

Mutant p53-R273H gains new function in sustained activation of EGFR signaling via suppressing miR-27a expression

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p53 is a major tumor suppressor whose function is pivotal for protection against cancer. In over half of human cancers, p53 is inactivated due to either point mutation or loss of *p53* gene. It has been well established that in addition to abrogating the tumor-suppressive function of wild-type p53, mutant p53 gains new functions and actively contributes to various stages of tumor progression. However, little is known about whether microRNA (miRNA) is involved in the gain-of-function of mutant p53. Here we report miR-27a as a novel downstream transcriptional target of mutant p53-273H. Mutant p53 binds to the miR-27a promoter region and suppresses its expression. We also identify epidermal growth factor receptor (EGFR) as a direct target of miR-27a. Via the miR-27a/EGFR axis, mutant p53-273H promotes a sustained EGF-induced extracellular signal-regulated kinase 1/2 activation, thereby facilitating cell proliferation and tumorigenesis. Collectively, this work reveals a direct link between the gain-of-function of mutant p53 and miRNA and uncovers a novel mutant p53-273H/miR-27a/EGFR pathway that has an important role in promoting tumor development.

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Subject Category: Cancer

p53, one of the most important tumor suppressors, has a pivotal role in preventing tumor development. Under oncogenic stress, wild-type p53 is activated to inhibit cell growth through inducing either cell-cycle arrest or apoptotic cell death.¹ In almost all human cancers, the normal p53 pathway is compromised.² Somatic mutation of p53 occurs in >50% of human cancers. Even in those cancers which retain wild-type p53, the function of p53 is often inactivated due to alterations in its regulators and/or mediators.² Unlike most other tumor-suppressor genes, whose inactivation occurs mainly by biallelic deletions or truncations, *p53* gene always carries a single monoallelic missense mutation that mainly resides in its DNA-binding domain.³ Most *p53* gene mutations in human cancers abolish its ability to bind to specific DNA sequences recognized by wild-type p53.⁴ Thus, these mutant p53 lose their tumor-suppressive function that is mostly dependent on the transcriptional activity.⁵ Moreover, the mutant p53 proteins frequently exhibit a dominant-negative activity over the wild-type p53 allele by interacting with wild-type p53 and reducing cellular concentration of functional wild-type p53.^{6–8}

However, as the field of p53 research evolves, increasing evidence demonstrates that mutant p53 proteins not only lose their tumor-suppressive functions and acquire dominant-negative activities but also gain new transforming abilities that promote tumorigenesis, which are independent of wild-type p53.^{9–11} In support of this notion, *in vivo* knock-in

mice harboring tumor-derived mutants of p53 tend to develop multiple types of tumors as well as more metastatic and invasive tumors compared with p53 null mice.^{12,13} Several potential mechanisms leading to gain of oncogenic function of mutant p53 have been proposed.^{8,10,14,15} For instance, although most missense mutations in DNA-binding domain are supposed to abolish the transcriptional activity of p53,⁴ mutant p53 is still able to modulate gene transcription, thereby contributing to its gain-of-function.^{14,16,17} On the one hand, several mutant p53 obtain the ability to bind specific non-B DNA structure with high affinity.¹⁸ On the other hand, many mutant p53 acquire transcriptional activities by interacting with and modulating other sequence-specific transcription factors, such as p53 family members p63 and p73,^{19,20} NF- κ B,²¹ and vitamin D receptor.²² However, the molecular details involved in the gain-of-function of mutant p53 still remains largely unknown.

microRNAs (miRNAs), which regulate the stability and translational efficiency of partially complementary target mRNAs, are small RNA molecules, typically 19–23 nucleotides in length.^{23,24} It has been shown that more than half of miRNA genes are located in cancer-associated genomic regions or in fragile sites.²⁵ Increasing evidence has documented nearly ubiquitous dysregulation of miRNA expression in cancer cells.^{26–28} Altered expression of specific miRNAs has been shown to promote tumorigenesis.^{27,28} It has been recently reported that miRNA also has an important

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Keywords: mutant p53; miR-27a; EGFR

Abbreviations: ChIP, chromatin immunoprecipitation; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; miR-27a, microRNA-27a; p-ERK, phosphorylated extracellular signal-regulated kinase; shRNA, small harpin RNA; 3'-UTR, 3' untranslated region

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role in mutant p53 gain-of-function.^{29–31} However, the details of how mutant p53 promotes tumorigenesis through miRNA are still largely unknown.

Here, we report that miR-27a, an miRNA that exhibits altered expression in various disease states including carcinoma,^{32–35} is transcriptionally repressed by the human mutant p53-273H. Epidermal growth factor receptor (EGFR) is identified as a novel target of miR-27a. We also demonstrate that p53-273H-mediated suppression of miR-27a expression increases EGFR levels and enhances EGF-induced sustained extracellular signal-regulated kinase 1/2 (ERK1/2) activation, thus facilitating cell proliferation and tumor growth. Taken together, our data reveal a novel miR-27a/EGFR pathway that contributes to the gain-of-function of mutant p53 in promoting tumorigenesis.

Results

Mutant p53 represses expression of miR-27a. To identify the novel miRNA(s) involved in the gain-of-function of mutant p53, we established a p53-inducible system where wild-type p53 (H1299-Tet-On-p53) or mutant p53-273H (H1299-Tet-On-p53-273H) can be induced by the addition of doxycycline. After incubation of the cells with doxycycline, p53 expression was markedly increased (Figure 1a). Along with the induced expression of wild-type of p53, levels of its downstream target gene p21 was strongly upregulated (Figure 1a); however, induced expression of mutant p53-273H failed to stimulate p21 expression (Figure 1a), indicating the specificity of these two p53-inducible H1299 cell lines. We next performed custom miRNA microarray analysis to compare the miRNA expression profiles between wild-type p53- and mutant p53-expressing H1299 cells. Compared with wild-type p53, mutant p53-273H exhibited differential expression of multiple miRNAs (Supplementary Table S1). One of these miRNAs, miR-27a, has been implicated in the suppression of certain types of cancers.^{34,36–38} We therefore focused on the study of miR-27a. The subsequent real-time reverse transcriptase-PCR analysis showed that miR-27a was indeed repressed by mutant p53-273H (Figure 1b). In contrast to mutant p53-273H, wild-type p53 did not repress miR-27a expression (Figure 1b). To further confirm the repressive effect of p53-273H on miR-27a expression, we knocked down p53-273H in MDA-MB-468 breast cancer cells that harbor mutant p53-273H. This led to the increased expression of miR-27a (Figure 1c). These results demonstrate that miR-27a is specifically downregulated by p53-273H. We also examined the effects of different tumor-associated p53 mutants on miR-27a expression. All three p53 mutants p53-175H, p53-273H and p53-G279E, but not wild-type p53, exhibited the inhibitory effects on miR-27a expression (Figure 1d), indicating that acquisition of the repressive effect on miR-27a is specific to mutant p53.

