Smad7 Regulates the Adult Neural Stem/Progenitor Cell Pool in a Transforming Growth Factor β- and Bone Morphogenetic Protein-Independent Manner

Monika Krampert,1,2 Sridhar Reddy Chirasani,2 Frank-Peter Wachs,2 Robert Aigner,2 Ulrich Bogdahn,2 Jonathan M. Yingling,3 Carl-Henrik Heldin,1 Ludwig Aigner,2,4 #* and Rainer Heuchel1,5 #*

Ludwig Institute for Cancer Research, Uppsala University, Box 595, BMC, 75124 Uppsala, Sweden1; Department of Neurology, University of Regensburg, Regensburg, Germany2; Eli Lilly, Lilly Research Laboratories, Cancer Research, Indianapolis, Indiana 462853; Institute of Molecular Regenerative Medicine, Paracelsus Medical University Salzburg, 5020 Salzburg, Austria4; and Karolinska Institutet, Department of Clinical Science, Intervention and Technology (CLINTEC) K53, 14186 Stockholm, Sweden5

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Members of the transforming growth factor β (TGF-β) family of proteins modulate the proliferation, differentiation, and survival of many different cell types. Neural stem and progenitor cells (NPCs) in the adult brain are inhibited in their proliferation by TGF-β and by bone morphogenetic proteins (BMPs). Here, we investigated neurogenesis in a hypomorphic mouse model for the TGF-β and BMP inhibitor Smad7, with the hypothesis that NPC proliferation might be reduced due to increased TGF-β and BMP signaling. Unexpectedly, we found enhanced NPC proliferation as well as an increased number of label-retaining cells in vivo. The enhanced proliferation potential of mutant cells was retained in vitro in neurosphere cultures. We observed a higher sphere-forming capacity as well as faster growth and cell cycle progression. Use of specific inhibitors revealed that these effects were independent of TGF-β and BMP signaling. The enhanced proliferation might be at least partially mediated by elevated signaling via epidermal growth factor (EGF) receptor, as mutant cells showed higher expression and activation levels of the EGF receptor. Conversely, an EGF receptor inhibitor reduced the proliferation of these cells. Our data indicate that endogenous Smad7 regulates neural stem/progenitor cell proliferation in a TGF-β- and BMP-independent manner.

The transforming growth factor β (TGF-β) superfamily of cytokines, consisting of TGF-βs, activins, and bone morphogenetic proteins (BMPs), participates in the regulation of a multitude of cellular processes, such as proliferation, apoptosis, differentiation, and extracellular matrix production. In consequence, TGF-β has important functions in embryonic development as well as in the adult organism (reviewed in references 22 and 26).

Recently, it was shown that TGF-β is involved in the regulation of adult neural stem cells located in two neurogenic niches, the dentate gyrus of the hippocampus (HC) and the subventricular zone (SVZ) of the lateral ventricles (reviewed in references 1 and 2). Treating adult neural stem/progenitor cells in culture, we have been able to demonstrate that TGF-β strongly suppresses proliferation of these cells. Moreover, intracerebroventricular infusion of TGF-β reduced the proliferation of neural progenitor cells in vivo (33). Similar results were reported in a transgenic approach involving overexpression of TGF-β in astrocytes (5).

The cellular functions of TGF-β are mediated via ligand-induced hetero-oligomerization of type I (TβRI) and type II (TβRII) serine/threonine kinase receptors and subsequent phosphorylation of TβRI by the constitutively active TβRII. Receptor-regulated Smad proteins (R-Smads), that is, Smad2 and -3 for TGF-β and activin and Smad1, -5, and -8 for BMPs, are phosphorylated by TβRI and heterotrimersize with the coreceptor Smad4. This complex then translocates to the nucleus, where it regulates gene transcription in association with coactivators or repressors (reviewed in references 27, 30, and 31). Negative feedback regulation of TGF-β signaling is provided by the inhibitory Smads, Smad6 and Smad7 (4, 16, 28). Upon TGF-β stimulation, Smad7 translocates from the nucleus to the cell membrane, where it interacts with the activated TβRII, thereby competitively inhibiting the activation of R-Smads (27). However, besides this inhibitory role, Smad7 can also act as a positive effector of TGF-β, mediating the interaction with alternative signaling pathways (11, 12; reviewed in reference 25).

Here we investigated the role of Smad7 in neural stem/progenitor cell proliferation and fate and in adult neurogenesis, using mice lacking exon 1 of the Smad7 gene. We demonstrated that, in contrast to our expectations, proliferation of adult neural stem/progenitor cells is strongly enhanced in mutant mice, an effect that is independent of TGF-β and BMP signaling.

