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Identification and characterization of human ARIP2 and its relation to breast cancer

Zhan Dong Li [a,b], Yin Wu [b], Yong Li Bao [a,*], Chun Lei Yu [a], Li Li Guan [a], Yue Zeng Wang [a,c], Xiang Ying Meng [a], Yu Xin Li [b,*]

[a] Institute of Genetics and Cytology, Northeast Normal University, 5268 Renmin St., Changchun 130024, China
[b] Research Center of Agriculture and Medicine Gene Engineering of Ministry of Education, Northeast Normal University, Changchun 130024, China
[c] Department of Pathology and Pathophysiology, School of Basic Medical Sciences, Jilin University, Changchun 130021, China

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ABSTRACT

Activin, a member of the TGF-β superfamily, inhibits the proliferation of breast cancer cells. Activin interacts with its type I and type II receptors to induce phosphorylation of intracellular signaling molecules known as Smads. Previous studies showed that mouse ARIP2 can reduce activin signaling by interacting with activin type II receptors (ActRIIs); however, the activity of ARIP2 in breast cancer is still unclear. In this study, we used RT-PCR to obtain a human homologue of mouse ARIP2, human activin receptor-interacting protein 2 (hARIP2). Like murine ARIP2, hARIP2 has a PDZ domain in its NH2-terminal region and can interact specifically with ActRIIs. Overexpression of hARIP2 reduced activin-induced transcriptional activity and enhanced cell proliferation and colony formation in human breast adenocarcinoma MCF-7 cells and MDA-MB-231 cells. However, down-regulation of hARIP2 expression by RNAi enhanced activin-induced transcriptional activity and reduced cell proliferation and colony formation. Immunohistochemistry revealed that hARIP2 was expressed more frequently and much more intensely in malignant breast tissues such as simple carcinoma, invasive ductal carcinoma and mucinous adenocarcinoma than in benign hyperplasia or fibroadenoma cases. These results suggest that hARIP2 is a putative growth-promoting factor involved in breast tumorigenesis and tumor development.

1. Introduction

Activin is a member of the transforming growth factor beta (TGF-β) superfamily of extracellular signaling proteins. It has numerous regulatory functions such as cell proliferation and differentiation, apoptosis, metabolism, homeostasis, immune responses, wound repair and various endocrine functions [1–4]. Activins elicit diverse biological responses by signaling mediated through type I and type II serine/threonine kinase receptors. Activin binds directly to the type II receptor, leading to the recruitment, phosphorylation and subsequent activation of the type I receptor. Upon activation, the type I receptor binds to and phosphorylates a subset of cytoplasmic Smad proteins. These phosphorylated Smads then translocate to the nucleus where they control the transcription of target genes [5,6].

So far, the known functions of the ActRIIs are limited to ligand binding, type I receptor recruitment, and transphosphorylation. The type II activin receptors include two subtypes of activin type II receptors: type IIA (ActRIIA) and IIB (ActRIIB), each of which is encoded by individual genes. Moreover, two splice variants of ActRIIA and five splice variants of ActRIIB have been found [7–9]. It is speculated that the ActRIIs play specific roles in activin signaling.

Breast cancer is one of the leading causes of mortality among women and there is a tenfold variation in breast cancer incidence among different countries [10–15]. Studies indicate that activin has been associated with both normal mammary gland development and breast carcinogenesis [16–18]. Activin signal transduction components have been identified in normal cell lines, breast cancer cell lines, and benign and malignant mammary tissues [19–21]. The loss or reduction of activin signaling components, specifically nuclear localization or phosphorylation of Smad2 and Smad3, has been documented as breast cancer becomes more aggressive [21–23]. Moreover, activin has been reported to inhibit growth of breast cancer cells by activating both Smad proteins and the p38 MAPK pathway. Since ARIP2 is capable of regulating activin signaling, we hypothesized that ARIP2 may play a role in the development of human breast cancer.

Recent investigations revealed that a series of PDZ domain-containing molecules can regulate activin signal transduction via transmembrane receptors through interacting with the ActRIIs. In the present study, we cloned and characterized human activin receptor-interacting protein 2 (hARIP2) and investigated its effect on activin signal transduction, cell proliferation, and colony formation. The results showed that overexpression of hARIP2 decreased activin-induced transcriptional activity and promoted cell

* Corresponding authors. Fax: 86 0431 8509 9502.
E-mail addresses: baoyl800@nenu.edu.cn (Y.L. Bao), liyx486@nenu.edu.cn (Y.X. Li).

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proliferation and colony formation. Furthermore, the cellular distribution of hARIP2 was characterized in mammary tissues including simple carcinoma, invasive ductal carcinoma, mucinous adenocarcinoma, benign hyperplasia and fibroadenoma. Immunohistochemistry revealed that the protein expression of hARIP2 was significantly higher in breast cancer tissues compared to benign breast tissues.

2. Materials and methods

2.1. Plasmids construction

Total RNA from normal human placenta tissue was extracted with the Trizol reagent according to the manufacturer's protocol (Life Technologies). The RNA samples were then reverse transcribed using SUPERSCRIPT II reverse transcriptase (Gibco–BRL). The ARIP2 gene containing a complete coding sequence was generated by PCR. The primers were forward, 5′-CCGAATTCATGAACGGAAGAGTG-3′ and reverse, 5′-CGGATCCTCAAGTTGTCTTAGTC-3′. The sense or reverse primer contained the EcoRI or the BamHI restriction enzyme sites, respectively. Amplified PCR products were cloned into the EcoRI and BamHI enzyme sites of a pcDNA3-FLAG eukaryotic expression vector.

