p53 Regulates Neural Stem Cell Proliferation and Differentiation via BMP-Smad1 Signaling and Id1

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Neural stem cells (NSCs) play essential roles in nervous system development and postnatal neuroregeneration and their deregulation underlies the development of neurodegenerative disorders. Yet how NSC proliferation and differentiation are controlled is not fully understood. Here we present evidence that tumor suppressor p53 regulates NSC proliferation and differentiation via the bone morphogenetic proteins (BMP)-Smad1 pathway and its target gene inhibitor of DNA binding 1 (Id1). p53 deficiency led to increased neurogenesis in vivo, and biased neuronal differentiation and augmented NSC proliferation of ex vivo NSCs. This is accompanied by elevated Smad1 expression/activation in the brain and NSC, which contributes to accelerated neuronal differentiation of *p53*^{-/-} NSCs. p53 deficiency also leads to upregulation of Id1, whose expression is repressed by p53 in BMP-Smad1-dependent and -independent manners. Elevated Id1 expression contributes to augmented proliferation and, unexpectedly, accelerated neuronal differentiation of *p53*^{-/-} NSCs as well. This study reveals a molecular mechanism by which tumor suppressor p53 controls NSC proliferation and differentiation and establishes a connection between p53 and Id1.

Introduction

P⁵³ IS A PROMINENT TUMOR suppressor that is mutated in more than 50% of the primary tumors in human [1]. Its anti-proliferation function is well studied, with its target gene p21 mediating the cytostatic effect and Bax/Puma mediating the cytotoxic effects [2]. In addition, p53 also plays a role in somatic stem cell maintenance as well as the reprogramming of induced pluripotent stem cells [3]. p53 deficiency usually leads to overproliferation of adult stem cells, whereas upregulation of p53 results in depletion of the stem cells. In accordance with its roles in adult stem cells, recent studies suggest that p53 has a pro-aging function [2].

Neural stem cells (NSCs) are responsible for early nervous system development and postnatal nervous tissue regeneration and repair [4–6]. They are located in several regions of the adult brain—in particular, the subventricular zone and the hippocampus. Upon the stimulation of unknown cues, NSCs differentiate into neurons or glia cells and thus provide new parts for the brain [6]. Defects in NSC compartments, including maintenance/renewal, migration, differentiation, and death, are believed to underlie various diseases of the

nervous system, including neurodegeneration and psychiatric disorders [7].

One of the major pathways controlling neurogenesis is the bone morphogenetic proteins (BMP)-Smad1/5/8 pathway [8,9]. They execute their functions by binding to and activating BMP receptors I and II, with the former phosphorylating Smad1, 5, and 8 at the C' terminal SXS motif. Smad1/5/8 then associate with Smad4, move into the nucleus, and turn on BMP target genes. BMPs play various and sometimes distinct roles throughout the development of the nervous system, often in a context and stage dependent manner [10]. In mouse embryonic stem (ES) cells, BMPs act to inhibit neuronal differentiation and help self-renewal [11]. In early embryogenesis, BMPs inhibit neuroectoderm formation [12,13], whereas in later neural differentiation, BMPs promote the differentiation of both neural cell types and astroglial cells [14–16]. Recent studies show that BMPs help to keep NSC cells at quiescent status in adult mice. Blockade of BMPs action with Noggin- or tissue-specific inactivation of BMPRIA led to eventual decrease of the NSCs in hippocampus compartment [17]. However, little is known about the BMP target genes that control neurogenesis, with the

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exception of inhibitor of DNA binding 1 (Id1). Id1 binds to the pro-neuronal transcription factors such as Mash1 to inhibit their functions. Thus, Id1 appears to mediate the inhibitory effects of BMPs on neuronal differentiation, at least in mouse ES cells [11].

p53 has been implicated in the nervous development [18,19]. In vivo studies have shown that p53 mRNA levels and promoter activity are increased during neuronal progenitor cell differentiation [20,21]. p53 null mice (about 16%) develop exencephaly, which is caused by overproliferation of neurons or lack of apoptosis of neurons [22]. It is possible that other members of the p53 family, p63 and p73, provide compensatory functions in the unaffected p53 null mice. Recently, p53 has been shown to negatively control the proliferation of NSCs and p53 deficiency facilitates glioblastoma development via Myc and p21 [23]. However, it is still uncertain how p53 regulates neural differentiation as conflicting results have been reported (reviewed in [18,24,25]). While several studies show that p53 inhibits neuronal lineage, there are studies showing that p53 plays no effect on neuronal differentiation [23,26–30].

Here we further analyzed neurogenesis in $p53^{-/-}$ embryo/mouse and NSC proliferation and differentiation and found that p53 is highly expressed during neuronal differentiation, and that p53 appears to inhibit NSC proliferation and NSC neural differentiation. p53 deficiency results in an increase in 2 critical neurogenesis regulators, Smad1 and Id1, which contribute to the effects of p53 deficiency on NSC proliferation and differentiation. These findings demonstrate that p53 is a critical regulator of neural differentiation in vivo and uncover a potential mechanism by which p53 regulates neurogenesis.

