Opposite Roles of Wnt7a and Sfrp1 in Modulating Proper Development of Neural Progenitors in the Mouse Cerebral Cortex

Nan Miao1†, Shan Bian2†, Trevor Lee2, Taufif Mubarak2, Shiyong Huang3, Zhihong Wen4, Ghulam Hussain5 and Tao Sun1,2*

1 Center for Precision Medicine, School of Medicine and School of Biomedical Sciences, Huaqiao University, Xiamen, China, 2 Department of Cell and Developmental Biology, Weill Cornell Medicine, Cornell University, New York, NY, United States, 3 College of Oceanology and Food Science, Quanzhou Normal University, Quanzhou, China, 4 Marine Biomedical Laboratory and Center for Translational Biopharmaceuticals, Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung, Taiwan, 5 Department of Physiology, Government College University, Faisalabad, Pakistan

The Wingless (Wnt)-mediated signals are involved in many important aspects of development of the mammalian cerebral cortex. How Wnts interact with their modulators in cortical development is still unclear. Here, we show that Wnt7a and secreted frizzled-related protein 1 (Sfrp1), a soluble modulator of Wnts, are co-expressed in mouse embryonic cortical neural progenitors (NPs). Knockout of Wnt7a in mice causes microcephaly due to reduced NP population and neurogenesis, and Sfrp1 has an opposing effect compared to Wnt7a. Similar to Dkk1, Sfrp1 decreases the Wnt1 and Wnt7a activity in vitro. Our results suggest that Wnt7a and Sfrp1 play opposite roles to ensure proper NP progeny in the developing cortex.

Keywords: Wnt7a, Sfrp1, cerebral cortex, neural progenitors, antagonist

INTRODUCTION

During development of the mammalian CNS, billions of neurons are produced from proliferating NPs (Rakic, 2009). In the cerebral cortex, NPs are expanded through symmetric and asymmetric division at the VZ and SVZ (Haubensak et al., 2004; Gotz and Huttner, 2005; Homem et al., 2015). The proper control of proliferation, survival and differentiation of NPs is the key step for normal cortical formation (Rakic, 2007, 2009; Zhao et al., 2008; Sun and Hevner, 2014).

A number of signaling pathways that regulate the switch and balance between proliferation and differentiation of NPs have been defined, including the Notch, Sonic hedgehog, fibroblast growth factor, TGF-β/Smads, and Wnt pathways (Chenn and Walsh, 1999; Rowitch et al., 1999; Hirabayashi et al., 2004; Joksimovic et al., 2009; Aguirre et al., 2010; Menendez et al., 2011; Rash et al., 2011). Wnt signaling pathways play crucial roles in neurogenesis (Kuwabara et al., 2009; Durak et al., 2016). For example, the canonical Wnt/β-catenin pathway is required for NP self-renewal and differentiation (Chenn and Walsh, 2003; Kalani et al., 2008;
Bengoa-Vergniory et al., 2014; Delaunay et al., 2014; Bengoa-Vergniory and Kypa, 2015; Garriock et al., 2015). Among the Wnt signaling molecules, Wnt7a has been shown to be critical in axonal remodeling, guidance, synaptogenesis and neurotransmitter release in the hippocampus (Hall et al., 2000; Cerpa et al., 2008; Ciani et al., 2011, 2015). Wnt7a controls neurogenesis through regulating genes involved in both cell cycle control and neuronal differentiation (Qu et al., 2013; Long et al., 2016).

Furthermore, three distinct receptor families have been reported to mediate the Wnt signaling: Fz, RoR, and Ryk (van Amerongen et al., 2008; Angers and Moon, 2009). In the nervous system, Fzs regulate a range of functions from neuronal differentiation to cell polarity, axon guidance, and cell survival (Van Raay et al., 2005; Prasad and Clark, 2006; Liu et al., 2008; Kilander et al., 2014; Zhou and Nathans, 2014; Morello et al., 2015; Chailangkarn et al., 2016). Moreover, Sfrps are a family of secreted factors that modulate Wnt-induced β-catenin pathway through selectively sequestering specific Wnts in different neurons by possessing the Wnt-binding frizzled CRD (Dann et al., 2001; Bovolenta et al., 2008; Nathan and Tzahor, 2009; Lavergne et al., 2011). For example, both Sfrp1 and Sfrp2 can be the dominant negative inhibitors of Wnt7a to inhibit proliferation in the developing chick neural tube (Galli et al., 2006), and Sfrp2 can negatively regulate the Wnt signaling in the CNS of Pax6 mutant mice via inhibiting Wnt7b (Kim et al., 2001a). Sfrp1 knockout mice display abnormal cortical morphogenesis (Esteve et al., 2018). However, the precise regulation of Wnts and their antagonist Sfrps in mammalian cortical neurogenesis is still unclear.

