p53 inhibits angiogenesis by inducing the production of Arresten.

Sarah Assadian1,2, Wissal El-Assaad1, Xue Q.D. Wang2, Phillipe O. Gannon3, Veronique Barrès3, Mathieu Latour4, Anne-Marie Mes-Masson3,5, Fred Saad3,6, Yoshikazu Sado7, Josée Dostie1,2 and Jose G. Teodoro1,2

1Goodman Cancer Research Center, McGill University, Montréal, Québec, Canada
2Department of Biochemistry, McGill University, Montréal, Québec, Canada
3Research Center of the Université de Montréal Hospital Center (CRCHUM), Institut du cancer de Montréal, and Faculty of Medicine, Université de Montréal, Montréal, Canada
4Department of Pathology, CHUM, Université de Montréal, Montréal, Québec
5Department of Medicine, Université de Montréal, Montréal, Québec, Canada
6Department of Urology, CHUM, Université de Montréal, Montréal, Québec, Canada
7Shigei Medical Research Institute, Yamada, Okayama 701-0202, Japan

Running title: p53 inhibits angiogenesis by inducing Arresten.

Key words: p53, angiogenesis, Arresten, collagen type 4

Financial support: This research was supported by operating grants from the Canadian Institute of Health Research (CIHR) to J.G.T. (MOP-86752 and MOP-115195) and (MOP-86716) to J.D.

Corresponding author:
Jose G. Teodoro, Ph.D (Corresponding Author)
Goodman Cancer Center and Department of Biochemistry
McGill University
3655 Promenade Sir William Osler
Montreal, QC, Canada, H3G 1Y6
Phone: (514) 398-3273
Fax: (514) 398-6769
E-mail: jose.teodoro@mcgill.ca

Conflict-of-interest: The authors have no conflict-of-interest to declare.

Word count: 4979
Total number of figures and tables: Seven Figures, one supplemental table, six supplemental figures
Abstract

Several types of collagen contain cryptic antiangiogenic non-collagenous domains that are released upon proteolysis of extracellular matrix (ECM). Among those is Arresten, a collagen-derived antiangiogenic factor (CDAF) that is processed from α1 collagen 4. However, the conditions under which Arresten is released from collagen 4 in vivo or whether the protein functions in tumor suppressor pathways remain unknown. Here we show that p53 induces the expression of α1 collagen 4 and release of Arresten containing fragments from the ECM. Comparison of the transcriptional activation of COL4A1 with other CDAF containing genes revealed that COL4A1 is a major antiangiogenic gene induced by p53 in human adenocarcinoma cells. p53 directly activated transcription of the COL4A1 gene by binding to an enhancer region 26kbp downstream of its 3’ end. p53 also stabilized the expression of full length α1 collagen 4 by upregulation of α(ΙΙ) prolyl-hydroxylase and increased the release of Arresten in the ECM through an MMP-dependent mechanism. The resulting upregulation of α1 collagen 4 and production of Arresten by the tumor cells significantly inhibited angiogenesis and limited tumor growth in vivo. Furthermore, we demonstrate that immunostaining of Arresten correlated with p53 status in human prostate cancer specimens. Our findings therefore link the production of Arresten to the p53 tumor suppressor pathway and demonstrate a novel mechanism through which p53 can inhibit angiogenesis.
**Introduction**

The tumor suppressor functions of p53 are primarily derived from its ability to act as a sequence specific transcription factor and regulate expression of a diverse array of genes. The most extensively studied p53 target genes are those that mediate cell autonomous effects such as cell cycle arrest, senescence and apoptosis (1). However, p53 can also upregulate genes that limit tumor growth by inhibiting effects such as metastasis and angiogenesis.

