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Defective p53 antiangiogenic signaling in glioblastoma

Abstract

Previous findings suggest an angiogenesis-regulating function of the p53 tumor suppressor protein in various malignancies. With several antiangiogenic agents entering the clinic, we assessed the value of the TP53 status in predicting angiogenesis in glioblastoma in vivo and examined underlying angiogenic-signaling pathways in vitro. We identified 26 TP53 wild-type and 9 TP53 mutated treatment-naïve, primary, isocitrate dehydrogenase 1 (IDH1) wild-type glioblastoma specimens by sequence analysis and quantified vascularization. P53 responsiveness of the angiogenesis-related target genes, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), thrombospondin 1 (TSP-1), brain-specific angiogenesis inhibitor 1 (BAI1), and collagen prolyl-4-hydroxylase alpha 2 (P4HA2), was evaluated by (i) overexpression of wild-type p53 in homozgyously TP53-deleted LN-308 cells; (ii) shRNA-mediated p53 knockdown in the TP53 wild-type LNT-229 cells; and (iii) chemical induction of wild-type p53 expression in LNT-229 cells by camptothecin. Irrespective of the TP53 status, vascularization did not differ significantly between the two groups of glioblastoma specimens. Of all target genes, only P4HA2 mRNA was upregulated through wild-type p53. As opposed to several nonglial tumors, in glioblastoma cells, p53-mediated transcriptional induction of P4HA2 mRNA neither resulted in increased levels of P4HA2 protein or antiangiogenic endostatin nor did it influence endothelial cell sprouting, viability, or transmigration in vitro. Moreover, p53-uncoupled stable overexpression of P4HA2 in LN-308 cells did not affect endothelial cell viability. These data challenge the view of p53 as an angiogenesis-regulator in glioblastoma in that relevant signaling pathways are silenced, potentially contributing to the angiogenic switch during malignant progression.
p53 antiangiogenic signaling is defective in glioblastoma

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Running title:
P53 and angiogenesis in glioblastoma
Abstract

Previous findings hint at a potential angiogenesis-regulating function of the p53 tumor suppressor protein in various malignancies. With the antiangiogenic agents currently being under clinical investigation, we assessed the value of p53 in predicting angiogenesis in glioblastoma and examined potential underlying angiogenic signaling pathways. We identified 26 TP53 wild-type and 9 TP53 mutated treatment-naïve, primary, isocitrate dehydrogenase 1 (IDH1) wild-type glioblastoma specimens by sequence analysis, and quantitated vascularization using automated image analysis of CD31-immunoreactive tumor vessels. For signaling analyses, we analyzed the p53-inducibility of known angiogenesis-related target genes, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), thrombospondin 1 (TSP1), brain-specific angiogenesis inhibitor 1 (BAI1), and collagen prolyl-4-hydroxylase alpha 2 (P4HA2), using three different approaches: i) stable overexpression of wild-type p53 in the homozygously TP53-deleted human glioblastoma cell line LN-308; ii) shRNA-mediated p53 knock-down in the TP53 wild-type glioblastoma cell line LNT-229; and iii) chemical induction of wild-type p53 expression in LNT-229 cells by the DNA-damaging agent camptothecin. We found that, irrespective of the underlying TP53 status, vascularization did not differ significantly between glioblastoma specimens. Unlike other candidate factors, only P4HA2 mRNA became robustly upregulated through wild-type p53. As opposed to several non-glial tumors, in glioblastoma cells, p53-mediated transcriptional induction of P4HA2 mRNA was neither paralleled by increased P4HA2 protein and endostatin concentrations nor influenced endothelial cell function in vitro. Moreover, p53-uncoupled stable overexpression of P4HA2 in LN-308 cells did not affect endothelial cell function either. In conclusion, our data challenge the view of p53 as an angiogenesis-regulating molecule in glioblastoma in that relevant signaling pathways are transcriptionally, translationally and post-translationally shut down potentially contributing to the angiogenic switch during malignant progression.
Key words:

Angiogenesis, collagen prolyl hydroxylase, glioblastoma, p53
Introduction

The tumor vasculature of glioblastomas has long been recognized as a promising therapeutic target, and consequently, a number of antiangiogenic treatment regimens have currently been under clinical investigation recently leading to the approval of bevacizumab for recurrent glioblastomas.\textsuperscript{1-5} Identifying and establishing angiogenesis-related molecular alterations as diagnostic, prognostic or predictive biomarkers as well as potential stratification factors will therefore be essential for future clinical trials that test antiangiogenic or vessel-modifying agents, and for selecting patient subpopulations in whom an antiangiogenic-based regimen will be beneficial.

The most devastating glioma grade, glioblastomas are highly vascularized malignancies, and the presence of microvascular proliferation is a key feature for their diagnosis.\textsuperscript{6} Tumor angiogenesis is fundamental to the propagation of glioblastomas as it results in the formation of new blood vessels providing supply of oxygen and nutrients to cells when the tumor size exceeds a few millimeters in diameter.\textsuperscript{7} The process of angiogenesis is tightly governed by a balance between pro- and antiangiogenic factors that regulate the growth of endothelial cells, the cellular precursors of blood vessels. Remarkably, as vessel formation is most required by rapidly growing tissues such as cancers, growth-promoting factors, i.e. oncogenes, frequently induce or propagate angiogenesis whereas tumor suppressor genes are often likely to contribute to the inhibition of angiogenesis.\textsuperscript{8}

Since its discovery in 1979, extensive research into the role of TP53 has revealed its function as a multimodally acting tumor suppressor being the most frequently altered gene in human cancers and mutationally inactivated in half of all tumors.\textsuperscript{9,10} Basal activities of wild-type p53 become markedly increased in response to oncogenic signaling and stress or damage signals. The crucial tumor suppressive function of p53 is impressively documented by the phenotype of the first Trp53 knockout mouse that, after developing normally, had early spontaneous tumors of various types.\textsuperscript{11} The p53 protein functions, at least in part, as a transcription factor controlling the expression of target genes that act at most steps in cancer biology, predominantly concerning cell cycle arrest, DNA repair, and apoptosis.\textsuperscript{12} In addition
to these classical ‘genome-guarding’ roles, p53 has also been attributed to an angiogenesis-controlling function as supported by findings from clinical studies in various human carcinomas demonstrating that tumors carrying p53 mutations were more highly vascularized than tumors harboring wild-type p53.\textsuperscript{13-18} \textit{In vitro} work on the molecular linkage between p53 and the regulatory pathways of angiogenesis has since discovered three basic mechanisms underlying the angiogenesis-limiting effects of wild-type p53: (1) interference with hypoxia-induced factor (HIF)-1\textalpha as a central responder to hypoxia and potent promoter of angiogenesis, (2) downregulation of proangiogenic factors, e.g. vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), and (3) upregulation of endogenous angiogenesis inhibitors, e.g. thrombospondin-1 (TSP-1), brain-specific angiogenesis inhibitor 1 (BAI1), \textalpha 1 collagen 18 (COL18A1), \textalpha 1 collagen 4 (COL4A1), or collagen prolyl hydroxylase 2 (P4HA2).\textsuperscript{19}

