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Disruption of Neurogenesis and Cortical Development in Transgenic Mice Misexpressing Olig2, A Gene In The Down Syndrome Critical Region

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Abstract

The basic helix-loop-helix (bHLH) transcription factor Olig2 is crucial for mammalian central nervous system development. Human ortholog *OLIG2* is located in the Down syndrome critical region in trisomy 21. To investigate the effect of *Olig2* misexpression on brain development, we generated a developmentally regulated Olig2-overexpressing transgenic line with a Cre/loxP system. The transgenic mice with Olig2 misexpression in cortical neural stem/progenitor cells exhibited microcephaly, cortical dyslamination, hippocampus malformation, and profound motor

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deficits. Ectopic misexpression of Olig2 impaired cortical progenitor proliferation and caused precocious cell cycle exit. Massive neuronal cell death was detected in the developing cortex of Olig2-misexpressing mice. In addition, Olig2 misexpression led to a significant downregulation of neuronal specification factors including *Ngn1*, *Ngn2* and *Pax6*, and a defect in cortical neurogenesis. Chromatin-immunoprecipitation and sequencing (ChIP-Seq) analysis indicates that Olig2 directly targets the promoter and/or enhancer regions of *Nfatc4*, *Dscr1/Rcan1* and *Dyrk1a*, the critical neurogenic genes that contribute to Down syndrome phenotypes, and inhibits their expression. Together, our study suggests that Olig2 misexpression in neural stem cells elicits neurogenesis defects and neuronal cell death, which may contribute to developmental disorders including Down syndrome, where *OLIG2* is triplicated on chromosomal 21.

Introduction

The bHLH transcription factor Olig2 plays an important role in the development of the mammalian central nervous system. Olig2 is required for oligodendrocyte fate specification and motor neuron formation from neural progenitor cells during embryogenesis (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002), and critical for glial development in postnatal stages (Cai et al., 2007; Komitova et al., 2011; Ono et al., 2009; Yue et al., 2006). Although telencephalic Olig2-expressing precursors give rise to subtypes of cortical interneurons (Miyoshi et al., 2007), the role of Olig2 in cortical neurogenesis is not fully understood.

The human ortholog *OLIG2* maps to the Down Syndrome Critical Region (DSCR) on chromosome 21. Recent studies suggest that triplication of *Olig2* and its paralog *Olig1* contributes to developmental brain defects in Down syndrome (DS) (Chakrabarti et al., 2010). DS is associated with severe mental retardation and caused by triplication (trisomy) of human chromosome 21 (HSA21) that occur in ~1-750 live births. Trisomies can be classified into different categories (whole-chromosome trisomy, partial trisomy, microtrisomy, or single gene triplication). Although the severity and range of DS phenotypes vary from patient to patient (Belichenko et al., 2009; Epstein, 2006; Fillat et al., 2010), the common neuropathological changes in DS include neuronal cell death, delayed and disorganized cortical lamination (Golden and Hyman, 1994; Roper and Reeves, 2006), reduction of neuronal number in the cerebral cortex and hippocampus (Antonarakis et al., 2004; Chakrabarti et al., 2007; Gardiner et al., 2010; Sylvester, 1983), and hypomorphic cerebellum with developmental delay and premature termination (Aylward et al., 1997; Baxter et al., 2000). While several mouse DS models including Ts65Dn and Ts1Cje mice are widely used in DS research, these models reflect a dosage increase of ~132 genes, in a partial trisomy 16, syntenic to human chromosome 21 (Antonarakis et al., 2004; Haydar and Reeves, 2012). At present, however, the contribution of an individual gene in the DS critical region to major neurological phenotypes of DS remains elusive.

Gene expression profiling on human DS fetal brain neural precursors and mRNA expression levels showed that *OLIG2* is upregulated in the DS cell lines (Esposito, *et al*, 2008) and fetal brain of DS patients (Lu et al., 2012). In addition, Olig2 and its paralog Olig1 are over-expressed in the mouse DS model Ts65Dn (Chakrabarti et al., 2010). Furthermore, *OLIG2*

over-expression impairs proliferation of human DS neural progenitors (Lu et al., 2012). These observations raise the possibility that excessive OLIG2 may contribute to the DS phenotype. Recent studies show that retrovirus-mediated overexpression of Olig1 in cortical progenitor cells causes neuronal cell death in the murine developing cortex (Lu et al., 2001). Dosage reduction of genetically linked Olig1 and Olig2 genes can rescue the inhibitory neuron defects in the brain of Ts65Dn mouse model of DS (Chakrabarti et al., 2010). These observations suggest a potential co-relationship of *Olig* dosage and DS phenotypes. At present it is not clear whether the increased dosage of the individual *Olig2* gene in the developing brain would lead to neurological phenotypes resembling those seen in DS patients.

To decipher the contribution of Olig2 dosage to certain aspects of neurological disorders like DS, we utilize an *in vivo* Cre/loxP system to generate new transgenic lines for conditional Olig2 misexpression and study the effects of Olig2 misexpression on brain development. The transgenic mice with Olig2 overexpression in nestin-expressing neural stem/progenitor cells exhibit a severe defect in cortical development and motor behavior. We show that ectopic Olig2 misexpression inhibits cortical progenitor proliferation and neurogenesis, and causes massive apoptosis in the developing brain. Thus, our studies suggest that ectopic or misexpression of Olig2, a gene in the DSCR, in neural progenitor cells, could lead to developmental brain defects and neuronal cell death, a neurological phenotype seen in DS, pointing to an important contribution of Olig2 dosage to developmental brain defects in DS.