The ability of p53 to limit angiogenesis has been demonstrated in several studies in which p53 mutation in prostate (2, 3), colon (3-5), head and neck (6) and breast cancers (7) was shown to correlate with increased microvessel density (MVD). This notion was further supported by animal models showing that reversal of the angiogenic switch required p53 (8), and that p53 could induce tumor dormancy by limiting angiogenesis (9, 10).

p53 negatively regulates angiogenesis by both inhibiting the production of pro-angiogenic factors such as VEGF, and by increasing production of antiangiogenic ones such as TSP-1 (11). p53 has also been shown to induce production of antiangiogenic factors derived from collagen such as Endostatin and Tumstatin (12). There are seven collagens identified to date that contain antiangiogenic activity in their non-collagenous 1 (NC1) domains (13-20). Experiments using transgenic mice have suggested that even modest increases in the levels of these factors can significantly slow tumor growth (21). Conversely, mice lacking expression of the precursor of tumstatin, α3 Collagen 4, demonstrated increased rates of tumor growth (22).
Several studies suggest that production of collagen-derived antiangiogenic factors (CDAFs) may function in the p53 tumor suppressor pathway. A rate limiting enzyme in collagen biosynthesis, $\alpha_2$ prolyl hydroxylase, was shown to be a transcriptional target of p53 (12). In addition, genome-wide screens for p53 binding sites have suggested that two collagen genes, $COL18A1$ (23) and $COL4A1$ (24), are targets of p53. These genes encode $\alpha_1$ collagen 18 and $\alpha_1$ collagen 4 that include the CDAFs Endostatin and Arresten respectively. In the current study we demonstrate that p53 upregulates the expression of the $COL4A1$ gene leading to release of the C-terminal non-collagenous domain of $\alpha_1$ collagen 4, which contains Arresten. We propose these effects to be a p53-mediated response to limit tumor angiogenesis.

**Materials and Methods**

Additional details are provided in the Supplemental Online Materials.

**Cell lines and virus**

H1299 and PC3 cells were purchased from ATCC. HCT116 cells (p53 wt and null) were a gift from Bert Vogelstein (25). Cell lines were routinely authenticated by immunoblot analysis of p53. HUVECs were bought from Clonetics. Ad-p53 and Ad-lacZ adenovirus have been described previously (12).

**Western Blots**

Whole cell extracts were obtained by harvesting cells and boiling in 1X Laemmli buffer. Endogenous proteins in conditioned media (CM) were precipitated with 15% TCA. V5-His-tagged collagen fragments in the CM were precipitated with TALON Superflow Metal Affinity Resin (BD Biosciences).
Quantitative real time PCR

RNA was isolated using TRIZOL (Sigma). Reverse transcription was performed with Quantitect Reverse Transcription kit (Qiagen) and PCR reactions were carried out using Quantitect SYBR Green PCR kit (Qiagen). The 18S ribosomal RNA was used as internal control.

Chromatin immunoprecipitation (ChIP)

ChIP experiments were conducted as previously described (26).

Analysis of tumors in mice

Animal experiments were approved by the McGill Animal Ethics Committee. 10x10⁶ H1299-COL4A1-V5 or control (EV) cells were injected subcutaneously into the back of nu/nu mice (Charles River). Tumor growth in vivo was measured as previously described (12). Microvessel density was measured as previously described (27).

Collagen deposition assay

Immunofluorescence was performed using a rabbit polyclonal antibody against total collagen 4 (Abcam ab6586) or normal rabbit gamma globulin (Innovative Research).

Endothelial tube formation assay

HUVECs were plated onto Matrigel (BD Bioscience) in the presence of EGM-2 (Lonza) media conditioned on H1299-COL4A1-V5 or H1299-EV stable cell lines. Tube complexity was measured as the number of branches in each field.
Tissue microarrays (TMAs)

Ethics approval for sample collection included in this study was obtained from the ethics board of the Research Center of Université de Montréal Hospital Center. Patients signed an informed consent form.

Statistical analysis

Two-tailed student t-test was performed for most experiments. One-way ANOVA was used for analysis of the qRT-PCR data. Analysis of the 3C data was done using the Kolmogorov-Smirnov test. For TMAs Mann-Whitney and Kruskal Wallis tests were used to demonstrate mean expression differences between groups. Correlations between the various variables were evaluated using Spearman’s rho. For all statistical tests, differences were considered significant when $P$ value $<$ 0.05.

