

# LncRNA AIRN influences the proliferation and apoptosis of hepatocellular carcinoma cells by regulating STAT1 ubiquitination

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**Abstract** Long non-coding RNAs (LncRNAs) have been implicated in the pathogenesis of various human diseases. In this study, we probed into the role and potential mechanisms of the antisense of IGF2R non-protein coding RNA (LncRNA AIRN) in the progression of hepatocellular carcinoma (HCC). Using a quantitative real-time polymerase chain reaction, we corroborated that LncRNA AIRN expression was raised in the HCC tissues and cells. The bioinformatic analysis revealed that a potential interaction between LncRNA AIRN and STAT1, which was verified by the RNA pull-down and RNA immunoprecipitation. In the cycloheximide-chase assay, the knockdown of LncRNA AIRN enhanced the stability of STAT1 protein. In the immunoprecipitation assay, the knockdown of LncRNA AIRN restrained the cullin 4A (CUL4A)-mediated ubiquitination of STAT1 protein. The cell transfection, MTT and flow cytometry assays expounded that the LncRNA AIRN/STAT1 axis was bound up with the regulation of the proliferation and apoptosis of HCC cells. The *in vivo* experiments corroborated that the knockdown of LncRNA AIRN restrained the tumor growth of HCC. Our data expounded that the knockdown of LncRNA AIRN restrained HCC cell proliferation and boosted cell apoptosis by restraining the CUL4A-mediated ubiquitination of STAT1 protein.

**Keywords** LncRNA AIRN · HCC · CUL4A · STAT1 · Ubiquitination

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## Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies (Thomas et al. 2010). Due to its high incidence and metastasis rate, HCC is a serious health problem in society (Shariff et al. 2009). Although some advancements have been gained in the treatment of HCC, the overall five-year survival rate of HCC patients is still low (El-Serag 2011). HCC-related molecular pathways have been considered as the potential therapeutic targets in HCC (Gnoni et al. 2015). Therefore, there is an urgent need for new insights into the progression of HCC to discover potential targets and ameliorate HCC outcomes.

Long non-coding RNA (LncRNA), with a length greater than 200 nucleotides, is a type of RNA that cannot encode proteins (He et al. 2014). An increasing number of abnormally expressed LncRNAs have been corroborated to regulate the progression of HCC. For instance, the highly expressed LncRNA MCM3AP-AS1 boosts the growth of HCC by targeting the miR-194-5p/FOXA1 axis (Wang et al. 2019), and LncRNA LNC473 recruits the deubiquitinase USP9X to restrain the ubiquitination level of survivin and then raises the expression of the latter, thereby functioning as an oncogene in HCC (Chen et al. 2018). Notably, Oliva et al. (2009) corroborated that LncRNA AIRN, also known as LncRNA antisense IGF2r or LncRNA AIR, is an imprinted gene transcribed from the paternal chromosome that is raised in the progression of liver tumors (Seidl et al. 2006), and we had previously predicted that LncRNA AIRN was bound up with liver cancer both in humans and mice through the MNDR database. However, the function of LncRNA AIRN in HCC and its potential mechanism remains unclear.

STAT1 is a member of the family of signal transduction and transcription factors that can be activated in various malignancies (Lim et al. 2006). STAT1 is a tumor

suppressor, while STAT3 has cancer-promoting functions, STAT1 and STAT3 activation are reciprocally regulated and their imbalanced expression or phosphorylation disorder may transfer cytokine/growth factor signals from proliferative to apoptotic, or from inflammatory to anti-inflammatory (Regis et al. 2008). Notably, a research expounds that the high expression of STAT1 restrains HCC cell proliferation and boosts cell apoptosis (Chen et al. 2013). Therefore, raising the expression of STAT1 to ameliorate HCC has become a focus of this research. Recently, increasing evidence has expounded that the ubiquitin-proteasome system has important regulatory functions in tumor progression (Pallante et al. 2013; Wu et al. 2014). As reported, E3 ubiquitin ligase SLIM interacts with STAT1 and lessens the latter's expression via mediating STAT1 ubiquitination (Tanaka et al. 2005). Notably, CUL4A is a member of the E3 ubiquitin ligase that decreases STAT1 expression by mediating STAT1 ubiquitination (Ulane et al. 2002). Therefore, the restraint of the CUL4A-mediated ubiquitination of STAT1 protein to raise STAT1 might contribute to restraining HCC progression.

In the present study, LncRNA AIRN expression was raised in the HCC tissues and cells and the knockdown of LncRNA AIRN restrained HCC cell proliferation and boosted cell apoptosis via restraining the CUL4A-mediated ubiquitination of STAT1 protein.

## Materials and methods

### Ethical approval

Our research was approved by the Medical Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (No. 2017029). All subjects provided signed informed consent and followed the Declaration of Helsinki, except for registration in a database (clause 35). The inclusion criteria for this study are as follows: (a) Patients diagnosed with HCC given the American Association for the Study of Liver Disease and the European Association for the Study of Liver HCC management guidelines (Bruix et al. 2011). (b) HCC patients with extrahepatic metastases; and (c) patients aged 18–70 years old. The exclusion criteria are as follows: (a) pregnancy; (b) cardiac insufficiency and severe pulmonary dysfunction; and (c) life expectancy of fewer than 3 months.

All the animal experiments were conducted under the approval of the Institutional Animal Care and Use Committees of the National Health Research Institutes. Our research was approved by the Medical Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (No. 2017029).

### Patients and tissue samples

Cancer tissues and adjacent normal tissues in patients with HCC ( $n = 20$ ) were acquired from the First Affiliated Hospital of Wenzhou Medical University. All tissue samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The clinicopathological characteristics of the patients are exhibited in Table 1.

### Cell culture

Normal hepatocytes QSG-7701, human HCC cells HepG2 and Huh7 were from Procell (Wuhan City, Hubei Province, China). All cells applied for the experiments underwent 1 to 3 passaging. The QSG-7701 cells were put in RPMI-1640 medium with the addition of 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, California, USA), and cultured in an environment of 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . The HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the addition of 10% FBS in a humidified incubator at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ . The Huh7 cells were seeded in a DMEM medium with the addition of 10% FBS, penicillin, and streptomycin, and then cultured at  $37^{\circ}\text{C}$  in a humid chamber with the addition of 5%  $\text{CO}_2$ . The 293T cells were put in a high glucose DMEM medium with the

**Table 1** The clinicopathological characteristics of 20 HCC patients

Variable	$n = 20$	
	Number	Percentage (%)
Age (years)		
$\leq 55$	15	75.0
$> 55$	5	25.0
Sex		
Male	18	90.0
Female	2	10.0
Tumor differentiation		
I–II	17	85.0
III–IV	3	15.0
TNM stage		
I–II	14	70.0
III–IV	6	30.0
Tumor size (cm)		
5	9	45.0
$> 5$	11	55.0
Tumor number		
Solitary	16	80.0
Multiple	4	20.0
Liver cirrhosis		
Without	6	30.0
With	14	70.0

addition of 10% FBS, and cultured in an environment of 5% CO<sub>2</sub> at 37 °C.

