The isolation of an RNA aptamer targeting to p53 protein with single amino acid mutation

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p53, known as a tumor suppressor, is a DNA binding protein that regulates cell cycle, activates DNA repair proteins, and triggers apoptosis in multicellular animals. More than 50% of human cancers contain a mutation or deletion of the p53 gene, and p53R175 is one of the hot spots of p53 mutation. Nucleic acid aptamers are short single-stranded oligonucleotides that are able to bind various targets, and they are typically isolated from an experimental procedure called systematic evolution of ligand exponential enrichment (SELEX). Using a previously unidentified strategy of contrast screening with SELEX, we have isolated an RNA aptamer targeting p53R175H. This RNA aptamer (p53R175H-APT) has a significantly stronger affinity to p53R175H than to the wild-type p53 in both in vitro and in vivo assays. p53R175H-APT decreased the growth rate, weakened the migration capability, and triggered apoptosis in human lung cancer cells harboring p53R175H. Further analysis actually indicated that p53R175H-APT might partially rescue or correct the p53R175H to function more like the wild-type p53. In situ injections of p53R175H-APT to the tumor xenografts confirmed the effects of this RNA aptamer on p53R175H mutation in mice.

p53 | RNA aptamer | contrast screening | SELEX | tumor

Nucleic acid aptamers, as single-stranded DNA or RNA oligonucleotides that are able to bind various targets with high specificity, were first isolated from a pool of random sequences with a process called systematic evolution of ligand exponential enrichment (SELEX) in 1990 by two laboratories (1, 2). Over the years, an array of methods have been invented to facilitate SELEX screening, and specific aptamers binding to partners ranging from small molecules to large proteins have been isolated. However, an RNA aptamer that can distinguish a protein with a single amino acid mutation from the wild-type (WT) protein remains absent (3–11).

Protein with a single amino acid substitution is the cause of a plethora of human diseases (12–14). A well-known example is sickle-cell anemia, which is caused by a point mutation in the β-globin chain of hemoglobin (15). Also, point mutations in multiple tumor suppressor proteins cause cancer (16–18). The protein p53 is a tumor suppressor and functions as a transcription factor to regulate the expression of genes involved in DNA repair, cell cycle, and apoptosis. A mutation within one allele of this gene can result in inactivation of the remaining WT allele in a dominant-negative manner, and mutations from six mutation hot spots located in the DNA-binding surface of p53 are frequently found in almost all cancer types (19). Actually, more than half of human cancer cases relate to mutations in p53, and the single amino acid substitution p53R175H is one of the mutations at the p53R175 hot spot (20, 21). R175H mutation abolishes the p53 WT functions in both MEF cells and thymocytes (22). p53R175H possesses a marked anti-apoptotic gain-of-function in lung cancer cells (23). Also, p53R175H cooperates better than any other mutant in foci transformation and promotes metastasis in immortalized prostate cells (24–26). Actually, patients with a mutation at position 175 show the poorest prognosis (27).

Considering the almost unlimited potential of nucleic acid aptamers in binding specificity and the biomedical significance of single amino acid mutations, we set up to isolate an RNA aptamer specific to mutant p53. We have developed a new strategy of contrast SELEX to screen for RNA aptamers that can distinguish p53R175H from WT p53. After an efficient one-pass examination, we isolated an aptamer that binds specifically to p53R175H in vitro. With further experiments in cell cultures, we showed that this RNA aptamer could bind to the p53R175H protein and further demonstrated rescue effects in cells expressing p53R175H. The effectiveness of this RNA aptamer to p53R175H was also held when tested with tumor xenografts.

Results

Contrast Screening of RNA Aptamer Targeting to p53R175H. To isolate an RNA aptamer, we first obtained purified WT p53 and p53R175H protein expressed with a prokaryotic system. WT p53 and p53R175H were chemically immobilized to agarose beads and magnetic beads, respectively. Both kinds of beads conjugated with an equal amount of protein were then incubated with the RNA library followed with cycles of contrast screening (Fig. 1*A*). In each cycle, the agarose beads coupled with WT p53 would be discarded after the incubation, and the RNAs binding to the p53R175H-coupled magnetic beads were recovered and amplified through reverse transcription PCR (RT-PCR) followed by in vitro transcription. The resulting RNAs were then subjected to the next cycle of incubation and SELEX screening. The starting RNA library was prepared from a DNA library through in vitro transcription (Fig. 1*B* and Fig. S1*A*). The in vitro synthesized RNA sequences in the library consist of two fixed regions at the 5′ and 3′ end, respectively, and a 25-nt random region. This contrast screening was greatly facilitated and made quickly by the easy separation of magnetic beads from the agarose beads with a magnet.

