

Original Article

Impaired TGF- β induced growth inhibition contributes to the increased proliferation rate of neural stem cells harboring mutant p53

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Abstract: Gliomas have been classified according to their histological properties. However, their respective cells of origin are still unknown. Neural progenitor cells (NPC) from the subventricular zone (SVZ) can initiate tumors in murine models of glioma and are likely cells of origin in the human disease. In both, p53 signaling is often functionally impaired which may contribute to tumor formation. Also, TGF-beta, which under physiological conditions exerts a strong control on the proliferation of NPCs in the SVZ, is a potent mitogen on glioma cells. Here, we approach on the crosstalk between p53 and TGF-beta by loss of function experiments using NPCs derived from p53 mutant mice, as well as pharmacological inhibition of TGF-beta signaling using TGF-beta receptor inhibitors. NPC derived from p53 mutant mice showed increased clonogenicity and more rapid proliferation than their wildtype counterparts. Further, NPC derived from p53^{mut/mut} mice were insensitive to TGF-beta induced growth arrest. Still, the canonical TGF-beta signaling pathway remained functional in the absence of p53 signaling and expression of key proteins as well as phosphorylation and nuclear translocation of SMAD2 were unaltered. TGF-beta-induced p21 expression could, in contrast, only be detected in p53^{wt/wt} but not in p53^{mut/mut} NPC. Conversely, inhibition of TGF-beta signaling using SB431542 increased proliferation of p53^{wt/wt} but not of p53^{mut/mut} NPC. In conclusion, our data suggest that the TGF-beta induced growth arrest in NPC depends on functional p53. Mutational inactivation of p53 hence contributes to increased proliferation of NPC and likely to the formation of hyperplasia of the SVZ observed in p53 deficient mice *in vivo*.

Keywords: Neural stem cell, p53, TGF-beta, glioblastoma stem cell, premalignant lesion, subventricular zone

Introduction

Glioblastoma multiforme (GBM) is both the most common and the most lethal human brain tumor. In spite of histological similarities between GBM cells and astrocytes, the cell of origin and the events causing malignant transformation are still matter of debate [1]. Several lines of evidence mainly based on data from animal models indicate that cells of the neurogenic areas in the adult murine brain [2-5], i.e. the subventricular zone and the hippocampus can give rise to GBM even though other publications also report the transformation of mature

brain cells including neurons [6]. In human GBM, p53 signaling is mostly dysfunctional, either directly due to mutations (in ~28% of primary and 65% of secondary GBM) [7, 8] or indirect due to dysfunctional regulatory proteins like MDM2 [9]. According to the TCGA (Total Cancer Genome Atlas) project which analyzed mainly primary GBM, the overall frequency of genetic alterations in the TP53/MDM2/MDM4/p14^{ARF} pathway was 87%, with TP53 mutations or homozygous deletion being observed in 35%, MDM2 amplification in 14%, MDM4 amplification in 7% and p14^{ARF} homozygous deletion or mutation in 49% of the sequenced GBM [10]. In

line with the crucial role for p53 in the genesis of GBM, most murine models of spontaneous GBM require mutant/dysfunctional p53. In a mouse model using conditionally inactivated p53 in the brain, tumors developed from neural progenitor cells (NPC) in the subventricular zone (SVZ) [4, 5, 11-13]. This is corroborated by a study reporting increased proliferation and hyperplasia in the SVZ of mice with mutated p53 [14]. To enhance tumor formation, murine GBM models use a "second hit" like PTEN inactivation or indirect activation of ras signaling via NF-1 inactivation [5, 12], which maximally increases the proliferation of malignant and premalignant cells in the hyperplastic SVZ and elsewhere [11]. In human, p53 mutations predispose for GBM formation in patients with Li-Fraumeni Fanconi syndrome [15]. However, the reasons why p53 mutations result in increased proliferation of cells within the SVZ have not been elucidated in detail.

TGF-beta 1 is a very potent inhibitor of NPC proliferation *in vitro* and abundantly expressed in the brain *in vivo*. Treatment of murine NPC cultures with TGF-beta 1 results in a long-term proliferation arrest that does not affect major stem cell properties [16, 17]. Furthermore, it regulates neurogenesis *in vivo* and contributes to the age-associated reduction of NPC proliferation in the SVZ [18, 19]. In GBM, TGF-beta 1/2 exerts a variety of pro-tumorigenic functions and is likely the most potent contributor to local immunosuppression in at least a subgroup of GBM [20, 21].