In this study, we show that Wnt7a and Sfrp1 are co-expressed in the VZ of mouse embryonic cerebral cortices. Knockout of Wnt7a causes microcephaly due to reduced numbers of NPs and decreased neurogenesis. Sfrp1 showed overexpression leads to a decrease in the NP population. Similar to the known Wnt antagonist Dkk1, Sfrp1 directly blocks the Wnt1 and Wnt7a activity in vitro. Our results indicate that opposite effects of Wnt7a and Sfrp1 play an important role in expansion of cortical NPs.

MATERIALS AND METHODS

Animals and Genotyping

The Wnt7a knockout mice (Wnt7a KO, Wnt7a−/−) were generated by mating female Wnt7a heterozygous mice (Wnt7a+/−) with male Wnt7a heterozygous mice (Wnt7a+/−). Mice that only have the mutant allele (Wnt7a-/+−) were Wnt7a KO mice, wild-type (WT) mice were used as controls. To achieve knockout of Wnt7a, a double-selection gene-replacement construct was designed to insert a neo gene into a Nael site in the second exon of the Wnt7a gene (Parr and McMahon, 1995; Ashrafi et al., 2012).

For staging of embryos, midday of the day of vaginal-plug formation was considered as E0.5; the first 24 h after birth were defined as P0. Animal use was overseen by the Animal Facility at Weill Cornell Medical College (Protocol number #2011-0062), and was performed according to the institutional ethical guidelines for animal experiments.

Mouse tail-tip biopsies were used for genotyping by PCR reactions using the following primers: for Wnt7a KO, forward: 5-CTCTTCCGTTGCTAGACTTG-3 and reverse-1: 5-TACGTCCTGACACGCGGAGCTG-3 (product size: 350 bp); for WT, reverse-2: 5-TCTTTCGAAGACAGTACG-3 (product sizes: 560 bp).

RNA Sequencing (RNA-Seq)

Total RNAs for RNA-seq were extracted from three individual E12.5 mouse cerebral cortices using TRIzol (Invitrogen, United States) according to manufacturer's instructions. The ribosome RNA (rRNA) removal, generation of cDNA library and high-throughput sequencing were performed on the Ion proton platform (Life Technologies, United States) from the NovelBio Bio-Pharm Technology Company (Shanghai, China). Three sets of raw reads were obtained, and data were deposited in Gene Expression Omnibus (GEO1) under the series number GSE116056. After removing contaminated and low-quality sequences, all reads were mapped onto the Ensembl mouse reference genome. Gene expression level were calculated by RPKM (reads per kilo-bases per million mapped reads).

In Situ Hybridization

In situ hybridization was performed as described: following fixation with 4% PFA, acetylation with acetylation buffer (1.3% triethanolamine, 0.25% acetic anhydride, 20 mM HCl), treatment with proteinase K (5 μg/ml, IBI Scientific) and pre-hybridization (1 × SSC, 50% formamide, 0.1 mg/ml Salmon Sperm DNA Solution, 1 × Denhart, 5 mM EDTA, pH 7.5), brain sections were hybridized with DIG-labeled LNA probes at Tm −22°C overnight. After washing with pre-cooled wash buffer (1 × SSC, 50% formamide, 0.1% Tween-20) and 1 × MABT, sections were blocked with blocking buffer (1 × MABT, 2% blocking solution, 20% heat-inactivated sheep serum) and incubated with anti-DIG antibody (1:1, 500, Roche) at 4°C overnight. Brain sections were washed with 1 × MABT and Staining buffer (0.1 M NaCl, 50 mM MgCl2, 0.1 M Tris-HCl, pH 9.5), stained with BM purple (Roche) at room temperature until ideal intensity was reached. The antisense RNA probe (Sfrp1, Wnt7a, Wnt7b, Pax6, Ngn2, and Hes5) was labeled using the DIG DNA labeling Kit (Roche, Switzerland) following the manufacturer's instructions.

Nissl Staining and Measuring Brain Size

Brain sections (14 μm) were processed through incubation in the subsequent solutions in the following order: ethanol/chloroform (1:1, overnight), 100% ethanol (30 s), 95% ethanol (30 s), distilled water (30 s, twice), cresyl violet (3–5 min), distilled water (2 min, three times), 50% ethanol (2 min), 95% ethanol (5–30 min), 100% ethanol (5 min, twice), xylene (3 min, twice). Thereafter, the sections were mounted with a coverslip.

1http://www.ncbi.nlm.nih.gov/geo/
The Wnt7a KO and WT brain images were captured in one picture, and the thickness of the cortex and CP was measured separately. The relative thickness of the cortex and CP in the KO was normalized from dividing the mean length of KO by that of the WT groups. At least three brains, and two chosen areas in each brain section were measured and averaged in each group. All data are presented as mean ± SEM. P-values were calculated using unpaired Student’s t-test.