Mutant p53 binds to and represses the miR-27a promoter. To determine whether miR-27a is a *bona fide* target gene of mutant p53-273H *in vivo*, we first attempted to identify the potential binding site(s) responsible for activation by mutant p53-273H. The consensus p53-responsible

element has been well defined for wild-type p53,^{39,40} yet the defined responsible element for mutant p53 has not been characterized. We therefore performed chromatin immunoprecipitation (ChIP) assays with five pairs of primers to survey for the potential binding site(s) within the *miR-27a* promoter region (Figure 2a). Mutant p53 exhibited the strong binding to the amplicon C located within the *miR-27a* promoter (nt –2899 to –2675), but showed the relatively weak binding to the amplicons A, B, D and E (Figure 2b). The subsequent analysis further revealed that mutant p53 but not wild-type p53 was capable of interacting with the fragment between nt –2899 and –2675 of the *miR-27a* promoter (amplicon C; Figure 2c). To confirm the physiological association of endogenous mutant p53 and *miR-27a* promoter, MDA-MB-468 cells that retain mutant p53-273H were used for ChIP assays. The amplicon C was readily amplified from chromatin in anti-p53 immunoprecipitates but not in control immunoglobulin G immunoprecipitates (Figure 2d, lane 4 versus 5). Together, these results suggest that mutant p53 specifically and preferentially binds to the *miR-27a* promoter region from nucleotides –2899 to –2675.

Next, we performed a luciferase reporter assay to determine whether the *miR-27a* promoter is responsive to mutant p53-273H. The *miR-27a* promoter region (nts –3556 to –2675), which was shown to interact with p53-273H by ChIP assays, was cloned into the pGL3-based luciferase reporter plasmid (Figure 2e). This construct was introduced into H1299 cells together with either mutant or wild-type p53. We found that the transcriptional activity of the *miR-27a* promoter was repressed by p53-273H but not by wild-type p53 in a dose-dependent manner (Figure 2f). Taken together, these data strongly suggest that mutant p53-273H specifically binds to the *miR-27a* promoter and transcriptionally represses its expression.

miR-27a targets EGFR and downregulates its cellular levels. We next searched the TargetScan 5.1 database for the potential targets of miR-27a. We found that the 3' untranslated region (3'-UTR) of the *egfr* gene contains two putative sites (nucleotides 200–207 and 430–436) that matched to the miR-27a seed region (Figure 3a). We thus constructed a luciferase reporter plasmid containing the wild-type 3'-UTR of EGFR and also three mutant reporter constructs in which putative binding sites 1 and 2 were either singly (mut1 and mut2) or doubly (mut1/2) mutated (Figures 3a and b). These reporter constructs were transfected into H1299 cells together with miR-27a mimics. Induction of miR-27a reduced the reporter activity of the construct containing wild type 3'-UTR of EGFR. MiR-27a also showed the similar repressive effect on mut2 reporter activity. However, the mut1 or mut1/2 reporter activity was little affected by miR-27a induction (Figure 3c). These indicate that miR-27a is targeted to the region 1 (nucleotides 200–207) of EGFR 3'-UTR.

To determine whether miR-27a downregulates EGFR expression, miR-27a mimics or inhibitors were utilized. Treatment of miR-27a inhibitors resulted in the elevated protein levels of EGFR, whereas miR-27a mimics showed the opposite effect (Figure 3d). However, neither miR-27a inhibitors nor miR-27a mimics caused any change in EGFR

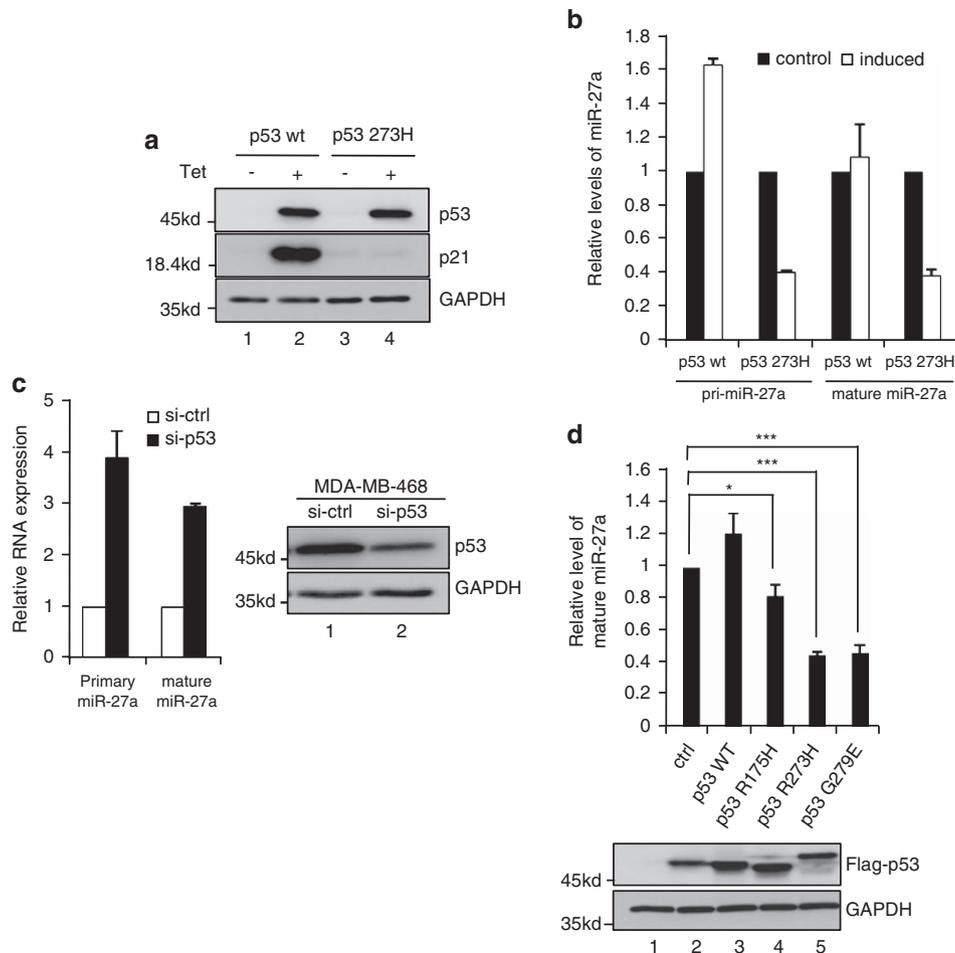


Figure 1 miR-27a is downregulated by p53 R273H mutant. (a) H1299 cells with doxycycline-inducible expression of either wild-type p53 (H1299-Tet-On-p53) or mutant p53 273 (H1299-Tet-On-p53 273H) were incubated with doxycycline to induce expression of p53. Twenty-four hours after induction, cell lysates were subjected to western blot analysis with the indicated antibodies. (b) H1299-Tet-On-p53 and H1299-Tet-On-p53 273H cells were treated with or without doxycycline for 24 h, followed by quantitative reverse transcriptase-PCR (RT-PCR) analysis to examine the expression levels of primary transcript and mature form of miR-27a. (c) MDA-MB-468 cells were transfected with either control or p53-specific siRNA. Forty-eight hours after transfection, expression levels of primary transcript and mature form of miR-27a in these cells were determined by quantitative RT-PCR. (d) The plasmids encoding wild-type p53 and the indicated mutant p53 were individually transfected into H1299 cells. Twenty-four hours after transfection, cells were harvested and subjected to quantitative RT-PCR analysis to examine the expression levels of mature form of miR-27a. The data are represented as mean \pm S.D. of three independent experiments. * $P < 0.05$ and *** $P < 0.001$

mRNA levels. These results suggest that EGFR is post-transcriptionally regulated by miR-27a.