Significance

We have developed a contrast screening strategy of systematic evolution of ligand exponential enrichment to isolate an RNA aptamer targeting specifically to p53R175H. This RNA aptamer partially rescued p53R175H in both cell cultures and tumor xenografts. p53 mutations are found in more than half of human cancers, and it is of biomedical significance to be able to isolate an RNA aptamer against a p53 mutation. This may also be the first report about an RNA aptamer specific to a mutant protein with a single amino acid change, and a plethora of human diseases are actually caused by single amino acid mutation. Thus, we present here a proof-of-concept study in isolating a specific and physiologically functional RNA aptamer against mutant protein with single amino acid substitution.

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Conflict of interest statement: G.S. and L.C. have an ownership interest in a patent related to this research.

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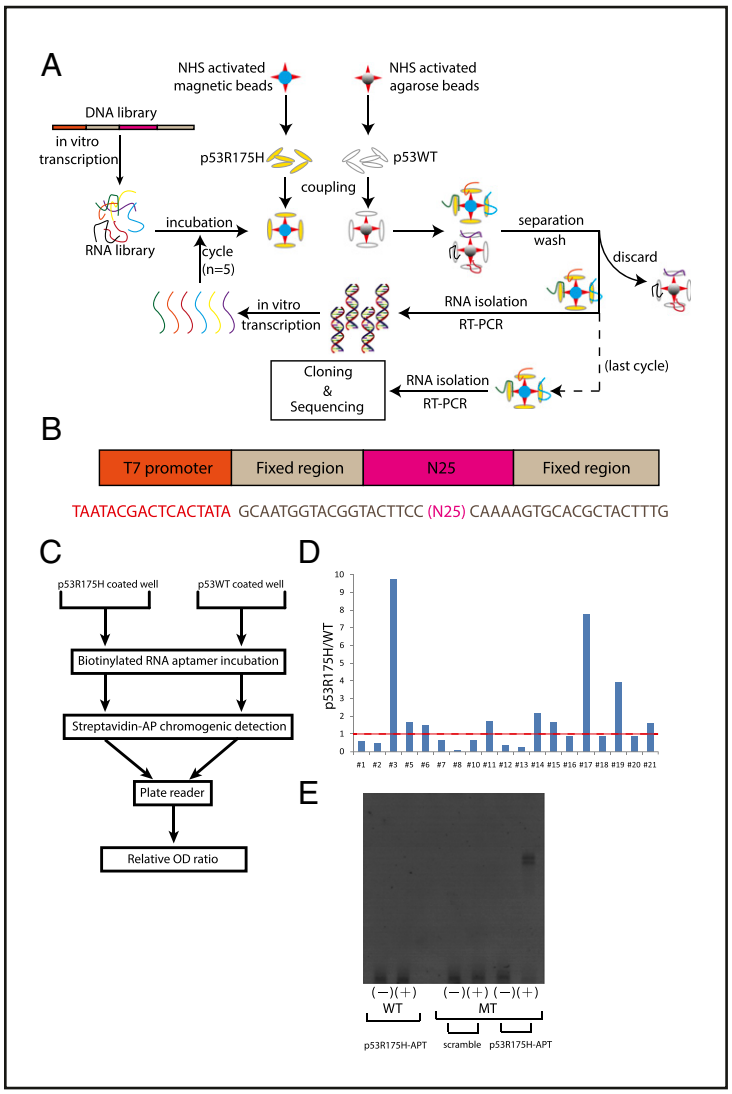

Fig. 1. Contrast SELEX screening followed with ELISA verification yielded RNA aptamers for p53R175H. (*A*) Workflow of the contrast SELEX screening. WT p53 protein-coupled agarose beads and p53R175H-coupled magnetic beads were incubated with the in vitro transcribed RNA pool. After washing, magnetic beads and agarose beads were separated, and RNAs on the magnetic beads were isolated and subjected to RT-PCR and in vitro transcription. After five SELEX cycles, RNAs on p53R175H-coupled magnetic beads were isolated, cloned, and sequenced for further analysis. (*B*) The starting ssDNA library. This library consists of 10^{15} unique sequences, each containing a 25-base internal randomized region flanked by two fixed regions with a T7 promoter sequence. (*C*) Workflow of the ELISA affinity assay. WT p53 and p53R175H were coated, respectively, to individual wells. The biotinylated RNA aptamer was incubated with p53 WT and p53R175H, respectively. (*D*) Relative ratio of reading from p53R175H- and WT p53-coated wells for each individual RNA aptamer. (*E*) Gel shift assay confirmed that p53R175H-APT interacts specifically with the p53R175H mutant protein (MT) but not the WT p53 (WT). (–), without the protein; (+), with the protein.

