

A Single Nucleotide Polymorphism in Promoter of Porcine *ARHGAP24* Gene Regulates Aggressive Behavior of Weaned Pigs After Mixing by Affecting the Binding of Transcription Factor P53

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Abstract

Background: Weaned pigs often have more aggressive behavior after mixing, which has negative effects on animal welfare and growth performance. Identification of functional single nucleotide polymorphisms (SNPs) related to aggressive behavior of pigs would provide valuable molecular markers of aggressive behavioral trait for genetic improvement program. Rho GTPase Activating Protein 24 (*ARHGAP24*) gene plays an important role in regulating the process of axon guidance, which may impact aggressive behavior of pigs.

Results: By re-sequencing the entire coding region, partially adjacent introns and the 5' and 3' flanking regions, 6 and 4 SNPs were identified in the 5' flanking region and 5' untranslated region (UTR) of porcine *ARHGAP24* gene, respectively. Association analyses revealed that 9 SNPs were significantly associated with aggressive behavioral traits ($P = < 1.00 \times 10^{-4} - 4.51 \times 10^{-2}$), and their haplotypes were significantly associated with aggressive behavior ($P = < 1.00 \times 10^{-4} - 2.99 \times 10^{-2}$). The core promoter region of *ARHGAP24* gene was identified between -670 bp and -1113 bp. Furthermore, the luciferase activity of allele A of rs335052970 was significantly less than that of allele G, suggesting the transcriptional activity of *ARHGAP24* gene was inhibited by allele A of rs335052970. It was identified that the transcription factor p53 bound to the transcription factor binding sites (TFBSs) containing allele A of rs335052970. In porcine primary neural cells, p53 bind to the target promoter region of *ARHGAP24* gene, reduce its promoter transcriptional activity, and then reduce its messenger RNA (mRNA) and protein expression through axon guidance pathway.

Conclusion: The results demonstrated that *ARHGAP24* gene had significant genetic effects on aggressive behavioral traits of pigs. Therefore, rs335052970 in *ARHGAP24* gene can be used as a molecular marker to select less aggressive pigs and improve animal welfare.

Introduction

Aggressive behavior of pigs after mixing, an important animal welfare issue, causes negative impacts on the growth performance, feed conversion ratio, immunity and meat quality, which affects the economic benefits of pig industries [1, 2]. Previous studies have shown that aggressive behavior was affected by environmental factors, such as stocking density [3], mixing [4], feeding space, and environmental enrichment [5]. However, genetic factors also have an important influence on the aggressive behavior of pigs [6]. Due to their complicated assessment process, aggressive behavioral traits are rarely included in traditional pig breeding selection programs. Phenotypic determination of individual animal aggressive behavior is challenging and limits the improvement of behavioral traits through genetic selection. Therefore, identification of molecular genetic markers of aggressive behavior could contribute to the genetic selection of less aggressive pigs.

In the process of screening candidate genes for aggressive behavior in pigs, the porcine Rho GTPase activating protein 24 (*ARHGAP24*) gene attracted our attention. The *ARHGAP24* gene encodes Rho

GTPase activating protein (RhoGAP), which stimulates the GTPase activities of Rho family of small GTPases, terminates the binding of Rho with GTP, thus inactivating the activity of Rho family proteins [7, 8]. Rho GTPase family members, including RhoA, Rac, and Cdc42 proteins [9], play important roles during the development of the nervous system [10, 11]. RhoA and Rac1 are regulated by GTPase-activating proteins and serve as their downstream targets [12]. Previous studies also have shown that *ARHGAP24* gene is implicated in axon and dendrite outgrowth and branching [13]. Meanwhile, it has been reported that aggressive behavior is associated with axon guidance signaling pathway in humans [14, 15]. Therefore, the *ARHGAP24* gene may play an important role in regulating the process of axon guidance, which then impacts aggressive behavior.

In this study, we hypothesized that the aggressive behavior of pigs is associated with the expression and function of the *ARHGAP24* gene. We aimed to identify the functional SNPs of the *ARHGAP24* gene and investigate their molecular mechanisms for aggressive behavior regulation in weaned pigs after mixing. This research could provide valuable molecular markers of aggressive behavior for the genetic improvement of pigs. At the same time, this study also facilitated adoption of the pig as a biomedical model of human mental diseases and reveal the molecular bases of abnormal animal behavior traits.

Materials And Methods

Animals, housing and sample collection

This study was approved by the Animal Care and Use Committee of Nanjing Agricultural University (SYXK Su 2017-0007). A total of 500 piglets from 65 litters were selected in the Huaiyin pig breeding farm (Huaian, Jiangsu, China). Piglets were weaned at 35 d of age and moved into new empty pens with their original littermates in a nursery room at two days before mixing. Then, 9 or 10 weaned pigs with the same sex and similar body weight from different litters were mixed in pens of dimension 2.5 m × 2.2 m. The pens were equipped with slatted floors, stainless-steel feeders and nipple drinkers to allow ad libitum access to feed and water. Ear tissues of weaned piglet were collected and genomic DNA was extracted by a standard phenol/chloroform method.

Behavioral assessment

A digital video recording system (Hikvision DS-2CE56C2P-IT3 3.6 mm; Hikvision network hard disk video recorder DS-7808HW-E1/M; Hikvision Digital Technology Co. Ltd., Hangzhou, Zhejiang, China) was used to continuously record the behavior of piglets for 72 h after mixing. A video camera was installed over each pen. To individually identify pigs in the video recording, all pigs in each pen were marked different numbers on the back of pigs using a spray paint (7CF, Shenzhen Zhaoxin Energy Co., Ltd, Shenzhen, Guangdong, China) before mixing. The definitions of aggressive behavioral traits used were described in our previous studies [16, 17] with some additional new traits. Specifically, 9 indicators were used to quantify aggression, and their description and definition are shown in Table 1. A fighting behavior was

The promoter region of the porcine *ARHGAP24* gene was amplified by PCR using Vazyme LAmp Master Mix (Vazyme Biotech, Nanjing, Jiangsu, China). Subsequently, plasmids containing variable lengths of truncated the porcine *ARHGAP24* promoter were individually amplified using different forward primers and a common reverse primer (*ARHGAP24*-P1: -33/+352, *ARHGAP24*-P2: -308/+352; *ARHGAP24*-P3: -670/+352; *ARHGAP24*-P4: -1113/+352; *ARHGAP24*-P5: -1572/+352; *ARHGAP24*-P6: -1976/+352), and the primers contained MluI and XhoI (Takara Bio Inc., Dalian, Liaoning, China) recognition sequences, respectively (Table S2). Subsequently, the amplified fragments were inserted into the multiple cloning sites of the pGL3-basic vector to generate luciferase reporter plasmids. Moreover, specific regions containing rs335052970, rs344700648, and rs339198696 were amplified using *ARHGAP24*-Haplotype primers contained recognition sequences of MluI and XhoI (Table S2). The DNA samples of piglets were amplified using primers (Table S2) targeting the promoter region of porcine *ARHGAP24* gene containing p53 transcription factor binding element (PBE) motif and then cloned into pGL3-basic vector by MluI and XhoI. The plasmid with PBE was used as DNA template, amplified by point mutation primer-*ARHGAP24*-PBE-MUT (Table S2). The cDNA fragments of p53 were amplified using a forward primer containing an EcoRI (Takara Bio Inc., Dalian, Liaoning, China) site and a reverse primer containing a XhoI site (Table S2) and connected to the eukaryotic expression vector pcDNA3.1 (+) (pcDNA3.1-p53). The plasmid structures were sequenced to confirm the integrity of the constructed fragments.