Mutations of the \textit{TP53} gene occur as early events in two-thirds of precursor low-grade diffuse astrocytomas and play a crucial role in the development of secondary glioblastomas derived thereof, whereas, in primary, i.e. \textit{de novo} glioblastomas, \textit{TP53} mutations are detected at a considerably lower frequency (<30% of cases).\textsuperscript{20} The goal of the present study was to define the value of the \textit{TP53} mutational status in indicating the extent of vascularization in glioblastoma, analyze potentially underlying angiogenic signaling pathways, and thus identify glioblastoma subgroups that are likely to respond differentially towards antiangiogenic treatment.
Materials and Methods

Glioblastoma specimens and vessel quantitation

For analyses of TP53 mutations, 40 previously untreated primary glioblastomas with available tumor tissue of at least one cm in diameter were included. All cases were diagnosed at the Department of Neuropathology, University Hospital Heidelberg, Germany. Informed consent for molecular analysis was given by the patient. DNA was isolated from paraffin tissue and analyzed for TP53 mutations in exons 5a, 5b, 6, 7 and 8 as previously described. To exclude glioblastomas with the genetic profile of secondary glioblastomas, we screened for the most frequent isocitrate dehydrogenase 1 (IDH1) mutation (R132H) with IDH1 mutation specific antibody mIDH1R132H as recently described. Specimens with conclusive sequencing of TP53 and negative mIDH1R132H immunostaining were further analyzed for vascularization.

To minimize observer bias, analysis of vascularization was performed by the following predefined criteria. For all specimens selected for vessel quantitation (n=35), consecutive sections stained for hematoxylin and eosin (H&E), proliferation marker Ki-67 (clone MIB1, dilution 1:100; Dako Denmark A/S, Glostrup, Denmark) and endothelial marker CD31 (clone JC70A, dilution 1:10; Dako) were prepared according to routine protocols. H&E-stained slides were evaluated to identify regions consisting of solid tumor tissue distant from necroses. Within these tumor regions, the region of highest proliferation marked by positive Ki-67 staining was determined in each specimen as observed under a light microscope and marked on the glass slides. The markings were transferred to CD31-immunostained slides and one random photomicrograph of the marked area was taken at 100-fold magnification on a Zeiss Axioplan2 microscope (Zeiss, Jena, Germany) with a Zeiss AxioCam color digital camera. The investigator (D.C.) was blinded for the results of the TP53 mutation analyses until photomicrographs were taken and submitted for analysis. Unprocessed Tagged Image File (tif) photomicrographs were then analyzed by an automated image analysis algorithm provided by S.CO for vascularity (S.CO LifeScience Company, Garching, Germany). The image analysis provides data of total area and total number of vascular structures.
Reagents, cell culture and transfections

The human malignant glioma cell lines LN-308 and LN-229 were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland). The p53 status of these cells was assessed previously. Importantly, LN-229 cells still harbor the wild-type TP53 gene and were therefore renamed LNT-229 (T for Tübingen) for clarification. The non-small cell lung carcinoma cell line H1299 was received by courtesy of Michael R. Green, University of Massachusetts Medical School, Worcester, MA, USA. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 4.5g/l glucose, 2 mM glutamine and 0.1 mg/l Fe(III)-nitrate-9-hydrate (PAA Laboratories, Pasching, Austria), supplemented with 10% fetal bovine serum (FBS) (Perbio, Bonn, Germany), 100 IU/ml penicillin (PAA Laboratories), 100 mg/ml streptomycin (PAA Laboratories), and 100 µM ascorbic acid (Sigma-Aldrich). Primary human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell (Heidelberg, Germany) and kept in endothelial cell growth medium (ECGM) supplemented with Supplement Mix C-39215 (Promocell), 10% FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin. HUVEC were only used between passages three and five. Human cerebral microvascular endothelial cells (hCMEC) were kindly provided by F. Miller (Institut Cochin, Paris, France) and grown in EBM-2 (Lonza, Basel, Switzerland) supplemented with EGM-2 MV SingleQuots (Lonza), HEPES (PAA Laboratories), 100 IU/ml penicillin and 100 mg/ml streptomycin in cell culture dishes coated with rat tail collagen I (BD, Heidelberg, Germany). For experiments, hCMEC were only used between passages 27 and 32. Cells were cultured at 37°C, 5% CO₂ and 21% O₂. All cell cultures were routinely tested for contamination with Mycoplasma as described previously.

Chemical induction of p53. For chemical induction of p53, LNT-229 cells were treated with 1, 10 and 100 nM of the DNA-damaging agent camptothecin (BioVision, Mountain View, CA, USA) or dimethyl sulfoxide (DMSO) as a vehicle control and harvested 36 h thereafter.

Transient overexpression of p53. p53-overexpressing cells were generated by transiently transfecting LN-308 and H1299 cells with the expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA) containing either cDNA encoding human p53 wild-type or no insert
DNA\textsuperscript{26} using the FuGENE\textsuperscript{®} HD transfection reagent (Roche, Mannheim, Germany) with the provided protocol. 

\textit{p53 silencing}. LNT-229 p53 knock-down cells were described before\textsuperscript{23} and cultured in puromycin-containing medium (10 µg/ml) (Sigma-Aldrich, Steinheim, Germany). 