DNA constructs for the mammalian two-hybrid assay were made using the plasmid pBIND to express the fusion protein with the GAL4 DNA binding domain and using pACT to express the fusion protein with the VP16 activation domain [9]. pBIND-ActRIIA, pBIND-ActRIIB, pBIND-TGF-β type II receptor (TGF-βRII), and pBIND-bone morphogenetic protein type II receptor (BMPRII), encoding the cytoplasmic regions of mouse ActRIIA, mouse ActRIIB, human TGF-βRII, and human BMPRII, respectively, have been described previously [9]. To make pACT/pBIND-ARIP2, cDNA fragments composed of nucleotides 119–676 of ARIP2 (GenBank Accession No. BM563433) were prepared by PCR and ligated into pACT and/or pBIND.

To create the hARIP2 siRNA expression vector, the pSilencer 2.0-U6 plasmid (Ambion) was used for DNA vector-based siRNA synthesis. hARIP2 siRNA corresponds to nucleotides 638–656. The sequences of the synthesized oligonucleotides are: 5′-GATCCCCCAGAAAGCCTTAAGACTATTCAAGAGATAGTCTTAAGGCTTTCTGGTTTTTTGGAAA-3′ (forward) and 5′-AGCTTTTCCAAAAAACCAGAAAGCCTTAAGACTATCTCTTGAATAGTCTTAAGGCTTTCTGGGG-3′ (reverse). The paired oligonucleotides were annealed in buffer [100 mmol/L Kacetate, 30 mmol/L Hepes–KOH (pH 7.4), and 2 mmol/L Mg acetate]. The mixture was first incubated at 90 °C for 3 min, then at 37 °C for 1 h. The oligonucleotide containing terminal BamHI–HindIII sites was subcloned into the BamHI–HindIII sites of the pSilencer 2.0-U6 vector to generate the pSilencer2.0-U6-hARIP2 siRNA vectors. A negative control scrambled siRNA (Ambion), which has no significant homology to mouse or human gene sequences, was designed to detect any non-specific effects.

2.2. Cell culture and transfection

Human embryonic kidney (HEK)293T cells, human hepatocellular liver carcinoma (HepG2) cells, MDA-MB-231 cells and human breast adenocarcinoma MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The cells were transfected with the indicated plasmids using the Gene Companion™ II cell transfection reagent (Genetrans, Changchun, China) according to the manufacturer's protocol for transient transfection.

2.3. Mammalian two-hybrid assay

Mammalian two-hybrid assay was performed using the CheckMate mammalian two-hybrid system (Promega, Madison, WI) according to the manufacturer's protocol [9]. In brief, 293 cells were cotransfected with the plasmids of interest, a cytomegalovirus promoter-driven β-galactosidase and a reporter plasmid, pG5luc, which drives the luciferase gene under the control of the GAL4-responsive promoter. Luciferase activity was measured using FLUOstar OPTIMA (BMG LABTECH, Offenburg, Germany) and normalized to the β-galactosidase activity (Promega, Madison, WI) as described previously [24].

2.4. Activin-responsive promoter assay

The CAGA-lux construct has been described previously [25]. CAGA-lux, cytomegalovirus β-galactosidase, and pcDNA3, pcDNA3-FLAG-hAIRP2, control siRNA or hARIP2 siRNA were introduced into 293T cells and MCF-7 cells using the Calcium phosphate cell transfection reagent according to the manufacturer's protocol. Stimulation by activin (50 ng/mL) and measurement of luciferase activity using FLUOstar OPTIMA (BMG LABTECH, Offenburg, Germany) were performed as described previously [24].

2.5. Antibodies

To make polyclonal anti-hARIP2 antibodies, recombinant COOH-terminal hARIP2 (nucleotides 416–676) was purified and used for immunization of New Zealand white rabbits. The specific antisera were purified by passing through protein A Sepharose (Amersham Biosciences) and used for immunohistochemical analysis. The anti-FLAG (M2) antibody was purchased from Sigma and anti-GAPDH antibody was purchased from Kangcheng Company (China).

2.6. Western blot analysis

MCF-7 cells and MDA-MB-231 cells were transfected with indicated plasmids using Gene Companion™ II cell transfection reagent (Genetrans, China) according to the manufacturer's protocol. Two days after transfection, MCF-7 cells and MDA-MB-231 cells were washed three times in PBS, followed by lysis in a lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM NaF, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin and 2 μg/mL aprotinin]. The lysate was centrifuged and the protein concentration of each supernatant was determined by Coomassie protein assay reagent (Beyotime Biotechnology, Jiangsu, China). Equal amounts of proteins were then separated by SDS–PAGE and transferred onto a polyvinylidene difluoride membrane. Then the membranes were incubated with anti-GAPDH and anti-hARIP2 antibodies followed by incubation with horseradish peroxidase conjugated secondary antibodies. The specific proteins were detected by ECL (Beyotime Biotechnology, Jiangsu, China).

2.7. Cell proliferation analysis

MCF-7 cells and HepG2 cells were transfected with pcDNA3 (control), pcDNA3-FLAG-hARIP2, control siRNA, and hARIP2 siRNA using the Gene Companion™ II cell transfection reagent. 24 h after transfection, the cells were seeded in triplicate in 96-well plates at a density of 1×10^4 cells per well with DMEM medium (Gibco) containing 10% FBS. The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays were done at 24, 48, 72 and 96 h. Twenty microliters of MTT stock solution (5 mg/mL in PBS, Sigma) were then added to each well and the plates were further incubated for 4 h. After incubation, 150 μl of dimethyl sulphoxide (DMSO) was added to each well, mixed by vigorous pipetting

Table 1
Characteristics of breast tumors.

Characteristics	Data
Lymph-node status, *n* (%)	
Negative	23(48.9)
Positive	24(51.1)
Unknown	0(0)
Tumor size, *n* (%)	
0–2 (cm)	13(27.7)
2–5 (cm)	29(61.7)
>5 (cm)	5(10.6)
Histology, *n* (%)	
SC	26(55.3)
IK	15(31.9)
MA	6(12.8)

Abbreviations: SC, simple carcinoma; IDC, infiltrating ductal carcinoma; MA, mucinous adenocarcinoma; BH, benign hyperplasia; FA, fibroadenoma.

and incubated for 15 min at 37 °C. Absorbances were measured immediately at 570 nm.