Mutant p53-273H promotes sustained EGF-induced ERK activation via the miR-27a/EGFR axis. As mutant p53 suppresses miR-27a expression and miR-27a, in turn, downregulates EGFR cellular levels, we sought to investigate whether mutant p53 regulates function of EGFR through miR-27a. As shown in Figure 4a, induction of mutant p53 resulted in an increase in the levels of EGFR; however, this was reversed by introduction of miR-27a mimics, indicating that mutant p53 upregulates EGFR expression via miR-27a. This was further confirmed by the finding that protein levels, but not mRNA levels, of EGFR were decreased by p53 knockdown in MDA-MB-468 cells (Figure 4b). To determine whether the effect of mutant p53-273H on miR-27a expression is confined to the EGFR, we examined the protein levels of zinc finger and BTB domain containing 10 (ZBTB10), forkhead box O1 (FOXO1)

and myelin transcription factor 1 (Myt-1), which were previously reported as the targets of miR-27a,^{41,42} in p53-273H-expressing H1299 cells and p53-273H knockdown MDA-MB-468 cells. As shown in Supplementary Figure S1, the protein levels of ZBTB10, FOXO1, and Myt-1 were not affected by either over-expression or knockdown of mutant p53-273H.

Given the important role of EGFR in mediating EGF-initiated cellular signaling cascade, we next determined whether mutant p53-273H regulates EGF-induced AKT or ERK activation. H1299-Tet-On-p53-273H cells were incubated with doxycycline to induce p53-273H expression and then treated with EGF for a prolonged period of time. p53-273H-expressing H1299 cells displayed, as compared with the control cells, a prolonged activation of ERK1/2 but not of AKT upon EGF stimulation (Figures 4c and d). By contrast, wild-type p53 failed to promote EGF-induced sustained ERK1/2 activation (Figure 4e). Furthermore, in MDA-MB-468 cells, the sustained ERK1/2 activation upon EGF