Results

*COL4A1 is a major transcriptional target of p53.*

Several types of collagen have been reported to contain cryptic antiangiogenic domains (13-20). We have previously shown that p53 can enhance the secretion of these factors, however the relative importance of the various collagens in the p53 pathway has yet to be elucidated. In figure 1A, qRT-PCR analysis was used to compare the ability of p53 to induce expression of collagens known to contain antiangiogenic activity as well as the well-characterized antiangiogenic p53 target, TSP-1. An adenoviral vector expressing p53 (Ad-p53wt) was used to reintroduce p53 into H1299 cells that lack p53 expression.
Figure 1A and C show that *COL4A1* expression measured by qRT-PCR was induced over 200 fold by p53 relative to controls, which was far greater than all other antiangiogenic collagens tested.

*COL4A1* expression was also compared in HCT116 cells that express *wt* p53 relative to HCT116 cells in which p53 has been deleted (28). Addition of 5-Fluorouracil (5-FU) to these cells results in the stabilization of p53 and induction of p53-dependent genes such as p21 (Figure 1D). Figure 1B shows that *COL4A1* mRNA was the most highly induced antiangiogenic collagen in p53 *wt* but not p53−/− cells following treatment with 5-FU, confirming that induction occurs under conditions using endogenous p53. Surprisingly, induction of *COL4A1* far exceeded that of the well-known antiangiogenic p53 target *TSP1* in both H1299 and HCT116 cells.

The mechanism by which p53 enhances expression of *COL4A1* appears to be unique from other p53 targets. The genomic structure of the six collagen 4 genes in the human genome are arranged in pairs, with a head-to-head orientation. The *COL4A1* and *COL4A2* gene are structured in this manner and share a common promoter sequence (29). Sequence analysis of the promoter of *COL4A1/A2* did not reveal any canonical p53 binding site. However, genome-wide analysis for p53 binding sites reported the presence of a perfect p53 binding site 26 kbp downstream from the 3′ end of the *COL4A1* gene (24) (Figure 2B). To confirm the occupancy of p53 on the putative binding site, chromatin immunoprecipitation (ChIP) was performed. Binding of p53 to the putative binding site was significantly enriched compared to a gene desert control region (Figure 2A). Since the identified p53 binding site is 26 kbp away from the 3′ end of *COL4A1*, we then asked if the distant p53 site interacts with the promoter through long range
lopping interactions as are often observed with transcriptional enhancers. Using chromosome conformation capture (3C) (30), a greater proximity between the promoter of COL4A1 and its 3’ p53 binding site was observed upon p53 expression (Figure 2B). This result suggests that p53 induces COL4A1 expression from a 3’ end enhancer by a long-range mechanism.

**p53 expression stimulates the production of Arresten.**

We then examined the effect of p53 expression on the production of the full-length \( \alpha_1 \) collagen 4. Figure 3A shows that expression of p53 in H1299 cells results in no significant change in full-length \( \alpha_1 \) collagen 4 protein levels in whole cell extracts. However, since previous studies have shown that soluble antiangiogenic fragments of \( \alpha_1 \) collagen 4 can be released from the ECM (15), we analyzed the conditioned media (CM) by western blot. Interestingly, the CM from p53-expressing cells showed the appearance of NC1 containing proteins of approximately 80 and 30kDa in size (Figure 3A- right). To confirm that the bands in the CM are derived from \( \alpha_1 \) collagen 4, a stable cell line was derived expressing full-length COL4A1 containing the His-V5 epitope tag fused to the C-terminus. Using this cell line (H1299/COL4A1-V5), NC1 containing peptides were purified from CM using metal affinity resin and detected with anti-V5 antibody. Figure 3B shows that COL4A1 expressing cells produce a 80kDa product similar to that observed with the endogenous protein. Expression of p53 in the H1299/COL4A1-V5 cells results in enhanced production of Arresten (Figure 3B), suggesting that p53 can also stimulate the production of the protein transcriptionally since the transgene is under the
control of an exogenous promoter. These results show that p53 expression can enhance the expression and processing of Arresten.

In order to confirm that p53 dependent induction of Arresten could be observed without the need for overexpression of p53 or COL4A1, we utilized the p53 wt and p53⁻/⁻ HCT116 cell lines described above. Figure 3C shows that when wt HCT116 cells are treated with 5-FU, an 80kDa Arresten band is observed in the CM, which is barely detectable in CM from p53⁻/⁻ cells. The increase in α1 collagen 4 protein is specific to the soluble C-terminal fragment since no significant increase in the full-length protein is observed in whole cell extracts (Figure 3C, top).