Results

Generation and characterization of conditional *Olig2* expressing transgenic mice

We used a Cre-loxP transgenic system to investigate effects of Olig2 overexpression in a cell type-specific manner (Figure 1A). To construct the conditional *Olig2* expression transgenic vector, we placed the myc-tagged-*Olig2* gene downstream of a floxed *LacZ*-carrying transcriptional 'stop' polyadenylation signal (Fukuda et al., 2006), which prevents transcription read-through from the CAG promoter and keeps the *Olig2* transgene in a transcriptionally silent state (Farley et al., 2000). The inducible transgenic mouse lines generated with this construct were designated as iTg. Sections of iTg mice exhibited widespread beta-galactosidase activity derived from the *LacZ* transgene in the entire brain, indicating the integration of a functional transgenic cassette (data not shown).

To examine the effect of Olig2 overexpression on cortical development, we bred iTg mice with cortical neural progenitor expressing nestin-Cre line (Dubois et al., 2006), in which the Cre activity has been observed in multipotent progenitors in the developing brain. The resulting bigenic mice with Olig2 expression in nestin+ neural stem/progenitor cells were designated as iTg-Nes. In iTg-Nes animals, staining of Myc tag of the Olig2 transgene product at embryonic day 13.5 (E13.5) showed expression of Myc positive cells spreading out in the ventricular zone and entire cortex, suggesting that ectopic Olig2 expression occurs in the neural stem/progenitor cells since endogenous *Olig2* mRNA expression was hardly detectable in the cortex before the embryonic stage at E14.5 (Yue et al., 2006). In contrast, no expression of ectopic Myc tag was detected in control iTg (Figure 1B). Expression of Myc-Olig2 persisted in postnatal stages in the cortex of iTg-Nes (Figure 1C).

Immunostaining showed a robust Olig2 expression in the cortex of iTg-Nes at E18.5 as compared to that of iTg controls (Figure 1D). Quantitative RT-PCR (qRT-PCR) assays further confirmed a significant increase in Olig2 expression at different developmental stages with ~3 to 6 fold increase over the age-matched control (Figure 1E). These results indicate generation of inducible Olig2 misexpression mice that permit ectopic Olig2 transgene expression specifically in neural stem/progenitor cells in the developing cortex. Despite gross motor deficits, iTg-Nes animals had comparable size and body weight to age-matching iTg control and wildtype mice.

Given that Olig2 is an oligodendrocyte lineage specification gene, we then analyzed the formation PDGFR α ⁺ oligodendrocyte progenitor (OPCs) and Sox10⁺ oligodendrocyte lineage cells in the developing brain of Olig2 misexpressing animals. In iTg-Nes mice at P7, we observed a substantial increase of PDGFR α ⁺ precursors in the developing cortex compared to the control (Figure 1F). Similarly, we detected a drastic increase of PDGFR α ⁺ OPCs and Sox10⁺ oligodendrocyte lineage cells in the midbrain (Figure 1G), consistent with the notion that Olig2 overexpression promotes the differentiation of neural stem/progenitor cells into oligodendrocyte lineage cells (Coprav et al., 2006; Maire et al., 2009; Yao et al., 2014).

Mice with Olig2 misexpression in neural progenitors exhibit microcephaly during cortical development

Despite an increase of oligodendrocyte lineage cell generation, iTg-Nes mice exhibited severe reduction in forebrain size during development and at adulthood (Figure 2A,B), while the midbrain appeared to be enlarged compared to the control. When examining the cortex at prenatal and postnatal stages, we observed a decrease in the thickness of the cortex in iTg-Nes mice compared to controls at E18.5, P7, P14 and adulthood P60 (Figure 2C,D). Similarly, we detected a diminution of the hippocampus (Figure 2E). In addition, the cerebellum of the iTg-Nes mice was smaller with impaired foliation and cerebellar architecture (Figure 2F). The results suggest that Olig2 misexpression causes global defects in brain development. Similar phenotypes were observed in three additional iTg transgenic founder lines when crossing with the nestin-Cre line or cortical progenitor cell-expressing hGFAP-cre line (data not shown). These results suggest that misexpression of Olig2 in cortical stem/progenitor cells develops microcephaly beginning at the late gestation stage and persisting into adulthood.