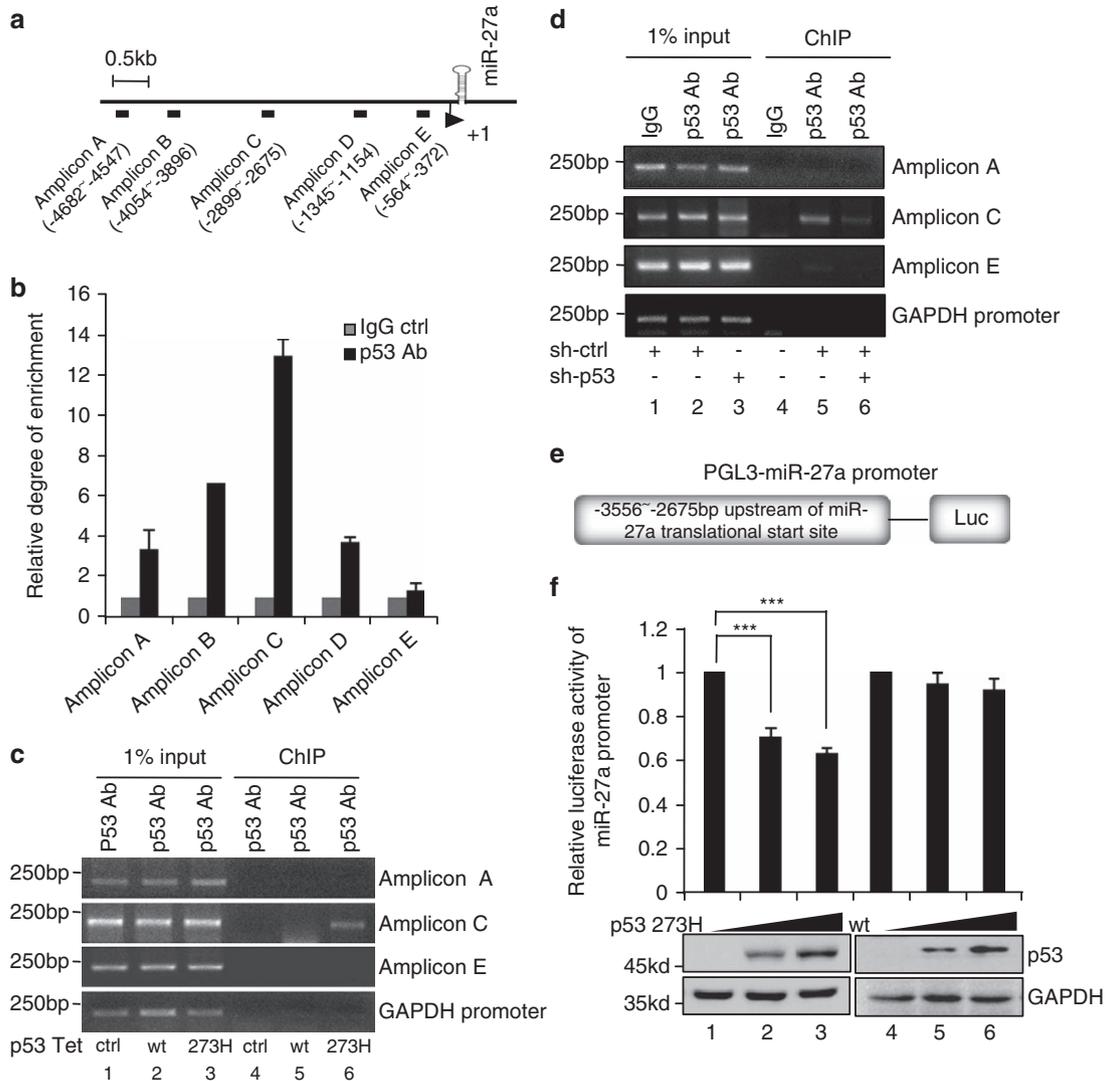


Figure 2 miR-27a is a transcriptional target of p53 273H. **(a)** Schematic illustration of miR-27a promoter and the positions of PCR amplicons used in ChIP assays. **(b)** ChIP analysis showing p53 273H binding to the promoter region of miR-27a. Input and immunoprecipitated DNA (by an antibody against p53) from H1299-Tet-On-p53 273H cells after treatment with doxycycline for 24 h were amplified by quantitative PCR to examine abundance of the indicated amplicons. An isotope-matched anti-IgG (anti-immunoglobulin G) antibody was also used as a negative control. **(c)** ChIP analysis showing p53 273H but not wild-type p53 binding to the -3056 to -2832 region of miR-27a promoter. Input and immunoprecipitated DNA (by an antibody against p53) from H1299-Tet-On-p53, H1299-Tet-On-p53 273H, or control cells after treatment with doxycycline for 24 h were amplified by PCR to detect abundance of the indicated amplicons. Amplification for GAPDH was used as a negative control. **(d)** MDA-MB-468 cells stably expressing either control or p53-specific shRNA were subjected to ChIP assays using anti-p53 antibody or an isotope-matched control IgG. ChIP products were amplified by PCR to determine abundance of the indicated amplicons. **(e)** A schematic illustration of pGL3-basic-based reporter construct used in luciferase assays to examine the transcriptional activity of the -3556 to -2675 region in response to wild-type or mutant p53 expression. **(f)** The increasing amounts of constructs encoding either wild-type or mutant p53 were cotransfected into H1299 cells together with pGL3-miR-27a and Renilla luciferase plasmids. Twenty-four hours after transfection, reporter activity was measured by luciferase assays and plotted after normalizing with respect to Renilla luciferase activity. The data are represented as mean \pm S.D. of three independent experiments. *** $P < 0.001$

stimulation was weakened by p53-273H knockdown (Figure 4f). To determine whether p53-273H-promoted sustained ERK1/2 activation upon EGF stimulation is EGFR dependent, we knocked down EGFR expression in p53-273H-expressing H1299 cells. As shown in Supplementary Figure S2, EGF-induced sustained ERK1/2 activation in p53-273H-expressing H1299 cells was largely abolished by EGFR knockdown, indicating that mutant p53-273H promotes sustained EGF-induced ERK1/2 activation through EGFR.

To further examine whether the promoting effect of mutant p53 on EGF-induced sustained ERK1/2 activation is due to

enhanced phosphorylation of ERK1/2 or decreased dephosphorylation of phosphorylated-ERK1/2 (p-ERK1/2), p53-273H-expressing or control H1299 cells were pre-treated with EGF before the addition of MEK-1 (MAP ERK kinase) inhibitor U0126 at the different time points. As shown in Figure 4g, EGF treatment resulted in the expected ERK1/2 activation in both p53-273H-expressing and control cells. Interestingly, after U0126 treatment, p53-273H-expressing and control H1299 cells exhibited comparable levels of activated ERK1/2 (Figure 4g). These results indicate that upstream activators of ERK1/2 likely have a primary role for the observed

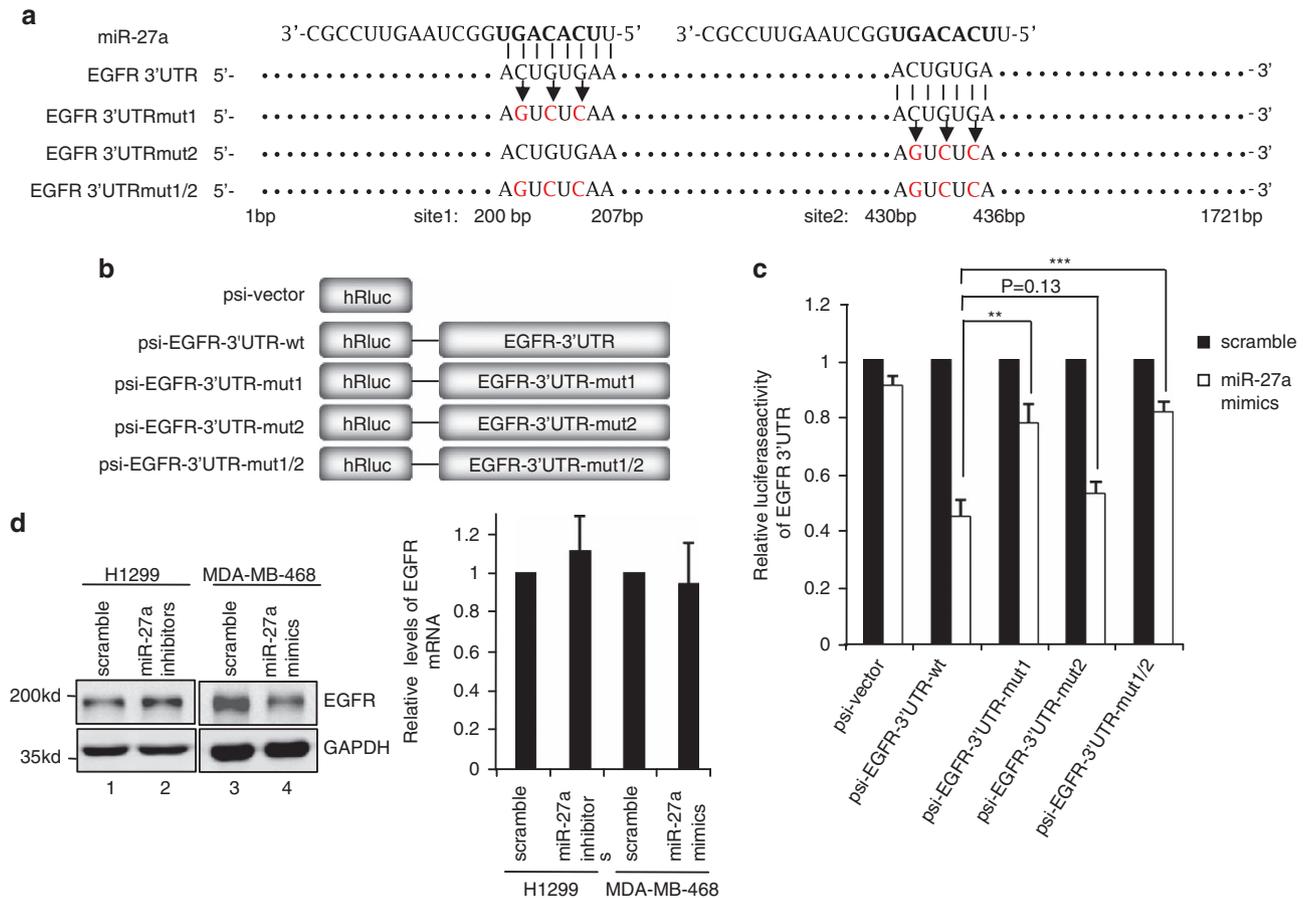


Figure 3 miR-27a targets EGFR expression. (a) Illustration of base pairing between miR-27a and the 3'-UTR of EGFR. Substitution of cytosine bases with guanine or substitution of guanine bases with cytosine (C to G or G to C) for the mutant reporter construct is also shown. (b) Schematic illustration of psi-CHECK2-based luciferase reporter constructs used for examining the effect of miR-27a on the 3'-UTR of EGFR. (c) miR-27a mimics were transfected into H1299 cells together with the indicated reporter constructs. Renilla luciferase plasmid was also introduced as an internal control. Twenty-four hours after transfection, reporter activity was measured and plotted after normalizing with respect to Renilla luciferase activity. The data are represented as mean \pm S.D. of three independent experiments. ** $P < 0.01$ and *** $P < 0.001$. (d) H1299 and MDA-MB-468 cells were transfected with scramble, miR-27a inhibitors or miR-27a mimics as indicated. Forty-eight hours after transfection, cell lysates and total RNA were subjected to western blot and quantitative reverse transcriptase-PCR analyses, respectively

sustained activation of ERK1/2. This notion is consistent with the finding that mutant p53-273H is able to upregulate EGFR protein expression (Figures 4a and b).