### Cell transfection

The HCC cells HepG2 and HUH7 ( $1 \times 10^5$ ) were seeded in a 24-well plate until the confluence reached nearly 70%. Next, the synthetic shRNA-AIRN and/or shRNA-STAT1, and their corresponding controls (GenePharma, Shanghai, China) were transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) given the reagent manufacturer's instructions. After 48 h of transfection, the transfection efficiency was tested through quantitative real-time polymerase chain reaction (qRT-PCR). The sequences of shRNA AIRN and shRNA STAT1 were exhibited: shRNA AIRN: sense: 5'- CAUGUUACUGG UGAUCAAGG-3', Antisense: 5'- UUGAUCACCAGU AACAUUGAA-3'; shRNA STAT1: sense: 5'- GCGUAA UCUUCAGGAUAAUTT-3', Antisense: 5'- AUUAUC CUGAAGAUAACGCTT-3'.

### qRT-PCR

The total RNAs in the HCC patient tissue samples, normal liver cells, and HCC cells applied in this study were extracted by TRIzol reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the quality and concentration of the RNA were tested. The cDNA was synthesized by reverse transcription using the iScript cDNA synthesis kit (Roche, Shanghai, China). GAPDH was applied as an internal reference. Real-time PCR was conducted using the SYBR Green PCR Master Mix (TaKaRa, Beijing, China) and ABI 7500 Fast Real-Time PCR system. The 2 delta-delta threshold cycle ( $2^{-\Delta\Delta C_t}$ ) method was applied to test the relative expression. The primers applied for the qRT-PCR are exhibited in Table 2.

**Table 2** The sequence of the primers used for the qRT-PCR

Gene name	Primer sequence
AIRN	Forward: 5'-TGGCAGCCCATAGTGGTGTGA-3' Reverse: 5'-CTCGCATTGCCGCGCTTAC-3'
STAT1	Forward: 5'-CGCTCTGCTGTCTCCGCTTCCACTCC-3' Reverse: 5'-AGTGTGATCCAAGCAGCATTGG-3'
$\beta$ -actin	Forward: 5'-AGTGTGACGTGGACATCCGCAAAG-3' Reverse: 5'-ATCCACATCTGCTGGAAGGTGGAC-3'

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT was applied to test the viability of HCC cells. The shRNA, shRNA-AIRN and/or shRNA-STAT1 were transfected into HCC cells HepG2 and Huh7, respectively. After 48 h of transfection, the HCC cells ( $1 \times 10^3$ ) were grown in a 96-well plate for 24 h, and then the cells were gently washed with PBS. The MTT solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was put in each well and incubated for 4 h. Thereafter, Dimethyl sulfoxide (DMSO) was added to each well. The optical density (OD) value was tested at 570 nm.

### Clone formation assay

The differently treated HCC cells HepG2 and HUH7 ( $1 \times 10^3$ ) were put in a six-well plate and cultured for another 2 weeks. Next, the colonies were fixed with 4% paraformaldehyde for nearly 10 min and then stained with 0.4% crystal violet (Solarbio, Beijing, China) for nearly 5 min. The number of colonies was counted using ImageJ and photographed for preservation.

### Flow cytometry analysis

To assess the apoptosis of HCC cells HepG2 and Huh7, the cells ( $1 \times 10^5$ ) were put in six-well plates, and then the cells were harvested after 24 h of culture. The apoptosis of HCC cells was tested using the annexin V-FITC apoptosis assay kit (KeyGen, Nanjing, China) given the manufacturer's protocol. Briefly, after gently washing the cells three times with PBS, fluorescein isothiocyanate-labeled annexin V (FITC) and PI were added to each well, which was then incubated for about 15 min at 37 °C in the dark. The apoptosis rate of HCC cells was tested using flow cytometry and CellQuest software.

### Western blot

The HCC cells applied in this experiment were gathered and the cells were lysed on ice for nearly 30 min with RIPA lysate with the addition of a protease inhibitor. The Bradford method (Bio-Rad, Philadelphia, PA, USA) was applied to test the concentrations of proteins in the cell lysates. Proteins of different molecular weights were separated through SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk for nearly 1 h at room temperature and then the membranes were incubated overnight in TBST with the addition of the primary antibody and 5% bovine serum albumin. The membranes were then washed three times with TBST and incubated with the secondary antibody for about 2 h. The protein bands

were visualized using ECL chemiluminescent reagents. The antibodies applied in this experiment were exhibited: rabbit anti-STAT1 (ab234400, 1: 1000, Abcam) and rabbit anti- $\beta$ -actin (ab8226, 1: 1000, Abcam).

### RNA pull-down assay

An RNA pull-down assay was conducted using the Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) given the manufacturer's instructions. After gathering a large number of HepG2 cells transfected with biotinylated LncRNA AIRN, the cells were lysed with a lysis buffer at room temperature and incubated with biotinylated probes. The above cell lysate was mixed with streptavidin agarose beads (Life Technologies, Gaithersburg, MD, USA) overnight. The RNA-protein mixture was boiled in SDS buffer for nearly 10 min, and the STAT1 protein level in the LncRNA AIRN pull-down complex was tested by western blot.

### RNA immunoprecipitation (RIP)

The EZ-Magna RIP kit (Millipore, Birrica, Massachusetts, USA) was applied for RIP assay in this study and the experiment was conducted given the manufacturer's instructions. The gathered HepG2 cells were put in RIP buffer for lysis. The HepG2 cell extract was then incubated with magnetic beads conjugated to the STAT1 antibody (Abcam, ab26859) for nearly 6 h. The expression of LncRNA AIRN in the coprecipitation was tested by qRT-PCR.

### Cycloheximide (CHX)-chase assay

CHX is a protein synthesis inhibitor (Schneider-Poetsch et al. 2010). After shRNA-AIRN was transfected into HCC cells HepG2 and Huh7, each group of cells was treated with 10  $\mu$ g/mL CHX, a protein synthesis inhibitor (Widelitz et al. 1986), and the protein level of STAT1 was tested through western blot at 0, 3, 6, and 9 h, respectively.

### Immunoprecipitation (IP) and ubiquitination assay

HA-Ub + FLAG-STAT1 + shRNA and HA-Ub + FLAG-STAT1 + shRNA-AIRN were transfected into 293T cells, respectively, and the cells were treated with 2 mM MG132 for 16 h, and whole-cell lysates were prepared using a cell lysis buffer. The above whole cell lysate and antibody were incubated in immunoprecipitation buffer for 4 h and then incubated with immunomagnetic beads (Millipore, Birrica, Massachusetts, USA) overnight. The eluted protein was tested by western blot.