**p53-induced Arresten production is independent of caspase activation and requires MMP activity.**

Expression of p53 can result in the induction of apoptosis and therefore the activation of caspases. To determine if the processing of α1 collagen 4 into Arresten was dependent upon caspase activity, H1299 cells were treated with the caspase inhibitor zVAD-fmk. Figure S1 shows that zVAD-fmk prevented the activation of caspase-3 but had no effect on the appearance of Arresten in the CM in response to p53.

Release of the antiangiogenic peptides from parent collagen requires proteolysis. Previous studies have implicated MMPs in the production of tumstatin, which is derived from α3 collagen 4 (22). To determine if MMP activity is required for Arresten production, cells were treated with the MMP inhibitor, GM6001. Figure 3D shows that addition of GM6001 reduced levels of Arresten in the CM of cells expressing p53 but did not affect full-length collagen in whole cell extract. These data indicate that production
of Arresten is not a consequence of apoptosis induced by p53 but rather is part of an antiangiogenic program whereby α1 collagen 4 expression is increased and processed into Arresten by MMP activity.

**Prolyl hydroxylase activity stimulates the production of α1 collagen 4 and Arresten.**

We have previously reported that a rate limiting enzyme in collagen production, α2 collagen prolyl hydroxylase [α(II)PH], is a target gene of p53 (12). We therefore asked if α(II)PH could potentiate the production of Arresten. Figure 4A shows that cell lines overexpressing α(II)PH were capable of producing more full-length α1 collagen 4 than control cells when transfected with a plasmid encoding the COL4A1 gene. Moreover, the CM of α(II)PH expressing cells contained more of the fully processed Arresten peptide (Figure 4A-right).

To confirm that prolyl hydroxylase activity is essential for Arresten production we examined the effect of an inhibitor of α(II)PH, ethyl-3,4-dihydroxy benzoate (EDHB), on α1 collagen 4 and Arresten production. Figure 4B shows that EDHB reduced levels of Arresten in CM after p53 expression. Taken together, these results suggest that p53 increases production of Arresten in a process that is dependent upon the α(II)PH enzyme.

**α1 collagen 4 expression is antiangiogenic and inhibits tumor formation.**

The H1299/COL4A1-V5 cells release a NC1 containing peptide of approximately 80kDa in size into the CM (Figure 3B). We therefore determined if these cells secrete antiangiogenic activity. Figure 5A&B show that CM from H1299/COL4A1-V5 cells
significantly inhibited tube formation of HUVECs and increased HUVEC cell death in vitro. It should be noted that even though this is a stable population of COL4A1 expressing cells, the level of Arresten released by these cells is less than that observed when endogenous Arresten is induced by p53 (see Figure S2). The antiangiogenic effect of COL4A1 expression in this system is therefore unlikely to be an artifact of overexpression.

The p53-dependent induction of COL4A1 expression and production of Arresten may represent a mechanism to limit tumor angiogenesis and growth. To test this possibility, H1299/COL4A1V5 or vector control cells were transplanted on the backs of nude mice. Figure 5D shows that H1299/COL4A1V5 tumors displayed significantly slower rates of tumor growth relative to vector alone (EV). This effect was not due to differences in cell growth or apoptosis as no major changes in these parameters were observed (Figure 5C). Histological examination of tumors revealed that MVD within the α1 collagen 4 expressing tumors was significantly lower suggesting that the slower tumor growth was due to an antiangiogenic effect (Figure 5E).

Expression of p53 remolds collagen 4 matrix and inhibits tube formation.