Materials and Methods

Mice, primary NSC isolation, and culture

$p53^{-/-}$ mice (on 129S genetic background) were obtained from The Jackson Laboratory. $p53^{+/+}$ mice on C57BL/6 genetic background were generated by successive crosses to normal C57BL/6 mice. NSCs were isolated from E13.5 embryos following a standard protocol. Briefly, the brains were taken out from the embryos and the bodies were used for genotyping. The telencephalon was separated from the rest and was trypsinized for 5 min at 37°C. After 3 times washing in 5 mL Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (Invitrogen) supplemented with 2 mM L-glutamine, the cells were resuspended in 1 mL DMEM/F12 (1:1) and the cell number was counted. Cells were seeded at 2×10^5 cells/mL in DMEM/F12 (1:1) supplemented with 2% B27 (Gibco BRL), basic fibroblast growth factor (bFGF; 20 ng/mL; R&D), and epidermal growth factor (EGF; 20 ng/mL; R&D). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. After 5 days of incubation, neurospheres were harvested by centrifugation, dissociated using trypsin and EDTA (Sigma-Aldrich), and re-plated. NSCs were expanded as neurospheres. For differentiation studies, the neurospheres were dissociated and the cells were plated in poly-L-lysine-coated chamber slides or dishes in the presence of 2% fetal bovine serum (FBS). To compare the clonogenic potential of the $p53^{+/+}$ and $p53^{-/-}$ NSCs, soft agarose assays were performed. Briefly, 1×10^4 cells were suspended in 1 mL of 0.3% low-melting-point agarose (Sigma-Aldrich) dissolved in the DMEM/F12 containing bFGF, EGF, and B27 supplement and

plated on the top of a 1-mL underlayer of 0.6% agarose in DMEM/F12 in 12-well culture plates. One mL of the proliferation medium was added on the top of the solidified agarose. After 3 weeks of incubation in the humidified incubator with 5% CO₂ at 37°C, the cell colonies were visualized by staining with 150 μ L 3-(4,5)-dimethylthiazolyl-2,5-diphenyltetrazolium bromide (5 mg/mL) (Ameresco, Solon). To quantitate the clonogenicity, the colonies in each well were counted under a microscope and the percentage of cells forming colonies (>20 μ m in diameter) were calculated.

NSC siRNA transfection

Cell transfection with specific siRNAs (ON-TARGETplus Smart pool, SiGenome) was performed 24 h after seeding; for this purpose, cells were changed to antibiotic-free medium and transfected using DharmaFECT transfection reagent, following the manufacturer's instructions. DharmaFECT transfection reagent was removed 48 h later and cells were maintained for 1–3 additional days before being counted, fixed for immunocytochemistry or western blot, or used for further experiments.

Tissue and cell immunocytochemistry

E11.5, E13.5, and E17.5 embryos and day 3 pups were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight, and then equilibrated in 30% sucrose. The tissues were then frozen and coronally sectioned (8 μ m) using a cryostat microtome. Sections were dried on poly-L-lysine-coated slides and processed immediately for immunocytochemistry. These slides as well as cultured NSCs were blocked in 5% bovine serum albumin in PBS with 0.5% Triton X-100 for 30 min. Primary antibody were diluted in the same blocking solution and applied overnight at 4°C. Slides were then washed 3 times in PBS for 10 min and incubated with secondary antibodies at 1:100 in PBS for 1 h at room temperature. After washing 3 times in PBS for 10 min, the slides were incubated with 3,3'-diaminobenzidine at room temperature without light for 10 min. Slides were then dehydrated and mounted with neutral gums. For cell culture slides, secondary antibodies conjugated with fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate (Sigma-Aldrich) were used. The following antibodies were used: anti-beta-III Tubulin (Tuj1) (ab78078, 1:100, Abcam), anti-microtubule-associated protein 2 (MAP2) (05-346, 1:200, Millipore), anti-gial fibrillary acidic protein (GFAP) (mAB3402, 1:500, Millipore), anti-Nestin (ab11306, 1:100, Abcam), anti-NeuN (ABN78, 1:50, Millipore), and anti-O4 (mAB345, 1:200, Millipore).

Western blot analysis

Cells or mouse brains were lysed in TNEN buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 0.1% Triton X-100) supplemented with 1 mM NaF, Na₂VO₃, 1 mM phenylmethanesulfonyl fluoride, and 1 μ g/mL of aprotinin, leupeptin, and pepstatin. Protein concentration was determined using an assay (Bio-Rad Laboratories). Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). The antibodies against the following proteins were used for western blot: GFAP (mAB3402,

1:1000, Millipore), MAP2 (05-346, 1:1000, Millipore), Nestin (mAB5326, 1:1000, Millipore), p53 (2524, 1:1000, Cell Signaling), Id1 (sc-488, 1:500, Santa Cruz), p21 (556430, 1:500, BD), Actin (sc-81178, 1:1000, Santa Cruz), p-Smad1 (9511, 1:1000, Cell Signaling), Smad1 (9743, 1:1000, Cell signaling), and Tuj1 (ab78078, 1:500, Abcam).

Luciferase assay

The Smad1 promoter (fragments 1.0 or 2.0 kb from the start of transcription) was cloned into pGL3 (luciferase basic vector; Promega). P53 expression constructs, the promoter plasmid, and renilla plasmid (internal control) were co-transfected into HCT116 or MC3T3-E1 cells. Cells were harvested 24 h later, washed with PBS, and lysed with reporter lysis buffer (Promega). The luciferase activities were measured following the manufacturer's procedures and were normalized against the renilla activity. All transient expression in this assay was performed in triplicate.

RNA isolation and real-time PCR

Total RNA was isolated from cells growing on 60 mm dishes using TRIzol reagent (GIBCO BRL). Total RNA was subjected to DNase treatment (Ambion) and quantitated. Five μ g of total mRNA was reverse transcribed into complementary DNA (cDNA) using AMV (Roche) reverse transcriptase. The total reaction was used in the real-time polymerase chain reaction (PCR) to assay. The primers were designed following the instructions from Roche.

