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The p53 isoform, $\Delta 133p53\alpha$, stimulates angiogenesis and tumour progression

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ABSTRACT

The tumour suppressor p53 involved in DNA repair, cell cycle arrest and apoptosis, also inhibits blood vessel formation, i.e. angiogenesis, a process strongly contributing to tumour development. The *p53* gene expresses 12 different proteins (isoforms), including TAp53 (p53 (or p53 α), p53 β and p53 γ) and Δ 133p53 isoforms (Δ 133p53 α , Δ 133p53 β and Δ 133p53 γ). Δ 133p53 α was shown to modulate p53 transcriptional activity and is overexpressed in various human tumours. However its role in tumour progression is still unexplored. In the present study, we examined the involvement of Δ 133p53 isoforms in tumoural angiogenesis and tumour growth in the highly angiogenic human glioblastoma U87. Our data show that conditioned media from U87 cells depleted for Δ 133p53 isoforms block endothelial cell migration and tubulogenesis without affecting endothelial cell proliferation *in vitro*. Δ 133p53 depletion in U2OS osteosarcoma cells resulted in a similar angiogenesis blockade. Furthermore, using conditioned media from U87 cells ectopically expressing each Δ 133p53 isoform, we determined that Δ 133p53 α and Δ 133p53 γ but not Δ 133p53 β stimulate angiogenesis. Our *in vivo* data using the chicken chorio-allantoic membrane and mice xenografts establish that angiogenesis and growth of glioblastoma U87 tumours are inhibited upon depletion of Δ 133p53 isoforms. By TaqMan Low Density Array, we show that alteration of expression ratio of Δ 133p53 and TAp53 isoforms differentially regulates angiogenic gene expression with Δ 133p53 isoforms inducing pro-angiogenic gene expression and repressing anti-angiogenic gene expression.

Keywords: angiogenesis/ p53/ isoform/ cancer/ glioblastoma

INTRODUCTION

The well-known tumour suppressor p53 called "the guardian of the genome" was first described as a single protein. However, we have shown that the *p53* gene expresses 12 protein isoforms (1, 2). Alternative splicing of intron 9 generates p53 isoforms bearing different C-terminal domains (α , β and γ) (Fig. 1A). The presence of an alternative promoter in intron 4 generates $\Delta 133p53$ isoforms lacking the transactivation domain and part of the DNA-binding domain. In addition, initiation of translation at alternative AUG codons, codon 40 or codon 160, leads to $\Delta 40p53$ and $\Delta 160p53$ isoforms, respectively. p53 protein isoforms can be categorized into four subclasses: TAp53, $\Delta 40p53$, $\Delta 133p53$ and $\Delta 160p53$ protein isoforms. Each subclass is composed of the three isoforms α , β and γ .

p53 isoforms are conserved in *Drosophila*, zebrafish, mouse and human, but little is known about their functions (2, 3). A gain of expression of $\Delta 133p53$ mRNAs in human breast tumours versus normal breast tissue suggests a protumoural role of these isoforms (2). Indeed, $\Delta 113p53$ the zebrafish ortholog of human $\Delta 133p53\alpha$ is able to regulate wild-type p53 (WTp53) pro-apoptotic function (3). This activity is conserved in human cells since $\Delta 133p53\alpha$ can inhibit p53-mediated apoptosis and G1 cell cycle arrest without inhibiting p53-mediated G2 cell cycle arrest (4). Furthermore, increased expression of $\Delta 133p53\alpha$ is associated with inhibition of replicative senescence in normal human fibroblasts and colon carcinoma (5). However, no data are available about a direct role of $\Delta 133p53\alpha$ in tumour progression.

In addition to its ability to control DNA repair, cell cycle arrest and apoptosis, p53 is involved in inhibition of angiogenesis, a critical mechanism in tumour progression and metastatic dissemination(6). This process is induced in most solid tumours, whose centers become hypoxic following an increase of the diffusion distance between the nutritive blood vessels and tumour cells. Hypoxia is one of the principal angiogenic stimuli leading to synthesis of angiogenic growth factors and inducing formation of new blood vessels (7, 8). Such vessels, in addition to their ability to feed tumour cells, provide a way for cells to disseminate and form metastases. p53 inhibits angiogenesis by at least three mechanisms: 1) by regulating expression and activity of the central regulator of hypoxia, the hypoxia-induced transcription factor HIF1- α , 2) by inhibiting production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2), and 3) by increasing production of anti-angiogenic factors (6, 9-14).p53 is able to induce tumour dormancy and the loss of WTp53 reverses the tumour switch from a dormant non-angiogenic state to an

angiogenic invasive phenotype (15, 16). Clinical data also suggest that p53 plays a crucial role in controlling tumour vascularization. However, nothing is known about the role of $\Delta 133p53$ isoforms (α , β and γ) in this process.

Among all solid tumours, glioblastoma multiforme (GBM) are the most angiogenic by displaying the highest degree of vascular proliferation and endothelial cell hyperplasia (for review (17)). Such intense vascularization plays a critical role in the pathological features of GBM, including peritumoural oedema resulting from the defective blood brain barrier (BBB) in the newly formed vasculature. Thus, human glioblastoma U87, expressing WT p53, has been chosen here to address the role of $\Delta 133p53$ isoforms in angiogenesis and tumour progression.

