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Pitx2a Binds to Human Papillomavirus Type 18 E6 Protein and Inhibits E6-mediated P53 Degradation in HeLa Cells*

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Abstract

Binding of high risk human papillomavirus (HPV) E6 protein to E6-associated protein (E6AP), a cellular ubiquitin-protein ligase, enables E6AP to ubiquitinate p53, leading to p53 degradation in cervical cancer cells such as HeLa cells. Here we report that Pitx2a, a bicoid-type homeodomain transcription factor, can bind to HPV E6 protein and inhibit E6/E6AP-mediated p53 degradation. Deletion of the Pitx2a homeodomain abrogates its ability to bind to HPV E6 protein and to induce p53 accumulation in HeLa cells, suggesting that the homeodomain of Pitx2a is essential for inhibition of E6/E6AP-mediated p53 degradation. Recombinant Pitx2a can also block E6/E6AP-mediated p53 degradation *in vitro*, indicating that this function of Pitx2a is independent of its transcription activity. Pitx2a does not regulate Hdm2-mediated p53 degradation, because Pitx2a does not affect p53 protein levels in HPV-negative cells, such as HCT116, U2OS, and C33A cells. In addition, Pitx2a-induced p53 is transcriptionally active and maintains its specific DNA binding activity in HeLa cells. Taken together, these findings suggest that, by binding to E6, Pitx2a interferes with E6/E6AP-mediated p53 degradation, leading to the accumulation of functional p53 protein in HeLa cells.

Pitx2 is a bicoid-type homeodomain transcription factor, which was originally identified as a candidate gene for Rieger syndrome, an autosomal dominant genetic disease characterized by craniofacial dysmorphologies as well as defects in the heart, limb, and pituitary (1). Ablation of *Pitx2* in mice resulted in abnormal left-right development, such as lung isomerism, as well as defects in the heart, teeth, pituitary, and eyes (2–5). Pitx2 has also been suggested as an important regulator of GABAergic neuron differentiation (6,7), as well as a downstream target for the acute leukemia *ALL1* gene (8). Recently, Pitx2 was identified as a component of the Wnt signaling pathway, controlling cell proliferation in a tissue-specific manner via regulation of its downstream target genes, such as *cyclin D1*, *D2*, and *Myc* (9,10). Therefore, Pitx2 is likely to execute its multibiological functions by regulating cell proliferation and differentiation.

Infection by high risk human papilloma viruses (HPVs),² such as HPV types 16 and 18, is associated with >90% of cervical carcinomas, the second leading cause of cancer-related deaths among women worldwide. HeLa cells, derived from cervical carcinoma, express wild-type p53 but only at a very low level, because p53 is targeted for ubiquitination and degradation by

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²The abbreviations used are: HPV, human papillomavirus; GFP, green fluorescent protein; EGFP, enhanced GFP; E6AP, E6-associated protein; GST, glutathione *S*-transferase; RNAi, RNA interference; Luc, luciferase.

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HPV E6 protein. Each HPV E6 protein consists of ~150 amino acids and has a molecular mass of ~18 kDa. E6 is the initiator of p53 degradation in cervical cancer cells. Integration of HPVs into the genome and continuous expression of HPV E6 protein are required for the maintenance of the transformed phenotype of cell lines derived from cervical carcinoma. Binding of HPV E6 protein to E6-associated protein (E6AP), a cellular ubiquitin-protein ligase, enables E6AP to ubiquitinate p53, leading to p53 degradation. E6/E6AP-mediated p53 degradation was believed to be the major mechanism leading to cervical carcinoma associated with high risk HPVs (11–14). However, it has been shown recently that the PDZ domain of E6 is responsible for the development of cancer in transgenic mice (15), indicating that not only does E6-mediated p53 degradation, but also E6-PDZ partner interaction, play critical roles in the development of HPV E6-mediated cancer.

We have previously reported that expression of *Pitx2a* in HeLa cells resulted in the accumulation of p53 and p21, leading to cell cycle arrest at G₁/G₀ (16). Here we demonstrate that Pitx2a can bind to HPV type 18 E6 protein and inhibit p53 degradation *in vitro*. The homeodomain of Pitx2a is important for Pitx2a-induced p53 accumulation as well as the E6-Pitx2a interaction in HeLa cells. Pitx2a-induced p53 is transcriptionally active and maintains its specific DNA binding activity. These findings indicate that binding of Pitx2a to HPV type 18 E6 protein interferes with E6/E6AP-mediated p53 degradation, leading to the accumulation of functional p53 protein and the induction of cell cycle arrest in HeLa cells.

MATERIALS AND METHODS

Cell Culture and Transfection

The inducible HeLa cell line expressing GFP-tagged Pitx2a has been described previously (16). We will refer to this stable cell line as Pitx2a cells in this article. The Pitx2a cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in the presence of hygromycin B and geneticin (each 100 µg/ml) (Invitrogen). The Pitx2a cells express GFP-Pitx2a in the presence (but not in the absence) of doxycycline. The HeLa Tet-On cells (BD Biosciences) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in the presence of geneticin (100 µg/ml) (both from Invitrogen). The expression plasmids were transfected into HeLa Tet-On cells using a Lipofectamine 2000 kit (Invitrogen) according to the manufacturer's instructions.

Plasmids

GFP-*Pitx2a* plasmid has been described previously (16). *Pitx2a*ΔN-(39–271) and *Pitx2a*ΔHD-(99–271) fragments were generated by PCR using GFP-*Pitx2a* as a template. Both fragments were then inserted into pEGFP-C3 and pCMV-Tag3B (Myc-tagged vector) to generate pEGFP-*Pitx2a*ΔN, pEGFP-*Pitx2a*ΔHD, pCMV-Tag3B-*Pitx2a*ΔN, and pCMV-Tag3B-*Pitx2a*ΔHD, respectively. Hemagglutinin-tagged E6AP II plasmids were kindly provided by Dr. P. M. Howley (17). E6AP II full-length cDNA was also cloned into pEGFP-C3 (BD Biosciences) to generate GFP-tagged E6AP II. The DNA fragment encoding HPV type 18 E6 protein was cloned into pCS3+MT (provided Dr. Y. S. Kim, NHLBI, National Institutes of Health) and pEGFP-C3 (BD Biosciences) to generate Myc-tagged and GFP-tagged HPV 18 E6, respectively. pcDNA3-F:11E6 was kindly provided by Dr. Cheng-Ming Chiang (18).

p53 RNA interference (RNAi) expression plasmid was purchased from Imgenex Corporation (San Diego, CA). Mouse *Pitx2a* RNAi expression plasmid was constructed using the "PCR-SHAGging" strategy as described in katahdin.cshl.org:9331/RNAi/html/rnai.html (19,20). The DNA sequence for the mouse Pitx2a RNAi is 5'-GAGAGGACAGGGGATTGACGTTTCATGGAGGAAGCTTG

CTCCGTGGACGTCGATCCCTTGTCTCTC-3' (the underlined sequence will become the loop when the short hairpin is formed).