Quantitation of western blot results

Western blot results were scanned with a Molecular Dynamics scanning densitometer. The relative levels of protein of interest were then determined by measuring the intensity of the corresponding bands. All values were averages of cell cultures isolated from at least 3 $p53^{-/-}$ mice and their control littermates and were normalized to the constitutive expression of Actin. In all the quantitation data, the values of wild type (WT) were set as 1.0.

Statistical analysis

Each experiment was repeated with 3 or more mutant and control mice. Statistical analysis was performed using an unpaired *t* test (STATISTICA Software; StatSoft, Inc.). *P* values were provided for all in vivo results. Significant association was defined when $*P < 0.05$ compared with control.

Results

p53 plays an active role in neurogenesis

To reexamine a possible role for p53 in neural development, we first analyzed the expression of neuron and astrocyte-specific markers in the brain homogenates of 13.5-, 15.5-, and 17.5-day embryos of $p53^{-/-}$ and WT (of the same litter) on C57BL/6 background. The initiation of embryonic neurogenesis and gliogenesis is controlled by multiple developmental cues [4–6]. We found that in WT embryos, neuron markers Tuj1 and MAP2 and astrocyte marker GFAP were expressed at low levels at day 13.5, and the expression of Tuj1 and MAP2 was increased at day 15.5, while the ex-

pression of GFAP was increased at day 17.5 (Fig. 1A), confirming that neuron differentiation is earlier than that of glia. $p53^{-/-}$ brains showed much higher levels of Tuj1 and MAP2 but lower levels of GFAP than WT embryos (Fig. 1A), suggesting that p53 deficiency promotes neuron differentiation but inhibits astrocyte differentiation at this stage of development.

To substantiate these findings, we carried out immunohistochemical staining of neuronal and glial markers on brain sections. Although only 16% of the $p53^{-/-}$ embryos were reported to develop exencephaly, we found that all the 9 $p53^{-/-}$ brain sections showed more Tuj1 at day 11.5 embryonic cerebral cortex region (MAP2 undetectable) and more NeuN (another neuron marker) at the day 13.5 embryonic cerebral cortex region than WT littermates (Fig. 1B and 1C). On the other hand, a decrease in GFAP staining was observed on brain sections of E17.5 embryos compared to control littermates (Fig. 1D). Moreover, a 30% decrease in the number of GFAP-positive cells in the corpus callosum region of day 3 $p53^{-/-}$ pups and the CA3 region of the hippocampus of 2-month-old $p53^{-/-}$ mice were also observed compared to WT controls (Supplementary Fig. S1 and S2; Supplementary Data are available online at www.liebertonline.com/scd). The difference in the reduction of gliogenesis between embryos and pups/adult mice may be caused by compensatory mechanisms activated by p53 deficiency. Nevertheless, these in vivo results (Fig. 1A–C) indicate that p53 negatively regulates neurogenesis but permits gliogenesis.

We also found that p53 protein levels went down from day 15.5 and were almost undetectable at day 17.5 (Fig. 1A), consistent with the expression pattern of p53 mRNA [21]. This p53 expression pattern, together with our observation that $p53^{-/-}$ embryos showed an increase in neurogenesis, suggests that p53 might help to curtail neurogenesis before day 15.5 but permit neurogenesis afterward. Alternatively, the p53 action after day 15.5 may be taken over by other members of the p53 superfamily such as p63 and p73 [24]. The observations that the start of GFAP expression at day 17.5 was associated with a decline of p53 and that p53 deficiency impeded gliogenesis suggest that the p53 action may be taken over by other members of the p53 superfamily, or the decline of p53 may serve other purposes at this stage, for example, promoting cell expansion.

An active role for p53 in NSC proliferation and cell fate determination

A role for p53 in NSC differentiation is still debatable as some studies showed that p53 inhibits neural differentiation, whereas the others did not find such a role [26–28]. To further clarify this issue, we isolated NSCs from brains of day 13.5 $p53^{-/-}$ and WT embryos (C57BL/6 genetic background) of the same litter with a standard protocol [23,26,28,30]. NSCs from individual embryos formed spheres and were further expanded in proliferation medium containing bFGFs and EGFs. These cells stained positive for NSC marker Nestin in spheres or monolayers, regardless of the p53 status (Fig. 2A); once cultured in the differentiation medium, they started to express markers for neuron or glia (Fig. 2B), suggesting that these freshly isolated cells are undifferentiated NSCs. Judged from the spheres size, it is obvious that p53