We examined the efficiency of the contrast screening method by sampling RNA aptamers (~20 randomly selected) obtained from the p53R175H-coupled magnetic beads from the third round and checked for their affinity to p53 WT and p53R175H protein with a one-pass ELISA (Fig. 1*C*, Fig. S1 *B* and *C*, and Table S1). Individual biotin-labeled RNA aptamers were incubated with either WT or the p53R175H protein coated in the wells. The relative binding affinity of each RNA aptamer to the p53R175H or the WT p53 was then calculated based on the readings from ELISA. None of the aptamers from round 3 and round 4 showed one magnitude of difference between p53R175H and p53 WT (Fig. S1 *B* and *C*). After the completion of five cycles of screening, RNAs from the p53R175H beads were cloned, and 21 sequences were obtained from the same number of clones analyzed (Table 1). There was no clone with identical sequences, indicating that the yielded RNAs were still a complex pool of mixed sequences.

In Vitro Verification of Isolated RNA Aptamers. The same one-pass ELISA affinity assay was carried out to verify the specific binding of the isolated RNA aptamer to p53R175H from round 5 (Fig. 1*C*). Nineteen RNA aptamers of the total 21 sequences analyzed showed higher readings in protein-coated wells than in the background, and out of these 19 aptamers, nine of them gave higher readings in the p53R175H-coated well than in the well coated with WT protein (Fig. 1*D*). The aptamer 3 showed the strongest relative affinity toward p53R175H compared to the WT p53, by ~10-fold. We then named this aptamer p53R175H-APT and chose it for further characterizations.

Gel shift assay with in vitro synthesized p53R175H-APT with the RNA of scrambled sequences as the control showed that the p53R175H mutant protein but not the WT p53 could bind to p53R175H-APT specifically (Fig. 1*E* and Fig. S1*D*). It seems that the contrast screening is efficient and one could start to examine the aptamers after about five cycles of screening.

Table 1. The RNA aptamer sequences cloned from the p53R175H-coupled beads after the fifth round of contrast screening

No.	p53R175H aptamers
1	TAGTGCTTACGAGTGTCCGCAGAGGTCCCGATCTG
2	TGCGTGTTCGCAGGGGAGTTGTGTTATCTTGTATG
3	ATTAGCGCATTTTAACATAGGGTGC
4	AACCTGGCAGGACAGATCGTCACGT
5	AACGCAATTCGGAAACGCGTTCTGACTGCTTGAAG
6	AGGGACATTAAATCCAGGGCCCGCG
7	TGACTAGCTTTTTTTCACAGGTTG
8	AGTCTGGTACACTGAAAGCCGCACC
9	TGGTACTATTAGTTTATGGGTTTGAGAAACACTT
10	GATGTGAGTTCACGCATCGTCGTCCTCAATCTATG
11	TCGAGCGAATAAAGAAAGTACGCTG
12	TGATACACCTCTCCCACTCGGAGCCAGTTCGGCGT
13	TACCCCTCCTGCGGCTCTTCTAATATGTTCAACAC
14	CTAGAGCCAGAGAGCTAGGTCTGGT
15	GGCCGGTAACAAAGTCGGGGGGTGA
16	CTCGCCTCTGTAGGTATCAATCCCATCGCCTGTGC
17	ATTCAGCTTGGTAGATCTTAGTTTCTTACTGTGTG
18	AACGCAATTCGGAAACGCGTTCTGACTGCTTGAAG
19	GCAGTGGCGACGAGCCTTGGTTGCGTTTGTGTTGG
20	TCGCGCCCATTCGCGGACACCCTTTTTCTGTTTGG
21	TTTTCCTGGGGTTGCGTTCTCCAGATTGT

p53R175H-APT Did Not Affect Cells with WT p53 or p53R273H Mutant. We started in vivo analysis of p53R175H-APT with HEK293T and HeLa cells, which are cells harboring WT p53 only. Gross feature and cell growth of p53 WT cells were unaffected by the application of p53R175H-APT (Fig. 2 *A* and *B* and Fig. S2*A*). Flow cytometry verified that transfection of plasmid expressing p53R175H-APT had no significant effect on apoptosis of 293 cells (Fig. 2*C*). This lack of effect was seen for even much higher concentrations of plasmids transfected (Fig. S2*B*).

We also tested the effect of p53R175H-APT in p53 WT cells with a nanoparticle delivery system of unmodified RNA (28). This nanoparticle keeps small RNA enclosed inside from degradation and is sensitive to and sheddable at a lower pH found in the microenvironment of tumor cells (28). Nanoparticles at a final concentration of 400 nM (with p53R175H-APT RNA enclosed) had no effect on cell growth and apoptosis based on the [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) and flow cytometry assays with 293 cells (Fig. 2*D*). Nanoparticles at a final concentration of 200 nM (with p53R175H-APT RNA enclosed) showed an inhibitory effect on cells with p53R175H expression (see Fig. S3*C*). Also, p53R175H-APT did not bind WT p53 compared with a control with a scrambled sequence when examined with p53 RNA immunoprecipitation (RIP) (Fig. 2*E* and Fig. S2*C*).