RNA and qRT-PCR

The RNAs for RT-PCR from five stages of samples (E12.5, E13.5, E14.5, E15.5, and E17.5), were extracted by TRIzol (Invitrogen, United States), with three mouse cerebral cortices from each age group. Experimental protocols of embryo treatment used here were approved by Weill Cornell Medical College’s animal care and use committee. The procedures were carried out in accordance with the approved guidelines. After RNA extraction, the cDNA was synthesized using high-capacity cDNA Reverse Transcription kit (Applied Biosystems). The qRT-PCR reactions were carried out in the Bio-Rad CFX-384 system, using the reaction mixture SYBR Green Mix (Bio-Rad, United States) with the aforementioned cDNA samples.

β-Actin was used as an internal control, and was used to normalize the relative mRNA expression level. Each group had three biological repetitions, and all experiments were performed in triplicate, and each experiment was repeated at least twice. The qRT-PCR primers were: Wnt7a, forward: 5'-CCGAATGTCGCGTGGG-3' and reverse: 5'-CGATGCAGGCTGGAGG-3' (PCR product: 251 bp); Sfrp1, forward: 5'-CAACCTTGGGCTACAAGAAGAT-3' and reverse: 5'-GGCCAGTAGAAGCCGAGGCGG-3' and reverse: 5'-CATTCATTGAGCTAGCAGCAGCTCCAG-3' (PCR product: 1,053 bp); Dkk1, forward: 5'-GACCTCGAGGCACTGGAGGGAT-3' and reverse: 5'-GACCTCGAGGCACTGGAGGGAT-3' and reverse: 5'-CATTCATTGAGCTAGCAGCAGCTCCAG-3' (PCR product: 1,053 bp); DKK1, forward: 5'-GAGAGAGCAGGATGAGAAAGTTGTGGCTTTTTTG-3' and reverse: 5'-GGTTTACTGTCGCGTGGGAGGAAGCAGCAAGCAGG-3' (Target site: GCCACAACTTTCTCATCATGG, 1,077–1,098); shRNA1, 5'-ACCACTTGCATCTTCTCATCG-3' (product size: 249 bp); shRNA2, 5'-ACCACTTGCATCTTCTCATCG-3' (product size: 245 bp). All data are presented as mean ± SEM. P-values were calculated using unpaired Student’s t-test.

Plasmid DNA Constructs

To clone Sfrp1, Dkk1 and Wnt7a coding sequences into pcAGIG for IUE, Sfrp1, Dkk1 and Wnt7a coding sequences from pGEM-T was attached to dEGFP, a destabilized variant of the wild-type GFP, and then subcloned d2EGFP-Sfrp1, -Dkk1 and -Wnt7a coding sequence fragments into pcAGIG.

Full length coding sequences (CDs) for Sfrp1, Dkk1 and Wnt7a were cloned using the following primers: Sfrp1, forward: 5'-ATTCCGGTCTCGAGGCGACCGCGAAGCTGTA-3' and reverse: 5'-GGTTTACTGTCGCGTGGGAGGAAGCAGCAAGCAGG-3' (PCR product: 1,053 bp); Wnt7a, forward: 5'-GCACCTCGAGGCACTGGAGGGAT-3' and reverse: 5'-GGTTTACTGTCGCGTGGGAGGAAGCAGCAAGCAGG-3' and reverse: 5'-CATTCATTGAGCTAGCAGCAGCTCCAG-3' (PCR product: 1,053 bp); DKK1, forward: 5'-GCACCTCGAGGCACTGGAGGGAT-3' and reverse: 5'-GGTTTACTGTCGCGTGGGAGGAAGCAGCAAGCAGG-3' (PCR product: 1,053 bp); DKK1, forward: 5'-GCACCTCGAGGCACTGGAGGGAT-3' and reverse: 5'-GGTTTACTGTCGCGTGGGAGGAAGCAGCAAGCAGG-3' (PCR product: 1,053 bp). The Sfrp1, Dkk1 coding sequences were subcloned into the pcDNA3.1 vector for the TOPflash and FOPflash luciferase reporter (Promega, United States) assay.

RNA Interference Design and Efficiency Analysis

To knockdown Sfrp1, 4 different Sfrp1 specific short hairpin RNA (Sfrp1-shRNA) were designed and cloned into the pSilencer vector, separately. To analyze interference efficiency, Neuro2A cells were plated into 6-well plates in triplicate, and were transfected with four Sfrp1-shRNA using Lipofectamine 3000 (Invitrogen, United States). Cells were cultured for 2 days and the endogenous Sfrp1 knockdown efficiency was verified by qRT-PCR. The shRNA with the highest knockdown efficiency was selected to perform further IUE in cerebral cortices.