deficiency led to increased NSC proliferation (see later results). Immunocytochemistry staining of NSC cultured in the differentiation medium showed that all 4 $p53^{-/-}$ NSC cultures analyzed differentiated into more MAP2-positive cells but fewer GFAP- or O4-positive cells than WT NSC cultures (Fig. 2B, and 2C). However, the percentage of differentiated cells was similar (49%) in WT and $p53^{-/-}$ NSC cultures, suggesting that p53 deficiency led to a shift in differentiation from glia to neuron (Fig. 2C). We did not find significant difference in apoptotic cells between $p53^{-/-}$ and WT NSC cultures under our experimental settings (Supplementary Fig. S3). We also cultured NSCs in medium containing only B27 to exclude possible influence of growth difference on differentiation as cell proliferation is limited in B27 medium. Similarly, we found that $p53^{-/-}$ NSC tend to differentiate into neurons, justified by western blot results of NSC differentiation markers, especially at 72 h (Fig. 2D), confirming an inhibitory role for p53 in neuronal differentiation, which might involve compensatory mechanisms due to the loss of p53. Note that, in general, NSC differentiation was analyzed after placing the cells in the differentiation medium for 3 or more days. We included early time points in our experiments to analyze Smad1 expression and phosphorylation and Id1 expression. Surprisingly, we detected low levels of Tuj1 in WT NSCs (but not in $p53^{-/-}$ NSCs) at early time points. One explanation for this is that GFAP expression might transiently respond to the components of B27 medium in a p53-dependent manner, which warrants further investigation. Although day 13.5 embryo rarely express GFAP in the brain, justified by western blot analysis of the brain homogenate (Fig. 1A), GFAP expression in the NSC cultures isolated from day 13.5 embryos is readily detectable. One possible explanation is that NSC themselves and the radial glia cells that exist in the in vitro NSC cultures express GFAP [31,32], whereas these cells are rather rare in day 13.5 brains. Alternatively, B27 medium (used in Fig. 2D) may affect gliogenesis and/or GFAP expression. The GFAP difference between $p53^{-/-}$ and WT NSC cultures appears to be greater when cultured in B27 than that with serum (Fig. 2C and 2D). These results, taken together, indicate that $p53^{-/-}$ NSCs differentiated into neurons at the sacrifice of astrocytes, supporting the conclusion drawn by Armesilla-Diaz et al. that p53 plays a negative role in NSC neural differentiation but a positive role in gliogenesis [26,28,30], but are inconsistent with 2 reports showing that p53 deficiency did not alter neural or glia differentiation of NSCs [23,27]. This discrepancy is not less likely to be caused by genetic backgrounds, as similar

results were obtained from NSCs isolated from $p53^{-/-}$ mice on 129 and C57BL/6 background (Supplementary Fig. S4 and Fig. 3).

We also observed that the levels of p53 were increased up to day 3 once cultured in the differentiation medium (Fig. 3A). This result, together with the p53 expression pattern during embryonic neurogenesis (Fig. 1A), suggests that one function of elevated p53 expression is to curb neuronal differentiation but promote gliogenesis. However, this finding is not consistent with a recent study showing that the level of p53 is not altered during NSC in vitro differentiation [33]. Further studies are needed to determine what causes this discrepancy.

$p53^{-/-}$ NSCs showed an increase in Smad1 expression and activation

How does p53 deficiency promote neuronal differentiation? A clue came from our mouse genetic studies of p53 in osteoblastogenesis and bone formation [34,35]. $p53^{-/-}$ mice show osteosclerotic phenotypes, with accelerated osteoblast differentiation due to upregulation of Runx2 and Osterix, 2 transcription factors essential for osteoblastogenesis [34,35]. Both Runx2 and Osterix were recently shown to be target genes of BMPs [36], which are critical regulators of the development of bone as well as the nervous system. This link encouraged us to test whether $p53^{-/-}$ NSCs have any alteration in BMP-Smad1 signaling during differentiation. We indeed found that WT NSCs display considerable levels of Smad1/5/8 phosphorylation when cultured in the differentiation medium, which quickly goes down, accompanied by a downregulation of Smad1 (Fig. 3A). The cues that elicit this change, as well as the change in p53 levels, are currently unclear. However, $p53^{-/-}$ NSCs maintained high levels of Smad1 protein and Smad1/5/8 activation at 6, 24, and 72 h in the differentiation medium, without showing alterations in the protein levels of Smad5 or Smad8 (Fig. 3A and Supplementary Fig. S5). Moreover, $p53^{-/-}$ embryos also displayed an upregulation of Smad1 and hyperactivation of Smad1 compared to WT littermates, justified by western blot analysis of the brain homogenates and immunohistochemical staining of p-Smad1 on brain sections (Fig. 3B and 3C). Comparison of the levels of p-Smad1 and the levels of Smad1 suggests that elevated expression of Smad1 contributes to the enhanced Smad1 activation (Fig. 3B).

Pervious studies showed BMP2 treatment did not alter the protein levels of p53 or the protein levels of p53 target gene p21 in several cell types [37]. However, we showed that the

FIG. 1. p53 deficiency led to an increase in neurogenesis at the sacrifice of gliogenesis during embryonic development. **(A)** Western blot analysis of the brain homogenates showed increased expression of neuron markers and decreased expression of astrocyte marker in the absence of p53, accompanied with an alteration in p53 expression. The brains of E13.5, 15.5, and 17.5 embryos were harvested and were homogenized. The protein levels of p53, glial fibrillary acidic protein (GFAP), neuron-specific class III beta-tubulin (Tuj1), and microtubule-associated protein 2 (MAP2) were analyzed by western blot. *Right panel:* quantitation data, with the value of normal cells set at 1.0. * $P < 0.05$ when $p53^{-/-}$ mice were compared to $p53^{+/+}$ mice. **(B,C)** The brain sections of $p53^{-/-}$ embryo showed more Tuj1- and MAP2-positive cells but fewer glia cells. The brains of E11.5 and E13.5 embryos were harvested, embedded, and sectioned. **(B)** Expression of Tuj1 (cerebral cortex, E11.5) and **(C)** NeuN (cerebral cortex, E13.5) were determined with immunocytochemistry. *Lower panels:* higher magnification of the inset (red box). *Arrow,* Tuj1 positive cells. **(D)** The brain (SVZ) sections of $p53^{-/-}$ embryo (E17.5) (*left panel*) showed a decrease in GFAP staining compared to control (*right panel*). The bigger red boxes at the *right bottom* show the lower magnification of SVZ region. Fig. 1D shows the higher magnification of the inset (*smaller red boxes*). Color images available online at www.liebertpub.com/scd