We further investigated whether p53R175H-APT had any effect on cells with another p53 mutant, p53R273H, and again nanoparticles at a final concentration of 400 nM (with p53R175H-APT RNA enclosed) had no effect on cell growth and apoptosis based on the MTT and flow cytometry assays (Fig. 2*F*).

These results indicated that p53R175H-APT did not interact with WT p53 and most likely did not elicit any physiology function in cells with normal p53 protein or an irrelevant p53R273H mutant.

p53R175H-APT Affected p53R175H Cells. To examine whether the aptamer can exhibit physiological functions in vivo, we first established a stable cell line expressing p53R175H in H1299 cells (H1299-p53R175H) (Fig. 3*A*). H1299 is a p53-null lung cancer cell line (29). We noticed that the growth of H1299-p53R175H cells was significantly inhibited by the application of p53R175H-APT plasmid (Fig. 3*B*). Flow cytometry demonstrated that p53R175H-APT transfection resulted in significantly more cells in early and late apoptosis in H1299-p53R175H cells (Fig. 3*C* and Fig. S3 *A* and *B*). Nanoparticles at a final concentration of 200 nM (with p53R175H-APT RNA enclosed) also showed an inhibitory effect on H1299-p53R175H cells (Fig. S3*C*). In clonogenic assays, p53R175H-APT–treated cells showed significantly fewer colonies than the control group with scrambled RNA (Fig. 3*D*). In soft agar assays, the same inhibitory effect was observed for p53R175H-APT (Fig. 3*E*). These data demonstrated that p53R175H-APT treatment inhibited or reversed the promoting effect of p53R175H on cell growth (30, 31).

Migration and invasion are basic characteristics of cancer cells. To investigate the migration, we performed scratch assays with or without the application of the p53R175H-APT plasmid using H1299-p53R175H cells (Fig. 3*F*). The gap in the p53R175H-APT group healed at a rate significantly slower than the control group (Fig. 3*F*). In the transwell assays to examine migration of individual cells, the p53R175H-APT plasmid-treated group again showed a significant decrease in cell migration (Fig. 3*G*). These results showed