The following oligos were used to clone Sfrp1-shRNA: Sfrp1-shRNA1, 5'-CACCACTTACACAAGAGATGTGCTGC TTTAAAGAGACCGCACACTTCTCGGTATAGGTGTATTTTT-3' (Target site: GCTACAAGAAGATGTGCTGC, 498–519); Sfrp1 shRNA2, 5'-CACCACTTACACAAGAGATGTGCTGC TTTAAAGAGACCGCACACTTCTCGGTATAGGTGTATTTTT-3' (Target site: GCCACAACTTTCTCATGTTGG, 1,077–1,098); Sfrp1 shRNA3, 5'-CACCACTTACACAAGAGATGTGCTGC TTTAAAGAGACCGCACACTTCTCGGTATAGGTGTATTTTT-3' (Target site: GCCACAACTTTCTCATGTTGG, 1,077–1,098); Sfrp1 shRNA4, 5'-CACCACTTACACAAGAGATGTGCTGC TTTAAAGAGACCGCACACTTCTCGGTATAGGTGTATTTTT-3' (Target site: GCCACAACTTTCTCATGTTGG, 1,077–1,098).
G-3' (Target site: GCCACAACCTTCTCATCAGG, 1,130–1,151); Sfrp1-shRNA4, 5'-CACCCGACCTTCTCCGCTCCTCA CTGTTCAGAGACTGAAAGCGCAAGAATGCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTTCAAGAGACGTGACACGCTGAGAATTTTTTTGG-3'.

In Utero Electroporation

In utero electroporation was performed in E12.5 embryos according to the published protocol (Saito and Nakatsuji, 2001; Saito, 2006; Ito et al., 2014). Briefly, plasmid DNA was prepared using the EndoFree Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions, and diluted to 2 µg/µl. DNA solution was injected into the lateral ventricle of the cerebral cortex, and electroporated with five 50-ms pulses at 35V using an ECM830 electro square porator (BTX). Embryos were allowed to develop to E13.5. Animals with their brains of interest were fixed with 10% formalin at P0 and subjected to whole brain ISH (ISH) and IHC.  Cerebral wall thickness was measured with ImageJ software. 

RESULTS

Wnt7a and Sfrp1 Are Co-expressed in NPs in the VZ

To screen genes that are highly expressed in the mouse E12.5 cerebral cortices, RNA sequencing (RNA-seq) was performed. 30,827,078 and 29,345,746 and 32,038,652 raw sequencing reads, and 28,547,544 and 27,289,172 and 29,753,653 clean reads, respectively, were obtained from three individual E12.5 cortices (Supplementary Table S1). The mapping rates of clean reads were 92.2%, 93.4%, and 92.6% (Supplementary Table S2). Among these genes, Wnt7a, Wnt7b, and Sfrp1 showed high expression (RPKM > 500) (Supplementary Figure S1A and Supplementary Table S4). Moreover, Wnt7b, Wnt7a, and Wnt5a displayed higher abundance expression levels than other Wnt genes (Supplementary Tables S3, S4).

To verify the RNA-seq data, we examined expression patterns of Wnt7a, Wnt7b, and Sfrp1, and compared them with those of NP markers such as Pax6, Ngn2, and Hex5, and other Sfrps such as Sfrp2, Sfrp4, and Sfrp5 in the mouse cortex at E12.5 using ISH (Figure 1A and Supplementary Figure S1B). We found that both Wnt7a and Sfrp1 are co-expressed in the VZ of the E12.5 cortex (Figure 1A). Moreover, expression of Wnt7a and Sfrp1 was co-localized with that of Pax6, Ngn2 and Hex5, suggesting that Wnt7a and Sfrp1 are largely expressed in NPs (Figure 1A). Conversely, Wnt7b was highly expressed in newborn neurons, and other Sfrps such as Sfrp2 displayed low expression in the cortex (Figure 1A and Supplementary Figure S1B).

Next, we investigated whether expression levels of Wnt7a and Sfrp1 progressively change over embryonic stages at E12.5, E13.5, E14.5, E15.5, and E17.5 using qRT-PCR. Wnt7a displayed ascending expression from E12.5 to E15.5 (Figure 1B). Sfrp1 expression showed a gradual decline from E12.5 to E17.5 (Figure 1C). Compared to Wnt7a, Sfrp1 displays overlapping expression with Wnt7a in the VZ and opposite expression levels, implying distinct roles of Wnt7a and Sfrp1 in cortical development.