Cell culture, cell transfection, and luciferase assays

Human embryonic kidney 293T (HEK 293T) cells were used for promoter activity analysis. Firstly, HEK 293T cells were cultured in an incubator at 37 °C and 5% CO₂. Cells were plated in 12-well plates and 3 wells for each group. On the following day, the plasmids contained the variable length of *ARHGAP24* promoter fragments, the rs335052970 A or G allele and haplotypes were individually co-transfected into the HEK 293T cells with pRL-TK Renilla luciferase reporter vector (Promega, Madison, WI, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Controls were the pGL3-basic and pGL3-control luciferase reporter gene vector. After 24 h, cells were harvested with passive lysis buffer (Promega, Madison, WI, USA). The cell lysates were assayed for reporter gene activity using a dual-luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Primary neural cells were prepared from cerebral cortices of weaned piglets, as previously described [23]. In brief, cerebral cortices were removed from the piglet's brains. Then, the meninges and microvessels were carefully removed in ice precooled D-Hanks' Balanced Salt Solution (HBSS, Gibco, Grand Island, NY, USA), and the brain tissue were minced into small pieces of about 1 mm³. After papain (Biofroxx, Einhausen, Germany) and DNase 1 (BioFroxx, Einhausen, Germany) were added respectively, they were digested in an incubator at 37 °C for 30 min. After the digestion was terminated with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA), they were sub packed into 15 mL centrifuge tubes for centrifugation for 10 min. The porcine neural cells were cultured in DMEM supplemented with 20% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) at 37 °C in a humidified atmosphere with 5% CO₂ for 48 h. Endotoxin-free plasmids of pcDNA3.1-p53 and pcDNA3.1 were transfected into primary neural

cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), respectively. The siRNA of p53 and ARHGAP24 were designed and chemically synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd. The primer sequence is shown in Table S2. Either scrambled siRNA or p53 siRNA plasmids were transfected into primary neural cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using the ChIP assay Kit (ab156907, Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, a total of 2×10^6 neural cells were fixed with 1% formaldehyde (Aladdin, Shanghai, China) via gentle swirling at room temperature for 10 min. Then, the fixing reaction was stopped by the addition of 1 mL of 2.5 M glycine solution (Beyotime, Shanghai, China). Nuclei were digested into 300-500 bp fragments. Inputs and immunoprecipitation (IP) samples were incubated with 2 μ L of p53 antibody (AF0879, Affinity, Suzhou, Jiangsu, China) or 0.8 μ L non-immune IgG overnight at 4°C on a rocking platform, and precipitated DNA fragments containing PBE motifs were detected using PCR with the specific primers listed in Table S2.

Immunofluorescence staining

After 10 days of culture, the porcine neural cells were identified by immunofluorescence staining. Briefly, the cells were washed three times with phosphate-buffered saline (PBS, HyClone, Logan, UT, USA) and fixed for 30 min in 4% paraformaldehyde (Beyotime, Shanghai, China). The cells were subsequently incubated in ice-cold 0.5% Triton X-100 (Beyotime, Shanghai, China) for 20 min and washed a further three times with PBS. Next, the cells were incubated with QuickBlock blocking buffer (Beyotime, Shanghai, China) at room temperature for 1 h. The cells were incubated with anti-beta III Tubulin (Tuj1) antibody (dilution 1:100; Abcam, Cambridge, UK) at room temperature for 1 h. The cells were then washed three times and incubated with fluorescein conjugated goat anti-rabbit IgG (H+L) antibody (dilution 1:500; Proteintech, Wuhan, Hubei, China) in the dark. After 1 h of incubation at room temperature, the cells were washed three times. The cell nuclei were stained with 4',6-Diamidino-2-Phenylindole (DAPI, Beyotime, Shanghai, China) in the dark. After washing three times, the glass slides were sealed with fluorescence anti-quenching agents (Beyotime, Shanghai, China). Images were captured using confocal microscopy (Zeiss, LSM 700; Oberkochen, Germany).

RNA extraction and quantitative reverse transcription-PCR (RT-qPCR)

The cells were harvested at day 1 post-transfection. Total RNA of porcine neural cells was extracted by using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity of RNA was detected by the NanoDrop 2000 (Thermo Fisher Scientific, Fremont, CA, USA). To

quantify the mRNA expression level of *ARHGAP24* and *p53*, total RNA was reverse-transcribed into cDNA using the HiScript III RT SuperMix (Vazyme Biotech, Nanjing, Jiangsu, China). The RT-qPCR was performed on Quantum Studio 5 quantitative PCR instrument (Applied Biosystems, Foster, CA, USA) using SYBR Green master mix (Vazyme Biotech, Nanjing, Jiangsu, China) and the specific primers (Table S2). Relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method [24]. Coding gene expression levels were normalized to the expression of *GAPDH*. For the RT-qPCR reaction, each treatment had at least 3 biological replicates.

Western blotting

Cell protein lysates were harvested using 200 μ L ice-cold radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) with 1% phenylmethyl sulfonyl fluoride (PMSF, Beyotime, Shanghai, China). Total protein extracts were separated on 4–20% SDS–PAGE gels (Genscript Biotech, Nanjing, Jiangsu, China) and then blotted onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). After blocking with QuickBlock™ blocking buffer (Beyotime, Shanghai, China) for 30 min, the PVDF membranes were incubated overnight with the following primary antibodies: immunoreactive proteins were detected with a rabbit polyclonal antibody for anti-p53 (1:1,000; AF0879, Affinity, China), anti-ARHGAP24 (1:1,000; DF9858, Affinity, China) and a rabbit polyclonal antibody for anti-GAPDH (1:5,000; AF7021, Affinity, China). The appropriate anti-rabbit secondary antibody (1:8,000; S0001, Affinity, China) was used to incubate the membranes. ECL Peroxidase Color Development Kit (Vazyme, Nanjing, Jiangsu, China) was used in chromogenic reaction according to the manufacturer's instructions. The protein bands visualization was performed by the ChemiDoc XRS + system (Bio-Rad, Hercules, CA, USA). The band density was analyzed using ImageJ software.

Statistical analyses

Data were analyzed using SAS 9.4 (SAS Institute Inc, Cary, NC, USA). Chi-square tests were used to analyze the difference of allele frequency between the most aggressive and the least aggressive pigs. The behavioral data were analyzed using GLIMMIX procedure with a model option DIST = EXPO. The relative fluorescence activity value was normalized by negative control pGL3-basic. The significance of luciferase activity statistics was analyzed by unpaired two-sided student's t-test and one-way ANOVA analysis. The results are presented as mean \pm standard error of the mean (SEM), and $P < 0.05$ was considered significant.

Results

Identification of SNPs in porcine *ARHGAP24* gene

A total of 10 SNPs were identified by sequencing on the entire coding region, 5'-and 3'-flanking regions of porcine *ARHGAP24* gene in 178 pigs (Table 2). Six of SNPs (rs339198696, rs344700648, rs335052970,

rs344498203, rs323776551, and rs342083908) are located in 5'-flanking region, and 4 SNPs (rs333053350, rs342210686, rs328435752, and rs787973778) are located in the 5'-UTR of porcine *ARHGAP24* gene. Five SNPs (rs344700648, rs335052970, rs323776551, rs342083908, and rs787973778) showed significant difference ($P < 0.05$) in allele frequencies between the most aggressive and the least aggressive pigs.

Association analyses between genotype of *ARHGAP24* gene and aggressive behavior in pigs

Association analyses between genotype and aggressive behaviors during the first 2, 24, 48, or 72 h after mixing are presented in Table S3. Three SNPs (rs339198696, rs344700648, and rs335052970) in 5'-flanking regions were significantly associated with aggressive behaviors during the first 2, 24, 48, or 72 h after mixing ($P < 0.05$). Interestingly, all SNPs had a strong association with multiple aggressive behaviors during the first 2 h after mixing. Moreover, CAS, duration of active attacks, frequency of active attack, frequency of standoff and win were greater in the pigs with the mutant AA genotype of rs335052970 than those in the pigs with the wild GG genotype during the first 2, 24, 48, or 72 h after mixing, respectively ($P < 0.05$, Fig 1). Similarly, 4 SNPs (rs333053350, rs342210686, rs328435752, and rs787973778) in the 5'-UTR were significantly associated with duration of active attacks and duration of standoff ($P < 0.05$) (See Table S3 for details).