2.8. Clonogenic assay

The effects of hARIP2 on the proliferation of single MCF-7, MDA-MB-231 or HepG2 cells were assessed by the colony formation assay which was performed as described [26]. Briefly, MCF-7, MDA-MB-231 and HepG2 cells were transfected with control, hARIP2, control siRNA or hARIP2 siRNA for 24 h and collected by trypsinization. The cells were counted and seeded into 6-well plates with 1000 cells/well. The cells were cultured for 14 days with growth media being replaced every 3 days. The cells were then stained with 0.5% crystal violet (in methanol–water, 1:1) and counted under the microscope (Olympus, Japan). An aggregate composed of more than 50 cells was recognized as a colony.

2.9. Immunohistochemical analysis of hARIP2 expression in benign and cancerous breast tissues

Paraffin embedded breast tissue sample blocks were collected from the Department of Pathology and Pathophysiology of Jilin University. The tissue materials included 27 cases of simple carcinoma, 14 cases of invasive ductal carcinoma, six cases of breast mucinous adenocarcinoma, 34 cases of breast hyperplasia and 43 cases of breast fibroadenoma. Patient information and tumor pathology have been summarized in Table 1. Immunohistochemical images of the sections were captured under a microscope and photographed. Immunoreactivity was scored on an ordinal scale of 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining) for the protein of hARIP2 evaluated. In order to diminish interobserver variability, all of the cases were scored in a blinded fashion.

