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ARID1A, a factor that promotes formation of SWI/SNF-mediated chromatin remodeling, is a tumor suppressor in gynecologic cancers

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Abstract

ARID1A (BAF250A) promotes the formation of SWI/SNF chromatin remodeling complexes containing BRG1 or BRM. ARID1A has emerged as a candidate tumor suppressor based on its frequent mutations in ovarian clear cell and endometrioid cancers and in uterine endometrioid carcinomas. Here we report that restoring wild-type ARID1A expression in ovarian cancer cells that harbor ARID1A mutations is sufficient to suppress cell proliferation and tumor growth in mice, whereas RNAi-mediated silencing of ARID1A in non-transformed epithelial cells is sufficient to enhance cellular proliferation and tumorigenicity. Gene expression analysis identified several downstream targets of ARID1A including CDKN1A and SMAD3, which are well known p53 target genes. In support of the likelihood that p53 mediates the effects of ARID1A on these genes, we demonstrated that p53 was required and sufficient for their regulation by ARID1A. Further, we showed that CDKN1A (encoding p21) acted in part to mediate growth suppression by ARID1A. Lastly, we obtained evidence that the ARID1A/BRG1 complex interacts directly with p53 and that mutations in the ARID1A and TP53 genes were mutually exclusive in tumor specimens. Our results provide functional evidence in support of the hypothesis that ARID1A is a bona fide tumor suppressor that collaborates with p53 to regulate CDKN1A and SMAD3 transcription and tumor growth in gynecological cancers.

Introduction

It has been well established that chromatin remodeling is critical for essentially all aspects of nuclear activities including transcription, DNA replication and DNA damage repair (1–3) and molecular genetic changes in chromatin remodeling genes have emerged as a new mechanism in cancer pathogenesis. Amplification of *Rsf-1*, a gene participating in ISWI chromatin remodeling, has been demonstrated to promote chromosomal instability, propel tumor progression, and contribute to disease aggressiveness in ovarian and oral cancer (4–8). As well, somatic inactivating mutations have been detected in several SWI/SNF chromatin remodeling genes including *PBRM1* (*BAF180*) (9) in renal cell carcinoma, *BRG1* (*SMARCA4*) in lung carcinoma (10, 11), and *ARID1A* (*BAF250a*) in endometrium-related carcinomas including uterine endometrioid carcinoma, ovarian clear cell carcinoma, and ovarian endometrioid carcinoma (12–14). In previous studies (12–14), we have found that 46%–57% of ovarian clear cell carcinomas, 40% of uterine endometrioid carcinomas, and

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30% of ovarian endometrioid carcinomas harbor somatic sequence mutations in *ARID1A*. *ARID1A*, a homolog of yeast *SWI1*, encodes a large nuclear protein, p270 (also known as BAF250a), which participates in forming a SWI/SNF chromatin remodeling complex (15). Coordinate activity of the proteins of the SWI/SNF complex is responsible for altering chromatin structure which is required to facilitate several cellular functions including transcription, DNA synthesis and DNA damage repair (16–21). ARID1A binds to DNA non-selectively *In vitro* and recruits other components to the complex, a process that may confer specificity of the SWI/SNF (22, 23). The core protein and the ATPases, BRG1 or BRM, are responsible for moving or dispersing nucleosomes surrounding specific chromosomal regions such as transcription initiation sites (17, 24). It has been proposed that SWI/SNF gene members play an important role in embryonic development, tissue regeneration, cell senescence, apoptosis and oncogenesis.

Because the majority of somatic mutations involving *ARID1A* in human cancers are insertions/deletions causing frameshift or nonsense mutations, *ARID1A* was thought to behave as a tumor suppressor gene, but direct evidence of its tumor suppressor activity has not been demonstrated. In the present study, we examined the effects of ARID1A expression or loss of expression on carcinoma cells and non-transformed cells, respectively. Additionally, we identified *ARID1A*-regulated genes as a primary step toward unveiling the mechanisms by which ARID1A inhibits tumor growth. Demonstration of tumor suppressor functions of *ARID1A* should be fundamental for future studies aimed at elucidating the biological roles of *ARID1A* and SWI/SNF complexes in endometrium-related tumorigenesis and should help clarify how aberrant chromatin remodeling activity participates in cancer development.

Materials and Methods

Plasmid construction and lentivirus production

The full-length cDNA of ARID1A (coding sequence of NM_006015) was sub-cloned from plasmids CMV-T7-hOsa1 (Addgene plasmid 17986) (25) and pCI-neo-BAF250 (2) into pCDNA6-V5/His.b (Invitrogen) and pLenti-puro, with V5/His tags at the C-terminus. The tetracycline-inducible lentiviral vector pLenti-puro was constructed using pLenti4/TO/V5-DEST vector (Invitrogen) as a backbone by removing the Gateway elements between the two EcoRV sites, and by replacing the Zeocin resistance gene with a puromycin resistance gene. The tetracycline repressor was introduced into cells using virus produced from pLenti6/TR (Invitrogen). The control plasmid, pLenti-puro-LacZ, was constructed by cloning lacZ from pLenti6/V5-GW/lacZ (Invitrogen) into the pLenti-puro vector. The shRNALentiviral plasmids were obtained from the RNAi consortium. The short hairpin RNA (shRNA) sequences for ARID1A were: sh1 (TRCN0000059090), CCTCTCTTATACACAGCAGAT, and sh2 (TRCN0000059091), CCGTTGATGAACTCATTGGTT. The shRNA sequences for p21 were: sh1 (TRCN0000040123), CGCTCTACATCTTCTGCCTTA, and sh2 (TRCN0000040126), GACAGATTTCTACCACTCCAA. The shRNA sequence for p53 was (TRCN0000003754) TCAGACCTATGGAAACTACTT. Lentivirus was produced using HEK293FT cells (Invitrogen) with the second generation packaging system pSPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259). Lentiviral titer was determined using a real-time qPCR method by measuring viral RNA content in viral supernatant as described (26). The firefly luciferase plasmids for the p21 promoter, WWP-Luc (27), and for SMAD3 (28) have been reported previously. The primers used in this study are listed in Table S3.

