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Research

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AXIN2 reduces the survival of porcine induced pluripotent stem cells (piPSCs)

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Abstract

Background: The establishment of porcine pluripotent stem cells (piPSCs) is still a critical topic and challenging issue. However, all piPSCs are extremely sensitive to changes of the culture conditions.In addition , the side effect of inhibitors in culture medium confine the pluripotency and practicability. This study aimed to investigate the roles of AXIN in piPSCs and further explore the mechanism. Here, porcine *AXIN1* gene and *AXIN2* were knockdown, cloned, and overexpressed in piPSCs. Digital RNA-seq was performed to explore the mechanism of cell proliferation and anti-apoptosis.

Results: Here, we found (1): overexpression of the porcine *AXIN2* gene significantly reduce the survivability of piPSCs, meanwhile wreck the pluripotency of piPSCs; (2): The Digital RNA-seq analysis reveals that *AXIN2*, as a negative effector of the WNT signaling pathway, whom after knockdown enhances the expression of genes involved in cell cycle such as *CCND1*, and reduced the expression of genes related to cell differentiation, cell death, and cell apoptosis.

Conclusion: AXIN2 could reduce the pluripotency and survival of piPSCs and also provided a potential to simplify the cultrue medium.

Introduction

The Wnt/β-catenin signaling pathway is a critical signal pathway for embryonic development and has function in many cellular processes including tumorigenesis, cell proliferation, differentiation, pluripotency, migration, and apoptosis [1]. CHIR99021, an inhibitor of GSK3, is wildly used in induced pluripotent stem cells (iPSCs) culture condition, and activate the Wnt/β-catenin signaling pathway to achieve the ability to maintain pluripotency [2]. However, the use of inhibitors could compromise epigenetic and genomic stability [3, 30]. As a result, it is needed to find other ways to activate the Wnt/β-catenin signaling pathway without inhibitors involving in.

β-catenin (CTNNB1) is the effector molecule of the Wnt/β-catenin signaling pathway [4]. CTNNB1 is degraded by a destruction complex that includes AXIN, APC,

GSK3β, and CK1 [5]. CTNNB1 is not only in favor of the self-renewal, pluripotent state of stem cells, but also makes for the reprogramming process. To be detailed, three groups have independently studied CTNNB1 in mESCs and concluded that CTNNB1 is dispensable for mESC self-renewal [8, 9, 10]. In addition, CTNNB1 has been linked to the maintenance of the pluripotent state in human and mouse ESCs [11]. Notably, CTNNB1 interacts with reprogramming factors Klf4, Oct4, and Sox2, further enhancing the expression of pluripotency circuitry genes [12]. Wnt/β-catenin signaling has also been shown to enhance the induction of pluripotency in somatic cells, obtaining through viral transduction or cell fusion [13].

AXIN provides a crucial scaffold for this destruction complex [6]. Upon receptor activation by Wnt ligands, AXIN1 and/or AXIN2 are recruited to the phosphorylated tail of LRP and the re-localization inhibits CTNNB1 ubiquitination, then causing CTNNB1 accumulation and translocation to the nucleus [6, 7]. Thus, AXIN1 and/or AXIN2 are pivotal in controlling canonical Wnt signaling [7]. While it was cleared in human AXIN2, but not AXIN1, worked as a negative effector for the Wnt/β-catenin signaling pathway [15, 16, 17]. Moreover, the maintenance of the pluripotent state of many reported piPSCs was dependent on the regulation of the Wnt/β-catenin signaling pathway [18]. However, there was no evidence to prove that AXIN has the function of the Wnt/β-catenin signaling pathway in piPSCs .

Here, we confirmed AXIN2 worked as a negative effector for the Wnt/β-catenin signaling pathway in piPSCs, and a low level of AXIN2 gained an improved ability to proliferate and resist apoptosis as well, the most important, which could replace CHIR99021. Our data not only suggested that AXIN2 had a stronger effect on the pluripotency of piPSCs than AXIN1, and explained the potential feedback mechanism in this process.