In the present study we establish by knockdown and ectopic expression experiments that angiogenesis can be regulated by manipulating the expression ratio of $\Delta 133p53\alpha$ and p53.

RESULTS

Characterization of p53 isoforms expression in human glioblastoma U87.

p53 isoforms expression was analyzed at the mRNA level by nested RT-PCR in U87 human glioblastoma cells, which express wild-type p53 gene. We determined that U87 cells express TAp53 isoforms (p53 ($p53\alpha$) and $p53\gamma$) and $\Delta 133p53$ isoforms ($\Delta 133p53\alpha$ and $\Delta 133p53\beta$). The $\Delta 40p53$ isoforms subclass as well as $p53\beta$ and $\Delta 133p53\gamma$ were not detected at the mRNA level (Fig. 1B).

To investigate the role of endogenous p53 isoforms in angiogenesis, U87 cells were transfected with the siRNA si $\Delta 133p53$ which targets specifically the $\Delta 133p53$ isoforms subclass or with the siRNA siTAp53 which targets specifically TAp53 isoforms subclass (Fig. 1A). The specificity of the above siRNAs has previously been assessed and validated (4, 18). Efficiency of si $\Delta 133p53$ and siTAp53 in U87 cells was confirmed by qRT-PCR and Western blotting, as compared to the control siRNA (siNON) (Fig. 1C1, 1C2, 1D1 and 1D2). As expected, transfection of siTAp53 inhibits p53 mRNA expression but has no effect on $\Delta 133p53$ mRNA expression. Transfection of si $\Delta 133p53$ in U87 cells represses $\Delta 133p53$ mRNA expression. However, transfection of si $\Delta 133p53$ induces p53 expression at both protein and mRNA levels in U87 cells (Fig. 1C2 and 1D2). Similar results were obtained with another siRNA specific for $\Delta 133p53$ isoforms, si $\Delta 133p53$ -2 (Suppl Fig. S1) (4). Thus induction of p53 in response to si $\Delta 133p53$ transfection seems to be due to inhibition of p53 mRNA expression by $\Delta 133p53$ isoforms. Co-transfection of U87 cells with siTAp53 and

si Δ 133p53 leads to inhibition of Δ 133p53 expression and prevents induction of p53, maintaining p53 expression at its basal level. Therefore, the expression ratio of endogenous Δ 133p53 and p53 isoforms can be manipulated by transfection of siTAp53 and/or si Δ 133p53.

Tumour conditioned medium from U87 glioblastoma cells or U2OS osteosarcoma cells transfected with si Δ 133p53 impairs angiogenesis in vitro.

Anti-angiogenic activities reported for p53 prompted us to look at the effect of Δ 133p53 isoforms in angiogenesis. U87 cells were transfected with si Δ 133p53, siTAp53 or siNON and the corresponding conditioned medium was added onto human umbilical vein endothelial cells (HUVECs). Migration of HUVECs was then analyzed in the wound healing assay (Fig. 2A1 and 2A2). The ability of cultured HUVECs to form vessel-like structures (tubulogenesis) on matrigel was also investigated (Fig. 2B1 and 2B2). Conditioned medium from U87 cells transfected with si Δ 133p53 inhibited HUVEC migration as well as their ability to form vessel-like structures (Fig. 2B1 and 2B2) whereas conditioned medium from U87 cells transfected with siTAp53 had no effect. Interestingly, conditioned medium from U87 cells co-transfected with siTAp53 and si Δ 133p53 had no effect on HUVEC migration and tube formation as well as proliferation (Fig. 2A1-2B2). Cell proliferation was also assessed: neither si Δ 133p53, nor siTAp53, nor the double siRNA treatment had any effect on U87 proliferation (Suppl. Fig. S2) and the different conditioned media had no effect on HUVEC proliferation (Fig. 2C).

Endothelial cell migration was then studied in a transwell migration assay. In this experiment adult bovine aortic endothelial cells (ABAE) were seeded in Boyden Chambers and their migration quantified after treatment with conditioned media of siRNA-transfected U87 cells (Suppl. Fig. S3). Here, the knockdown of Δ 133p53 in U87 cells again resulted in a significant decrease of endothelial cell migration whereas the knockdown of p53 generated a significant increase, suggesting that the absence of increase of HUVEC migration and tubulogenesis following treatment with siTAp53-conditioned media is probably due to experimental conditions. Such an increase would probably have been observed as for ABAE if migration and tube formation would have been quantified at earlier times.

Altogether these data show that the secretion of angiogenic and/or anti-angiogenic factors by U87 cells into the conditioned medium is regulated by the expression ratio of p53 and Δ 133p53 and suggest that the knockdown of Δ 133p53 in U87 cells impairs the ability of these tumour cells to stimulate angiogenesis.

To determine whether this effect of $\Delta 133p53$ can be generalized to other tumours, HUVEC tubulogenesis was performed using conditioned media of U2OS osteosarcoma cells (p53 wild type) treated with the different siRNAs. The same data were obtained as for U87 cells, showing that the pro-angiogenic effect of $\Delta 133p53$ is not restricted to U87 glioblastoma (Fig. 2D).

