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SHORT COMMUNICATION

Gain-of-function mutant p53 downregulates miR-223 contributing to chemoresistance of cultured tumor cells

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Mutant p53 proteins are expressed at high frequency in human tumors and are associated with poor clinical prognosis and resistance to chemotherapeutic treatments. Here we show that mutant p53 proteins downregulate micro-RNA (miR)-223 expression in breast and colon cancer cell lines. Mutant p53 binds the miR-223 promoter and reduces its transcriptional activity. This requires the transcriptional repressor ZEB-1. We found that miR-223 exogenous expression sensitizes breast and colon cancer cell lines expressing mutant p53 to treatment with DNA-damaging drugs. Among the putative miR-223 targets, we focused on stathmin-1 (STMN-1), an oncoprotein known to confer resistance to chemotherapeutic drugs associated with poor clinical prognosis. Mutant p53 silencing or miR-223 exogenous expression lowers the levels of STMN-1 and knockdown of STMN-1 by small interfering RNA increases cell death of mutant p53-expressing cell lines. On the basis of these findings, we propose that one of the pathways affected by mutant p53 to increase cellular resistance to chemotherapeutic agents involves miR-223 downregulation and the consequent upregulation of STMN-1.

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INTRODUCTION

P53, encoded by the human gene *TP53*, is the most frequently mutated protein in human cancer.[1,2] It mainly acts as a tetrameric transcription factor regulating a plethora of genes in response to genotoxic insults, resulting in cell cycle arrest, senescence, apoptosis, DNA repair and other processes.[3–5] Most of the p53 mutations are missense mutations in the DNA-binding domain that produce full-length p53 proteins that can no longer bind the wild-type (wt) p53 consensus sequence.[6] Mutant p53 forms lose their wt activity, show a dominant-negative (DB) effect by binding to and inhibiting the activity of the wt protein encoded by the remaining normal allele and some mutants show 'gain-of-function' oncogenic properties that go beyond the functional inactivation of wt p53. These mutations have been linked to poor clinical prognosis and are associated with drug resistance in several tumors and malignant cell lines.[7–12] Different molecular mechanisms of action underlying mutant p53 gain-of-function have been described. These include binding and inhibition of proteins with antitumoral activity, as shown in the case of p63 and p73,[13,14] and transcriptional regulation of target genes either by interacting with other transcription factors (such as NF-Y, E2F1, Sp1 and others[15–17]) or by directly binding to a wide range of secondary DNA structures (such as non-B DNA).[18]

In the past decade, micro-RNAs (miRNAs) were identified as a novel class of pivotal regulators of gene expression. miRNAs are small (~22 nucleotides), non-coding single-stranded RNA that regulate target mRNAs' expression. Many studies demonstrate that miRNAs' misexpression correlate with various human cancers.[19,20] As each miRNA can control hundreds of gene targets, we explored the possibility that mutant p53 could execute gain-of-function activities by regulating miRNAs' expression. With this aim, we performed a screening in an inducible clone of the non-small-cell lung cancer cell line H1299, which expresses gain-of-function mutant $p53^{R175H}$, and identified miR-128-2 and miR-223 as $p53^{R175H}$ transcriptional targets. We recently published a study illustrating that miRNA-128-2, upregulated by $p53^{R175H}$, increases resistance of non-small-cell lung cancer cell lines to DNA-damaging agents by targeting E2F5, thus relieving repression on $p21^{WAF}$, an antiapoptotic protein that prevents procaspase 3 cleavage.[21]

Here, we show that induction of mutant $p53^{R175H}$ in H1299 cells decreases miR-223 expression. Accordingly, mutant p53 down-regulation by small interfering RNA (siRNA) determines an increase of miR-223 expression in different breast and colon cancer cell lines. We found mutant p53, together with the transcriptional repressor ZEB-1, bound to miR-223 promoter whose transcriptional activity is higher when mutant p53 levels are decreased by siRNA and lower when $p53^{R175H}$ is exogenously overexpressed. Exogenous overexpression of miR-223 sensitizes breast and colon cancer cell lines bearing mutant p53 to DNA-damaging treatments. The microtubule-regulatory protein stathmin-1 (STMN-1), a miR-223 target, is strongly downregulated upon miR-223 overexpression. Silencing of mutant p53 by siRNA decreases STMN-1 expression as well. STMN-1 is a well-recognized oncoprotein; likewise, we observed that its downregulation by siRNA decreases the viability of cells expressing mutant p53 upon induction of DNA damage.

The findings we present here indicate that modulation of the pathway miR-223/STMN-1 is yet another means by which mutant p53 increases cellular resistance to chemotherapeutic drugs.

RESULTS AND DISCUSSION

Mutant p53 negatively regulates miR-223 expression

We set out to investigate the possibility that regulation of miRNAs by mutant p53 could contribute to its gain-of-function properties.