2 Methods

2.1 Cell culture

The piPSCs constructed by [41] were used in this study. piPSCs were cultured on feeder maintained in LB2i medium [31], which consisted DMEM (Hyclone) supplemented with 15% FBS (VIS), 10 ng/mL LIF (Sino Biological, 14890-HNAE), 10 ng/mL bFGF (Sino Biological, 10014-HNAE), 0.1 mmol/L NEAA,1 mmol/L L-glutaMAX, 0.1 mmol/L β-mercaptoethanol (Sigma-Aldrich, M3148), 3 μmol/L CHIR99021 (MCE, HY-10182), 2 μmol/L SB431542 (Selleck, S1067), 4 μg/mL Doxycycline (Sigma-Aldrich, D9891), and 100 μg/mL streptomycin ,100 units/mL penicillin. The piPSCs were passaged using TrypLE™ Select (Invitrogen) into a single cell at a ratio of 1:50 every 5 or 6 days.

2.2 Plasmid and cloning

To investigate the function of AXIN, the genes, *AXIN1* and *AXIN2* were PCR-amplified from piPSCs and then were subcloned into EF1-3FLAG-MCS-T2A-puromycin Lentiviral vectors [35].

In this study, two shRNA vectors of both *Axin1* and *Axin2* were designed by using the online shRNA design tool from Invitrogen (http://rnaidesigner.thermofisher.com/rnaiexpress)and subcloned into pCDH-U6-MCS-EF1-GFP-T2A-puromycin vector [31]. The potential off-target sites were filtered out using BLAST based on the porcine genome. The shRNA vectors of *AXIN1* or *AXIN2* were infected in piPSCs and the knockdown efficiencies were 80% and 57% respectively were used in this study.

2.3 Lentivirus packaging and transduction

HEK293T cells used in this study were cultured in DMEM supplemented with

10% FBS, 1 mM L-glutaMAX (Gibco), 0.1 mM NEAA (Gibco, United States),0.1 mM β-mercaptoethanol (Sigma-Aldrich, United States), 100 μg/ml of streptomycin, and 100 U/ml of penicillin.

HEK293T cells were seeded onto a 6-well plate and grown to 70% or 80% confluence [33]. Then the package vector and lentivirus backbone (psPAX2 and pVSV-G) were transfected into HEK293T cells using PEI (polyethyleneimine, Sigma) according to the manufacturer's instructions.

Then the medium contained viral particles was obtained from each individual and filtered through a 0.45 μm filter (Millipore, USA). For the lentiviral transduction, 2×10^4 cells were plated on feeder covered 12 well plate per well. Then the virus particles and fresh media were added at a ratio of 1:1 supplemented with 4 μg/mL polybrene for 12 h -16 h. The infected piPSCs were then plated on a feeder-coated 6-well plate after 12 h and cultured by the piPSCs culture medium for 5-7 days. After 1 week, stably infected colonies were screened with puromycin (10 μg/mL) for 24 hours, and then the cell lines were successfully established.

2.4 AP staining

Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 minutes at room temperature, washed twice using ice-cold PBS, and developed with AST Fast Red TR and α-Naphthol AS-MX Phosphate (Sigma-Aldrich) according to the instructions [31，33，34]. The cells were incubated with the mixture (1.0 mg/mLFast Red TR, 0.4 mg/mL Naphthol AS-MX in 0.1 mol/L Tris-HCL Buffer) at room temperature for 20 minutes. Then the AP-positive colonies showed in red color. The images were captured by Nikon phase-contrast microscope.

2.5 RNA extraction, reverse transcription and qRT-PCR detection

Total RNA was extracted by using RNAiso Plus reagent (TaKaRa, Japan) according to protocol. Then by using the PrimeSript™ RT reagent Kit (Tiangen, China)

according to its protocol the cDNA was synthesized as Reverse transcription PCR (RT-PCR). The qRT-PCR reaction system was 20 μL in volume: 10 μL SYBR® Premix Ex Taq II (Vazyme), 0.5 μL cDNA, 0.4 μL PCR Forward Primer (10 μM), 0.4 μL PCR Reverse Primer (10 μM) and added RNase-free water to a total volume of 20 μL. The specific primers of genes used in this study were shown in Table 2.