Tumorigenicity in immunocompromised mice

OSE4 cells were transduced by lentivirus expressing shRNA targeting ARID1A (sh1) or GFP, and two days later the transduced cells were injected into the subcutaneous tissue on the right and left flanks, respectively, of 4–6 week-old nu/nu mice. For each injection site, 4×10^6 cells mixed 1:1 (v:v) with Matrigel (BD Biosciences) were injected. The tumors were excised and weighted 6 weeks after inoculation. All tumors were confirmed by histopathologic analysis. To restore ARID1A expression in the xenograft model, HEC-1-AARID1A or LacZ inducible cells (2×10^6 cells/site mixed 1:1 (v:v) with Matrigel) were injected onto the left and right flanks, respectively, of 4–6 week-old nu/nu mice. Starting ten days after inoculation, 125 μg doxycycline per mouse was administered every other day by intraperitoneal injection. Tumors were excised and weighted 17 days after inoculation. Student's *t* test was used for statistical analysis using Prism software (GraphPad). Care of experimental animals was in accordance with institutional guidelines (Johns Hopkins Medical Institutions).

Chromatin Immunoprecipitation

Cells were cross-linked with 1% formaldehyde (Sigma) diluted in culture media at room temperature for 10 minutes followed by quenching with 125 mM Glycine for 5 minutes. The chromatin was extracted with ChIP lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 7.5). After one freeze-thaw cycle, the DNA was fragmented twice for 10 minutes using a Bioruptor (Diagenode) sonicator at 30 second on/off cycles set at the highest intensity. The chromatin was precipitated using 2 μg antibody pre-absorbed onto 20 μl Protein A/G (1:1) DYNAL magnetic beads (Invitrogen). After reverse cross-linking, the DNA was purified using a PCR purification kit (Qiagen). Binding to promoter regions was analyzed by qPCR and reported as percent of input. In ChIP-reChIP, immunocomplex from the 1st ChIP was eluted with 10mM DTT in TE at 37 °C. The eluates were then diluted with ten volumes of ChIP lysis buffer and used for the 2nd round ChIP.

Cell Lines

The nature of ovarian surface epithelial cells OSE4 (29) and IOSE-80PC (30), as well as the ovarian clear cell carcinoma cell line, OVISE, (31) were previously described. All the cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. HEC-1-A cells were obtained from ATCC (Rockville, Maryland). These four cell lines exhibited distinct morphological features, and were also authenticated by the Fragment Analysis Facility at the Johns Hopkins University using the short tandem repeat (STR) DNA profiling (PowerPlex 1.2 System, Promega). The STR profiles of HEC-1-A and OVISE were matched to their original profiles in the cell line database at the Japanese Collection of Research Bioresources (JCRB, http://cellbank.nibio.go.jp/cellbank_e.html). The STR profiles of OSE4 and IOSE-80PC cell lines have not yet been deposited but they were distinct from any cell line reported in the current STR Profile Databases maintained by JCRB and ATCC (http://www.atcc.org). Mycoplasma was tested negative in all cell lines (PlasmoTest, InvivoGen).

Results

Restoring ARID1A expression suppresses tumor growth

To determine the tumor suppressive function of *ARID1A*, we restored the expression of wild-type *ARID1A* in cancer cells harboring *ARID1A* mutations. Based on mutation information and Western blot analysis (Fig. S1), we selected the HEC-1-A uterine endometrioid carcinoma cell line and the OVISE ovarian clear cell carcinoma cell line as the models. Both cell lines contained homozygous *ARID1A* mutations and did not express

detectable ARID1A protein. A Tet-On tetracycline inducible system was established to control ARID1A expression in both cell lines. Expression of ARID1A in those cells was induced by treating cells with doxycycline, a derivative of tetracycline (Fig. S2). As a control, we also established Tet-On inducible LacZ expression in both cell lines (Fig. S2). Cellular proliferation and the percentage of cells in the S phase of cell cycle were markedly decreased in ARID1A-induced cells as compared to the control groups of cells lacking ARID1A expression (Fig. 1A and 1B). In contrast, induced expression of ARID1A in the ovarian clear cell line, JHOC5, which has wild-type *ARID1A* and abundantly expressed the protein, had minimal effects on cellular proliferation and cell cycle progression (Fig. S3).

To extrapolate the above *in vitro* findings to an *in vivo* setting, we established Tet-On inducible HEC-1-A tumor xenografts in athymic *nu/nu* mice. In the experimental group, ARID1A expression was induced 10 days after tumor cell inoculation, when small subcutaneous tumors were palpable. We found that expression of wild-type *ARID1A* significantly reduced tumor weights as compared to the control group without restoration of wild-type ARID1A ($p= 0.025$) (Fig. 1C). As expected, tumor growth in mice was not affected by induction of the control gene, LacZ (Fig. 1C). Immunohistochemistry performed on the excised tumors showed ARID1A nuclear immunoreactivity in ARID1A induced tumors (+Tet) but not in mock induced tumors (−Tet), confirming tetracycline-regulation of ARID1A expression in the tumor xenografts (Fig. S4). Based on immunostaining, the level of ARID1A induction in tumor cells, although varying in intensity, was comparable to mouse stromal cells and endometrium, indicating a physiological level of ARID1A expression in our Tet-On inducible system (Fig. S4).