### Tumor formation assay in nude mice

Twelve male BALB/C nude mice aged 6 weeks were from the Shanghai Experimental Animal Research Center. The mice were placed in a temperature and humidity controlled environment and subjected to a 12 h light and dark cycle. All mice were always free to get water and food, and animal health and behavior were tested every 24 h. Subsequently, the HepG2 cells transfected with shRNA or shRNA-AIRN were resuspended in PBS. Mice were randomly divided into two groups, and each group was assigned to six mice. The above HepG2 cells ( $6 \times 10^6$ ) were subcutaneously inoculated into the left axilla of the mice. One week later, the diameter of the tumor was measured every other week with a vernier caliper. When the tumor volume was greater than 1000 mm<sup>3</sup>, the mice were euthanized. After 5 weeks, the mice were euthanized through an intraperitoneal injection of 150 mg/kg pentobarbital sodium and examined to determine whether they did not breathe spontaneously for 2–3 min and had no blink reflex. The tumor tissues were gathered for the subsequent experiments.

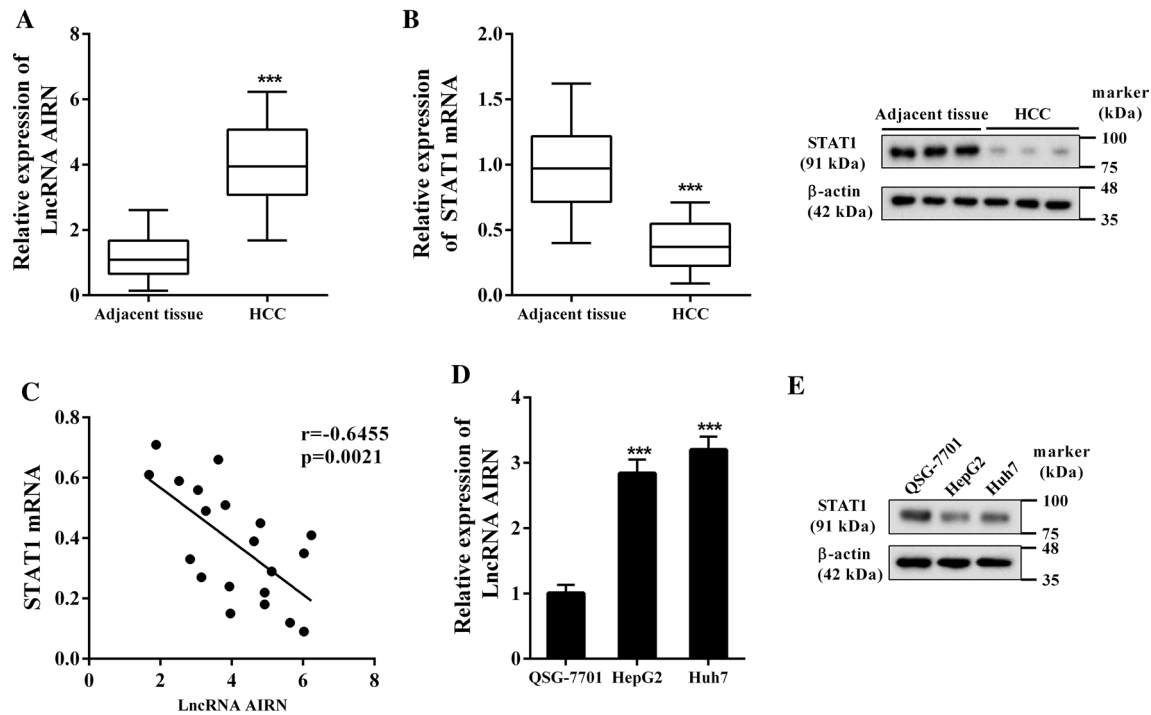
### Statistical analysis

SPSS software 16.0 (SPSS, Chicago, USA) was applied for all statistical analyses in this experiment. All data were expressed as mean  $\pm$  standard deviation of the values gathered from three independent experiments. The student's *t*-test was applied to assess the difference between the two groups, and ANOVA was applied to assess the difference when there were more than two groups. Moreover, for more than two samples, we applied the Kruskal–Wallis test analysis. The post hoc tests of the experimental group were assessed using Dunn's test. Pearson correlation coefficient analysis was applied to assess the correlation between LncRNA AIRN and STAT1 and the *r*-value was applied to evaluate the correlation of two variables. A *P* value < 0.05 was considered to be significantly different.

## Results

### Expressions of LncRNA AIRN and STAT1 in HCC tissues and cells

In the present study, qRT-PCR results demonstrated that LncRNA AIRN expression was raised in the HCC tumor tissues compared with the adjacent tissues of HCC patients (Fig. 1a). The qRT-PCR and western blot assays expounded that the mRNA and protein levels of STAT1 were lessened in the HCC tumor tissues compared with the adjacent tissues (Fig. 1b). Pearson correlation analysis revealed that the expression of LncRNA AIRN was negatively correlated



**Fig. 1** Different expressions of LncRNA AIRN and STAT1 in the hepatocellular carcinoma (HCC) tissues and cells. **a** Quantitative real-time PCR (qRT-PCR) was conducted to measure the expression of LncRNA AIRN in the HCC tissues. **b** qRT-PCR and western blot were applied to quantify the mRNA and protein levels of STAT1 in HCC tissues. **c** Pearson correlation analysis was conducted to assess the correlation between STAT1 and LncRNA AIRN in the tumor tissues of HCC patients. **d** qRT-PCR was conducted to test LncRNA AIRN expression in HCC cells. **e** Western blot was applied to measure the protein level of STAT1 in HCC cells. \*\*\* $P < 0.001$  (compared to the Adjacent tissue group or QSG-7701 group)

with the expression of STAT1 in the tumor tissues of HCC patients (Fig. 1c). Also, LncRNA AIRN expression was raised in the HCC cells HepG2 and Huh7 compared with the normal hepatocyte QSG-7701 (Fig. 1d). The results of western blot expounded that the protein level of STAT1 was lessened in the HCC cells compared with the normal hepatocyte cell (Fig. 1e). The above results corroborated that LncRNA AIRN expression was raised in the HCC tissues and cells.