* Corresponding author. Mailing address for Rainer Heuchel (Smad7/TGF-β/BMP): Karolinska University Hospital, KFC, Novum 4th floor, 14186 Stockholm, Sweden. Phone: 46 (0)8-5858 3033. Fax: 46 (0)8-5858 3850. E-mail: rainer.heuchel@ki.se. Mailing address for Ludwig Aigner (neural stem cells): Institute of Molecular Regenerative Medicine, Paracelsus Medical University Salzburg, Strabergasse 21, 5020 Salzburg, Austria. Phone: 43 (0)662 442002 1280. Fax: 43 (0)662 442002 1209. E-mail: ludwig.aigner@pmu.ac.at. # These authors contributed equally to the work. # Published ahead of print on 17 May 2010.
**MATERIALS AND METHODS**

**Animals.** Mutant mice deficient in exon 1 of the Smad7 gene (termed **Smad7**+) have been described recently (21). All mice were from a C57 background. All animal experiments were approved by the local ethical committee (Uppsala Tingsraett).

**BrdU labeling, tissue processing, and immunohistochemistry.** Three-month-old male mice were injected with 50 mg/kg bromodeoxyuridine (BrdU) (Fluka, Germany) once daily for 4 consecutive days. Four weeks later, animals were deeply anesthetized (20.38 mg/ml ketamine, 5.38 mg/ml xylazine, and 0.29 mg/ml acepromazine) and perfused transcardially with 4% paraformaldehyde. Tissue processing and immunostaining were described as previously (19). Tissue was cut into 25-μm sagittal sections. The following primary antibodies were used: rat anti-BrdU, 1:250 (Oxford Biotechnology, United Kingdom); goat anti-doublecortin (anti-DCX), 1:500 (Santa Cruz); mouse anti-NeuN, 1:500 (Dako, Germany); mouse anti-proliferating cell nuclear antigen (anti-PCNA), 1:500 (Santa Cruz); rabbit anti-glial fibrillary acidic protein (anti-GFAP), 1:1,000 (Dako, Germany); mouse anti-NeuN, 1:500 (Dako, Germany); mouse anti-polymerizing cell nuclear antigen (anti-PCNA), 1:500 (Santa Cruz). Secondary antibodies were donkey anti-goat, anti-mouse, anti-rabbit, and anti-rat antibodies conjugated with fluorescein isothiocyanate (FITC), rhodamine X (ROX), or biotin 1:500 (Jackson Immuno Research).

**Counting procedures and statistical analysis.** To determine the number of PCNA-, BrdU-, and DCX-positive cells, every twelfth section of one cerebral hemisphere was selected from each animal and processed for immunohistochemistry. The volume of the granule cell layer of the hippocampal dentate gyrus or the subventricular zone was determined by tracing the area using a semiautomatic stereology system (Stereoinvestigator; MicroBrightField, Colchester, VT) and multiplying with the section thickness and interval. Positive cells were counted on each section. The total counts of positive cells of one structure were multiplied by the section interval, divided by the determined volume, and depicted as positive cells per volume.

To determine the differentiation pattern of newborn cells, a series of every sixth section (150-μm interval) was double stained with BrdU and either NeuN or GFAP and examined using a confocal laser microscope (LSM 5, Zeiss, Germany). On average, 50 BrdU-labeled cells per animal (n = 8 per group) and structure were analyzed for colabeling.

**Adult neural progenitor cultures, proliferation and differentiation assays, and growth factors.** Adult neural stem and progenitor cells (NPCs) were isolated from the ventricle walls and hippocampi of 4-month-old mice. The dissected tissue was dissociated mechanically, resuspended in PPD solution containing 0.1% papain (Worthington Biochemicals), 0.1% dispase II (Roche, Switzerland), 0.01% DNase I (Worthington Biochemicals), and 12.4 mM MgSO4 in Hank’s balanced salt solution (HBSS) without Mg2+/Ca2+ (PAA, Germany), and digested for 30 min at 37°C. The single-cell suspension was resuspended in NB medium (Invitrogen) supplemented with B27 (Invitrogen), 2 mM L-glutamine, 100 μM penicillin, and 100 μg/ml streptomycin (Sigma, Germany), 2 μg/ml heparin (Sigma), 100 μg/ml fibroblast growth factor 2 (Peprotech, United Kingdom), and 20 mg/ml epidermal growth factor (EGF) (R&D Systems, United Kingdom). Cells were seeded in T-25 culture flasks, and cultures were maintained at 37°C in an incubator with 5% CO2. Cells were passaged weekly as described previously (33) and used for experiments from passages 2 to 6.

For proliferation assays, NPCs were seeded at a density of 105 cells/ml in 24-well plates and counted after 4 days. Alternatively, cells were plated in 96-well plates at the same density and the MTS assay (Promega GmbH, Germany) was used as a readout for proliferation. Growth factors and/or inhibitors were added 3 to 5 h after plating. Growth factor tested were TGF-β1 and -β2 (Peprotech Inc.), BMP2 (ImmuNoTools Inc.), and BMP7 (kindly provided by K. Sampath, Curis Inc., Boston, MA); inhibitors used were TIR1/kinase inhibitors LY2190761 (Eli Lilly) and SB431542 (Sigma), recombining noggin (BMP antagonist), Piraprotech, p38 inhibitor SB203580 (Calbiochem, CA), and EGF receptor (EGFR) inhibitor AG1478 (Sigma).