TGF-beta belongs to a cytokine superfamily and exerts its effects by canonical signaling via SMAD proteins. Still, non-canonical signaling e.g. via ras and PI-3 kinase can also be initiated by the TGF-beta receptor complex. In the case of TGF-beta 1/2, binding to its receptor results in phosphorylation of SMAD2, which dimerizes with SMAD4 and translocates into the nucleus [22, 23]. For DNA binding, the SMAD2/4 complex requires transcriptional cofactors which determine the manifold and highly cell type-specific biological effects of canonical TGF-beta signaling. P53 is one of these cofactors involved in TGF-beta induced cell cycle arrest, in transcription of certain target genes, and in translocation of the SMAD2/4 complex into the nucleus [24]. A mutant-p53/Smad complex was described to differentially regulate 5 genes (*Sharp-1/DEC2/BHLHB3, Cyclin*

G2/CCNG2, ADAMTS9, Follistatin, and GPR87), to oppose the transcription of p53-dependent genes and thereby to empower TGF-beta-induced metastasis [25]. Still, the role of p53 in regulating and mediating TGF-beta induced proliferation arrest in NPC is unknown. We now hypothesized that lack of functional active p53 may impair TGF-beta-induced growth arrest and thereby provide an explanation for the hyperproliferation of NPC in the SVZ of p53 mutant mice.

Materials and methods

Animals and genotyping

B6.129S2-Trp53^{tm1Tyj}/J heterozygous breeding pairs were obtained from Charles River. For genotyping, DNA was isolated from clipped tails and PCR was performed using the primers 5'-ACAGCGTGGTGGTACCTTAT-3', 5'-TATACTCAG-AGCCGGCCT-3' (oIMR037), 5'-CTATCAGGACATAGCGTTGG-3', yielding a fragment of 400 bp for p53^{+/+} and 600 bp for p53^{-/-} mice. 2 different clones per condition were investigated; mice were littermates from heterozygous^{wt/mut} breeding couples.

Neural progenitor cell culture

Mice (Charles River Deutschland GmbH, Germany) were decapitated and SVZs were dissected as previously described [26]. The tissue was homogenized and cells were resuspended in Neurobasal (NB) medium (Gibco BRL, Germany) containing B27 supplement (Gibco BRL, Germany), 2 mM L-glutamine (PAN, Germany), 100 U/ml penicillin/100 µg/ml streptomycin (PAN, Germany). For expansion, the NB/B27 medium was supplemented with 2 ng/ml heparin (Sigma, Germany), 20 ng/ml FGF-2 (R&D Systems, Germany), and 20 ng/ml EGF (R&D Systems, Germany), yielding an efficient proliferation medium. For all experiments, NPC were treated with recombinant human TGF-beta 1 and recombinant human TGF-beta 2 (both from Peprotech) using 10 ng/ml of each unless indicated otherwise.

Reverse transcriptase PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. 1 µg of total RNA was used to synthesize single stranded cDNAs using reverse transcription kit (Pro-

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mega, USA). PCRs were performed for 30 cycles (30 seconds at 94°C, 2 minutes at 66°C and 1 minute at 72°C) using the following primers.

SMAD2: 5'-AGTATTAAGCTCAGATGGGATCCCCTTC-3', 5'-CTTTAATTGATGAGACCTCAAGTGCTG-3';
SMAD7: 5'-TTTGCCTCGGACAGCTCAATTCGG-3';
5'-CTCGTAGTCGAAAGCCTTGATGGAG-3'

SDS-PAGE and western blot analysis

Total cell lysates were prepared in the presence of phosphatase inhibitor cocktail 1 (Sigma) according to the manufacturer's instructions. For Western Blot analysis of TFGR2, p21, and phosphorylated Smad2 protein, 20-30 µg of total cell lysates were separated by 10-15% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed overnight at 4°C with antibodies against TGFBR2 (1:1000 Upstate), p21 (1:1000 Santa Cruz Biotechnology), pSmad2 (1:1000 Calbiochem), SMAD2 (1:1000, BioNordica), and beta-actin (1:5000, Abcam, Cambridge, UK).

Alamar blue assay

This assay measures metabolic activity, which is a measure for cell proliferation. It is based on oxidation-reduction reaction (REDOX) and incorporates a fluorometric/colorimetric REDOX indicator that fluoresces and changes color. The non-toxic REDOX indicator is cell-permeant and serves as electron acceptor in the respiratory chain. The ensuing reduction causes the REDOX indicator to change from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form. The ratio of both forms was photo-metrically quantified.

Clonogenicity assay

Cells were seeded as indicated in 96 well plates and treated as indicated. 10 wells with 50 cells were plated for each condition. Depending on the growth rate of various cell lines, the medium supplemented with TGF-beta was replaced weekly. At the end of 1-2 weeks the number of spheres/patches formed was determined.