Olig2-misexpressing mice develop cortical dyslamination

Given that Olig2 misexpression in neural progenitors caused a defect in brain development, we then examined cortical neuron development with cortical layer-specific markers Cux1 (layers II–III), Brn1 (Layers II–III), Ctip2 (V) and Er81 (V–VI) at neonatal stages. The population of Cux1-expressing and Brn1 expressing layers II/III neurons was substantially reduced in the cortex of iTg-Nes mice as compared to the control at P7 (Figure 3A–C, arrows). In contrast, the deep layers labeled by Ctip2 and Er81 were expanded into superficial cortical layers (Figure 3A–C, arrowheads). Quantification indicates that the decrease in the upper cortical layers is accompanied by a concomitant increase of neuronal subtypes in the deep cortical layers of iTg-Nes mice (Figure 3D,E). In addition, the

calretinin expressing interneuron in layers II-III is substantially disrupted in the cortex and leading to apparent dispersal of calretinin expressing cells in the superficial cortical layers of the iTg-Nes cortex (Figure 3F). Conversely, we observed an expansion of parvalbumin⁺ and calbindin⁺ inhibitory interneurons in the cortex of iTg-Nes mice (Figure 3G,H), consistent with the increase of inhibitory neurons in the Ts65Dn brain (Chakrabarti et al., 2010). The cortical dyslamination did not simply reflect a developmental delay since cortical defects were observed from prenatal stages to adulthood. Consistent with reduced superficial cortical layers that are the principal origin of callosal projection neurons (Jensen and Altman, 1982), the iTg-Nes brain displays frank agenesis of the corpus callosum (Figure 3I). Furthermore, the thickness of the external capsule white matter tract of which cortical efferent axons are a principal component is much reduced in the iTg-Nes cortex (Figure 3J).

Olig2-misexpression causes a proliferation defect of neural progenitor cell and precocious cell cycle exit in the developing cortex

The reduction in cortical size suggested that generation, differentiation, and/or survival of cortical cell precursors were impaired in the iTg-Nes mice. To determine the proportion of proliferating cells in the cortex, we examined expression of a proliferative marker, Ki67, a protein expressed in all phases of cell cycle except resting cells (G0) (Scholzen and Gerdes, 2000). We found a significant reduction of Ki67⁺ progenitors in the iTg-Nes cortex compared to the control at E13.5 (Figure 4A,B). Similarly, the number of 5-bromo-2'-deoxyuridine (BrdU)-positive proliferative cells after BrdU pulse labeling for 30 min was also reduced in iTg-Nes cortex (Figure 4A,C), suggesting that Olig2 misexpression results in a defect in cortical progenitor proliferation.

To further investigate effects of Olig2-misexpression on cell cycle progression of cortical progenitors, we analyzed the retention of progenitor cells in the cell cycle of control and iTg-Nes cortices. We performed sequential injections of BrdU and 5-ethynyl-2'-deoxyuridine (EdU) at E13.5. At t = 0h, BrdU was intraperitoneally injected and labeled all proliferative cells at the beginning of the experiment. At t=1.5h, EdU was injected to label all cells in S phase at the end of the experiment. The mouse was sacrificed at t = 2h and embryos were dissected out, processed for immunostaining with a BrdU specific antibody and EdU detection assay. With this system, cells labeled with BrdU and not EdU, have left S phase from the initial cohort of cells (Figure 4D). In the iTg-Nes cortex, we found that the percentage of cells that remained in the S-phase (the ratio of EdU⁺ cells among BrdU⁺ cells) was reduced (Figure 4E), however, the proportion of the cells that have exited S phase (the ratio of BrdU⁺/EdU⁻ cells among BrdU⁺ cells) increased (Figure 4F). BrdU⁺/EdU⁻ cells that migrated into the upper cortical layers were much more extensive compared to the control (Figure 4G). These observations suggest that misexpression of Olig2 accelerates the cell cycle exit of cortical progenitors, causing a precocious completion of S phase and proliferation defects.

Massive neuronal cell death in the cortex of Olig2-misexpressing mice

To determine the potential cause of developmental defects in the brain of iTg-Nes mice, we analyzed neural cell survival during cortical development. The neocortices of control and iTg-Nes mice at E14.5 were examined by immunostaining with an antibody to the cleaved

active form of Caspase-3, a marker for apoptotic cells. Caspase 3-positive apoptotic cells were hardly detectable in either wild-type or control iTg cortex at E14.5, in contrast, a large population of Caspase 3-positive cells were observed in the cortex of iTg-Nes embryos (Figure 5A,B).

Since neurogenesis is the major event in the developing cortex at E14.5, we then examined whether apoptosis in the iTg-Nes cortex occurred neuronal cells. Co-immunostaining of Caspase3 with a pan-neuronal marker Tuj1 demonstrated that majority of Caspase 3⁺ apoptotic cells were Tuj1⁺ neuronal cells (Figure 5A,B).

Strikingly, DAPI (4'-6-Diamidino-2-phenylindole) nuclear counterstaining revealed that the majority of cells in the VZ of Myc-Olig2 expressing iTg-Nes embryos had condensed and small nuclei compared to the control (Figure 5C-E). The cells with condensed nuclei were detected with activated Caspase 3 expression (Figure 5E, F), suggesting that they were undergoing an apoptotic process. Gene expression profiling analysis of the developing cortex of control and Olig2-misexpressing embryos at E14.5 by Affymetrix microarray (Supplementary table 1) indicated that there was a significant increase in pathways associated with apoptosis (Figure 5G). We accordingly detected that the pro-apoptotic genes, including *Casp3*, were substantially upregulated in Olig2-misexpressing cortices (Figure 5H), consistent with extensive Caspase 3 immunolabeling as shown in Figure 5C. These observations suggest that ectopic expression of Olig2 in neural stem/progenitor cells activates expression of pro-apoptotic genes and leads to a cell death process, thereby affecting the survival of neural progenitor cells.

