Expression pattern of β-catenin during the development of human fetal spinal cord

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Abstract
Development of the human fetal spinal cord is a very complicated process involving numerous signaling pathways including Wnt signaling pathways. These pathways are critical for the development and function of the mammalian nervous system. β-Catenin is a key molecule in the canonical Wnt signaling pathway. However, the distributions of β-catenin during development of the human fetal spinal cord have not been well characterized. Therefore, in this study, we performed immunohistochemical analysis of the β-catenin distribution in the developing human spinal cord from 35 fetuses at three weeks to eight months of gestation. As early as E3W and E4W, β-catenin was mainly expressed in the internal limiting membrane of the neural tube and neuroepithelium (E: Embryos; W: Weeks). During developmental stages, β-catenin was widely expressed in various structures and cells including the neuroepithelium, internal limiting membrane, mantle layer, marginal layer, basal plate, alar plate, ependyma, gray matter, white matter, neurons with multiple processes, glial cells, and nerve fibers. This study clarifies the morphological developmental characteristics of the human fetal spinal cord as well as the distribution and expression pattern of β-catenin in chronological and spatial aspects. Our results suggest that the Wnt/β-catenin signaling pathway might play a crucial role in various stages of the formation and differentiation of the human fetal spinal cord.

Keywords: β-catenin expression pattern, morphology, human fetal spinal cord.

Introduction
Development of the fetal spinal cord is a very complex process with multiple signaling pathways such as Wnt signaling pathways. The canonical Wnt signaling pathway involves stabilization and nuclear translocation of β-catenin. As a signal transduction protein, β-catenin contributes to cell adhesion, axis determination, organogenesis, as well as cell proliferation and differentiation during early development [1–4]. In addition, the Wnt/β-catenin signaling pathway plays a significant role in stem cell proliferation, tissue regeneration, and neurodegenerative diseases [5–7]. β-Catenin serves as a key factor in embryonic developmental processes such as cellular transport, apoptosis, and abnormal tumor activation [8–10]. In vertebrate posterior neural development, Wnt/β-catenin signaling initially induces the posterior regions of the nervous system, including the mid-hindbrain border, hindbrain, spinal cord, and neural crest, and then subsequently fine tunes the pattern of each region and determines the different cell fates within them [11, 12]. The Wnt/β-catenin pathway also regulates various developmental aspects of spinal cord neural precursors [13]. Proliferation and patterning in the developing spinal cord are separate events regulated independently by Wnt signaling [14]. A previous study found that Wnt signaling is a key regulator of the proliferation and differentiation of dopaminergic precursors during ventral midbrain neurogenesis [15]. Tightly regulated stable expression of β-catenin is required for spontaneous spinal cord regeneration [11]. Wnt has been also shown to mediate neural crest induction [16].

Most studies on the molecular mechanisms underlying the development of spinal cord were conducted in mice; however, the effect and distribution of β-catenin at various developmental stages in the human fetal spinal cord are not well reported.

In the current study, we systematically detected the distributions of β-catenin by immunohistochemistry (IHC) to assess detailed developmental changes in a spatio-temporal manner, and further demonstrate the role of β-catenin in human fetal spinal cord development.

Materials and Methods
Sample collection and preparation
A total of 35 spinal cords were obtained from human fetuses at gestational ages of three weeks to eight months from the First People’s Hospital of Yunnan Province with approval of the local ethics committee (Table 1, the approval date is October 15, 2007). Fetuses were obtained immediately from spontaneous or therapeutic abortions for social/legal reasons and had no visible developmental abnormalities. The gestational ages of fetuses were estimated based on the maternal menstrual history and measurement of the fetal size. The spinal cord tissues were fixed in 4% phosphate-buffered formalin, dehydrated...
through graded ethanol solutions, and embedded in paraffin. Four μm-thick serial sections were cut and mounted on Superfrost Plus glass sides (Fisher; Atlanta, GA, USA).

<table>
<thead>
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<th>Table 1 – Samples of human fetuses used in this study</th>
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<tr>
<td><strong>Gestational age</strong></td>
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<td>CRL [mm]</td>
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<td>Sample quantity</td>
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*E: Embryos; W: Weeks; M: Months; CRL: Crown-rump length.*

**Immunohistochemistry**

After deparaffinization and rehydration, endogenous peroxidase activity was blocked with 0.3% H2O2 followed by incubation with non-immune serum for 20 minutes at 37°C to block non-specific binding. The sections were then incubated overnight at 4°C with mouse anti-human β-catenin anti-serum (Santa Cruz Biotechnology, Inc., Catalog No. sc-7963, 1:500 dilution) in 0.3% Triton X-100. After washing in phosphate-buffered saline (PBS, pH 7.2) three times for 5 minutes each, the sections were incubated with a Biotin-labeled rabbit anti-mouse secondary antibody and then applied to the Avidin–Biotin–Peroxidase detection system (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). The sections were washed and incubated with the Avidin–Biotinylated Peroxidase complex for 30 minutes. After washing, the peroxidase activity was visualized with 0.5% 3,3'-diaminobenzidine tetrahydrochloride (DAB Peroxidase Substrate Kit; Zhong Shan Jing Qiao Biotechnologies, Inc., Beijing, China). After counterstaining with Hematoxylin, the sections were dehydrated, coverslipped, and examined under a Reichert–Jung microscope equipped with a Photostar automatic camera system (DMIRB, Leica, Germany). Control of immunostaining specificity was performed by either omitting the primary antibody or pre-adsorbing antibodies with the appropriate immunogens. Scoring of immunoreactive intensities was performed by three observers independently according to immunoreactive staining, and the average values of the scores derived from each observers were calculated, as follows: + (pale yellow, average values: 60–70), weak staining; ++ (yellow, average values: 71–89), moderate staining; +++ (deep yellow, average values: 90–100), intense staining.