Immunocytochemistry

Cells were plated in 24 well plates in neurobasal medium. After 5 days, cells were fixed using 4% PFA. Fixed cells were washed in TBS (0.15

M NaCl, 0.1 M Tris-HCl, pH 7.5), blocked with TBS supplemented with 0.1% Triton-X100 (only for intracellular antigens), 1% bovine serum albumin (BSA), and 0.2% teleostean gelatin (Sigma, Taufkirchen, Germany). This solution was also used during the incubations with antibodies. Smad2 primary antibody (1:1000 Santa Cruz, USA) was applied overnight at 4°C. Stainings were visualized using anti-rabbit Alexa Fluor 488 (1:100, Molecular Probes, Leiden and NL) conjugates. Nuclei were counter-stained using DAPI (1:10000, Sigma-Aldrich). Specimens were mounted on microscope slides using Prolong Antifade kit (Molecular Probes, U.S.A.). Epifluorescence observation and photo-documentation were realized using a Leica microscope (Leica Mikroskopie und Systeme GmbH, Germany) equipped with a Spot™ digital camera (Diagnostic Instrument Inc, U.S.A.).

Statistics

Data analysis was performed using Graphpad Prism 5. The statistical tests used are indicated in the figure legends. All data are given as Mean and Standard Error of the Mean (SEM). The statistical significance was assessed using a two-sided student's t-test. A *p*-value <0.05 was considered significant.

Results

p53^{mut/mut} NPC show enhanced proliferation compared to p53^{wt/wt}

TGF-beta 1 which is abundantly expressed in the brain is a potent inhibitor of proliferation of NPC *in vitro* and *in vivo* [16, 27]. Given that crosstalk between TGF-beta and p53 signaling has been described, we wondered whether the increased *in vitro* and *in vivo* proliferation rate of p53^{mut/mut} NPC could be connected to differential responses towards TGF-beta. We therefore compared the effects of TGF-beta 1/2 on murine NPC cells derived from the SVZ of 10-14 week old wildtype and p53^{mut/mut} mice. Plating of single cells from p53^{wt/wt} and p53^{mut/mut} NPC cultures revealed that mutant cells give rise to much larger neurospheres (**Figure 1A, 1B**) and display higher clonogenicity (**Figure 1C**). In line with previous reports, p53^{mut/mut} NPC also showed increased proliferation as measured via the amplification rate (i.e. the number of cells after 5 days compared to the number of cells plated, **Figure 1D**) [14].