The human E6AP small interfering RNA oligo was purchased from Dharmacon (Chicago, IL). The sequence for its sense oligo is 5'-CUUUCUCA AUGCACUUGUAUU-3'. Transfection of E6AP small interfering RNA into U2OS cells was done using a Lipofectamine 2000 kit (Invitrogen) according to the manufacturer's instructions.

GST Fusion Proteins and *In Vitro* Translated Proteins

cDNA fragments encoding mouse *Pitx2a* was cloned into pGEX-5x-1 expression vector, and the GST-Pitx2a protein was expressed in BL21 cells. Baculoviruses expressing GST-E6AP and GST-p53 were purchased from Orbigen, Inc. (San Diego, CA). The GST fusion proteins were purified using glutathione beads (Sigma). Myc-E6 was translated *in vitro* using a T_NT kit (Promega, Madison, WI), and the *in vitro* translated proteins were confirmed by immunoblot analysis using anti-Myc (9E10) antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

In Vitro Binding Assay

GST-Pitx2a immobilized on glutathione agarose beads was incubated with 5 μ l of *in vitro* translated HPV type 18 E6 rabbit reticulocyte lysate in binding buffer (0.15 M NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 1.0 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) at 4 °C overnight. The glutathione-agarose beads were then washed five times with binding buffer. The washed glutathione-agarose beads were boiled in SDS loading buffer for 10 min, and proteins were resolved on 10% SDS-polyacrylamide gels, followed by immunoblot to detect Myc-tagged HPV E6 protein using Myc (9E10) antibody (Santa Cruz).

Co-immunoprecipitation and Immunoblot Analysis

The transfected cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, leupeptin, and pepstatin) on ice for 10 min. The cell lysates were clarified at 11,750 \times g at 4 °C for 12 min and were incubated with protein G-agarose at 4 °C for 30 min, followed by collection of the supernatant. Antibodies were added to the supernatant and incubated at 4 °C for 2 h followed by the addition of protein G-agarose. After another 12 h of incubation at 4 °C, the beads were washed in radioimmune precipitation assay buffer four times, boiled in SDS loading buffer, and proteins were resolved on 10% SDS-PAGE. Proteins were then transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA), blocked with 5% nonfat milk for 1 h at 23 °C, and incubated with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology) for 1 h at 23 °C. The blots were visualized by SuperSignal West Pico Luminol/Enhancer solution (Pierce).

The following primary antibodies were used: polyclonal rabbit antibody to GFP (1:1000, Santa Cruz Biotechnology); polyclonal rabbit antibody to E6AP (1:1000, Upstate Biotechnology); monoclonal antibodies to p53 (DO-1, 1:1000, Santa Cruz Biotechnology), and Myc (9E10, 1:1000, Santa Cruz Biotechnology); polyclonal goat antibody to p53 (1:1000, Santa Cruz Biotechnology); and polyclonal rabbit antibody to Pitx2 (21).

p53 *In Vitro* Degradation Assay

p53 degradation assay was performed as described previously (22). Briefly, *in vitro* translated Myc-E6 and GST-p53 were incubated at 23 °C for 0 and 90 min with or without GST-Pitx2a. 10 μ g of ubiquitin was also included in the degradation assay mix. The incubation was done in 25 mM Tris (pH 7.5), 100 mM NaCl, and 3 mM dithiothreitol. The mixed proteins were resolved

on a 10% SDS-polyacrylamide gel, followed by immunoblot analysis to assess the extent of p53 degradation.

Luciferase Assay

The Pitx2a cells were transfected with reporter plasmids p53-TA-Luc or ppTA-Luc (BD Biosciences), together with the β -galactosidase expression plasmid. 24 h after the transfection, the transfected cells were split and cultured for another 24 h in the absence or presence of doxocycline. The cells were lysed in reporter lysis buffer, followed by centrifugation to remove the cell debris. The luciferase activity was assessed using a TR717 microplate luminometer (Tropix). The light units were normalized by β -galactosidase activity. The p53 response element in p53-TA-Luc is as follows: 5'-ACGTTTGCCTTGCCTGGACTTGCCTGGCCTTGCCTTGGACATGCCCGGGCTGTC-3'.

Assay of p53 Binding to Its Consensus Site

The Pitx2a cells were cultured in the presence or absence of doxycycline for 2 days. The cells were lysed in binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 μ g/ml poly(dI-dC)) and clarified by centrifugation to remove cell debris. The clarified supernatant was incubated with agarose-conjugated double-stranded DNA oligos with wild-type or mutant p53 consensus binding sites at 4 °C overnight. The agarose beads were washed with binding buffer five times and boiled in SDS loading buffer, and the eluted proteins were resolved on 10% SDS-polyacrylamide gels, followed by immunoblot analysis using an antibody specific for p53 (Santa Cruz Biotechnology).

RESULTS

We previously generated a stable HeLa cell line inducibly expressing GFP-tagged Pitx2a. The cell line expresses Pitx2a in the presence (but not in the absence) of doxycycline (16). We will refer to this stable cell line as Pitx2a cells in this article. As shown in Fig. 1A, expression of Pitx2a in HeLa cells resulted in the accumulation of p53. In addition, transient expression of GFP-Pitx2a (Fig. 1B, *a-c*) (but not GFP-vector (Fig. 1B, *d-f*)) in HeLa cells induced p53 accumulation in the nucleus.

Because Pitx2a is a transcription factor, we also tested whether Pitx2a up-regulated p53 expression at the transcriptional level. Total RNA was isolated from the Pitx2a cells cultured in the presence or absence of doxycycline, and reverse transcription-PCR was performed to examine the p53 mRNA level. The result showed that the p53 mRNA level was not significantly affected by Pitx2a, suggesting that Pitx2a does not regulate p53 expression at the transcriptional level (Fig. 1C). This result is consistent with the notion that the p53 level is mainly regulated by ubiquitination and the proteasome-dependent proteolysis pathway (23). In addition, the mRNA level for one of the p53 downstream target genes, *p21*, was up-regulated in the presence of doxycycline (Fig. 1C).

