YFP and β -cateninEX3^{loxP/+} [9] mice were bought from Jackson lab. ROSA-YFP mice are crossed with ICR mice for breeding. All β -cateninEX3^{loxP/+} mice used are heterozygous for the floxed exon-3. Adult mice (8-10 weeks old) were used for sampling. All animal experiments were carried out under protocols approved by

the Animal Care and Use Committee of Fourth Military Medical University.

2.2 Immunohistochemistry

Serial coronal sections (16 μ m-in-thickness) were prepared on a cryostat. After blocking with 3% BSA in PBS, sections were incubated with the following primary antibodies overnight at room temperature: rabbit anti-GFAP(1:1000, Dako), rabbit anti-NG2(1:200, Millipore), mouse anti-CC1(1:30, Calbiochem), rabbit anti-NeuN(1:1000, Millipore), goat anti-YFP(1:500, Rockland), mouse anti-GAD67(1:1000, Millipore).Corresponding secondary antibodies were incubated for 3 hours at room temperature protected from light. Alexa Fluor 594 (donkey anti-rabbit, or anti-mouse IgG, 1:800) and Alexa Fluor 488 (donkey anti-goat IgG, 1:800) were all purchased from Jackson ImmunoResearch. Nuclei were counterstained by Hoechst 33342(1:1000, Sigma).Images were taken by confocal microscope (FV300, Olympus).

2.3 Cell counting and statistical analysis

Cells were counted from every eighth section and three mice were included for comparison in each group. To make the data comparable, all the cell counting was performed in the M1 area of neocortex as showed in Fig 1A. The cell counting was performed in a design-blind manner. Data are presented as mean $\pm$ standard error. Student's t-test was used to analyze differences of the means of cell counting. P values less than 0.05 were considered as statistically significant.

3. Results:

3.1 Fate mapping of Olig1-positive cells in the forebrain cortex with or without β -catenin over-expression.

To examine the effects of over-expressing β -catenin on the differentiation of Olig1-positive cells, we analyzed the fate of YFP-labeled cells in the primary motor cortex (M1) of Olig1-Cre:ROSA-YFP (Olig1/YFP) mice and Olig1-Cre: β -cateninEX3^{loxp/+}:ROSA-YFP (β -cateninEX3 CKO) mice (Fig 1A). In β -cateninEX3 CKO mice, the exon-3 of β -catenin is deleted by Olig1-Cre, and thus leading to stabilization of β -catenin in Olig1-positive cells. Most of the YFP-positive cells in the cortex of Olig1/YFP mice exhibited glial-like morphology, while most of the YFP-positive cells in the cortex of β -cateninEX3 CKO mice showed neuronal morphology (Fig 1B), indicating that β -catenin over-expression may result in more neuronal production.

3.2 Inhibition of glial differentiation of Olig1-positive cells by β -catenin over-expression.

Detailed analysis of the fate of olig1-positive cells was performed by double-staining YFP with markers of different neural cells as below. Double-immunostaining of YFP with GFAP, the most widely used astrocyte marker showed that in the cortex of

Olig1-Cre:ROSA-YFP (Olig1/YFP) mice, approximately $28.59 \pm 2.18\%$ of YFP-labeled cells are GFAP-positive. In the cortex of β -cateninEX3 CKO mice, only approximately $2.02 \pm 0.54\%$ of YFP-labeled cells are GFAP-positive (Fig. 2A). Double staining of YFP with NG2 and CC1, the markers of oligodendrocyte progenitor and mature oligodendrocyte, showed that, in the cortex of Olig1/YFP mice, approximately $43.75 \pm 2.58\%$ of YFP-labeled cells were NG2-positive, and $23.36 \pm 2.80\%$ were CC1-positive. However, in the cortex of β -cateninEX3 CKO mice, only approximately $0.38 \pm 0.01\%$ were NG2-positive and $0.53 \pm 0.12\%$ were CC1-positive (Fig. 2B, 2C). These data suggested that over-expressing β -catenin dramatically suppressed the differentiation of Olig1-positive cells towards glial lineage cells.

3.3 Increase of neuronal differentiation from Olig1-positive cells by β -catenin over-expression.

Since the differentiation of both oligodendrocytes and astrocytes from Olig1-positive cells were inhibited, we then examined the neuronal differentiation. Double-immunostaining of YFP with NeuN showed that in the cortex of Olig1/YFP mice, approximately $3.23 \pm 0.63\%$ of YFP-labeled cells were NeuN-positive. Interestingly, the percentage of YFP-labeled NeuN-positive cells increased to approximately $95.48 \pm 0.49\%$ in the cortex of β -cateninEX3 CKO mice (Fig 3A). Considering previous report that Olig1-positive cells give rise to a small portion of GABAergic neurons, we double-stained YFP with GAD67, a marker of GABAergic

neurons. The results showed that in the cortex of *Olig1/YFP* mice, *Olig1*-positive cells contributed to approximately $25.09 \pm 3.98\%$ of GAD67-positive cells, but in *β -cateninEX3* CKO mice, approximately $93.43 \pm 2.99\%$ of GAD67-positive cells were YFP-labeled (Fig 3B). These data suggested that over-expressing β -catenin dramatically biased the differentiation of *Olig1*-positive progenitors towards neurons, and increased the contribution of *Olig1*-derived GABAergic neurons in cortex.