To determine whether misexpression of Olig2 in other cell types could also induce cell death, we bred iTg mice with Desert hedgehog (Dhh)-Cre line, in which Dhh promoter directed the Cre expression in the Schwann cell precursors (Mirsky et al., 1999). In the sciatic nerves of iTg-Dhh-Cre mice, Schwann cells were developed normally; the number of cells expressing Schwann cell markers Krox20 and Sox10 was comparable to the control (Figure 6A). We did not detect any significant Schwann cell death assayed by the active form of Caspase 3 (Figure 6B), suggesting that cell death induced by Olig2 misexpression is a cell-type specific event.

Olig2 overexpression in the cortex leads to downregulation of proneural and neuronal differentiation genes

To investigate the impact of Olig2 overexpression on cortical neurogenesis, we analyzed gene expression profiling from the developing cortex of control and iTg-Nes embryos at E14.5 by Affymetrix microarray analysis. We detected a substantial alteration of the genes that regulate neurogenesis (Figure 7A). qRT-PCR analysis showed a significant downregulation of proneural and neuronal differentiation genes including *Ngn1*, *Ngn2*, *Neurod1*, *Neurod4*, *Neurod6*, *Pax6*, *Bhlhb5*, *Tbr2/Eomes*, *Fzf2/Zfp312* and *Lhx2*, as well as *Nfatc4* (Figure 7B and Supplemental Table 1). *Nfatc4* dysregulation could lead to neurological features of Down syndrome (Arron et al., 2006). *In situ* hybridization analysis further confirmed that expression of pro-neurogenic genes such as *Ngn1*, *Ngn2* and *Pax6* was severely reduced in the cortex (Figure 7C-E). To determine the direct target of Olig2, we analyzed genomic occupancy by Olig2 in OPCs using ChIP-seq (Yu et al., 2013). We

found that Olig2 occupied the promoter and/or enhancer regions of *Nfatc4*, *Pax6*, *Dyrk1a*, and *Dscr1/Rcan1*, the key genes that regulate neurogenesis and contribute to DS phenotypes (Figure 7F-I). This suggests that Olig2 could directly target the enhancer/promoters of neurogenic genes and inhibits their expression. In addition, many of downregulated genes encode bHLH proneural factors such as *Ngn1*, *Ngn2* and *NeuroD1*, which likely share common binding partners, like E proteins. Excessive and ectopic bHLH Olig2 might titrate away these essential binding partners of neuronal bHLH factors in neural progenitors (Ross et al., 2003; Sun et al., 2003), thereby inhibiting their activity for neurogenesis. Our data suggest that misexpression of Olig2 in neural progenitor cells impairs cortical neurogenesis at least in part by inhibiting expression of proneural and neuronal differentiation genes.

Locomotion and behavior abnormalities in Olig2-misexpressing transgenic mice

Because abnormal cortical development in rodents is well known to cause impaired locomotor activity (Fillat et al., 2010), we evaluated motor function in control and iTg-Nes transgenic mice. Mice (8-10 wk old) were trained on the Rotarod (Shiotsuki et al., 2010) and then tested for their ability to stay on the accelerating rod. The iTg-Nes animals had a significantly reduced capacity of coordinated motor performance compared to controls (Figure 7J). No significant difference was seen between wild type and control mice (data not shown). To further confirm motor impairment, we carried out the hanging wire test. Control mice could perform the task robustly, while iTg-Nes mice performed poorly in this test (Figure 7K), suggesting a deficit in motor control in iTg-Nes mice. The locomotor dysfunction observed in the iTg-Nes mice appears to correlate with persistent defects in cortical and cerebellum development in adult mice. Since the mice were subject to 3 training days before the Rotarod test, the control group displays a gradual improvement in latency to fall. In contrast, the iTg-Nes mice fail to improve. These behavior defects in locomotor functions are consistent with severe cortical, hippocampal and cerebellar defects in iTg-Nes transgenic mice.

Discussion

In this study, we show that transgenic mice with Olig2 misexpression in cortical neural stem/progenitor cells develop microcephaly through triggering neural cell death and inhibiting cortical neurogenesis. iTg-Nes mice develop characteristic neurological deficits seen in other DS animal models including neuronal degeneration alongside reduced cell proliferation and differentiation in the neocortex, cerebellum and hippocampus, as well as learning and behavioral deficits (Amano et al., 2004; Chakrabarti et al., 2010; Clark et al., 2006; Contestabile et al., 2007; Haydar and Reeves, 2011; Reeves and Garner, 2007). Given the location of Olig2 in the DSCR (Chakrabarti et al., 2010) and phenotypic similarities between iTg-Nes transgenic mice with Ts65Dn mice and DS patients, and the fact that OLIG2 is significantly upregulated in DS frontal cortices at 14 week and 18 week gestational ages (Lu et al., 2012), our studies suggest that ectopically increased Olig2 dosage in cortical neural progenitors may contribute to the neurological phenotypes observed in DS patients.