Type 4 collagen serves as a major reservoir for antiangiogenic factors that can be released by proteolysis, but conversely, stable collagen 4 matrix is an essential component of vascular basement membranes and is therefore essential for angiogenesis (31, 32). Since p53 expression in tumor cells increases the production of peptides derived from α1 collagen 4, this suggests that p53 may promote remodeling of collagen 4 matrix to prevent angiogenesis. In order to form stable protomers and assemble into a
matrix, both $\alpha_1$ and $\alpha_2$ collagen 4 must be present. These proteins associate in a heterotrimeric triple-helix and subsequently assemble into a large-scale matrix through interactions with the globular NC1 domains (32). We therefore examined the effect of p53 on collagen 4 deposition of tumor cells. H1299 cells do not form any appreciable collagen 4 matrix due to lack of baseline expression of both $\alpha_1$ and $\alpha_2$ collagen 4 (see Figure 3 & S3). The prostate cancer cell line PC3, however, expresses both $\alpha_1$ and $\alpha_2$ collagen 4 and forms a robust collagen 4 matrix (Figure 6B). Importantly, PC3 cells also completely lack expression of p53, which allows the effects of p53 re-expression to be addressed.

Figure 6A shows that both $\alpha_1$ and $\alpha_2$ collagen 4 are present in PC3 cells and when p53 is reintroduced, NC1 fragments are released in the CM. The extent of collagen deposition on the surface of these cells was examined by immunofluorescence. Figure 6B shows that p53 expression following matrix deposition completely abolished the collagen 4 matrix surrounding these cells. In this experimental system, p53 expression did not result in any appreciable induction of apoptosis, or changes in cell morphology (Figure S4).

Remodeling of the collagen 4 matrix by p53 could represent a potential antiangiogenic mechanism destabilizing the basement membrane essential for angiogenesis. We therefore investigated the effect of p53-CM on tube formation of endothelial cells growing on reconstituted basement membranes (Matrigel). Matrigel monolayers were incubated with CM from p53 expressing or control cells. After 72 hours the CM was removed and HUVECs were added to the treated matrigel and assessed for tube formation. Figure 6C shows that matrigel treated with p53-CM was
unable to support tube formation. Intriguingly, addition of the MMP inhibitor GM6001 to the p53-CM during matrigel treatment blocked this effect, suggesting that the inhibition of tube formation was dependent upon MMP activity.

A previous study has shown that p53 can transcriptionally activate expression of the MMP2 gene, which encodes a collagenase well known to degrade type IV collagen (33). We therefore determined if p53 is able induce MMP2 expression in PC3 cells. Figure S5A shows that infection of PC3 cells with Ad-p53 resulted in a 4-fold induction of the MMP2 gene. In order to determine if MMP2 activity is able to release the Arresten NC1 domain from PC3 cells, a HA-tagged cDNA expressing MMP was transfected into PC3 cells. Figure S5B shows that expression of MM2 induced the appearance of a similar 80kDa Arresten band observed upon p53 expression.

These data suggest that p53 affects collagen 4 by two mechanisms that can inhibit angiogenesis. First, p53 enhances production of the antiangiogenic factor Arresten. Secondly, p53-dependent induction of MMP2 or other ECM proteases remodel existing collagen 4 networks into a destabilized state that cannot support attachment and migration of endothelial cells.

**p53 mutation correlates with Arresten expression in human prostate cancer.**

Since p53 increases production of Arresten in vitro, we then asked whether p53 status in primary human cancers correlate with expression of Arresten. A TMA containing 99 cases of prostate cancer was utilized for these studies. A summary of the clinico-pathological parameters of the patient cohort present on this TMA is shown in Table S1. Even though p53 mutations are not as common in prostate cancer as in other
carcinomas, when present they correlate with poor patient survival (34). Prostate cancers carrying p53 mutations are believed to be a small but highly aggressive sub-group that has a high risk of progression even after radical prostectomy. In order to identify tumors that contain p53 mutations, immunohistochemistry was performed to visualize nuclear accumulation of p53. Nuclear accumulation of p53 correlates with the presence of missense mutations in the TP53 gene (35-37). A duplicate set of TMAs was also stained with the same monoclonal antibody against Arresten used in the above experiments.

In agreement with previous studies, we find that the p53 mutation correlates with several negative diagnostic outcomes including bone metastasis (coeff=0.345, \( P<0.001 \)) and overall mortality (coeff=0.348, \( P<0.001 \)). Mutant p53 was detected in 17% of the cases examined, which fits well with previously reported p53 mutation frequencies in prostate cancer (36). Interestingly, presence of mutant p53 significantly correlated with lack of Arresten staining within the tumor (coeff=-0.211, \( P=0.036 \)). In normal prostate tissue very little staining was observed for either p53 or Arresten (Figure S6A). Representative tumors are shown in Figure 7. These data provide clinical relevance to the in vitro findings we have described thus far and demonstrate that loss of p53 activity in human tumors results is reduced levels of Arresten.