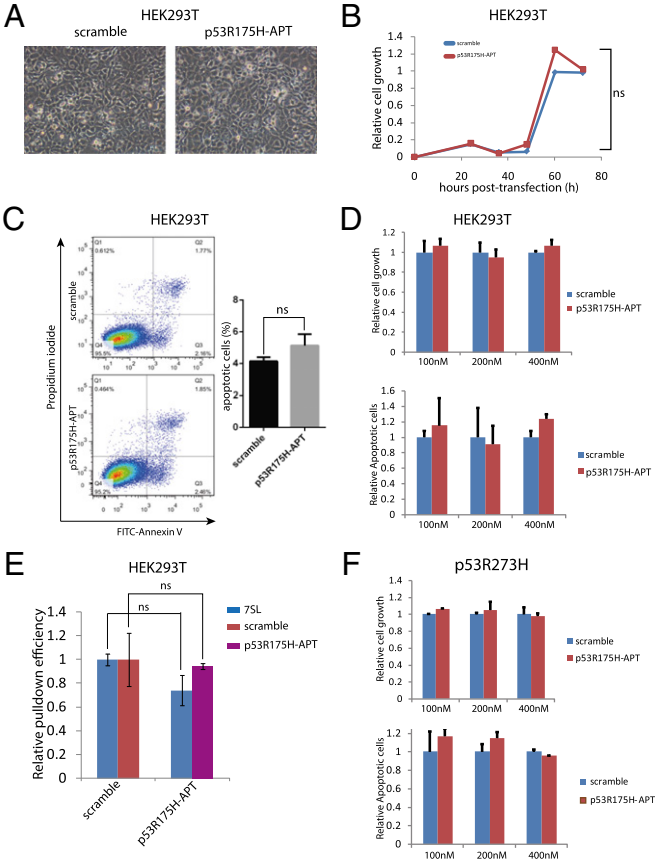

Fig. 2. p53R175H-APT did not affect cells with WT p53 or p53R273H. (*A*) Images of HEK293T after transfection with either p53R175H-APT or the RNA control with scrambled sequences. (*B*) Cell growth curve plotted with MTT assay. Growth of HEK293T cells was unaffected by the application of p53R175H-APT. (*C*) Detection of apoptotic cells by FITC-annexin V staining and propidium iodide (PI) staining with FACS. Representative ($n = 3$) FACS data and bar graph summarizing the FACS data (Q2 + Q3 for early and late apoptosis) are shown. (*D*) HEK293 cells treated with various concentrations of nanoparticles with enclosed p53R175H-APT RNA or scramble control. Cell growth and apoptosis were measured with MTT assays and FACS, respectively. (*E*) p53R175H RIP followed with qPCR for p53R175H-APT RNA and the scramble control using cells transfected with either p53R175H-APT or scramble control plasmid. 7SL was examined as an irrelevant noncoding RNA as an assessment of nonspecific binding. (*F*) Cells with p53R273H expression (H1299-p53R273H cells) treated with various concentrations of nanoparticles with enclosed p53R175H-APT RNA or scramble control. Cell growth and apoptosis were measured with MTT assays and FACS, respectively. All data were from three repeats. Error bars represent SD. ns, difference between groups of data are not significant. *P* values were determined with two-tailed Student's *t* test.

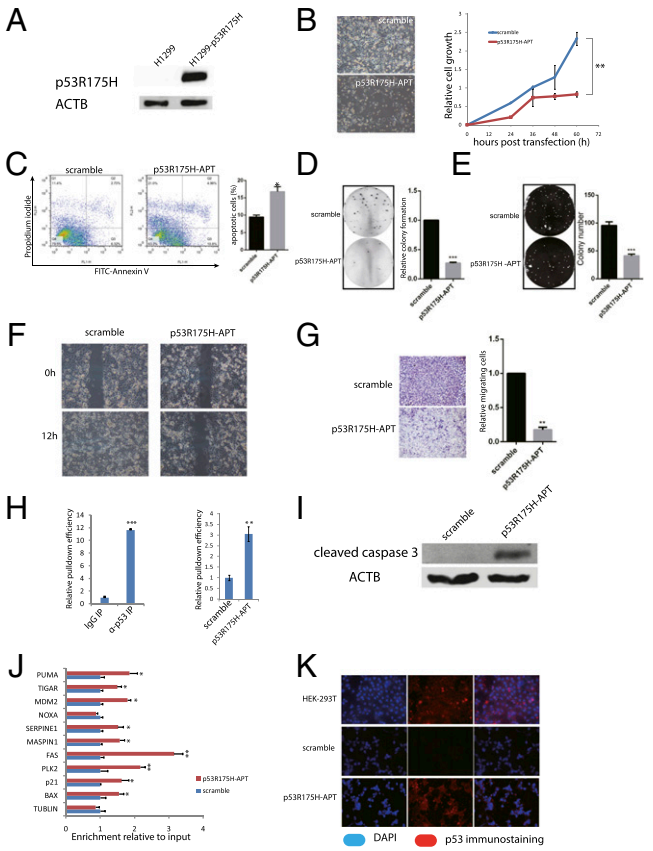

Fig. 3. p53R175H-APT rescued p53R175H in cell cultures. (*A*) Western blot of p53R175H verified the successful establishment of the H1299-p53R175H stable cell line. (*B*) Gross appearance of H1299-p53R175H cells transfected with p53R175H-APT along with the scramble control. Relative cell growth of p53R175H-APT–transfected cells was determined by MTT assay. (*C*) Detection of apoptotic cells by FITC-annexin V staining and PI staining with FACS. Representative ($n = 3$) FACS data and bar graph summarizing the FACS data (Q2 + Q3 for early and late apoptosis) are shown. (*D*) Clonogenic assay and (*E*) soft agar assay with p53R175H-APT– and scramble control-transfected cells. (*F*) Representative images ($n = 5$) of scratch assay for cells transfected with either p53R175H-APT or the scramble control. (*G*) Transwell migration assay using H1299-p53R175H cells transfected with p53R175H-APT or the scramble control. Representative images ($n = 3$) of crystal violet staining of migrated cells and bar figure of relative migrating cells are shown. (*H*) p53R175H RIP assay followed with qPCR. IgG was the negative control of p53 antibody (α-P53) to show the relative pull-down efficiency of p53R175H-APT RNA by the α-P53 (*Left*). Scramble was the negative control of p53R175H-APT to show the relative pull-down efficiency of p53R175H-APT RNA by the α-P53 (*Right*). (*I*) Western blot of cleaved caspase 3 in p53R175H-APT– and the scramble control-transfected cells. (*J*) p53R175H ChIP assay followed by qPCR to examine 10 known p53 target genes. Promoter of tubulin (TUBB) was a negative control. The known binding sites of p53 for each of these genes examined are shown in Fig. S3*H*. (*K*) Immunostaining (red signal) of cells with the antibody PAb1620 specific for the WT p53 structure. HEK293T is a cell line with WT p53. p53R175H-APT, H1299-p53R175H cells treated with the p53R175H-APT aptamer; scramble, H1299-p53R175H cells treated with the scramble control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *P* values were determined with two-tailed Student's *t* test. All data were from at least three repeats. Error bars represent SD.