FIG. 2. p53 deficiency promoted neural differentiation of neural stem cells (NSCs). **(A)** Both $p53^{-/-}$ and wild-type (WT) NSC cultures showed Nestin staining in spheres (*upper panel*) and monolayer (*lower panel*). **(B)** Immunocytochemical staining showed that $p53^{-/-}$ NSC cultures tend to differentiate into neurons but not glia cells. WT and $p53^{-/-}$ NSC cells were cultured in differentiation medium for 5 days and then stained for cells positive for MAP2, GFAP, or O4. **(C)** Quantitation data of Fig. 2B showing the numbers of cells positive for MAP2, GFAP, or O4 per view under 10×20 ($n=4$). $*P<0.05$ when $p53^{-/-}$ cells were compared to $p53^{+/+}$ cells. **(D)** Western blot shows that $p53^{-/-}$ NSC cultures expressed more neuronal marker Tuj1 and less astrocyte marker GFAP, when cultured in the presence of B27. NSC isolated from E13.5 embryos were expanded in proliferation medium and then switched to B27 medium for different periods of time. Cells were harvested and the differentiation markers were determined by western blot. *Lower panel*: quantitation data, with the value of normal cells set at 1.0. $*p<0.05$ when $p53^{-/-}$ cells were compared to $p53^{+/+}$ cells. Only values at 24 and 72 h were shown. Color images available online at www.liebertpub.com/scd

loss of p53 leads to Smad1 upregulation (Fig. 3A and 3B), suggesting that p53 represses the expression of Smad1 under normal conditions. We found that in osteoblasts, p53 represses Smad1 expression via the well-established p53-p21-CDK-E2F1 pathway, and that Smad1 promoter has multiple binding sites for E2F1 (G. Ma et al., unpublished results). It is likely that p53 represses Smad1 expression with the same mechanism in NSCs and this needs further investigation.

Enhanced BMP-Smad1 signaling contributes to accelerated neuronal differentiation of $p53^{-/-}$ NSCs

The enhanced activation of Smad1 signaling is predicted to participate in neuronal differentiation of $p53^{-/-}$ NSCs, as BMPs (the main activator of Smad1/5/8) are known to regulate neurogenesis in cell context, localization, and stage-

FIG. 3. p53 deficiency led to upregulation of Smad1, hyperactivation of Smad1, and upregulation of inhibitor of DNA binding 1 (Id1) in NSCs and mouse brains. **(A)** Western blot shows an increase in Smad1 expression, Smad1/5/8 activation, and Id1 expression in p53^{-/-} NSCs compared to WT NSCs. NSCs were cultured in differentiation medium and the levels of Smad1, Id1, Actin, and p-Smad1/5/8 were determined by western blot. **Lower panel:** quantitation data, with the value of normal cells set at 1.0. *P < 0.05 when p53^{-/-} cells were compared to p53^{+/+} cells. Only values at 6, 24, and 72 h were shown. **(B)** p53^{-/-} brains also showed enhanced expression of Smad1 and Smad1/5/8 activation and Id1 expression. The experiments were carried out like Fig. 1A and the levels of Smad1, Id1, Actin, and p-Smad1 were determined by western blot. **Lower panel:** quantitation data, with the value of normal mouse set at 1.0. *P < 0.05 when p53^{-/-} brains were compared to p53^{+/+} brains. **(C)** The brain sections of E17.5 p53^{-/-} embryo showed more p-Smad1/5/8. The levels of p-Smad1/5/8 were determined with immunocytochemistry. The bigger red boxes at the right bottom show lower magnification of the cortical plate. Fig. 3C shows the higher magnification of the inset (smaller red boxes). Color images available online at www.liebertpub.com/scd

dependent manners [9,38–40]. To determine the function of BMPs in NSCs, we treated WT and p53^{-/-} NSCs with 50 ng/mL of BMP2, which led to an increase in Smad1/5/8 phosphorylation (Supplementary Fig. S6), and followed the differentiation process. Immunocytochemistry staining showed that BMP2 treatment increased the number of Tuj1-positive

cells and decreased the number of GFAP-positive cells, in the presence or absence of p53 (Fig. 4A). The greater effect of BMP2 on GFAP-positive cells in WT than in p53^{-/-} NSCs could be due to the pre-existing hyperactivation of Smad1/5/8 in p53^{-/-} NSCs. To corroborate these findings, we inhibited BMPs action with both Noggin and Chordin in the

FIG. 4. Bone morphogenetic protein 2 (BMP2) treatment promoted, while Noggin and Chordin inhibited, neural differentiation of NSC. **(A)** $p53^{-/-}$ and WT NSC cultures were treated with 50 ng/mL BMP2 for 4 days, and the differentiation markers were determined by immunocytochemistry staining (upper panel). Lower panel: the numbers of cells positive for Tuj1 or GFAP ($n=4$). * $P < 0.05$ when $p53^{-/-}$ cells were compared to $p53^{+/+}$ cells. **(B)** $p53^{-/-}$ and WT NSC cultures were treated with 50 ng/mL of Noggin and 25 ng/mL of Chordin (N+C) for 4 days, and the differentiation markers were determined by immunocytochemistry staining. Lower panel: the numbers of cells positive for Tuj1 or GFAP ($n=4$). * $P < 0.05$ when $p53^{-/-}$ cells were compared to $p53^{+/+}$ cells. Color images available online at www.liebertpub.com/scd

differentiation assays. Noggin and Chordin are protein antagonists specific for BMPs and the combinational use is intended to efficiently block BMPs' action. Immunocytochemistry staining showed that Noggin and Chordin treatment led to a decrease in the number of Tuj1-positive cells but an increase in the number of GFAP-positive cells (Fig. 4B). Western blot analysis showed that Noggin and Chordin decreased Smad1/5/8 activation although they somehow led to an upregulation of Smad1 (Supplementary Fig. S7). However, the effects of Noggin/Chordin treatment on GFAP were minimal in $p53^{-/-}$ NSCs (Fig. 4B). Although inconsistent results regarding BMPs' role in neuronal differentiation has been reported, our findings support the conclusion that hyperactivated BMP-Smad1 signaling contributes to accelerated neuronal differentiation of $p53^{-/-}$ NSCs. The differential effects of Noggin and Chordin on $p53^{-/-}$ and WT NSCs suggest that factors other than BMP-Smad1 may also play an important role in $p53^{-/-}$ NSCs.

