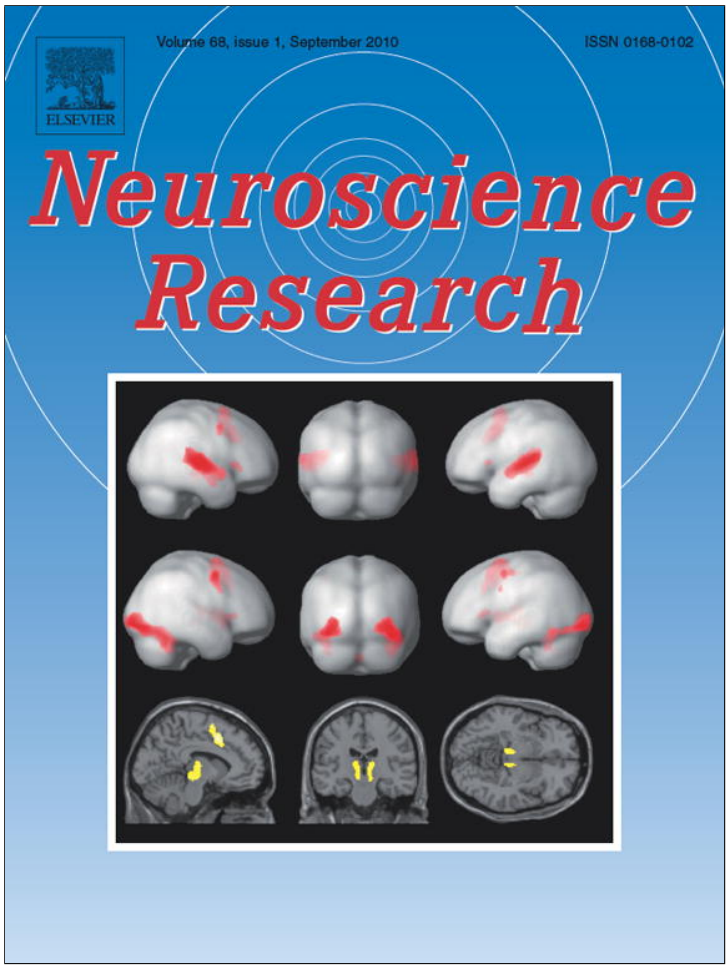

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Rapid communication

Area dependent expression of ZNF312 in human fetal cerebral cortex

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ABSTRACT

The human cerebral neocortex is divided into six layers consisting of specific neuronal cell types and connections. To determine the distribution of cortical neurons during early development, we examined the expressions of layer-specific markers in human midterm fetal brains. Layer V marker ZNF312 is expressed in most cortical areas, but not in the prospective somatosensory association area. Expression of layer IV marker RORβ is also diminished in this region but increased in the primary visual cortex, where expression of ZNF312 is reduced. Our results indicate that ZNF312 and other layer markers have area dependent expressions in the human fetal cortex.

The mammalian neocortex gives rise to perception and initiates voluntary motor responses. The cortical laminae, comprised of six distinct cellular layers of local circuit neurons and projection neurons, were initially characterized by classical histochemical staining. Genetic markers for delineating the cortical layers have recently been identified in rodents (Molyneaux et al., 2007). For example, Layer IV, the recipient of thalamocortical fibers, expresses RAR-related orphan receptor β (RORβ), and layer V is marked by the transcription factor Fezf2 that plays a critical role for specification of subcortical projections in the mouse (Arlotta et al., 2005; Chen et al., 2005a,b).

A homolog of Fezf2, ZNF312, is expressed in the deep layer of a human fetal cortex (Kwan et al., 2008). To determine the distribution of ZNF312-expressing neurons during human cortical development, we examined the expression of ZNF312 across cortical areas of the human neocortex at gestation week (GW) 21 and 27. Expressions of MAP1B and RORβ were also determined as comparative markers. In the 27 GW developmental stage, the cortical areas of the human brain are discernable—specifically, the gyri and sulci. These are useful anatomic reference systems (Zilles and Amunts, 2010), which can facilitate the study of area dependent gene expression.