**Results**

As early as E3W, β-catenin was detected in the neuroepithelium and internal limiting membrane (Figure 1A) and subsequently appeared in the external limiting membrane at E4W. Then, upon formation of the basal and alar plates (Figure 2), a few positive cells were observed in the apical and basal plates at E5W (Figure 1C). Subsequently, a large number of positive nerve fibers appeared in the mantle and marginal layers, but more nerve fibers were found in the marginal layer. In addition, positive cells with multiple processes were seen in the mantle layer from E6W to E7W (Figure 1, D and E). Positive fibers were obviously detected in the basal plate at E8W when the anterior median fissure has been formed (Figures 1F and 2). From E9W to E10W, the two alar plates undergo fusion and the posterior median septum has been formed (Figure 2), at which time some positive processes were present in the boundary area between the mantle and marginal layers and some processes extended to the marginal layer (Figure 1, G and H). When the shape of the spinal cord was similar to that of an adult from E11W to E12W (Figure 2), positive

<table>
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<th>Table 2 – Immunoreactive intensities of β-catenin in human fetal spinal cord at different gestational ages</th>
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<td><strong>Gestational age</strong></td>
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<td><strong>Internal limiting membrane</strong></td>
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<td><strong>Neuroepithelium</strong></td>
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<td><strong>External limiting membrane</strong></td>
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<td><strong>Mantle layer</strong></td>
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<td><strong>Marginal layer</strong></td>
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<td><strong>Basal plate or ventral horn</strong></td>
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<td><strong>Alar plate or dorsal horn</strong></td>
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<td><strong>Ependymal</strong></td>
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<td><strong>Neuron in gray matter</strong></td>
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<td><strong>Glia in gray matter</strong></td>
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<td><strong>Glia in white matter</strong></td>
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<td><strong>Nerve fiber in white matter</strong></td>
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*E: Embryos; W: Weeks; M: Months. Immunoreactive intensities: intense (+++), moderate (++), and weak (+).*
Expression pattern of β-catenin during the development of human fetal spinal cord

699
cells were found in the gray matter (Figure 1, I and J). At E4M, larger positive neurons with multiple processes were observed in the gray matter, and the immunopositive staining of their cell bodies and processes was more intense (Figure 1K). At E5M, in addition to neurons with positive cytoplasm and neurites, microglial cells with positive nuclei were seen in the gray matter (Figure 1, L and M). From E6M to E8M, these cells were still present in the gray matter, and neurons exhibited a mature polygonal morphology with multiple processes and their immunopositive staining was more intense (Figure 1, N and O). Controls did not exhibit any specific immunostaining in the spinal cord (Figure 1P). In conclusion, β-catenin was widely detected in various structures and cells during the investigated periods, including the neuroepithelium, internal and external limiting membrane, mantle and marginal layers, basal and alar plates, ependyma, gray and white matters, neurons, glial cells, and nerve fibers.

Figure 1 – Immunohistochemical staining of β-catenin in spinal cord at different gestational ages. Gestational ages: (A) E3W, 400×; (B) E4W, 400×; (C) E5W, 400×; (D) E6W–E7W, 100×; (E) E6W–E7W, 200×; (F) E8W, 200×; (G) E9W, 200×; (H) E10W, 200×; (I) E11W, 200×; (J) E12W, 200×; (K) E4M, 400×; (L) E5M, 400×; (M) E5M, 400×; (N) E6W–E7M, 400×; (O) E8M, 400×; (P) Negative control of the spinal cord stained with Hematoxylin (E5W, 100×). “L, m” are magnifications with the microglial cells of “L, M”. E: Embryos; W: Weeks; M: Months; EP: Epithelial cell; ILM: Internal limiting membrane; ELM: External limiting membrane; MEL: Mantle layer; EM: Ependyma; AP: Apical plate; BAP: Basal plate; MAL: Marginal layer; GM: Gray matter; WM: White matter; LH: Lateral horn. Arrows indicate representative immunoreactions of β-catenin. Black arrows indicate an immunopositive neuron. Red arrows indicate an immunopositive nerve fiber. Blue arrows indicate an immunopositive glial cell.