$\Delta 133p53\alpha$ and $\Delta 133p53\gamma$ overexpression stimulates endothelial cell tubulogenesis.

In order to determine which $\Delta 133p53$ isoform (α, β or γ) is responsible for endothelial cell migration and tubulogenesis, lentivectors expressing each of these isoforms under the control of a tetracyclin-inducible promoter were used to transduce U87 cells (Suppl Fig. S4 and S5). To measure tubulogenesis, HUVECs were seeded on matrigel as above and incubated in conditioned media produced by doxycycline-treated U87 cells (Fig. 3). Data clearly showed that overexpression of $\Delta 133p53\alpha$ and $\Delta 133p53\gamma$, but not of $\Delta 133p53\beta$, are able to stimulate endothelial cell tubulogenesis, suggesting that $\Delta 133p53\alpha$ and $\Delta 133p53\gamma$ isoforms have a pro-angiogenic activity. Since U87 cells express $\Delta 133p53\alpha$ and $\Delta 133p53\beta$ but not $\Delta 133p53\gamma$ (Fig. 1B), we concluded that only $\Delta 133p53\alpha$ is responsible for the secretion of factors by U87 cells that regulate endothelial cell migration and tubulogenesis of HUVECs.

Si $\Delta 133p53$ inhibits angiogenesis and tumour growth *in vivo* on the chorio-allantoic membrane (CAM).

The effect of $\Delta 133p53\alpha$ on angiogenesis and tumour growth was addressed *in vivo* using the experimental glioma assay developed on the chicken CAM, as previously described (19). This assay recapitulates hallmarks of human GBM and allows following the first steps of tumoural angiogenesis. siRNA-transfected U87 cells were deposited on the CAM of fertilized eggs and tumours were analyzed 4 days later (Fig. 4A). Transfection of siTAp53 resulted in an increase of tumour vascularization, whereas transfection of si $\Delta 133p53$ led to the formation of avascular tumours, showing that angiogenesis can be regulated by change in expression ratio of p53 and $\Delta 133p53\alpha$ (Fig. 4B and 4C, left panel). Similar results were obtained with si $\Delta 133p53$ -2 (Suppl Fig. S6). Angiogenesis inhibition upon si $\Delta 133p53$ transfection was assessed by counting chick blood vessels in tumour sections stained with SNA-1 lectin conjugated to FITC (Fig. 4C, middle panel). The anti-angiogenic effect upon si $\Delta 133p53$ transfection was also correlated with smaller size of tumours at day 4 (Fig. 4C, right panel). Western blot analyses confirmed that siRNAs were still efficient in tumours 4 days after transfection: tumours treated with siTAp53 or si $\Delta 133p53$ showed an decrease or an increase

of p53 expression, respectively (Suppl. Fig. S7). Interestingly, tumours resulting from the co-transfection of siTAp53 and si Δ 133p53 looked similar to the ones obtained after transfection of siNON (control). This confirms *in vitro* data and indicates that angiogenesis can be regulated by altering the expression ratio of p53 and Δ 133p53 α .

si Δ 133p53 inhibits angiogenesis and tumour growth in mouse xenografts.

To study Δ 133p53 α involvement in tumour progression, siRNA-transfected glioblastoma U87 cells were subcutaneously implanted in nude mice, and followed during 44 days (Fig. 5 and Table I). At day 7 after xenograft, U87 cells transfected with si Δ 133p53 generated significantly smaller tumours compared to U87 cells transfected with siNON (Fig. 5A). However, tumours generated from U87 cells transfected with siTAp53 or cotransfected with siTAp53 and si Δ 133p53 had similar sizes compared to tumours generated from U87 cells transfected with siNON. The mice were followed until day 44, which clearly showed that tumours generated after si Δ 133p53 treatment of U87 grew significantly more slowly than the control and were drastically smaller than the control at day 35 as well as day 44 (Fig. 5B and Table I). However, in contrast to the data obtained at day 7, the size of tumours from U87 cells co-transfected with si Δ 133p53 and siTAp53 was similar to the size of tumours from U87 cells transfected with si Δ 133p53.

Angiogenesis was quantified at day 7 by staining of the endothelial cell marker, CD31. Our data showed that tumours from si Δ 133p53-transfected U87 cells were significantly less vascularized at day 7, as compared to tumours from U87 cells transfected with siNON or siTAp53 or co-transfected with siTAp53 and si Δ 133p53 (Fig. 5C).

These results are consistent with the ones obtained in CAM and confirm that change in expression ratio of p53 and Δ 133p53 α regulates angiogenesis and tumour growth in both *in vivo* models. Hence an increase of Δ 133p53 α compared to p53 would stimulate angiogenesis while an increase of p53 compared to Δ 133p53 α would inhibit angiogenesis.

p53 and Δ 133p53 α regulate angiogenesis by differentially modulating expression of angiogenesis-related genes.