Ectopic Olig2 overexpression disrupts cortical neurogenesis

The analysis of progenitor development of Olig2-misexpressing embryos reveals a significant reduction in expansion and differentiation of cortical progenitor cells, suggesting that cortical development defects begin at embryonic stages. The high proportion of progenitor cells precociously exits cell cycle may lead to a depletion of progenitor pools in the Olig2-overexpressing cortex. These data suggest that Olig2 misexpression impairs the cell cycle progression of VZ progenitor cells and causes a reduction of neural progenitor cells. Although Olig2 is expressed only in a small set of neural stem cells during normal brain development, under certain pathophysiological conditions, such as trisomy 21, Olig2 upregulation was observed in human DS neural stem cells (Lu et al., 2012). Thus, our transgenic mice with Olig2 overexpression in neural stem/progenitors may be relevant to the certain aspect of brain disorders with OLIG2 upregulation in neural stem cells such as DS.

In the brain of Olig2-misexpressing transgenic mice, the reduced cortical size persists to adulthood, and normal cortical layering and lamination are disrupted. Particularly, we observe the number of Cux1- or Brn1-expressing excitatory neurons in the superficial cortical layer II and III is substantially reduced. Conversely, we observed an increase in the number of cortical inhibitory neurons marked by parvalbumin and calbindin in Olig2-misexpressing animals, consistent with the observation in Ts65dn mice (Chakrabarti et al., 2010). These observations suggest that Olig2 might have a role in asymmetric cortical lamination, or that the progenitor pool undergo apoptosis considerably such that there are too few progenitors to generate the upper cortical layers, leading to laminar disorganization. This phenotype is consistent with the observation of underproduction of excitatory neurons, which comprise the bulk of cortical neurons, in DS fetal brain (Contestabile et al., 2007; Guidi et al., 2008) and Ts65Dn cortex (Chakrabarti et al., 2010; Haydar and Reeves, 2011). These observations are in keeping with the notion that the imbalance between excitation and inhibition activities of cortical neurons may contribute to behavioral abnormalities in DS. In addition, we observed hippocampal hypocellularity and reduced volume of the dentate gyrus. Hippocampal volume is markedly reduced in infants and young adults with DS (Pinter et al., 2001; Sylvester, 1983) and in Ts65Dn mice (Chakrabarti et al., 2007). The comparable morphometric changes noted in the brain of Olig2-misexpressing mice with Ts65Dn mice (Chakrabarti et al., 2007) and DS patients (Pinter et al., 2001), with respect to cortical and hippocampal development, suggest that ectopic Olig2 misexpression in neural stem/progenitors may contribute to neurogenesis defects seen in DS. In contrast to cortical neurogenesis defects, we observed a substantial increase of OPCs and oligodendrocyte lineage cells in the Olig2-overexpressing brain, which is consistent with a critical role of Olig2 in oligodendrocyte specification. The increase of oligodendroglial genesis might, at least in part, contribute to the enlarged midbrain detected in the Olig2-overexpressing mice.

Olig2 misexpression induces apoptosis in cortical progenitors

A universal feature of the Down syndrome phenotype is mental retardation resulting, in part, from age-related neuronal degeneration (Holtzman et al., 1996). Neuronal degeneration and neuronal cell death are the major contributing factors in the pathogenesis of DS (Busciglio and Yankner, 1995) and in trisomy 16 DS mice (Bambrick and Krueger, 1999; Hallam and Maroun, 1998; Stabel-Burow et al., 1997). Strikingly, we observe massive neuronal

apoptosis in the cortex of Olig2-overexpressing transgenic mice during early corticogenesis, which provides the first *in vivo* evidence that Olig2 misexpression causes cortical neuronal cell death during mammalian brain development. Although the phenotype of Olig2 misexpressing transgenic mice does not completely recapitulate that in DS, our study indicates that Olig2 misexpression in neural stem/progenitor cells can cause cortical neuronal cell death during mammalian brain development, thereby contributing to the reduced neuronal numbers and brain size in DS caused by trisomy 21. At present, the mechanisms underlying Olig2-misexpression induced neuronal death are not known. Since Olig2 is a glial cell specification factor, when ectopic overexpression in the neural stem/progenitors at active neurogenesis phases, it might present as a conflict cue for neurogenesis and cause abnormal neuronal development and cell death. Considering retrovirus-mediated misexpression of *Olig1*, which links with *Olig2* within 36 kb in the DS critical region, could also cause neuronal cell death, trisomic DS may represent excessive dosage of both *OLIG* genes, which may produce a significant high level of OLIG proteins including OLIG2 (Lu et al., 2012). Thus, the increase in the dosage of Olig1 and Olig2 may act synergistically to induce neuronal cell death and impair brain development.