High risk HPV E6 protein and ubiquitin-protein ligase E6AP play an important role in the regulation of p53 degradation in HPV-positive cells such as HeLa cells. In the presence of HPV E6 protein, E6AP acts as a p53 ubiquitin-protein ligase to target p53 protein for degradation (24). It is therefore conceivable that down-regulation of E6AP or E6 would result in p53 accumulation in HeLa cells. Pitx2a is a transcription factor. We therefore asked whether Pitx2a could down-regulate the expression of HPV E6 and E6AP in HeLa cells. Reverse transcription-PCR indicated that E6 and E6AP mRNA levels in HeLa cells were not significantly affected by Pitx2a (Fig. 1C). Northern blot also revealed that the E6AP mRNA level did not significantly change (Fig. 1D). Therefore, expression of Pitx2a in HeLa cells did not affect the mRNA levels

of E6AP and HPV E6, suggesting that Pitx2a-induced p53 accumulation was not because of the repression of E6 and E6AP transcription.

HPV E6 can bind to E6AP, forming an E6-E6AP complex, which in turn binds to and ubiquitinates p53 (24). Recently, it was also shown that E6 protein can bind to p53 in an E6AP-independent fashion to regulate p53-dependent gene activation. Interestingly, this E6-p53 interaction was not involved in p53 degradation (18). Expression of Pitx2a in HeLa cells did not affect the mRNA levels of HPV E6 and E6AP (Fig. 1, C and D). We therefore hypothesized that Pitx2a could bind to HPV 18 E6 in HeLa cells, interfering with E6/E6AP-mediated p53 ubiquitination and degradation. As indicated in Fig. 2A, immobilized GST-Pitx2a protein (but not GST alone) could pull down the *in vitro* translated Myc-tagged HPV E6 protein (compare lane 2 with lane 3). This *in vitro* E6-Pitx2a interaction was also confirmed by a co-immunoprecipitation experiment. As shown in Fig. 2B, Myc-E6 could pull down GFP-Pitx2a in transfected HeLa cells (compare lane 2 with lane 3). Additionally, the HPV E6-Pitx2a interaction was further supported by the similar intra-cellular localization of HPV E6 and Pitx2a proteins. GFP-tagged Pitx2a was only expressed in the nucleus, and GFP-tagged HPV 18E6 also localized predominantly to the nucleus of this HeLa cell line (data not shown), consistent with previous reports (21,25,26). Another possibility is that Pitx2a can bind to E6AP and directly inhibit the activity of E6AP ubiquitin-protein ligase toward p53. To test this possibility, we transfected GFP-E6AP into Saos-2 cells, and GST pull-down assay was performed to test whether GST-Pitx2a interacted with GFP-E6AP from transfected Saos-2 cells. The Saos-2 cell line was used because it does not express E6 and p53 proteins. We were not able to detect the interactions between GST-Pitx2a and GFP-tagged full-length E6AP in Saos-2 cells (Fig. 2C), suggesting that Pitx2a did not interact with full-length E6AP protein.

The Pitx2a homeodomain contains both DNA binding and transcriptional activation domains, which can bind to bicoid DNA binding sites and regulate the transcription of its target genes. We therefore asked whether the homeodomain is required for Pitx2a-induced p53 accumulation. We deleted amino acids 1–38 and 1–98 from the amino terminus of Pitx2a protein, generating truncated Pitx2a polypeptides, Pitx2a Δ N (containing amino acids 39–271, with the homeodomain) and Pitx2a Δ HHD (containing amino acids 99–271, without the homeodomain) (Fig. 3A). The expression plasmids encoding Pitx2a, Pitx2a Δ N, and Pitx2a Δ HHD polypeptides were transfected into HeLa cells, and the p53 protein level was examined 24 h following the transfection. As shown in Fig. 3B, deletion of 38 amino acids from the amino terminus of Pitx2a, *i.e.* Pitx2a Δ N, did not affect the ability of Pitx2a to induce p53 accumulation in HeLa cells. However, deletion of 98 amino acids (including the homeodomain) from the amino terminus of Pitx2a, *i.e.* Pitx2a Δ HHD, almost completely abrogated the ability of Pitx2a to induce p53 accumulation, suggesting that the homeodomain of Pitx2a is essential for Pitx2a-induced p53 accumulation.

We then asked whether the homeodomain of Pitx2a is essential for its binding to HPV E6. Myc-tagged full-length *Pitx2a*, *Pitx2a Δ N*, and *Pitx2a Δ HHD* were cotransfected into HeLa cells with GFP-tagged HPV E6, respectively. As shown in Fig. 3C, a co-immunoprecipitation experiment indicated that Myc-tagged full-length Pitx2a and Pitx2a Δ N (but not Pitx2a Δ HHD) could bind to GFP-tagged HPV E6. Therefore, deletion of the Pitx2a homeodomain abrogated its ability to bind to HPV E6 and to induce p53 accumulation, suggesting that binding to HPV E6 is likely the cause for Pitx2a to induce p53 accumulation in HeLa cells.

HPV-positive cells, such as HeLa cells, use E6AP as an ubiquitin-protein ligase to ubiquitinate p53, resulting in a low level of p53 protein. However, HPV-negative cells, such as HCT116, U2OS, and C33A, use ubiquitin-protein ligase Hdm2 to ubiquitinate p53 to maintain an appropriate p53 level. Hdm2-mediated p53 degradation is not operating in HPV-positive cervical cancer cells (27). We therefore asked whether Pitx2a could regulate Hdm2-mediated

p53 degradation in HPV-negative cells. GFP-tagged full-length *Pitx2a*, *Pitx2a* Δ N, and *Pitx2a* Δ HD were transfected into HCT116, U2OS, and C33A cells. p53 protein levels were examined 24 h after the transfection. Both full-length and truncated *Pitx2a* did not affect p53 protein levels in HCT116 (Fig. 4A), U2OS, and C33A (data not shown) cells, indicating that *Pitx2a* did not regulate Hdm2-mediated p53 degradation. In contrast, nutlin-3, an inhibitor of Hdm2, could significantly induce p53 accumulation in U2OS cells (Fig. 4B).