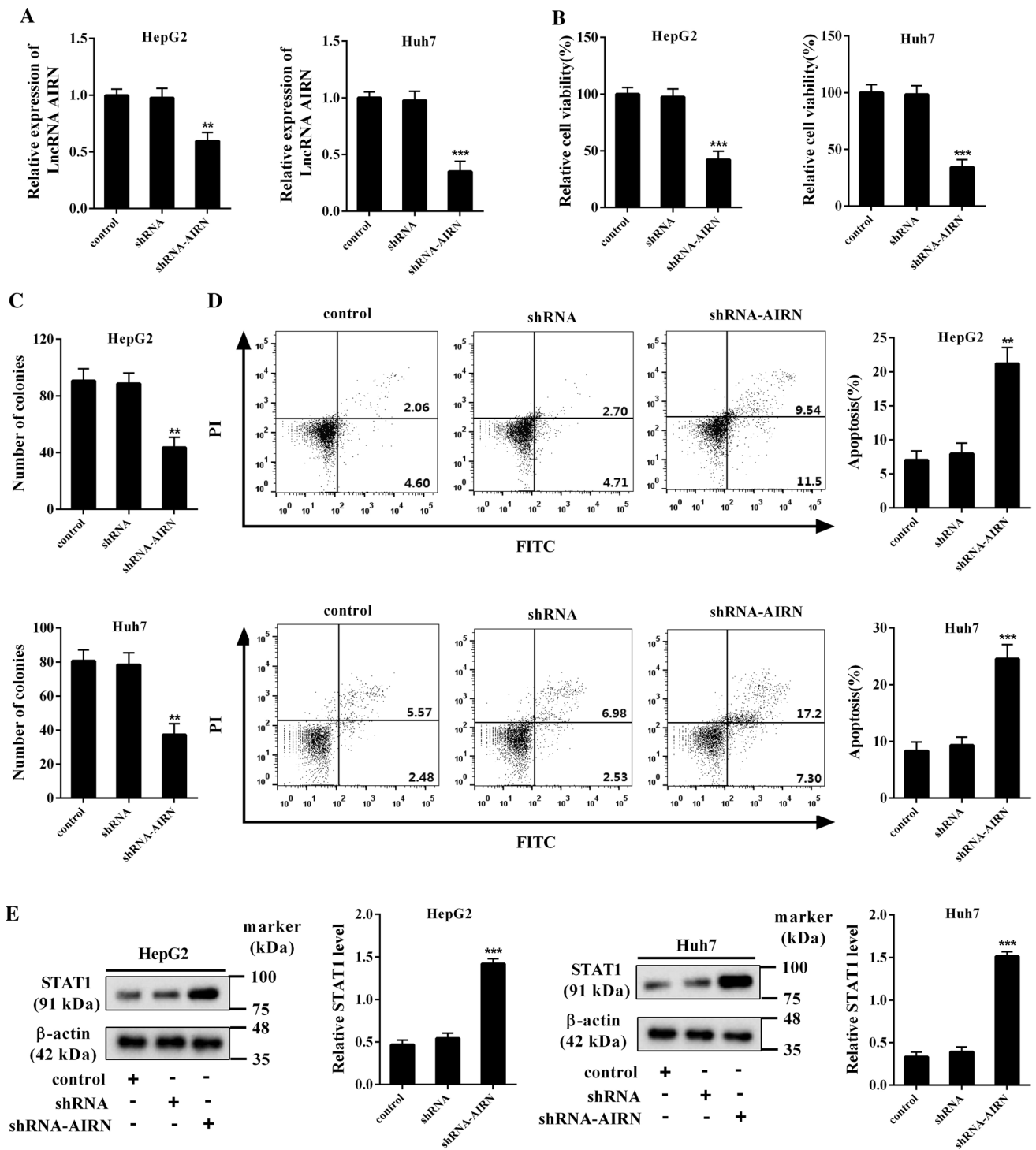
#### Effect of the interference with LncRNA AIRN on the proliferation and apoptosis of HCC cells

shRNA-AIRN were transfected into HCC cells HepG2 and Huh7, respectively. The results of qRT-PCR expounded that the transfection of shRNA-AIRN lessened the expression of LncRNA AIRN (Fig. 2a). MTT analysis corroborated that the transfection of shRNA-AIRN reduced HCC cell viability (Fig. 2b). Colony formation assay expounded that the transfection of shRNA-AIRN reduced the number of HCC cell clones (Fig. 2c). Flow cytometry results corroborated that the transfection of shRNA-AIRN boosted the apoptosis of HCC cells (Fig. 2d). Western blot analysis corroborated that the transfection of shRNA-AIRN raised the protein level of STAT1 (Fig. 2e). The above findings expounded that the

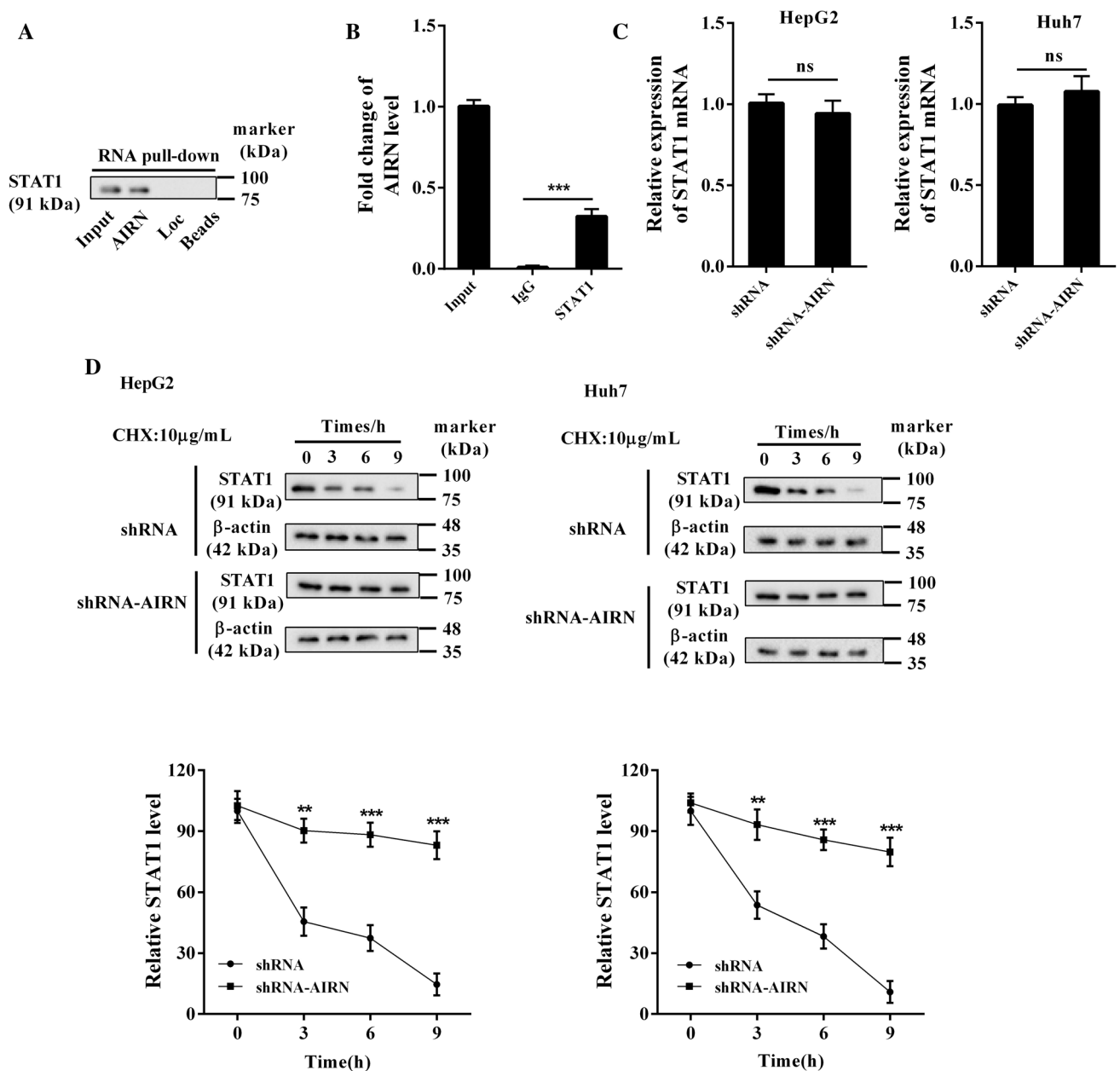
interference with LncRNA AIRN in HCC cells restrained HCC cell proliferation and boosted cell apoptosis.