Association analyses between haplotype of *ARHGAP24* gene and aggressive behavior in pigs

We estimated the LD among the 10 SNPs of *ARHGAP24* gene using Haploview 4.2. Seven SNPs (rs333053350, rs342210686, rs328435752, rs787973778, rs335052970, rs344700648, and rs339198696) were highly linked ($D' > 0.69$; Fig. 2) in two haplotype blocks. The first haplotype block (block 1) consisted of three haplotypes: H1 (GGAA), H2 (TAGC) and H3 (GAAA). The second haplotype block (block 2) consisted of three haplotypes: H1 (AAC), H2 (GAA), H3 (GTA). The two haplotype blocks were significantly associated with aggressive behavior ($P < 0.05$) (Table S4). In the haplotype block 1, pigs with haplotype H1 (GGAA) were more aggressive than pigs with haplotype H2 (TAGC) or H3 (GAAA). Similarly, pigs with the haplotype H1 (AAC) in haplotype block 2 were more aggressive than those with haplotype H2 (GAA) or H3 (GTA) ($P < 0.05$).

Promoter prediction and identification of porcine *ARHGAP24* gene

Two promoter regions (-1364/-1314 bp and +89/+139 bp), and three transcription initiation sites (-1700, -800, and +100 bp) of the porcine *ARHGAP24* gene were predicted by Promoter 2.0 and neural network

promoter prediction. Transcription factor potential binding sites for RUNX2, RREB1, IRF2, IRF1, p53, CREBBP, POLR3A, and GLI1 were predicted in the 5' flanking region of the porcine *ARHGAP24* gene (Table S5). In the promoter activity analyses, the luciferase activity of plasmids contained the promoter fragments of *ARHGAP24* gene was greater than that of the pGL3-basic plasmid ($P < 0.01$) but less than that of the pGL3-control plasmid ($P < 0.01$). Moreover, the luciferase activity of pGL3-basic-P4, pGL3-basic-P5, and pGL3-basic-P6 was greater than that of pGL3-basic-P1, pGL3-basic-P2, and pGL3-basic-P3 ($P < 0.01$). The luciferase activity of pGL3-basic-P3 and pGL3-basic-P1 was greater than that of pGL3-basic-P2 ($P < 0.05$) (Figure 3a). These results revealed that the core promoter region of *ARHGAP24* gene is located between -670 bp and -1113 bp, whereas a negative regulatory promoter region is located between -308 bp and -33 bp.

Promoter activity analyses of porcine *ARHGAP24* gene

The luciferase activity was greater in plasmids contained the G allele of rs335052970 than that of plasmids contained the A allele ($P < 0.01$) (Fig. 3b). Moreover, there are three linked SNPs (rs335052970, rs344700648, and rs339198696) in the core promoter region (-670/-1113 bp) of *ARHGAP24* gene. They form only three haplotypes: H1 (AAC), H2 (GAA), and H3 (GTA). The luciferase activity of plasmids contained the haplotypes of the core promoter region was greater than that of pGL3-basic but less than that of pGL3-control ($P < 0.0001$). The relative luciferase activity of plasmids contained haplotype GAA was the greatest, while that of plasmids contained haplotype AAC was the least ($P < 0.01$) (Fig. 3c). It indicates that the site affecting promoter activity is rs335052970 (-744G > A).

Transcription factor p53 regulate *ARHGAP24* gene expression in porcine neural cells

The effects of SNP rs335052970 (-744G > A) on TFBSs was predicted through the Animal TFDB online website. The allele A of rs335052970 was found to be located in the potential binding sequence of transcription factor p53 (TP53) (Table S5). To verify the binding sequence of transcription factor p53 in the upstream region of *ARHGAP24* gene contains SNP rs335052970, a chromatin immunoprecipitation (ChIP) assay was used to demonstrate that p53 binds to the transcription factor binding element (PBE) motif directly in vivo (Figure 4a). To investigate whether p53 regulates the expression of *ARHGAP24* through the PBE site, we cloned the PBE site into a pGL3 vector (Promega, USA) to construct PBE-allele A (pGL3-WT) and PBE-allele G (pGL3-MUT) reporter vectors (Figure 4b). Reporter vectors and p53 over-expression vector (pcDNA3.1-p53) were co-transfected into porcine neural cells. The results of immunofluorescence identification are shown in Figure 4c. Porcine primary neural cells treated with TuJ1 antibody showed red fluorescence, indicating that TuJ1 detection is positive, the cell neurites are connected with each other, and the dendritic contour is clearly visible. The mRNA expression level of *p53* in the pcDNA3.1-p53 group was greater than that in the control group ($P < 0.05$) (Figure 5a). The mRNA expression level of *p53* in the siRNA-p53 group was less than that in scrambled group ($P < 0.05$) (Figure

5b). The luciferase activity of pcDNA3.1-WT group was less than that of the pcDNA3.1-MUT group ($P < 0.05$) when p53 was overexpressed (Figure 5c). Moreover, the luciferase activity of siRNA-p53 group was greater than that of the control group ($P < 0.05$) (Figure 5d). The over-expression of p53 reduced the mRNA and protein expression level of *ARHGAP24* ($P < 0.01$) (Figure 5e, 5g), but interfering p53 increased the mRNA expression level of *ARHGAP24* ($P < 0.01$) (Figure 5f, 5h).

Transcription factor p53 regulate aggression in pigs through axon guidance pathway

In order to explore how the signal pathway involved in *ARHGAP24* gene regulates aggressive behavior and the expression of related genes in the signal pathway when p53 is overexpressed or inhibited. We connected the eukaryotic expression vector pcDNA3.1 (+) (pcDNA3.1-p53) and chemically synthesized siRNA-p53, siRNA-*ARHGAP24*, and then transfected into porcine neural cells to detected the expression level of related genes in the axon guidance pathway. The mRNA expression level of *RHOA* in the pcDNA3.1-p53 group was greater than that in the control group ($P < 0.05$) (Figure 6a), while the mRNA expression level of *RHOA* in the siRNA-p53 group was less than that in scrambled group ($P < 0.05$) (Figure 6b). Similarly, the mRNA expression level of *ROCK1* in the pcDNA3.1-p53 group had an increased tendency than that in the control group ($P = 0.0567$) (Figure 6c), while the mRNA expression level of *ROCK1* in the siRNA-p53 group was less than that in scrambled group ($P < 0.05$) (Figure 6d). By contrast, the mRNA expression level of *RAC1* in the pcDNA3.1-p53 group was less than that in the control group ($P < 0.05$) (Figure 6e), while the mRNA expression level of *RAC1* in the siRNA-p53 group was greater than that in scrambled group (Figure 6f). Moreover, the mRNA expression level of *RHOA* and *ROCK1* in the siRNA-*ARHGAP24* group was greater than that in the Scrambled group ($P < 0.05$) (Figure 6g, 6h). However, the mRNA expression level of *RAC1* was not different between the siRNA-*ARHGAP24* group and Scrambled group (Figure 3i).

Discussion

To our knowledge, *ARHGAP24* gene encodes a GTPase-activating protein. RhoGAPs are important negative regulators of Rho signaling pathway [25]. Studies have also revealed that several members of the Rho family of GTPase activators have neuronal functions, including regulating dendritic morphology and synaptic plasticity [26]. *ARHGAP24* has been found to be a genetic marker to distinguish patients with major depression from healthy people [27]. In this study, we first demonstrated that the SNPs in *ARHGAP24* gene were associated with several aggressive behavioral traits. In addition, haplotypes was used as markers in association analysis to explain important genetic variation [28]. LD was used to locate causal mutation sites that could not be precisely located by simple single-marker association [29]. Association analyses between haplotypes and aggressive behavior traits revealed that three haplotype blocks were all significantly associated with aggressive behavioral traits, which is consistent with the association between SNPs and aggressive behavior trait in weaned pigs after mixing.