Wnt7a Positively Regulates Proliferation of NPs and Promotes Neurogenesis

Because of Wnt7a expression in the cortical VZ, we investigated whether Wnt7a regulates NP proliferation by analyzing cortical development in Wnt7a knockout mice (Wnt7a KO). The body size of Wnt7a KO was indistinguishable from that of WT mice. The cortical size and brain size were measured at P0, P5, and P20 (Figures 2A–C and Supplementary Figure S2). Compared to WT, the cortical size and brain size of Wnt7a KO mice were greatly reduced from P0 to P20, suggesting a progressive brain deterioration (Figures 2A–C and Supplementary Figure S2). Moreover, the thickness of the cortical wall was significantly reduced in the brain sections with Nissl staining in Wnt7a KO mice (Figures 2B,C). Interestingly, the ratios of cortical size versus brain size were similar between WT and KO, suggesting that the overall brain size is reduced in Wnt7a KO mice (Figure 2C and Supplementary Figure S2).
We then examined whether the NP population was changed in E13.5 Wnt7a KO mice using immunohistochemistry. NPs can be detected by labeling cells in the G1, S, G2, and M phases using the anti-Ki67 antibody. The number of Ki67+ cells was significantly decreased in the E13.5 Wnt7a KO cortex, compared to the control (Figures 2D,E). The numbers of Sox2+ and Pax6+ radial glial cells (RGCs), and Tbr2+ IPs were also reduced, suggesting an early reduction of NPs (Figures 2F–K). Moreover, because Pax6+/Tbr2+ cells are under transition from RGCs to IPs, we quantified the number of Pax6+/Tbr2+ cells. While a significant decrease in the number of Pax6+/Tbr2+ cells was detected in E13.5 Wnt7a KO cortex, the percentages of Pax6+/Tbr2+ cells versus total Pax6+ cells and Pax6+/Tbr2+ cells versus total Tbr2+ cells were unchanged, indicating that Wnt7a deletion doesn’t affect transition of RGCs to IPs (Supplementary Figures S3A–D). In addition, even though the total number of Tbr2+ cells was reduced, the percentage of Tbr2+ cells versus total DAPI+ cells remained the same in WT and Wnt7a KO cortices, suggesting that reduction in IPs is in proportion with that of total cells (Supplementary Figures S3E,F).

Next, we examined whether the early loss of NP population is maintained at E15.5. Compared to the controls, the numbers of BrdU+, Ki67+, Sox2+, Tbr1+, Pax6+, and Tbr2+ cells were greatly reduced in E15.5 Wnt7a KO cortices, suggesting that the deletion of Wnt7a causes a progressive loss of NPs (Figures 3A–F and Supplementary Figures S4A,B).

Because the overall organization of cortical layers is becoming clear, and neuronal production is evident at P0, P0 pups were collected to analyze brain phenotypes without sacrifice of the mother. We examined the expression of Tbr1 (layer VI), Ctip2 (layer V) and Satb2 (layer II, III, and IV) in P0 Wnt7a KO and control cortices (Molyneaux et al., 2007). The relative positioning of layer markers in the CP was similar to that of the WT, suggesting that overall cortical layer organization is not greatly affected by Wnt7a deletion (Figures 3G–J). Despite concordance of the position of layer markers, each layer examined was thinner in the Wnt7a KO cortex than that in the control, with significantly fewer mature NeuN+ neurons found, and great reductions in the number of Tbr1+ and Satb2+ neurons (Figures 3G–J). The Ctip2+ neurons showed no appreciable decrease in Wnt7a KO mice (Figures 3J). Moreover, the percentages of Tbr1+ and Satb2+ cells versus DAPI+ cells were unchanged in WT and KO cortices, indicating that the reduction in newborn neurons is in proportion with that of total cells (Supplementary Figure S4C).

Taken together, our results indicate that knockout of Wnt7a causes reduced NPs and production of newborn neurons.

**Sfrp1 Negatively Regulates Proliferation of NPs**

We next examined whether altering Sfrp1 expression in the cortex has a similar or an opposite effect on NPs as deleting Wnt7a expression. The full length cDNA for Sfrp1 was cloned (pCAGIG-Sfrp1) and was ectopically expressed in E12.5 cortices by using IUE, and embryos were analyzed after 24 h. Overexpression of Sfrp1 resulted in a decreased number of GFP+ NPs that are...
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FIGURE 2 | Wnt7a positively regulates brain size and proliferation of NPs. (A) The cortex of P0 Wnt7a knockout (KO) mice was greatly reduced compared to wild type (WT) controls. The arrowheads show the most rostral and caudal regions in the cortex. “L1” represent the cortical length, and “L2” represent the brain length.

(B) The cortical wall in P0 Wnt7a KO mice were thinner than that in WT mice, detected by Nissl staining. CP: cortical plate.

(C) The relative thickness of the cortex and cortical plate in the KO was normalized from dividing the mean length of Wnt7a KO by that of the WT groups. Values of histogram represent mean ± SEM, and each dot represents a data point of the relative thickness in each section or length in the brain images. n = 3 brains, at least two sections from each brain.

∗P < 0.05; ∗∗P < 0.01; ∗∗∗P < 0.001; ns, non-significant; unpaired Student’s t-test. 

(D–K) The numbers of Pax6+ and Tbr2+ neural progenitors were greatly reduced in the E13.5 Wnt7a KO cortex. Values of histogram represent mean ± SEM, and each dot represents a data point of the counting number in each section (200 µm bin). n = 3 brains, at least four sections from each brain.