Silencing of ARID1A expression enhances cellular proliferation and tumorigenicity

Our previous studies (14, 32) together with the Western blot analysis (Fig. S1) demonstrates that *ARID1A* mutations correlate with loss of protein expression in human endometrium-related cancers. Thus, to simulate the effect of *ARID1A* mutations, we knocked down *ARID1A* in two epithelial cell lines, IOSE-80PC and OSE4, which were derived from normal ovarian surface epithelium. It has been demonstrated in an engineered mouse model that ovarian surface epithelium may undergo Mullerian metaplasia to become endometrium tissue and develop ovarian endometrioid carcinoma after genetic inactivation of Wnt/β-catenin and PIK3/Pten pathways (33). Furthermore, ovarian clear cell carcinoma has long been thought to derive from endometriosis. Since normal epithelial cell lines established from uterine endometrium have not been available, ovarian surface epithelial cell lines may represent the relevant models currently available to prove the principle if loss of ARID1A expression promotes oncogenesis.

As compared to cells transduced with control virus carrying GFP-targeting shRNA, cells transduced with ARID1A shRNA virus demonstrated an elevated proliferation rate and an increased cell population in S phase (Fig. 1D, 1E). To determine if ARID1A knockdown affected tumorigenicity *in vivo*, OSE4 cells, in which ARID1A expression had been knocked down by shRNA, were injected into athymic nu/nu mice. Although OSE4 cells are poorly tumorigenic, they became highly tumorigenic when ARID1A expression was reduced by shRNA (Fig. 1F).

ARID1A regulates p53-controlled genes

The above results indicate that ARID1A controls cell cycle progression, and downregulation of ARID1A expression leads to increased cellular proliferation. To determine the possible mechanisms, we compared the transcriptomes of IOSE-80PC and OSE4 cells between ARID1A knockdown and control groups using Illumina BeadChip arrays. We identified a total of 104 genes in which transcript levels were up- or down-regulated by at least two folds

in both cell lines (Table S1 and Fig. 2A). Those genes downregulated by ARID1A shRNAs included CDKN1A, SMAD3, SMAD5, Nag1 (of TGF-βsuperfamily), TRIM8 and SMARCD1 (BAF60a). Pathway analysis of these AIRD1A-regulated genes demonstrated an enrichment of pathways in cell cycle regulation (Table S2), and, in fact, the top two pathways involved cell cycle regulation. Interestingly, even though the expression levels of p53 remained stable, several p53-related genes were down-regulated by ARID1A knockdown (Fig. 2A). Among them, two prominent molecular hubs, *CDKN1A*, encoding p21 (Cip1 or WAF1), and *SMAD3*, were identified. Because of the known biological roles of *CDKN1A* and *SMAD3* in pathways related to cell cycle and p53, we selected both for further characterization in this study. Down-regulation of expression of *CDKN1A* and *SMAD3* by *ARID1A* knockdown was validated using quantitative RT-PCR or Western blot analyses (Fig. S5A, Fig. 2B). In addition, induction of ARID1A expression led to upregulation of p21 protein in HEC-1-A and OVISE Tet-on cells (Fig. 2C) and to an increase of SMAD3 mRNA levels in OVISE Tet-on cells (Fig. S6A).

To further determine if p21 mediated the growth suppressive effects of ARID1A, we knocked down *CDKN1A* in the OVISE cell line carrying tet-inducible ARID1A. As expected, ARID1A induction resulted in growth suppression and p21 overexpression, and more importantly, two different p21 shRNAs significantly increased cellular proliferation in cells with ARID1A induction as compared to control (GFP) shRNA treated cells (Fig. 2E), indicating that p21 was responsible, at least in part, for growth suppression following ARID1A induction.

Binding of ARID1A/BRG1/p53 complex to the promoters of *CDKN1A* and *SMAD3*

It has been established that ARID1A interacts with BRG1 ATPase to form a SWI/SNF chromatin remodeling protein complex (2, 22). It has also been reported that p53 interacts with SWI/SNF subunits BAF60a, BRG1 and BAF47 (34–36). To determine if p53 bound to ARID1A/BRG1 complex in our experimental system, we performed co-immunoprecipitation in OSE4 cells by pulling down endogenous p53 then blotted with either ARID1A or BRG1 antibodies. As shown in Fig. 3A, both ARID1A and BRG1 co-immunoprecipitated with p53. We further showed that the C-terminus (amino acid 1759–2285) but not the N-terminus (amino acid 1–1758) of ARID1A mediates the interaction with p53 (Fig. 3B). Next, we addressed whether ARID1A and p53 could directly interact with each other. Recombinant GST-p53 (both wild-type p53 and R175H mutant) and the C-terminus of ARID1A (amino acid 1759–2285 with C-terminal V5-tag) were purified from *Escherichia coli* and used in immunoprecipitation assay (Fig. 3C). Recombinant p53 but not GST pulled down the C-terminus of ARID1A. Moreover, the ARID1A/BRG1 SWI/SNF complex and p53 occupied the same promoter regions of *CDKN1A* and *SMAD3*, as demonstrated by sequential and reciprocal chromatin immunoprecipitation (ChIP)-reChIP using p53 and BRG1 antibodies, respectively (Fig. 3D). The above results provide new evidence that p53 is recruited to the ARID1A/BRG1 complex that binds to *CDKN1A* and *SMAD3* promoters.