Fetal samples were collected by the Department of Forensic Medicine, at less than one hour post-mortal delay, from the hospital of Wenzhou Medical College in China. Clearance from the College's ethical committee and consent of the parents involved were sought prior to use of the fetuses in this study. Saggital sections from fifteen frozen blocks of 27 GW brains were hybridized with anti-sense RNA probes, which were prepared from a cDNA library template of a human fetal brain. We found that ZNF312 was clearly expressed in most cortical areas as determined by *in situ* hybridization (ISH). The expression of ZNF312 was apparent in the deep layer of the prospective Brodmann's area (PBA) 3, 1, 2, the primary somatosensory cortex located on the post-central gyrus. In contrast, ZNF312 expression was diminished in the somatosensory association cortex (PBA 7) located in the parietal lobe. In the visual cortex of the occipital lobe, the expression pattern was reversed in that ZNF312 expression was high in the association cortex (PBA 18 and 19), but low in the primary cortex (PBA 17) (Fig. 1).

To verify that ZNF312 is localized in the layer V in humans, we compared ZNF312 labeling with well-known human layer markers MAP1B and RORβ. MAP1B is a microtubule-associated protein that has been detected by immunohistochemistry in cortical pyramidal neurons of human midterm fetuses (Ohyu et al., 1997) and in subsets of layer V neurons in a human fetal brain of 32 GW (Hevner, 2007). The expression of MAP1B was determined in the 27 GW brains by ISH (Fig. 2), and the marker was found to be expressed in the cortical plates where both upper and lower layers were strongly stained. ZNF312 labeling aligned with the lower band of MAP1B, suggesting specific expression of ZNF312 in the inner pyramidal layer (layer V) of the developing cortex. As expected, layer

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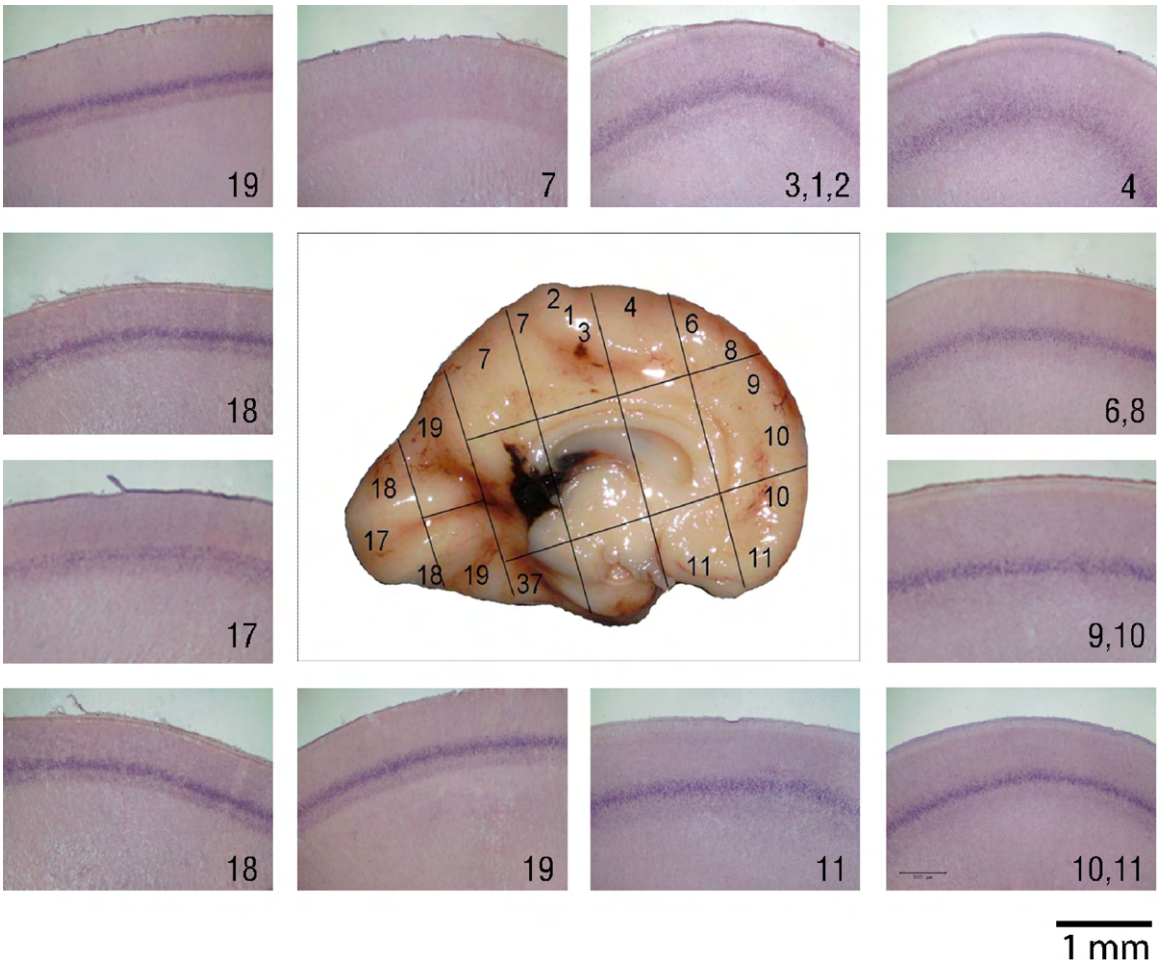