**For differentiation assays, 5 × 104 cells/ml were plated on poly-ornithine (100 μg/ml; Sigma)-laminin (5 μg/ml; Sigma)-coated coverslips. Differentiation was induced by withdrawal of EGF/FGF and addition of 5% fetal calf serum (FCS) (PAN, Germany) to the medium. Cells were fixed with 4% paraformaldehyde after 7 days. For immunostaining, fixed cells were washed in Tris-buffered saline (TBS) (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) and incubated in blocking buffer (0.1% Triton X-100, 0.1% tricine serum albumin [BSA], and 0.2% Telencephalic gelatin) in TBS. All antibody incubations and washing steps were performed with blocking buffer. The following primary antibodies were used: rabbit anti-GFAP, 1:1,000 (Dako, Germany); mouse anti-MAb(2a+2b), 1:250 (Sigma); mouse anti-2',3'-cyclic dinucleotide 3'-phosphodiesterase, 1:200 (Sigma). The secondary fluorochrome-conjugated antibodies were diluted 1:500 (donkey anti-mouse, -rabbit, or -goat antibody; Dianova, Germany). Nuclear counterstaining was performed with 4',6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI, Sigma, Germany) at 0.25 μg/μl. For analysis, pictures were taken at 200× magnification on a fluorescent microscope (Leica DMR; Leica, Germany) equipped with a Spot charge-coupled device (CCD) camera (model 2.2.1; Diagnostic Instruments). Three independently performed immunocytochemical stains were analyzed. Immunoreactive cells were determined in 10 random fields (total number of cells between 250 and 300).

**RNA isolation and real-time PCR (RT-PCR).** (i) RNA extraction. Total RNA was extracted from mouse SVZ-derived neurospheres treated with transforming growth factor β (TGF-β) (10 μM) and also from various tissues from neurogenic and nonneurogenic areas of brain. Three independent sets of RNA were isolated for each treatment condition using the RNasy kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions. All the samples were treated with DNase I to eliminate genomic DNA contamination. RNA quantification was determined spectrophotometrically. The ratio of optical density at 260 nm (OD260/OD280) was used to evaluate the purity of the nucleic acid samples, and the quality of the extracted total RNA was determined using agarose gel electrophoresis.

(ii) Quantification of mRNA by RT-PCR analysis. For cDNA synthesis, 1 μg total RNA from SVZ-derived neurospheres or from tissues was reverse transcribed using a cDNA synthesis kit (Promega, CA). Amplification was performed on a cDNA amount equivalent to 25 ng total RNA. PCRs were performed on three independent sets of template, and cycling parameters were 95°C for 2 min, 55°C for 45 s, and 72°C for 5 min for 40 cycles. RT-PCR experiments were performed using the Stratagene detection system and with the SYBRGreen mouse Smad7 QuantiTect primer assay kit (catalog no. QT0124607; Qiagen, Germantown, MD). Standard curves were generated using serial dilutions of mouse SVZ-derived or tissue cDNAs. All parameters calculated by linear regression analysis of the comparative threshold cycle (Ct) versus log[template] blots using Graph Pad Prism 3.0 software. The amount of studied cDNA in each sample was calculated by the 2^-ΔΔCt method and expressed as 2^exp (Ct) using 18S rRNA as an internal control.

**Sphere-forming and self-renewal analysis.** To determine the initial number of spheres obtained from hippocampus and SVZ tissue, we applied the “neural colony-forming-cell assay” kit (Stem Cell Technologies, Inc.) according to the manufacturer’s recommendations. Briefly, cells isolated from six 3-month-old mice were pooled in 1 ml medium; 75 μl of SVZ cells or 200 μl of hippocampus cells was suspended in 1.5 ml of a collagen gel and plated into 12-well plates (500 μl/well). When this assay was performed with established NPC cultures, 1 × 104 cells were used per 1.5 ml gel. Growth factors were supplemented weekly, and spheres were counted after 3 weeks. In addition, clonal analysis was used to analyze the cell’s potential to form spheres. Here, NPCs were seeded into 96-well plates at a density of 0.5 cells per well. Fresh medium was added after 1 week, and spheres were counted on day 7. The cell density of 2 × 10^6 cells/ml was determined for each treatment condition using the RNeasy kit (Qiagen, Germantown, MD) according to the manufacturer’s recommendations. Briefly, 4 × 10^6 cells were mixed with 4 μg of either a pcDNA3-FLAG-Smad7 vector (21) or the pmaxGFP vector (AMAXA) as a control and electroporated with the AMAXA Nucleofector II device. Cells were resuspended in 4 ml of growth medium and replated for further experiments 24 to 40 h after transfection at a density of 2 × 10^4 cells/ml.