For immunohistochemistry assays, breast tissues were cut into 5 μm thick sections and mounted on poly-(L-lysine)-coated slides. After deparaffinization and epitope retrieval in 10 mM citrate buffer, the non-specific binding sites were blocked by incubating the section in 5% normal rabbit serum in PBS (pH 7.4) for 15–20 min. After washing in PBS, the sections were incubated with the primary antibody anti-hARIP2 at a 1:500 dilution in PBS/1% BSA for 12 h at 4 °C or mouse IgG as control. After extensive washing with PBS, the biotinylated secondary antibody was added onto the sections and incubated at room temperature for 20 min. After washing in PBS, the sections were incubated with a peroxidase conjugated avidin-biotin complex for 20 min at room temperature. Then the reaction product was developed using 3,3-diaminobenzidine tetrahydrochloride and hydrogen peroxide in PBS. The sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in histosol and coverslips mounted using neutral balsam.

```
ATGAACGGAA GAGTGGATTA TTTGGTCACT GAGGAAGAGA TCAATCTTAC CAGAGGGCCC    60
M  N  G  R  V  D  Y  L  V  T  E  E  E  I  N  L  T  R  G  P
TCAGGGCTGG GCTTCAACAT CGTCGGTGGG ACAGATCAGC AGTATGTCTC CAACGACAGT   120
S  G  L  G  F  N  I  V  F  F  T  D  Q  Q  Y  V  S  N  D  S
GGCATCTACG TCAGCCGCAT CAAAGAAAAT GGGGCTGCGG CCCTGGATGG GCGGCTCCAG   180
G  I  Y  V  S  R  I  K  E  N  G  A  A  A  L  D  G  R  L  Q
GAGGGTGATA AGATCCTTTC GGTAAATGGC CAAGACCTAA AGAACCTGCT GCACCAGGAT   240
E  G  D  K  I  L  S  V  N  G  Q  D  L  K  N  L  L  H  Q  D
GCTGTAGACC TCTTTCGTAA TGCAGGCTAT GCTGTGTCTC TGAGAGTGCA GCACAGGTTG   300
A  V  D  L  F  R  N  A  G  Y  A  V  S  L  R  V  Q  H  R  L
CTGATTGTTG GAGGTTCTTT TGGTCTTCGT GAGTTTTCTC AAATCCGATA TGATGCTGTG   360
L  I  V  G  G  S  F  G  L  R  E  F  S  Q  I  R  Y  D  A  V
AAGAGTAAAA TGGATCCTGA GCTTGAAAAA AAACTGAAAG AGAATAAAAT ATCTTTAGAG   420
K  S  K  M  D  P  E  L  E  K  K  L  K  E  N  K  I  S  L  E
TCGGAATATG AGAAAATCAA AGACTCCAAG TTTGATGACT GGAAGAATAT TCGAGGACCC   480
S  E  Y  E  K  I  K  D  S  K  F  D  D  W  K  N  I  R  G  P
AGGCCTTGGG AAGATCCTGA CCTCCTCCAA GGAAGAAATC CAGAAAGCCT TAAGACTAAG   540
R  P  W  E  D  P  D  L  L  Q  G  R  N  P  E  S  L  K  T  K
ACAACTTGAC TCTGCTGA                                                 558
T  T
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Fig. 1. Nucleotide and amino acid sequences of hARIP2. The amino acid sequence is shown below in the single-letter code. The PDZ domain of hARIP2 is indicated by the underline. Nucleotides are numbered on the right.

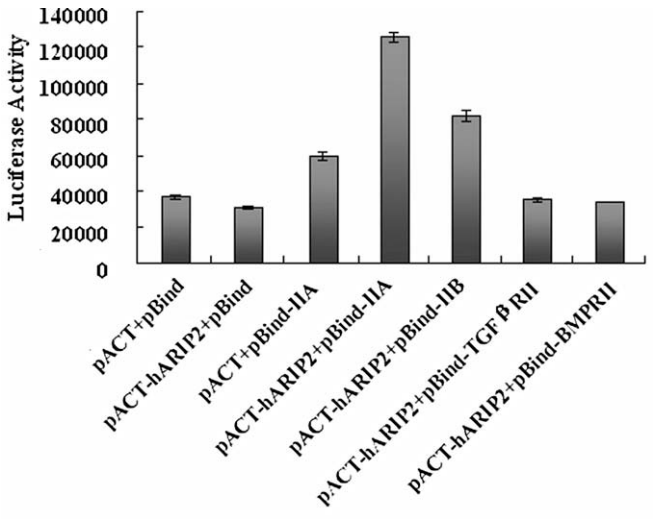

Fig. 2. hARIP2 Interacts with ActRIIs. 293 cells were cotransfected with the plasmids of interest, a cytomegalovirus promoter-driven β-galactosidase and a reporter plasmid, pG5luc, which drives the luciferase gene under the control of GAL4-responsive promoter. Luciferase activity was measured and normalized to the β-galactosidase activity.

2.10. Statistical analysis

All in vitro experiments were repeated at least three times. Each result was the reflection of the mean of three independent experiments. The statistical significance of the differences between groups analysed was determined by the Student's *t*-test. Comparisons resulting in a *P* value of less than 0.05 were considered statistically significant and identified in the figures with an asterisk (∗).

3. Results

3.1. Identification of hARIP2