$p53^{-/-}$ NSCs showed an upregulation of Id1

Id1 is a BMPs target gene and has been shown to inhibit neuronal differentiation but promote NSC self-renewal [41,42]. As a helix-loop-helix protein, Id1 binds to helix-loop-helix transcription factors such as Mash1 to inhibit their activities [11,43]. Since BMP-Smad1 pathway is hyperactivated in $p53^{-/-}$ NSCs, we determined the protein levels of Id1 in $p53^{-/-}$ and WT NSCs during differentiation and found that Id1 levels were initially very low, went up at 2, 4, 6 h, and went down at 72 h in WT NSCs, and that $p53^{-/-}$ NSCs showed an increase in Id1, except at 72 h after cultured in differentiation medium (Fig. 3A). A decline of Id1 levels from day 1 to day 8 during NSC differentiation was previously reported [33]. Moreover, the brain tissue of $p53^{-/-}$ embryos also showed an upregulation of Id1 compared to WT littermates, justified by western blot analysis (immunohistochemical staining did not work with the available antibodies) (Fig. 3B). Realtime PCR analysis showed that the mRNA levels of Id1 are also much higher in $p53^{-/-}$ NSCs than in WT cells (Fig. 5A), suggesting that p53 might repress Id1 transcription.

Moreover, $p53^{-/-}$ mouse embryonic fibroblast also showed an upregulation of Id1 at the protein levels (Fig. 5B). On the other hand, Id1 was downregulated when p53 is transiently upregulated by nutlin-3 in PC-12 and NSC cells (Fig. 5C, 5D), a small molecule compound that disrupts Mdm2-p53 interaction and stabilizes p53 [44]. These results, taken together, suggest that p53 is a repressor of Id1 transcription.

p53 represses Id1 expression in BMP-Smad1-dependent and -independent manners

It has been established that Id1 is a target gene of BMP-Smad1 pathway [42,45,46]. We found that $p53^{-/-}$ cells show enhanced Smad1 activation and increased Id1 expression (Fig. 3A). In a reporter assay using Id1 promoter fused to luciferase, p53 was able to inhibit the promoter activity of Id1, while co-expression of Smad1 or Smad1 and Smad4 was able to activate Smad1 promoter (Fig. 5E). However, Id1 promoter has no typical p53-binding site, suggesting that p53-mediated repression of Id1 transcription does not involve direct binding to the promoter. Alternatively, p53 may

interfere with transactivators of Id1 gene such as Smad1. We indeed found that co-expression of p53 abolished Smad1-mediated Id1 promoter activation (Fig. 5E). This result, together with the finding that loss of p53 leads to an increase in Smad1 expression and activation, suggests that p53 is a transcription repressor of Id1.

However, regulation of Id1 expression during neural differentiation seems to be rather complex. We noticed that Id1 expression pattern was not always positively correlated to that of BMP-Smad1 activation (Fig. 3A), suggesting that there exist other signals that control Id1 transcription. Moreover, Id1 is expressed at higher levels in $p53^{-/-}$ NSCs, especially 2.5 and 4 h after being placed in the differentiation medium, when the BMP-Smad1 signaling is comparable between WT and $p53^{-/-}$ NSCs (Fig. 3A), suggesting that p53 may also regulate Id1 independently of the BMP-Smad1 pathway. This is further supported by our observation that $p53^{-/-}$ cells still showed a much higher induction of Id1 than WT cells in response to 50 ng/mL of BMP2 (Fig. 5F). Id1 promoter has binding sites for YY1, Egr1, Sp1, ATF/CREB, and other transcription factors [47]. It is possible that p53 might interfere with the transactivation activity of 1 or more of these transcription factors to repress Id1 transcription.

Elevated Id1 expression contributes to increased self-renewal, proliferation, and differentiation of $p53^{-/-}$ NSC

To determine the function of elevated Id1 in $p53^{-/-}$ NSCs, we tried to knock down Id1 with siRNA. Pooled siRNA could efficiently lower the protein levels of Id1 (to the levels similar to that of WT NSCs) when transiently transfected into WT and $p53^{-/-}$ NSCs for 3 days. We then tested a possible role of Id1 in neuronal differentiation of WT and $p53^{-/-}$ NSCs and found that knockdown of Id1 enhanced neuronal differentiation of WT NSCs, evidenced by an increase in Tuj1 and MAP2 expression (Fig. 6A), consistent with previous studies showing that Id1 is a negative regulator of neural differentiation [41,42]. Unexpectedly, Id1 knockdown resulted in a decrease in the expression of neuronal markers in $p53^{-/-}$ NSCs (Fig. 6A). This finding was further validated by immunocytochemistry experiments that show that Id1 knockdown increased the number of Tuj1-positive cells in WT cultures but decreased the number of Tuj1-positive cells in $p53^{-/-}$ cultures (Fig. 6B). Thus, loss of p53 might have altered the cell context and as such, highly elevated Id1 seems to promote neural differentiation under this condition. Further studies are needed to understand how p53 status makes such a switch in determining the function of Id1. These data, taken together, suggest that Id1 elevation contributes to the increase in neuronal differentiation of $p53^{-/-}$ NSCs.