**Immunoblotting.** For immunoblotting, cells or tissues were homogenized in ura buffer (10 mM Tris, pH 8.0, 9.5 M urea, 2 mM EDTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose filters. Antibody incubations were performed in 5% bovine serum albumin in Tris-buffered saline. Antibodies against phospho-Akt (1:1,000), Akt (1:1,000), and phospho-ERK1/2 (1:1,000) were obtained from Cell Signaling Technology, the anti-β-actin antibody (1:5,000) was from Sigma, and antibodies against phospho-EGFR (1:1,000) and EGFR (1:5,000) were from Santa Cruz Biotechnology Inc.

**Cell cycle assay.** For cell cycle analysis, 3 × 10^6 cells/ml were plated in T25 flasks and grown for 4 days, dissociated into single cells, and fixed with 70% ethanol at −20°C for 24 h. After being washed with phosphate-buffered saline (PBS), cells were incubated with propidium iodide staining solution (0.1% Triton X-100, 0.2 mg/ml RNase, 25 μg/ml propidium iodide) for 30 min. Samples were analyzed on a Guava PCA 96 system fluorescence-activated cell sorter (FACS) machine (Guava Technologies Inc.).

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and expressed as $2^{\text{expt}} (C_T)$ using 18S rRNA as an internal control.

(B) Smad7 mRNA levels were determined in the tissues indicated in vivo. (A) Suspension cultures of NPCs were treated with 10 ng/ml TGF-β1 for the time points indicated. mRNA levels were quantified in triplicate using RT-PCR. The amount of single-copy DNA was calculated by the comparative threshold cycle ($C_T$) method and expressed as $2^{\text{expt}} (C_T)$ using 18S rRNA as an internal control.

FIG. 1. Smad7 expression after TGF-β treatment in vitro and in different tissues in vivo. (A) Suspension cultures of NPCs were treated with 10 ng/ml TGF-β1 for the time points indicated. mRNA levels were quantified in triplicate using RT-PCR. The amount of single-copy DNA was calculated by the comparative threshold cycle ($C_T$) method and expressed as $2^{\text{expt}} (C_T)$ using 18S rRNA as an internal control. (B) Smad7 mRNA levels were determined in the tissues indicated using RT-PCR as described in Materials and Methods.

RESULTS

Enhanced cell proliferation in the neurogenic regions of mice lacking exon 1 of the Smad7 gene. To elucidate the physiological role of Smad7 in the regulation of adult neural stem cell biology, we used mice carrying a hypomorphic Smad7 allele. In these mice, exon 1 of the Smad7 gene, including the start codon and the first half of the coding region for the Smad7 protein, was replaced by a neomycin expression cassette. This resulted in deletion of the N terminus of the Smad7 protein and subsequently in a strong reduction of its ability to suppress TGF-β activity (7, 13, 21). These mice will be referred to as S7ΔEx1 mice in the rest of this report.

We first tested if Smad7 is induced in the same way by TGF-β in neural stem/progenitor cultures as it has been observed to be induced in other cell lines. Therefore, we stimulated NPCs with TGF-β in vitro and analyzed the kinetics of Smad7 expression at different time points by RT-PCR. As seen in Fig. 1A, TGF-β induces its inhibitor Smad7, suggesting that the TGF-Smad7 feedback loop is active in these cells. The maximum of Smad7 expression was found already after 1.5 h, a kinetic parameter similar to that observed in other cell types.

Next, we analyzed the expression levels of Smad7 in vivo, performing RT-PCR with mRNA derived from tissue from various neurogenic and nonneurogenic brain regions. As seen in Fig. 1B, substantial amounts of Smad7 mRNA were found in hippocampus, SVZ, and olfactory bulb, comparable to those found in other tissues, e.g., kidney. In contrast, the nonneurogenic brain regions cortex and striatum had rather low Smad7 expression levels.

Based on the previous findings that TGF-β inhibits adult neural stem cell proliferation in vivo (5, 33), we hypothesized that, if relevant amounts of endogenous TGF-β or BMPs were present in the unchallenged adult brain, S7ΔEx1 mice should display reduced proliferation of neural stem and progenitor cells due to a higher growth-suppressive effect of these factors. Proliferation in the neurogenic regions was analyzed by immunostainings of brain sections for the marker protein PCNA. Against our expectations, a markedly higher density of PCNA-positive cells was found in the dentate gyrus (HC) and the subventricular zone (SVZ) of the mutant mice (Fig. 2A and B). PCNA-positive cells were found primarily in the two neural stem cell niches, the subgranular region of the dentate gyrus, and the ventricular lining of the lateral ventricles (Fig. 2A and B), irrespective of the animals’ genotype.