**P < 0.01; ***P < 0.001; unpaired Student’s t-test). Scale bar: 50 µm.

double-positive for BrdU+, Pax6+, Sox2+ and Tbr2+, compared to those of electroporation of the control (pCAGIG) in E13.5 cortices, suggesting a decrease of NPs after Sfrp1 overexpression (Figure 4).

To test whether the endogenous Sfrp1 limits the NP numbers in vivo, we used shRNA designed to outcompete endogenous Sfrp1 transcripts. The Sfrp1 knockdown efficiency were verified in mouse Neuro2A cell by qRT-PCR (Supplementary Figure S5). The construct of shRNA (Sfrp1-sh4) that shows the highest knockdown efficiency among four tested shRNAs was used to perform IUE. Greater proportions of GFP+ NPs expressed BrdU, Pax6 and Sox2 were found in the VZ/SVZ following electroporation of the Sfrp1-sh4 (Supplementary Figures S6A–F). Tbr2+ NPs displayed no appreciable increase (Supplementary...
FIGURE 3 | Wnt7a promotes neurogenesis at E15.5 and P0. (A–F) Compared to controls (WT), Wnt7a knockout (KO) cortices at E15.5 displayed a reduction in the numbers of BrdU+, Ki67+, Sox2+, Tbr1+, Pax6+, and Tbr2+ cells. The dashed box represents the cell counting area. Values of histogram represent mean ± SEM, and each dot represents a data point of the counting number in each section (200 µm bin). n = 3, at least four sections from each brain. *P < 0.05; unpaired Student’s t-test. (G–J) In P0 Wnt7a KO cortices, the numbers of Tbr1+ and Stab2+ neurons were greatly reduced. NeuN+ neurons but not Citp2+ neurons were also reduced. The dashed box represents the cell counting area. Values of histogram represent mean ± SEM, and each dot represents a data point of the counting number in each section (200 µm bin). n = 3, at least five sections from each brain. *P < 0.05; **P < 0.01; ns, non-significant; unpaired Student’s t-test. Scale bar: 100 µm.

Figures S6G,H). These results indicate that Sfrp1 negatively modulates NP proliferation.

**Sfrp1 Has an Opposite Role of Wnt7a in Regulating NP Proliferation**

Based on opposite effect of Wnt7a and Sfrp1 on NP development, we suspected that Wnt7a might be regulated by its antagonists during cortical development. Previous studies have shown that Dkk1 is an antagonist of Wnt7a (Fortress et al., 2013). To examine how the Wnt7a antagonist may regulate NP development in the cortex, we over-expressed both Wnt7a and Dkk1 in the VZ of cortex using IUE. While Wnt7a promoted expansion of NPs, as shown by an increased number of BrdU+ and Pax6+ cells, over-expression of Dkk1 and Wnt7a in the VZ dampened Wnt7a effects, suggesting an antagonistic regulation of Dkk1
**FIGURE 4** | Sfrp1 negatively regulates proliferation of NPs at E13.5. (A,C,E,G) Overexpression of Sfrp1 in E12.5 cortices using in utero electroporation, analyzed at E13.5, caused the reduction of BrdU+/GFP+, Pax6+/GFP+, Sox2+/GFP+, and Tbr2+/GFP+ neural progenitors. (B,D,F,H) The proportion of cells labeled with individual progenitor markers and GFP versus cells labeled with GFP was quantified. Values represent mean ± SEM, and each dot represents a data point of the marker+GFP+/GFP% in each section (200 µm × 200 µm). n = 3, at least two sections from each brain. **P < 0.01; ***P < 0.001; unpaired Student’s t-test. Scale bar: 50 µm. 

(Supplementary Figure S7). Moreover, increasing Dkk1 dosage caused a greater decrease in the number of BrdU+ and Pax6+ cells, suggesting a dosage-dependent antagonistic regulation of Dkk1 on Wnt7a (Supplementary Figure S7).

If Sfrp1 also has the functions as a Wnt7a antagonist, it should have a similar effect to Dkk1 in NP development. With this in mind, Wnt7a and Sfrp1 were both overexpressed in the cortex using IUE. Similar to Dkk1, Wnt7a-Sfrp1 overexpressed in the VZ caused a reduction of BrdU+ and Pax6+ cells (Figure 5). Moreover, increasing the dosage of Sfrp1 had a more profound activity in suppressing Wnt7a effect on NP expansion (Figure 5).

Our results suggest that similar to Dkk1, Sfrp1 acts as an antagonist of Wnt7a and negatively regulates expansion of NPs. 

**Sfrp1 Inhibits Wnt7a Activity in TOPflash Luciferase Reporter Assay**

Based on the dosage-dependent regulation of Sfrp1 on Wnt7a, we tested whether Sfrp1 could down-regulate the Wnt7a activity. To validate Sfrp1-Wnt7a interaction, we used the TOPflash luciferase reporter assay containing the active TCF/LEF binding sites, which is the classical method to identify canonical Wnt/ß-catenin activity (Figure 6A) (Veeman et al., 2003). If the canonical Wnt signaling is activated, the ß-catenin will be associated with the TCF/LEF transcription factors to promote the Firefly luciferase activity. The mutant TCF/LEF binding site of FOPflash was used as the control (Figure 6A).