To further delineate the role of ARID1A/BRG1/p53 complex in regulating transcription of *CDKN1A* and *SMAD3*, we performed ChIP using antibodies reacting to BRG1. Antibody against p53 was used in the ChIP assay as a control. As shown in Fig. 3E, both BRG1 and p53 bound to the promoter regions of *CDKN1A* and *SMAD3*, and, more importantly, knockdown of *ARID1A* significantly reduced BRG1 binding to both promoter sequences. In contrast, interaction of p53 with *CDKN1A* and *SMAD3* promoter regions was unaffected by reduced expression of *ARID1A*. Besides, promoter reporter (luciferase) analysis demonstrated that the transcriptional activity of p21 and SMAD3 was decreased by *ARID1A* knockdown (Fig. S5B).

ARID1A regulates p21 in a p53-dependent fashion

Because p21 is a well established downstream target of p53 and ARID1A interacts with p53, we asked whether ARID1A-regulated p21 transcription depended on the p53 pathway in three cell models. First, two different *TP53* knockout cell lines, HCT116$^{TP53-/-}$ and MCF10A$^{TP53-/-}$, were used. These cells, as well as their parental wild-type counterparts, were transfected with a plasmid expressing ARID1A-V5. We observed that ectopic expression of ARID1A-V5 was associated with increased mRNA levels of p21 in p53 wild-type cells and to a lesser extent in p53 null cells (Fig. 4A and B). Next, we knocked down p53 levels with shRNA in HEC-1-A ARID1A-inducible cells and measured its effect on the transcription level of *CDKN1A*. The results demonstrated that induction of p21 by ARID1A was significantly reduced by p53-specific shRNA as compared to control shRNA (Fig. 4C). Similarly, SMAD3 upregulation by ectopic expression of ARID1A was compromised in MCF10A$^{TP53-/-}$ cells as compared to MCF10A$^{TP53+/+}$ cells (Fig. S6B).

Correlation of *ARID1A* mutation and *TP53* mutation in tumor tissues

The above results suggest that inactivating mutations in either *ARID1A* or *TP53* result in loss of transcriptional regulation of *CDKN1A* and *SMAD3*. To this end, we analyzed the mutational status of *ARID1A* and *TP53* in 77 ovarian clear cell carcinomas and uterine endometrioid carcinomas which were known to have *ARID1A* and *TP53* mutations. Our data showed a mutually exclusive pattern of *ARID1A* and *TP53* mutations ($p= 0.031$, Fisher's exact test) (Table 1). Specifically, all 34 tumors with *ARID1A* mutations were *TP53* wild-type, and all 6 carcinomas harboring *TP53* mutations contained wild-type *ARID1A*.

Discussion

In this study, we demonstrated the tumor suppressive role of *ARID1A* and proposed the possible mechanisms involved. The evidence for tumor suppressor function of *ARID1A* comes from its ability to inhibit cellular proliferation and tumor growth when expressed in cancer cells harboring mutated *ARID1A* and to enhance cellular proliferation and tumorigenecity when its expression is silenced in epithelial cells with wild-type *ARID1A*. Although *ARID1A* mutations appear to predominate in endometrium-related carcinomas, inactivation of the ARID1A-containing BAF complex, as a consequence of genetic or epigenetic alterations, may be common in development of other human neoplastic diseases including carcinomas arising from kidney, breast, lung, and stomach (14, 37, 38). For example, deletion of the chromosome 1p35 region harboring *ARID1A* has been found in more than half of pancreatic carcinomas (39).

It has been established that SWI/SNF chromatin remodeling genes are required for several nuclear activities including transcription. It remains unclear, however, whether they act globally to facilitate transcriptional activity in transcriptionally active domains, or if they locally target a set of selected genes that work in concert for specific cellular functions. The data presented in this study, together with a recent report (40), favor the latter view, as evidenced by downregulation of specific genes upon *ARID1A* silencing. Analysis of ARID1A downstream genes reveals that the tumor suppressor role of ARID1A mainly involves negative regulation of cell cycle progression and identifies two p53-regulated genes, *CDKN1A* and *SMAD3*, which may serve as the major target genes to mediate its tumor suppressor functions. The induction of p21 by ARID1A, and negative regulation of ARID1A on cell-cycle progression in our cell models are in agreement with previous reports which showed that ARID1A is required for cell-cycle arrest after induced differentiation in mouse osteoblastic cells (41, 42).

The regulation of p53-related genes by ARID1A raises the possibility if ARID1A molecularly cooperates with p53 to inhibit tumor growth. In fact, this view is supported by several pieces of evidence reported in this study. Most importantly, both protein co-immunoprecipitation and ChIP-reChIP analyses demonstrate that p53 interacts with ARID1A/BRG1 and the ARID1A/BRG1/p53 complex binds to the promoter regions of *CDKN1A* and *SMAD3*. By employing a *TP53* knockout cell system and RNA interference strategy, we observed that ARID1A-induced p21 and SMAD3 expression required the presence of p53. Therefore, it is possible that in non-transformed cells, ARID1A and p53 collaborate as a pair of gatekeepers that prevent tumorigenesis by transcriptional activation of tumor-inhibiting downstream genes such as *CDKN1A* and *SMAD3*, since the tumor suppressor role of *CDKN1A* that encodes for p21 has been well established (43) and deficiency of SMAD3 has been reported to propel tumor progression in a mouse model (44). Besides, we found that all tumors with mutated *ARID1A* contained wild-type *TP53*, and tumors with mutated *TP53* harbored wild-type *ARID1A*. Because both *ARID1A* and *TP53* appear to be essential for tumor suppression of endometrium-related cancer, concurrent mutations in both genes are thus not required for tumorigenesis. In other words, mutation in either *ARID1A* or *TP53* is sufficient to inactivate the ARID1A/BRG1/p53 complex and silence transcription of *CDKN1A* and *SMAD3*.