Fig. 1. ZNF312 expression in the cerebral cortex of the human fetus. A human fetal brain (27 GW) was cut roughly according to Brodmann's area, and the blocks were fixed in 4% paraformaldehyde. After cyro-protection by sucrose, 50 μm sections (in floating) were processed for digoxigenin ISH at 65 °C (Chen et al., 2005b). All blocks in the figure were processed by identical procedures including the time for color development. ZNF312 was expressed in the deep layer of the cortex. However, expression of ZNF312 was largely reduced in PBA 7 as well as in PBA 17. The ISH probe for ZNF312 corresponds to 850–1357 of the coding sequence.

IV marker RORβ was expressed in the layer slightly above that of MAP1B and ZNF312 (Fig. 2A).

The cytoarchitecture of the human cortex was examined by Nissl staining, which labels the cell-dense cortical plate. Although major laminar segregation was hard to distinguish at this stage of development, Nissl staining revealed that the cortical plates in rostral areas were thicker than those at the caudal area, which may reflect regional differences in cell cycle kinetics in the germinal zone. Similarly, MAP1B had a wide expression in area 4 of Brodmann, the developing primary motor cortex located on the precentral gyrus. Although ZNF312 was not expressed in PBA 7, MAP1B had a clear labeling in the layer V, indicating the presence of subsets of pyramidal neurons in the developing somatosensory association cortex (Fig. 2C).

Sensory cortices are generally marked by a well-developed layer IV, the major recipient layer of thalamocortical fibers. Therefore, we also examined the expression of RORβ, a marker for the granular layer IV (Hevner, 2007), across the cortical areas. Our results indicate that RORβ expression was reduced in PBA 4 (Fig. 2C), consistent with a previous observation suggesting that the granular layer in area 4 withdraws during the postnatal development (Amunts et al., 1995). As expected, RORβ was strongly expressed in the layer IV of PBA 17, as it is a prominent thalamocortical projection target. However, RORβ expression was significantly reduced in the visual association cortex (PBA 18 and 19). In the somatosensory cortices, RORβ was labeled in the primary cortex (PBA 3, 1, 2) but diminished in the association area 7 (Fig. 2C). To determine the changes of ZNF312 and RORβ expression at the single cell level, fluorescent ISH was performed. This approach allowed us to compare gene expressions in detail by confocal microscopy. Our data indicate that ZNF312-expressing neurons and RORβ neurons were largely reduced in PBA 7 (Fig. 2B).

Area 7 processes and integrates somatosensory and visual information and conveys the signals to the motor areas within the cerebral cortex. It is notable that both RORβ and ZNF312 expressions were diminished in the prospective area 7. To validate these observations, expressions of RORβ and ZNF312 were assessed in a human fetal brain of 21 GW (Fig. 3). Unfortunately, we were not able to distinguish the different areas in the cortex of 21 GW as gyri and sulci have not developed yet. The cortex was approximately divided into 12 blocks, as indicated in Fig. 3C, and the gene expressions were determined by ISH. Similar to the observation from the 27 GW brain, RORβ and ZNF312 were not expressed in the region of the parietal lobe (PL) that develops into the somatosensory association area later (Fig. 3A). Diminished ZNF312 expressions in prospective somatosensory association area were further confirmed in additional four fetal brains at 17, 19, 21, and 22 GW each (data not shown). In the occipital lobe, changes in labeling intensities