To decipher how p53 and Δ 133p53 α regulate angiogenesis, expression of 96 angiogenesis-related genes was analyzed by TaqMan Low Density Array (TLDA) in siRNA-transfected U87 cells, as indicated (Fig. 6). We used a Taqman human angiogenesis array providing a panel of 96 angiogenesis-related genes (Suppl Table I). In the control, expression of 14/39

anti-angiogenic genes (35%) and 20/28 pro-angiogenic genes (71%) was detected (Fig. 6A). Results with si Δ 133p53 and siTAp53 were normalized to siNON-transfected U87 cells.

Regarding the anti-angiogenic factors, transfection of si Δ 133p53 significantly induced interleukin 12A (IL12A) and matrix metalloproteinase 2 (MMP2), whereas collagen IV (COL4A2) was significantly repressed (Fig. 6B and Table II). Transfection of siTAp53 induced also MMP2 expression, while no change of expression was noted for collagens IV (COL4A1, -A2 or -A3) and XVIII (COL18A1) known as targets of p53 (6). In contrast, IL12A was repressed upon TAp53 isoforms depletion. Interestingly, only IL12A expression was still significantly induced upon cotransfection of U87 cells with siTAp53 and si Δ 133p53. This indicates that anti-angiogenic factors expression is differentially regulated by the expression ratio of Δ 133p53 α and p53. Moreover, it suggests that some genes such as IL12A are regulated by Δ 133p53 α independently of p53.

Regarding the pro-angiogenic factors, angiogenin (ANG), midkine (MDK, neurite growth-promoting factor 2), EGF-like repeats and discoidin I-like domains 3 (EDIL3) and hepatocyte growth factor (HGF) were significantly down-regulated upon transfection of si Δ 133p53. In contrast, transfection of siTAp53 had no effect on the level of expression of these genes (Fig. 6C). Interestingly, the level of expression of the classical angiogenic growth factors VEGF, FGF1 and FGF2 was not significantly affected by any of the siRNAs. Moreover, expression of MDK, EDIL3 and HGF genes remained unchanged in U87 cells co-transfected with siTAp53 and si Δ 133p53, while ANG expression was still induced (Fig. 6C). In addition, upon transfection of si Δ 133p53, the angiogenic marker angiopoietin-like 4 (ANGPTL4) is down-regulated, a repression that is not observed in U87 cells co-transfected with siTAp53 and si Δ 133p53 isoforms. This indicates that these pro-angiogenic factors and markers are differentially regulated by the expression ratio of Δ 133p53 α and p53 (Fig. 6D). Moreover, it suggests that some genes such as ANG are regulated by Δ 133p53 α independently of p53.

Altogether, our results indicate that upon transfection of U87 tumour cells with si Δ 133p53, which represses Δ 133p53 α expression and induces p53, anti-angiogenic factors are induced, while expression of secreted pro-angiogenic factors is repressed.

DISCUSSION

We and others have previously shown abnormal expression of p53 isoforms including p53 β , p53 γ or Δ 133p53 mRNA expression in human tumours compared to normal corresponding

tissue (20). p53 γ expression is associated with good prognosis in breast cancer patients expressing mutant p53 (21). Moreover, Δ 133p53 α inhibits replicative senescence, p53-mediated apoptosis and G1 cell cycle arrest without preventing p53-promoted G2 cell cycle arrest by regulating gene expression (2-5). Altogether, it suggests that p53 isoforms play a key role in carcinogenesis. However, the role of p53 isoforms in angiogenesis was unknown. The present study reveals that Δ 133p53 is responsible for a strong angiogenesis stimulation that mainly contributes to acceleration of human glioblastoma progression. Δ 133p53 angiogenic effect is due to Δ 133p53 α and γ , but not β isoform.

To study the role of p53 isoforms on angiogenesis, we used the glioblastoma U87 model expressing WTp53. Glioblastoma multiforme (GBM) are the most angiogenic by displaying the highest degree of vascular proliferation and endothelial cell hyperplasia. We have shown that U87 cells express p53 and p53 γ (TAp53 isoforms) as well as Δ 133p53 α and Δ 133p53 β (Δ 133p53 isoforms) at the mRNA level. Only p53 protein could be detected at the protein level because of the low affinity of current p53 antibodies and the low expression level of p53 protein isoforms. However, it is important to note that several studies have reported that p53 protein isoforms are biologically active at low expression level (3-5, 18, 22).

By a knockdown approach using siRNAs specific of TAp53 isoforms or of Δ 133p53 isoforms, we have noticed that si Δ 133p53 concomitantly represses Δ 133p53 mRNAs and induces p53 at the mRNA and protein levels in U87 cells, while siTAp53 represses p53 expression at the mRNA and protein level without altering expression of Δ 133p53 mRNAs. Interestingly, co-transfection of si Δ 133p53 and siTAp53 represses Δ 133p53 mRNAs expression without inducing p53 expression. Since the specificity of siTAp53 and si Δ 133p53 has been previously validated, it suggests that Δ 133p53 isoforms repress p53 expression in U87 cells cultured under standard conditions. We and others have shown that p53 transactivates in response to stress the internal p53 promoter giving rise to Δ 133p53 isoforms, thus providing a negative feedback regulation of the p53 response (3, 4, 18, 23). Altogether, it suggests that p53 and Δ 133p53 isoforms regulate each other expression in U87 cells. Further experiments will be required to decipher the Δ 133p53-mediated repression of p53 in U87 cells.