that p53R175H-APT inhibited or reversed the promoting effect of the p53R175H mutant on cell migration and invasion (32, 33).

p53R175H-APT Interacted with the p53R175H Protein and Might Partially Correct the Mutant in Vivo. The phenotypic "rescue" of p53R175H by p53R175H-APT in cultured cells led us to investigate the possible "correction" of p53R175H at the molecular level. First, we investigated whether p53R175H binded to p53R175H-APT with RIP (Fig. 3*H* and Fig. S3 *D*–*F*). Indeed, the p53R175H mutant protein could bind to p53R175H-APT specifically (Fig. 3*H*). Interestingly, RIP with the mutated part of p53 (a fragment of 150 amino acids with the p53R175H mutation in the middle) showed that the mutated part of p53 interacted with the p53R175H-APT, although with a weaker ability compared with the full mutant protein (Fig. S3*G*). This result indicates that the selected aptamer may not only bind to the mutated part of p53 but also recognize the region outside of the mutation. We knew already that p53R175H-APT did not bind to WT p53 (Fig. 2*E* and Fig. S2*C*). Also, the resulting apoptosis in H1299-p53R175H cells upon the treatment of p53R175H-APT was mediated by the apoptotic pathway involved in the cleavage of caspase 3 (Fig. 3*I*) (34, 35).

As a transcriptional factor, WT p53 functions at the transcriptional level by interacting with the promoter of target genes (36). Defective p53 proteins have decreased or destroyed regulatory ability (21, 37). To investigate whether p53R175H-APT could rescue the regulation of p53 target genes by p53, we performed p53 chromatin immunoprecipitation (ChIP assay). Data from the ChIP assay revealed that p53R175H-APT enhanced the binding of p53R175H to the promoter of multiple p53 target genes such as FAS, PLK2, BAX, SERPINE1, maspin, P21, MDM2, PUMA, and TIGAR (Fig. 3*J* and Fig. S3*H*). These genes are all known p53 targets involved in apoptosis, cell cycle control, and cell migration (36, 38–43). Almost all genes tested showed a moderate increase in the ChIP assay, and for unknown reasons, we did not see a change for NOXA (Fig. 3*J* and Fig. S3*H*).

The ability of the p53R175H-APT aptamer to rescue partially the function of the p53R175H mutant raised the possibility that it could promote correct folding of the p53 mutant. Using the WT p53-specific antibody PAb1620, we performed immunostaining to examine this issue (44). The results showed that indeed the p53R175H-APT–treated but not the scramble control-treated cells (with p53R175H expression) gave rise to immunostaining signals (Fig. 3*K*). Also, consistent with the partial rescue effect, the immunostaining signals from p53R175H-APT–treated cells were lower than the signals from cells with WT p53 (Fig. 3*K*).

These results together showed that p53R175H-APT binds to the p53R175H protein, partially rescues the function of the mutant protein, and may aid in the folding of mutant protein to restore its structure so it is more close to that of the WT protein in cultured cells.

p53R175H-APT Had an Effect on Tumor Xenografts with the p53R175H Mutant. The impressive effects of p53R175H-APT in cell cultures promoted us to examine it further with tumor xenografts. We injected s.c. H1299-p53R175H cells to the nude mice to generate tumors, and treatment started 5 d after seeding when the tumor became palpable. Mice were treated with in vitro synthesized p53R175H-APT or the scramble control conjugated with nanoparticles at day 5 and day 8 by s.c. injection directly to the tumor (28). The volume of tumors was measured in the following 3 wk. Tumors treated with p53R175H-APT but not the scrambled control showed significantly slower growth after day 18 (Fig. 4*A*). Tumors in the p53R175H-APT treatment group were much smaller, as shown with in vivo fluorescent imaging and when dissected out (Fig. 4 *B* and *C*). TUNEL staining with tumor sections confirmed that there were more apoptotic cells in the p53R175H-APT treatment group (Fig. 4*D*). We have also performed tail vein i.v. administration of p53R175H-APT or the scramble control conjugated with nanoparticles, and similar inhibitory effects of the aptamer on tumor growth were observed (Fig. 4*E* and Fig. S4).

Discussion

We have isolated an RNA aptamer with physiological function using a new strategy of contrast SELEX screening and a quick ELISA verification. This RNA aptamer can bind specifically to the p53R175H mutant but not the WT p53 in both test tubes and cells. More fascinating, p53R175H-APT seems to rescue, at least to a certain degree, the function of p53R175H both in cells and in tumor xenografts.