We next investigated whether miR-27a regulates EGF-induced ERK1/2 activation. Introduction of miR-27a inhibitors into H1299 cells was shown to recapitulate the promoting effect of mutant p53 on EGF-induced sustained ERK1/2 activation by increasing the expression levels of EGFR (Figures 4c and h). This result suggests that mutant p53 may promote EGF-induced sustained ERK1/2 activation via the miR-27a/EGFR axis. In support of this, in p53-273H-expressing H1299 cells, induction of miR-27a by its mimics substantially attenuated EGF-initiated sustained ERK1/2 activation, which was correlated with the decreased levels of EGFR (Figure 4i).

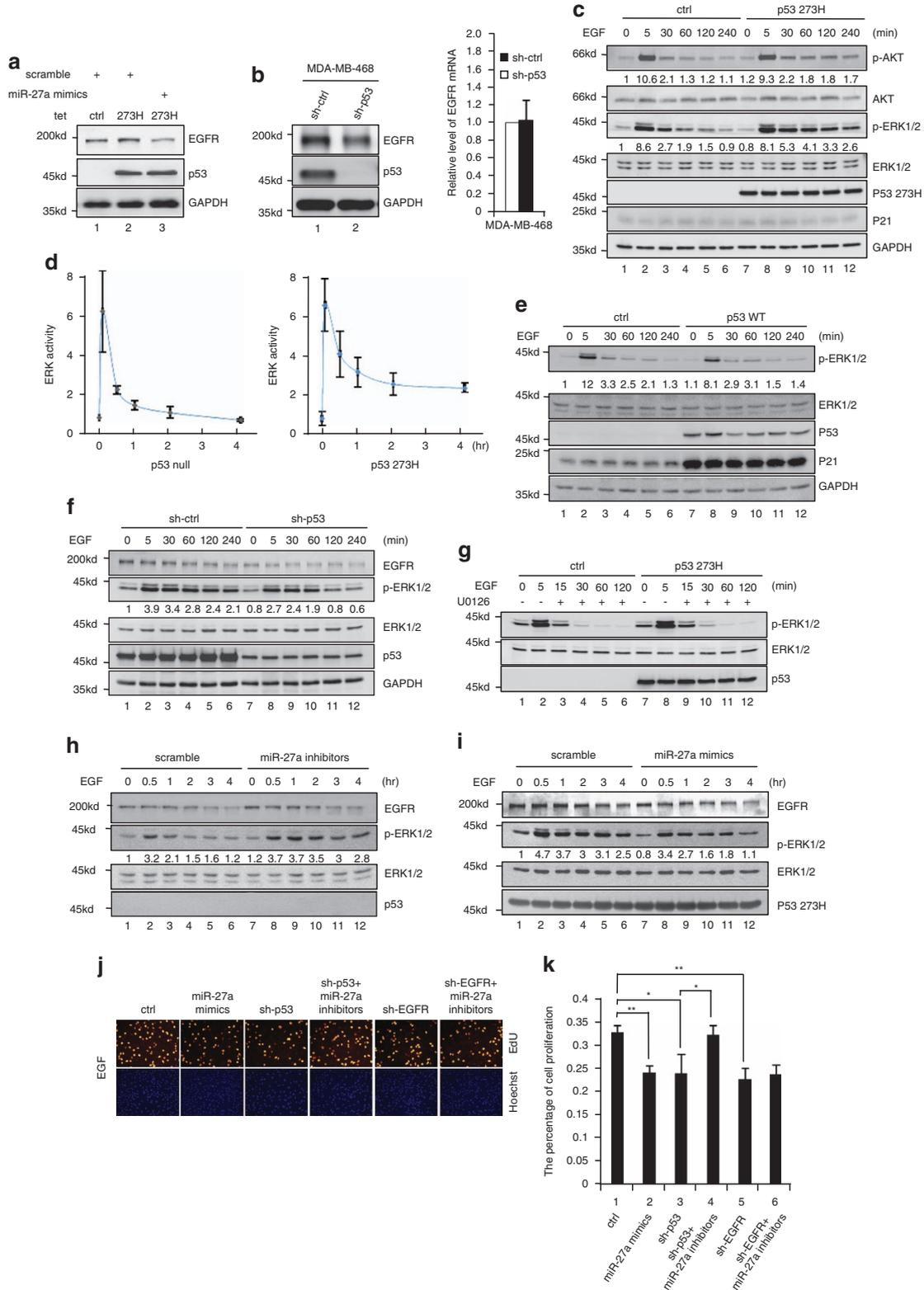
Previous studies have demonstrated that sustained ERK1/2 activation is required for EGF-promoted cell proliferation.^{43–46} To study the effect of mutant p53-mediated miR27a/EGFR regulation on cell proliferation, we performed a 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay. MDA-MB-468 cells stably expressing control small harpin RNA

(shRNA), p53- or EGFR-specific shRNA were transfected with miR27a mimics or inhibitors as indicated, followed by EGF stimulation. Induction of miR-27a by its mimics resulted in a noticeable decrease in cell proliferation (Figures 4j and k). Knock down of p53 also inhibited cell proliferation in a similar way as miR-27a (Figures 4j and k). However, addition of miR-27a inhibitors greatly recovered the growth retardation of p53 knockdown cells (Figures 4j and k). These results indicate that p53-273H supports cell proliferation via miR-27a. We also showed that miR-27a inhibitors showed no effect on cell proliferation inhibition caused by EGFR knock down (Figures 4j and k), indicating that EGFR mediates the downstream effect of miR-27a on cell proliferation. Taken together, these results suggest that mutant p53-273H may promote EGF-stimulated cell proliferation via the miR-27a/EGFR axis.

The miR-27a/EGFR axis contributes to the gain-of-function of p53-273H in promoting tumor formation. To evaluate the effects of miR-27a and EGFR on anchorage-independent growth of mutant p53-expressing tumor cells, we performed a soft agar assay. We used MDA-MB-468 cells individually

expressing miR-27a mimics, p53 shRNA, p53 shRNA plus anti-miR-27a, EGFR shRNA, or EGFR shRNA plus anti-miR-27a. These cells were separately plated in six-well plates coated with soft agar. After 30 days of incubation, the developed colonies were counted. MDA-MB-468 cells expressing miR-

27a mimics, p53 shRNA, or EGFR shRNA formed fewer colonies than control cells (Figure 5b) and was accompanied by the decreased levels of EGFR (Figure 5a). Anti-miR-27a induction greatly recovered the colony-forming ability of p53 knockdown cells (Figure 5b), correlating well with the increased



levels of EGFR in these cells (Figure 5a). However, when EGFR was knocked down in MDA-MB-468 cells, induction of anti-miR-27a did not show any effect on their colony-forming ability (Figure 5b). These combined results indicate the important role of miR-27a and EGFR as downstream effectors of mutant p53 in promoting cell growth.