It is possible that Myc-tag may interfere Olig2 function, however, we observe an increase of OPCs in the developing cortex of iTg-Nes mice, suggesting that Myc-Olig2 is functional to promote oligodendrogenesis. Our data suggest that Olig2 misexpression inhibits neurogenesis from cortical stem/progenitor cells at least in part by inhibiting proneural bHLH factors and neuronal differentiation genes including *Nfatc4*, a crucial gene for DS phenotypes (Arron et al., 2006). This is consistent with a role of Olig2 as a repressor of neurogenesis in cells reacting to brain injury (Buffo et al., 2005). Thus, the Olig2 ectopic gain-of-function approach reveals a new complex role of Olig2 misexpression in affecting corticogenesis during embryonic development. The severe defects in cortical neurogenesis, hippocampal and cerebellar development in iTg-Nes mice may predispose animals to cognitive, motor disabilities in adulthood. In conclusion, the present study provides *in vivo* evidence that ectopic increase of Olig2 dosage in neural stem cells causes neuronal apoptosis and neurogenesis defects, suggesting that the increase of Olig2 dosage in trisomy 21 may contribute to neurodegeneration and DS brain pathology. The dosage increase of genes such as *Ets2* and *Dyrk1A* in DSCR regions in mice was shown to confer behavioral, neurophysiologic phenotypes characteristic of DS (Park et al., 2007; Wolvetang et al., 2003). Thus, dosage imbalances of one or a few genes in trisomy 21 may make a conspicuous contribution to neurobiological and behavior deficits in DS patients (Belichenko et al., 2009; Chakrabarti et al., 2010; Dowjat et al., 2007; Haydar and Reeves, 2011; Reeves et al., 1995). The new inducible Olig2 transgenic mice may provide an important resource to elucidate both simple and complex genetic contributions to DS neurological phenotypes. Further elucidation of the molecular pathways that control the biologically significant and additive genes including Olig2 mediated neuronal apoptosis may open new avenues for the treatment of neurodegenerative disorders including DS.

Materials and Methods

Generation of inducible *Olig2* overexpressing transgenic mice

To generate the transgenic construct, *Myc*-tagged *Olig2* was placed after the ubiquitous CAG promoter. The transgene segment is followed by loxP-flanked *LacZ* stop segment, which maintains the *Olig2* transgene in a transcriptionally-silent state. The *NotI* fragment carrying the transgene was purified by elutip-D columns and injected into pronuclei of mouse fertilized B6SJLF1/J oocytes to produce transgenic founders. *Olig2* expression is triggered by mating the transgenic mice to a Cre mouse line under the control of tissue specific promoters. Progeny from four founder mice carrying *Olig2* transgene gave rise to the same phenotype after mating with hGFAP-Cre or Nestin-cre driver lines (Jax laboratories). Dhh-Cre line is kindly provided by Dr. Dies Meijer. The data presented are derived from the progeny of a single transgenic line. All protocols involving the use of animals were approved by the Institutional Animal Care and Research Advisory Committee (IACUC) at the UT Southwestern Medical Center at Dallas and the Cincinnati Children's Hospital Medical Center.

Immunohistochemistry and Nissl staining

Control and iTg-Nes brains from embryonic and postnatal stages were harvested from anesthetized mice. They were fixed in 4% paraformaldehyde at 4°C for 1 hour, cryoprotected in 25% sucrose in PBS overnight, embedded in OCT, and cryosectioned at 12 μ m. Immunostaining methods with tissue sections from mouse brains were as described previously (Lu et al., 2002). Double immunostaining was performed by simultaneous incubation with the antibodies of interest. The following antibodies were used: Myc (Millipore, 06-340), *Olig2* (Millipore), Active caspase3 (Cell Signaling Technology), Tuj1 (Babco), *Cux1* (Sigma) *Ctip2* (Abcam, ab18465), *Brn1* (Santa Cruz, sc-6028-R), *Er81* (Covance, PRB-362C), *Calretinin* (Abcam, ab702), *Parvalbumin* (Swant, PV25), *Calbindin* (Chemicon, ab1778), *PDGFR α* (BD Pharmingen, 558774), *Sox10* (Santa Cruz, sc-17342), *BrdU* (mouse anti-BrdU, BD sciences, 347580; rat anti-BrdU, Abcam, ab6326), *Ki67* (rabbit anti-Ki67, Thermo scientific, RM-9106-S1), *Krox20* (Covance, PRB236B). Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to Cy2 and Cy3 (Jackson ImmunoResearch, West Grove, PA) were used for double-labeling experiments. For *BrdU* staining, *BrdU* were injected with 100 mg/kg body weight at defined periods prior to sacrifice. For Nissl staining, the sections were stained with cresyl violet acetate for 5 min. *EdU* staining was performed with Click-iT® Assay Kits according to the manufacture instruction (Life technologies, Inc.).

RNA *in situ* hybridization

Control and iTg-Nes brains from embryonic and postnatal stages were harvested from anesthetized mice. They were fixed in 4% paraformaldehyde at 4°C overnight, cryoprotected in 25% sucrose in PBS overnight, embedded in OCT, and cryosectioned at 16 μ m. Digoxigenin-labeled riboprobes were used to perform RNA *in situ* hybridization as described in Yue et al. (2006). Probes used were as follows: *Olig2*, *Ngn1*, *Pax6* and *Mash1*.