\textit{Stable overexpression of P4HA2}. H1299 cells with stably integrated P4HA2-encoding or no insert cDNA-containing p3xFLAG-myc-CMV-26 (Sigma-Aldrich) expression vectors were kindly provided by Michael R. Green, University of Massachusetts Medical School, Worcester, MA, USA.\textsuperscript{24} Glioma cells stably overexpressing P4HA2 and empty vector control cells, respectively, were generated by transfecting LN-308 cells with either vectors using FUGENE\textsuperscript{®} HD and selecting with 700 µg/ml G418 (Sigma-Aldrich).

\textbf{Quantitative reverse transcription polymerase chain reaction}

For isolation of total RNA, cells were cultured for 48 h and harvested according to the Qiagen RNAeasy RNA isolation kit (Qiagen, Hilden, Germany). cDNA synthesis was carried out following the instructions of the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in an ABI 7000 thermal cycler using the SYBR Green PCR Mastermix (Applied Biosystems) according to standard protocols. Reactions were checked by including no-RT-controls, by omission of templates, and by both melting curve and gel analysis. The sizes of the amplicons were assessed by loading the PCR products on an ethidium bromide-stained 1.5% agarose gel analyzed under ultraviolet light. Standard curves were generated for each transcript demonstrating 90-100% amplification efficiency. Relative quantification of gene expression was determined by comparison of threshold values. All results were normalized to \textit{glyceraldehyde-3-phosphate dehydrogenase} which varied neither with overexpression nor silencing of p53. To exclude amplification of \textit{GAPDH} pseudogenes, previously described \textit{GAPDH}-specific primer sequences were used.\textsuperscript{27} All qRT-PCR primers were purchased from Sigma-Aldrich.

Primer sequences were (forward, reverse):
**BAI1**: 5'-GCAAACCAAGTTCTGCAACAT-3', 5'-CTCCAGCTGACCACCACAATATT-3';

**bFGF**: 5'-AGCGGGCTGTACTGCAAAAAC-3', 5'-TGCTTGAAGTTGTAGCTTGATGT-3';

**GAPDH**: 5'-CTCTCTGCTCCTCCTGTTTGAC-3', 5'-TGAGCGGATGGCTCGGCT-3';

**P4HA1**: 5'-GGCAGCCAAAGCTCTGTTA-3', 5'-AGTCCTCAGCCGTTAGAAAG-3';

**P4HA2**: 5'-AGCAGCTAAACACAGACTGG-3', 5'-GGCAGCTCCTATCTGCTCCT-3';

**p21CIP1/WAF1**: 5'-TCACTGTCTTTGTACCTTGTGC-3', 5'-GGCGTTTGGAGTGGTAGAAA-3';

**p53**: 5'-CTGCAGCAAGATGTT-3', 5'-CCACTCGGAATAAGATGCTGA-3';

**TSP1**: 5'-CAATTGCCACAGTTCTGTGA-3', 5'-TGGAGACCAAGCATCCTGC-3';

**VEGF**: 5'-TCTTCAAGCCATCTGTTG-3', 5'-GGTGAGGTTTGATCCGCATA-3'.

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**P4HA2 promoter analysis**

For sequence analysis of the P4HA2 promoter region, genomic DNA was extracted from H1299 and LN-308 cells using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. Genomic DNA spanning 1470 bp of the *P4HA2* promoter region (-1141 to +329, relative to the transcription start site) covering the three putative p53-binding half sites at positions -442 to -450, -449 to -457 and -458 to -46724 was amplified as two partially overlapping DNA fragments, 550 base pairs (bp) and 990 bp of length, by polymerase chain reaction (PCR) using the following PCR primer pairs (forward, reverse):

5'-CAGGGGTCGCAGAGGACGGC-3', 5'-CCCGGGAGCCTGACAGGGAA-3';

5'-CTTCGTGCAGTTCCCTCGGC-3', 5'-TTGGCTCACTGCAACCTCTCC-3'.

Using the same primers, both strands of each PCR product were sequenced, aligned and analyzed for mutations by comparison with the data base entry for the *P4HA2* 5' flanking sequences and exon 1.28

Methylation-specific PCR (MSP) was used to analyze the p53-binding sites within the *P4HA2* promoter region for potential methylation-mediated epigenetic silencing. One µg of genomic DNA derived from LN-308 or LNT-229 cells was treated with sodium bisulfite using the Qiagen EpiTect Kit (Qiagen) with the provided protocol. The primer sequences used to
detect methylated \(P4HA2\) promoter sequences were 5'-TGGTCGTGTTTTTTTGTTAGTTTTC-3' and 5'-CGCAACTACAAAAAAACACG-3'. The primer sequences used to detect unmethylated \(P4HA2\) promoter sequences were 5'-GGTTGTGTGTGTTAGTTTTTTG-3' and 5'-CCACAACTACAAAAAAACACAC-3'. Both primer combinations amplify 271 bp fragments from methylated and unmethylated DNA, respectively. After an initial denaturation step at 95°C for 10 min, 38 PCR cycles were run using the following conditions: denaturation, 94°C for 30 s; annealing, 60°C for 60 s; extension, 72°C for 55 s; followed by a final extension at 72°C for 10 min. As positive or negative controls for methylation, CpGenome Universal Methylated or Unmethylated DNA, Vial A, (Millipore, Temecula, CA, USA) were used. In addition, a control reaction without any template DNA was performed for both PCR experiments. The PCR products were separated on a 3% agarose gel, extracted from the gel and sequenced with the respective primer pair used in the MSP. MSP primers were purchased from Eurofins MWG Operon (Ebersberg, Germany).

**Immunoblot**

Immunoblots were performed as described.\(^{29}\) Briefly, cell lysates were prepared in 50 mM Tris-HCl (pH 8.0) containing 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 2 mg/ml aprotinin, 10 mg/ml leupeptin and 100 mg/ml phenylmethylsulfonyl fluoride. 20 \(\mu\)g aliquots of cell lysates were electrophoresed on 10% SDS-PAGE gels under reducing conditions and subsequently transferred onto a nitrocellulose membrane (Bio-Rad, Munich, Germany) in all cases except for c-P4HA1 that was blotted using PVDF membranes (Millipore, Schwalbach, Germany). After blocking nonspecific-binding sites in 5% (w/v) dried milk in TBS containing 0.1% tween for 2 h, membranes were incubated with specific primary antibodies towards p53, p21\(^{\text{CIP1/WAF1}}\), c-P4HA1 or P4HA2 overnight at 4°C, washed and incubated with the respective horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Equal protein loading was ascertained by detecting expression of housekeeping genes (GAPDH, actin, or \(\alpha\)-tubulin) accordingly. Protein bands were visualized
using Enhanced Chemiluminescence Plus (GE Healthcare UK Limited, Buckinghamshire, UK).