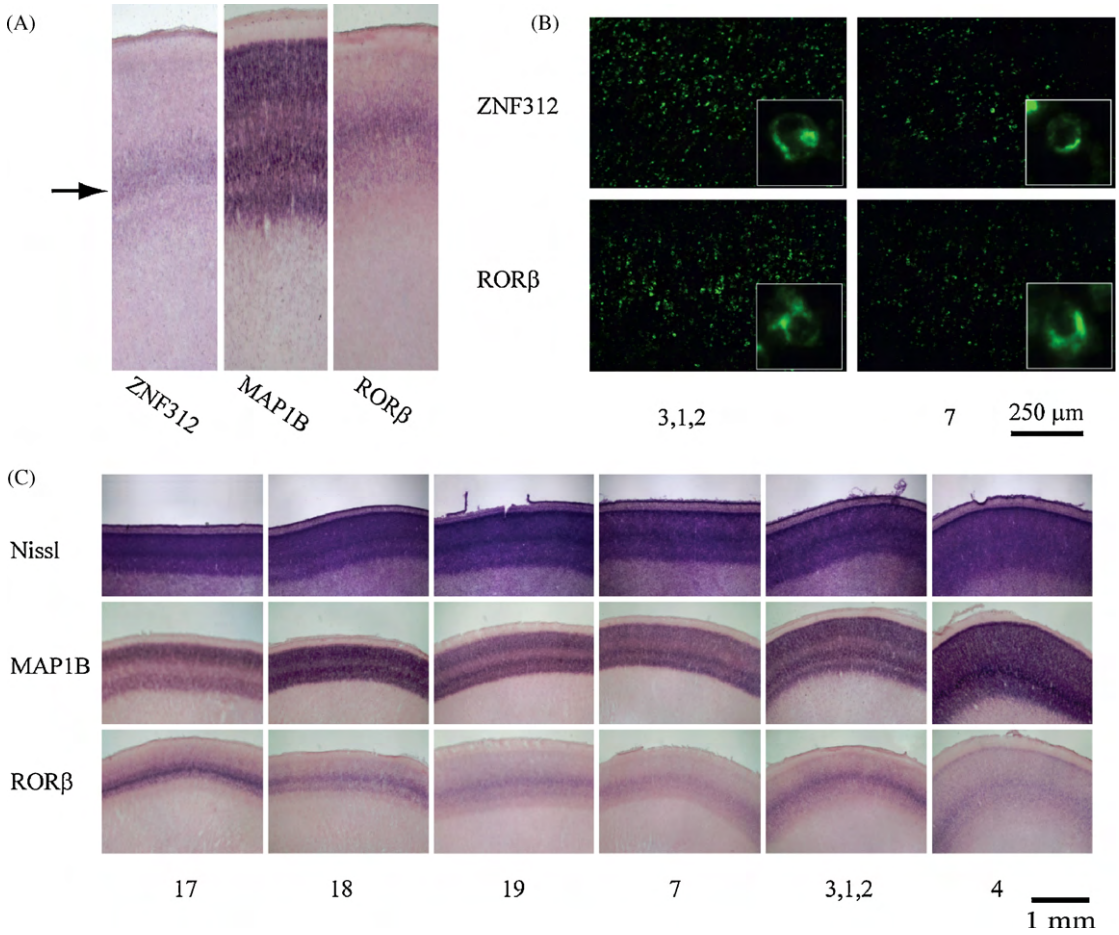

Fig. 2. Comparative expression of ZNF312, MAP1B and RORβ at 27 GW. (A) Alignment of ISH labeling for ZNF312, MAP1B, and RORβ. (B) Fluorescence ISH performed with TSA™ system (Roche) for signal amplification. Both ZNF312-expressing neurons and the RORβ cells were largely reduced in PBA 7. The inserts are magnified pictures showing perinuclear labeling of mRNA. (C) Nissl staining, MAP1B, and RORβ expressions were determined in the motor and sensory areas of the cortex. MAP1B was dominantly expressed in pyramidal cell layers across the cortical areas. The expression was weak, however, in the inner layer of PBA 17. Layer IV marker RORβ had a strong expression in PBA 17, but low expression in PBA 7 and PBA 4. ISH probes for MAP1B cover nucleotide 2352–2915, and those for RORβ cover nucleotide 233–787 of the mRNA sequences.