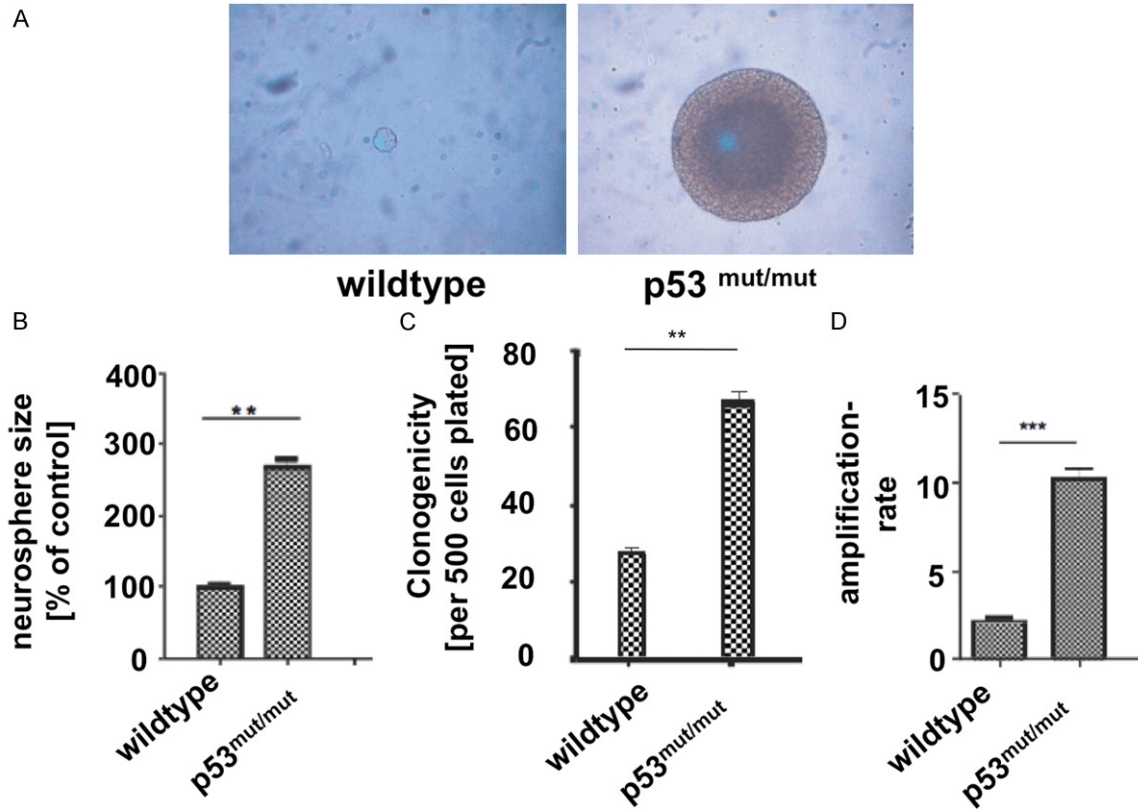


Figure 1. P53^{mut/mut} NPC show enhanced proliferation compared to p53^{wt/wt} NPC. A. Neurospheres formed from p53^{wt/wt} and p53^{mut/mut} NPCs were photographed after 5 days in culture. B. Based on the recorded images, the diameter of neurospheres was assessed. Shown are median values and standard deviations for the size of spheres formed from p53^{wt/wt} and p53^{mut/mut} NPC (**P<0.01). C. Clonogenicity of untreated p53^{wt/wt} NPC as compared to p53^{mut/mut} NPC was determined (**P<0.01). D. The amplification rate (i.e. the number of viable cells divided by the number of cells plated) was determined for p53^{mut/mut} and p53^{wt/wt} NPCs (***P<0.001).

TGF-beta-induced growth inhibition of NPC depends on functional p53

In line with a report by Wachs et al. [16, 27], addition of TGF-beta 1/2 substantially impaired growth and proliferation of NPC cultures from wildtype mice (Figure 2A, 2B). In contrast, proliferation of p53^{mut/mut} NPC did not change in response to TGF-beta 1/2 (Figure 2A, 2B). This effect was specific since inhibition of TGF-beta signaling by the specific TGF-beta receptor kinase inhibitor SB431542 prevented TGF-beta induced growth inhibition in p53^{wt/wt} NPC whereas p53^{mut/mut} NPC were not only insensitive to TGF-beta itself, but also to the inhibitor (Figure 2C, 2D). Consequently, autocrine TGF-beta signaling (which would also be affected by SB431542) does not seem to be relevant for p53^{mut/mut} NPC. Insensitivity to the exogenous cytokine hence cannot be explained by saturating levels of autocrine TGF-beta. As previously described, TGF-beta did not affect the clono-

genicity of NPCs which was found to be enhanced when p53 was mutated (Figure 3A).

P53^{mut/mut} NPC resisted TGF-beta-induced growth arrest even though the components of the TGF-beta signaling were intact

Cordenonsi et al. identified p53 as co-factor for TGF-beta signaling in Xenopus embryos and could show that p53 is required for TGF-beta induced growth arrest in Hep2G cells [28]. To investigate how p53 contributes to TGF-beta induced growth arrest in NPC, we investigated the expression of key proteins of TGF-beta signaling. There was no difference in the expression of TGF-beta receptor 2 (Figure 3B). Likewise, SMAD2 expression was similar. SMAD7, which acts as a TGF-beta receptor antagonist and prevents the phosphorylation of SMAD2 was not amplified, either (Figure 3C). Rapid and equal phosphorylation of SMAD2 in p53^{wt/wt}, p53^{wt/mut}, and p53^{mut/mut} NPC con-