The following antibodies were used:

**Actin:** anti-actin goat polyclonal antibody (pAb) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 0.2 µg/ml;

**GAPDH:** anti-GAPDH goat pAb (abcam, Cambridge, UK), 1:1,000;

**P4HA1:** whole serum from guinea pig immunized with P4HA1 peptide (gift from Tore Kempf, PhD, German Cancer Research Center, Heidelberg, Germany), 1:5,000;

**P4HA2:** anti-P4HA2 rabbit pAb (Bethyl Laboratories, TX, USA), 2.0 µg/ml;

**p21<sup>CIP1/WAF1</sup>** anti-p21<sup>CIP1/WAF1</sup> mouse monoclonal antibody (mAb) (Merck), 0.1 mg/ml;

**p53:** anti-p53 mouse mAb (Merck, Darmstadt, Germany), 1 µg/ml;

**α-tubulin:** anti-α-tubulin mouse mAb (Sigma), 1:5,000;

**anti-goat:** peroxidase-conjugated donkey anti-goat (Invitrogen), 0.4 µg/ml;

**anti-guinea pig:** peroxidase-conjugated goat anti-guinea pig (Jackson ImmunoResearch, Suffolk, UK), 0.16 µg/ml;

**anti-mouse:** peroxidase-conjugated sheep anti-mouse (GE Healthcare UK Limited), 1:5,000;

**anti-rabbit:** peroxidase-conjugated donkey anti-rabbit (GE Healthcare UK Limited), 1:2,000.

**Endostatin enzyme-linked immunosorbent assay**

For the quantitative determination of human endostatin concentrations, serum-free glioblastoma cell-conditioned supernatants supplemented with 100 µM ascorbic acid (Sigma-Aldrich) were freed from cellular debris, nonadherent and dead cells by centrifugation (3 min; 4,370 g) and subjected to a solid-phase enzyme-linked immunosorbent assay specific for human endostatin (Quantikine<sup>®</sup> Human Endostatin Immunoassay, R&D Systems,
Minneapolis, MN, USA). Duplicates of each sample were examined according to the manufacturer’s protocol.

**In vitro angiogenesis assays**

Conditioned supernatants derived from adherent LN-308, LNT-229 and H1299 tumor cells were tested in functional in vitro angiogenesis paradigms. LN-308 glioma cells, 8 h after transient transfection with p53 wild-type or control plasmids, p53-silenced LNT-229 cells and LN-308 or H1299 cells stably overexpressing P4HA2, following adherence overnight, were washed in PBS and cultured in endothelial cell basal medium (ECBM) including 1 µM Fe(III) (Promocell) supplemented with 100 µM ascorbic acid without FBS or antibiotics for 48 h. For hCMEC viability, DMEM was used instead of ECBM. Supernatants were collected and freed from debris, nonadherent and dead cells by centrifugation (3 min; 4,370 g), supplemented with 2-10% FBS and recombinant VEGF (R&D Systems) at 25 ng/ml, and tested in the in vitro angiogenesis assays described below (a-c). Recombinant human endostatin (Calbiochem, Darmstadt, Germany) at 10 µg/ml compared with its vehicle control (66 mM sodium phosphate, 59 mM sodium chloride, 17 mM citric acid, pH 6.2) diluted in ECBM served as a positive control in all in vitro angiogenesis experiments.

a) HUVEC sprouting assay. Spheroid formation and sprouting of HUVEC was carried out as described previously. In brief, spheroids were generated by resuspending 400 HUVEC per spheroid in 25 µl culture medium containing 0.24% (w/v) carboxymethylcellulose (Sigma-Aldrich) followed by an incubation for 24 h as hanging drops. After 24 h, spheroids were embedded into collagen gels. A collagen stock solution was prepared prior to use by mixing 8 volumes collagen (rat tail collagen equilibrated to 2 mg/ml in 0.1% acidic acid) with 1 volume Medium 199 (Sigma-Aldrich), ∼1 volume 0.2 M NaOH to adjust pH to 7.4 and 1 M HEPES buffer (Roth, Karlsruhe, Germany) at a final dilution of 1:50. Fifty HUVEC spheroids were resuspended into a collagen gel consisting of 0.5 ml collagen stock solution mixed with 0.5 ml medium (ECBM including 20% FBS) containing 0.5% (w/v) carboxymethylcellulose to prevent sedimentation of spheroids prior to polymerization. One ml per well of the spheroid-
containing gel was rapidly transferred into a 24-well plate prewarmed to 37°C and allowed to polymerize for 30 min. One hundred µl of glioblastoma cell-conditioned supernatant were then pipetted on top of each gel. After incubation for 24 h, *in vitro* angiogenesis was digitally quantitated by measuring the lengths of the sprouts grown out of each spheroid with an ocular grid at 100x magnification using Image J (http://rsbweb.nih.gov/ij/index.html). At least 30 spheroids per experimental group and experiment were analyzed. The average cumulative sprout length of all spheroids belonging to one treatment group was calculated.

b) *Cell viability assay*. Endothelial cell viability assays were performed using both HUVEC and hCMEC. Cells were seeded into 96-well plates at 2,000 cells suspended in 100 µl growth medium. After attachment (~ 8 h), endothelial cells were starved for 16 h by replacing the growth medium with ECBM (for HUVEC) or DMEM (for hCMEC) containing 2% FBS. Following starvation, endothelial cells were incubated in 100 µl of tumor cell-derived supernatant for 96 h conditioned as described above. Absorbance of viable endothelial cells was colorimetrically measured in a 96-well plate reader (Thermo Scientific, Karlsruhe, Germany) at 490 nm using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) as a substrate provided in the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer’s recommendations. Each supernatant was tested in triplicates.

c) *HUVEC transmigration assay*. HUVEC transmigration assays were performed in 6.5 mm Transwells® with gelatine-coated 8.0 µm pore polycarbonate membrane inserts (Corning Incorporated, Lowell, MA, USA). These inserts were placed into 24-well plates containing 600 µl glioma cell supernatants conditioned as described above and supplemented with VEGF as chemoattractant. HUVEC were seeded at 10⁵ cells suspended in 100 µl of the same supernatants without VEGF on top of each membrane into the upper chamber. Cells were allowed to migrate through the pores for 4 h, fixed with ice-cold methanol and stained with Hoechst dye (Sigma-Aldrich). Migrated cells were counted after removal of non-migrated cells from the upper surface of the membrane using a cotton swab. Quantitation was performed using the particle analysis Cell-F software (Olympus, Hamburg, Germany) by
counting labeled cells within seven high-power fields at 100-fold magnification. Each supernatant was tested in triplicates.