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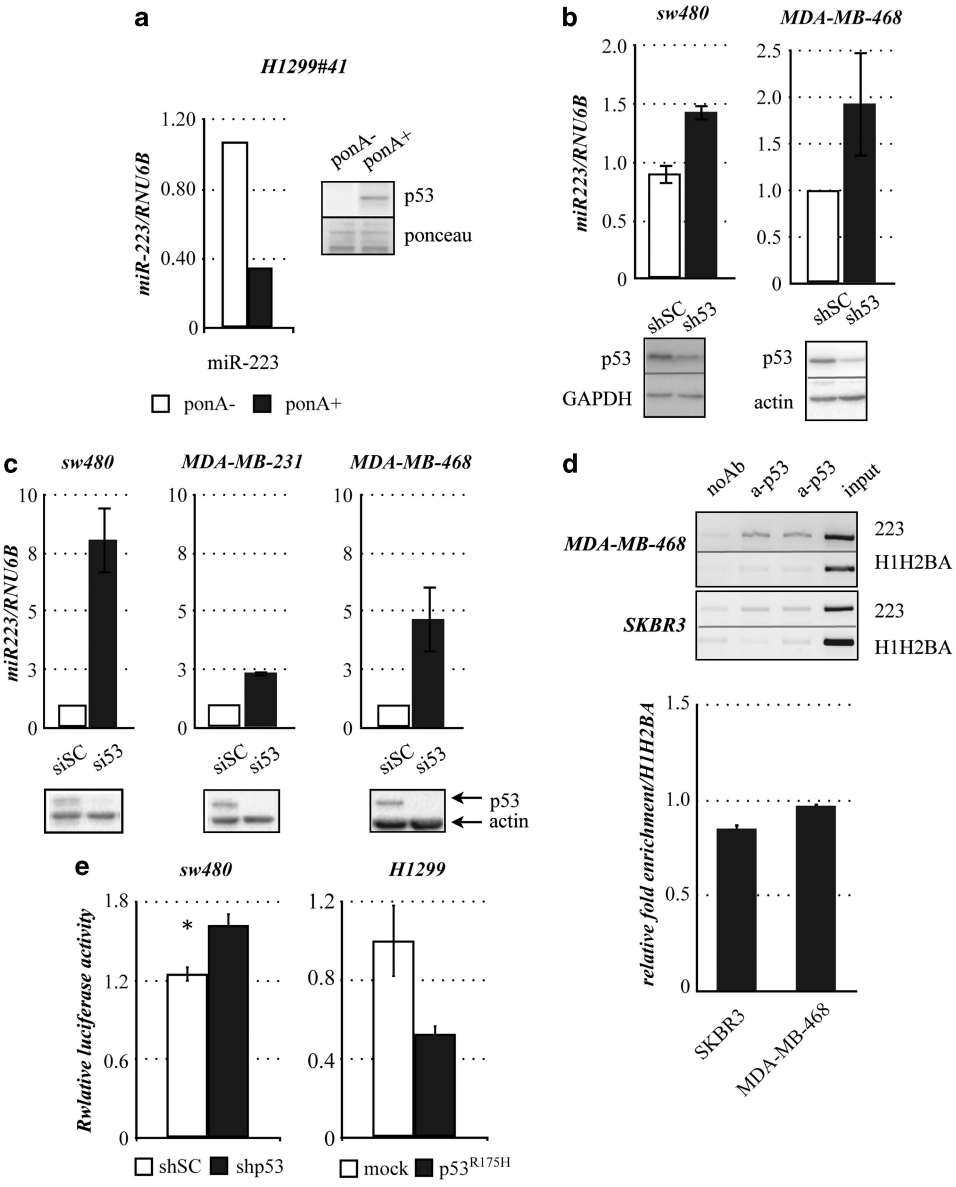
We thus performed a screening for miRNAs belonging to a cancer signature[22] in the lung cancer cell line H1299 clone no. 41, which is a ponasterone A-inducible clone expressing $p53^{R175H}$ (described in Donzelli *et al.*[21]). miR-223 was downregulated upon expression of $p53^{R175H}$ in the presence of DNA damage (Figure 1a).

We next wondered whether we could observe miR-223 modulation by mutant p53 in different cancer cell lines in a more physiological context. We thus silenced mutant p53 in the colon cancer cell line (SW480) and breast cancer lines (MDA-MB-468 and MDA-MB-231) by siRNA, and then evaluated miR-223 expression. miR-223 was upregulated either upon stable (Figure 1b) or transient (Figure 1c) mutant p53 downregulation. These observations indicate that mutant p53 represses miR-223 expression in different cancer cell lines.

We investigated if miR-223 regulation by mutant p53 was at the transcriptional level. We thus performed chromatin immunoprecipitation (ChIP) assays with an anti-p53 serum to assess if p53 was bound to the miR-223 promoter. Two regulatory regions of miR-223 have been described, one closely upstream of the transcription start site of the miR-223 primary transcript[23] and the other one internal to the pri-miR-223.[24] We focused on the regulatory region identified by Fukao and co-workers[23] and found that mutant p53 bound it in different cancer cell lines (Figures 1d and 2a, upper panel). We next studied the transactivation of the miR-223 regulatory region bound by mutant p53 with luciferase assays. We observed an increase in miR-223 regulatory region activity when mutant p53 was downregulated in SW480 cells, whereas its activity decreased when $p53^{R175H}$ was exogenously expressed in H1299 cells (Figure 1e). We then performed an *in silico* analysis (PATCH 1.0, http://www.gene-regulation.com/pub/programs.html) of the miR-223 regulatory region bound by mutant p53 looking for a repressive transcription factor that could cooperate with mutant p53 in miR-223 downregulation. Interestingly, we found various binding site for ZEB-1, a transcription factor deeply involved in epithelial-to-mesenchymal transition and implicated in resistance to chemotherapeutics.[25–28] Performing ChIP analysis of SW480-derived chromatin with an anti-ZEB-1 antibody, we found ZEB-1 bound to the same miR-223 regulatory region where mutant p53 is recruited (Figure 2a, upper and