**Discussion**

Several types of collagen have been shown to have potent antiangiogenic domains that can be released by proteolysis of ECM (31). Our previous work has suggested that these factors can be mobilized downstream of the p53 pathway (12). The relative importance of each of the various types of antiangiogenic collagen in the p53 pathway
remains unclear. In the current study we show that the collagen gene COL4A1, which contains the antiangiogenic peptide Arresten, is the most highly induced CDAF containing collagen in response to p53. We demonstrate that p53 is able to increase Arresten production, through three distinct mechanisms. First, p53 is able to directly increase transcription of the COL4A1 gene that contains Arresten. Second, collagen α2 prolyl hydroxylase, which we have previously characterized as a p53 target gene also potentiates the production of full-length α1 collagen 4 and Arresten (12). Lastly, p53 promotes the MMP-dependent remodeling of the collagen 4 matrix in the ECM, which further enhances the processing of α1 collagen 4. Since a stable collagen 4 matrix in vascular basement membranes is required to form vessels (31, 32), remodeling of collagen 4 in the ECM would prevent the association of endothelial cells with the destabilized matrix and be potentially antiangiogenic.

We observe a predominant 80 kDa C-terminal fragment of α1 collagen 4 released upon p53 activation in H1299, HCT116 or PC3 cells. This peptide is considerably larger than the 26 kDa NC1 domain that has been previously shown to have antiangiogenic activity (15). The actual size of Arresten present in vivo has never been reported. Although the 80 kDa species is the predominant form of Arresten released from cells in culture, we cannot rule out that additional processing steps could take place in vivo.

Our data supports an antiangiogenic function for p53 in which collagen 4 is shed from cells into the tumor microenvironment. This would result in Arresten and possibly other collagen 4-derived antiangiogenic factors inhibiting endothelial cell growth and tube formation. Of the six type 4 collagens, five of these have been reported to possess domains that are antiangiogenic (18). These collagens represent an enormous quantity of
antiangiogenic potential that can be released by proteolysis of BM with MMPs or other 
ECM-degrading enzymes. Measurement of physiological serum concentrations of these 
factors in humans has never been reported and it would be interesting to determine if 
serum levels of Arresten or other factors correlate with clinical parameters such as overall 
survival in cancer patients.

Acknowledgements.
We thank Bert Vogelstein for cell lines and Isabelle Gamache for invaluable technical 
assistance.

Grant Support.
This research was supported by grants from the Canadian Institute of Health Research 
(CIHR) to J.G.T. (MOP-86752 and MOP-115195) and to J.D (MOP-86716). J.G.T and 
J.D. are both CIHR New Investigators and FRSQ Research Scholars. S.A. was supported 
by a Canderel/FRSQ studentship and CIHR doctoral award. W.E-A was supported by a 
post-doctoral fellowship from the CIHR cancer training program. X.Q.D.W. is supported 
by a scholarship from the Cole Foundation.

Figure legends

Figure 1. COL4A1 is the major CDAF parent collagen induced by p53. 
(A) qRT-PCR quantification of the induction of CDAF parent collagens and TSP-1 in 
H1299 cells at 30h following Ad-p53 or Ad-LacZ infection. \( P_{COL4A1}=0.03, P_{COL15A1}= 
0.039, n=3 \) 
(B) qRT-PCR quantification of the induction of CDAF parent collagens and TSP-1 
following activation of endogenous p53 after 30h of 5-FU treatment in HCT116 WT or 
p53 -/- cells. \( P=0.033, n=3 \)
(C) (Top) Expression of COL4A1 and p21 by RT-PCR in H1299 cells following infection with Ad-p53 or Ad-LacZ at 0, 12, 24 and 30 hours post infection (t.p.i). GPDH was used as a loading control. Immunoblot against p53 on the whole-cell extract (WCE) was performed to confirm expression of p53. Tubulin was used as a loading control. (Bottom) qRT-PCR quantifying induction of COL4A1 relative to time 0h in Ad-lacZ or Ad-p53 infected H1299 cells ($P = 0.005$ (24h), $P = 0.030$ (30h), $n=3$)