**Statistical analysis**

Quantitative data obtained for vessel quantitations, qRT-PCR, ELISA and *in vitro* angiogenesis assays are expressed as mean +/- standard deviation, as indicated. Statistical analyses were performed using Student's t-test (Excel, Microsoft, Seattle, WA, USA). Values of *p* < 0.05 were considered significant and marked with an asterisk.
Results

The TP53 Mutational Status Does Not Affect Tumor Angiogenesis in Primary Glioblastoma

Wild-type p53 has been reported to be associated with suppressed angiogenesis in human tumors and cancer cell lines originating from different tissues.\textsuperscript{24,32-35} Based on these previous findings, we intended to assess the potential value of p53 as a molecular marker to indicate the extent of vascularization in glioblastoma. To this end, we selected formalin-fixed, paraffin-embedded tissues of 40 newly diagnosed, treatment-naïve glioblastoma specimens. Though mutations in the \textit{TP53} gene are observed more frequently in secondary than in primary glioblastomas,\textsuperscript{36} we focused our study exclusively on newly diagnosed glioblastomas thereby avoiding that potential therapy-induced genetic and tissue alterations bias our findings. IDH1 mutations have recently been identified as a marker for the genetic profile of secondary glioblastomas.\textsuperscript{37} To exclude glioblastomas with the genetic profile of secondary glioblastomas we screened for the most frequent IDH1 mutation (R132H) using the IDH1 mutation-specific antibody mIDH1R132H as recently described.\textsuperscript{22} In this set of primary glioblastomas, we determined the \textit{TP53} mutational status by sequence analysis of exons 5 to 8. In four cases, DNA quality was too low to perform sequence analysis. One case was excluded because of positive immunoreactivity with mIDH1R132H. In the remaining cases, we identified 26/35 (74\%) \textit{TP53} wild-type (p53wt) and 9/35 (26\%) \textit{TP53} mutated (p53mut) specimens (see Table 1 for details). For standardization, automated image analysis of CD31-positive vascular structures was performed in solid tumor tissue distant from necroses within regions of highest tumor proliferation. Representative examples of analyzed tumor regions are given in Fig. 1A. As a result, neither total area nor total number of vascular structures differed significantly between p53wt and p53mut glioblastomas (Fig. 1B and C; $p = 0.31$ and $p = 0.4$, respectively).

\textit{P4HA2 Is Transcriptionally Activated through p53 in Glioblastoma Cells}

The results from these comparative analyses demonstrated a lacking correlation between the \textit{TP53} mutational status and the extent of vascularization in primary glioblastoma. As these
observations seemed to conflict in part with data from the literature attributing an angiogenesis-regulating function to p53, we next investigated whether factors encoded by p53-dependent target genes that have been described previously to be involved in controlling angiogenesis of various malignancies were functional in glioblastoma cells. This literature-guided candidate approach comprised the two proangiogenic molecules VEGF and bFGF and the three antiangiogenic factors TSP1, BAI1, and P4HA2. We overexpressed wild-type p53 in the human glioblastoma cell line LN-308 that natively expresses no p53 protein due to a homozygous deletion of the TP53 gene (p53 -/-), and checked p53-dependent transcription of the aforementioned angiogenesis-associated factors by qRT-PCR (Fig. 2A). We confirmed wild-type activities of p53 (Fig. 2A, upper left panel) in that it was able to upregulate the levels of mRNA for the known p53-responsive downstream target gene, p21CIP1/WAF1 (Fig. 2A, upper right panel). As opposed to all other candidate transcripts included in our study, only mRNA for P4HA2 was significantly induced by overexpression of wild-type p53. By contrast, expression of wild-type p53 had no effect on transcription of another collagen prolyl-4-hydroxylase isoform, P4HA1 (Fig. 2A, lower panel).

Based on these observations, we focused our study hence on the role of P4HA2 in regulating glioblastoma angiogenesis. The P4HA2 gene encodes a component of a collagen prolyl 4-hydroxylase, a key enzyme in collagen biosynthesis composed of two identical alpha subunits and two beta subunits. P4HA2 provides the major part of the catalytic site of the active enzyme that catalyzes, in collagen and related proteins, the formation of 4-hydroxyproline being essential to the proper three-dimensional folding of newly synthesized procollagen chains. While the second rate-limiting enzyme of collagen biosynthesis, P4HA1, is ubiquitously expressed and represents the major isoform in connective tissues, P4HA2 is expressed in a more restricted manner in chondrocytes and endothelial cells. Aside from p53-/- Saos-2 osteosarcoma cells, transcriptional activation of P4HA2 through stimulation of endogenous wild-type p53 or gene transfer of wild-type p53 was first described for the human p53-/- non-small cell lung carcinoma cell line H1299. In these cells, P4HA2 induction by wild-type p53 resulted in increased synthesis and secretion of full-length collagen 18 and
4, which were proteolytically processed in the extracellular matrix to produce respective C-terminal antiangiogenic peptides, endostatin and tumstatin, leading to reduced angiogenesis in vitro and in vivo.\textsuperscript{24}

We first confirmed the p53-responsiveness of $P4HA2$ in H1299 carcinoma cells (Fig. 2B) and included them as positive controls in further experiments with glioblastoma cells. To foster our findings from LN-308 cells, we used, as an additional model, the p53wt glioblastoma cell line LNT-229 in which expression of p53 was silenced by single hairpin RNA (shRNA) technology.\textsuperscript{23} The resulting knock-down of p53, as controlled by proving reduced expression of $p21^{CIP1/WAF1}$, was paralleled by a decrease in the transcription of $P4HA2$, but not of $P4HA1$ (Fig. 2B). Conversely, up-regulation of $P4HA2$, but not of $P4HA1$, was observed when endogenous p53 expression was pharmacologically induced in a concentration-dependent manner in LNT-229 cells by applying the DNA damage-inducing topoisomerase I inhibitor camptothecin.\textsuperscript{42} Again, p53-mediated, concentration-dependent induction of $p21^{CIP1/WAF1}$ in response to camptothecin served as an internal positive control in this model (Fig. 2B).