2.6 Western blot

The cells were lysed by cold RIPA buffer (Beyotime, China) for 30 min on ice, added to 5× SDS-PAGE loading buffer (Beyotime), and heated at 100 °C for 10 min. Then the lysates were then separated by 8-12% SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes by Trans-Blot SD Cell and Systems (Bio-Rad) for 45 min at 15 V. After blocked with 5% non-fat milk in TBS-T buffer (20 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20), PVDF membranes were incubated with the primary antibodies [36]. After incubation with a secondary antibody, the signals were measured using Chemiluminescent Imaging System (Tanon, China). Beta-ACTIN was used as an endogenous loading control. Antibodies used in this study were: Cyclin D1 (WL01435a, Wanleibio), Caspase3 (WL02117, Wanleibio), β-Catenin （51067-2-AP, Proteintech） and β-Actin (AC028, ABclonal).

2.8 Apoptosis detection

The apoptosis detection was performed by Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, A211) according to the manufacturer's instruction [29]. In brief, the cell suspension was washed twice with pre-cooled PBS and centrifuged at 1000 g for 5 minutes. Adding 100 μL 1× Binding Buffer and blowing gently to make a single-cell suspension. Then add 5 μL Annexin V-FITC and 5 μL PI Staining Solution, and incubate for 10 minutes at room temperature. Finally, add 400 μL 1× Binding Buffer and mix gently. Then the images were captured with the phase-contrast

microscope (Nikon).

2.9 RNA-seq analysis

Total RNA of the RNA samples was isolated from cell pellets by Trizol reagent (TaKaRa) and RNA-Seq library was generated according to the manufacturer's recommendations [34]. Paired-end 150-bp sequencing was performed on Illumina HiSeq2500 PE150 by SEQHEALTH Technology Corporation (China). Clean reads were aligned to pig genome Ssc11.1, and the expression level was normalized as RPKM with gene annotation file. Differential expression genes and functional enrichment for Gene Ontology (GO) and KEGG were performed with the GO stats package.

2.10 Immunofluorescence staining

The cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 10 minutes [36]. Fixed cells were washed three times using ice-cold PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and then subsequently blocked for 1 hour at room temperature in PBS containing 10% FBS. The cells were incubated with a blocking buffer containing CTNNB1 antibodies (51067-2-AP, Proteintech) at 4°C overnight. The Alexa Fluor® 594 conjugate goat anti-rabbit IgG (H + L; #ZF-0516; ZSGB-BIO) was diluted in blocking buffer and incubated at room temperature for 1 hour. After washing with PBS for three times, the nuclei were stained with 10 μg/mL DAPI for 10 minutes. Finally, the images were collected by an EVOS fluorescence microscope.

2.11 Bimolecular Fluorescence Complementation

Ctnnb1 was PCR-amplified from piPSCs and then were subcloned into pBiFC-VC155 vectors [37]. *Tcf3, Tcf5* were PCR-amplified from piPSCs and then

were subcloned into pBiFC-VN173 vectors. HEK293T cells were seeded onto a 6-well plate and grown to 70%-80% confluence. Then vectors were transfected into HEK293T cells using PEI (polyethyleneimine, Sigma) according to the manufacturer's instruction. There were three groups: HEK293T cells with VN173TCF3-VC155CTNNB1, HEK293T cells with VN173TCF5- VC155CTNNB1, and HEK293T cells with VN173-VC155 as control (NC).

3 RESULTS

3.1 The absence of CHIR99021 affected the pluripotency and proliferation of piPSCs

To determine whether WNT signals are essential for the survival of piPSCs, we withdrew the WNT activator CHIR99021 for 3 passages, so that the effect could fade away. We further investigated its effects on the pluripotency and proliferation of piPSCs. The results showed that the AP staining was weaker and the colony became smaller after CHIR99021 was withdrawn (Figure 1A, 1B). To gain a deeper understanding of what happened from the mRNA level, we tested the expression levels of *CTNNB1*, *APC*, *AXIN1,* and *AXIN2* which were other components of the WNT cascade as well. The results showed dropped amount of *APC and* a rose amount of *AXIN2* without CHIR99021 (Figure 1C, 1D), which meant the WNT signaling pathway was more blocked. Further Quantitative Real-time PCR (qPCR) analysis of pluripotent genes revealed a significant decrease in the expression levels of endogenous *OCT4, SOX2, KLF4,* and *MYC* between the two groups (Figure 1E). And we wanted to explore why a smaller colony occurred when CHIR99021 was withdrawn, we tested cell cycle-related genes. Although the expression level of *CCND2* was upregulated, the decrease of *CCNA2*, *CCND1,* and smaller morphology implicated a positive correlation among WNT signals and the growth of piPSCs. These results showed that CHIR99021 could maintain the pluripotency and proliferation of piPSCs via the WNT signaling pathway.