To further elucidate the relationships between *Pitx2a*, E6AP, and E6 in the regulation of p53 degradation, we examined whether *Pitx2a* and its truncated form, *Pitx2a* Δ HD, could inhibit p53 degradation induced by E6 protein in U2OS cells. Consistent with previous reports (28), expression of HPV type 18 (but not type 11) E6 proteins induced p53 degradation in U2OS cells (Fig. 4C, lanes 2 and 3). As expected, E6-mediated p53 degradation was abrogated by reduction of E6AP using RNAi (Fig. 4C, lane 5), confirming E6-mediated p53 degradation was dependent on E6AP. In addition, expression of *Pitx2a* (but not *Pitx2a* Δ HD) inhibited p53 degradation mediated by E6/E6AP (Fig. 4C, compare lane 8 with lane 9). These experiments further confirmed that *Pitx2a* could inhibit E6/E6AP-mediated p53 degradation.

Pitx2a is a homeodomain transcription factor, and its homeodomain is essential for p53 accumulation induced by *Pitx2a*. We therefore asked whether the transcription activity of *Pitx2a* is required for p53 accumulation in HeLa cells. To answer this question, we performed an *in vitro* p53 degradation assay. We incubated GST-p53 and *in vitro* translated HPV 18 E6 in the presence or absence of *Pitx2a*. It is not necessary to add E6AP, because the rabbit reticulocyte lysate provides functional E6AP (29). As shown in Fig. 5, in the absence of *Pitx2a*, p53 protein levels significantly decreased after 90 min of incubation at 23 °C (compare lane 1 with lane 3). In contrast, in the presence of *Pitx2a*, p53 protein levels did not significantly change (Fig. 5, compare lane 2 with lane 4), suggesting that *Pitx2a* was able to directly inhibit E6/E6AP-mediated p53 degradation. This result also further confirmed that binding of *Pitx2a* to HPV E6 and the subsequent interference with ubiquitination and degradation of p53 by the E6-E6AP complex is the cause of p53 accumulation induced by *Pitx2a* in HeLa cells. Importantly, there were no transcriptional and translational events in this assay system, suggesting that inhibition of p53 degradation by *Pitx2a* was independent of *Pitx2a* transcriptional activity.

The tumor suppressor function of p53 depends on its ability to activate the transcription of its downstream target genes, such as *p21/cip1*, *GADD45*, *NOXA*, *Bax*, and *14-3-3* (30). It has been demonstrated that p53-mediated growth suppression is critically dependent on the transcriptional activation of *p21/cip1* by p53 (23). We therefore examined whether p21 was up-regulated at the transcription level by *Pitx2a*. Reverse transcription-PCR revealed that the p21 mRNA level significantly increased when the *Pitx2a* cells were cultured in the presence of doxycycline (Fig. 1C), indicating that *Pitx2a*-induced p53 was most likely functional. To further confirm this result, we also examined the transcriptional activity of the accumulated p53 by using a p53-responsive luciferase reporter plasmid, p53-TA-Luc, in which a p53-responsive element was fused to the luciferase reporter gene. Functional p53 protein was expected to activate the transcription of the luciferase reporter gene from p53-TA-Luc. As shown in Fig. 6A, expression of *Pitx2a* (in the presence of doxycycline) significantly increased the luciferase activity from the p53-responsive luciferase reporter plasmid, p53-TA-Luc, but did not affect the luciferase activity from a control plasmid without the p53-responsive element pTA-Luc. This result suggested that *Pitx2a*-induced p53 is functional.

These results were further confirmed by RNAi experiments. Both p53 and *Pitx2a* RNAi significantly reduced activation of the p53-responsive promoter by *Pitx2a* in the presence of doxycycline (Fig. 6A). To confirm that p53 and *Pitx2a* RNAi could down-regulate the expression of p53 and *Pitx2a*, we transfected the *Pitx2a* cells with the respective RNAi

plasmids, followed by immunoblot analysis to detect the protein levels of p53 and Pitx2a. As shown in Fig. 6, B and C, p53 and *Pitx2a* RNAi significantly knocked down p53 and Pitx2a proteins, respectively.

We then asked whether the accumulated p53 could bind to its consensus DNA binding site. p53 is a transcription factor containing a DNA binding domain, which can bind to its consensus binding site on the promoter region and regulate the transcription of its downstream target genes. We used agarose-conjugated double-stranded DNA oligos containing the p53 consensus binding site to purify p53 from Pitx2a cells cultured in the presence or absence of doxycycline. If the accumulated p53 can bind to its consensus site, the agarose-conjugated p53 oligos should be able to enrich p53 from the cell lysates. As shown in Fig. 7, p53 protein was enriched by the agarose-conjugated double-stranded DNA oligo containing the wild-type p53 consensus binding site. In contrast, the agarose-conjugated oligo containing a mutated p53 binding site was not able to enrich p53 protein induced by Pitx2a (Fig. 7, compare *lane 4* with *lane 6*). This result suggested that Pitx2a-induced p53 maintained its DNA binding activity. This is also consistent with our previous results that indicated that the expression of Pitx2a in HeLa cells induced the accumulation of p21, a p53 downstream target gene, as well as cell cycle arrest at G₁/G₀ (16).

DISCUSSION

We have demonstrated in this article that Pitx2a, a homeodomain transcription factor, can bind to high risk HPV type 18 E6 protein and subsequently interfere with E6/E6AP-mediated p53 degradation, leading to p53 accumulation in HeLa cells. The homeodomain of Pitx2a is essential for both the Pitx2a-HPV E6 interaction and the induction of p53 accumulation in HeLa cells. The accumulation of p53 induced by Pitx2a is independent of Pitx2a transcription activity. In addition, Pitx2a-induced p53 is functional and maintains its DNA binding activity.