In a previous study a novel mouse protein named mouse ARIP2 was identified [27]. The mouse ARIP2 cDNA is composed of 862 bp, encodes a protein of 153 amino acids, and contains a single PDZ domain in its NH2-terminal region. The result of data base searching for ARIP2-related sequences revealed the presence of a highly homologous sequence in expressed sequence tags in human tissue (GenBank Accession No. BM563433) which is likely to be a human counterpart of ARIP2 [27]. In this study we cloned human ARIP2 encoding sequences from normal human placenta tissue by RT-PCR. The coding sequence of hARIP2 is composed of 558 bp, encodes a protein of 182 amino acids, and contains a single PDZ domain in its NH2-terminal region (Fig. 1).

3.2. Human ARIP2 interacts with ActRIIs

Since a previous study reported that mouse ARIP2 interact with ActRIIs via its PDZ domain, and hARIP2 has the same PDZ domain as mouse ARIP2 [27], we investigated the interaction of hARIP2 with ActRIIs by mammalian two-hybrid assay. The results showed

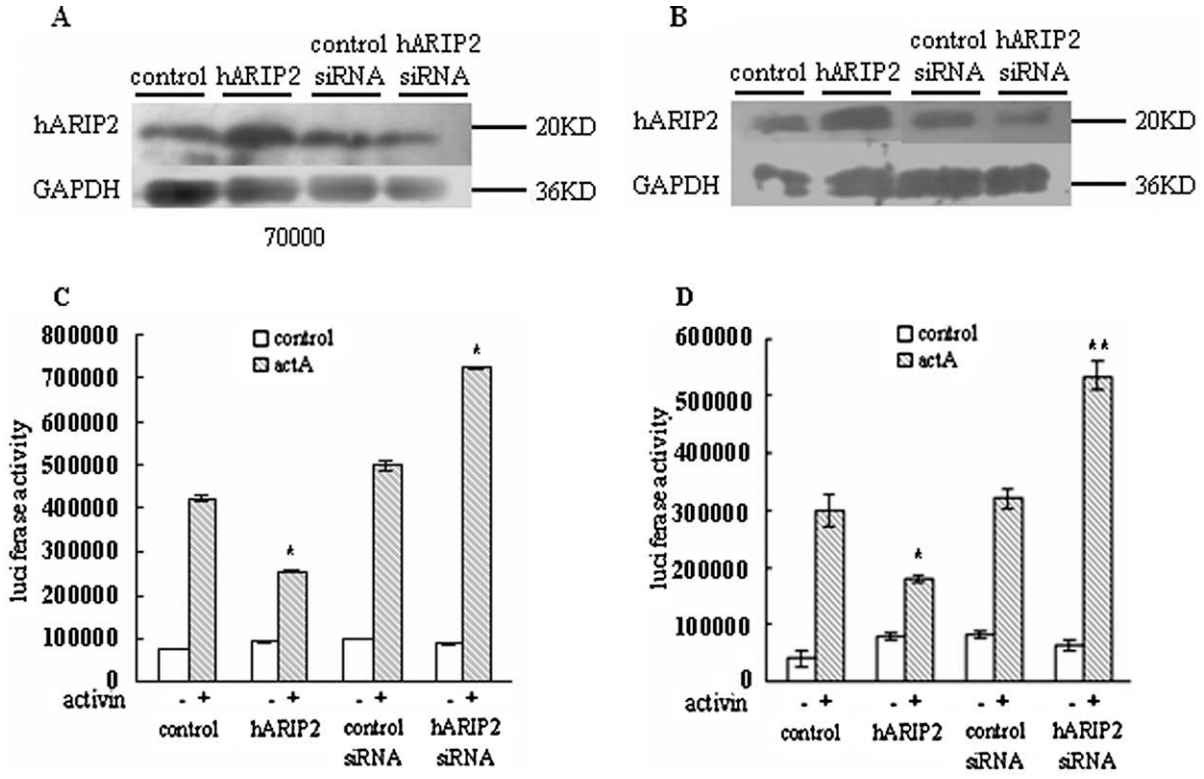

Fig. 3. Effect of hARIP2 on activin-induced transcription. pcDNA3-hARIP2 and hARIP2 siRNA have opposite effects on activin-induced transcription in MDA-MB-231 cells and MCF-7 cells. (A) MDA-MB-231 cells were transfected with control siRNA or hARIP2 siRNA and analysed by western blotting. Equal amounts of cell lysates were analysed by western blotting with anti-GAPDH and affinity-purified anti-hARIP2 antibodies. (B) MCF-7 cells were transfected with control siRNA or hARIP2 siRNA and analysed by western blotting. Equal amounts of cell lysates were analysed by western blotting with anti-GAPDH and affinity-purified anti-hARIP2 antibodies. (C) MDA-MB-231 cells were transfected with CAGA-lux, cytomegalovirus-β-galactosidase, and either control, pcDNA3-hARIP2, control siRNA or hARIP2 siRNA. (D) MCF-7 cells were transfected with CAGA-lux, cytomegalovirus-β-galactosidase, and either control, pcDNA3-ARIP2, control siRNA or hARIP2 siRNA then incubated with 50 ng/mL activin A for 24 h. The level of luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values in the figure represent the means and SD of triplicate determinations ($^{*}P < 0.05$ vs. activin-treated cells with control or control siRNA by using a *t*-test).

that human ARIP2 interacted with both ActRIIA and ActRIIB. In contrast, hARIP2 did not show interaction with either TGF-β type II receptor or bone morphogenetic protein type II receptor (Fig. 2). These results suggest that hARIP2 may have specific roles in activin signaling.

3.3. The effect of hARIP2 on activin-induced transcriptional response

HEK293T cells and MCF7 cells were transfected with either pcDNA3, pcDNA3-ARIP2, control siRNA or hARIP2 siRNA together with a reporter plasmid, CAGA-lux, and activin-induced luciferase activity was measured. Overexpression of ARIP2 in MDA-MB-231 cells and MCF7 cells (Fig. 3) decreased activin-induced transcriptional activity. In contrast, inhibiting human ARIP2 expression through RNA interference increased activin-induced transcriptional activity. These data suggest that hARIP2 has an inhibitory effect on activin-induced transcriptional response.

3.4. Effect of hARIP2 on tumor cell proliferation

To examine the roles of hARIP2 in human tumor cell proliferation, we transfected MCF-7 and MDA-MB-231 cells with pcDNA3,

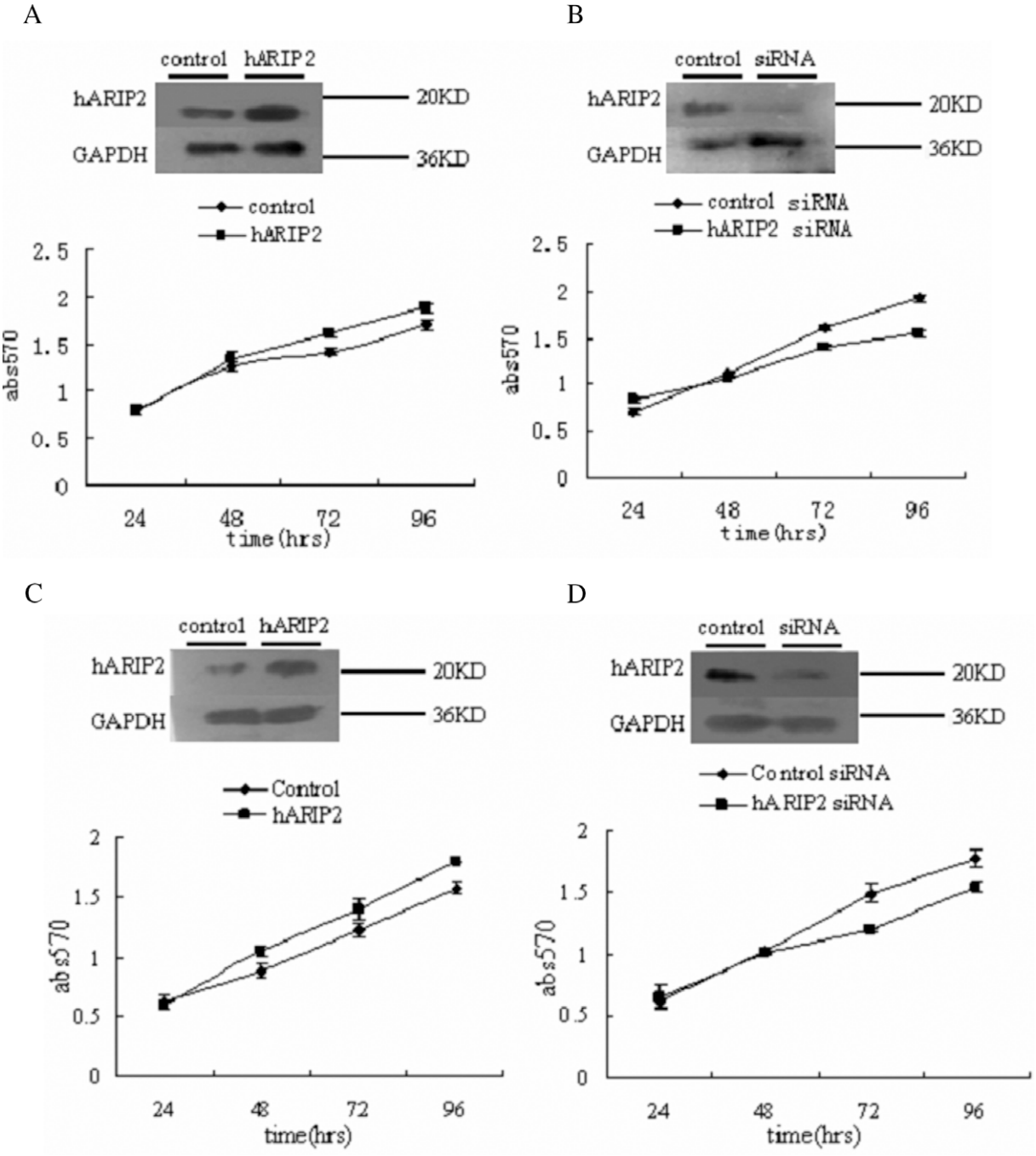

Fig. 4. In vitro effects of hARIP2 on the proliferation of MCF-7 and MDA-MB-231 cells. (A) Growth curves for control and hARIP2-transfected cells in vitro proliferation assays for MCF-7 cells by MTT assay. (B) Growth curves for control siRNA and hARIP2 siRNA-transfected cells in vitro proliferation assays for MCF-7 cells by MTT assay. (C) Growth curves for control and hARIP2-transfected cells in vitro proliferation assays for MDA-MB-231 cells by MTT assay. (D) Growth curves for control siRNA and hARIP2 siRNA-transfected cells in vitro proliferation assays for HepG2 cells by MTT assay. MTT assays were done at 24, 48, 72 and 96 h. The absorbances were measured immediately at 570 nm. Each data is expressed as the mean ± SD obtained from triplicate experiments.