middle panel). Re-ChIP analysis of chromatin of SW480 cells expressing a Flag-tagged ZEB-1 protein demonstrated direct binding between mutant p53 and ZEB-1 (Figure 2a, lower panel). Mutant p53 does not bind DNA by recognition of a specific binding site. We thus wondered if ZEB-1 binding to miR-223 promoter was necessary for p53 binding to the same region. To answer this question, we repeated the ChIP assay as in Figure 2a on chromatin of SW480 cells transfected with a construct coding for a ZEB-1 DB (ZEB-DB).[29] Inhibition of ZEB-1 by ZEB-DB displaced ZEB-1 and, more importantly, mutant p53 from the miR-223 regulatory region (Figure 2b). To assess whether ZEB-1 has a role in miR-223 downregulation, we transfected ZEB-DB in SW480 cells and observed that inhibition of ZEB-1 activity increased miR-223 expression (Figure 2c).

Altogether, these data suggest that mutant p53 binds to and modulates the miR-223 regulatory region upstream of the primary transcript transcription start site. As discussed above, mutant p53 is known to control transcription of target genes not via binding a consensus DNA sequence but either by recognizing different DNA secondary structures and/or by interacting with sequence-specific transcription factors. Interestingly, we observed an interaction between mutant p53 and the transcriptional repressor ZEB-1 on the miR-223 regulatory sequence in SW480. However, the identification of the repressive complex on the miR-223 promoter requires much more extensive investigation, as it will unveil other transcriptional regulators and complexes with which mutant p53 might interact.

miR-223 sensitizes cells expressing mutant p53 to chemotherapeutic drugs

Gain-of-function p53 mutant proteins are well known to confer resistance to chemotherapeutic agents in tumors and cancer cell lines.[3,7,9,10,21] As mutant p53 downregulates miR-223, we wondered if this micro-RNA could counteract the action of mutant p53 by sensitizing the cells to chemotherapeutic drugs. To investigate this issue, we overexpressed miR-223 in colon (SW480) and breast (MDA-MB-231) cell lines bearing mutant p53 (Figure 3a) and tested their sensitivity to DNA-damaging treatments. miR-223 overexpression increased cell death upon DNA damage relative to mock controls, as revealed by the propidium iodide exclusion assay (Figure 3b). Apoptotic cell death was confirmed by annexin V assay and by the presence of higher amounts of cleaved poly (ADP-ribose) polymerase in miR-223-overexpressing cells relative to mock-transfected controls (Figure 3c).

Previous work from our laboratory demonstrated that mutant p53 gain-of-function alters cell cycle progression upon DNA damage, causing an increased percentage of cells in the S phase.[16] We thus analyzed the cell cycle distribution in response to DNA damage in cell lines expressing mutant p53 and exogenous miR-223 and found that miR-223 overexpression significantly decreased the number of cells in the S phase (Figure 3d). In the same study, we showed that such alteration of the cell cycle was due to aberrant regulation of various genes involved in cell cycle control, among which is cdk1. Hence, we checked cdk1 activation status upon DNA damage in the presence of miR-223 overexpression. We found increased