Discussion

In this study, we investigated the effects of β -catenin over-expression on the differentiation of *Olig1*-positive cells in mouse forebrain. To over-express β -catenin in *Olig1*-positive cells, we crossed *Olig1*-Cre mice with *β -cateninEX3^{loxp/+}* mice. The Cre recombinase was knocked in the *Olig1* locus in genome and the Neo has been removed [21]. Therefore, the expression of Cre can authentically reflect the expression of *Olig1*. The exon-3 of β -catenin encodes the peptide involved in the phosphorylation of β -catenin, which leads to the degradation of β -catenin [9, 15]. Therefore, combination of these two mice lines will stabilize β -catenin in *Olig1*-positive cells. To visualize the changes of *Olig1*-positive cells, we crossed the *Olig1*-Cre; *β -cateninEX3^{loxp/+}* mice with ROSA-YFP mice, and thus all the *Olig1*-positive cells and their progenies could be labeled by YFP.

Previous study using the same *Olig1*-Cre and *β -cateninEX3^{loxp/+}* mice, reported the inhibition of oligodendrocyte differentiation in spinal cord[5], which was similar as what we observed (Fig. 2). Most of the *β -cateninEX3* CKO mice in their study died around 2 weeks old [5]. In our study, probably owing to the incorporation of ROSA-YFP mice, which is maintained in ICR background for easy breeding, the

β-cateninEX3 CKO mice can survive to two month. This offered us the opportunity to probe the progenies of Olig1-positive cells in later stages.

Our data showed that over 70% of the progenies of Olig1-positive cells are oligodendrocyte lineage cells and approximately 3% are neurons in normal mice. This is consistent with previous reports [14, 16]. The presence of YFP-labeled astrocytes indicates that Olig1-positive cells in forebrain are multi-potential, which has not been reported before.

The most striking finding of this study is that over-expressing β -catenin biased the differentiation of Olig1-positive progenitors towards a predominantly neuronal fate. The percentage of GABAergic neurons generated from Olig1-positive cells increased up to three times as compared to normal mice, consisting with previous reports and our observation in normal cortex that Olig1-positive cells contribute to the generation of GABAergic neurons. During the development of ventral telencephalon, Wnt receptor Ryk has been shown to switch the GABAergic neuron-oligodendrocyte progenitors towards neuronal fate [22]. In our study, the phenotype of β -catenin over-expression is more dramatic, possibly owing to its function in regulating multiple proneuronal transcription factors. Depleting β -catenin by Olig1-Cre will facilitate the dissection of the mechanisms of Olig1-positive progenitor differentiation, which is worthy to be further studied.

Another point which distinguishes our study from previous report using the same genetic mice model[5] is our focus on forebrain. During development, the morphogenic signals usually function in a spatial-temporal specific manner which ensures proper development of polarized tissues. Along the anterior-posterior axis, Wnt/ β -catenin signaling showed different effects on the development of rostral and caudal neural tube[1]. Compared to the temporal-specific effect of β -catenin on the

oligodendrocyte differentiation of Olig1-positive cells in spinal cord[5], our data, for the first time, demonstrated the powerful neurogenic effect of β -catenin on Olig1-positive cells in forebrain cortex. Since Olig1 is expressed by the type C cells in the SVZ of [20], it will be interesting to investigate whether more new-neurons could be found in olfactory bulb, and the phenomenon revealed here may be utilized for promoting adult neurogenesis after brain injury in future.

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Figure legends

Figure 1. Fate mapping strategy of Olig1-positive cells and quantification area.

(A) Regions of primary motor cortex (M1) in which YFP-positive cells were quantified. (B) Representative images of YFP in M1 area of Olig1-Cre:ROSA-YFP (Olig1/YFP) mice and Olig1-Cre: β -cateninEX3^{loxp/+}:ROSA-YFP (β -cateninEX3 CKO) mice. Notice the morphological changes of YFP-positive cells. Bars = 100 μ m.

Figure 2. Effects of over-expressing β -catenin on the glial differentiation of Olig1-positive cells.

(A) Double-immunostaining and quantification of YFP and GFAP in the cortex of Olig1-Cre:ROSA-YFP (Olig1/YFP) mice and Olig1-Cre: β -cateninEX3^{loxp/+}:ROSA-YFP (β -cateninEX3 CKO) mice. (B) Double-immunostaining and quantification of YFP and NG2 in the cortex of Olig1/YFP mice and β -cateninEX3 CKO mice. (C) Double-immunostaining and quantification of YFP and CC1 in the cortex of Olig1/YFP mice and β -cateninEX3 CKO mice. Notice the number of YFP-labeled astrocytes and oligodendrocytes decreased dramatically in the cortex of β -cateninEX3 CKO mice. Arrowheads point to representative double-stained cells. Bars=50 μ m in left panels and bars=10 μ m in right panels. **p<0.01.

Figure 3. Effects of over-expressing β -catenin on the neuronal differentiation of Olig1-positive cells.

(A) Double-immunostaining and quantification of YFP and NeuN in the cortex of Olig1-Cre:ROSA-YFP (Olig1/YFP) mice and Olig1-Cre: β -cateninEX3^{loxp/+}:ROSA-YFP (β -cateninEX3 CKO) mice. Arrowheads point to representative double-stained cells. Bars=50 μ m in left panels and bars=10 μ m in right panels.

****p<0.01.** (B) Double-immunostaining and quantification of YFP and GAD67 in the cortex of *Olig1/YFP* mice and *β -cateninEX3* CKO mice. Bars=100 μ m in left panels and bars=10 μ m in right panels. ****p<0.01.** Notice the dramatic increase of YFP-labeled neurons in the cortex of *β -cateninEX3* CKO mice.

Figure 1

A

A'

Olig1/YFP

β -catenin^{EX3} CKO

Figure 2

Figure 3