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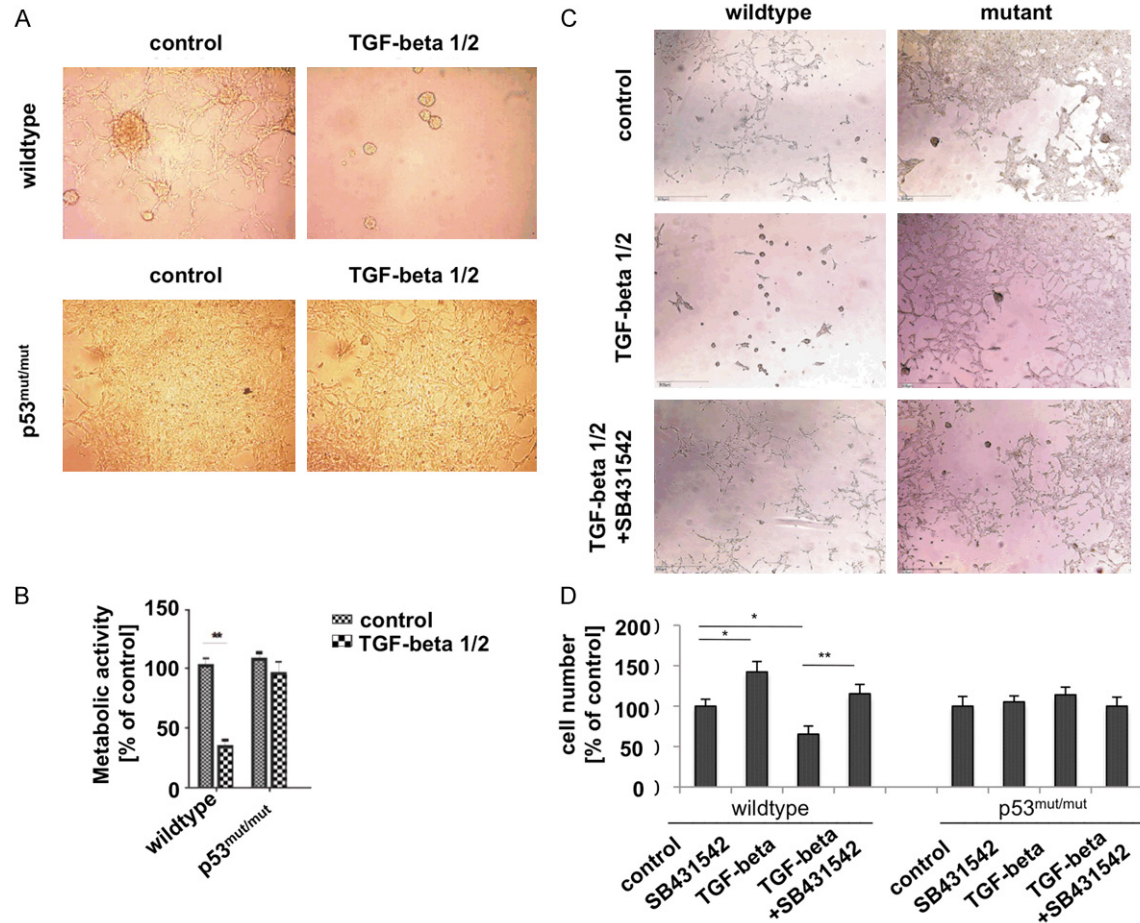


Figure 2. P53^{mut/mut} NPC does not respond to TGF-beta induced growth arrest. A. Shown are representative images depicting the change of growth pattern of p53^{wt/wt} and p53^{mut/mut} NPC in the absence or presence of TGF-beta (10 ng/ml TGF-beta 1 and 2). B. Using the Alamar Blue assay, the proliferation of p53^{wt/wt} and p53^{mut/mut} NPC was assessed in the presence of 10 ng/ml TGF-beta 1/2 (**P<0.01, ***P<0.001). C. Shown are representative images of p53^{wt/wt} and p53^{mut/mut} NPC in the absence or presence of 10 ng/ml TGF-beta 1/2 or 25 nM SB431542 (Scalebars: 500 μM). D. Cell number relative to control conditions of p53^{wt/wt} and p53^{mut/mut} NPC treated with 10 ng/ml TGF-beta 1/2 or 25 nM SB431542 or both (*P<0.05, **P<0.01).

firmly that SMAD2 activation by TGF-beta occurs independent of p53 function (Figure 3D) and without increased SMAD2 expression (Figure 3E). However, SMAD2 translocation into the nucleus was only observed in p53^{wt/wt} NPC but not in p53^{mut/mut} NPC (Figure 4A) suggesting that the canonical TGF-beta signaling cascade was impaired downstream of the receptor signaling complex. This is in line with previous reports describing that mutant p53 both attenuates and alters the TGF-beta-induced transcription activity of SMAD2/3 proteins [25, 28, 29]. A molecular explanation was offered by Cordenonsi et al. who found that the TGF-beta dependent interaction of p53 with Smads 2 and 3 and the subsequent recruitment of SMAD4 to the nuclear transloc-

tion complex depends on the phosphorylation (and hence on the functionality) of p53. As a consequence, TGF-beta addition failed to induce p21 expression in p53^{mut/mut} NPC whereas p21 was readily induced in p53^{wt/wt} NPC (Figure 5A). As this protein is a key effector for TGF-beta-mediated growth inhibition, the differential induction of p21 explains why functional p53 is a required co-factor for TGF-beta induced growth arrest.