#### The interaction between LncRNA AIRN and STAT1

RPISeq predicted that there might be an interaction between LncRNA AIRN and STAT1. To further validate the association between LncRNA AIRN and STAT1, we conducted an RNA pull-down assay and corroborated that STAT1 protein was enriched in the LncRNA AIRN pull-down complex. Moreover, the results of the RIP assay expounded that LncRNA AIRN was enriched in the protein sample precipitated by anti-STAT1 (Fig. 3a, b). The results of qRT-PCR corroborated that there were no significant changes in the mRNA level of STAT1 after the interference with LncRNA AIRN in the HepG2 and Huh7 cells (Fig. 3c). CHX is a protein synthesis inhibitor that restrains the synthesis of proteins in cells (Widelitz et al. 1986). Therefore, we evaluated the effect of LncRNA AIRN on the stability of STAT1 protein through the CHX chase assay. After the transfection of shRNA-AIRN into the HepG2 and Huh7 cells, the cells were treated with CHX for 0, 3, 6, and 9 h, respectively. Western blot analysis expounded that in the control (shRNA) group, the



**Fig. 2** Interfering with LncRNA AIRN restrains HCC cell proliferation and boosts cell apoptosis. shRNA and shRNA-AIRN were transfected into HCC cells HepG2 and Huh7, respectively. **a** qRT-PCR was applied to test the expression of LncRNA AIRN. **b** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was applied to quantify the viability of HCC cells. **c** A colony formation assay was conducted to assess the proliferation of HCC cells. **d** Flow cytometry was conducted to quantify the apoptosis of HCC cells. **e** Western blot was applied to test the protein level of STAT1 in HCC cells. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (compared to the shRNA group)



**Fig. 3** LncRNA AIRN binds to STAT1 and enhances the stability of STAT1 protein. **a, b** RNA pull-down and RNA Immunoprecipitation (RIP) experiments were applied to verify the interaction between LncRNA AIRN and STAT1. **c** After the interference with LncRNA AIRN in HepG2 and Huh7 cells, qRT-PCR was conducted to test the mRNA level of STAT1. **d** After the interference with LncRNA AIRN in HepG2 and Huh7 cells, the cells were treated with cycloheximide (CHX) for 0, 3, 6, and 9 h, respectively. Western blot was conducted to test the protein level of STAT1. \*\* $P < 0.01$  (compared to the shRNA group), \*\*\* $P < 0.001$  (compared to the IgG or shRNA group)

protein level of STAT1 was gradually lessened with the prolonged CHX treatment time, while the protein level of STAT1 in the shRNA-AIRN group had no significant changes (Fig. 3d). These results corroborated that LncRNA AIRN bound to STAT1 and that the interference with LncRNA AIRN enhanced the stability of STAT1 protein.

#### Effect of the interference with LncRNA AIRN on STAT1 expression

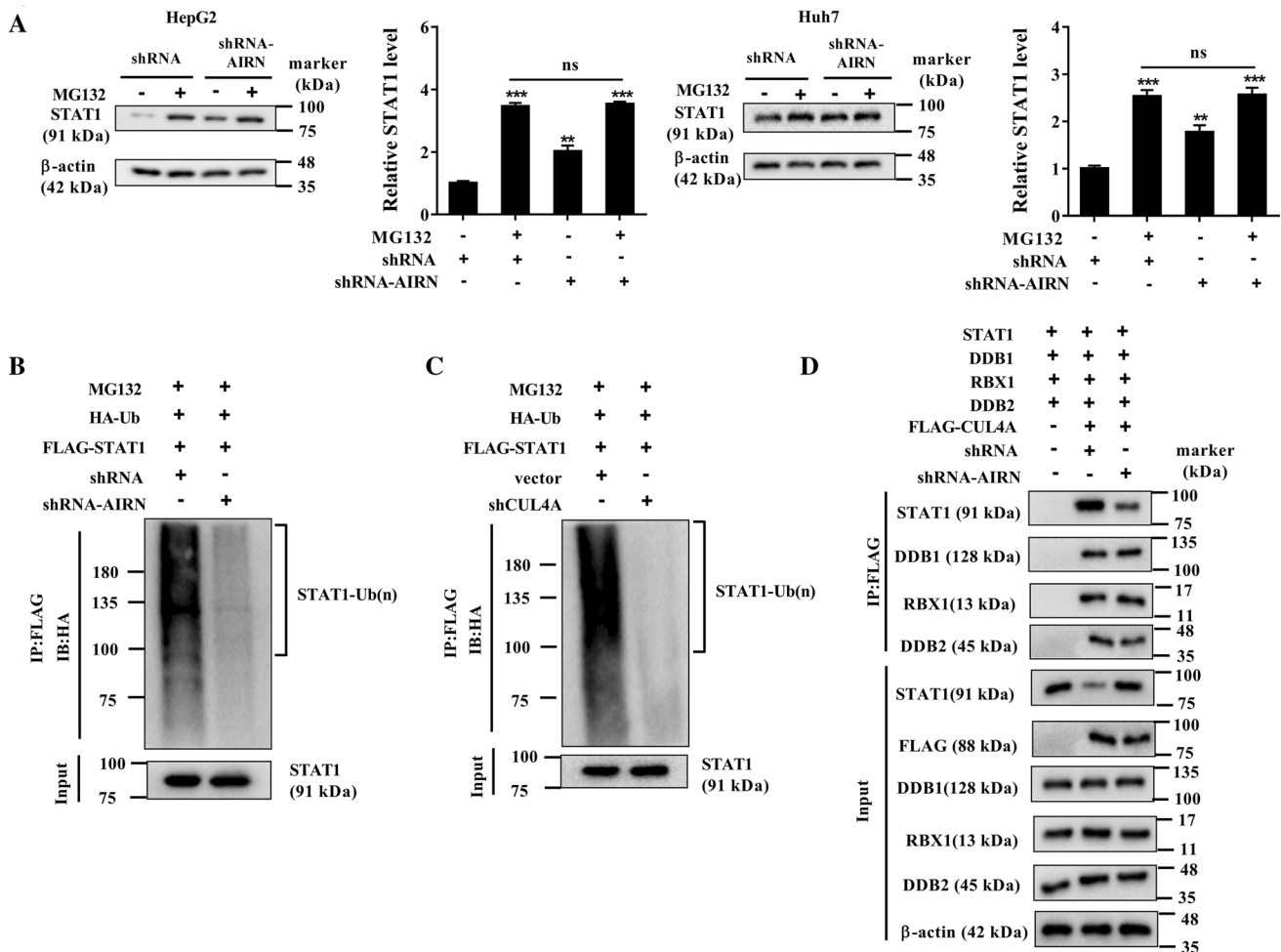
shRNA-AIRN was transfected into HCC cells HepG2 and Huh7, and then the cells were treated with 0.3 mol/L MG132, which is a commonly used proteasome inhibitor (Yan et al. 2014). Western blot analysis expounded that