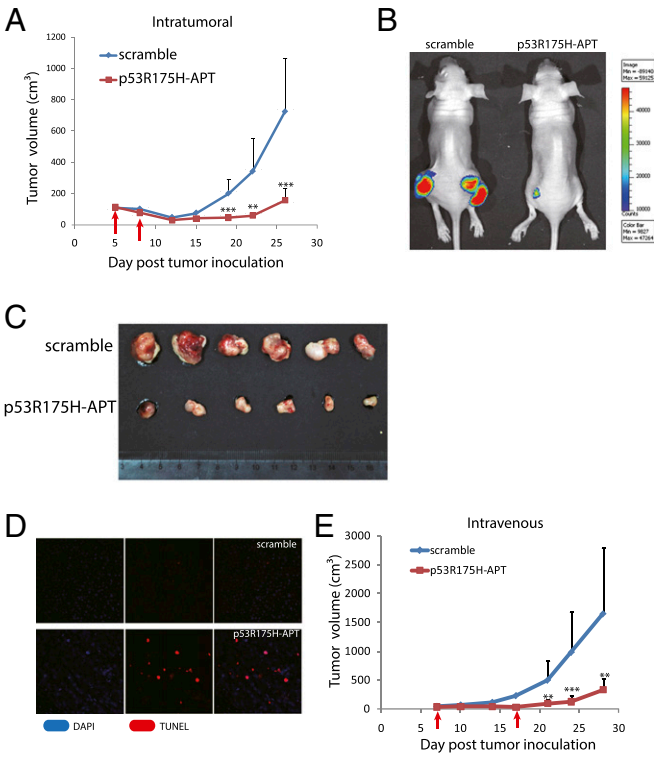

Fig. 4. p53R175H-APT inhibited tumor growth. (*A*) Mice were treated with in vitro synthesized p53R175H-APT coated with nanoparticles at day 5 and day 8 by s.c. injection directly to the tumor. Tumor volumes were measured twice a week. (*B*) Bioluminescence imaging of representative tumors was shown. (*C*) Tumor dissected out from the p53R175H-APT– and the scramble control-treated groups. (*D*) TUNEL staining of tumor sections of p53R175H-APT– and the scramble control-treated groups. (*E*) Mice were treated with in vitro synthesized p53R175H-APT coated with nanoparticles at day 7 and day 17 by tail vein i.v. administration. Tumor volumes were measured twice a week. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. *P* values were determined with two-tailed Student's *t* test. All data were from at least three repeats. Error bars represent SD.

The contrast SELEX screening is easy and efficient. The agarose beads and magnetic beads coupled with WT and mutant proteins, respectively, can be easily separated, and it only needs a relatively low number of SELEX cycles to be able to isolate aptamers specific to the mutant protein (Fig. 1). Actually, with only five cycles, we isolated nine aptamers with bias to the mutant protein out of the 21 sequences cloned (Fig. 1*D*). Presumably, more contrast SELEX cycles may further enrich RNA aptamers with high binding affinity to the mutant protein, and it should be convenient to just add more cycles. Also, this strategy can be easily adopted to screen for a nucleic acid aptamer specific to the other mutant protein against the WT protein.

We suspect that PCR errors during the multiple rounds of amplification might be the major factor for the variable length, although rare copies of shorter or longer template DNA in the original pool might also contribute. Sequences from the third and fourth round of selection actually showed less variability (Table S1). Some other studies also noticed and discussed this kind of phenomenon when selecting for RNA and DNA aptamers (45–47).

The RNA aptamer p53R175H-APT not only binds specifically to p53R175H but also rescues the mutant protein at least partially (Fig. 3). p53R175H is a conformational mutant, as the mutation may affect the positioning of L2 and L3, two loops in p53 that interact with the minor groove of DNA (20). It would be tempting to speculate that the binding of p53R175H-APT to p53R175H enhances the interaction between the protein and DNA by adjusting the structure of the mutant protein. A detailed view of the structure of the p53R175H-APT/p53R175H complex may bring more insights to this issue.

As a hot spot mutant, p53R175H comprises 5.5% of all mis-sense p53 mutants (48) and is a druggable target (49–51). One chemical compound, NSC319726, has been screened to target p53R175H with great effect; actually, it is believed that NSC319726 could restore WT p53 structure and act as a p53R175H reactivator (52). To be able to isolate an RNA aptamer binding specifically to and rescuing in part the mutant protein is encouraging. Furthermore, p53R175H-APT delivered with nanoparticles had an impressive inhibitory effect on tumor xenografts when administered either intratumorally or i.v. (Fig. 4) (52). Another concern about using unmodified RNA coated by nanoparticles rather than using chemically modified RNA is the feature of the binding between the RNA aptamer and the protein. We were worried that chemical modification on RNA may interfere with the binding, as this is not a base-pairing binding like that between two molecules of nucleic acid.