Figure 1. Mutant p53 negatively regulates miR-223 expression. (**a**) Expression of mutant $p53^{R175H}$ in the lung cell line H1299 leads to miR-223 downregulation. The cell line H1299 clone no. 41 was treated with 2.5 μM ponasterone A (ponA) and 10 μg/ml cisplatin (CDDP) to induce the expression of and activate mutant $p53^{R175H}$, as described previously.[14,17,21] An aliquot of cells treated or not with ponA were lysed to obtain protein extracts and run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to confirm $p53^{R175H}$ induction. A representative western blot, in which ponceau staining of the membrane was used as loading control, is shown in the right panel. Upon induction of $p53^{R175H}$, the expression of mature miR-223 substantially decreased. miR-223 expression levels were measured by real-time polymerase chain reaction (PCR) with the TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Analysis of the data was performed by the $\Delta\Delta C_t$ method, using RNU6B (TaqMan MircroRNA assay; Applied Biosystems) as endogenous control for standardization. (**b** and **c**) Downregulation of mutant p53 in different cancer cell lines increases miR-223 expression. (**b**) The colon and breast cancer cell lines SW480 and MDA-MB-468, which express, respectively, mutant $p53^{R273H,P309S}$ and $p53^{R273H}$, were stably transfected with plasmids expressing short-hairpin RNAs against p53 (sh53) or with a scrambled control (shSC).[16] Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Cells were selected with 2 μg/ml puromycin. (**c**) Mutant p53 was transiently silenced in the same cell lines plus the breast cancer line MDA-MB-231 (expressing $p53^{R280K}$) by transfection of siRNA against p53 (sip53) or against the green fluorescent protein as a control (siGFP) (MWG, Ebersberg, Germany; siRNA p53: 5′-GACUCCAGUGGUAAUCUAC-3′; siRNA GFP: 5′-AAGUUCAGCGUGUCCGGGGAG-3′). Downregulation of mutant p53 was checked by western blot with an anti-p53 antibody (mouse monoclonal DO1) using actin (anti-actin AC74; Sigma-Aldrich, St Louis, MO, USA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (anti-GAPDH; Santa Cruz Biotechnology, Dallas, TX, USA; SC32233) for standardization; secondary antibodies, horse radish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse, were obtained by Bio-Rad Laboratories (Hercules, CA, USA). Detection of the western blots was performed with the Immobilon Western reagent (Millipore, Hayward, CA, USA) and images of the blots were obtained with VersaDoc Model 4000, using the Quantity One software (Bio-Rad Laboratories). Representative western blot are shown in lower panels. Upon RNA isolation by Trizol reagent (Invitrogen), miR-223 expression levels were measured by real-time PCR using the miScript RT kit and miScript PCR system (Qiagen, Hilden, Germany) (upper panels). Analysis of the data was performed by the $\Delta\Delta C_t$ method, using RNU6B (miScript PCR system; Qiagen) as an endogenous control for standardization ($n = 2 \pm$ s.e.m.). In all the experimental settings, mutant p53 downregulation determined the upregulation of miR-223. (**d**) Mutant p53 binds miR-223 promoter in breast cancer cell lines. ChIP analysis revealed direct binding of mutant p53 to the miR-223 promoter in SKBR3 (breast cancer cell line expressing $p53^{R175H}$) and in MDA-MB-468. Crosslinked and sonicated chromatin was immunoprecipitated with anti-p53 serum (5 μl sheep serum Ab7 JA1308; Calbiochem/Millipore) or with a mix of protein A/G-agarose beads without an antibody as negative control (noAb) as described previously.[16] After purification, immunoprecipitated DNA was analyzed by PCR. The primers used amplified a region spanning the transcription start site (TSS) of the pri-miR-223 (-105 to $+108$) (described in Fukao *et al.*[23]) (FW: 5′-GCATCCAGATTTCCGTTGGCTAAC-3′; RW: 5′-GCAAATGGATACC ATACCTGTCAGTG-3′). A heterochromatin region of the gene Hist1H2BA was used as an internal control (hTSH2B primers; Diagenode sa, Liège, Belgium). The same immunoprecipitated DNA was also analyzed by real-time PCR using costumed Primetime qPCR Probe and Primers (IdT, Coralville, IA, USA; probe: FAM/5′-AGGGCAGTG-3′/ZEN/5′-GCCTGCTACTATTGCT-3′/3IABkFQ FW primer: 5′-TCTGTTGAAGGTCAGCTGGGAGTT-3′; RW primer: 5′-TGCTGTTGTGAAAGGGTCTGCTAC-3′) designed in the same region as above (-105 to $+108$). Analysis of the data was performed by the percent of input method, using the gene Hist1H2BA as an internal control gene. The enrichment of the ChIP samples with no antibody was zero. (**e**) Mutant p53 inhibits miR-223 promoter activity in SW480 cells. A luciferase reporter construct (ppri-hmiR-223^{-669}-Luc) containing the miR-223 regulatory region (-669 to $+91$,[23] a kind gift form Dr Taro Fukao) was transfected in SW480, where mutant p53 was stably silenced (sh53) or in the scrambled control cells (shSC) as in (**b**). Cells were co-transfected with a β-gal-expressing plasmid and the β-gal assay was used for standardization. Decreased expression of mutant p53 determined higher miR-223 promoter activity ($n = 6 \pm$ s.e.m., **P*-value < 0.002) (left panel). H1299 cells were co-transfected with a plasmid expressing mutant $p53^{R175H}$ or the empty vector (mock) and the ppri-hmiR-223^{-669}-Luc reporter plasmid.[23] pRL-TK reporter (Promega, Madison, WI, USA), constitutively expressing the *Renilla* luciferase, was included for normalization. After 48 h, cells were lysed and assayed for luciferase activity using the Dual Luciferase kit (Promega) to measure both *Renilla* and Firefly luciferase activities. Overexpression of mutant p53 decreased miR-223 promoter activity (right panel).