Impaired autocrine TGF-beta signaling may contribute to the increased proliferation of p53^{mut/mut} NPC

P53^{mut/mut} mice show increased proliferation in the subventricular zone [14] which further pro-

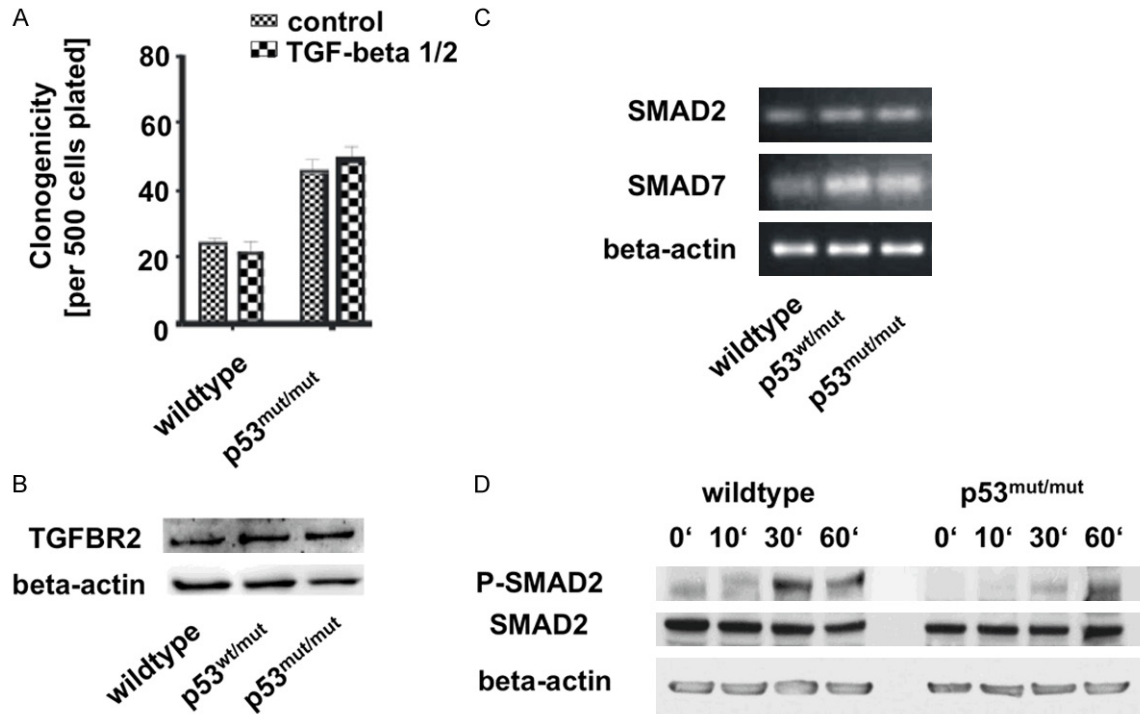


Figure 3. In spite of being refractory to the growth-inhibitory effects of TGF-beta, p53^{mut/mut} NPC do not lack any components of the canonical TGF-beta signaling pathway. A. p53^{wt/wt} and p53^{mut/mut} NPC were treated with 10 ng/ml TGF-beta 1/2 and clonogenicity was determined after 1-2 weeks. B. Expression of TGFBR2 was assessed by Western Blotting, using beta-actin as loading control. C. SMAD2 and SMAD7 mRNA expression was assessed by PCR. D. Cells were treated with 10 ng/ml TGF-beta 1/2 and analyzed for SMAD2 phosphorylation, beta-actin and SMAD2 expression at the indicated time points.

gresses into glioma-like tumors in mice [11]. We wondered whether impaired autocrine TGF-beta signaling may contribute to the increased proliferation of NPC. To assess effects of autocrine TGF-beta signaling in NPC, NPC were treated with the specific TGF-beta receptor kinase inhibitor SB431542, resulting in increased proliferation of p53^{wt/wt} NPC (Figure 2C, 2D) but not in p53^{mut/mut} NPC (Figure 5B-D). Autocrine TGF-beta signaling thus seems to be active in NPC. Anti-proliferative effects, however, appear to be restricted to p53^{wt/wt} cells.

Discussion

NPC derived from neurogenic zones within the adult brain are likely cells of origin for GBM. Mice with conditional mutations in the brain develop hyperplasia within the SVZ and later-GBM-like tumors that develop from these cells in the SVZ [11]. This suggests that p53 mutations result in premalignant lesions in the brain that later (likely upon acquisition of further genetic hits) progress to highly malignant

tumors. In our experiments, we could confirm the increased proliferation rate of p53^{mut/mut} cells *in vitro*. Loss of p53 function specifically prevented TGF-beta-induced growth arrest due to impaired translocation of SMAD2/4 into the nucleus. This resulted in the loss of TGF-beta mediated p21 induction, which likely contributes to increased proliferation of NPC *in vitro* and *in vivo*. The *in vivo* proof-of-concept of this hypothesis in a relevant tumor model would have been beyond the scope of this study. In addition, p53^{mut/mut} NPC still proliferated slightly faster than p53^{wt/wt} NPC even when the TGF-beta receptor kinase inhibitor SB431542 was present. This suggests that p53 also limits the proliferation of NPC by TGF-beta independent signaling pathways not explored in this study.