A promoter is necessary for the initiation of gene transcription and one of the upstream cis-acting elements for gene expression regulation [30, 31]. A core promoter initiates transcription, including transcription initiation sites (TSS) and upstream elements [32]. Based on luciferase activity analyses, the porcine *ARHGAP24* gene had not only a positive regulatory promoter region (from -670 bp to -1113 bp, but also a negative regulatory promoter region located (from -308 bp to -33 bp). The core promoter region has specific transcription factors binding sites and initiates the expression of downstream genes [33]. In addition, the SNPs located in the core promoter region affected mRNA transcription by affecting the binding to transcription factors [34]. In the present study, three SNPs in the core promoter region were in LD. Moreover, the relative luciferase activity of plasmids with haplotype AAC was the least than those of the other two haplotypes. Meanwhile, the luciferase activity was greater in plasmids with genotype GG than that of plasmids with genotype AA of rs335052970, implying that the promoter with allele G of rs335052970 might have higher transcriptional activity than the promoter with allele A. Our results demonstrated that SNP rs335052970 (-744G > A) as a functional mutation site can regulate the expression of *ARHGAP24* gene by affecting promoter activity.

Previous studies revealed that SNPs located in the core promoter region changed the activity of transcription factor binding sites [35, 36]. Transcription factors can activate or inhibit gene expression, which could result in a change of phenotype [37, 38]. In the present study, the allele A of rs335052970 located in the core promoter region of *ARHGAP24* gene was predicted to invent the TFBSs for p53. Subsequently, a ChIP analysis demonstrated that p53 directly binds to the transcription factor binding element (PBE) motif containing allele A of rs335052970 *in vitro*. p53, a tumor suppressor gene [39], promoted or inhibited cell growth [40], cell proliferation, senescence, and apoptosis directly or indirectly by regulating its target genes [41]. A recent study found that p53 may be a central regulator of neurodegeneration [42]. In the present study, the mRNA and protein expression level of *ARHGAP24* was decreased after over-expression of p53. Furthermore, the mRNA and protein expression level of *ARHGAP24* was increased by interfering p53. Previous study presented that p53 acted as a repressor to down-regulate PRR11-SKA2 to inhibit tumor formation [43], which is similar to our present study. Therefore, we found that p53 binds to TFBSs containing allele A of rs335052970 in the core promoter region of *ARHGAP24* gene, reduces the transcriptional activity of promoter, and then inhibits the mRNA and protein expression level of *ARHGAP24* gene in porcine neural cells.

The pathways regulating aggressive behavior include G-protein coupled receptor (GPCR) signaling pathway, axon guidance, and ERK/MAPK signaling [15]. The small GTPase Rho, including RhoA, Rac and Cdc42, as downstream regulators of RhoGAPs, regulates the development of nervous system by participating in axon guidance pathway [10]. Rho kinase (ROCK), a downstream target of small GTPase Rho, is associated with a variety of neural functions, such as dendritic development and axon extension [44]. In the present study, the mRNA expression level of *RHOA* was increased after over-expression of p53. While, the mRNA expression level of *RHOA* and *ROCK1* was decreased by interfering p53. Whereas, the mRNA expression level of *RAC1* was decreased after over-expression of p53, while was increased after by interfering p53. In general, Rac1 and Cdc42 are positive regulators of axon growth and

guidance, while RhoA is a negative regulator [45]. Moreover, the mRNA expression level of *RHOA* and *ROCK1* was greater than that in the Scrambled group when *ARHGAP24* was inhibited in the present study. It has been reported that p53 was transcriptionally activated and participates in neural growth factor mediated neurite growth [46, 47]. Meanwhile, the deregulation of *ARHGAP24* gene inhibited the growth and branching of axons and dendrites [13]. Repeated stress in rats resulted in atrophy of dendrites in hippocampal and medial prefrontal cortex neurons and increased aggression [48]. Therefore, p53 might reduce the axonal outgrowth and dendritic arborization by inhibiting the expression of *ARHGAP24* gene, which makes pigs are more aggressive after weaning (Figure 7). Thus, SNP rs335052970 may be a potential causal mutation of porcine aggressive behavioral traits. It changes the transcriptional activity of *ARHGAP24* gene, and regulates genes expression of axon guidance in combination with transcription factor p53. However, further functional studies are needed to verify how the transcription factors affect the aggressive behavior of pigs.

Conclusion

In conclusion, our results revealed the significant genetic effects of *ARHGAP24* gene on aggressive behavioral traits in weaned pigs after mixing. In addition, the rs335052970 was highlighted as a functional mutation for aggressive behavioral traits that changed the transcriptional activity of *ARHGAP24* gene by affecting the binding of transcription factor p53. The functional SNP rs335052970 can be used for the genetic selection for less aggressive pigs and improve animal welfare.

Abbreviations

SNPs: single nucleotide polymorphisms; ARHGAP24: Rho GTPase Activating Protein 24; UTR: untranslated region; TFBSs: transcription factor binding sites; mRNA: messenger RNA; RhoGAP: GTPases activating protein; PCR: polymerase chain reactions; PBE: p53 transcription factor binding element; HEK 293T: human embryonic kidney 293T; TP53: transcription factor p53; ChIP: chromatin immunoprecipitation; IP: Inputs and immunoprecipitation; PBS: phosphate-buffered solution; Tuj1: beta III Tubulin; DAPI: 4',6-Diamidino-2-Phenylindole; TSS: transcription initiation sites; GPCR: G-protein coupled receptor; ROCK: Rho kinase.

Declarations

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Author's contributions

Conceptualization, Q.X., B.Z.; investigation, statistical analysis and writing-original draft preparation, Q.X.; data curation, investigation, Y.G., J.Z.; visualization, M.L.; methodology and funding acquisition, B.Z.; writing—review and editing, A.P.S, B.Z. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The original contributions presented in the study are included in the article/Supplementary information, further inquiries can be directed to the corresponding author.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest in this research.

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Tables

Table 1 Description of indicators used to evaluate aggressiveness.

Trait	Description
Duration of active attack	In a fight, one pig actively bites, collides and chases another pig, which is considered to have launched an active attack [49]. The duration is defined as the "Duration of active attack", which uses seconds as a unit of time.
Frequency of active attack	As mentioned above, the number of active attacks is launched by pigs for 72 h after mixing, which is defined as "Frequency of active attack".
Duration of being bullied	When the recipient pig suffers from biting and head-knocking performed by the aggressive pig and the recipient pig moves away without retaliation, which is regarded as a being bullied [50]. Similarly, the duration is defined as the "Duration of being bullied", which uses seconds as a unit of time.
Frequency of being bullied	As mentioned above, the number of bullying behaviors is initiated by the aggressive pig for 72 h after mixing, which is defined as the "Frequency of being bullied" of the recipient pig.
Duration of standoff	If two pigs stand in parallel or head to head, shoulder to shoulder, colliding, squeezing and chasing each other during the fight, and the two individuals are about equal in strength and form a single dyadic interchange, but there is no avoidance behavior [51]. The duration is defined as "Duration of standoff", which uses seconds as a unit of time.
Frequency of standoff	As mentioned above, the number of standoff behaviors is launched by two pigs for 72 h after mixing, which is defined as "Frequency of standoff".
CAS	The composite aggressive score (CAS) is defined as follows: $CAS = \text{frequency of active attack} + 0.07 \times \text{duration of active attack[s]}$ [52].
Duration of fight	The fighting of pigs includes active attack, bullying and standoff. The total duration of the three types of fighting behavior is defined as "Duration of fight", with seconds as the unit of time.
Win	If a pig continues to attack other pigs during the fight, and the attacked pig dodges, stops fighting and tries to escape, but the aggressive pig still has intention to continue to attack, it is deemed to have won the fight [53]. The number of victories achieved by the aggressive pig during fighting for 72 h after mixing is defined as "win".

Table 2 The allele frequencies of single nucleotide polymorphisms (SNPs) in the porcine *ARHGAP24* gene.