Several unique properties make small RNA and RNA aptamers attractive to biomedical research and even clinical application (53, 54). The first FDA-approved aptamer drug was an RNA aptamer called Macugen (55, 56). Several RNA aptamers are also in clinical trials (57).

To our knowledge, our work may be the first report about an RNA aptamer specific to a mutant protein with a single amino acid substitution. Our screening strategy would be efficient and convenient to adopt, and the successful isolation of p53R175H-APT with a partial rescuing effect provides a proof-of-concept example in isolating a specific and physiologically functional RNA aptamer against protein with a single amino acid mutation.

Materials and Methods

Contrast SELEX Screening. p53R175H and p53 WT were first immobilized to magnetic beads (NHS Mag Sepharose, GE Healthcare) and agarose beads (NHS-Activated Sefinose 4 Fast Flow, Sangon), respectively, according to the manufacturer's instructions. An initial RNA library was generated by in vitro transcription of the DNA random library. p53R175H-coupled magnetic beads and WT p53-coupled agarose beads were washed once by SELEX binding buffer (20 mM Hepes pH 7.35, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) before incubation. Then both of the beads were incubated with the initial RNA library in 500 μL SHMCK buffer system (1 μg of RNA with 1 μg of each protein on 2.5 μL of each kind of beads) for 2 h in 37 °C. After competitive binding, only those RNA on the magnetic beads would be separated from the incubation system and extracted by TRIzol. Extracted RNA was subjected to RT (TaKaRa Biotechnologies) into single-stranded cDNA and further double-stranded DNA by PCR for 30 cycles with the corresponding primer pairs (Table S1). The amplified DNA was used as a template for the in vitro transcription, and the resulting RNA was used for the next round with the same SHMCK buffer system. The quantity of both p53R175H and WT p53 protein was decreased to 0.5 μg at the fourth and fifth round of selection for higher stringency.

Random DNA Library. The random DNA library was synthesized by TaKaRa Biotechnology, containing 25 randomized nucleotides flanked by two fixed primer-binding sequences for PCR and a T7 promoter at the 5′ end. The library contains ~10^{15} random sequences.

RIP Assay. We incubated 3 μg of p53 antibody (Cell Signaling Technology, cat. no. 2524S) with 50 μL of Dynabeads Protein G (Invitrogen) according to the manufacturer's recommendations, with modifications (58). Cells were transfected with either p53R175H-APT or scramble control in two 10-cm plates with Lipofectamine 2000 according to the manufacturer's protocols (Life Technologies). At 48 h posttransfection, cells were washed twice with PBS and fixed with 1% formaldehyde. RIPA buffer (50 mM Tris•Cl pH 8.0, 500 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS) was used for cell lysis, followed by sonication and centrifugation. Cell lysates were preserved and then incubated with p53 antibody-coupled beads for the immunoprecipitation procedures. After 2 h of incubation, beads were washed three times with high-salt RIPA buffer (50 mM Tris•Cl pH 8.0, 500 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS), and 100 μL of the samples were preserved for Western blot analysis. The rest was subjected to TRIzol RNA extraction followed by standard RT and real-time quantitative PCR (qPCR). To calculate the relative pull-down efficiency of scramble RNA versus the p53R175H-APT RNA, the percentage of each RNA pull-down was calculated against the corresponding input (pull-down percentage), and then the pull-down percentage of

p53R175H-APT RNA was normalized to the pull-down percentage of scrambled RNA. The oligos used are shown in Table S2.

Aptamer–Nanoparticle Conjugate Preparation. Nanoparticles loaded with RNA were prepared by a double emulsion–solvent evaporation technique. Briefly, an aqueous solution of RNA (10 μg) in 25 μL of RNase-free water was emulsified by sonication for 60 s over an ice bath in 0.5 mL of chloroform containing 1.0 mg of 1,2-Dioleoyl-3-triMethylaMMoniuM-propane and 25 mg of PEG5k-PLGA10k. This primary emulsion was further emulsified in 5 mL of H_2O by sonication (80 W for 1 min) over an ice bath to form a water-in-oil-in-water emulsion. The mixture was then added to a 50-mL round-bottom flask, and the solvent (chloroform and RNase-free H_2O) was concentrated under reduced pressure by a rotary evaporator to a volume of 1 mL (28).

All mice used in this study were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (59), and protocols were approved by the Institutional Animal Care and Use Committee at The University of Science and Technology of China (USTCACUC1401008 and USTCACUC1401010).

Detailed information describing cell lines, plasmids, transfection, animal procedures, and other methods used in this study is provided in *SI Materials and Methods*.

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