Our co-immunoprecipitation study using recombinant p53 and ARID1A indicates that ARID1A directly binds to p53 proteins and thus may recruit p53 to the BAF complex for transcriptional regulation of its downstream targets. In this way, p53 may target the SWI/SNF complex to specific promoters with p53 binding sites, while the SWI/SNF complex alters the local chromatin structure by displacing nucleosomes and creating naked DNA sites to be occupied by transcriptional machinery including RNA polymerase II during transcription (Fig. 4C). The mutually exclusive pattern of *ARID1A* and *TP53* mutations supports our view that these tumors do not require concurrent mutations in both genes because as shown in this study mutation of either gene is sufficient to turn off tumor suppressor activity by transcriptionally inactivating the set of genes co-regulated by *ARID1A* and *TP53*. It has been established that p53 mutant such as R175H failed to induce p21 expression and cell-cycle arrest (27, 45). One of the functions of mutant p53 is to decrease wild-type p53 binding to its target genes including p21 in a dominant-negative manner (46). Recently it has been reported that mutant p53 protein may bind to a set of unique genes that are not the conventional p53-regulated genes such as *GRO1* (47). Thus, the observation that mutant p53 can bind to ARID1A raises the possibility that p53/ARID1A/BRG1 complex might regulate a different set of genes depending on the mutation status of *TP53*.

It should be noted that OSE4 and IOSE-80PC were immortalized by SV40 large T antigen (29, 48), and one of the functions of T antigen (Tag) is to interact with p53 and inactivate its transcriptional activity (49). Interestingly, it has also been demonstrated that the p53-Tag complex purified from human cells but not from mouse cells still retains p53 transcriptional activity (50), and p53 is required for the transcription of p53-responsive genes including the insulin-like growth factor I and p21 in SV40 Tag immortalized cells (51). Therefore, it warrants further study to determine whether ARID1A binds to p53 alone and/or p53-Tag complex in OSE4 and IOSE-80PC cells. Nevertheless, our data show that ARID1A is required for the expression of several p53-responsive genes, and the interaction between ARID1A and p53 can be further supported by our experiments performed in four cell lines (OVISE, HEC-1-A, HCT116, and MCF10A) that do not express Tag.

Similar to any newly identified cancer genes, there are several important questions remaining to be addressed in order to fully understand their molecular functions and before translational applications in cancer can be proposed. For example, future studies are required to determine the roles of *ARID1A* mutations in the context of pathway networks, including

PI3K/Pten and canonical Wnt pathways, in which alterations have been identified in endometrium-related cancers (52). It will also be important to determine differences in response to therapy, risk for disease recurrence, and overall prognosis between tumors with and without *ARID1A* mutations. Such information should be valuable in assisting clinical management of patients who suffer from endometrium-related cancer.

In summary, the above data suggest that *ARID1A* is a negative cell cycle regulator and a tumor suppressor gene. One of the mechanisms involving tumor suppressor function of *ARID1A* is through the molecular collaboration between ARID1A and p53, as the complex formation is required and sufficient to transcriptionally regulate several p53-related genes, including those with known tumor suppressor functions. We demonstrate that in the presence of wild-type *TP53*, tumor cells may take advantage of the disruption of the ARID1A/BRG1/p53 complex caused by *ARID1A* mutations to downregulate p53-regulated genes including p21, SMAD3, and perhaps other genes with tumor suppressor functions. The findings presented in this study suggest a close collaboration between genetic and epigenetic alterations in cancer pathogenesis and provide a new molecular mechanism that contributes to tumor suppression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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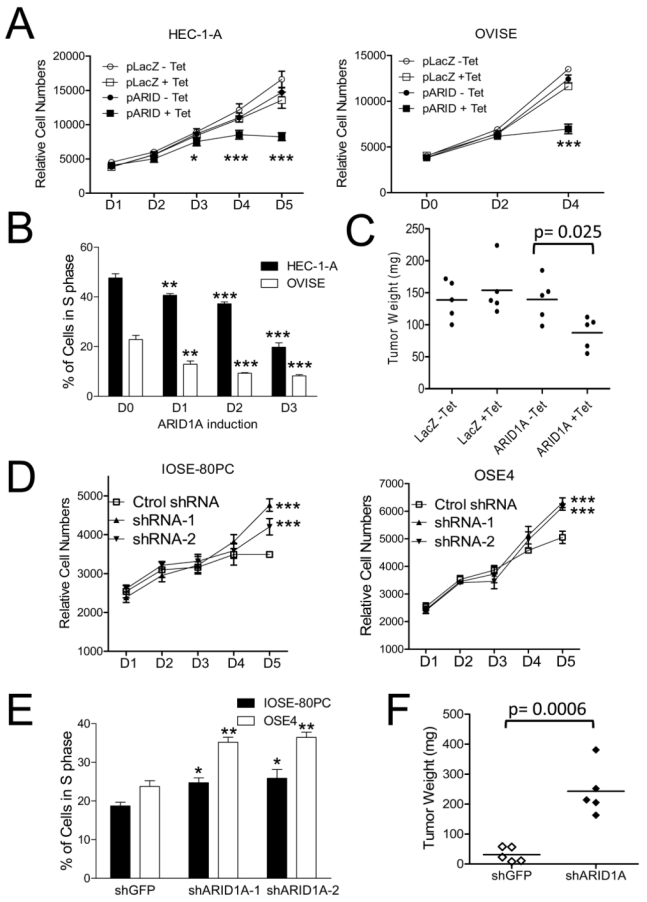

Fig. 1.