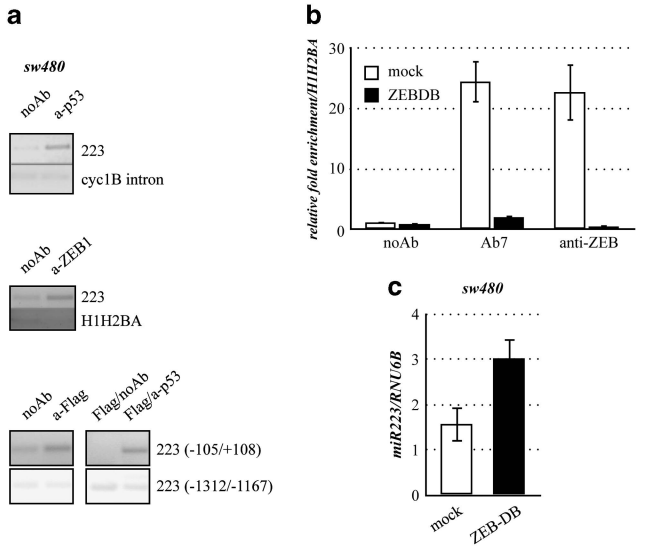


Figure 2. The transcriptional repressor ZEB-1 contributes to miR-223 regulation. (**a**) ZEB-1 binds the miR-223 regulatory region together with mutant p53. Binding of mutant p53 to the miR-223 promoter was demonstrated in SW480 cells by ChIP assay following the methods described in Figure 1d (upper panel). The first intron of the *cyclin B1* gene was used as internal control.[16] ChIP assay using an anti-ZEB-1 antibody for ChIP (E20; Santa Cruz Biotechnology; SC-10572) revealed binding of ZEB-1 to the miR-223 promoter (middle panel). SW480 cells were transfected with a Flag-tagged ZEB-1-expressing construct. At 48 h upon transfection, chromatin was processed as in Figure 1d and ChIP was performed with an anti-Flag antibody (F7425; Sigma) (a-Flag) or with A/G-agarose beads without antibody as a negative control (noAb) (lower left panel). After elution from the agarose beads with 10 mM dithiothreitol (DTT), ChIP with anti-Flag antibody was immunoprecipitated again with anti-p53 serum (Flag/a-p53) or with beads alone (Flag/noAb) (ReChIP) (lower right panel). Detection of the miR-223 regulatory region (-105 to $+108$) in the ReChIP chromatin demonstrates mutant p53 and ZEB-1 interaction in this region. Amplification of a miR-223 DNA area further upstream of the pri-miR-223 transcription start site (TSS) (-1312 to -1167) served as an internal control. (**b**) ZEB-1 contributes to mutant p53 binding to the miR-223 regulatory region. SW480 cells were transfected with an empty vector (mock) or with a construct expressing the DNA-binding domain of ZEB-1, able to inhibit ZEB-1 activity (ZEB-DB).[29] ChIP assay was performed 48 h upon transfection with the anti-p53 or the anti-ZEB antibody as in Figures 1d and 2a. Immunoprecipated DNA was analyzed by real-time polymerase chain reaction (PCR) and analysis was performed by the percent of input method, using the gene Hist1H2BA as an internal control. Competition of ZEB-1 DNA binding by overexpression of ZEB-DB impaired ZEB-1 and, importantly, mutant p53 binding to the miR223 regulatory region. (**c**) ZEB-1 contributes to miR-223 downregulation. SW480 cells were transfected with the DNA-binding domain of ZEB-1 (ZEB-DB) as in (**b**). At 48 h upon transfection, cells were harvested and RNA was extracted to analyze the levels of expression of miR-223 as in Figure 1b. Ectopic expression of ZEB-DB released negative regulation of miR-223.

phosphorylation on residues Thr14/Tyr15, which is a modification that inhibits cdk1 activity, blocking the entrance in the M phase.[30] The cdk1 activating phosphorylation on Thr161 was instead not affected by miR-223 overexpression, either in the absence or in the presence of DNA damage (Figure 3e).

Our results lead to the conclusion that miR-223 plays an antagonistic role to mutant p53, and it is indeed repressed by the latter. miR-223 was mainly characterized in the hematopoietic system. It is an important player in granulocytic differentiation and maturation in physiological conditions and in acute promyelocytic leukemia.[23,24,31] The role of miR-223 in cancer remains to be clarified, as opposing observations have been published. It was found to be highly expressed in recurrent ovarian cancer[32] and bladder cancer,[33] but was instead repressed in hepatocellular carcinomas.[34] Very recently, Jia and co-workers[35] proved that miR-223 suppresses cell proliferation and tumorigenicity of HeLa cells by downregulating IGF-1R, consequently shutting down the PI3K/Akt/mTOR/p70S6k pathway. They also observed IGF-1R downregulation in hepatoma and leukemia cells. However, we did not observe hypophosphorylation of p70S6k in the presence of mutant p53 upon miR-223 overexpression, which suggests that in the cell lines we tested, this pathway is not affected (data not shown).

Our data in colon and breast cancer cell lines expressing mutant p53 indicate that miR-223 increases sensitivity to chemotherapeutic treatments.

The miR-223 target STMN-1 confers chemoresistance to cells expressing mutant p53

Having established that miR-223 affects resistance of mutant p53-expressing cancer cells to chemotherapeutic drugs, we wanted to identify the target(s) mediating its functional role. We thus selected a short list of 14 miR-223 putative targets by the GoMir application (http://www.bioacademy.gr/bioinformatics/projects/GOmir/) that integrates four different prediction algorithms (TargetScan, miRanda, RNAhybrid and PicTar) and we searched for genes that could be involved in chemoresistance. Among the selected genes, we decided to focus our attention on STMN-1, which is a key microtubule-regulatory protein whose regulation is essential for cell cycle progression.[36–39] STMN-1 is a well-recognized oncoprotein that is overexpressed in a variety of human cancers (including breast, colorectal, head and neck, gastric, prostate, ovarian and sarcomas). It is associated with increased histological grading, shorter patient survival times, metastasis, recurrence and increased drug resistance (see refs 40–45 for a comprehensive review) and it is indeed studied as a therapeutic target.[46,47] STMN-1 expression has been linked to wt and mutant p53. According to its oncogenic features, STMN-1 is downregulated by wt p53.[48,49] On the contrary, high levels of STMN-1 are found in breast, colon and liver cancer cells and in gastric primary tumors in association with mutant p53 expression.[50–53]

We observed that downregulation of mutant p53, in breast and colon tumor cell lines, caused a decrease in STMN-1 expression (Figure 4a). Negative regulation of STMN-1 by miR-223 has been demonstrated in hepatocellular carcinoma.[34] We determined indeed that miR-223 overexpression strongly decreased STMN-1 protein levels in colon and breast cancer cell lines expressing mutant p53 (Figure 4b). If STMN-1 had a role in conferring chemoresistance to mutant p53-expressing cell lines, its down-regulation should sensitize them to chemotherapeutic drugs. We found that upon downregulation of STMN-1 by siRNA, cancer cell lines expressing mutant p53 display slightly but significantly increased death (sub-G1 population) and a decrease of the S phase in the presence or in the absence of DNA damage (Figure 4c). According to the findings obtained by miR-223 overexpression, we found that STMN-1 silencing determined inhibition of cdk1 activity via increased phosphorylation on Thr14/Tyr15 (Figure 4d).