TGF-beta makes an important contribution to the decrease of neurogenesis in the aging brain [18, 19]. Our data suggest that this effect may strongly depend on p53. This could also explain why mutation or inactivation of p53 - which occurs early during gliomagenesis [9] - fosters

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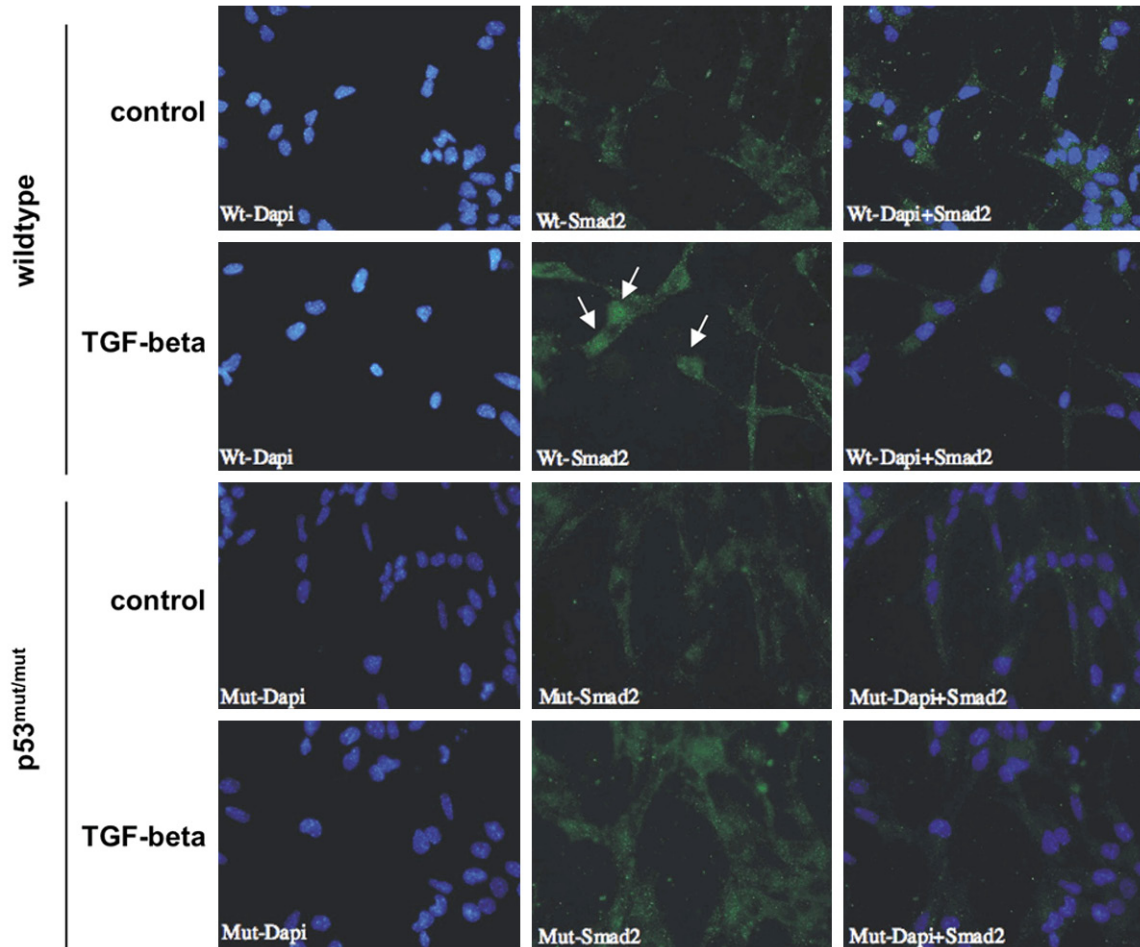


Figure 4. Intracellular translocation of SMAD2. Using epifluorescence microscopy, translocation of SMAD2 into the nucleus was monitored 30 min after addition of 10 ng/ml TGF-beta 1/2. DAPI was used as nuclear counterstain.

the growth and migration of genetically altered NPC in the brain. It is therefore tempting to speculate that p53 dysfunction may predispose NPC towards the formation of glial tumors. Possible effects of p53 inactivation on neurogenesis were not investigated in our study.

A classical step in the malignisation of epithelial derived cancers like breast cancer is the conversion of TGF-beta from tumor suppressor to tumor promoter. Together with published data [25] our findings offer a possible explanation for this shift. Inactivation of p53 is typically an early event in the genesis of gliomas, which directly protects the putative cells of origin against the anti-proliferative functions of TGF-beta. Consequently, TGF-beta would lose its tumor suppressor function at an early stage during gliomagenesis. The premalignant tumors could therefore upregulate TGF-beta and there-

by induce local immunosuppression without having to acquire additional mutations in the TGF-beta pathway.