Elevated numbers of label-retaining cells in S7ΔEx1 animals. Next, we analyzed whether the increased number of proliferating cells present in the neurogenic niches of S7ΔEx1 mice in vivo might originate from an increase in the stem/progenitor cell pool. To test that, we used the ex vivo neural colony-forming-cell assay and the in vivo BrdU label-retaining paradigm. The first one makes use of the characteristic potential of neural stem and progenitor cells to form clonal spheres in a collagen matrix (6). Freshly isolated cells from the hippocampus and SVZ of wild-type (wt) and S7ΔEx1 mice were plated directly into a collagen gel in order to ensure clonal sphere formation. The number of spheres was analyzed 3 weeks later. As a result, we obtained a higher number of spheres from the hippocampus (Fig. 2C) and from the SVZ (Fig. 2D) of S7ΔEx1 mice, indicating a higher number of stem/progenitor cells with proliferative capacity in mutant mice.

The BrdU label-retaining paradigm is based on the fact that cells in the stem cell niche that retain detectable levels of BrdU over a longer time are usually considered to be slowly dividing stem cells, as the fast-cycling progenitor cells dilute out the label over this time period (10, 23, 24). For analysis of label-retaining cells in the stem cell niche, mice were injected with BrdU for 4 consecutive days, and tissue samples were taken 4 weeks later (Fig. 2E). Mutant mice showed significantly elevated levels of BrdU-positive cells in the subgranular region of the dentate gyrus (HC) and in the SVZ but not in the olfactory bulb (OF) (Fig. 2F). This indicates that (i) the newly generated cells survived and (ii) the excess of labeled cells in the mutant mice remained in the neurogenic niches rather than migrating to their usual site of integration. To exclude that this is an effect of impaired migration or abnormal differentiation, we costained the BrdU-labeled cells with the neural marker NeuN. However, no NeuN-positive cells were found in the SVZ, suggesting that the label-retaining cells were undifferentiated stem/progenitor cells (data not shown).

Neural stem/progenitor cultures derived from S7ΔEx1 mice show higher proliferation and sphere-forming capacity. To further investigate the effects of Smad7 deletion on the biology of neural stem and progenitor cells (NPCs), we analyzed sphere formation, proliferation, and differentiation of NPC cultures that were already established as neurospheres. Six days after reseeding of SVZ-derived NPCs in growth medium, S7ΔEx1 spheres were markedly bigger than wild-type spheres.
Moreover, the cell number in S7^Ex1 sphere cultures was significantly higher already at 2 days after reseeding (Fig. 3B). Similar results were obtained with hippocampus-derived cells (data not shown). As an additional proliferation assay, we stained NPCs, grown in adhesion on coated slides for 1 day, for the cell cycle marker Ki67. The percentage of Ki67-positive cells was higher in S7^Ex1 cells (Fig. 3C). In accordance with these results, FACS analysis of propidium iodide-stained cells revealed a higher number of S7^Ex1 cells than of wild-type cells in S and G2/M phases (Fig. 3D).

Besides the proliferative potential, the self-renewal potential of S7^Ex1 NPCs was also enhanced. In clonal dilution assays, the percentage of mutant cells able to form new spheres was markedly higher than that of control cells (Fig. 3E). Similar results were obtained using the collagen sphere assay described above with established NPC cultures (data not shown). No difference was found in the rate of apoptosis between wild-type and mutant cells (data not shown).

To determine whether the observed difference in proliferation was a direct effect of Smad7, we tried to rescue the phenotype by reintroducing Smad7 into NPCs derived from wild-type and S7^Ex1 mice. Electroporation of these cells with a FLAG-tagged Smad7 construct and a GFP control vector resulted in approximately 30% transfection efficiency (as revealed with the GFP control) (data not shown). Smad7-transfected mutant cells showed indeed a growth reduction of about 20% in the MTS assay compared to the GFP control (Fig. 3F). The effect of Smad7 transfection in wild-type cells was less pronounced but statistically significant.

The differentiation fate of S7^Ex1 neural stem/progenitor cells. Next we investigated the differentiation fate and potential of S7^Ex1 neural stem/progenitor cells in vivo and in vitro. First, we analyzed neurogenesis using the neuronal precursor and immature neuron-specific marker doublecortin (DCX). Staining of brain sections from wild-type and S7^Ex1 brains revealed that the number of young neurons was not altered in the dentate gyrus of mutant mice (Fig. 4A and B), suggesting that the higher proliferation of neural progenitors in S7^Ex1 mice was not followed by an increased generation of new neurons.