MG132 treatment raised the protein level of STAT1 compared with the untreated group, implying that proteasome-mediated STAT1 protein degradation was successfully blocked. Furthermore, the transfection of shRNA-AIRN raised the protein level of STAT1, but the transfection of shRNA-AIRN had no significant effect on STAT1 protein level in MG132 pretreated cells (Fig. 4a). The IP and ubiquitination assays corroborated that the interference with AIRN restrained the ubiquitination of STAT1 protein and restrained the interaction of STAT1 and E3 ubiquitin ligase CUL4A, and the interference with CUL4A also inhibited the ubiquitination of STAT1 protein (Fig. 4b, c). Besides, we detected the expressions of the binding protein DDB1, catalytic subunit RBX1 and substrate receptors DDB2 and the results indicated that the interference with AIRN restrained the binding of CUL4A to STAT1, but had no significant effect on the binding of CUL4A to DDB1, CUL4A

to RBX1, and CUL4A to DDB2 (Fig. 4d). The above experimental results expounded that the interference with LncRNA AIRN restrained the CUL4A-mediated ubiquitination of STAT1 protein.

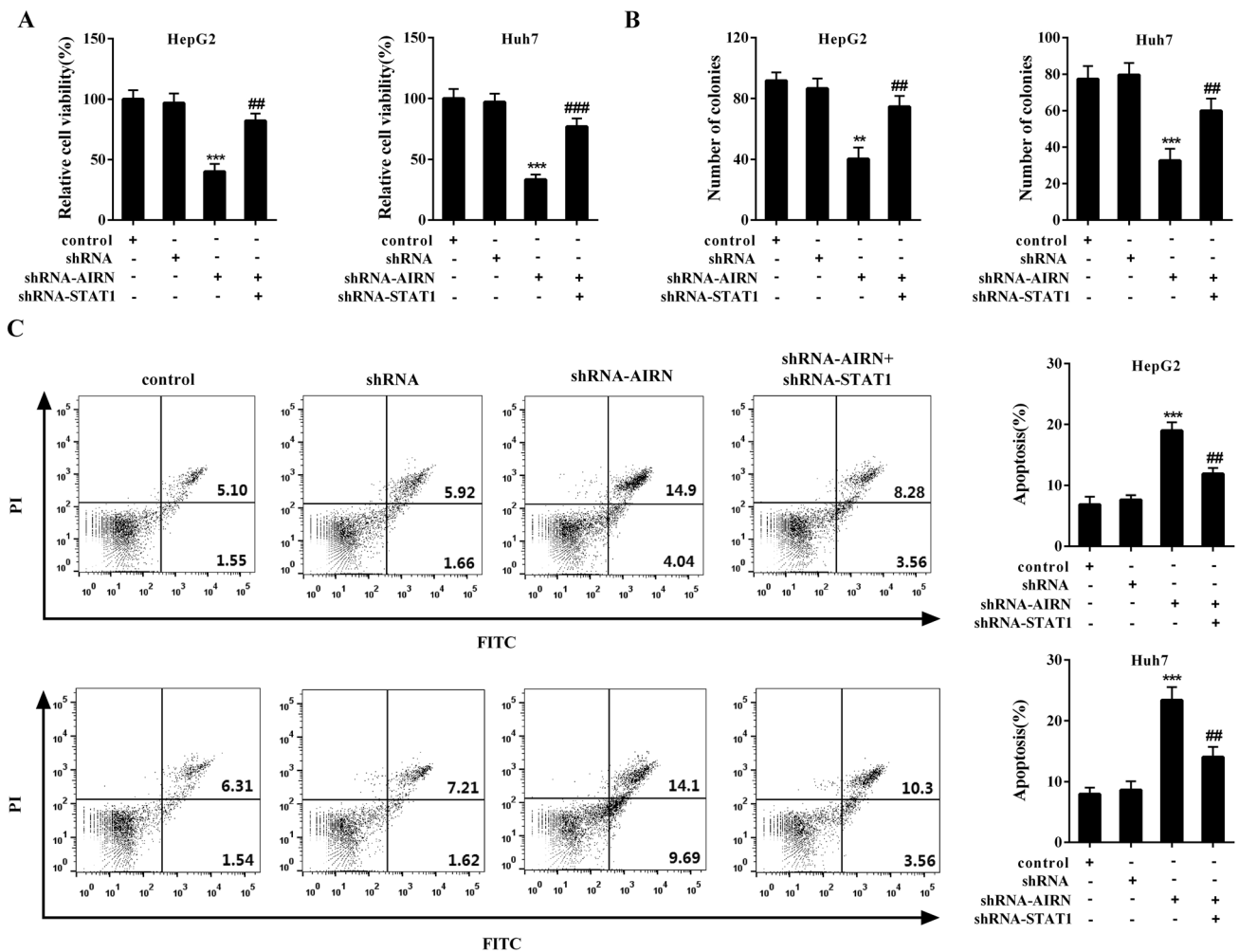
**LncRNA AIRN/STAT1 axis is bound up with the proliferation and apoptosis of HCC cells**

shRNA-AIRN and/or shRNA-STAT1 were transfected into HCC cells HepG2 and Huh7, respectively. The results of MTT expounded that the transfection of shRNA-AIRN restrained the viability of HCC cells, while this restraint was reversed after the transfection of shRNA-STAT1 (Fig. 5a). The colony formation assay expounded that the transfection of shRNA-AIRN lessened the number of HCC cell clones, while this lessening was reversed after the transfection of shRNA-STAT1 (Fig. 5b). The flow cytometry analysis