By incubating conditioned media from siRNA-transfected U87 cells with primary human or bovine endothelial cells, we have demonstrated that the expression ratio of p53 and Δ 133p53 isoforms regulate endothelial cell migration and tubulogenesis. Furthermore their ectopic expression has revealed that Δ 133p53 α and Δ 133p53 γ but not Δ 133p53 β are able to stimulate angiogenesis. Since Δ 133p53 α and Δ 133p53 β but not Δ 133p53 γ are endogenously expressed

in U87 cells, we conclude that stimulation of angiogenesis is only due to endogenous $\Delta 133p53\alpha$ in these cells. Using *in vitro* and *in vivo* models, we have then established that the p53 and $\Delta 133p53\alpha$ regulate angiogenesis by modulating expression of angiogenic-related genes. Hence, an expression ratio of p53 and $\Delta 133p53\alpha$ in favor of $\Delta 133p53\alpha$ would stimulate angiogenesis while an expression ratio of p53 and $\Delta 133p53\alpha$ in favour of p53 would inhibit angiogenesis.

A previous report showed that $\Delta 113p53$, the zebrafish ortholog of human $\Delta 133p53\alpha$, antagonizes p53-induced apoptosis via Bcl2 activation, and differentially modulates p53 target gene expression (3). Moreover, $\Delta 133p53\alpha$ prevents replicative senescence by inhibiting transactivation of miR-34a by p53 (5). We have recently established that $\Delta 133p53\alpha$ inhibits p53-mediated apoptosis and G1 cell cycle arrest without inhibiting p53-mediated G2 cell cycle arrest. This suggests that $\Delta 133p53\alpha$ does not exclusively act by inhibiting p53. This has been confirmed by the fact that *p21*, *Bcl-2* and *HDM2* genes are differentially regulated by $\Delta 133p53\alpha$ in response to DNA damage. Indeed, $\Delta 133p53\alpha$ represses p53 transcriptional activity on p21 promoter, while it increases p53-dependent induction of *HDM2* expression and induces *Bcl-2* expression independently of p53 (4). Therefore, we concluded that $\Delta 133p53\alpha$ modulate apoptosis and cell cycle progression in response to stress by regulating gene expression in a p53-dependent and independent manner.

Our present study of angiogenesis-related gene expression by TLDA has figured out that upon si $\Delta 133p53$ transfection, which represses $\Delta 133p53\alpha$ and induces p53, several anti- and pro-angiogenic factors are differentially regulated leading to inhibition of angiogenesis. Reciprocally, upon co-transfection of U87 tumour cells with si $\Delta 133p53$ and siTAp53, which represses (not abolishes) $\Delta 133p53\alpha$ expression and maintains p53 expression at basal level, one can expect that anti-angiogenic factors are repressed, while expression of secreted pro-angiogenic factors is induced, leading to stimulation of endothelial cell migration and blood vessel formation (tubulogenesis). Our data suggest that $\Delta 133p53\alpha$ upregulation affects the angiogenic balance in favour of angiogenesis, and provides an explanation to inhibition of HUVEC tubulogenesis *in vitro* and tumours angiogenesis *in vivo* observed in our study. Therefore, angiogenesis through regulation of angiogenesis-related gene expression could be controlled by modulating the expression ratio of p53 and $\Delta 133p53\alpha$ using specific siRNAs.

Moreover, the fact that some pro and anti-angiogenic gene factors (such as IL12A and ANG) are still regulated upon co-transfection of siTAp53 and si $\Delta 133p53$, suggests that such genes are regulated by $\Delta 133p53\alpha$ independently of p53. Furthermore, it is important to note that expression of the classical angiogenic VEGF or FGF genes is not significantly altered upon

transfection by si Δ 133p53, suggesting a regulation of a distinct signaling pathway involving other angiogenic factors including ANG, HGF and ANGPTL4.

The present study demonstrates for the first time the direct role of Δ 133p53 α isoform in tumour progression. Such a feature may be critical in tumours exhibiting WTp53, which represent about 72% of grade I and 35% of grade II glioblastoma and for which angiogenesis has been recognized as a key event in their progression to malignancy (17, 24). Our data may find an important relevance in anti-angiogenic therapeutics of cancer, presently based on angiogenesis inhibitors targeting the VEGF signaling pathway. Such treatments have been proven to be efficacious in patient survival, associated with a chemotherapy. However, while antitumoural effects and survival benefit are often evident, relapse to progressive tumour growth has been recently reported, reflecting multiple mechanisms of adaptation to antiangiogenic therapies (25, 26). In this context, Δ 133p53 may have a critical role by stimulating angiogenesis through pathways involving several angiogenic factors distinct from VEGF. Thus, targeting Δ 133p53 may have a strong impact in cancer therapeutics.

MATERIALS AND METHODS

Cells and embryos.

HUVECs (Promocell) were cultivated in endothelial growth medium (EGM-2) containing 2% fetal bovine serum (Cambrex/Lonza). **ABAE (primary cells) were cultivated as previously (28).** Humanglioblastoma U87-MG (No ATCC : HTB-14, provided by LGCpromochem, Molsheim, France), osteosarcoma U2OS (No ATCC HTB-96) and fertilized chicken eggs (E.A.R.L. Morizeau, Dangers, France) were handled as described (19).