SNPs	location	Mutation type	Allele	Aggressive/docile frequency	χ^2	<i>P</i> -Value
rs339198696	5' flanking region	A > C	A	0.54/0.71	1.39	0.238
			C	0.46/0.29		
rs344700648	5' flanking region	T > A	T	0.00/0.25	6.86	0.009**
			A	1.00/0.75		
rs335052970	5' flanking region	G > A	G	0.92/0.50	10.08	0.001**
			A	0.08/0.50		
rs344498203	5' flanking region	C > G	C	0.88/0.71	2.02	0.155
			G	0.13/0.29		
rs323776551	5' flanking region	C > A	C	0.21/0.50	4.46	0.035*
			A	0.79/0.50		
rs342083908	5' flanking region	G > A	G	0.88/0.58	5.17	0.023*
			A	0.13/0.42		
rs333053350	5' UTR	G > T	G	0.79/0.54	3.38	0.066
			T	0.21/0.46		
rs342210686	5' UTR	G > A	G	0.58/0.33	3.02	0.082
			A	0.42/0.67		
rs328435752	5' UTR	A > G	A	0.79/0.54	3.38	0.066
			G	0.21/0.46		
rs787973778	5' UTR	A > C	A	0.83/0.54	4.75	0.029*
			C	0.17/0.46		

Note: χ^2 : chi-square value; * Statistically significant, * $P < 0.05$, ** $P < 0.01$.

Figures

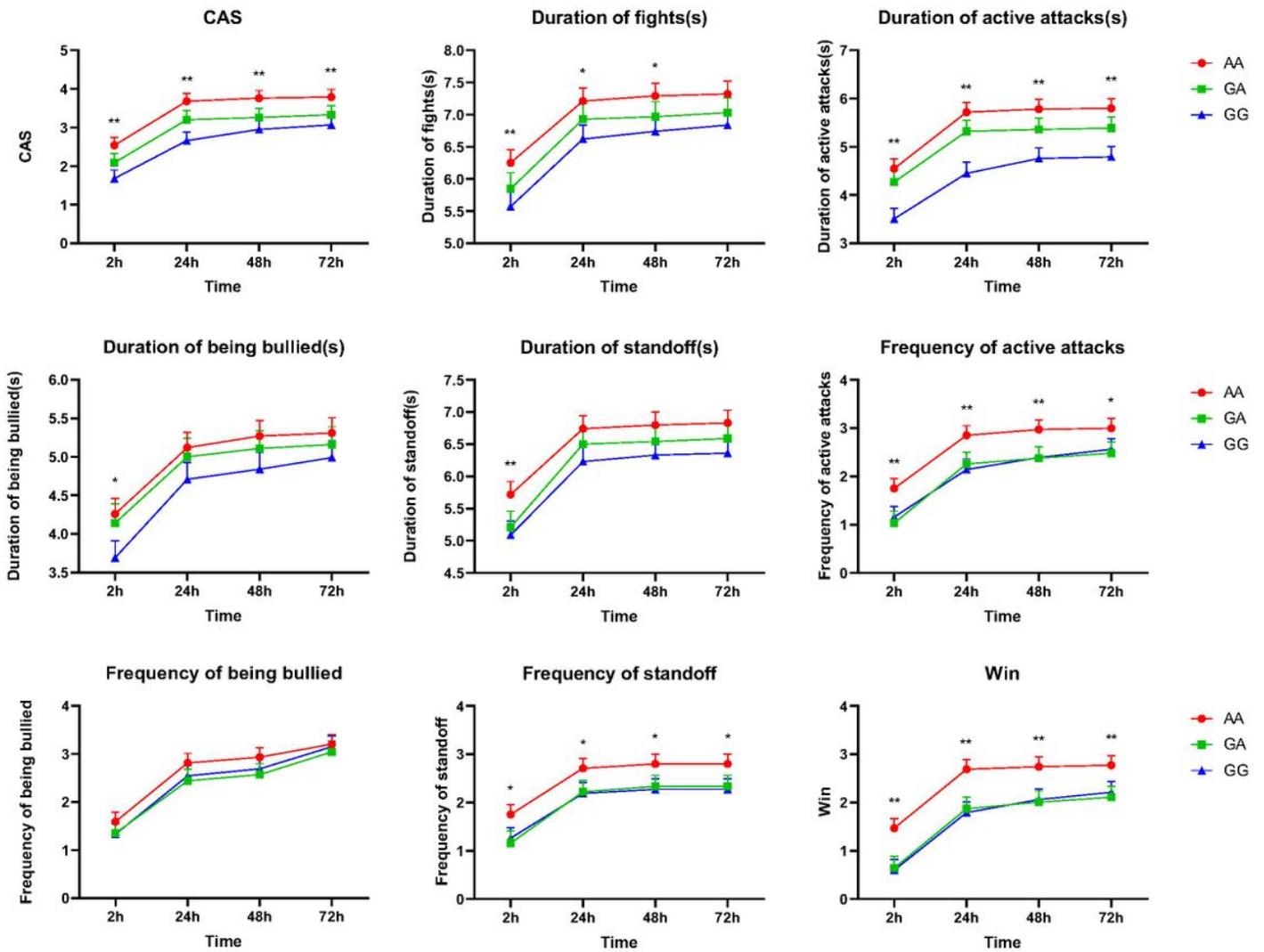


Figure 1

Associations of the SNP rs335052970 in *ARHGAP24* gene with aggressive behavioral traits at the 2, 24, 48, or 72 h after mixing in weaned pigs (LSM \pm SE). * $P < 0.05$; ** $P < 0.01$, indicate that the difference is significant.