**Fig. 4** Interference with LncRNA AIRN restrains CUL4A-mediated ubiquitination of STAT1 protein. **a** After MG132 treatment of HCC cells HepG2 and Huh7 transfected with shRNA-AIRN, the protein level of STAT1 was tested by western blot. **b, c** Immunoprecipitation (IP) and ubiquitination assays were conducted to assess the influence of LncRNA AIRN or CUL4A on CUL4A-mediated ubiquitination of STAT1 protein. **d** Western blot was conducted to analyze the protein levels of STAT1, binding protein DDB1, catalytic subunit RBX1 and substrate receptors DDB2. **\*\*P** < 0.01, **\*\*\*P** < 0.001 (compared to the shRNA group)





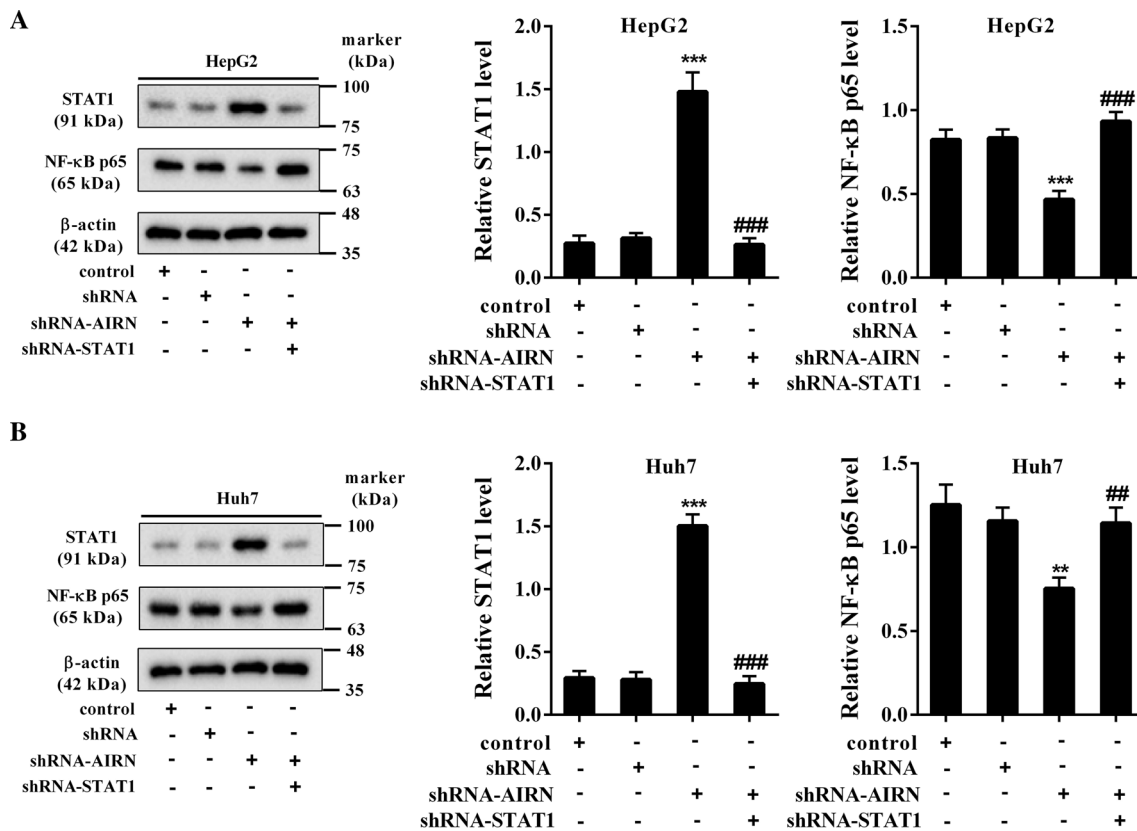
**Fig. 5** LncRNA AIRN/STAT1 regulates the proliferation and apoptosis of HCC cells. shRNA-AIRN and/or shRNA-STAT1 were transfected into HCC cells HepG2 and Huh7. **a** MTT was applied to test the viability of HCC cells. **b** Colony formation assay was applied to test the proliferation of HCC cells. **c** Flow cytometry was conducted to test the apoptosis of HCC cells. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (compared to the shRNA group), ## $P < 0.01$ , ### $P < 0.001$  (compared to the shRNA-AIRN group)

corroborated that the transfection of shRNA-AIRN boosted the apoptosis of HCC cells, while this boost was reversed after the transfection of shRNA-STAT1 (Fig. 5c). Moreover, we further probed into the role and mechanism of LncRNA AIRN in key signaling pathways during the progression of HCC. Western blot analysis expounded that the transfection of shRNA-AIRN lessened the protein level of NF- $\kappa$ B p65, while this lessening was reversed after the transfection of shRNA-STAT1 (Fig. 6). These data corroborated that the LncRNA AIRN/STAT1 axis was bound up with the regulation of the proliferation and apoptosis of HCC cells.

#### Interfering with LncRNA AIRN affects the tumor growth of HCC

shRNA-AIRN was transfected into HepG2 cells, and the cells were subcutaneously inoculated into the left axilla

of mice. After 5 weeks, the mice were sacrificed and the tumor tissues were gathered. Vernier calipers were applied to measure the tumor size and the results corroborated that the growth of mouse tumors was more restrained in the shRNA-AIRN group compared with the shRNA group (Fig. 7a). The results of the mouse body weight test expounded that there was no significant difference between the body-weight of the mice in the shRNA-AIRN group and the shRNA group (Fig. 7b). qRT-PCR results expounded that compared with the shRNA group, the expression of LncRNA AIRN was lessened in the shRNA-AIRN group (Fig. 7c). Western blot analysis corroborated that compared with the shRNA group, the protein level of STAT1 was raised in the shRNA-AIRN group (Fig. 7d). Conclusively, the interference with LncRNA AIRN restrained the tumor growth of HCC.



**Fig. 6** Detection of key signal pathways of LncRNA AIRN in the progress of HCC. shRNA-AIRN and/or shRNA-STAT1 were transfected into HCC cells HepG2 and Huh7. Western blot was applied to test the protein levels of STAT1 and NF-κB p65 in HCC cells HepG2 (a) and Huh7 (b). \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (compared to the shRNA group), ## $P < 0.01$ , ### $P < 0.001$  (compared to the shRNA-AIRN group)