HPV E6-E6AP complex formation is required for p53 degradation in cervical cancer cells (11–14). Our results showed that Pitx2a could bind to the E6 protein and interfere with E6/E6AP-mediated p53 degradation in HeLa cells. Deletion of the Pitx2 homeodomain disrupted both the Pitx2a-E6 interaction and the induction of p53 accumulation in HeLa cells. We therefore speculate that there was a link between Pitx2a-E6 interaction and the inhibition of E6/E6AP-mediated p53 degradation, *i.e.* the Pitx2a-E6 interaction interferes with E6/E6AP-mediated p53 degradation, leading to p53 accumulation in HeLa cells. We do not know at present how the Pitx2a-E6 interaction leads to the inhibition of E6/E6AP-mediated p53 degradation. However, two possibilities must be considered. First, binding of Pitx2a to E6 protein can block the further interactions between HPV E6 and E6AP. Therefore, E6AP, without binding to HPV E6, will not be able to degrade p53 protein. Second, binding of Pitx2a to E6 protein can cause a conformational change of the E6-E6AP complex, which subsequently loses its ability to degrade p53. We favor the first possibility, because we were not able to detect the formation of the trimolecular complex E6-E6AP-Pitx2a (data not shown).

In addition to E6AP, many interacting partners for high risk HPV E6 protein have been identified, such as p300/CBP (31,32), Bak (33), E6BP/ERC55 (34), E6TP1 (35), interferon regulatory factor-3 (36), Paxillin (37,38), hDlg (39,40), MUPP-1 (41), and hScrib (40). Binding of HPV E6 protein to these interacting partners results in different biological effects, such as inhibition of apoptosis (Bak), disruption of the actin cytoskeleton (Paxillin), and inhibition of β -interferon transcription (interferon regulatory factor-3). Only a low level of HPV E6 expression was present in cervical cancer cells, but this amount was sufficient to maintain the transformation phenotype. Do these E6-interacting partners compete with each other for the limited amount of HPV E6 protein in cervical cancer cells? For instance, does binding of p300/CBP to HPV E6 interfere with E6-E6AP-p53 complex formation? This question remains to be

answered. At least, however, it has not been previously reported that these E6 interacting partners had an effect on E6/E6AP-mediated p53 degradation in HPV E6-positive cells. We have demonstrated in this article that, not only can Pitx2a bind to HPV E6 protein, but it also can inhibit E6/E6AP-mediated p53 degradation.

High risk HPVs play a critical role in the development of cervical carcinoma. HPV E6 protein is sufficient to induce immortalization of a number of human normal epithelial cell types. In addition, continuous expression of HPV E6 protein is required for the maintenance of the transformed phenotype of cervical cancer cells. Degradation of p53 by E6/E6AP in HPV-positive cells is the underlying mechanism leading to cervical carcinoma associated with high risk HPVs (11–14). Therefore, HPV E6 is an ideal target for molecular anti-cancer strategies to cervical carcinoma. Many approaches, such as antisense oligos and ribozymes directed against HPV E6 mRNA, have been examined to block the expression of HPV E6 protein in HPV-positive cervical cancer cells (42–47). All of these approaches have been shown to inhibit the expression of HPV E6 mRNA and protein, leading to cell growth suppression and a reduction of tumorigenicity *in vivo*. In addition, blocking E6 activity by using a therapeutic peptide was shown to induce apoptosis of HPV-positive cancer cells and to inhibit E6-induced degradation of its cellular substrates, such as p53, Dlg, and the membrane-associated guanylate kinase inverted family of proteins (48,49).

Pitx2a, a bicoid-type homeodomain transcription factor, can bind to HPV E6 protein and inhibit p53 degradation in HPV-positive HeLa cells, resulting in p53 accumulation and cell cycle arrest at G₁/G₀ (16). Pitx2 was also originally isolated as a downstream target for the human acute leukemia *ALL1* gene. Loss of function of the *ALL1* gene has been implicated in the development of human acute leukemia associated with abnormalities at 11q23 (50). Pitx2 is expressed in normal human bone marrow and leukemic cell lines with a normal *ALL1* allele but is not expressed in the leukemic cell lines in which *ALL1* is rearranged (8). Additionally, both Pitx2 mRNA and protein are up-regulated by treatment with lithium chloride and expression of constitutively active β -catenin, suggesting that the Pitx2 level is regulated by the Wnt signaling pathway (9). Although the physiological roles for the Pitx2a-E6 interaction remain to be determined, further dissection of the E6-binding domains on the Pitx2a molecule could help in designing strategies for the molecular therapy of cervical carcinoma.

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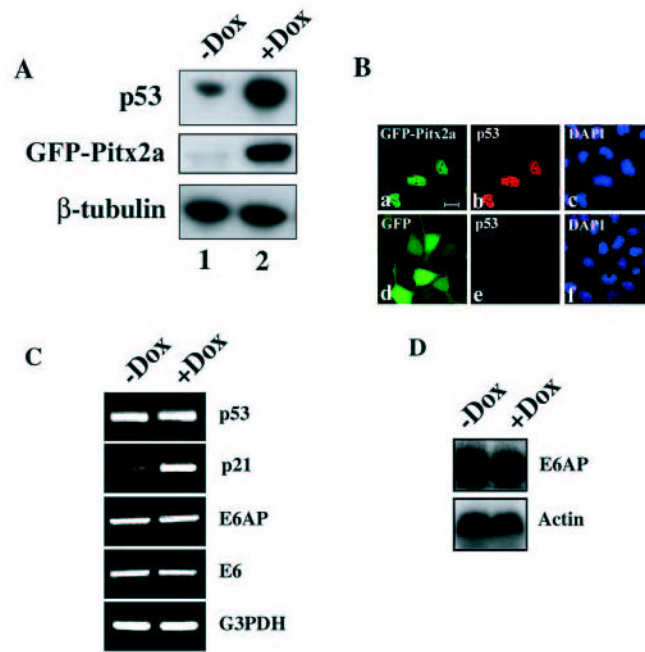


FIGURE 1. Effect of Pitx2a on the expression levels of p53, E6AP, E6, and p21 in HeLa cells
A, inducible expression of GFP-Pitx2a in HeLa cells induces p53 accumulation. Immunoblot analysis for the protein levels of p53, GFP-Pitx2a, and β -tubulin using specific antibodies. Note that the addition of doxycycline (+Dox) leads to the expression of GFP-Pitx2a and the accumulation of p53 protein. **B**, transient expression of GFP-Pitx2a (*a–c*), but not GFP-vector (*d–f*), in HeLa cells induces p53 accumulation in the nucleus. Scale bar, 20 μ m. **C**, reverse transcription-PCR analysis of p53, p21, E6AP, and E6 mRNA expression in the presence (+Dox) or absence (–Dox) of doxycycline. **D**, Northern-blot analysis of E6AP mRNA expression in the presence or absence of doxycycline.