There is evidence that the growth advantage of $p53^{-/-}$ cells is attributable to decreased expression of p53 target gene p21, a CDK inhibitor [23]. Consistent with previous studies, we found that $p53^{-/-}$ NSCs showed enhanced proliferation (Fig. 7A). To test the potential effect of elevated expression of Id1 in NSC proliferation, we knocked down Id1 with siRNA and found that Id1 knockdown led to a decrease in the sphere size of $p53^{-/-}$ NSCs, whereas it has a minimal effect on WT NSCs (Fig. 7A). This could be because Id1 knockdown was transient and less than complete in our

FIG. 5. p53 represses Id1 expression in BMP-Smad1-dependent and independent manners. **(A)** $p53^{-/-}$ NSC cultures showed an increase in the mRNA levels of Id1. RNA was isolated from WT and $p53^{-/-}$ NSC cultures and converted to cDNA with reverse transcription, which was used to determine the Id1 mRNA levels by real-time polymerase chain reaction. $*P < 0.05$ when $p53^{-/-}$ cells were compared to $p53^{+/+}$ cells. **(B)** $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) also showed elevated protein levels of Id1. MEFs grown in log phase were harvested and the Id1 protein levels were determined by western blot. **(C)** Upregulation of p53 induced by Nutlin-3 repressed the expression of Id1 in PC-12 cells. Cells were treated with nutlin3 for different periods of time. The levels of Id1, p53, p21, and Actin were determined by western blot. **(D)** Upregulation of p53 induced by Nutlin-3 repressed the expression of Id1 in NSCs. **(E)** Luciferase reporter assay shows that p53 inhibits the basal levels of and Smad1-stimulated Id1 promoter activity. **(F)** p53 deficiency promoted Id1 expression in response to BMP2. WT and $p53^{-/-}$ NSCs were cultured in proliferation medium, and 50 ng/mL of BMP was added to the cultures for different periods of time. The levels of Id1, Smad1, Actin, and p-Smad1 were determined by western blot.

experimental settings. Counting the cell numbers in the NSC spheres transfected with Id1 or control siRNA also confirmed that Id1 promoted the proliferation of $p53^{-/-}$ NSCs, as the cell numbers were decreased by 29% in the presence of Id1 siRNA (Fig. 7B). BrdU labeling experiments also showed that $p53^{-/-}$ NSC cultures had more proliferating cells, which was reduced by Id1 knockdown (Fig. 7C). Moreover, we found that $p53^{-/-}$ NSC had increased self-renewal ability, which requires elevated expression of Id1 as Id1 knockdown decreased the self-renewal of $p53^{-/-}$ NSCs (Fig. 7D and Supplementary Fig. S8). These results, taken together, suggest that elevated Id1 protein mediates the role of p53 deficiency on NSC self-renewal and proliferation.

Discussion

There is increasing evidence that p53 regulates cell differentiation in addition to cell proliferation, apoptosis, and senescence. For instance, p53 has been reported to promote the differentiation of myogenic and hematopoietic cells but inhibit the differentiation of osteogenic and adipogenic cells, suggesting that the function of p53 in regulating differentiation is cell type specific [24,48]. Our in vivo results and ex vivo NSC culture results, by detecting cell marker with immunocytochemistry and western blot, show p53 deficiency led to enhanced proliferation and accelerated neuronal differentiation of NSCs, at the sacrifice of glia. This is similar to

FIG. 6. Knock-down of Id1 impeded neural differentiation of $p53^{-/-}$ NSCs, but not WT NSCs. **(A)** The pooled target siRNA led to a down-regulation of Id1, an increase in neural differentiation of normal NSC, but a decrease in neural differentiation of $p53^{-/-}$ NSCs. NSC cultures of monolayers were transfected with control or Id1 siRNA for 3 days and the differentiation markers were analyzed by western blot. *Right panel:* quantitation data, with the value of normal cells set at 1.0. * $P < 0.05$ when cells transfected with Id1 siRNA were compared to the same type cells transfected with control siRNA. **(B)** Immunocytochemistry staining showed that knock-down of Id1 led to an increase in the number of Tuj1-positive cells in WT NSC cultures but a decrease in the number of Tuj1-positive cells in the $p53^{-/-}$ NSC cultures. * $P < 0.05$ when cells transfected with Id1 siRNA were compared to the same type cells transfected with control siRNA. Color images available online at www.liebertpub.com/scd

mesenchymal stem cells, in which p53 deficiency leads to an increase in both proliferation and differentiation [49]. However, there are studies showing that p53 does not regulate NSC differentiation [18,23,27]. This discrepancy is not likely caused by the genetic background of the $p53^{-/-}$ mice as in most studies C57BL/6 or FvB/B6 mice were used [23,26–28]. Moreover, we found that NSC isolated from 129 background behaved like that isolated from C57BL/6 background. We suspect that the culture conditions might be a major factor causing the discrepancy. In various studies, NSC differentiation was assayed in the presence of serum on coated slides. Different coating materials (polylysine/laminin, GFR-Matrigel™, poly-ornithine-coated, fibronectin) and different sera (different concentrations of calf serum or FBS) were used in the above-mentioned studies [23,26–28], which may have effects on the expression of p53 and Id1 and therefore neural differentiation. For example, the concentrations of serum have been reported to affect the basal level and DNA damage-induced expression of p53 [50]. Moreover, the culture conditions might affect the activation of co-factors of Smad1 to influence NSC differentiation. For example, serum concentration and origin may affect the activation of Stat3, which has been reported to form a complex with Smad1 via p300 to regulate astrocyte differentiation [30,51–53].