Microarray and qRT-PCR analysis

For gene-chip microarray, RNAs from the cortices of control and iTg-Nes embryos at E14.5 were processed for microarray analysis (Affymetrix gene-chip, ST1.0) at the NIH consortium UCLA microarray core facility. qRT-PCR was performed using the ABI Prism 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems) as previously described with *Gapdh* (glyceraldehyde-3-phosphatase dehydrogenase, TaqMan kit, Applied Biosystems) as an internal control (Xin, *et al*, 2005). Primer sequences used for expression analyses are mouse Olig2 (F1: gcgagcacctcaaatctaattc, R1: aaaagatcatcgggttctggg); Ngn1 (F: atcccctttctccttctctg; R: cttcagccagttcccatc); Ngn2 (F: tcgccagggactgtatctag; R: ctgctctgtgaagtggagtc); Pax6 (F: cgggacttcagttaccagg; R: cttcatccgagtcttctccg); NeuroD1 (F: acgcagaaggcaaggtgtc; R: cgctctcgctgtatgatttg); NeuroD4 (F: gccagagactgtggtactga; R: ccaccatgtccttggtattc); NeuroD6 (F: gccacttccttacgacttac; R: ttgccttaattagagtgaggagg); BHLHb5 (F: cctattcaacagcgtctctg; R: gcagcttctcacttctctctag); Fezf2 (F: tttgtggcaaaggctttcac; R: tcttgctgtgtgggtgtg); Lhx2 (F: atgccaaggactgaagcag; R: gtaaaaggttgccctgaac); Tbr2 (F: aaacacggatatcaccagc; R: ggcaaagtgttgacaaagg); Nfatc4 (F: cttctccccttgcttggtc; R: tgctcactggctgggttaa), β -actin (F: ctggctggccgggacgtgaca; R: accgctcgttgccaatagtgatga).

Accelerating Rotarod test

Performance on an accelerating Rotarod was assessed using a four-station mouse Rotarod (*AccuScan Instruments Inc., Columbia, Ohio, USA*). In the test, mice were placed on a rotating cylinder, and the cylinder was gradually accelerated with a 5 min period. Latency to fall is reported in seconds (s). Test animals were trained for 3 days and tested three times each day on 4 consecutive days with 1 hour intervals between testing trials. The mean latency to fall (s) of three trials at each time point on the cylinder was reported.

Wire-hang test

Mice were placed on a horizontal wire and were allowed to grab the wire. Each mouse was tested three times on 3 consecutive days, with an intertrial interval of 1 hour. All the testing animals were trained for 3 days before the test. The latency to fall off the wire was recorded. The average performance for each session is presented as the average of the three trials.

Statistical analysis

Results were analyzed for statistical significance using two-tailed Student's *t* test and all error bars were expressed as standard deviations ($\pm$ s.d.). Data are shown as means $\pm$ s.d. Values of $p < 0.05$ were considered significant. *P* values were derived using Student's *t*-test. For multiple comparisons, which were done using one-way analysis of variance analysis (ANOVA) with posttest: Newman-Keuls Multiple comparison test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Olig2 misexpression in neural stem cells causes severe defects in brain development

Olig2 misexpression leads to massive neuronal cell death and cortical dyslamination

Olig2 misexpression impairs proliferation and cell cycle exit of cortical progenitors

Olig2 targets the enhancers of neurogenic genes and inhibits cortical neurogenesis

Olig2-misexpressing transgenic mice exhibit locomotion deficits

Figure 1. Generation of inducible Olig2 overexpressing transgenic mice

(A) Conditional misexpression of Olig2 in transgenic mice. In this system, the ubiquitous CAG promoter driven Myc-tagged Olig2 gene expression is interrupted by loxP-flanked stop segments, which maintains Olig2 transgene in a transcriptionally-silent state. Myc-Olig2 expression is triggered in the F1 progeny by mating the transgenic mice to a Cre mouse line under the control of tissue specific promoters.

(B, C) Expression of Olig2 transgene in transgenic mice is regulated by Cre activity. The inducible Olig2 transgenic mice were crossed with nestin-Cre mice generate iTg-Nes mice,

which permit Olig2 misexpression in neural stem/progenitor cells. Immunostaining of Myc tag in control and iTg-Nes mice cortex at e14.5 (B) and P7 (C).

(D) Expression of Olig2 in the cortex of control (iTg) and iTg-Nes animals at E18.5 was examined by immunostaining.

(E) qRT-PCR assay of Olig2 mRNA level in the cortex of control (iTg) and iTg-Nes animals at E14.5 and P8 and P38. Data represent the mean $\pm$ SEM ($n = 3$ animals). $**p < 0.01$; Student's t test.

(F) The Cortex of control and iTg-Nes mice at P7 were immunostained with PDGFR α . Boxed regions in left panels were shown in the right.

G) Midbrains of control and iTg-Nes mice at P7 were immunostained with PDGFR α or Sox10 as indicated.

Scale bars in A–D, 50 μ m; F,G; 100 μ m.

Figure 2. Developmental Brain Defects in iTg-Nes mice

(A,B) Brain images of control and iTg-Nes mice at P14 (A) and P60 (B).

(C) Relative thickness of the cortex in iTg-Nes vs. control littermates at the stages as indicated. (n =3, * $P < 0.05$; ** $P < 0.001$; Student's t -test).

(D-F) Nissl staining of coronal sections of control (iTg) and iTg-Nes cortices at prenatal and postnatal stages as indicated (D), and adult hippocampus (E) and cerebellum at P60 (F).

Scale bars in C, E, F: 100 μ m.