To further study the fate of newborn cells, we analyzed cells double labeled for BrdU and either the neural marker NeuN (Fig. 4C) or the astrocyte marker GFAP (Fig. 4D) in mice 4 weeks after BrdU treatment. As mentioned above, a higher total number of BrdU-positive cells was found in mutant animals. Out of those, a lower percentage of cells isolated from the HC or SVZ were plated directly into a collagen gel. Colonies were counted after 3 weeks. P values as revealed by Student’s t test: HC, P = 0.0038; SVZ, P = 0.012. (E and F) Increased maintenance of BrdU-labeled cells in the hippocampus and SVZ of S7^Ex1 mice. (E) Mice were injected with BrdU over 4 days and sacrificed 30 days later. (F) Brain sections were stained using an anti-BrdU antibody. Positive cells were quantified stereologically in the HC, the SVZ, and the olfactory bulb (OF). Data are presented as positive cells per volume (mean ± standard deviation). Statistical analysis was performed using Mann-Whitney’s nonparametric test; HC, P = 0.0022; SVZ, P = 0.0059; OF, P = 0.251. * P < 0.05; ** P < 0.01; *** P < 0.001; ns, not significant.
had differentiated into neurons in mutant mice than in wild-type mice in the dentate gyrus but not in the olfactory bulb (Fig. 4C). In contrast, no difference in the percentage of BrdU-GFAP double-positive cells was found in any of the regions analyzed (Fig. 4D).

In the next step we investigated the differentiation of cultured NPCs in vitro to find out whether there are cell-intrinsic differences in the differentiation potential of S7ΔEx1 NPCs. Under standard differentiation conditions, wild-type and S7ΔEx1 NPCs showed no difference in their differentiation pattern (“ctrl” in Fig. 4E and F), indicating that the S7ΔEx1 mutation did not cause a general impairment in the differentiation potential of these cells. In the presence of BMP2 and -4, the generation of neurons was strongly impaired, with the mutant cells reacting more strongly than the wild-type cells (see BMP in Fig. 4E), suggesting an enhanced responsiveness of S7ΔEx1 neural stem/progenitor cells toward BMPs.

**TGF-β and BMP responsiveness of S7ΔEx1 NPCs.** The best-studied function of Smad7 is its role as negative regulator of TGF-β and BMP signaling. We therefore investigated whether S7ΔEx1 NPCs show an increased sensitivity to stimulation with these growth factors. Expression levels of TGF-β and BMP receptors and Smad6 (mRNA levels) or Smad proteins 1 to 3 were not affected by the Smad7 mutation (data not shown). In the presence of TGF-β1 or BMP7, proliferation of NPCs was significantly reduced in both wild-type and S7ΔEx1 cells (Fig. 5A and B). However, wild-type and mutant cells responded to TGF-β1 equally, whereas the response toward BMP7 was much stronger in mutant cells. Likewise, TGF-β1 induced similar levels of phosphorylation of Smad2 in a dose-dependent manner in both wild-type and mutant cells (Fig. 5C; middle panel), while mutant cells showed stronger Smad1 phosphorylation in response to BMP7 (Fig. 5D). Similar results were obtained with BMP2 (data not shown). In vivo, no difference was seen in Smad2/3 phosphorylation in protein lysates derived from different neurogenic (SVZ, hippocampus, or olfactory bulb) and nonneurogenic (cerebellum, brain stem, or striatum) regions (data not shown).

The enhanced proliferation of S7ΔEx1 NPCs is independent of TGF-β and BMP signaling. The strong growth-suppressive effect of both TGF-β and BMP suggests that the enhanced basal proliferation of S7ΔEx1 NPCs is independent of Smad7’s function as an inhibitor of these pathways. To confirm this hypothesis, we performed proliferation assays in the presence of TGF-β and BMP inhibitors. We first ensured that the inhibitor concentrations we used indeed reversed the effect of exogenous TGF-β or BMP, respectively (data not shown). LY2109761, a chemical inhibitor of TβRII (Alk5) kinase activity (20), did not change the growth difference between wild-type and mutant cells (Fig. 5E). Results were confirmed with a second TβRI inhibitor (SB431542) (data not shown). As Alk5 inhibitors also block the activity of the activin receptors Alk4 and Alk7 (9, 17), we excluded the possibility that the observed phenotype was mediated via these pathways. Smad7 has also been described to be involved in TGF-β activation of non-Smad-signaling pathways such as the TAK1-p38 pathway (11). However, we could not detect activation of TAK1 or p38 in the absence or presence of TGF-β stimulation (data not shown). In accordance with this, a p38 inhibitor did not influence the proliferation of either wild-type or S7ΔEx1 cells (Fig. 5F). To address the involvement of BMP signaling, we applied recombinant noggin, an endogenous secreted BMP antagonist. As expected, noggin could not inhibit the enhanced proliferation of Smad7 mutant cells (Fig. 5G). The slightly enhanced growth of both wild-type and mutant cells in the presence of noggin can be explained with inhibition of endogenous or medium-
derived BMP that might be present in the cultures. Taken together, these results strongly suggest that the difference in proliferation between wild-type and S7ΔEx1 mutant cells does not depend on differential susceptibilities to TGF-β or BMP signaling.