More importantly, our study revealed 1 molecular mechanism by which p53 regulates NSC proliferation and differentiation

(Fig. 7E). We show that p53 deficiency altered the expression of 2 critical regulators of NSC homeostasis, Id1 and Smad1, which seem to mediate the effects of p53 deficiency on NSC proliferation and/or neural differentiation. The link of p53 deficiency to Id1 and Smad1 expression/activation is also observed in osteoblasts (Ma G et al., unpublished results). Previous studies have shown that p53 regulates the stemness of NSCs via altering the expression of Oct4 [54,55], and NSC proliferation via p21, Id2, and c-myc [23,56]. Thus, p53 might act on multiple pathways to control NSC proliferation and differentiation. The coordinated action of these pathways may help to fine-tune the output from p53 in cell proliferation and differentiation. To add to the complexity of p53 in neurogenesis regulation, we found that p53 status also acts like a switch in the action of Id1 in neuronal differentiation.

This study shows that the expression of Id1 is repressed by p53 in NSC, and more importantly, Id1 mediates, at least partially, the effects of p53 deficiency on NSC proliferation and neural differentiation. Lack of p53 led to an elevation of Id1, while upregulation of p53 led to a decrease in Id1. p53 seems to repress the promoter activity of Id1 in a p53-DNA binding independent manner. A recent study suggested that p53 might represses Id1 expression via DEC1, a p53 target gene in DNA damage response [57]. In addition, we show that p53 inhibits the Smad1's activity on Id1 promoter. Thus, p53 might repress Id1 expression via multiple

FIG. 7. Knock-down of Id1 inhibited proliferation of $p53^{-/-}$ NSCs. **(A, B)** Id1 knock-down decreased proliferation of $p53^{-/-}$ NSCs, but not WT NSCs, judged by the size of the neurosphere **(A)** and the number of cells **(B)**. NSC cultures of monolayers grown in proliferation medium were transfected with control or Id1 siRNA for 3 days and the cells were counted. * $P < 0.05$ compared with control, **when $P < 0.05$ compared with $p53^{-/-}$ NSC transfected with Id1 siRNA. **(C)** BrdU incorporation assays confirmed that Id1 knock-down decreased the proliferation of $p53^{-/-}$ NSCs, but not much of WT NSCs. * $P < 0.05$ compared with control, **when $P < 0.05$ compared with $p53^{-/-}$ NSC transfected with Id1 siRNA. **(D)** p53 deficiency increased the self-renewal of NSCs, which was impeded by Id1 knock-down. * $P < 0.05$ compared with control, **when $P < 0.05$ compared with $p53^{-/-}$ NSC transfected with Id1 siRNA. **(E)** A diagram showing how p53 regulates BMP-Smad1 signaling and Id1 expression in controlling NSC proliferation and differentiation. Color images available online at www.liebertpub.com/scd

mechanisms, including BMP-Smad1-dependent and -independent manners. The conclusion that Id1 contributes to the enhanced proliferation of $p53^{-/-}$ NSCs is consistent with previous studies showing that Id1 promotes self-renewal of mouse ES cells, extends the lifespan of mouse embryonic

fibroblasts [47], and augments proliferation of $p53^{-/-}$ cancer cells [58]. In addition, Id1 elevation is shown here to promote neuronal differentiation in $p53^{-/-}$ NSCs, whereas Id1 inhibits neuronal differentiation in normal NSCs. One possible explanation for this switch is that p53 deficiency

may have altered the cell context so that neuronal differentiation process differently responds to Id1, which warrants further investigation. Together with the findings that p53 also represses Id2, which also participates in NSC self-renewal [25,59], our results suggest an important link between p53 and Id proteins.

This study also establishes a connection between p53 and the BMP-Smad1 pathway in NSC. Previous studies have shown that p53 interacts with Smad2/3 to regulate several cellular and developmental processes [60], this study shows that p53 could repress Smad1 expression in NSCs and mouse brain. Furthermore, p53 seems to be able to inhibit the transcription activity of Smad1 on Id1. Recent studies revealed that p53 might inhibit Smad1 expression via the p21-CDK-Rb-E2F1 pathway (Ma G. et al., unpublished results), a well established route that is used by p53 to repress the transcription of some target genes. The link between p53 and BMP-Smad1 signaling might provide an explanation why *p53*^{-/-} cells are more sensitive to BMPs-induced differentiation of mesenchymal stem cells. In NSCs, the function of Smad1 elevation seems to promote neuronal differentiation and this effect is partially mediated by BMP target gene Id1. While these results suggest that p53 has an impact on BMP-Smad1 signaling (Fig. 7E), we found that BMPs do not activate p53 [37]. Thus, p53 is a unilateral regulator of the BMP-Smad1 pathway.

In summary, this study provides in vivo evidence that p53 plays roles in NSC differentiation and proliferation and uncovered an important mechanism by which p53 executes its functions. p53 expression is altered during NSC differentiation and in vivo neurogenesis, and p53 deficiency leads to increased expression of Smad1 and Id1, both of which contribute to accelerated neuronal differentiation of *p53*^{-/-} NSCs. Elevated Id1 expression also contributes to the increased proliferation of *p53*^{-/-} NSC. The link to BMP-Smad1 signaling and Id1 expression may also underlie other aspects of development regulated by p53.

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Author Disclosure Statement

No competing financial interests exist.

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