3.2 *Axin2* had a stronger influence on the pluripotency of piPSCs than *Axin1*

As AXIN provides a crucial scaffold for the WNT signaling pathway, then further investigate the role of AXIN1 and AXIN2 in piPSCs, we firstly established *AXIN1* and *AXIN2* knockdown (sh-AXIN1/AXIN2) cell lines. All piPSCs lines were generated using lentiviral vector. qPCR analysis showed that in sh-AXIN1 piPSCs lines the expression of *AXIN1* was about 80% reduced compared with that of the NC group and that *AXIN2* in sh-AXIN2 piPSCs lines was about 58% reduced than NC group (Figure 2A, 2B). qPCR analysis also displayed a significant decrease of *APC* in sh-AXIN2 group but no fluctuation in sh-AXIN1 group (Figure 2C). This result revealed *AXIN2* but not *AXIN1* might influence *APC*.

Then AP staining was used to determine the pluripotency of sh-AXIN1 and sh-AXIN2 piPSCs. The area of colonies was measured and the number of AP positive colonies was counted. The results showed that the sh-AXIN2 piPSCs formed the biggest colone with the strongest AP staining, while sh-AXIN1 piPSCs showed no significant change compared to NC group (Fig.2D, 2E). Then we examined the expression level of endogenous *OCT4, SOX2, KLF4, MYC* in sh-AXIN2, sh-AXIN1, and NC groups by qPCR, the results demonstrated significant up-regulated *KLF4* and *MYC* in sh-AXIN2 and no evident change in sh-AXIN1 group (Figure 2F,2G). This result revealed *Axin2* could affect the pluripotent state of piPSCs via regulating *KLF4* and *MYC*.

We next constructed *AXIN1* overexpression (OE-AXIN1) and *AXIN2* overexpression (OE-AXIN2) cell lines to investigate the role of *AXIN1* and *AXIN2* in piPSCs. qPCR showed that the expression of *AXIN1* in OE-AXIN1 piPSCs was significantly higher than that of the CON group and in OE-AXIN2 piPSCs lines the expression of *AXIN2* was about 80 times higher than NC group (Figure 3A, 3B). Moreover, the expression level of *APC* was also upregulated in OE-AXIN2 group (Figure 3C), which provided a shred of evidence to the previous conclusion: *APC* might be influenced by *AXIN2 but* not *AXIN1*. *CTNNB1* level also decreased in OE-AXIN2 group (Figure 3C). Then the pluripotency of OE-AXIN1 and OE-AXIN2

was examined with AP staining. The area of colonies was measured and the number of AP positive colonies was counted. The result showed that the AP staining was much weaker in OE-AXIN2 than that of CON group, while OE-AXIN1 piPSCs showed as normal as NC group (Figure 3D, 3E). The results demonstrated the RNA level of *MYC* was significantly down-regulated in OE-AXIN2 and OE-AXIN1 group, and the RNA level of *KLF4* was up-regulated in OE-AXIN1 group (Figure 3F, 3G). All results indicated that *AXIN2* had a stronger influence on the pluripotency of piPSCs than *AXIN1*.

According to previous research in our laboratory [32], feeder could influence the pluripotent state of piPSCs. Thus, because of the significant change in pluripotency in *sh*-AXIN2 and OE-AXIN2 group, we cultured CON, sh-AXIN2, and OE-AXIN2 cell lines without the feeder. The AP staining showed that OE-AXIN2 piPSCs with the weakest AP staining started to differentiate and lose the ability to yield colonies while sh-AXIN2 piPSCs could form bigger colonies with the strongest AP staining than that of CON group (Figure 3H). In a word, a low level of *AXIN2* was curial for piPSCs to maintain pluripotency.

3.3 AXIN2 affected the proliferation of piPSCs by CCND1

In the previous results, AXIN could affect the amount of CTNNB1 in the nucleus, and then influence the expression of WNT-related genes [1]. We further analyzed the mRNA and protein level of CTNNB1. There was no significant change in mRNA level of all groups, while Western blotting showed huge differences, especially in groups with changing AXIN2, higher level in sh-AXIN2, and lower level in OE-AXIN2 group respectively (Figure 2C, 3C, 4E, 4F). Immunofluorescence results showed that we could hardly detect CTNNB1 in nucleus in OE-AXIN2 group, while in sh-AXIN2 group, CTNNB1 diffusely distributed in whole-cell and more than NC group (Figure 4D). This result displayed a negative correlation between AXIN2 and CTNNB1 in protein level.