To determine the role of mutant p53-regulated miR-27a and EGFR in tumor formation, we used a xenograft mouse model. MDA-MB-468 cells stably expressing miR-27a mimics, p53-specific shRNA, p53-specific shRNA plus anti-miR-27a, EGFR-specific shRNA, or EGFR-specific shRNA plus anti-miR-27a were injected subcutaneously into the dorsal flanks of nude mice. Twenty days after injection, mice were killed and tumors were excised (Figures 5c and d). miR-27a mimics-expressing cells gave rise to smaller tumors than control cells. MDA-MB-468 cells with p53-273H knockdown also dramatically reduced tumor growth; however, this was partially reversed by induction of anti-miR-27a. In addition, knockdown of EGFR in MDA-MB-468 cells greatly attenuated tumor growth, recapitulating the effect of miR-27a induction or p53 knockdown. Nonetheless, this tumor growth inhibition by EGFR knockdown was unable to be rescued by expression of anti-miR-27a (Figures 5c and d). Taken together, these results suggest that the miR-27a/EGFR axis contributes to the gain-of-function of mutant p53-273H in promoting tumor formation.

To further validate the biological significance of the miR-27a/EGFR axis in tumorigenesis *in vivo*, we analyzed the expression levels of p53, miR-27a, EGFR, p-ERK1/2, and p-Akt in human primary gastric tumors (T) and their matched adjacent normal tissues (N). We found that five out of six examined gastric tumors (T1–T5) expressed high levels of EGFR and p-ERK1/2 along with the decreased levels of miR-27a compared with their matched adjacent normal tissues (N1–N5) (Figure 5e). Interestingly, high expression of p53 (an indicator of the presence of mutant p53⁴⁷) was also found in these tumor samples. However, only two out of six

examined gastric tumors (T2 and T3) exhibited increased expression of p-Akt compared with the control normal tissues (N2 and N3). These results indicate that dysregulation of the miR-27a/EGFR/ERK1/2 axis driven by p53-273H could have an important role in promoting human tumor formation.

Discussion

Previous studies have suggested that the gain-of-function of mutant p53 is largely attributed by the transcriptional regulation of some cancer-related genes, such as *MDR1*, *c-MYC* and *NFKB2*.^{48–50} In this study, we provide evidence demonstrating that miRNA also has an important role in the gain-of-function of mutant p53. We show that miR-27a is transcriptionally repressed by mutant p53, therefore contributing to the gain-of-function of mutant p53 in promoting cell proliferation and tumorigenesis. Mechanistically, mutant p53-mediated miR-27a suppression increases cellular levels of EGFR and promotes EGF-induced ERK1/2 sustained activation.

Although mutant p53 loses the activity to bind DNA sequence specific to wild-type p53, it is well accepted that mutant p53 is still able to influence gene expression by regulating function of other transcriptional factors, such as p63, p73, nuclear transcription factor Y (NF-Y), and vitamin D receptor.^{19–22} In this study, we show that mutant p53 binds to the –2899 to –2675 *miR-27a* promoter region and suppresses miR-27a expression, although the detailed mechanism still needs to be further characterized. Of note, a recent study has reported that in SaoS-2 sarcoma cells, mutant p53 is able to transcriptionally upregulate EGFR expression by binding to its promoter.⁵¹ However, EGFR appears not to be transcriptionally regulated by mutant p53 in MDA-MB-468 breast cancer cells. Instead, we find that mutant p53 increases EGFR expression via miR-27a in these cells. This discrepancy could be explained by the specific difference in the cellular context.

Figure 4 Role of the p53 273H/miR-27a/EGFR pathway in EGF-induced sustained ERK activation. (a) H1299-Tet-On-p53 273H or control cells were transfected with either miR-27a mimics or scramble RNA as indicated. Twenty-four hours after transfection, cells were incubated with doxycycline for another 24 h. Cell lysates were then subjected to western blot analysis with the indicated antibodies. (b) Lysates and total RNA from MDA-MB-468 cells expressing control or p53 shRNA were subjected to western blot and quantitative reverse transcriptase–PCR analyses, respectively. (c) H1299-Tet-On-p53 273H or control cells were treated with or without doxycycline for 24 h before they were serum-starved for an additional 12 h. The cells were then stimulated with EGF (100 ng/ml) for the indicated periods of time. Cell lysates were analyzed by western blot with the indicated antibodies. The blots were quantified using the Gel-Pro analyzer software (MediaCybernetics, Rockville, MD, USA). The relative p-Akt/Akt and p-ERK1/2/ERK1/2 ratios were also shown. (d) EGF-induced ERK activation in p53-273H-expressing and control cells were determined based on the expression levels of p-ERK1/2 (c) in these cells upon EGF stimulation. The data are represented as mean \pm S.D. of three independent experiments. (e) H1299-Tet-On-p53 or control cells were incubated with doxycycline for 24 h before they were starved for another 12 h. The cells were then stimulated with EGF for the indicated periods of time. Cell lysates were analyzed by western blot. The blots were quantified using the Gel-Pro analyzer software (USA). The relative p-ERK1/2/ERK1/2 ratio was also shown. (f) MDA-MB-468 cells expressing control or p53-specific shRNA were serum-starved for 12 h before they were stimulated with EGF for the indicated periods of time. Cell lysates were then subjected to western blot analysis with the indicated antibodies. The blots were quantified using the Gel-Pro analyzer software (USA). The relative p-ERK1/2/ERK1/2 ratio was also shown. (g) H1299-Tet-On-p53 273H or control cells were incubated with doxycycline for 24 h, followed by serum starvation for an additional 12 h. The cells were then treated with EGF and UO126 in the indicated combinations. Cell lysates were analyzed by western blot with the indicated antibodies. (h) H1299 cells were transfected with scramble or miR-27a inhibitors. Twenty-four hours after transfection, cells were serum-starved for another 12 h. The cells were then stimulated with EGF for the indicated periods of time, followed by western blot analysis with the indicated antibodies. The blots were quantified using the Gel-Pro analyzer software (USA). The relative p-ERK1/2/ERK1/2 ratio was also shown. (i) H1299-Tet-On-p53 273H cells were transfected with scramble or miR-27a mimics. Twenty-four hours after transfection, cells were treated with doxycycline for another 24 h. The cells were then serum-starved for 12 h before they were stimulated with EGF for the indicated periods of time. Cell lysates were then analyzed by western blot. The blots were quantified using the Gel-Pro analyzer software (USA). The relative p-ERK1/2/ERK1/2 ratio was also shown. (j and k) MDA-MB-468 cells expressing control, p53 or EGFR-specific shRNA were transfected with miR-27a mimics or inhibitors as indicated. Twenty-four hours after transfection, cells were serum-starved for 12 h. The cells were then incubated with EGF for 24 h. (j) Cells were stained with EdU. The nuclei were also visualized by Hoechst 33342 staining. (k) The percentage of proliferating cells was expressed as the ratio of EdU-positive cells to total Hoechst 33342-positive cells. The data are represented as mean \pm S.D. from three independent experiments. The data were also statistically analyzed. * $P < 0.05$ and ** $P < 0.01$