Sequence analysis of the promoter region of the $P4HA2$ gene revealed a CpG site at position -458/-457 critically located between two of three putative neighboring p53-binding half sites ranging from position -467 to -442 relative to the transcription start site (Fig. 2C, upper panel).\textsuperscript{24} As methylation of this CpG site might interfere with the transcriptional efficiency of the $P4HA2$ mRNA, we performed methylation analyses of this promoter region using bisulfite-treated genomic DNA from LN-308 and LNT-229 cells analyzed by methylation-specific polymerase chain reaction (MSP, Fig. 2C, middle panel) and methylation-specific sequencing (Fig. 2C, lower panel). As a result, we found that CpG site to be unmethylated showing that p53-induced transcriptional activation of $P4HA2$ would not be affected by methylation-mediated promoter silencing in glioblastoma cells.

\textbf{p53-Dependent Antiangiogenic Signaling via P4HA2 Is Defective in Glioblastoma Cells}

Our results from LN-308 and LNT-229 glioblastoma cells suggested that, of the angiogenesis-related factors examined in our study, only $P4HA2$ was transcriptionally
controlled by wild-type p53. Hence, it seemed consequent to figure out whether p53-induced mRNA levels of \( P4HA2 \) translated into an increase of biologically active P4HA2 enzyme and thus enhanced production of functionally relevant antiangiogenic collagen fragments. Unexpectedly, despite its transcriptional activation, ectopic overexpression of wild-type p53 in p53\(^{−/−}\) LN-308 cells did not lead to induced protein levels of P4HA2 (Fig. 3A, left panel). Consistently, p53-silencing in p53wt LNT-229 cells did not lead to a decrease of P4HA2 protein (Fig. 3A, middle panel), and camptothecin-mediated induction of endogenous p53 did not upregulate P4HA2 protein either (Fig. 3A, right panel). Wild-type activities of p53 were verified in all immunoblots by proving the regulation of p21\(^{\text{CIP1/WAF1}}\) protein (Fig. 3A). We next tested whether overexpression of wild-type p53 in p53\(^{−/−}\) LN-308 cells or p53-silencing in p53wt LNT-229 cells had an effect on extracellular concentrations of the antiangiogenic collagen 18 fragment endostatin using an endostatin-specific enzyme-linked immunosorbent assay (ELISA). We found concentrations of endostatin in supernatants of both glioblastoma cell lines were not altered by manipulating the expression levels of p53 compared with the respective controls (Fig. 3B). Consistent with these results, supernatants derived from p53-overexpressing LN-308 cells or p53-silenced LNT-229 cells did not influence endothelial cell function as examined in three independent in vitro angiogenesis assays: (i) sprouting of primary human umbilical vein endothelial cells (HUVEC) (Fig. 3C), (ii) cell viability of HUVEC and human cerebral microvascular endothelial cells (hCMEC) (Fig. 3D), and (iii) transmigration of HUVEC (Fig. 3E). As it has long been recognized to inhibit multiple endothelial cell functions, recombinant human endostatin served as a positive control in each angiogenesis assay (Fig. 3C-E).

In summary, the results from all three in vitro angiogenesis assays mirror the overall effect of wild-type p53 expressed in glioblastoma cells on endothelial cell function irrespective of underlying signaling mechanisms. In this regard, these data make clear that potentially p53-associated and angiogenesis-related mechanisms that would even be independent from P4HA2- and collagen-dependent signaling are inefficient in modifying endothelial cell function. After all, these data are in line with our results from the comparative vessel
quantitations in p53wt and p53mut primary glioblastoma specimens (Fig. 1) that also failed to show a difference.

*p53-Independent P4HA2 Antiangiogenic Signaling through Endostatin Is Functional in Carcinoma but Not in Glioblastoma Cells*

If, in glioblastoma cells, p53 does not trigger P4HA2-mediated production of antiangiogenic collagen fragments such as endostatin, then ectopic overexpression of P4HA2 should clarify whether antiangiogenic signaling downstream of P4HA2 is functional even in the absence of p53. To pursue this question, we generated stably P4HA2-overexpressing p53-/- LN-308 cells (Fig. 4A) and compared derived extracellular endostatin concentrations with control-transfected LN-308 cells. As opposed to H1299 lung carcinoma cells that showed a marked increase of extracellular endostatin leading to reduced HUVEC survival, LN-308 glioblastoma cells responded to p53-independent overexpression of P4HA2 neither with induced endostation production (Fig. 4B) nor with differential effects on endothelial function *in vitro* as measured by HUVEC viability (Fig. 4C). Thus, uncoupling of p53 and P4HA2 action still failed to restore the antiangiogenic program that seems to be functional in carcinoma cells. In summary, these data suggest that, in glioblastoma cells, p53-controlled antiangiogenic signaling through P4HA2, collagens and their proteolytically derived C-terminal fragments is shut down both at the translational and the post-translational level.
Discussion

With the present study, we demonstrate that (i) the tumor suppressor protein p53 does not have an impact on vascularization in glioblastomas and (ii) the reason for this is that, in these tumors, wild-type p53-dependent antiangiogenic signaling pathways known to be functional in other malignancies are shut down either at the transcriptional or the translational/posttranslational level.