While the morphology change followed by the expression level of *AXIN2* shocked us, we posited that the proliferation might involve in. To be detailed, the piPSCs with a

higher level of *AXIN2* exhibited much smaller colonies, and could not withstand the same ratio when went through passaging every 5 days. The cell growth curve showed that the sh-AXIN2 group had bigger colonies with faster proliferation than NC group, while the OE-AXIN2 group showed lower proliferation than CON, while sh-AXIN1 and OE-AXIN1 showed no significant change (Figure 4A, 4B). Moreover, the CCND1 level showed the same tendency as CTNNB1 after the changing of *Axin2* as well (Figure 4C, 4D, 4E, 4F). To be detailed, AXIN2 had a negative relationship with CTNNB1 and CCND1.

In the previous study, nuclear function of CTNNB1 could depend on transcription factor T-cell factor (TCF) and lymphocyte-enhancing factor (LEF) [19, 38]. The accumulated CTNNB1 is then translocated into the nucleus and bound to the TCF/LEF to promote the expression of downstream genes *CCND1* and *MYC* [20]. We further analyzed *TCF2, TCF 3, TCF4,* and *TCF5* by qPCR, and the results revealed only *TCF3* and *TCF5* changed followed on the *AXIN2* changing (Figure 4F). To identify whether CTNNB1 could team up with TCF3 or TCF5 in piPSCs, we applied split fluorescent protein to consolidate it. We could see TCF3 and TCF5 both combining CTNNB1, while TCF5 performed stronger binding with CTNNB1 (Figure 4G). These results indicated that *Axin2* has a certain effect on the proliferation through AXIN2/CTNNB1/TCF3,5/CCND1 axis.

3.4 AXIN2 knockdown enhanced anti-apoptotic ability of piPSCs through Caspase family and BAX

We attempted to gain insights into the molecular consequences that were triggered in response to the knockdown of *AXIN2*. RNA sequencing was performed on these two cell lines after 5 days of culture. Transcriptional changes in both directions were observed after the knockdown of AXIN2 (489 up vs. 910 down) indicating that knockdown of *AXIN2* mainly promoted the expression of some genes to promoting cell growth and improving the anti-apoptotic ability of piPSCs (Figure 5A). To further investigate this process, we applied KEGG analysis for the up-regulated and

down-regulated genes. The list of genes upregulated in sh-AXIN2 group showed that enriched KEGG pathways included Protein digestion and absorption, PI3K-Akt signaling pathway, Focal adhesion, ECM−receptor interaction, Cell adhesion molecules (CAMs). And the list of genes downregulated in sh-AXIN2 cells enriched KEGG pathways included Apoptosis, TGF-beta signaling pathways (Figure 5B, 5C).

Except for the proliferation, we also assumed that apoptosis might also determine the changed colony size. In a previous study, lower expression of *AXIN2* in embryo improved the ability to resist apoptosis by up-regulating *BCL2* [21, 22], however, *BCL2* did not be significantly influenced by *AXIN2* in this study. The qPCR analysis further found the expression levels of pro-apoptotic protein *BAX*, *CASPASE-3,* and *CASPASE-9* were significantly decreased in sh-AXIN2 and increased in OE-AXIN2 (Figure 5D). The Western blotting analysis further found that the expression level of Cleaved caspase-3 in the OE-AXIN2 group was significantly higher than that in the CON group, which also indicated that more severe apoptosis occurred in the OE-AXIN2 group (Figure 5E). Then, we further detected the death of piPSCs after overexpression of *AXIN2* by using PI (P) and Annexin V (A) staining. The results showed that the overexpression of *AXIN2* significantly promoting the apoptosis of piPSCs at the late stage of culture (Figure 5F).

All results indicated that *AXIN2* affected *CASPASE-3*, *CASPASE-9,* and *BAX but* not *BCL2*, then determine the survival of piPSCs.