Effects of ARID1A on cellular proliferation and tumor growth. (*A*) A Tet-on inducible system was established in HEC-1-Aand OVISE cancer cell lines which harbor *ARID1A* inactivating mutations. Growth curve analysis shows that induction of ARID1A expression significantly decreases cellular proliferation as compared with the control groups without ARID1A expression. (*B*) Ectopic expression of wild-type ARID1A in HEC-1-A and OVISE leads to a decreased population of cells in S-phase. (*C*) ARID1A expression (ARID1A + Tet) significantly reduces the tumor weight of HEC-1-Atumor xenografts as compared to control tumors without ARID1A induction. (*D*) As compared to IOSE-80PC and OSE4 epithelial cells transduced with control shRNA lentivirus, cells transduced with virus expressing two different ARID1A shRNAs demonstrate significantly greater proliferation by day 5. (*E*) The percentage of cells in S phase is increased in IOSE-80PC and OSE4 cells transduced by ARID1A shRNAs (shARID1A-1 and shARID1A-2). (*F*) OSE4 cells transduced with ARID1A shRNA virus (shRNA 1) were injected into the subcutaneous tissue on the right flank of nu/nu mice, and control (Cont) shRNA virus transduced cells were injected into the left flank. Six weeks later, tumors arising from cells transduced with ARID1A shRNA are larger than tumors arising from cells transduced with control shRNA ($p < 0.0006$). For panelsA and B, statistical analysis was performed between ARID1A-induced and un-induced cells using *t* test. For panels D and E, statistical analysis was performed between ARID1A knockdown samples and control samples. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (t test).

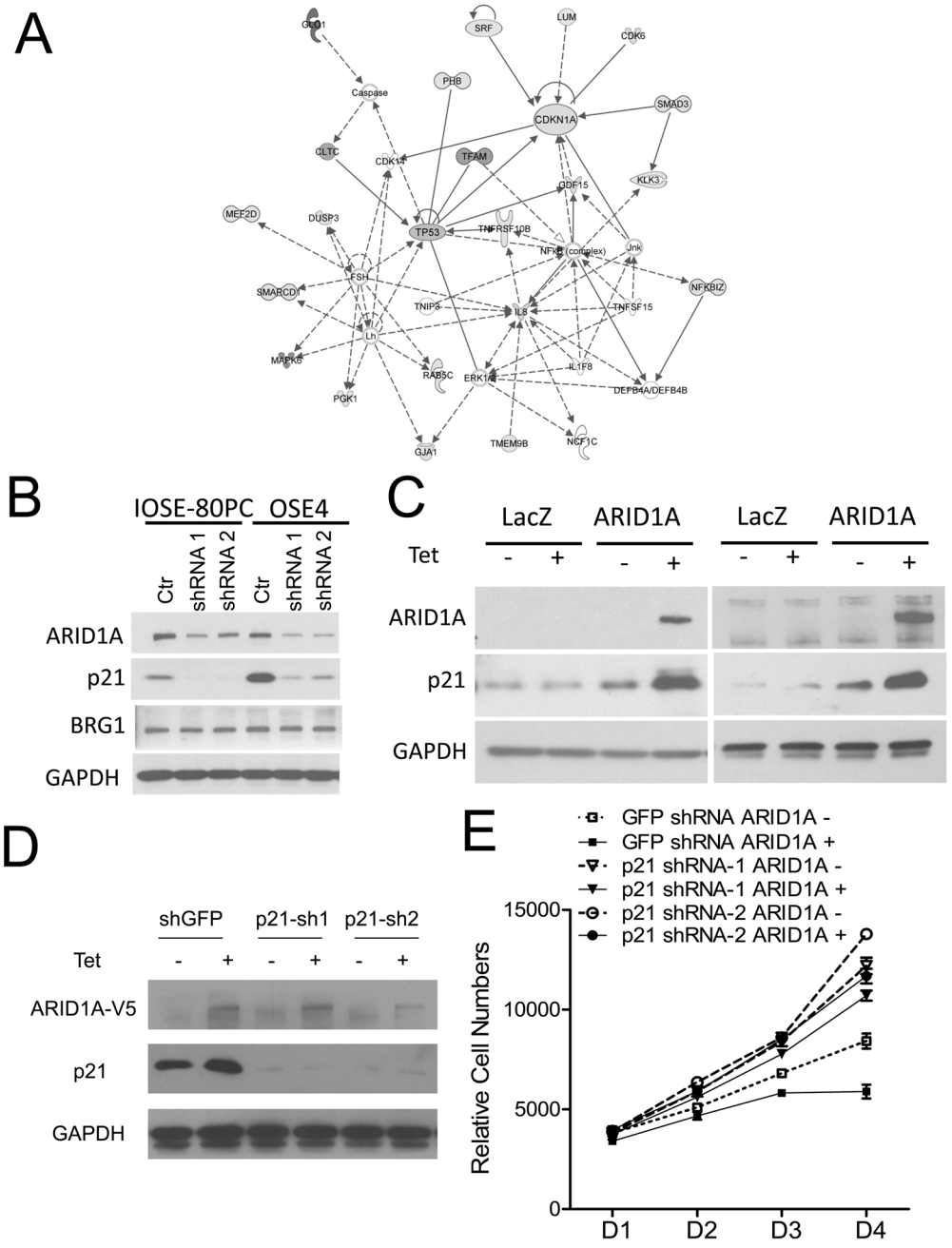

Fig. 2.