Our data support the findings reported by Alli *et al.*[51] and Singer *et al.*[52] who decreased cell proliferation, viability and clonogenicity in mutant p53 breast and liver cell lines by downregulating STMN-1 by siRNA. Alli *et al.*[51] observed that STMN-1 silencing increased sensitivity only to chemotherapeutic drugs acting on microtubules but not to those inducing DNA damage via topoisomerase blockage (camptothecin and doxorubicin), whereas Singer *et al.*[52] found that drugs affecting either the microtubules or damaging the DNA via crosslinking (cisplatin (CDDP)) were more effective in inducing liver cell death upon STMN-1 silencing. Here, we show that STMN-1 downregulation sensitizes mutant p53 colon cell lines to CDDP.

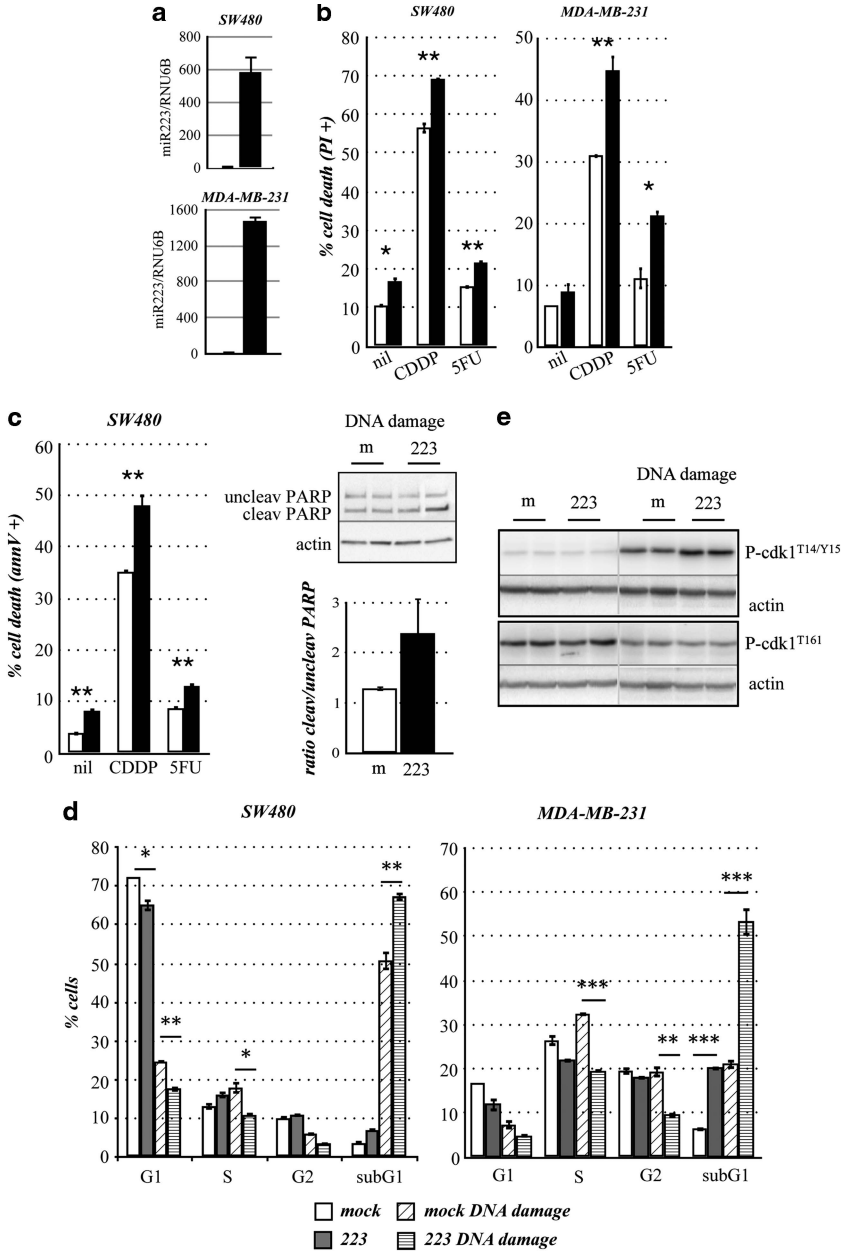


Figure 3. miR-223 antagonizes mutant p53 gain of function. (**a**) Mimic pre-miR-223 transfection results in the overexpression of mature miR-223. Mimic pre-miR-223 or mock control no. 2 (5 nM final concentration; Ambion, Applied Biosystems) were transfected in SW480 and MDA-MB-231 cells by Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. High levels of expression of mature miR-223 were confirmed by real-time PCR as in Figures 1b and c. (**b**) miR-223 overexpression augments chemotherapeutic drug-induced cell death of cells expressing mutant p53. Mimic pre-miR-223 or mock control no. 2 were transfected in SW480 and MDA-MB-231 cells as in (**a**). At 24 h after transfection, the medium was changed with or without the addition of chemotherapeutic drugs (7.5 μg/ml CDDP or 15 μg/ml 5-fluorouracil). Upon additional 48 h, cells were collected and cell death assessed by flow cytometry after staining of dead cells with propidium iodide ($n = 2 \pm$ s.e.m., $^{**}P$-value < 0.005, $^{*}P$-value < 0.02). Flow cytometry data were acquired and analyzed with the Guava EasyCyte System (Millipore). (**c**) Cells overexpressing miR-223 show increased levels of apoptosis. Death by apoptosis of SW480 cells overexpressing miR-223, after DNA damage, was assessed by flow cytometry, measuring the percentage of cells exposing annexin V on the plasma membrane after staining the cells with an anti-annexin V-FITC antibody (left panel). Further indications of apoptosis were obtained by observing an increase in cleaved poly (ADP-ribose) polymerase (PARP). SW480 cells were transfected with mimic pre-miR-223 as in (**a**). At 48 h after adding CDDP, cells were harvested, total protein extracts were prepared and run by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After blotting, the nitrocellulose filters were decorated with an anti-PARP antibody (Cell Signaling, Danvers, MA, USA; no. 9542). The ratio between the cleaved and the uncleaved forms of PARP was obtained by densitometric measurements using the Quantity One software (Bio-Rad) (right panel) (**d**) miR-223 overexpression reduces the DNA damage-induced accumulation in the S phase of the cell cycle typical of mutant p53 cell lines and increases the percentage of cells in sub-G1. SW480 and MDA-MB-231 cells were treated as in (**b**). At 48 h after adding the chemotherapeutic drug, the cell cycle was analyzed by flow cytometry after cell fixation (70% ethanol O/N at 4 °C), RNA digestion (1 mg/ml RNase in phosphate-buffered saline (PBS), 1 h at room temperature) and DNA staining with propidium iodide (40 μg/ml), ($n = 2 \pm$ s.e.m., $^{*}P$-value < 0.05, $^{**}P$-value < 0.02, $^{***}P$-value < 0.005). (**e**) miR-223 overexpression inhibits cdk1 via phosphorylation on Thr14/Tyr15, upon chemotherapeutic drug treatment. MDA-MB-231 cells were treated as in (**b**). After 48 h of treatment with 5-fluorouracil, cells were collected, lysed in 8 M urea and cdk1 phosphorylation on Thr14/Tyr15 or on Thr161 was assessed by western blot with anti-cdk1-P-T14/Y15 (Santa Cruz Technology; SC12340) or anti-cdk1-P-T161 (Santa Cruz Technology; SC12341). Actin was used for standardization.