pcDNA3-FLAG-hARIP2, control siRNA or hARIP2 siRNA. Proliferation activities shown in Fig. 4 were the means of three independent experiments. Results of the MTT assay showed that overexpression of hARIP2 increased cell proliferation, and inhibiting hARIP2 expression by RNA interference decreased cell proliferation. Similar results were obtained in SGC7901 cells (data not shown). These results indicate that hARIP2 enhanced human tumor cell proliferation.

3.5. Effect of hARIP2 on tumor cell colony formation

Next, we wanted to evaluate the effect of hARIP2 on the reproductive potential of a single cell by colony formation assay. We transfected MCF-7, HepG2 and MDA-MB-231 cells with pcDNA3, pcDNA3-FLAG-hARIP2, control siRNA or hARIP2 siRNA. As shown in Fig. 5, overexpression of hARIP2 increased cell colony formation ability and inhibition of hARIP2 expression by RNA interference decreased cell proliferation. These results suggest that hARIP2 stimulates colony formation in MCF-7, HepG2 and MDA-MB-231 cells.

3.6. Distribution of hARIP2 in benign and malignant breast tissues

hARIP2 was detected in both benign and malignant breast tissues. Omission of the primary antibody was used as a negative control (Fig. 6A). Intense staining was detected in cancers including simple carcinoma, invasive ductal carcinoma and mucinous adenocarcinoma (Fig. 6B–D). Moderate to low staining levels were found in benign hyperplasia and fibroadenoma (Fig. 6E–F). A statistical

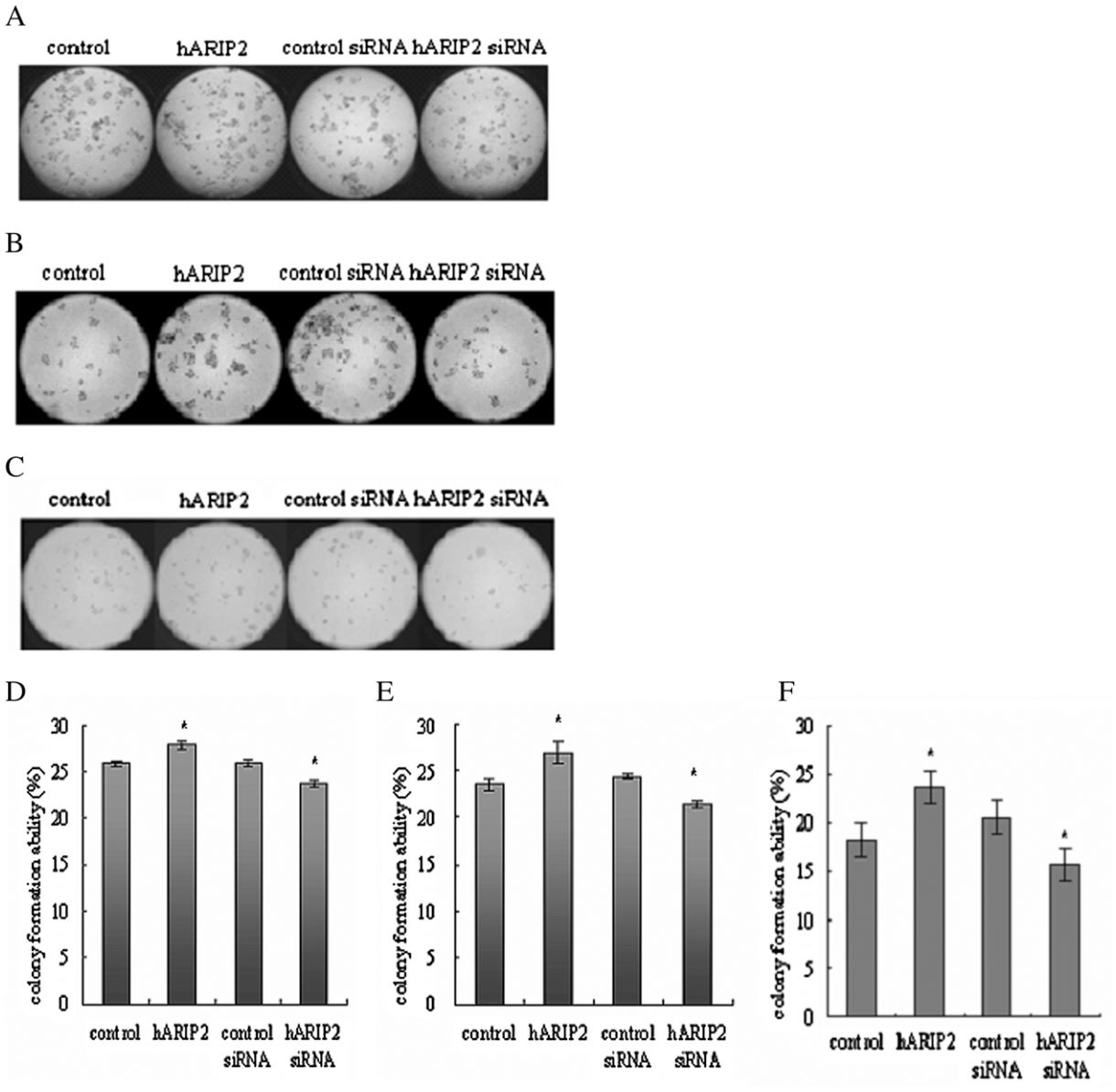

Fig. 5. Effects of hARIP2 on colony formation in MCF-7, HepG2 and MDA-MB-231 cells. The cells transfected with control, hARIP2, control siRNA and hARIP2 siRNA, respectively, were cultured for 14 days. Formed colonies were counted under the microscope after staining with 0.5% crystal violet. An aggregate consisting of more than 50 cells was counted as a colony. (A) MCF-7 cells were then replated in fresh media and assessed for clonogenic survival. (B) HepG2cells were then replated in fresh media and assessed for clonogenic survival. (C) MDA-MB-231 cells were then replated in fresh media and assessed for clonogenic survival. The colony formation rate was calculated by comparing the colony number of transfected cells. (D) The colony formation rate of MCF-7 cells transfected with control, hARIP2, control siRNA and hARIP2 siRNA, respectively. (E), The colony formation rate of HepG2 cells transfected with control, hARIP2, control siRNA and hARIP2 siRNA, respectively. (F) The colony formation rate of MDA-MB-231 cells transfected with control, hARIP2, control siRNA and hARIP2 siRNA, respectively. Each data is expressed as the mean ± SD obtained from triplicate experiments ($^{*}P < 0.05$ vs. control or control siRNA by using a *t*-test).

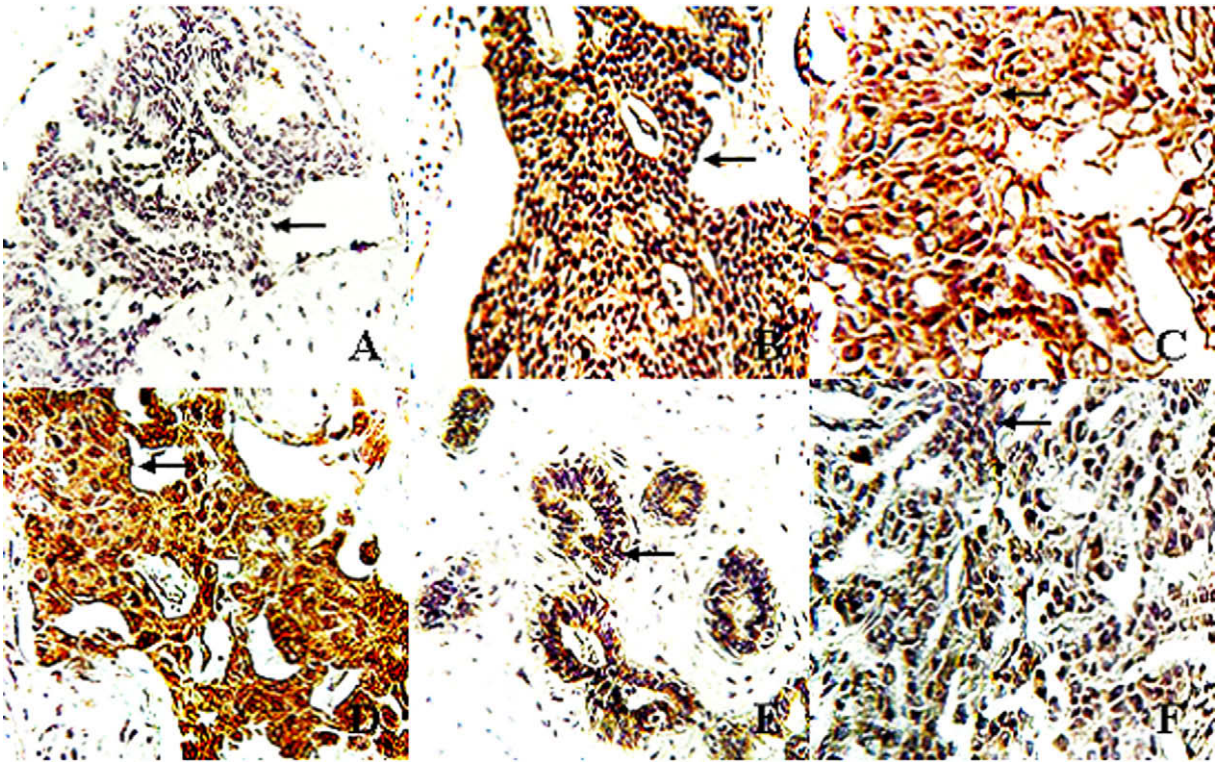

Fig. 6. hARIP2 immunostaining in malignant and normal tissues. Tissue stained brown is positive for immunoreactivity. Nuclei are stained blue. (A) Negative control treated without primary antibody. (B) Simplex carcinoma. (C) Mucinous adenocarcinoma. (D) Invasive ductal carcinoma. (E) Fibroadenoma. (F) Benign hyperplasia. Original magnification is 20× for all panels. Arrows denote the results of cell staining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

analysis of the intensity scores for hARIP2 immunoreactivity indicated that hARIP2 was more frequently and much more intensely expressed in malignant tissues (81.43% simple carcinoma; 80% invasive ductal carcinoma; and 100% mucinous adenocarcinoma; Table 2) than it was in benign hyperplasia and fibroadenoma cases (29.41% benign hyperplasia; 39.53% fibroadenoma; Table 2). Furthermore, our result shows that the abundance of hARIP2 is proportional to the presence of lymph-node metastasis and tumor size in breast cancer (Tables 3 and 4).