Identification of ARID1A regulated genes. (*A*) Ingenuity analysis of networks contributed by 104 differentially expressed ARID1A-regulated candidate genes identified two prominent molecular hubs, *CDKN1A* encoding p21 (Cip1 or WAF1) and *SMAD3*. A color copy is shown in Supplementary Fig. S7. (*B*) Western blot demonstrates that ARID1A knockdown results in reduced expression of ARID1A and p21. (*C*) Induction of ARID1A expression leads to upregulation of p21 protein in both HEC-1-A (*left panel*) and OVISE (*right panel*) cell lines. (*D*) Western blot shows significant reduction in p21 levels following knockdown with shRNA targeting p21. (*E*) Two different p21 shRNAs significantly increased cellular

proliferation in ARID1A-induced cells as compared to control (GFP) shRNA treated ARID1A-induced HEC-1-A cells ($p < 0.01$).

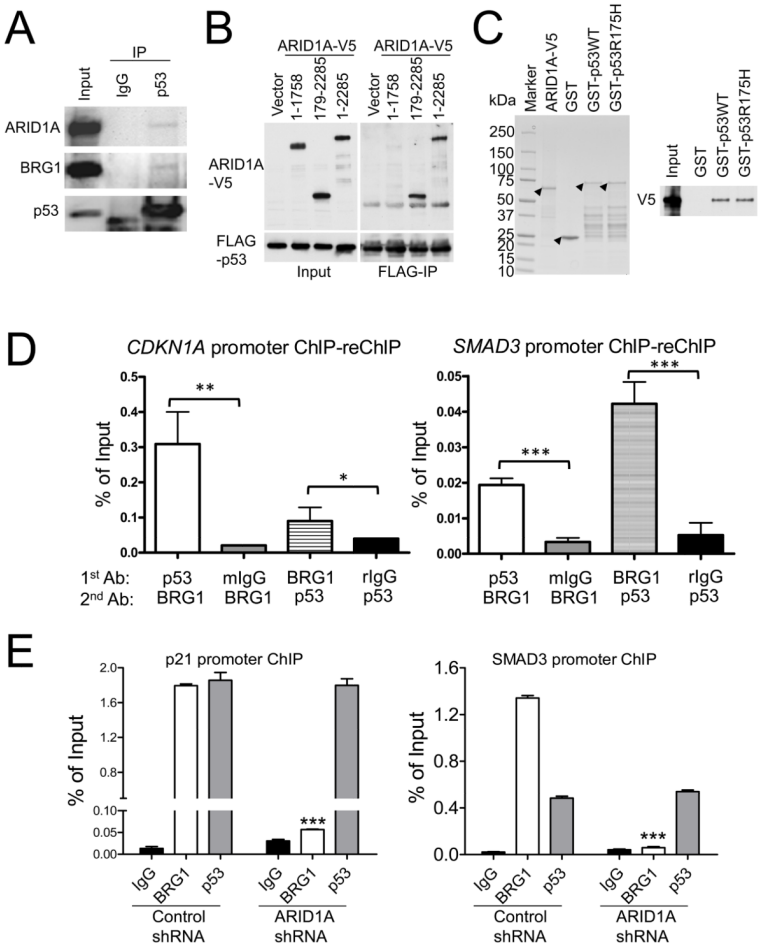

Fig. 3.

Interaction of p53 with ARID1A/BRG1 SWI/SNF chromatin remodeling complex. (A) Endogenous p53 co-immunoprecipitates with ARID1A and BRG1 in OSE4 cells. As a control, p53 was immunoprecipitated with a mouse anti-p53 antibody and was detected by a rabbit anti-p53 antibody in immunoblotting. (B) C-terminus of ARID1A interacts with p53. V5-tagged full-length (amino acid 1–2285) or fragments of ARID1A were co-stransfected into 293FT cells with FLAG-p53 construct and were applied for immunoprecipitation using FLAG-Ab beads. (C) Coomassie stain of recombinant ARID1A-V5 (amino acid 1759–2285), GST, GST-p53 wild-type (WT) and p53 R175H mutant (left); Western blot of ARID1A-V5 after pull-down with Glutathione beads (right). Arrow heads indicate the bands corresponding to respective proteins. (D) ChIP-reChIP experiments with anti-p53 and BRG1 antibodies. Mouse IgG (mIgG) and rabbit IgG (rIgG) were used as negative controls. (E) Chromatin immunoprecipitation demonstrates that both BRG1 and p53 bind to *CDKN1A* and *SMAD3* promoters, and that knockdown of ARID1A significantly reduces the binding of BRG1 but not p53 to these two promoters. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (*t* test).