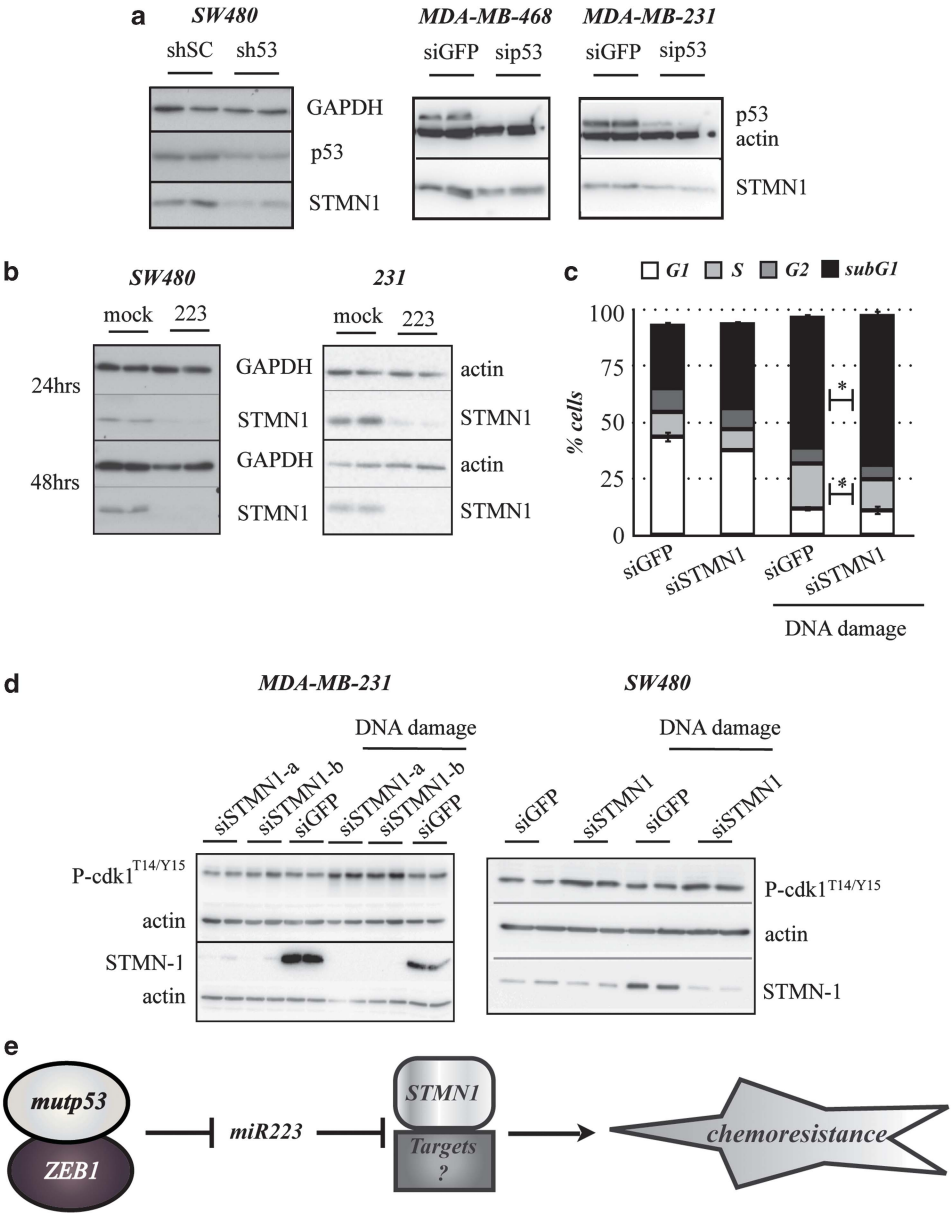


Figure 4. The miR-223 target STMN-1 confers chemoresistance to cells expressing mutant p53. (**a**) Mutant p53 downregulation lowers STMN1 expression. STMN-1 expression was analyzed by western blot (anti-STMN1 from Abcam, Cambridge, UK; ab52906) in SW480 in which p53 was stably silenced (as in Figure 1b) and MDA-MB-468 and MDA-MB-231 transiently transfected with siRNA targeting p53 mRNA (as in Figure 1c) for 72 h. In the tested cell lines, p53 downregulation lowered STMN-1 protein levels. (**b**) STMN-1 is an miR-223 target. Mimic pre-miR-223 was transfected in SW480 and MDA-MB-231 cells as in Figure 3a. At 48 h after transfection, cells were harvested and total protein extracts, obtained by cell lysis in 8 M urea, analyzed by western blot for STMN-1 expression. In the tested cell lines, miR-223 overexpression determined STMN-1 downregulation. (**c**) STMN-1 downregulation augments chemotherapeutic drug-induced cell death of cells expressing mutant p53. siRNAs against STMN-1 mRNA (siSTMN-a: 5′-GAAAGACGCAAGUCCCAUG-3′; siSTMN-b: 5′-UAAAGAGAACCGAGAGGCA-3′) or a mock control siRNA against GFP (siGFP) were transfected (as described in Figure 1c) in SW480 cells. At 24 h after transfection, the medium was changed with or without the addition of CDDP (7.5 μg/ml CDDP). Upon additional 48 h, cells were collected and the cell cycle was analyzed by flow cytometry after fixing and staining as described in Figure 3d ($n = 2 \pm$ s.e.m., ***P*-value < 0.02, **P*-value < 0.05). (**d**) STMN-1 downregulation increases the fraction of cdk1 phosphorylated on Thr14/Tyr15. SW480 and MDA-MB-231 cells in which STMN-1 was silenced by siRNA (as described in (**c**)) were treated for 48 h with 7.5 μg/ml CDDP or 15 μg/ml 5-fluorouracil, respectively. Cells were then harvested and total protein extracts were analyzed by western blot for cdk1 phosphorylation on Thr14/Tyr15 (as described in Figure 3e). Actin was used for standardization. (**e**) Mutant p53-driven miR-223 downregulation contributes to cancer cells chemoresistance. We present a schematic of the proposed model in which mutant p53, together with ZEB-1, inhibits miR-223 transcription, thus relieving translational inhibition of STMN-1. The consequent accumulation of STMN-1 and of other miR-223 targets, still to be identified, contribute to the resistance to DNA-damaging chemotherapeutic drugs.