4. Discussion

ARIP2. regulates endocytosis of activin type II receptors through a Ral/Ral-binding protein 1-dependent pathway [27]. Expression of ARIP2 enhances endocytosis of ActRIIs and suppresses activin-induced transcription. In this work we describe the cloning and characterization of a human homologue of mouse ARIP2, hARIP2. hARIP2 cDNA encodes a protein of 182 amino acids that contains a single PDZ domain in its NH2-terminal region.

Table 2
Correlations for intense hARIP2 and clinical and pathological markers of breast cancer.

Tissues	hAMP2 Staining intensity		hARIP2 positive rate (%)	*P*
	0, 1 (No or weak)	2, 3 (Moderate or strong)		
Simple carcinoma infiltrating ductal	4	22	84.61	0.002
Carcinoma	3	12	80	0.006
Mucinous adenocarcinoma	0	6	100	0.003
Benign hyperplasia	26	17	29.41	
Fibroadenoma	24	10	39.53	

Since a previous study [27] revealed that mouse ARIP2 interacts with ActRIIs via its PDZ domain, and hARIP2 has the same PDZ domain as mouse ARIP2, we investigated the interaction of hARIP2 with ActRIIs by mammalian two-hybrid assay. The results confirmed our hypothesis that hARIP2 interacted with both ActRIIA and ActRIIB. In contrast, hARIP2 did not show interaction with either TGF-β type II receptor or bone morphogenetic protein type II receptor (Fig. 2). These results suggest that human ARIP2 may have specific roles in activin signaling. Therefore, we further investigated the effect of hARIP2 on activin-induced transcriptional response. The results showed that overexpression of hARIP2 in MDA-MB-231 cells and MCF7 cells decreased activin-induced transcriptional activity (Fig. 3C and D) and inhibition of hARIP2 expression through RNA interference (Fig. 3A and B) increased activin-induced transcriptional activity (Fig. 3C and D). These data suggest that hARIP2 has an inhibitory effect on activin-induced transcriptional response.

Activin is a member of the TGF-β superfamily that inhibits the proliferation of breast cancer cells. Activin functions by interacting with its type I and type II receptors to induce phosphorylation of intracellular signaling molecules known as Smads. Since hARIP2 can interact with ActRIIs and inhibit activin-induced transcription response, we hypothesized that hARIP2 may have an effect on breast cell proliferation. To prove this hypothesis, we overexpressed hARIP2 in MCF7 cells and MDA-MB-231 cells and measured cell proliferation by MTT assay. The results revealed that hARIP2 can slightly induce cell proliferation. To confirm this result, we inhibited the expression of hARIP2 by introducing a hARIP2 specific siRNA expression vector into MCF7 cells and then measured cell

Table 3
Correlation for intense hARIP2 and lymph-node status of breast cancer.

Lymph-node status	hARIP2 Staining Intensity		Positive rate (%)
	0, 1 (No or weak)	2, 3 (Moderate or strong)	
0–3	9	28	75.68
4–90	0	4	100
⩾10	0	6	100

Table 4
Correlation for intense hARIP2 and tumor size of breast cancer.

Tumor size	hARIP2 Staining intensity		Positive rate (%)
	0, 1 (No or weak	2, 3 (Moderate or strong)	
<2 (cm)	7	6	46.15
2∼5 (cm)	2	27	93.10
>5 (cm)	0	5	100

proliferation. The result indicated the cell proliferation was slightly reduced. Those results suggested that hARIP2 may be partially involved in breast tumorigenesis and tumor development. In contrast to the dramatic differences in hARIP2 staining between malignant and non-malignant primary breast specimens, the effects of ectopic overexpression of hARIP2 (or its siRNA) on proliferation and colony formation of MCF-7 cells and MDA-MB-231cells in culture were relatively modest (Figs. 4 and 5). We conclude that transfection efficiency might be limiting the magnitude of the observed effect in these experiments. Further experiments are required to confirm this.