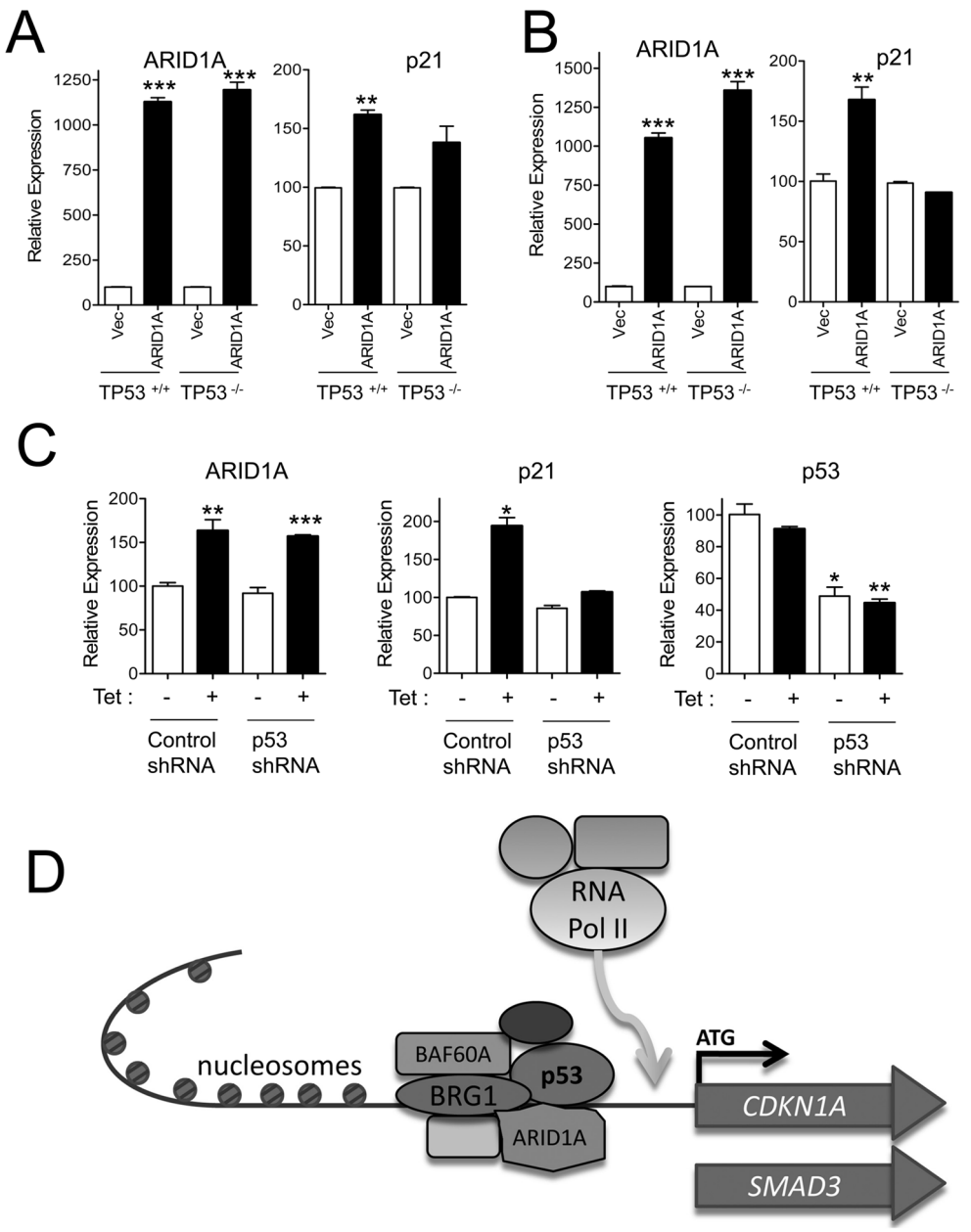

Fig. 4.

Regulation of p21 by ARID1A and p53. (*A*) HCT116$^{TP53-/-}$ and HCT116$^{TP53+/+}$ cells were transfected with ARID1A-V5. Ectopic expression of ARID1A-V5 is associated with increased mRNA levels of p21 in HCT116$^{TP53+/+}$ but to a lesser extent in HCT116$^{TP53-/-}$ cells. (*B*) MCF10A$^{TP53-/-}$ and MCF10A$^{TP53+/+}$ cells were transfected with ARID1A-V5. Ectopic expression of ARID1A-V5 upregulate p21mRNA levels in MCF10A$^{TP53+/+}$ cells but not in MCF10A$^{TP53-/-}$ cells. (*C*) ARID1A inducible HEC-1-A cells were treated with p53 shRNA. ARID1A induction by doxycycline (Tet) increases p21 transcript expression in cells treated with control shRNA but not in cells treated with p53 shRNA. (*D*) Schematic presentation of SWI/SNF complex containing ARID1A in regulating target gene expression. ARID1A can directly or indirectly recruit p53 to the BAF complex for transcriptional regulation of its downstream targets, e.g., CDKN1Aand SMAD3. p53 may target the SWI/

SNF complex to promoters containing a p53 binding site. Upon binding, SWI/SNF alters chromatin structure by displacing nucleosomes to create naked DNA sites to be occupied by transcriptional machinery including RNA polymerase II for transcriptional initiation. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$(*t* test).

Table 1

Mutation status of *ARID1A* and *TP53* in 77 ovarian clear cell and uterine endometrioid carcinomas

	TP53 MUT	TP53 WT	Total
ARID1A MUT	0	34	34
ARID1A WT	6	37	43
Total	6	71	77

MUT, mutation; WT, wild-type p=0.031