Figure 2 – Morphological characteristics of spinal cord at different gestational ages. Hematoxylin staining. Gestational ages: (A) E3W, 200×; (B) E4W, 200×; (C) E5W, 100×; (D) E8W, 100×; (E) E9W, 100×; (F) E10W, 100×; (G) E11W, 50×; (H) E12W, 100×; (I) E4M, 100×; (J) E5M, 50×; (K) E6M, 50×; (L) E7M, 50×; (M) E8M, 50×. E: Embryos; W: Weeks; M: Months.
Discussion

The Wnt/β-catenin pathway is a crucial regulator of cell growth and proliferation, and is involved in cell development, differentiation, migration, adhesion, polarization, and tumorigenesis [17–19]. During early development, Wnt signaling has a key role in patterning the prospective nervous system by regulation of cell fate specification, polarity, and migration. Wnt also coordinates the formation of neural circuits at multiple levels such as transcription, the cell cycle, and asymmetric cell division [20]. A recent study reported that modulation of Wnt/β-catenin signaling can improve cell replacement therapy for Parkinson’s disease [21]. Wnt/β-catenin signaling can also regulate hippocampus neurogenesis, and Wnt knockout mice show midbrain loss [22]. β-catenin signals are thus essential to maintain the proliferation of neuronal progenitors, control the size of the progenitor pool, and regulate the fate decision of neuronal progenitors to proliferate or differentiate [23].

Expression of β-catenin in neurons and glial cells

During early differentiation of the neural tube (E4W), we found that β-catenin was not only highly expressed in the internal limiting membrane and neural epithelial cytomembrane, but also present at the lumen surface of the central canal and ependyma in late embryogenesis. β-catenin is closely related to the development and differentiation of the early neural tube and neuroepithelium. When neuroblasts and glioblasts constitute the mantle and marginal layer along with differentiation of the neuroepithelium at E6W, β-catenin appeared in some cell nuclei of the neuroepithelium, mantle layer, which formed by apolar neuroblasts and glioblasts, and the cytoplasm of bipolar, unipolar, and multipolar neuroblasts as well as some neurites that extended to the cytoplasm of neurons at gray matter, while the immunoreactions were mostly seen in nuclei of glial cells. Studies of Drosophila subsequently led to the discovery of a second role for β-catenin in human cells, involving translocation of the protein from the cytoplasm into the nucleus [24, 25]. The canonical Wnt/β-catenin pathway is involved in controlling the neurogenic niche for dopamine neuron development and hippocampal neurogenesis [26, 27], rescues neurons from degeneration, and improves animal behavior following β-amyloid fibril insults [28]. Sustained β-catenin activity in neural crest cells promotes the formation of sensory neuronal cells in vivo at the expense of virtually all other neural crest derivatives [29]. β-Catenin is a crucial factor in the conical Wnt signaling pathway that plays a very important role in development of the central nervous system (CNS). In addition, Wnt plays an essential role in maintaining the development, growth, and proliferation of normal mouse embryonic cells [30]. The numbers of nerve cells in the CNS and precursor neurons are all observably decreased in β-catenin knockout mice [23, 31]. In addition, β-catenin is necessary for oligodendrocyte differentiation, and disruption of β-catenin signaling leads to a significant delay of oligodendrocyte maturation, and may regulate oligodendrocyte development in a stage-dependent manner [32]. In our study, we found that β-catenin was widely distributed in various structures and cell types, and expressed in the nucleus or cytoplasm of various cells at different developmental stages. Therefore, β-catenin may be involved in the proliferation, differentiation and migration of neuroblasts and glioblasts, and might play a crucial role during the development of the human fetal spinal cord.

Expression of β-catenin in neurites and nerve fibers

β-Catenin was detected in the cytoplasm and neurites of neurons in the mantle layer, alar and basal plates, and gray matter, and in nerve fibers in the white matter. Some studies of hippocampal neurons have shown that the levels of intracellular free β-catenin can affect the morphogenesis of neurites, and high levels of β-catenin can promote dendrite branching [33, 34]. In neural differentiation of P19 cells, β-catenin is widely expressed in the cell membrane and network-like nerve fibers [30]. N-cadherin is involved in the formation, elongation and clustering of neurites. β-Catenin can form a complex with α-catenin and γ-catenin, and combine with the N-cadherin intracellular domain for linkage to the cytoskeleton [35–37]. We assumed that β-catenin cooperated with N-cadherin to support neurite formation, elongation, and clustering of nerve fibers. Although differentiation of the neural tube is asymmetrical, the anterior–posterior and dorsal–ventral axes are symmetrical, which causes temporal and spatial distributions of neurons and glial cells, and constitutes the different phenotypes of the neural tube. This developmental process depends on the regulation of largely unknown signals. Our results showed that β-catenin was expressed in various cells of the developing human fetal spinal cord, indicating that β-catenin serves as a signaling molecule during development of the human fetal spinal cord.

Conclusions

Development of the human fetal spinal cord is a very complex process with multiple signaling pathways involved. β-Catenin is a key molecule in the canonical Wnt signal pathway. Our study demonstrated that β-catenin is widely expressed at various developmental periods and regions, and plays very important roles in the development and differentiation of the human fetal spinal cord. Our results should help advance our understanding of the developmental characteristics of the human spinal cord.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Expression pattern of β-catenin during the development of human fetal spinal cord

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