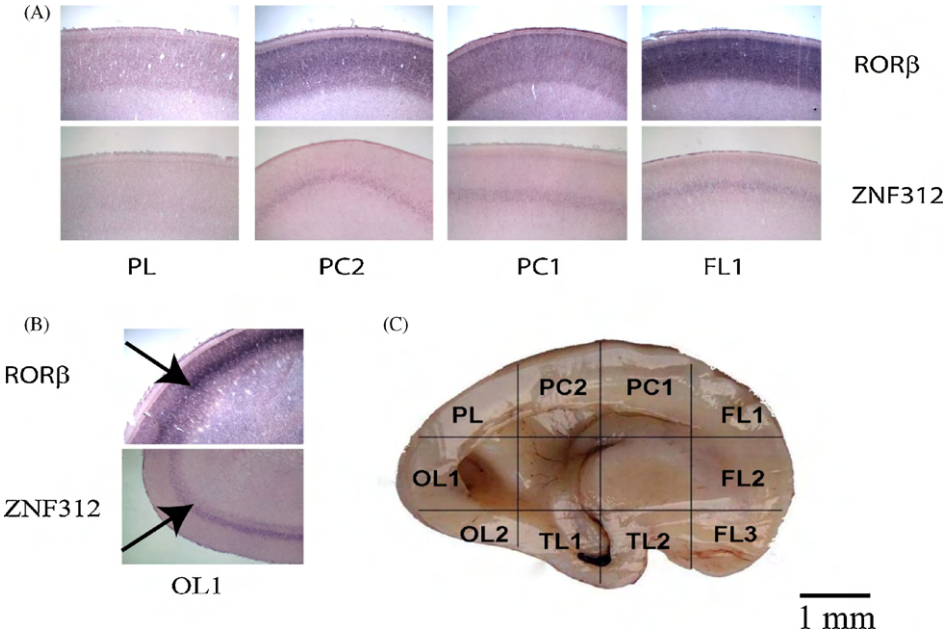

Fig. 3. ZNF312 expression in human fetal brains at 21 GW. (A) Expression of ZNF312 as determined by ISH in the cerebral cortex. ZNF312 expression was diminished in the parietal lobe (PL), the location for the prospective area 7 in matured brain. (B) ZNF312 and RORβ expressions in visual cortices. The arrows mark changes across the margin of the primary visual cortex. (C) Saggital section of the brain indicated cutting lines for the division of the cortex. FL: frontal lobe; PC: paracentral lobule; OL: occipital lobe; TL: temporal lobe; PL: parietal lobe. The scale bar is for ISH images.

were noticed. ZNF312 was reduced but RORβ increased toward the occipital pole of the cerebrum, the expected region for the primary visual cortex (PBA 17) (Fig. 3B).

Traditionally, functional areas in the mammalian cortex are identified largely on the basis of connections and distinctions in cytoarchitecture. However, it is difficult to delineate cortical areas in human fetal brains prior to emergence of the fully developed cytoarchitecture. Although different areas of the human cortex are highly similar by gene expression profiling, gene expression data can help define cortical functional areas at the molecular level (Khaitovich et al., 2004; Roth et al., 2006; O'Leary et al., 2007). Layer-specific markers have previously been used to identify cortical neurons in the human brain (Hevner et al., 2003; Hevner, 2007). Our study indicates that ZNF312 and other layer markers have area dependent expressions in the human fetal cortex, which in turn suggests that developing cortical regions may be identified by only a few layer markers. For example, prospective area 7 may be identified as the region where both ZNF312 and RORβ expressions are diminished, while the developing primary visual cortex displays reduced ZNF312 and increased RORβ expression.

The absence of ZNF312 staining in area 7 and area 17 can be expected if layer V neurons are not differentiated or do not exist in terms of the large pyramidal neurons expressing ZNF312. However, an analysis in a transiently laminated cortex limits definitive proof that ZNF312 is absent in the fully developed areas, as this marker may express later in development. Nevertheless, the accumulation of thalamocortical afferents in the transient subplate zone also shows a clear area distribution (Kostovic and Judas, 2007). The subplate zone may participate in the modeling of the cortical cytoarchitecture during development (Kostovic and Rakic, 1990). Future studies are needed to test this hypothesis and to provide a comprehensive molecular parcellation of the developing cortical areas.

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