Wnt1 is a known molecule of the Wnt signaling and is crucial for early development of the CNS (Leal et al., 2011; Cai et al., 2013). As the positive control, we first tested whether Dkk1 and Sfrp1 can block Wnt1 in Neuro2A cells. Compared to the FOPflash group, the luciferase activity of Wnt1 in Dkk1 overexpression treatment was significantly decreased in the TOPflash group (Figure 6B). Agreed with Dkk1, the luciferase activity of Sfrp1 overexpression showed a similar decrease (Figure 6B).

Next, we tested whether Sfrp1 can inhibit Wnt7a in a similar fashion to how Wnt1 is negatively regulated in the aforementioned experiment. We found that the luciferase activity of Wnt7a was decreased appreciably in both Sfrp1 and Dkk1 overexpression treatment, suggesting that Sfrp1 acts like the known antagonist Dkk1, and blocks the Wnt7a signal (Figure 6C).

In summary, Sfrp1 has an attenuating role in Wnt signaling by blocking Wnt1 and Wnt7a in vitro.
DISCUSSION

The maintenance of normal cortical formation and size is essential for brain function. The Wnt signaling plays critical roles to regulate cell cycle control, neuronal differentiation and tissue repair (Chenn and Walsh, 2003; Kalani et al., 2008; Piccin and Morshead, 2011; Delaunay et al., 2014). The precise antagonistic regulation of Wnt members by Wnt modulators also controls cortical neurogenesis. Our study shows that Wnt7a and Sfrp1 are co-expressed in cortical NPs and their opposite role is essential for controlling NP expansion and neuronal production.

Among the many signals known to influence the CNS development, the Wnt signal has attracted great attention. Wnt/β-catenin signaling acts upstream of a complex and dynamic temporal network to control progenitor fate (Draganova et al., 2015): long-term overexpression of Wnt3a leads to cortical dysplasia by inducing early differentiation of IPs into neurons and the heterotopias of these newborn neurons (Munji et al., 2011). Studies have shown the role of Wnt7a in axon development and guidance, as well as synapse formation and maintenance (Hall et al., 2000; Cerpa et al., 2008; Ciani et al., 2011, 2015). Investigations of Wnt7 in the early step of neurogenesis in the cerebral cortex have just begun (Qu et al., 2013; Long et al., 2016). Transcriptome sequencing data from us and others have shown that Wnt7b, Wnt7a, and Wnt5a are the most abundant Wnt factors in the E12.5, E16.5, and E17.5 cortices (Wang et al., 2016; Nguyen et al., 2018). Moreover, we have found that Wnt7a is highly expressed in the VZ and Wnt7b in the...
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FIGURE 6 | Sfrp1 inhibits Wnt7a activity in the TOPflash luciferase reporter assay. (A) TOPflash is a luciferase reporter of β-catenin-mediated transcriptional activation with active TCF/LEF binding sites, which affect the firefly luciferase expression. The control plasmid is FOPflash, which contains mutant TCF/LEF binding sites. (B,C) After transfection of the pcDNA3.1-Sfrp1 and pcDNA3.1-Dkk1, a statistically significant decrease in luciferase activity of Wnt1 and Wnt7a was observed in comparison with controls. Values represent mean ± SEM. n = 3, **P < 0.01; ***P < 0.001; unpaired Student’s t-test.

intermediate zone and CP, which is consistent with the RNA-seq results from isolating specific cellular zones and layers in E14.5 and E15.5 cortices (Ayoub et al., 2011; Belgard et al., 2011; Aprea et al., 2013; Liu et al., 2016). How distinct expression patterns of different Wnts are established in developing cortices remains unclear. Differential expression of Wnt7a and Wnt7b in the cortical layers may determine their different roles in cortical neurogenesis (Stenman et al., 2008; Durak et al., 2016): Wnt7a promotes neurogenesis by regulating genes involved in cell cycle control and neuronal differentiation (Qu et al., 2013); the increased Wnt7b modulates neuronal differentiation by regulating T-domain transcription factors Tbr1 and Tbr2 (Papachristou et al., 2014).