Altogether, these data suggest that high levels of STMN-1 induce resistance to chemotherapeutic drugs in mutant p53 context via different mechanisms, involving its role in not only microtubule dynamics but also other functions, still to be identified. The fact that STMN-1 interacts with proteins involved in different activities like HSP70b, $p27^{kip1}$, KIS kinase and STAT3

support this hypothesis.[54–57] Interestingly, STMN-1 has been found to be upregulated upon γ-irradiation, suggesting that it could be involved in DNA damage response.[58]

Some mutations in p53 confer the mutant protein 'gain-of-function' features, independently from the loss of wt p53 oncosuppressor activities, which range from augmented rates of proliferation and alteration of the cell cycle regulation to increased chemoresistance and higher migration and invasion capacity *in vitro* and *in vivo*. More recently, gain-of-function mutant p53 has been shown to affect angiogenesis via the transcriptional control of the transcription factor ID4.[17] Various molecular mechanisms of mutant p53 action have already been described and mostly involve transcriptional control via interaction with different partners.[6,15,16] We are now showing the ability of mutant p53 to affect a wide group of targets by transcriptional modulation of miRNAs. We recently published that mutant $p53^{R175H}$ increases non-small-cell lung cancer cells resistance to chemotherapeutic treatment by upregulating miR-128-2.[21] This study furthers our understanding and knowledge of miRNAs regulation by mutant p53 identifying another target, miR-223. It demonstrates that miR-223 downregulation by mutant p53 contributes to the well-recognized gain-of-function property of increased chemoresistance. We show that mutant p53 silencing in different breast and colon cancer cell lines upregulates miR-223 expression. We also illustrate that exogenous miR-223 overexpression sensitizes mutant p53 cancer cell lines to chemotherapeutic treatments. In this study, we focused on STMN-1 as a miR-223 target able to increase cancer cell chemoresistance. We show that its downregulation has similar effects on cell viability and cell cycle progression upon DNA damage as miR-223 overexpression. However, we need to stress that other miR-223 targets, yet to be identified, very likely contribute to the gain-of-function properties of mutant p53.

Altogether, our findings suggest a model in which mutant p53 upregulates STMN-1 expression via miR-223 downregulation, thus enhancing cancer cell resistance to chemotherapeutic drugs (Figure 4e).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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