(D) (Top) Immunoblot of WCE from 5-FU treated HCT116 WT and p53 -/- cells confirming p53 stabilization and induction of the p21 target gene. Tubulin was used as a loading control. (Bottom) qRT-PCR quantifying induction of COL4A1 relative to time 0h following activation of endogenous p53 with 5-FU (375μM) in HCT116 WT or p53 -/- cells. ($P= 0.022$ (24h), $P=0.033$ n=3)

**Figure 2. COL4A1 is a direct transcriptional target of p53.**

(A) ChIP analysis of the COL4A1 p53 binding site following infection of H1299 cells with Ad-LacZ or Ad-p53. Binding to the p21 promoter and Chromosome 22 were used as positive and negative controls respectively. Fold enrichment of COL4A1 p53 binding site over the Chromosome 22 region by ChIP-qPCR, normalized to mouse IgG. ($P=0.006$, $n=4$)

(B) Diagram demonstrating the COL4A1 and COL4A2 genes on Chromosome 13, as well as the putative p53 binding site 26kb downstream of the 3’ end of COL4A1 (highlighted in yellow). Graph demonstrating interaction frequencies of the COL4A1 promoter with various regions of chromosome 13 containing the COL4A1 and COL4A2 genes as determined by 3C. Values represent the ratio of interaction frequencies of Ad-p53 infected H1299 cells over Ad-LacZ controls in each region. ($P=0.015$, $n=2$)

**Figure 3. Expression of p53 stimulates the extra-cellular release of α1 collagen 4 NC1 fragments.**

(A) (Left) Immunoblot showing expression of α1 collagen 4 in the WCE of H1299 cells following infection with Ad-LacZ or Ad-p53. Expression of p53, p21 and tubulin were monitored as controls. Release of endogenous COL4A1-NC1 fragments were analyzed by immunoblotting the conditioned media (CM). Ponceau red staining was used as a loading control.

(B) Immunoblots of WCE and CM of H1299/COL4A1-V5 or EV cells infected with Ad-LacZ or Ad-p53. V5-tagged full-length or C-terminal α1 collagen 4 fragments were detected using an antibody against V5. Expression of p53 and tubulin were analyzed as controls.

(C) Immunoblots of WCE and CM of HCT116 WT and p53 null cells treated with or without 5-FU. Induction of p53 in the WCE was monitored as control and tubulin was used as loading control. Induction of endogenous COL4A1-NC1 in WCE and CM was examined as described in panel (a). Ponceau red staining of the membrane was used as a loading control for the CM.

(D) Immunoblots on WCE and CM of H1299 cells infected with Ad-lacZ or Ad-p53 in the presence of the global MMP inhibitor, GM6001, or DMSO as control. Expression of COL4A1-NC1 in the WCE and its NC1 fragments in the CM were detected as in panel
(A). Expression of p53 and tubulin in the WCE were monitored as controls. Ponceau red staining of the membrane was used as loading control for the CM.

**Figure 4.** α(ΙΙ) prolyl-4-hydroxylase stabilizes α1 collagen 4 expression and increases the extra-cellular release of its NC1 fragments.

(A) Immunoblots of WCE and CM from FLAG-tagged P4HA2 expressing cells lines (PH1, 2, 3) or controls (C1, 2, 3) transfected with COL4A1-V5 or empty vector (EV). Expression of EGFP was monitored to normalize for transfection efficiency. FLAG antibody was used to detect expression of P4HA2. Expression of COL4A1-V5 in the WCE and CM were detected using a V5 antibody. Tubulin was used as a loading control.

(B) Immunoblots of WCE and CM from H1299 cells infected with Ad-LacZ or Ad-p53 in the presence of EDHB or vehicle. Expression of full-length α1 collagen 4 in the WCE and its NC1 fragments in the CM were detected as in figure 3A. Expression of p53 and tubulin in the WCE were monitored as controls. Ponceau red staining of the membrane was used as loading control for the CM.

**Figure 5.** Release of α1 collagen 4 NC1 fragments result in an antiangiogenic effect *in vitro* and inhibits tumor growth *in vivo*.