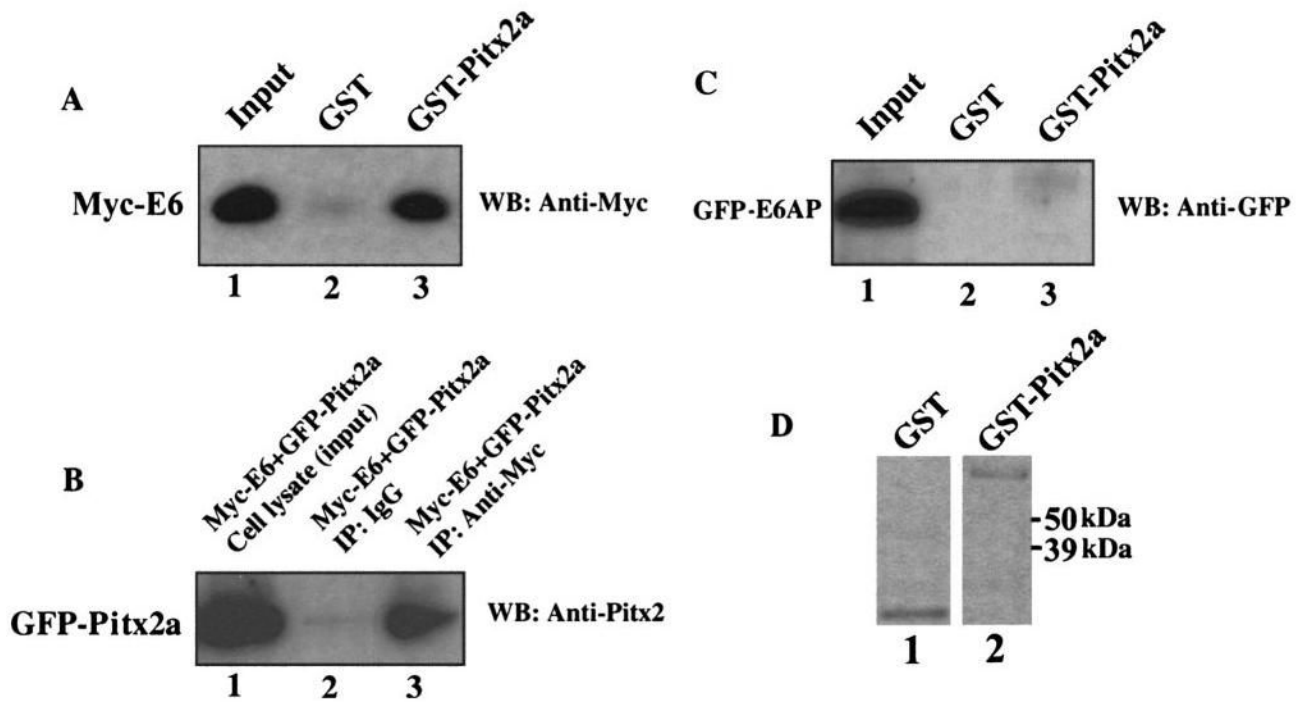


FIGURE 2. Pitx2a can binds to HPV and E6 but not E6AP

A, immobilized GST-Pitx2a can pull down *in vitro* translated Myc-tagged E6 protein. *B*, plasmid encoding GFP-Pitx2a was co-transfected with plasmid encoding Myc-E6. Co-immunoprecipitation (*IP*) was done using anti-Myc antibody, followed by immunoblot (*WB*) analysis using antibody specific for Pitx2. *C*, immobilized GST-Pitx2a cannot pull down GFP-E6AP protein from transfected Saos-2 cell lysate. *D*, Coomassie Blue staining gel of GST and GST-Pitx2a proteins used in *A* and *C*.