Moreover, we have shown that the deletion of Wnt7a expression causes microcephaly by reducing the population of NPs and newborn neurons. These data are consistent with previous reports demonstrating that Wnt7a positively regulates NPs and neurogenesis (Qu et al., 2013; Long et al., 2016; Wang et al., 2016). Recent research has shown that Wnt7a regulates the asymmetry of spindles in neuroepithelial cells in the VZ, which is linked to asymmetric cell division (Delaunay et al., 2014). The embryonic ventral midbrain of Wnt7a KO mice displays reduced Sox2+ progenitors (Fernando et al., 2014). We have also found that Sox2+ progenitors are decreased in the cerebral cortex at E13.5. Decreased expansion of cortical NPs is likely a major cause of microcephaly in Wnt7a KO mice. Among Wnt molecules, Wnt7a is a known regulator in the beta-catenin signal pathway (mmu04310) functioning in different biological processes (Daneman et al., 2009; Ciani et al., 2011; Qu et al., 2013; King et al., 2015). Wnt molecules are associated with Hippo signaling pathway, Integrin signaling and Notch signaling (Qu et al., 2013; Ciani et al., 2015; Wang et al., 2016). These pathways likely cooperate to regulate cortical development.
Sfrps are a family of receptors known to possess a Wnt-binding frizzled CRD, and abnormal expression of Sfrp1 leads to CNS functional disorders (Esteve et al., 2011, 2018). Sfrp1 is a key member of the Sfrp family that can bind directly to Wnts via their regions of homology to Fz. In the CNS, Sfrp1 can block dopamine neuron development, dendritic development and hippocampus formation (Rosso et al., 2005; Miquelajauregui et al., 2007; Kele et al., 2012). In this study, we have found that Sfrp1 is expressed in the VZ of the mouse embryonic cerebral cortex, which is consistent with the observation of its expression restricted to the proliferative zone in the CNS (Augustine et al., 2001). Similar to the known antagonist Dkk1, we have found that overexpression of Sfrp1 reduces the NP population, and Sfrp1 significantly decreases the number of NPs in a dosage-dependent manner, suggesting an opposite role of Sfrp1 in cortical development compared to Wnt7a (Adamska et al., 2004; Kim et al., 2008; Osada et al., 2010). In the recent study of Sfrp1 knockout mice, the authors observed an increase in the number of BrdU+/Tbr2+ cells in E12.5 Sfrp1−/− cortex (Esteve et al., 2018). We think that the reason we did not detect an increase of Tbr2+ cells when Sfrp1 is knocked down, is likely due to the efficiency of shRNA of Sfrp1, compared to the gene knockout. Moreover, recent studies have shown that Sfrps interact with the Wnt signaling, Hedgehog signaling, BMP and Notch signaling (Kato and Kato, 2006; Mii and Taira, 2009; Misra and Matise, 2010; Esteve et al., 2011, 2018). It is likely a combined effort of Sfrp1 with other signals contributes to cortical development.

Sfrps is a physiological Wnt-signaling scavenger that binds directly to Wnts due to their similarity to the receptor Frizzled, thus, it is capable of regulating the availability of Wnt proteins (Finch et al., 1997; Rattner et al., 1997; Baarsma et al., 2013; Cruciat and Niehrs, 2013). The exclusive repression of the Wnt pathway is possible by selective Sfrps in cortical development (Mikels and Nusse, 2006; Lacour et al., 2017). Sfrp1 and Sfrp3 are expressed in opposing anterolateral to caudomedial gradients, and regulate normal temporal advancement of neuronal birth and maturation in anterior and lateral cortical regions by selectively modulating Wnts (Kim et al., 2001b). Previous studies have shown that Dkks inhibit the canonical Wnt pathway by internalizing LRP5/6, whereas Sfrps inhibit both the canonical and non-canonical pathways by binding Wnt ligands or Frizzled (Dees et al., 2014; Majchrzak-Celinska et al., 2016). The future study will be to investigate whether Sfrp1 directly binds to Wnt7a or through other mechanisms in the cortex.

The reciprocal control of Wnt7a and Sfrp1 may be a dosage-dependent compensatory mechanism to maintain normal cortical formation during early development. Our study reveals that an optimal expression level of Wnt7a and Sfrp1 is critical for proper establishment of the NP population. Further work will be dedicated to explore the precise regulation of how different Sfrps mediate canonical Wnt signaling pathway in NP proliferation and differentiation during embryonic cortical development. Our findings suggest that dysregulation of the Wnt signaling can lead to developmental defects similar to human cortical malformation disorders such as microcephaly.

**AUTHOR CONTRIBUTIONS**

TS: conceived and designed the experiments. NM, SB, TL, and TM: experiment. NM, SB, SH, ZW, GH, and TS: result analysis. NM and TS: wrote the paper. NM and TS: edited paper.

**FUNDING**

This work was supported by an R01-MH083680 grant from the NIH/NIMH (TS), the National Natural Science Foundation of China (81471152, 31771141), and China Postdoctoral Science Foundation (2017M622053).

**ACKNOWLEDGMENTS**

We thank members of the Sun Laboratory for their valuable discussions and advice. We thank Dr. Tony Brown for providing reagents, Dr. Julia Kaltschmidt for providing mouse lines, and Drs. Y. Kawase-Koga and Q. Li for technical support.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00247/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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