(A) Endothelial tube formation assay performed with EGM-2 media conditioned on H1299/COL4A1-V5 or EV cells and quantification of tube complexity ($P=0.0093$, n=3).

(B) Trypan blue quantification of HUVEC cell death following treatment with EGM-2 media conditioned on H1299/COL4A1-V5 or EV cells ($P=0.012$, n=3).

(C) Growth rates of stable H1299/COL4A1-V5 and control (EV) cells *in vitro*.

(D) (Left) Representative images of tumors derived from H1299/COL4A1-V5 or control (EV) cells (Right) *In vivo* assessment of H1299/COL4A1-V5 (n=10) or EV (n=8) tumor volumes and growth rates following injection ($P=0.027$ and $P=0.042$).

(E) (Left) Representative images of H&E and CD31 stained sections of H1299/COL4A1-V5 or EV tumors.

(F) Quantification of MVD in H1299/COL4A1-V5 tumor sections and EV controls ($P=0.006$, n=5).

**Figure 6.** p53-induced remodeling of the collagen 4 matrix results in an antiangiogenic effect.

(a) (Left) Immunoblot showing expression of α1 collagen 4 (COL4A1-NC1) in the WCE of PC3 cells following infection with Ad-LacZ or Ad-p53. α1 and α2 collagen 4 were detected using antibodies raised against the NC1 domains. Expression of p53 and tubulin were monitored as controls. (Right) Release of endogenous COL4A1-NC1 fragments was analyzed by immunoblotting the CM using the same antibody used for the WCE. Ponceau red staining of the membrane was used as a loading control.

(b) Representative images of matrix deposition by PC3 cells, analyzed by immunofluorescence following infection with Ad-lacZ, Ad-p53 or mock infection. ECM was detected using an antibody against total collagen 4. Normal rabbit IgG was used to detect background staining.

(c) Tube formation assay of HUVEC cells, pretreated with CM from H1299 cells infected with Ad-p53, Ad-lacZ or mock, in the presence of GM6001 or vehicle control. Treatment of matrigel in media with no prior conditioning was also used as a control (NC).
Figure 7. Correlation of p53 status with COL4A1-NC1 staining in prostate cancer tissue microarrays (TMAs).
Representative prostate cancer specimens highlighting the correlation of p53 with COL4A1-NC1 staining. Upper panel: tumors with high (mutated) p53 score and low H11 staining. Lower panel: tumors with low p53 score indices and high H11 staining. Correlation coefficient=-0.211, \( P=0.036 \), n=99.
References

Figure 1

A

B

C

D

Author Manuscript Published OnlineFirst on January 17, 2012; DOI: 10.1158/0008-5472.CAN-11-2348

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 2
Figure 4

A

**WCE**

<table>
<thead>
<tr>
<th>COL4A1-V5</th>
<th>EV</th>
<th>COL4A1-N5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>C2</td>
<td>C3</td>
</tr>
<tr>
<td>PH1</td>
<td>PH2</td>
<td>PH3</td>
</tr>
</tbody>
</table>

250

VS

eGFP

PH4A10-FLAG

tubulin

**CM**

<table>
<thead>
<tr>
<th>COL4A1-V5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
</tr>
<tr>
<td>PH1</td>
</tr>
<tr>
<td>VS</td>
</tr>
</tbody>
</table>

B

**WCE**

<table>
<thead>
<tr>
<th>Ad-HscZ</th>
<th>Ad-β3</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL4A1-NC1</td>
<td>p60</td>
</tr>
<tr>
<td>tubulin</td>
<td></td>
</tr>
</tbody>
</table>

250

**CM**

<table>
<thead>
<tr>
<th>Ad-HscZ</th>
<th>Ad-β3</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL4A1-NC1</td>
<td>Porcine Red</td>
</tr>
<tr>
<td>COL4A1-NC1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 7
p53 inhibits angiogenesis by inducing the production of Arresten

Sarah Assadian, Wisal El-Assaad, Xue Q.D. Wang, et al.

Cancer Res  Published OnlineFirst January 17, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-11-2348

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2012/01/15/0008-5472.CAN-11-2348.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.