SiRNA knockdown, cell transfection, western blotting.

Small interfering RNAs (siRNAs) were purchased from Eurogentec (Liege, Belgium) and Sigma-Aldrich (Saint-Quentin Fallavier, France). Sequences are provided in **Suppl Table II**. SiNON served as a negative control. SiTAp53 targets the full transactivation domain (TA) of p53 and Si Δ 133p53 1 and 2 target the 5' untranslated region of Δ 133p53. U87 cells were transfected with siRNAs at 50nM using InterferIN (Polyplus transfection, New York, USA) or with p Δ 133p53, using FuGENE reagent (Roche, Meylan, France).

Western blots were performed as previously described (2). Primary antibodies were CM-1 and Sapu(2) for p53 and Δ 133p53, respectively, 2A10 (Abcam, Cambridge, UK) for mdm2, TUB2.1 (Sigma-aldrich) for β -Tubulin and AC-15 (Sigma-aldrich) for β -Actin. Horseradish

peroxidase-conjugated goat antibodies (Jackson ImmunoResearch, Baltimore), were used as secondary antibodies in immunoblots. Quantitative analysis of the immunoblot data was performed using the ImageJ 1.40g software.

Lentivector production and U87 cell transduction.

The cDNAs coding $\Delta 133P53\alpha$, $\Delta 133P53\beta$, or $\Delta 133P53\gamma$, respectively, were subcloned into the lentivector pTRIP-DU3-TRE-MCS, derived from the self-inactivating lentivector pTRIP-DU3-EF1a-EGFP(27). The EF1a promoter was replaced by the tetracyclin-inducible pTRE promoter coming from the vector pTRE-tight (Clontech) and a multiple cloning site was inserted in place of the GFP gene (Suppl Fig. S4). The resulting lentivector plasmids were pTRIP-TRE- $\Delta 133P53\alpha$, - $\Delta 133P53\beta$, and - $\Delta 133P53\gamma$. Lentivector particles were produced by the BIVIC vector facility (L. Van den Berghe, F. Gross) from HEK293 cell co-transfection by each plasmid pTRIP with plasmids pLvPack and pLvVSVg (SigmaAldrich) coding human immunodeficiency virus 1 capsid proteins and vesicular stomatitis virus G envelope protein, respectively, as previously described.

Endothelial cell migration, tube formation and proliferation.

HUVECs migration in the “wounding assay” was performed during 8 hours after treatment with conditioned media from siRNA-transfected U87 or U2OS cells, as described previously (28). Conditioned media were harvested 72h after cell transfection and administrated to HUVEC 48h after starvation.

ABAE migration in the transwell migration assay was performed in Boyden Chambers (Millipore) according to the manufacturer instruction. Quantification was performed 6h after treatment with conditioned media.

To assess the ability of cultured HUVECs to form vessel-like structures in culture (tube formation), Matrigel (BD Biosciences) was added to the wells of an 24-well plate in a volume of 300 μ l and allowed to solidify at 37°C for 30 min. After the Matrigel solidified, HUVECs (5×10^4 cells) were plated in 100 μ l of media EGM-2 with 0.5% serum and 300 μ l of siRNA tumour cells transfected supernatant containing 20 ng/ml FGF2 final. Cultures were photographed and the mean number of branch points (SEM) per microscopic field was determined. Studies were performed in triplicate.

Cell proliferation was determined by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. HUVECs (2×10^3 /well) were incubated with EBM-2 medium with 2% FBS in 96-well plates. HUVECs were serum starved overnight and

incubated in 50 μ l of media EGM-2 with 0.5% serum and 150 μ l of siRNA tumour cells transfected supernatant containing 3 ng/ml FGF2 final. The medium then was aspirated and MTT was added to each well (0.25 mg/ml). Cells then were incubated for a further 4 h at 37°C. The medium then was aspirated and the cells were lysed with DMSO and absorbance at 540 nm was measured.

Tumour in CAM and xenografts in mouse.

3 millions of siRNA-transfected U87 cells were deposited on CAM as described (19). 18 digital photos were taken on day 4 using a stereomicroscope (Nikon SMZ800).

For xenografts, 1 million cells were injected subcutaneously into nude background (n=8). Animals were sacrificed 10 days later. Six-week-old nude mice were purchased from Janvier. Mice were injected with 10^6 siRNA transfected U87 cells subcutaneously into the mid-back region. The tumour size was measured in three dimensions with calipers twice weekly starting at Day 3. Mice were observed for any change in behavior, appearance or weight. At the end of the experiment at Day 10, the mice were killed and xenograft specimens were harvested for further analyses.

Tumours volumes were measured according to the international formule $V = 1/2 (\text{length} \times \text{width}^2)$ (29). Length and width were estimated using a stereomicroscope and Adobe Photoshop software.

Histology and immunochemistry.