Elevated expression of epidermal growth factor receptor (EGFR) and higher phosphorylation levels in response to growth factor stimulation are found in S7ΔEx1 cells. To get a first insight into the mechanism that might underlie the enhanced proliferation we observed in S7ΔEx1 NPCs, we examined the cells’ response to the growth factors EGF and FGF-2, which are the major proliferation-promoting components in the growth medium used. Interestingly, we found a strong increase of both EGFR phosphorylation and the total EGFR protein levels in S7ΔEx1 cells (Fig. 6A). Among the known downstream targets of EGF/FGF-2, activation of Akt was also enhanced (Fig. 6B), whereas Erk1/2 phosphorylation was unaltered (Fig. 6C).

EGFR activity determines the proliferation rate of wild-type and S7ΔEx1 NPCs. To determine if the level of EGFR signaling is correlated to the proliferation rate of the NPCs, we used serial dilutions of the EGFR kinase inhibitor AG1478. As seen in Fig. 6E, the inhibitor suppressed the proliferation of both wild-type and S7ΔEx1 cells in a concentration-dependent manner. However, mutant cells were more strongly affected than the control cells, suggesting that the enhanced proliferation of mutant cells is at least partially mediated by upregulation of the EGFR and a stronger response to EGF. We tried to grow wild-type and S7ΔEx1 NPCs in the absence of EGF to further analyze the role of this growth factor and its receptor for the observed difference in proliferation. However, this was impeded by the fact that EGF was essential for the survival of both wild-type and mutant cells in culture.

**DISCUSSION**

We have previously shown that infusion of exogenous TGF-β1 strongly inhibits neurogenesis by arresting neural stem/progenitor cells in the cell cycle (33). This is a direct effect on the stem/progenitor cells, as Smad2/3 phosphorylation is
found in this population mainly after TGF-β1 treatment. However, it is not yet known if endogenous TGF-β1 is involved in regulating neural stem cell proliferation and/or differentiation under normal physiological conditions. We therefore asked whether the deletion of Smad7, the most potent inhibitor of TGF-β signaling, also inhibits neurogenesis, which would be an indirect proof of TGF-β action.

We used mice with a deletion of exon 1 of the Smad7 gene, resulting in an N-terminally truncated version of Smad7, due to an alternative start codon in exon 2 (21). The missing N domain has several important functions. It is essential for efficiently binding to the receptor and as such for competing with Smad2/3 recruitment (15). The N domain is also required for Smad7/Smurf1/2-mediated ubiquitination of the TGF-β type I
Unexpectedly, we observed a markedly higher proliferation of the neural stem/progenitor cells from S7\textsuperscript{AEx1} mice both \textit{in vivo} and \textit{in vitro}. As both TGF-\(\beta\) and BMPs inhibited the growth of these cells, we speculated that the observed effect was independent of the inhibitory role of Smad7 for these signaling pathways. Consistent with this hypothesis, inhibitors of TGF-\(\beta\), activin and BMP signaling did not inhibit the enhanced proliferation \textit{in vitro}. NPCs expressed similar levels of TGF-\(\beta\) and BMP receptors and Smad proteins in the two genotypes and responded to the addition of the respective ligands with Smad phosphorylation and growth suppression. Therefore, we exclude the possibility that the mutation we introduced interferes with TGF-\(\beta\)/BMP signaling or leads to compensatory differential Smad signaling of the cells. We conclude from our data that endogenous Smad7 has a TGF-\(\beta\)- and BMP-independent role in negatively regulating the proliferation of neural stem/progenitor cells. This is directly supported by the observation that loss of Smad4 expression in adult neural stem cells influences neurogenic fate decision but not neural stem/progenitor cell proliferation (8, 35).

Recently it was shown that Smad7 can influence epidermal stem cell fate via \(\beta\)-catenin degradation and subsequent suppression of wnt signaling (14). However, we did not find any changes in total cellular \(\beta\)-catenin levels. In luciferase assays with wnt reporter constructs, no significant reporter activity was found in either wild-type or mutant NPCs under standard growth conditions, suggesting that this pathway is not or is only very weakly active in unstimulated NPC s.

The TGF-\(\beta\)-independent antiproliferative effect of Smad7 on neural stem/progenitor cells is in contrast to results reported for other stem cell compartments. In hematopoietic stem cells, transgenic overexpression of Smad7 enhances proliferation of these cells by suppression of the canonical Smad signaling pathway (3). Thus, it seems that Smad7 can fulfill different functions in different stem cell compartments and under different conditions. Therefore, it will be very interesting to see which consequences the lack of Smad7 has on stem/progenitor cells in other tissues.