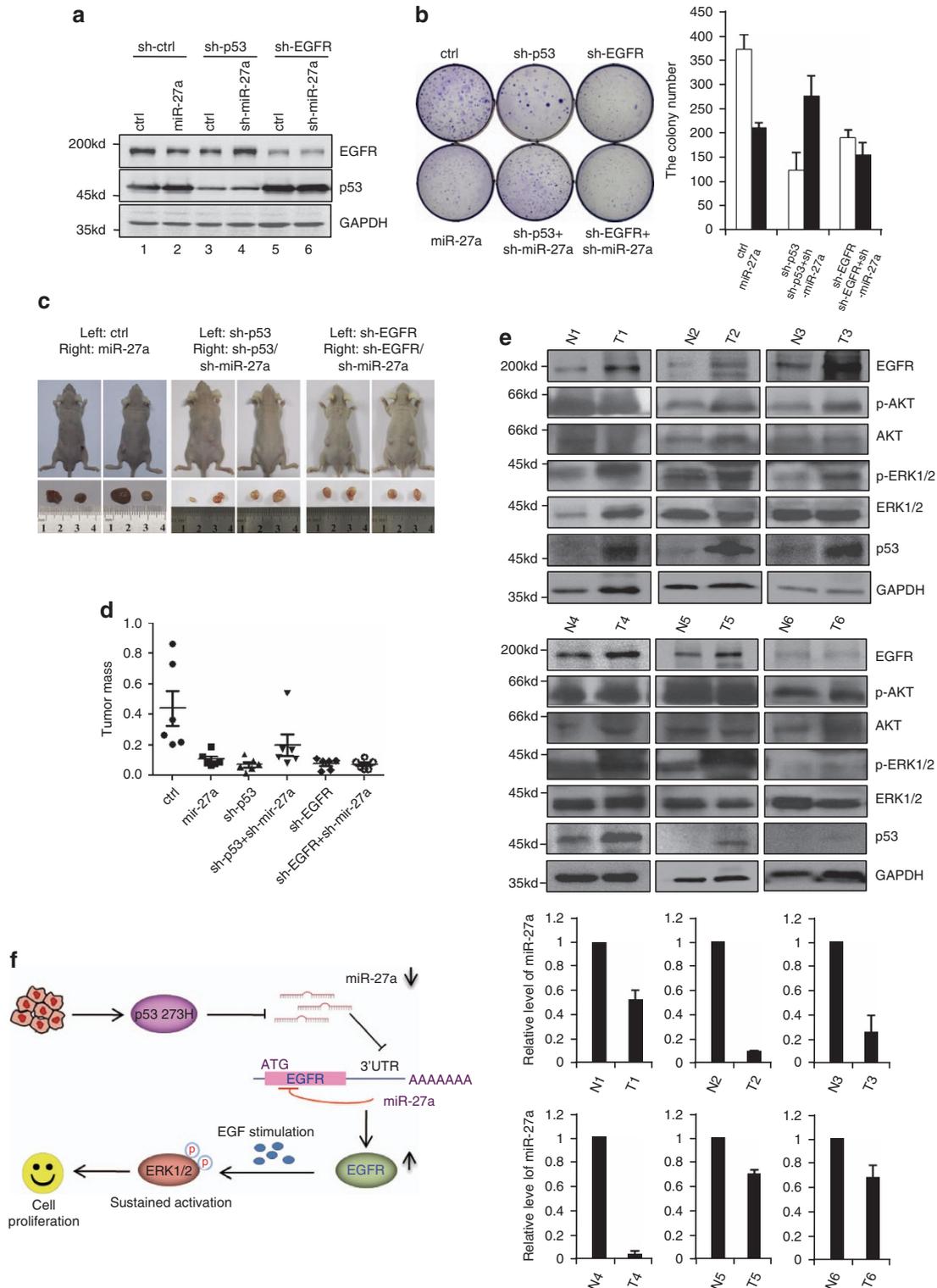


Figure 5 Role of the p53 273H-miR-27a-EGFR pathway in tumorigenesis. (a) MDA-MB-468 cells were infected with lentiviruses expressing control shRNA, p53 or EGFR-specific shRNA, miR-27a mimics or anti-miR-27a in the indicated combinations. Forty-eight hours after infection, cell lysates were analyzed by western blot. (b) The cells generated in (a) were subjected to soft agar assays. After growing in soft agar for 30 days, cells were fixed and stained with crystal violet. The numbers of colonies were counted and plotted. The mean numbers of colonies were from three independent experiments. (c) The cells generated in (a) were subcutaneously injected into the dorsal left (Left) or right flanks (Right) of nude mice as indicated ($n = 6$). Representative pictures of tumor-bearing nude mice were taken 20 days after injection. The volumes of the excised tumors were also compared. (d) Mass of the indicated xenografts excised from nude mice. (e) Expression levels of EGFR, p53 and miR-27a in gastric tumors (T) and their surrounding normal tissues (N) were determined by western blot and quantitative reverse transcriptase-PCR analyses, respectively. (f) A schematic illustration of the proposed model depicting a unique p53 mutant-mediated pro-survival pathway in tumor

miR-27a has been reported to be significantly upregulated in several types of human cancers such as renal, cervical, and breast cancers.^{42,52,53} Increased expression of miR-27a was shown to promote tumor development by targeting multiple cellular factors, including ZBTB10, FOXO1, and Myt-1.^{41,42} These studies support the oncogenic role of miR-27a. However, miR-27a was also shown to be downregulated in certain types of cancers, including acute promyelocytic leukemia, colorectal cancer, malignant melanoma, and gastric cancer.^{33,34,36–38} In addition, induction of miR-27a elicits caspase-dependent and -independent apoptosis in human embryonic kidney cells.⁵⁴ In agreement with these data, we have shown that induction of miR-27a strongly decreases cell proliferation *in vitro* and tumor formation *in vivo*. Our data add the complexity to miR-27a function in regulating tumorigenesis. Taken together, it is conceivable that miR-27a could act either as an oncogene or a tumor-suppressor gene by targeting different cellular proteins in different situations.

Previous reports have shown that mutant p53 is able to enhance EGFR/PI3K-AKT signaling pathway and promote cell migration.⁵⁵ Additionally, a recent report by Muller *et al.*⁵⁶ has demonstrated that mutant p53 enhances met proto-oncogene (MET)-dependent ERK1/2 phosphorylation in response to hepatocyte growth factor treatment.⁵⁶ Interestingly, our data reveal that in response to EGF stimulation, mutant p53-273H promotes sustained activation of ERK1/2 but not AKT and thus increases cell proliferation. Taken together, these findings indicate the complexity of the mechanisms underlying mutant p53 gain-of-function and suggest that activation of the EGFR/PI3K-AKT and EGFR/ERK1/2 pathways may have a distinct role in tumor development.