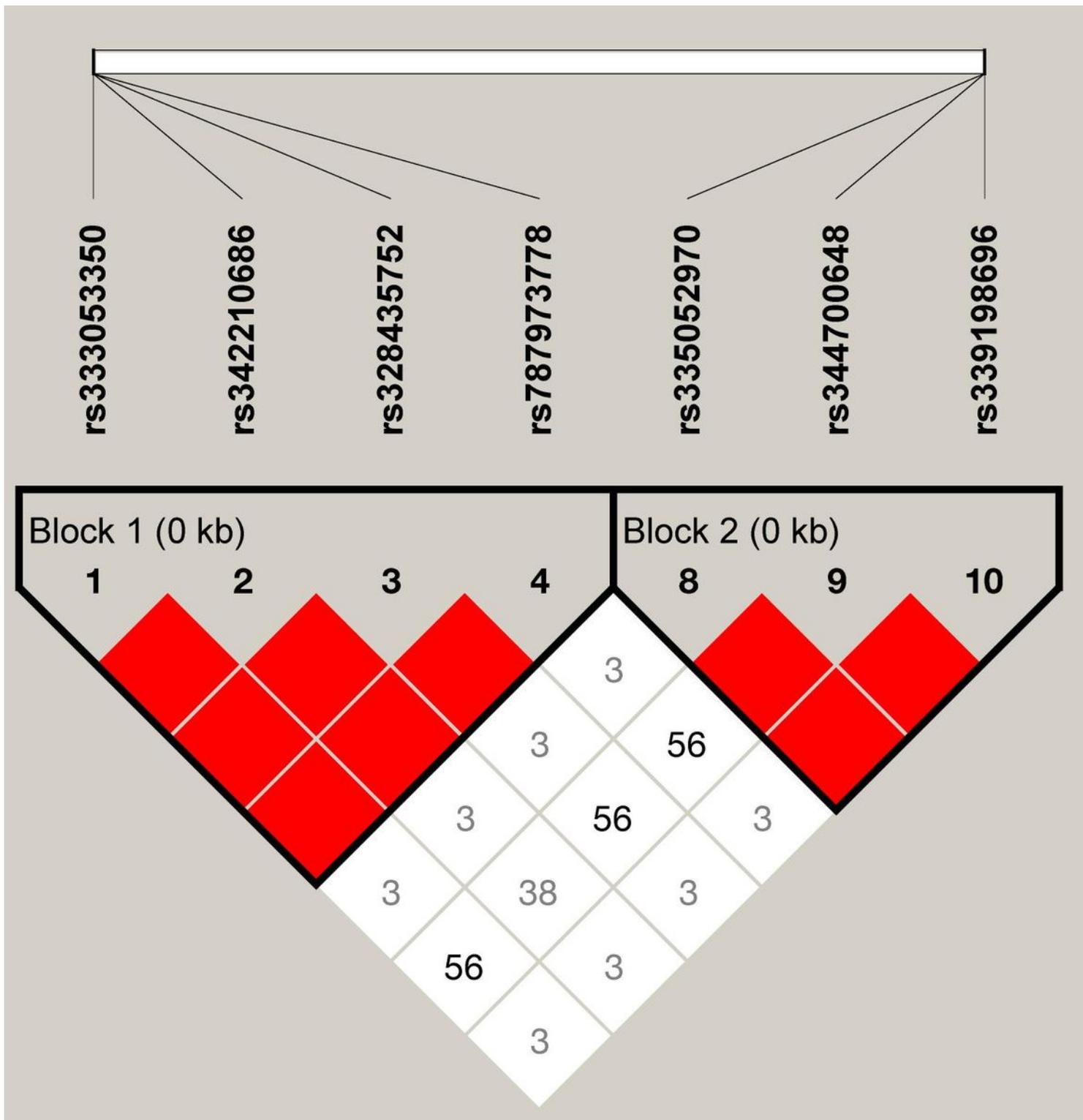


Figure 2

LD among the SNPs in porcine *ARHGAP24* gene ($D' = 0.03 - 1.00$). Note: The blocks indicate haplotype blocks and the text above the horizontal numbers is the SNPs name. The values in boxes are pairwise SNP correlations (D'), while bright red boxes without numbers represent complete LD ($D' = 1$).

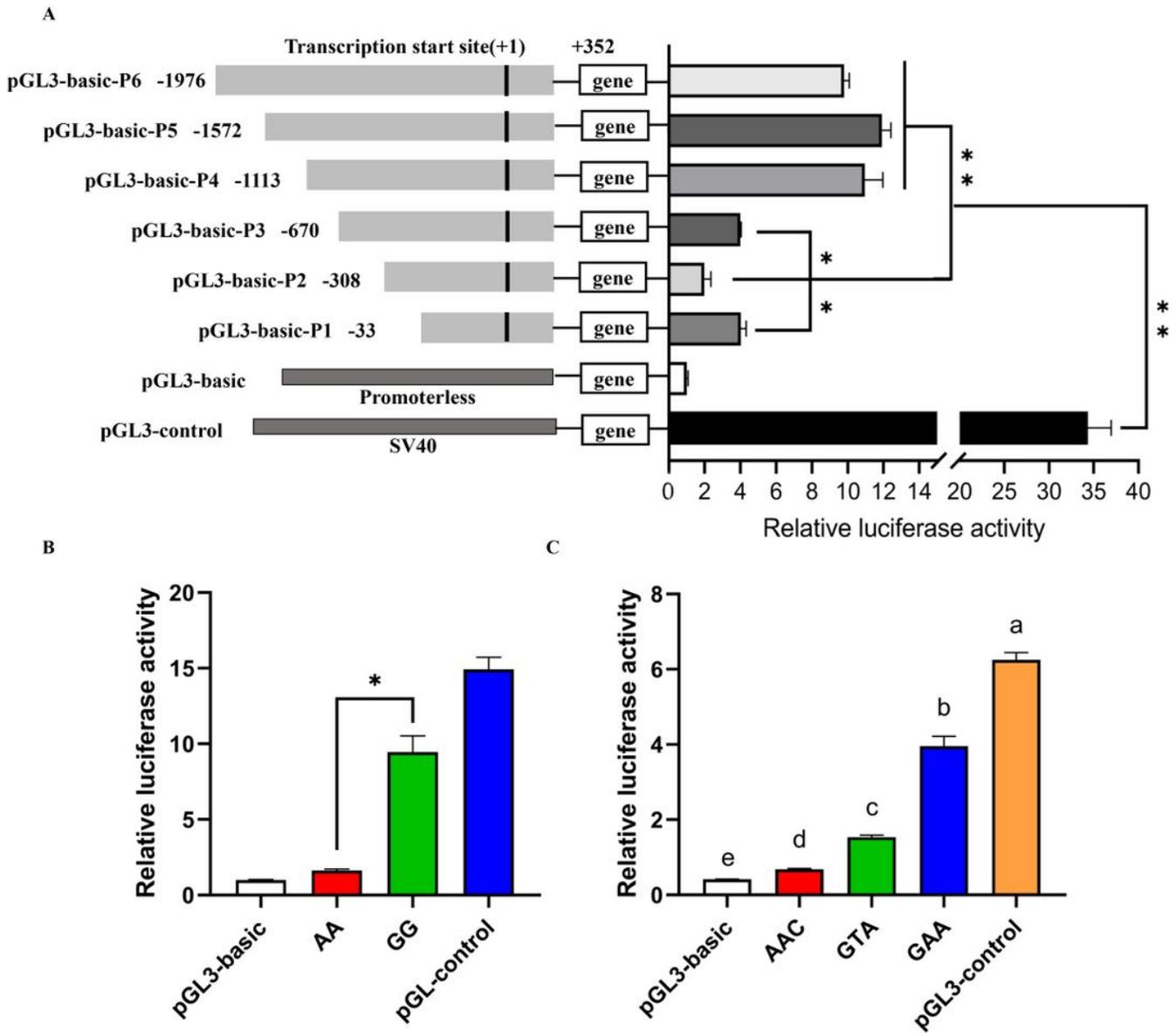


Figure 3

Luciferase assays for porcine *ARHGAP24* promoter activity analyses. PGL3-basic as a negative control and PGL3-control as a positive control. (A) Six luciferase reporter plasmids expressing successive truncations of the *ARHGAP24* promoter sequence were constructed and transfected into HEK 293T cells. (B) Luciferase reporter gene assays of porcine *ARHGAP24* alleles contained rs335052970 (-744G > A). (C) The Luciferase activities of plasmids contained three haplotypes of porcine *ARHGAP24* gene. The relative luciferase activity values represent the mean \pm SEM of three independent experiments. Statistical differences in luciferase activity were assessed using the one-way ANOVA analysis, * $P < 0.05$, ** $P < 0.01$. Different letters (a, b, c, etc.) indicate that the difference is significant ($P < 0.05$).

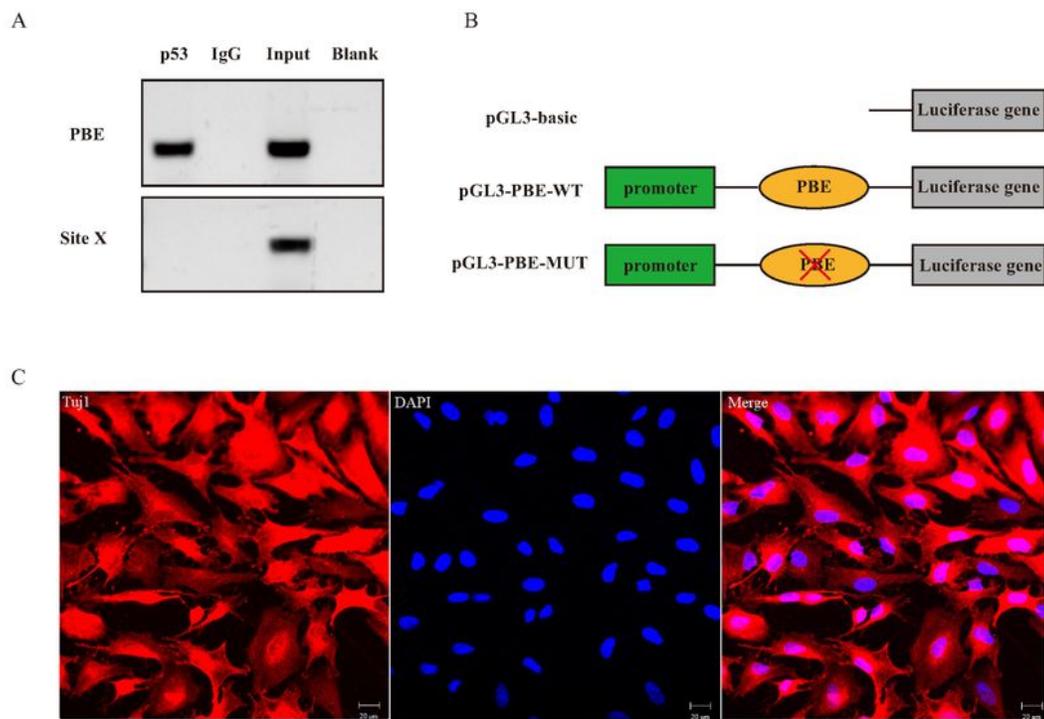


Figure 4

Transcription factor p53 directly targeted the binding element (PBE) motif of *ARHGAP24* gene in porcine neural cells. **(A)** Confirmation the direct interaction between p53 and *ARHGAP24* promoter. CHIP-PCR assay was performed with IgG as negative control. Site X, a negative control locus; Input, total DNA from