A recent study revealed that activin receptors and signaling molecules are present in human breast cancer cell lines. When there is the loss or reduction of activin signaling components, the breast cancer becomes more aggressive. However, the distribution of hARIP2 in the human breast is still unclear.

In the present study we collected five kinds of human breast tissue samples, including simple carcinoma, invasive ductal carcinoma, breast mucinous adenocarcinoma, breast hyperplasia and breast fibroadenoma. Then we investigated the expression of hARIP2 in these tissues by immunohistochemistry. The results show that hARIP2 was more frequently and much more intensely expressed in malignant tissues than it was in benign hyperplasia and fibroadenoma cases (Table 2). In addition, a significant association between hARIP2 staining and the presence of lymph-node metastasis in breast cancer was also observed (Table 3). However, we do not know whether hARIP2 is an accelerator of metastasis or merely a correlative product during progression of breast cancer. Likewise, our result shows that the abundance of hARIP2 is proportional to tumor size (Table 4). In short, the breast cancer stage correlation with hARIP2 is intriguing. hARIP2 staining score was much higher in higher degree of malignancy, increased number of lymph-node metastasis, and bigger tumor size. But we still do not understand the exact mechanism by which hARIP2 plays its role in the progression of breast cancer.

Currently, there is great interest in the development of clinical applications for the members of the TGF-β superfamily and their antagonists [1]. Clinical trials of activin or activin antagonists have not yet started; however, the efficacy of these factors in animal disease models suggests that they might also prove beneficial in treating human diseases [28,29]. According to our studies, hARIP2 is a regulator of activin signaling. Therefore, hARIP2 can also act as a modulator in some activin-induced cancer. Of particular interest, our results suggest that hARIP2 might even represent a potential target for treatment of some human diseases.

Although we identified hARIP2 and confirmed some functions of hARIP2, there are still questions to be answered. The detailed mechanism by which hARIP2 exerts its role in activin signaling is not yet clear. Our current data cannot exclude the possibility that hARIP2 has other roles. Therefore, future studies of hARIP2 might shed light on the exact molecular mechanisms of the activin signal transduction pathway. At the same time, further work is required in order to determine whether hARIP2 has other roles or is involved in other signaling pathways.

In summary, we have cloned the hARIP2 gene encoding region and have characterized its involvement in breast cancer. hARIP2 has a PDZ domain in its NH2-terminal region and this domain can interact specifically with ActRIIs. Further study revealed that hARIP2 reduces activin-induced transcriptional activity in human breast cancer (MCF7) cells and MDA-MB-231 cells and enhances cell proliferation and colony formation in human breast cancer (MCF7) cells, MDA-MB-231cells and human hepatocellular liver carcinoma (HepG2) cells. Immunohistochemistry results indicate that hARIP2 was more frequently and much more intensely expressed in malignant tissues compared to benign hyperplasia and fibroadenoma cases. These results suggest that hARIP2 is a putative growth-promoting factor that might be involved in breast tumorigenesis and tumor development.

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