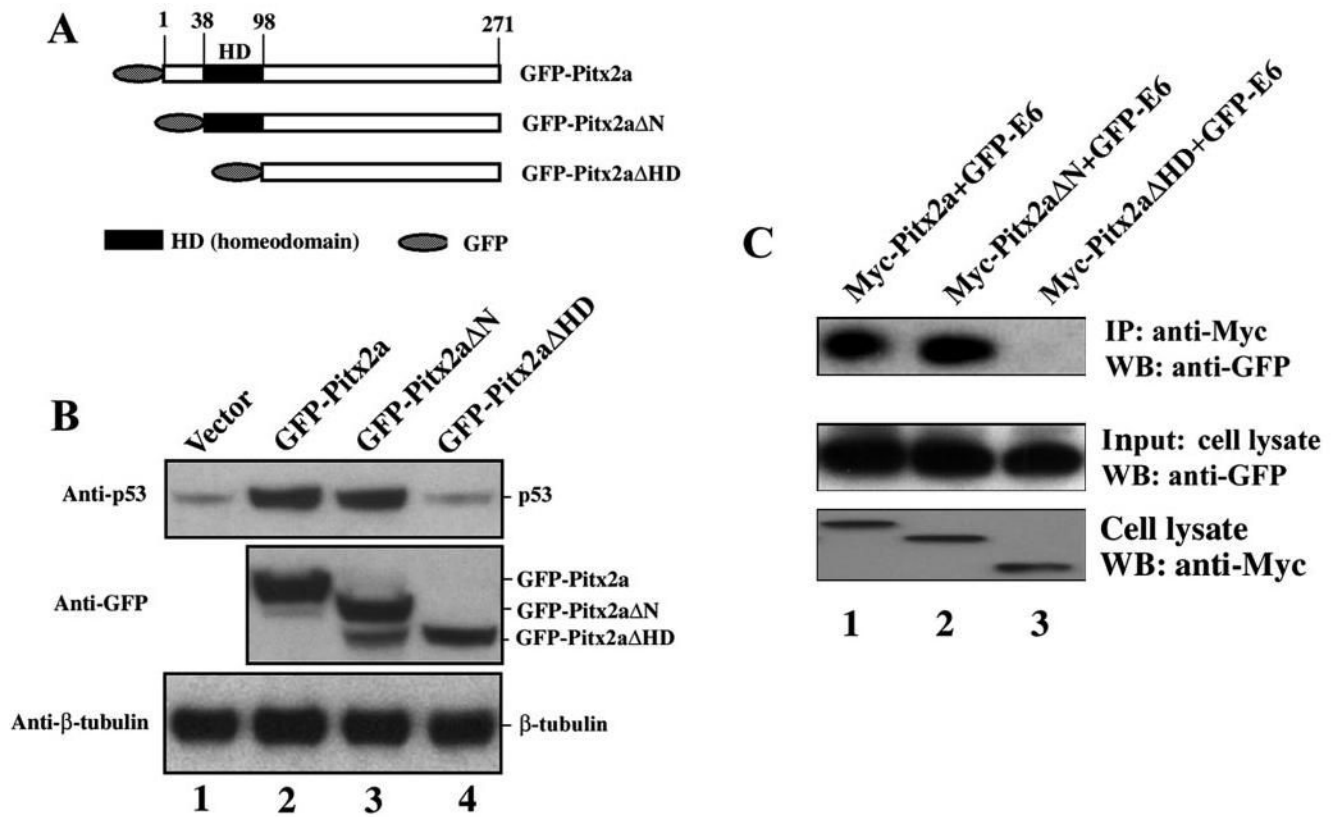


FIGURE 3. The homeodomain of Pitx2a is essential for p53 accumulation

A, schematic diagrams for GFP-tagged full-length Pitx2a (*GFP-Pitx2a*) and truncated Pitx2a polypeptides (*GFP-Pitx2aΔN* and *Pitx2aΔHD*). The number represents the amino acids in Pitx2a. *B*, HeLa cells were transfected with vector alone (*lane 1*) and plasmids encoding GFP-Pitx2a (*lane 2*), GFP-Pitx2aΔN (*lane 3*), and GFP-Pitx2aΔHD (*lane 4*). Immunoblot analysis of the transfected cell lysates was conducted to assess the p53 protein levels. β-tubulin was used as a loading control. *C*, plasmids encoding Myc-Pitx2a (*lane 1*), Myc-Pitx2aΔN (*lane 2*), and Myc-Pitx2aΔHD (*lane 3*) were co-transfected with plasmid encoding GFP-E6, respectively. Co-immunoprecipitation was done using anti-Myc antibody, followed by immunoblot analysis using antibody specific for GFP.