We included two genetically different human glioblastoma cell lines in our in vitro studies and applied three independent modes of regulating p53 activity in these cells: (i) ectopic overexpression of wild-type p53 in a homozygously p53-deleted genetic background (LN-308 and H1299 cells); (ii) shRNA-mediated silencing of wild-type p53 function (LNT-229 cells); and (iii) pharmacological induction (camptothecin) of endogenous wild-type p53 activity (LNT-229 cells). We then compared conditioned supernatants derived from these tumor cell lines in functional assays testing for human endothelial cell sprouting, viability and transmigration. Aside from otherwise commonly used human umbilical vein endothelial cells (HUVEC), we integrated human cerebral microvascular endothelial cells (hCMEC) in these studies as they appeared likely to be more meaningful for investigations of angiogenesis in cerebral tumors. Guided by precedent reports from the literature, we analyzed the p53-dependent transcriptional inducibility of five candidate genes implicated in angiogenesis: VEGF, bFGF, TSP1, BAI1, and P4HA2, and concluded that of these, only P4HA2 was transcriptionally activated through wild-type p53 (Fig. 2A). This collagen prolyl hydroxylase isoform was first described by Teodoro et al. as a genetic and biochemical linkage between the p53 tumor suppressor pathway and the synthesis of antiangiogenic collagen fragments, e.g., endostatin and tumstatin, in various cancer cell lines including H1299 lung carcinoma cells. We hence focused on this signaling pathway and found that, despite the p53-dependent induction of its transcript, the amounts of P4HA2 protein turned out not to vary in response to altering p53 activities in glioblastoma cells suggesting a translational blockade (Fig. 3A). In this context, another isoform of the protein family of collagen prolyl hydroxylases, P4HA1, has been reported to be translationally dependent on
two chaperon molecules, nucleolin and annexin A2, when cells were exposed to hypoxia. It is thus conceivable that wild-type p53 activates transcription of the P4HA2 isoform without leading to increased protein amounts due to reduced mRNA stability in the absence of required chaperones. However, even stable overexpression of P4HA2 in glioblastoma cells independent from p53 activities (Fig. 4A) was neither paralleled by induced extracellular endostatin levels (Fig. 4B) nor by altered endothelial functions (Fig. 4C) suggesting at least one additional posttranslational blockade in this pathway. The processing of full length collagen 18 into biologically active endostatin is a complex process that involves several matrix metalloproteinases (MMP) as well as cathepsin L and elastase. Regarding our results, it is possible that, in addition to translationally blocked P4HA2, a further step needed for the maturation and processing of collagen molecules to antiangiogenic C-terminal fragments is defective in glioblastoma cells.

Besides collagen-derived endostatin or tumstatin, other secretory factors have been reported to regulate angiogenesis in a p53-dependent manner in various cancers. To our knowledge, however, the undisputed identification and characterization of such a factor in malignant gliomas is pending. Brain-specific angiogenesis inhibitor 1 (BAI1) was identified as a promising antiangiogenic candidate in a library screen for p53-regulated genes in glioma. BAI1 contains vasculostatin, a proteolytic fragment of its extracellular domain, with known antiangiogenic properties. A subsequent study, however, that evaluated the relationship between p53 and BAI1 more thoroughly concluded that expression of BAI1 is independent from p53 activity. Consistent with this later work, we did not observe BAI1 to be transactivated by wild-type p53 in LN-308 cells (Fig. 2A), and BAI1-specific mRNA levels were proved to be absent in LNT-229 cells (data not shown).

Yet conflicting with our observations are data from an early work in this field describing an angiogenesis-suppressing function of wild-type p53 upon Tet-mediated conditional induction in one p53-deficient glioblastoma cell line, LN-Z308, using in vitro bovine adrenal gland capillary endothelial cell migration assays and in vivo neovascularization experiments in rat
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corneas as functional read-outs. The authors of that work postulated the existence of a secretory angiogenesis-suppressing factor released from wild-type p53 glioblastoma cells which they tentatively called glioma-derived angiogenesis inhibitor factor (GD-AIF).\textsuperscript{33} Albeit based on different experimental setups that might, at least in part, explain the difference to our results, that work did not provide tissue-based proofs corroborating the preclinical findings in glioblastoma cells. Proofs for a negative correlation between expression of wild-type p53 and tumor vascularization based on patient-derived tumor specimens are indeed documented for a variety of epithelial cancers including non-small cell lung, colorectal, breast, prostate, and esophageal carcinomas.\textsuperscript{15,16,51-53} By contrast, in some gynecologic malignancies, i.d. epithelial ovarian and endometrial cancers, such a correlation was not observed.\textsuperscript{54,55} Together with our results that confirmed the p53/P4HA2-mediated antiangiogenic pathway in a carcinoma cell line, H1299, it seems therefore likely that effects of p53 on tumor angiogenesis are cell or tumor type-specific.

Of note, a recently published work described increased microvessel densities and absolute vessel numbers in a set of TP53-mutated diffuse low-grade astrocytoma specimens when compared with their TP53 wild-type counterparts suggesting that p53 exerts an angiogenesis-suppressing function in low-grade gliomas.\textsuperscript{56} It might thus be tempting to speculate that the influence of p53 on glioma angiogenesis declines during the course of malignant transformation and that this might contribute to the angiogenic switch. This assumption would be in line with the notion that, in contrast to most carcinomas, in which p53 mutations occur late during tumor progression, p53 mutations represent an early event in the progression of gliomas.\textsuperscript{57,58} By contrast, pathological angiogenesis is a late event during glioma progression that primarily characterizes the phenotype of glioblastomas.\textsuperscript{59} Tumor cells lacking wild-type activities of p53 are genetically unstable and thus prone to accumulating additional molecular alterations enhancing the neoplastic phenotype including increased neovascularization. In addition, hypoxia becomes increasingly influential during tumor progression subsequently leading to the stabilization of HIF-1\textalpha (and HIF-2\textalpha) and the expression of proangiogenic target genes, e.g., VEGF. Hypoxia, in turn, has been
demonstrated to select for tumor cells with diminished apoptotic potential, e.g., due to loss of wild-type p53 function, in solid tumors.\textsuperscript{60} Being aware of the fact that the frequency of mutations in the $\text{TP53}$ gene is lower in primary (28\%) than in secondary glioblastomas (65\%), our study consistently focuses on the role of p53 in affecting angiogenesis in newly diagnosed, i.e. treatment-naïve, glioblastomas primarily in order to circumvent the potential bias caused by therapy-induced genetic and tissue alterations.\textsuperscript{20}