Figure 3. Disruption of cortical lamination in iTg-Nes mice

(A-C) Coronal sections of cortices of control and iTg-Nes mice at P7 were immunostained with different layers markers Cux1 (II–III), Brn1 (II–III), Ctip2 (V) and Er81 (V–VI) as indicated. The corresponding boxed regions in A and B were shown at a high magnification in the right panels. Arrows and arrowheads in B and C indicate Cux1 or Brn1 (arrows) and Ctip2 or Er81 (arrowheads) positive cells, respectively. Approximately layers were indicated in the right side of the panels A and B.

(D, E) Quantification of the number of labeling cells as indicated in A-C in the cortex/area (0.05 mm²) in control and iTg-Nes littermates at P7. (n =3; * $p < 0.05$; ** $p < 0.01$; t -test). (F-H) Cortices of control and iTg-Nes mice at P7 were immunostained with antibodies to calretinin, parvalbumin or calbindin. Boxed regions were shown in the right side of corresponding panels. Arrows indicate immuno-positive cells in the cortex. (I,J) Coronal sections of cortices of control and iTg-Nes mice at P14 were subject to Nissl staining. Arrows in H indicate the presumptive corpus callosum region. The vertical bar scales in I indicate the width of the cerebral white matter tract. Scale bars in A-C; 50 μ m; F-I: 100 μ m.

Figure 4. Reduction of progenitor cell proliferation and precocious cell cycle exit in the developing cortex of iTg-Nes mice

(A,B) Cortices of control and iTg-Nes mice at E13.5 after 30 min BrdU pulse-labeling were immunostained with antibodies to BrdU and Ki67.

(C) The bar chart showing the average number of Ki67 positive cells per unite area (0.05 mm^2) in the ventricular zone (VZ) of control and iTg-Nes cortices in A. ($n=3$; ** $p < 0.01$, t -test).

(D) A diagram showing BrdU and EdU injection scheme and cell cycle progression.

(E) The percentage of EdU-positive cells among BrdU⁺ progenitors in the cortex of control and iTg-Nes animals at E13.5 (n=3; * $p < 0.05$, *t*-test).

(F) The proportion of BrdU⁺ EdU⁻ cells among BrdU⁺ progenitors in the cortex of control and iTg-Nes animals at E13.5 (n=3; * $p < 0.05$, *t*-test).

(G) Cortical sections of control and iTg-Nes mice at E13.5 after sequential BrdU and EdU injections as in D were immunostained with the BrdU antibody and stained with EdU.

Arrow indicates the BrdU⁺ cells present in upper cortical layers.

Scale bars in A, G, 50 μm .

Figure 5. Massive neuronal cell death in the developing cortex of iTg-Nes animals

(A) The cortices of control and iTg-Nes embryos at E14.5 were immunostained with apoptotic cell marker, cleaved caspase-3 (red) and pan-neuronal marker Tuj1.

(B) The boxed region of panel A was shown in a high magnification in B. Arrows indicate the neuronal cells co-labeled with activated caspase-3 and Tuj1.

(C) The cortices of control and iTg-Nes embryos at E14.5 were immunostained with Myc (red), apoptotic cell marker cleaved caspase-3 and counterstaining of nuclei with DAPI (blue).

(D-F) The boxed regions in control and iTg-Nes cortices in panel C were shown in a high magnification in D and E, F as indicated. Arrows indicate the apoptotic cells stained with activated caspase-3 with small, condensed nuclei.

(G) ToppGene ontology analysis of expression profiling of the developing cortex of control and Olig-iTg embryos at E14.5 indicated a significant increase of pathway components associated with apoptosis.

(H) Pro-apoptotic genes, including *Casp3* and 26S protease subunits (*Psm*), were upregulated in Olig2-iTG through gene-chip microarray analysis of cortices of control and Olig-iTg embryos at E14.5 ($p < 0.05$)

Scale bars in A, C 50 μm ; in B, 20 μm ; in D 25 μm .

Figure 6. Normal Schwann cell development in mice with Olig2 misexpression in Schwann cell precursors

(A-B) The sciatic nerve sections of control of iTg-Dhh-Cre mice P70 were immunostained with antibodies to Schwann cell markers Krox20 and Sox10 (A) as well as caspase3 (B). Scale bars in A-B, 100 μ m.

Figure 7. Olig2 overexpression leads to downregulation of proneural and neuronal differentiation genes and behavior abnormalities

(A) Gene ontology analysis indicates a significant alteration of the genes that regulate neurogenesis.

(B) Expression of proneural and neuronal differentiation genes as indicated in the cortex of control and iTg-Nes animals at E14.5 were analyzed by qRT-PCR. Gapdh was used as an internal control. (n = 3, * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$; t -test).

(C-E) Expression level of *Ngn1*, *Ngn2* and *Pax6* in the cortex of control and iTg-Nes animals was assayed by mRNA *in situ* hybridization.

(F-I) Genome browser view of the distribution of Olig2 (red) and IgG control on the loci of *Nfatc4* (F), *Pax6* (G), *Dyrk1a* (H), and *Dscr1/Rcan1* (I) in OPCs by ChIP-seq.

(J,K) Behavior test for locomotion for control and iTg-Nes adult animals. Rotarod (J) and wire hanging (K) tests were performed in control and iTg-Nes mice (n=15) at 8-10 weeks old mice. The latency to fall off was recorded. ($p < 0.01$ in both behavior tests, one-way ANOVA)

Scale bars in C-E, 100 μ m