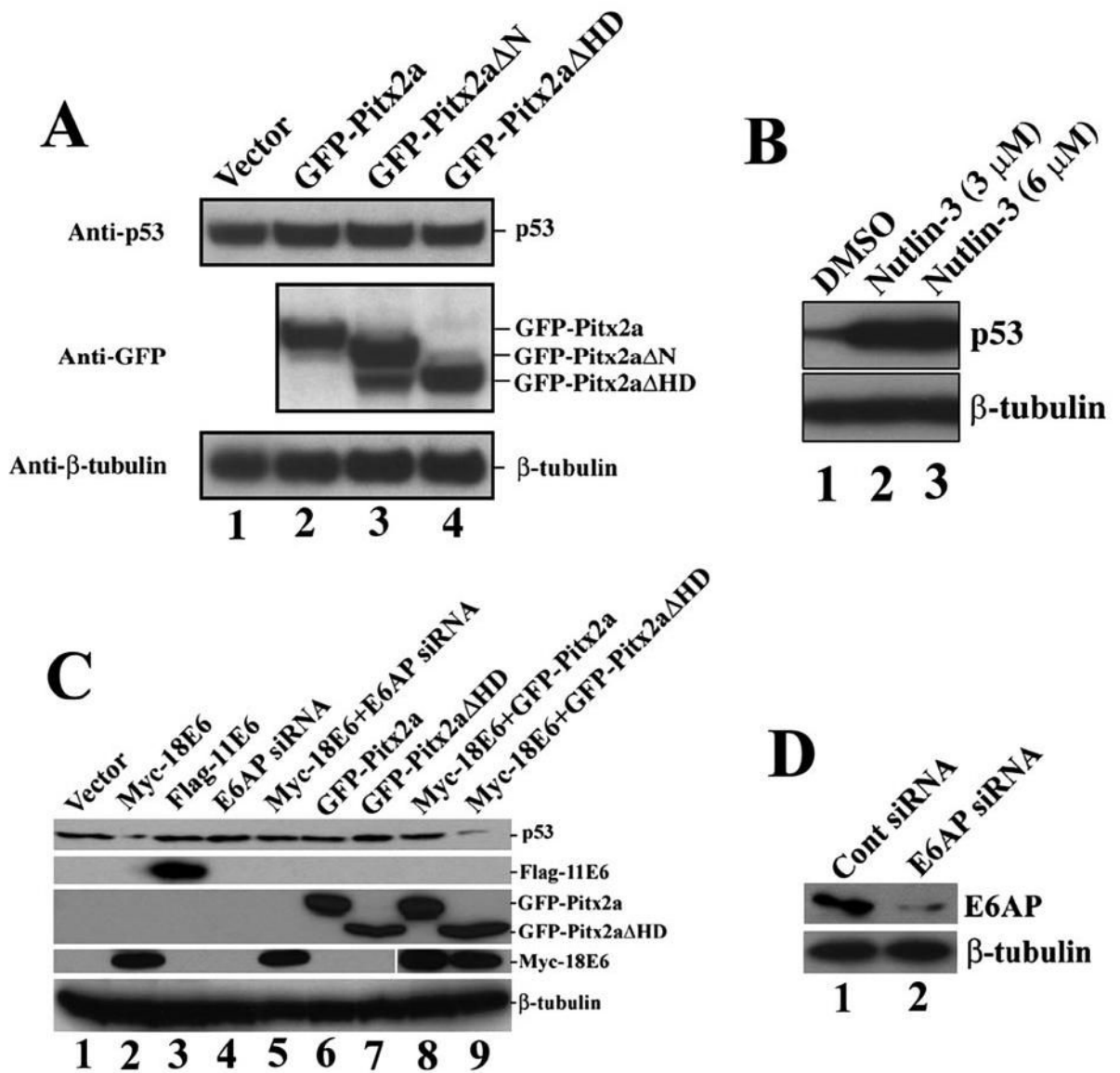


FIGURE 4. Effect of Pitx2a on Hdm2- and E6-mediated p53 degradation

A, Pitx2a did not induce p53 accumulation in HCT116 cells. Vector alone (lane 1) and plasmids encoding GFP-Pitx2a (lane 2), GFP-Pitx2aΔN (lane 3), and GFP-Pitx2aΔHD (lane 4) were transfected into HCT116 cells. Immunoblot analysis was done to assess the p53 protein levels by using an antibody specific for p53. GFP-Pitx2a, GFP-Pitx2aΔN, and GFP-Pitx2aΔHD were detected using an antibody specific for GFP. β-tubulin was detected using antibody specific for β-tubulin and was a control to assure equal loading of all lanes. **B**, Nutlin-3 induced p53 accumulation in U2OS cells. U2OS cells were cultured in the presence of Me₂SO (DMSO, lane 1) or nutlin-3 (lanes 2 and 3) for 24 h. The p53 protein level was detected using an antibody specific for p53. **C**, Pitx2a (but not Pitx2aΔHD) inhibited E6/E6AP-mediated p53 degradation. The indicated plasmids were transfected into U2OS cells. 48 h after transfection, p53 protein level was determined using a specific antibody for p53. Note that expression 18 E6 induced p53 degradation (lane 2), and this p53 degradation was inhibited by E6AP RNAi (lane 5) and Pitx2a (lane 8) but not Pitx2aΔHD (lane 9). The FLAG-11E6, GFP-Pitx2a/Pitx2aΔHD, Myc-18E6, and β-tubulin were detected using antibodies specific for FLAG, GFP, Myc, and

β -tubulin, respectively. *D*, E6AP RNAi reduced E6AP protein in U2OS cells. U2OS cells were transfected with E6AP small interfering RNA. 48 h after transfection, the E6AP protein level was determined using an antibody specific for E6AP.

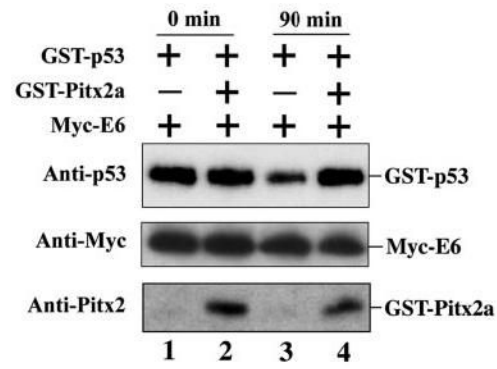


FIGURE 5. Effect of Pitx2a on p53 degradation *in vitro*

GST-p53 and *in vitro* translated Myc-E6 were incubated at 23 °C in the presence (*lanes 2 and 4*) or absence (*lanes 1 and 3*) of GST-Pitx2a for 0 or 90 min. Immunoblot analysis was done to assess the p53 protein level. Note that rabbit reticulocyte lysate contains active E6AP (29).

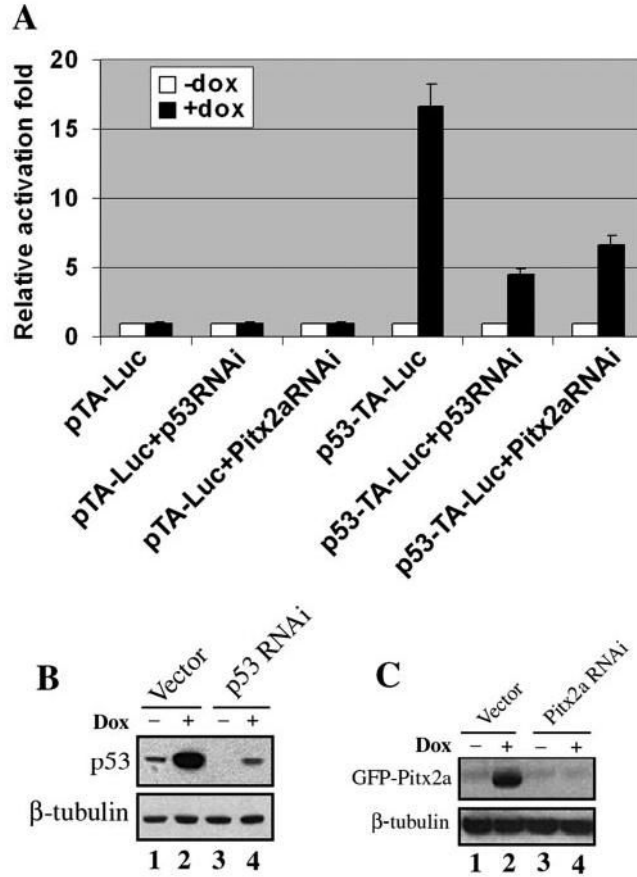


FIGURE 6. Pitx2a-induced p53 is functional

A, pTA-Luc alone, pTA-Luc plus p53 RNAi or *Pitx2a* RNAi, p53-TA-Luc alone, and p53-TA-Luc plus p53 RNAi or Pitx2a RNAi, were transfected into the Pitx2a cells, respectively. 24 h after the transfection, the cells were split and cultured in the presence or absence of doxycycline for another 24 h. The luciferase activity was measured according to the manufacturer's instructions. *pTA-Luc*, control luciferase reporter plasmid; *p53-TA-Luc*, p53-responsive luciferase reporter plasmid. **B**, vector alone (*lanes 1* and *2*) and plasmid expressing p53 RNAi were transfected into Pitx2a cells. 24 h after the transfection, the cells were split and cultured in the presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of doxycycline for another 24 h. Immunoblot analysis was done to assess the p53 protein level. **C**, vector alone (*lanes 1* and *2*) and plasmid expressing *Pitx2a* RNAi were transfected into Pitx2a cells. 24 h after the transfection, the cells were split and cultured in the presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of doxycycline for another 24 h. Immunoblot analysis was done to assess the GFP-Pitx2a protein level. β -tubulin was used as a loading control.

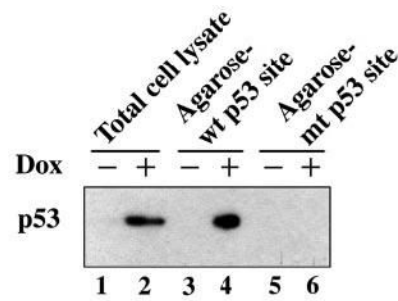


FIGURE 7. Pitx2a-induced p53 maintains its DNA binding activity

The addition of doxycycline (*Dox*) induced p53 accumulation (compare *lane 1* with *lane 2*) in the Pitx2a cells. The accumulated p53 was enriched by an agarose-conjugated double-stranded DNA oligo containing the p53 binding site (*lane 4*) but was not enriched by an agarose-conjugated double-stranded DNA oligo containing a mutated (*mt*) p53 binding site (compare *lane 4* with *lane 6*). *wt*, wild type.