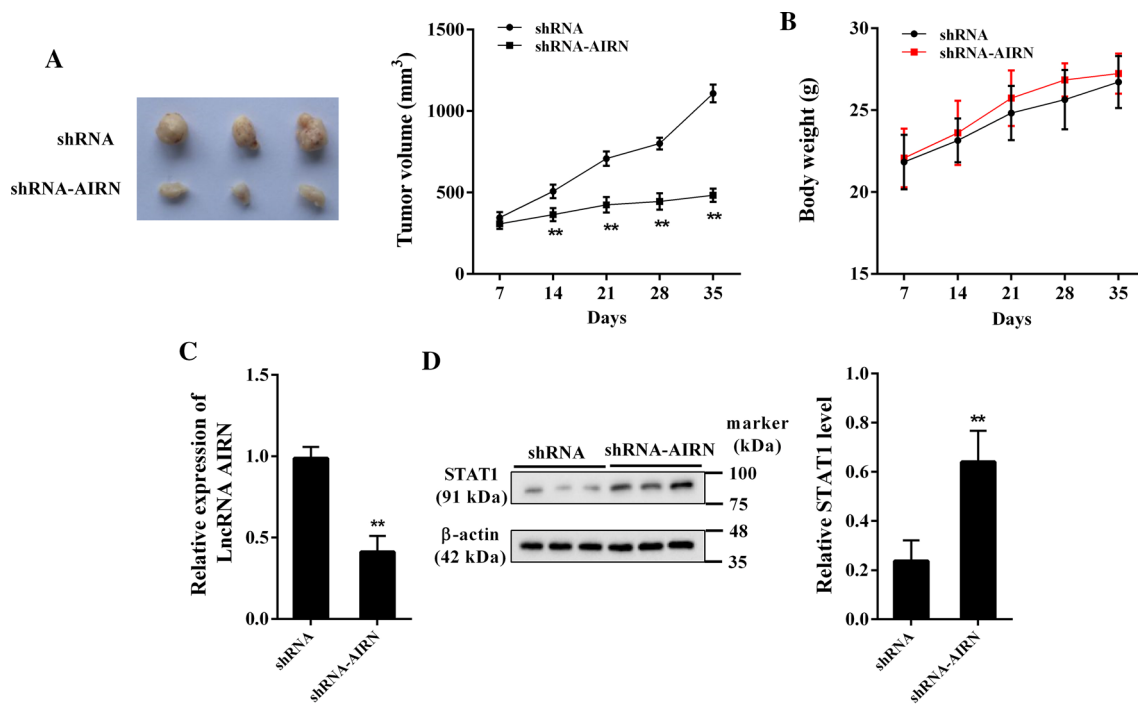
## Discussion

The present research expounded that LncRNA AIRN expression was raised in the HCC tissues and cells and our investigations expounded that the interference with LncRNA AIRN in HCC cells restrained cell proliferation and boosted cell apoptosis by restraining the CUL4A-mediated ubiquitination of STAT1 protein, and our data discovered a new regulatory axis LncRNA AIRN/STAT1 in HCC.

The functions of LncRNAs in cancer are mainly reflected in the regulation of cell biological processes, including cell proliferation and apoptosis, and in the process of the regulation, the raising LncRNAs have been proved to be dysregulated in HCC (Mercer et al. 2009; Li et al. 2013; Jin et al. 2020). Fang et al. expounded that knocking down RP11-284P20.2 restrains HCC cell viability, migration, invasion, and colony formation, and boosts cell apoptosis (Fang et al. 2020). Previous research has corroborated that in human and mouse genomes, AIRN is in the antisense direction corresponding to the imprinting, and the tumor suppressor gene insulin-like growth factor-2 receptor (IGF2R) raises the survival rate of HCC patients and restrains the recurrence of HCC and its expression is regulated by LncRNA AIRN

(Lautem et al. 2019), prompting that LncRNA AIRN might have a function in HCC by regulating IGF2R, which also warrants further in-depth exploration. Notably, the raised expression of LncRNA AIRN was also observed during the development of mouse liver tumors (Oliva et al. 2009). However, the role and underlying mechanism of LncRNA AIRN in HCC are not clear. In the present study, we also discovered that LncRNA AIRN expression was raised in the HCC tissues and cells and our investigations corroborated that the interference with LncRNA AIRN restrained the proliferation of HCC cells and boosted cell apoptosis, which was consistent with the above studies.

Increasing evidence expounds that the overexpression of STAT1 induces HCC cell cycle arrest and boosts cell apoptosis (Zhu et al. 2010). Recently, the role of ubiquitination modification in tumors has gradually drawn attention (Mansour 2018; Faktor et al. 2019). CUL4A is one of the E3 ubiquitin ligases that boosts cancer progression by mediating protein ubiquitination degradation of tumor suppressors (Kim et al. 2013), and CUL4A-mediated protein ubiquitination can be regulated by LncRNAs (Ni et al. 2017). Moreover, it is worth noting that CUL4A mediates STAT1 protein ubiquitination to lessen the protein level of STAT1 (Ulane



**Fig. 7** Interfering with LncRNA AIRN in HCC cells restrains the tumor growth of HCC. Mice received the injections of two different transfected cell types, and experiments were divided into two groups: shRNA ( $n = 6$ ), shRNA-AIRN ( $n = 6$ ). **a** Vernier calipers were applied to measure the tumor volume (Tumor volume =  $1/2$  length $\times$ width $^2$ ). **b** Detection of mouse body weight. **c** qRT-PCR was conducted to detect the expression of LncRNA AIRN in mouse tumor tissues. **d** The protein level of STAT1 in mouse tumor tissues was analyzed by western blot.  $**P < 0.01$  (compared to the shRNA group)

et al. 2002). In the present study, we corroborated that STAT1 bound to LncRNA AIRN, and our in-depth examinations expounded that CUL4A-mediated STAT1 ubiquitination was regulated by LncRNA AIRN. This was consistent with the following findings: DHX9 binds to E3 ubiquitin ligase MDM2, and this interaction is enhanced by LncRNA CCDST, indicating that LncRNA CCDST promotes DHX9 degradation by serving as a scaffold to facilitate the formation of MDM2 and DHX9 complexes (Ding et al. 2019). Our further study indicated that the LncRNA AIRN/STAT1 axis was bound up with HCC cell proliferation and apoptosis. Besides, the NF- $\kappa$ B signal transduction is considered to be the main pathway leading to the occurrence of cytokine-related cancers and is bound up with tumor cell growth and apoptosis (Baud et al. 2009). Li et al. discovered that lessening the level of NF- $\kappa$ B p65 in HCC cells boosts cell apoptosis and restrains cell growth (Li et al. 2017), and STAT1 restrains the expression of NF- $\kappa$ B p65 in HepG2 cells (Chen et al. 2015). Therefore, we further probed into the role and mechanism of LncRNA AIRN in key signaling pathways during the progression of HCC and corroborated that NF- $\kappa$ B p65 expression was lessened in the shRNA-AIRN group, while this lessening was reversed after the transfection of shRNA-STAT1, implying that LncRNA AIRN might play functions in HCC through the NF- $\kappa$ B signaling pathway.

Taken together, our data expounded that LncRNA AIRN expression was raised in the HCC tissues and cells, and our further studies corroborated that the interference with LncRNA AIRN ameliorated HCC, mainly by restraining the CUL4A-mediated ubiquitination of STAT1 protein to restrain HCC cell proliferation and boost cell apoptosis. LncRNA AIRN might play functions in HCC through the NF- $\kappa$ B signaling pathway. This research might provide new insights for ameliorating HCC.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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