The most striking alterations in signal transduction pathways in the S7\textsuperscript{AEx1} NPCs were related to EGF signaling. We found elevated EGF receptor protein levels in the mutant cells and subsequently stronger phosphorylation in response to ligand. Application of an EGF inhibitor reduced proliferation of both genotypes in a dose-dependent manner, indicating that the level of EGF signaling is a limiting factor for proliferation. This observation is in line with the recent finding that the presence of the EGF exclusively marks actively dividing neural stem/progenitor cells in vivo. NPCs expressed similar levels of EGF receptor with 20 ng/ml EGF and 10 ng/ml FGF-2 for the time indicated. Cell lysates were analyzed by Western blotting for the activation of EGFR, Akt, and Erk1/2 (A to C, upper panels) and for the expression of total Akt, and Erk1/2 (A to C, lower panels). (D) Levels of \(\beta\)-actin were determined as loading controls. (E) EGFR signaling is essential for proliferation of wild-type and S7\textsuperscript{AEx1} NPCs. Cells (10\(^4\) ml) were plated without growth factors in the presence of the EGFR inhibitor AG1478 at the concentrations indicated. After 30 min, 20 ng/ml EGF and 10 ng/ml FGF-2 were added. Cell numbers were determined after 4 days.

FIG. 6. Enhanced growth factor response and EGFR expression in S7\textsuperscript{AEx1} NPCs. (A to D) NPCs derived from wild-type (wt) and S7\textsuperscript{AEx1} mice were growth factor starved for 3 h and subsequently stimulated with 20 ng/ml EGF and 10 ng/ml FGF-2 for the time indicated. Cell lysates were analyzed by Western blotting for the activation of EGFR, Akt, and Erk1/2 (A to C, upper panels) and for the expression of total EGFR, Akt, and Erk1 (A to C, lower panels). (D) Levels of \(\beta\)-actin were determined as loading controls. (E) EGFR signaling is essential for proliferation of wild-type and S7\textsuperscript{AEx1} NPCs. Cells (10\(^4\) ml) were plated without growth factors in the presence of the EGFR inhibitor AG1478 at the concentrations indicated. After 30 min, 20 ng/ml EGF and 10 ng/ml FGF-2 were added. Cell numbers were determined after 4 days.

receptor and Smad7 itself (18, 32, 34). Altogether, these properties easily explain the increased TGF-\(\beta\) activity of S7\textsuperscript{AEx1} mutant cells that have been observed so far. For instance, B cells derived from S7\textsuperscript{AEx1} mice are characterized by reduced lipopolysaccharide-induced proliferation and increased immunoglobulin switching from IgD/M to IgA due to enhanced responsiveness (21). This phenotype of enhanced TGF-\(\beta\) responsiveness was furthermore confirmed and extended in experimental models for liver and kidney fibrosis, where a significantly higher TGF-\(\beta\) signaling-based fibrotic response was observed in Smad7 mutant mice (7, 13). Most likely, the truncated protein retains some of its ability to inhibit TGF-\(\beta\) signaling, which ensures that S7\textsuperscript{AEx1} mice survive into adulthood since complete loss of Smad7 is lethal to embryos (I. Kleiter and A. Weisman, personal communication). Thus, S7\textsuperscript{AEx1} mice are a suitable model to investigate Smad7 effects in the adult mouse.
pus in vivo and an enhanced proliferation of neurospheres in vitro associated with a shift toward the S and G2/M phase of the cell cycle. In contrast, no differences between wild-type and mutant cells were observed with respect to apoptosis. Several regulatory steps and cell types present in the neurogenic niche might contribute to the increase in proliferation: (i) activation of the quiescent pool of stem cells and an increase in the number of proliferating stem cells, (ii) activation of the pool of proliferating stem cells, (iii) activation of the transit-amplifying pool of progenitor cells, or (iv) an extended duration of cells in the amplifying status. Due to the restricted number of validated and specific tools to identify the particular cell types, the present study does not allow a final and conclusive statement regarding the specific cell types involved in the proliferation phenotype. The increase in the number of label-retaining cells in the SVZ in vivo and the increase in the number of CFU ex vivo suggest an increase in the pool of proliferating stem cells via the recruitment of a normally quiescent population.

Apparently, the overproduction of cells in the neurogenic niche in the S7ΔE31 mice does not translate into a higher number of DCX-expressing neuronal precursors, suggesting that the cells stay in an undifferentiated state. This is in accordance with the observation of a reduced percentage of BrdU-positive cells that colabel with NeuN. However, as NPCs from mutant mice generated neurons and astrocytes in amounts comparable to those from wild-type mice in vitro, the capability to differentiate into neurons is not generally impaired by the S7ΔE31 mutation. In summary, Smad7 is involved in the regulation of neural progenitor cell proliferation. Attenuating Smad7 gene function translates into an increase in proliferating cells which remain in the neurogenic niche and increase the pool of stem/progenitor cells. As the proliferating cells seem to survive, as indicated by the maintenance of BrdU-positive cells, it will be interesting to see in future experiments if under pathological conditions that require enhanced neurogenesis, such as stroke, enhanced proliferation in the S7ΔE31 mice can translate into enhanced neurogenesis and thus contribute to structural and functional recovery.

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