Upregulation of EGFR occurs in a range of cancers such as breast and lung cancers, which correlates with high metastases and a poor prognosis.^{57,58} Consistent with this, we have demonstrated that increased levels of EGFR were detected in a higher proportion of clinical gastric tumor samples, which was correlated well with the decreased expression of miR-27a. This result suggests that miR-27a inhibition may promote tumor development via upregulating EGFR. We also show that these tumor samples exhibit high expression of p53 compared with their adjacent normal tissues, indicating that these tumors may harbor mutant p53.⁴⁷ Therefore, these combined data suggest that the mutant p53/miR-27a/EGFR pathway may have an important role in promoting tumorigenesis and also indicate miR-27a as a valuable target for treatment of cancers with mutant p53. In summary, we propose a hypothetic model as shown in Figure 5f, which depicts miR-27a and EGFR as downstream effectors of mutant p53-273H-promoted proliferation of tumor cells.

Materials and Methods

Cell culture and reagents. H1299 and MDA-MB-468 cell lines were cultured in DMEM (Dulbecco's modified Eagle's medium) medium containing 10% fetal bovine serum. Antibodies against GAPDH (glyceraldehyde 3-phosphate dehydrogenase), phospho-AKT (S473), AKT, and p53 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against EGFR, phospho-ERK1/2, and ERK1/2 were obtained from Cell Signaling (Danvers, MA, USA). Anti-p21 antibody, EGF, and UO126 were ordered from Sigma Aldrich (St Louis, MO, USA).

Mimics and inhibitors of miR-27a and siRNA against p53 were purchased from GenePharma (Shanghai, China).

RNA isolation and miRNA profiling. H1299-Tet-On-p53 and H1299-Tet-On-p53 273H cells were treated with or without doxycycline for 24 h. Total RNA from these cells was isolated with TRIzol reagent (Invitrogen, Grand Island, NY, USA). To compare the miRNA expression profiles between wild-type p53- and mutant p53-expressing H1299 cells, the μ Paraflo MicroRNA microarray analysis was performed by LC Sciences (Houston, TX, USA). The fold change of each miRNA induced by mutant p53 (Fold-change cutoff: 2.5, *P*-value cutoff: 0.05) from the microarray was presented as the means of three biological replicates. The fold changes of these miRNAs on wild-type p53 induction were also shown.

Overexpression of miR-27a and knockdown of miR-27a, p53, and EGFR. To stably knock down p53 and EGFR, shRNAs against p53 and EGFR were first constructed into pLKO.1 vector. The targeted sequences are 5'-GACTCCAGTGGTAATCTAC-3' (p53) and 5'-GTGGAGCGAATTCCTTTGGA A-3' (EGFR). Similarly, to stably overexpress miR-27a, the fragments containing pre-miR-27a were amplified using primers 5'-GTACCGGTAATCACATTGCCAGG GATTCC-3' and 5'-GTGAATCTTTTGTGTTTCAGCTCAGTAGGCAC-3' and cloned into pLKO.1 vector. These pLKO.1 constructs were then individually transfected into HEK293T cells together with pREV, pGag/Pol/PRE, and VSVG for lentivirus generation. Twenty-four hours after transfection, cells were cultured with DMEM medium containing 20% fetal bovine serum for another 24 h. The culture medium containing lentivirus particles was cleaned by centrifugation to get rid of the cell debris at 12 000 \times g for 5 min and was used to infect the target cells. To stably knock down miR-27a, the method was used as previously described.

Quantitative reverse transcriptase-PCR analysis. Total RNA was isolated using TRIzol reagent (Invitrogen). The assay was performed as previously described. MiR-27a was detected by using Hairpin-it miRNAs qPCR Quantitation Kit (GenePharma, Shanghai, China).

ChIP. H1299 or MDA-MB-468 cells were cross-linked with 1% formaldehyde for 10 min. The ChIP assay was performed by using anti-p53 and the ChIP assay kit (Upstate/Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Anti-mouse immunoglobulin G was also used as a negative control. The bound DNA fragments were subjected to quantitative-PCR or PCR reactions using the following primer pair: Amplicon A, 5'-gcctgtctgtctgtcct-3' and 5'-gga acatctggcttccact-3'; Amplicon B, 5'-tgggaaggatgttgagcgg-3' and 5'-gccacagctc agtgaccac-3'; Amplicon C, 5'-acgcacacgcgtcttga-3' and 5'-caggtgccaggagaca g-3'; Amplicon D, 5'-ggtcaggaggtcgagacc-3' and 5'-ccaggctggatgcaatgg-3'; and Amplicon E, 5'-ggcctgtatcttggagcttgatc-3' and 5'-cgccactcctagaagcctg-3'. PCR products were separated by gel electrophoresis on 2% agarose gel.

Luciferase reporter assay. To determine the effect of wild-type or mutant p53 on miR-27a promoter, the increasing amounts of either pcDNA3.0-Flag-p53 or pcDNA3.0-Flag-p53 273H constructs were cotransfected into H1299 cells together with PGL3-miR-27a promoter and Renilla luciferase reporter plasmids by using lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activity were measured by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). To evaluate the effect of miR-27a on EGFR 3'-UTR, the psi-CHECK2 vectors containing EGFR 3'-UTR or different EGFR 3'-UTR-muts were cotransfected with miR-27a mimics into H1299 cells using oligofectamine (Invitrogen). The data were analyzed as previously described.⁵⁹

EdU incorporation assay. The EdU incorporation assay was performed with an EdU Assay Kit (Guangzhou RIBOBIO, Guangzhou, China) according to the manufacturer's instructions. Briefly, the cells were incubated with DMEM medium containing 50 μ M EdU for 2 h. The nuclei were also stained with Hoechst 33342 (Sigma, St Louis, MO, USA), and the images were acquired with an Olympus DP71X microscope (Olympus, Tokyo, Japan).

Xenograft mouse model. In all, 1×10^7 MDA-MB-468 cells, stably expressing miR-27a mimics, p53 shRNA, p53 shRNA plus anti-miR-27a, EGFR shRNA, or EGFR shRNA plus anti-miR-27a were subcutaneously injected into the dorsal flanks of male nude mice (MARC of Nanjing University; *n* = 6 mice per group). Mice were killed and tumors were excised at 20 days after transplantation.

Studies on animals were conducted with approval from the Animal Research Ethics Committee of the University of Science and Technology of China.

Reproducibility. All the data were repeated at least three times. The western blot and ChIP analyses were representatives of three independent experiments.

Statistical analysis. Statistical analysis was carried out using Microsoft Excel software (Microsoft, Redmond, WA, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA) to assess differences between experimental groups. Statistical significance was analyzed by Student's *t*-test and expressed as *P*-value. *P*-values <0.05 were considered to be statistical significant.

Conflict of Interest

The authors declared no conflict of interest.

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