untreated cells. **(B)** Construction of luciferase activity reporter vectors containing wild-type (WT) and mutant-type (MUT) PBE on the promoter of *ARHGAP24* gene. Blue boxes represent luciferase gene; green boxes represent pGL-3 promoter; orange ovals represent PBE; red fork represents mutation. **(C)** Immunofluorescence identification of porcine primary neural cells. Immunofluorescence staining of porcine neural cells with Tuj1 (red) and DAPI (blue), Scale bars represent 20 μ m.

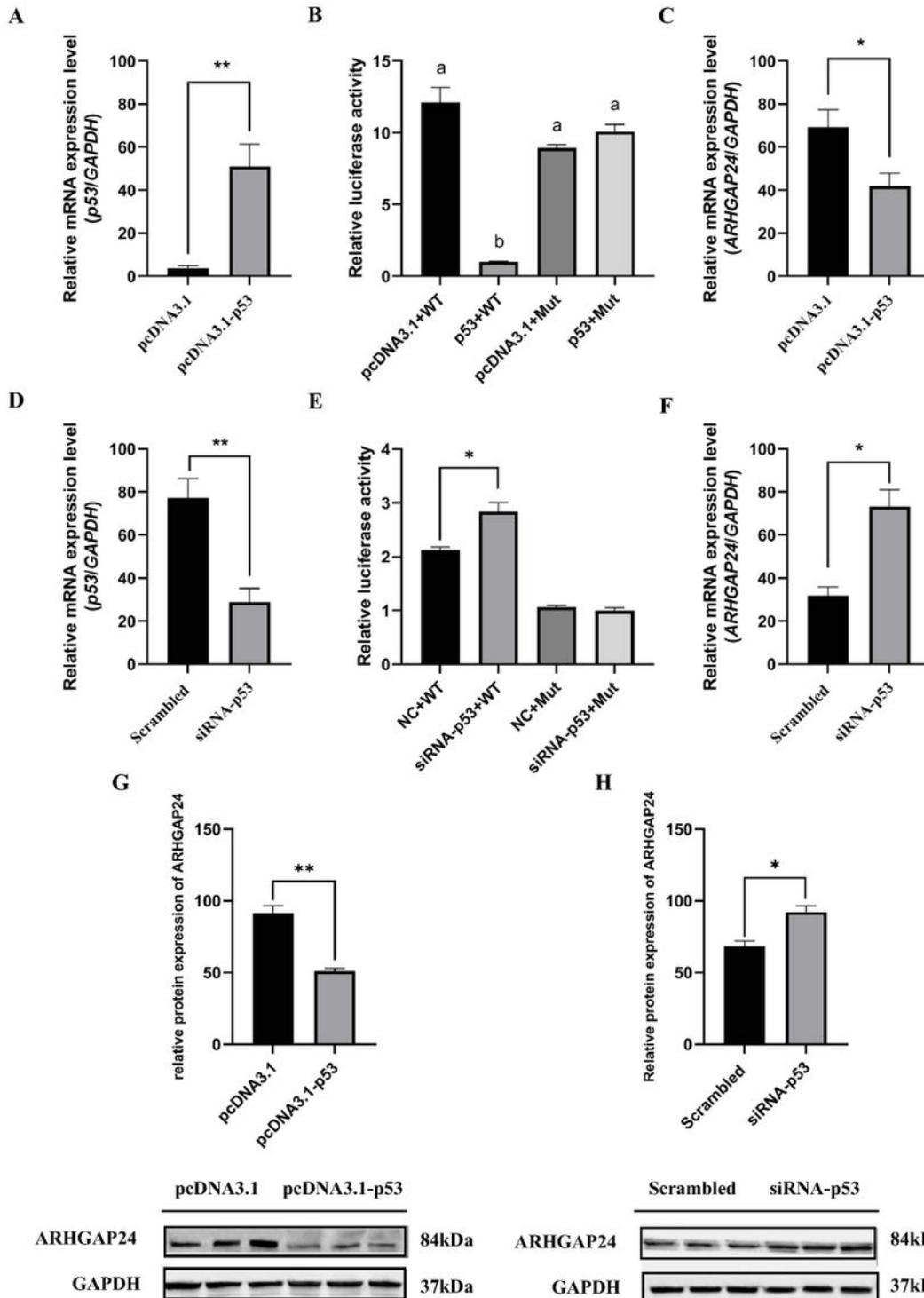


Figure 5

Transcription factor p53 regulate *ARHGAP24* gene expression in porcine neural cells. (A) The mRNA level of p53 after p53 over-expressed. (B) The mRNA level of p53 after p53 knockdown. (C) The luciferase activity of the *ARHGAP24* promoter region after p53 over-expressed. (D) The luciferase activity of *ARHGAP24* promoter region after p53 knockdown. (E) The mRNA level of *ARHGAP24* after p53 over-expressed. (F) The mRNA level of *ARHGAP24* after p53 knockdown. (G) Western blot analyses of *ARHGAP24* protein expression in porcine neural cells transfected with pcDNA3.1-p53 and pcDNA3.1(+). (H) Western blot analyses of *ARHGAP24* protein expression in porcine neural cells transfected with Scrambled and siRNA-p53. The protein levels were normalized to GAPDH. * $P < 0.05$, ** $P < 0.01$. Different letters (a, b, c, etc.) above the columns indicate that the difference is significant ($P < 0.05$)