Tumours from CAM xenografts were fixed and cryo-sectioned as described (30). Ten micrometer sections were stained with hematoxylin and eosin. For immunohistology studies, the following primary antibodies were used: Ab-2 (clone V9, NeoMarkers Ab, Montluçon, France) for vimentin, Anti Ki-67 (AnaSpec Inc., Fremont, CA) for Ki-67, fluorescein-coupled SNA-1 (AbCys, Paris, France) for Sambucus nigra lectin (SNA-lectin). Cell nuclei were visualized by DAPI dye. Corresponding secondary antibodies were from Molecular Probes (Invitrogen). SNA-lectin staining allowed quantification of the capillary network invading the tumour by counting the number of green pixels on each field with Adobe Photoshop software. The whole surface of tumours has been counted because of capillary staining heterogeneity (at least 5 tumours / group).

For mice tumour xenografts, five 5 μ m cryosections of tissues were prepared using a Leica cryostat CM3050. Slides were fixed in ice cold acetone for two minutes, permeabilized in 0.1% Triton X-100 in phosphate buffered saline (PBS), blocked in 5% bovine serum albumin in PBS for 1 hour at room temperature and then incubated with 5 μ g/ml primary antibodies

for 1 hour at room temperature. Blood vessels were detected with 5 μ g/ml anti-CD31 (BD Pharmingen) antibody. After extensive washing, slides were incubated with 1-2 μ g/ml cross-absorbed goat anti-rat DyLight 549 (Tebu-bio) secondary antibody for 1 hour at room temperature. Slides were counterstained with DAPI (Tebu-bio). Coverslips were mounted with DakoCytomation fluorescent mounting medium (Dako). For quantification, number of blood vessels in 5-10 microscopic fields per cryosection (per animal) were quantified and the mean number of vessels +/- s.e.m. for the entire treatment group determined.

RNA extraction, nested RT PCR, real time qRT-PCR and TLDA.

Total RNA was isolated from U87 cells using Nucleospin RNA II kit (Macherey Nagel, Hoerd, France), assessed and nested RT PCR was performed as described previously (2).

Quantitative PCR was performed on StepOne+ (Applied Biosystems) using 10 to 100 ng of cDNA and Power SYBER Green or TaqMan Universal PCR master mix (Applied Biosystems) for detection of FLP53, Δ 133p53 and 18S.

TaqManlow density array (Applied Biosystems) was performed following manufacturer instructions with 50 ng of cDNA. The results were treated with the StepOne Software v2.0 (Applied Biosystems). Primers sequences are provided in [Suppl Table II](#).

Statistical analysis

All statistical determination was performed with GraphPad Prism, version 4.0 (GraphPad, San Diego, CA). All data are presented as the mean \pm SEM. One-way and two-way analysis of variance was used to evaluate the significance of differences between groups. All statistical analyses were performed with a two-tailed Student's t-test. A p value of less than 0.05 was considered statistically significant.

CONFLICT OF INTEREST

There is no conflict of interest in relation to the work described.

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FIGURE LEGENDS

Figure 1. Regulation of expression of endogenous p53 isoforms in U87 cells.

(A) Human *p53* gene structure. Alternative splicing of intron 9 generates p53 isoforms bearing different C-terminal domains (α , β and γ). TAp53 isoforms include p53 (p53 α), p53 β and p53 γ , whereas Δ 133p53 isoforms include Δ 133p53 α , Δ 133p53 β and Δ 133p53 γ (2). The different siRNAs used (siTAp53 and si Δ 133p53) and the regions they target are indicated underneath the *p53* gene structure. The different p53 domains are shown: transactivation domains (TA), proline rich domain (PR), DNA binding domain (DNA BD), nuclear localization signal (NLS) and oligomerization domain (OD). (B) Gel electrophoresis (1% agarose) of p53 isoform mRNAs amplified by nested RT-PCR in U87 cells. Total RNA was extracted from U87 cells and nested RT-PCR were performed, as previously described (2).

(C) Δ 133p53 (C1) and p53 mRNAs (C2) relative expression was determined by qRT-PCR following siRNA transfection in U87 cells. Results shown are the average of two independent experiments performed in duplicate. Relative quantification of p53 isoforms was determined by the $2^{-\Delta\Delta Ct}$ by normalizing to 18S rRNA and control (siNON-transfected U87 cells)(4). Results are presented as percentage compared to control.

(D1) Δ 133p53 protein expression was analyzed by Western blotting after transfection of U87 cells with siRNA (si Δ 133p53 or siNON) and Δ 133p53 α expression vector, as indicated. (D2) p53 expression was analyzed by Western blotting after siRNA transfection (β -tubulin was used as a loading control). The histogram shows densitometry analysis of multiple scanned Western blot images to quantify the percentage increase over base line of p53 α (4 independent experiments).

Figure 2. Tumour conditioned medium from U87 cells transfected with si Δ 133p53 impairs HUVEC endothelial cell migration and tube formation, but not their proliferation.