Remarkably, molecular alterations in tumor cells, e.g. the presence or absence of functional p53, leading to their genetic instability and thus resistance towards apoptosis influence the response of adjacent (genetically stable) endothelial cells in the tumor vasculature representing the virtual target of antiangiogenic agents. Hints that p53 is not only instrumental in regulating tumor angiogenesis but also potentially predictive for responding to antiangiogenic therapy come from studies in mice bearing tumors derived from $\text{TP53}^{-/-}$ HCT116 human colorectal carcinoma cells that proved to be less responsive to antiangiogenic therapy than mice bearing isogenic $\text{TP53}^{+/+}$ tumors.\textsuperscript{61} p53 being well-established in neuropathological diagnostics could thus have been an attractive novel biomarker of angiogenesis and predictor of response to antiangiogenic treatment in high-grade gliomas, particularly given that a number of promising antiangiogenic and vasculature-modifying agents are currently under investigation for these tumors.\textsuperscript{62} In a recently published translational study conducted by the German Glioma Network analyzing 301 prospectively accrued patients with newly diagnosed glioblastomas, neither $\text{TP53}$ mutations nor p53 immunoreactivity turned out to be of prognostic or predictive value for primary glioblastomas treated according to current standards of care.\textsuperscript{21} Our findings extend this perception in that they challenge the view of p53 as an angiogenesis-regulating factor in glioblastoma and hence question its potential predictive value for any antiangiogenic regimen applied in this tumor entity.
Acknowledgments

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References


**Table 1.** *TP53* mutations (exons 5 to 8) identified in 9 of 35 primary glioblastomas. Het, heterozygous.
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**Fig. 1.** The extent of vascularization in primary glioblastomas is independent from the *TP53* mutational status. (A) Photomicrographs of CD31-stained primary glioblastoma specimens carrying either the *TP53* wild-type (*TP53*wt) or *TP53* mutated (*TP53*mut) gene. Micrographs were subjected to automated image analyses. The left panel shows a representative *TP53*wt tumor (specimen 3), the right panel represents a *TP53*mut tumor (specimen 25). Magnification 100-fold. (B and C) Vessel quantitation in *TP53*wt and *TP53*mut primary glioblastoma specimens depicted as total area of vascular structures as percentage of total analyzed tumor region (B, *p* = 0.31), and total number of vascular structures (C, *p* = 0.4). Data are mean ± standard deviation.

**Fig. 2.** *p53* activates transcription of *P4HA2* in glioblastoma cells. (A) Expression levels of *p53* (upper left panel), *p21^{CIP1/WAF1}* (upper right panel) and mRNAs of several proangiogenic (*VEGF* and *bFGF*) and antiangiogenic (*TSP1, BAI1, P4HA2*) factors plus *P4HA1* (lower panel) measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in homozygously *TP53*-deleted (*p53*^-/-^) LN-308 glioblastoma cells following transfection with a *p53*-encoding expression vector (*p53*) or the empty vector as a control (Ctrl.). (B) QRT-PCR-measured expression levels of *p53* (upper panel), *p21^{CIP1/WAF1}* (upper middle panel), *P4HA2* (lower middle panel) and *P4HA1* (lower panel) mRNAs in the *p53*^-/-^ H1299 lung carcinoma cell line transfected as LN-308 cells in (A), in *p53*-silenced (*p53*sh) or control (Ctrl.) LNT-229 glioblastoma cells (*TP53* wild type, *p53*wt), and in LNT-229 cells treated with increasing concentrations (1, 10 and 100 nM) of camptothecin or dimethyl sulfoxide as a vehicle control (0 nM). *GAPDH*-normalized expression of each transcript in (A) and (B) is expressed relative to control conditions set as 1. Data are mean ± standard deviation. *, *p* < 0.05. Experiments were repeated three times with similar results. (C) Methylation analysis of the *P4HA2* promoter region. **Upper panel,** schematic diagram of the *P4HA2* promoter region from position -467 to -442 showing the positions of the putative partially overlapping *p53*-binding half sites at positions -442 to -450, -449 to -457 and -458 to -467, as indicated with brackets,
and the genomic and deduced bisulfite DNA sequences in case of methylation (a) or not (b) including the critical CpG site at position -458/-457 (marked by red rectangle) relative to the transcription start site. Middle panel, methylation-specific polymerase chain reaction (MSP) products at 271 base pairs (bp) using bisulfite-treated genomic DNA derived from LN-308 and LNT-229 glioblastoma cells as a template and primer pairs specific for annealing to methylated (M) and unmethylated (U) bisulfite DNA. CpGenome Universal Methylated (Meth) or Unmethylated (Unmeth) DNA (Millipore, Temecula, CA, USA) served as positive or negative controls for both primer pairs. A control reaction without any template DNA (H₂O) was included as an additional negative control. PCR products were separated on a 3% agarose gel. First lane left, 100 bp DNA marker. Lower panel, methylation-specific sequence of bisulfite-treated DNA of the P4HA2 promoter region (position -467 to -442) in LN-308 and LNT-229 cells. The methylation status of the critical CpG site at position -458/-457 is marked by a red square corresponding to the deduced bisulfite DNA sequence depicted in the upper panel. TSS, transcription start site.

**Fig. 3.** p53-dependent antiangiogenic signaling via P4HA2 is defective in glioblastoma cells. (A) Immunoblot analyses demonstrating expression of p53, p21⁴⁳⁴¹⁺⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓
control cells as in (A) for HUVEC sprouting (C), HUVEC and hCMEC viability (D), and HUVEC transmigration (E). Treatment with 10 µg/ml recombinant human endostatin (rhEndo) served as a positive control in each assay. Responses of HUVEC and hCMEC towards supernatants from p53-overexpressing and p53-silenced glioblastoma cells or recombinant endostatin is expressed relative to supernatants derived from respective control cells or the vehicle for endostatin set as 100%. Data are mean ± standard deviation. *, p < 0.05. Experiments were repeated three times with similar results. Insets depict representative endothelial cell responses towards the respective treatment as indicated. Scale bars, 100 µm.

**Fig. 4.** p53-uncoupled P4HA2-mediated antiangiogenic signaling through endostatin is functional in carcinoma but not in glioblastoma cells. (A) Immunoblot analysis confirming overexpression of P4HA2 protein in both p53⁻/⁻ H1299 lung carcinoma (left panel) and p53⁻/⁻ LN-308 glioblastoma cells (right panel) following stable transfection with an expression vector encoding P4HA2 (P4HA2) and an empty vector (Ctrl.), respectively. α-tubulin or GAPDH served as loading controls. (B) ELISA-based quantitation of endostatin concentrations in conditioned supernatants of either H1299 or LN-308 cells transfected as in (A). (C) Viability of HUVEC exposed to conditioned supernatants from either H1299 or LN-308 cells transfected as in (A). HUVEC viability at exposure to supernatants derived from P4HA2-overexpressing tumor cells is expressed relative to supernatants from empty vector controls set as 100%. Data are mean ± standard deviation. *, p < 0.05. Experiments were repeated three times with similar results.