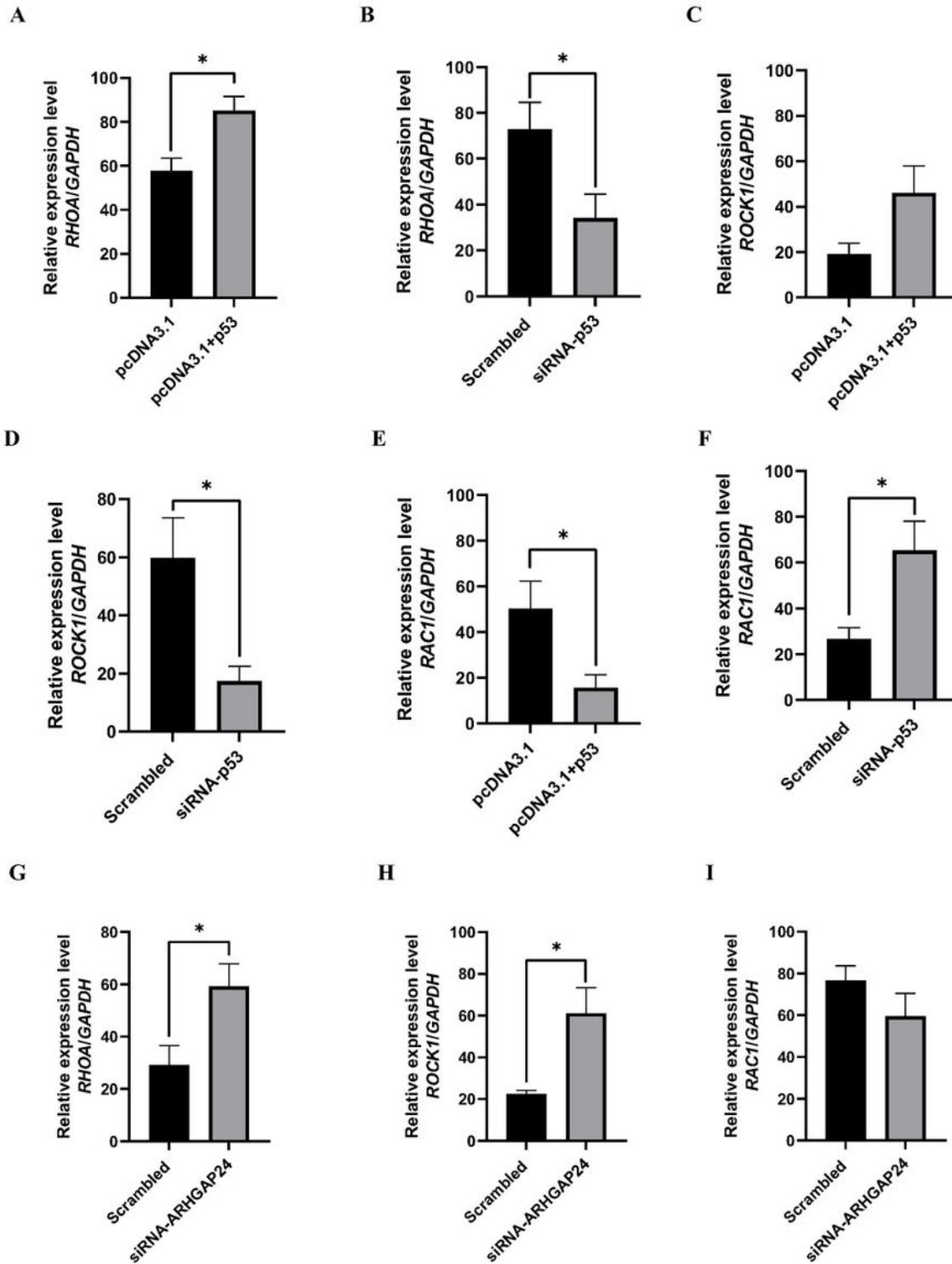


Figure 6

Transcription factor p53 Regulate Axon Guidance Pathway Related Gene Expression in Porcine Neural Cells. **(A)** The mRNA level of *RHOA* after p53 over-expressed. **(B)** The mRNA level of *RHOA* after p53 knockdown. **(C)** The mRNA level of *ROCK1* after p53 over-expressed. **(D)** The mRNA level of *ROCK1* after p53 knockdown. **(E)** The mRNA level of *RAC1* after p53 over-expressed. **(F)** The mRNA level of *RAC1* after p53 knockdown. **(G)** The mRNA level of *RHOA* after *ARHGAP24* knockdown. **(H)** The mRNA level of

ROCK1 after *ARHGAP24* knockdown. (I) The mRNA level of *RAC1* after *ARHGAP24* knockdown. * $P < 0.05$, ** $P < 0.01$. Different letters (a, b, c, etc.) above the columns indicate that the difference is significant ($P < 0.05$)

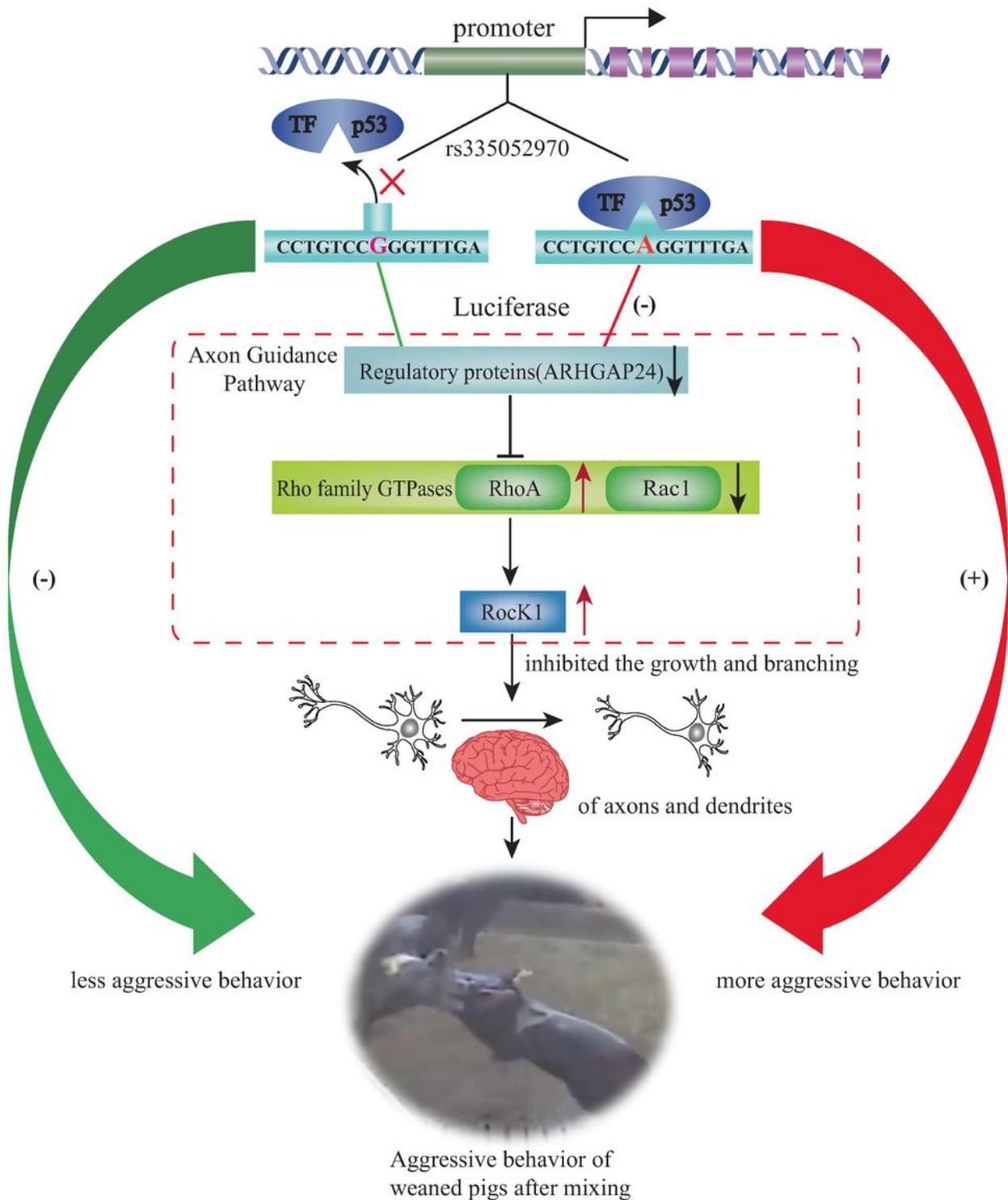


Figure 7

A hypothesized regulating pathway by rs335052970 on aggressive behavior of weaned pigs after mixing. Based on the results, we hypothesized a regulating pathway by rs335052970 in *ARHGAP24* gene on aggressive behavior of pigs. In this diagram, rs335052970 binds to transcription factor p53 to form a complex that regulates *ARHGAP24* gene expression. Specifically, the promoter with allele A is more capable of binding to the transcriptional repressor p53 and downregulates *ARHGAP24* gene expression. Since RhoA and Rock1 are the downstream targets of *ARHGAP24*, p53 also activates the expression of RhoA and Rock1. This might reduce the growth and branching of axons and dendrites, which makes pigs more aggressive after weaning. Consequently, the promoter with allele A of rs335052970 upregulates the aggression of weaned pigs after mixing.

Supplementary Files

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