Conditioned media from siRNA-transfected U87 cells were used to assess HUVEC migration, tube formation and proliferation. (A1) Micrographs of scratch wound healing assay of HUVEC grown in conditioned media from U87 cells transfected with siRNAs as indicated. (A2) Quantification of HUVEC migration by scratch wound healing assay. (B1) Micrographs (100x magnification) of tube formation by HUVEC grown on Matrigel containing 10ng/mL FGF2 (HUVEC migration must be stimulated to detect a possible inhibition by siRNAs) in

conditioned media from siRNA-transfected U87 cells as indicated. (B2) Quantification of the number of branch points formed during tubulogenesis. Asterisk indicates statistical significance. *, $P < 0.05$ vs control (conditioned medium from siNON-transfected U87 cells). (C) Effects of conditioned media from siRNA-transfected U87 cells supplemented with 3ng/mL FGF2 on HUVEC proliferation. HUVECs were incubated with conditioned media from siRNA-transfected U87 cells for 24h, 48h and 72 h. Cell proliferation was determined by MTT assay. (D) Conditioned media from U2OS cells transfected with siRNAs as above. Quantification of the number of branch points formed during tubulogenesis. Asterisk indicates statistical significance. *, $P < 0.05$ vs control (conditioned medium from siNON-transfected U2OS cells).

Figure 3. $\Delta 133p53\alpha$ and $\Delta 133p53\gamma$ stimulate endothelial cell tubulogenesis.

U87 cells were transduced with lentivectors expressing $\Delta 133p53\alpha$, $\Delta 133p53\beta$, or $\Delta 133p53\gamma$ under the control of a tetracyclin-inducible promoter. Conditioned media from transduced and doxycycline-treated U87 cells were used to assess HUVEC tube formation as in Figure 2B, except that FGF2 was not added to Matrigel.

Left panels: bright field images (100x magnification) of HUVEC *in vitro* “vessel” formation grown in conditioned media from U87 cells expressing $\Delta 133p53\alpha$ (A), $\Delta 133p53\beta$ (B), or $\Delta 133p53\gamma$ (C) in response to doxycycline treatment, as indicated. Right panels: Quantification of the number of branch points formed during tubulogenesis. Mean number of branching points per 100X field \pm SEM (* $p < 0.05$, $^{\$}p = 0.001$).

Figure 4. Transfection of si $\Delta 133p53$ inhibits angiogenesis and tumour growth *in vivo* on the chorio-allantoic membrane (CAM).

U87 cells transfected with siNON, siTAp53, si $\Delta 133p53$ or siTAp53 + si $\Delta 133p53$ (as indicated) were deposited on CAM of fertilized eggs and tumours were analyzed 4 days later. (A) Experimental protocol. (B) Tumour growth was assessed by biomicroscopy ($\times 3$ magnification) (I, II, III and IV), fluorescent immunohistochemistry ($\times 20$ magnification) (V, VI, VII and VIII) and H&E staining ($\times 20$ magnification) (IX, X, XI and XII). Tumour sections were co-stained for the mesenchymal marker vimentin (tumour cell marker, in red) and SNA-lectin (marker of endothelial cells, in green). Numerous irregular and dilated blood vessels are visible in all tumour sections (V, VI and VIII) except for tumours transfected with si $\Delta 133p53$ isoforms (VII). H&E staining of the corresponding tumour sections is shown and

blood vessels are indicated by an arrow (IX-XII). (C) Left panel :Angiogenic appearance presented as the percentage of absent (no visible tumour), vascular or avascular tumours following treatments with the different siRNAs (10-18 tumours for each siRNA treatment). Middle panel: SNA-lectin staining allowed quantification of the blood vessel network invading the tumour by counting the number of green pixels on each field with Adobe photoshop software (at least 5 tumours / group). Right panel: Tumour volume was quantified for the tumours obtained in CAM presented in Figure 4 (11 to 16 tumours / condition).

Figure 5. Transfection of si Δ 133p53 inhibits angiogenesis and tumour growth in mouse xenografts

U87 cells transfected with siNON, siTAp53, si Δ 133p53 or siTAp53 + si Δ 133p53 at 50nM were xenografted into nude mice. Tumour volumes were quantified from day 7 to day 44 (8 tumours /condition)(Table I). (A) Quantification at days 7. * = $p < 0,05$. (B) Kinetics of tumour growth until day 44 (C) Immunostaining to detect blood vessels in glioblastoma at day 7. Cryosections of xenografted tumour of U87 cells were immunostained using anti-CD31 antibody. Nuclei were stained with DAPI (blue), 200x magnification. Right panel: quantification of number of blood vessels/field. Asterisk indicates statistical significance. *, $P < 0.05$ vs the SiNON-treated group.

Figure 6. p53 and Δ 133p53 α regulate angiogenesis by differentially modulating expression of angiogenesis-related genes.

Fold change expression of anti- and pro-angiogenic genes upon transfection of U87 cells with siNON, siTAp53, si Δ 133p53 or siTAp53 + si Δ 133p53 as analyzed by TLDA. (A) Expression of anti- and pro-angiogenic factors in U87 cells represented as the number of genes amplified in basal conditions (SiNON). Fold change mRNA expression of 44 genes divided into 4 categories - pro-angiogenic (B), anti-angiogenic (C), markers and target genes (D) - according to Applied Biosystem classification form (Suppl. Table I). We considered as relevant repression (-) or induction (+) variations superior to two folds. Experiments were repeated twice. Details are presented in Table II.