While these considerations inevitably contain a certain amount of speculation on early events during tumorigenesis, our work provides new insights into the potentially first steps during glioma development. Based on the data presented in this manuscript we propose that inactivation of p53 in a single NPC does not only inhibit the guardian of the genome but also inactivate the TGF-beta dependent mechanism which would otherwise control neurogenesis in the ageing brain. The simultaneous disruption of these two important control mechanisms may therefore allow the formation of a premalignant lesion, which can later develop into full-blown glioma.

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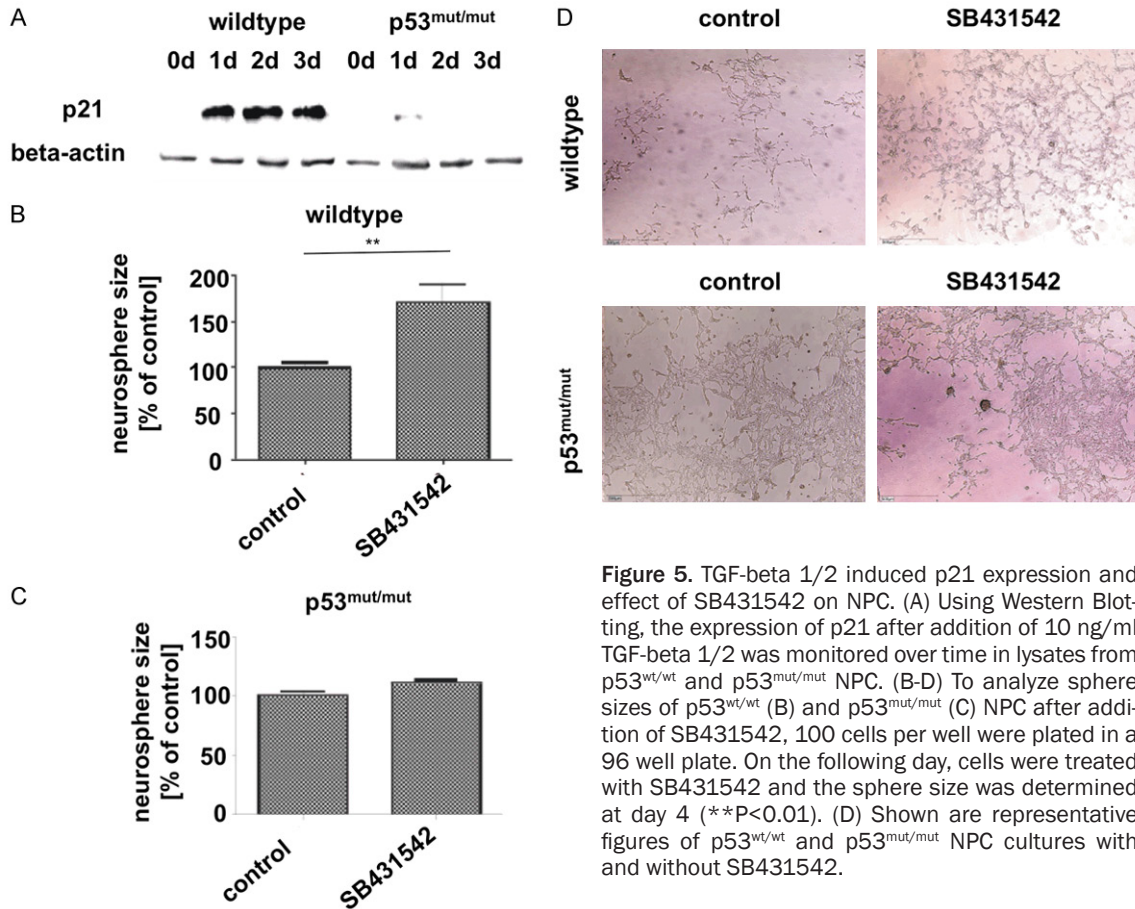


Figure 5. TGF-beta 1/2 induced p21 expression and effect of SB431542 on NPC. (A) Using Western Blotting, the expression of p21 after addition of 10 ng/ml TGF-beta 1/2 was monitored over time in lysates from p53^{wt/wt} and p53^{mut/mut} NPC. (B-D) To analyze sphere sizes of p53^{wt/wt} (B) and p53^{mut/mut} (C) NPC after addition of SB431542, 100 cells per well were plated in a 96 well plate. On the following day, cells were treated with SB431542 and the sphere size was determined at day 4 (**P<0.01). (D) Shown are representative figures of p53^{wt/wt} and p53^{mut/mut} NPC cultures with and without SB431542.

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Disclosure of conflict of interest

None.

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