3.5 piPSCs with a lower expression level of AXIN2 could grow well without CHIR99021

To verify whether the part of the activated WNT signal pathway effect produced after *AXIN2* knockdown was enough for piPSCs to grow, we withdrew CHIR99021 in the culture medium. As to the morphology, there was showing no significant change (Figure 6A, 6B). We further investigated its effects on the pluripotency and proliferation of piPSCs. AP staining was used to preliminarily determine the pluripotency of piPSCs. The result showed that the AP staining was as strong as the

group cultured in normal medium and qPCR revealed risen to the level of *MYC* (Figure 6C). The cell cycle-related genes expression levels were no differences (Figure 6D), which means sh-AXIN2 could withstand the slowed the growth in culture medium without CHIR99021. To sum up, piPSCs with the low expression level of AXIN2 could maintain without CHIR99021.

To conclude, AXIN2 degrades CTNNB1 then restrains the proliferation, meanwhile, AXIN2 improves the CASPASE family and BAX leading to cell death (Figure 6E).

DISCUSSION

In general, our study revealed that *AXIN2* participated in the WNT signaling pathway in piPSCs, and a low level of *AXIN2* was essential to maintain the proliferation and pluripotency of piPSCs. This surprisingly could not be consistent with the previous study which indicated XAV939, known as a stabilizer of AXIN1 and AXIN2, could enhance the pluripotency of iPSCs [17]. Moreover, knockdown *AXIN2* could be enhanced the expression of genes involved in the cell cycle such as *CCND1*, and reduced the expression of genes related to cell differentiation, cell death, and apoptosis, thus maintaining the pluripotency and survival of piPSCs.

AXIN family could promote β-catenin ubiquitination and promote β-catenin degrading so that the WNT signaling pathway would play a role in the pluripotency and differentiation, but which factor of *AXIN* is unknown. In this study, we found that *AXIN2*, but not *AXIN1*, could regulate the expression level of *KLF4* and *MYC* so that the piPSCs could be maintained without CHIR99021. The result is consistent with the studies of mouse iPSCs[39,40].

The endogenous OCT4, which was the most central factor related to pluripotency [32], was not activated in piPSCs. Plentiful studies were trying to boost the expression of *OCT4*, and increases in the levels of *CTNNB1* regulate the activity of *OCT4* [12，23, 24], and enhance the pluripotency. As CTNNB1 could accumulate when AXIN2 was knockdown, we had examined the effect of *CTNNB1* on the *OCT4* promoter activity by

dual-luciferase reporter assay, while the outcome disappointed us. Up to now, the slumber of OCT4 was still a big puzzle to piPSCs [32].

Previous studies showed that the AXIN family could take a part in the regulation of other signaling pathways, such as the TGFβ signaling pathway which is also a core signaling pathway in pluripotency maintenance of piPSCs [25, 26]. AXIN activates TGF-beta signaling by forming a multimeric complex consisting of Smad7 and ubiquitin E3 ligase Arkadia [27]. Our results suggested a possibility of a simplified culture medium for piPSCs, however, we did not perform function researches by using sh-AXIN2 piPSCs cultured without CHIR99021. The results inspired us to find a new way to replace the use of SB431542, an inhibitor to the TGFβ signaling pathway because the list of genes downregulated in sh-AXIN2 cells enriched KEGG pathways included the TGF−beta signaling pathways. As we demonstrated before, the using of small molecule inhibitors could impair the potential of stem cells when differentiation [3], then one important future direction of culture medium was to as simple as possible. There might be other genes could maintain pluripotent work as pathway inhibitor.

The biggest drawback to the present piPSCs is that it is still hard to contribute to efficient chimeras and was extremely sensitive to changes culture conditions, previous studies showed overexpression of *BCL2* enables piPSCs to enhance the survival under stress and contribute to forming chimeras, and this effect was mediated mainly by increased resistance to apoptosis [28, 29, 35]. In our study, we also confirmed that apoptotic family CASPASE3, CASPASE9, and apoptotic protein BAX were downregulated after AXIN2 knockdown. We did not examine whether sh-AXIIN2 cells contribute a bigger ratio when forming chimeras, but we could make a hypothesis, and test it in the future.

Conclusions

In summary, our study not only suggested that porcine AXIN2 had a stronger effect than AXIN1 on piPSCs, which affects the proliferation and survivability as well but also explained the potential feedback mechanism in this process.

Availability of data

The raw sequencing reads of this study have been deposited in the NCBI Sequence Read Archive (SRA) database under PRJNA772804.

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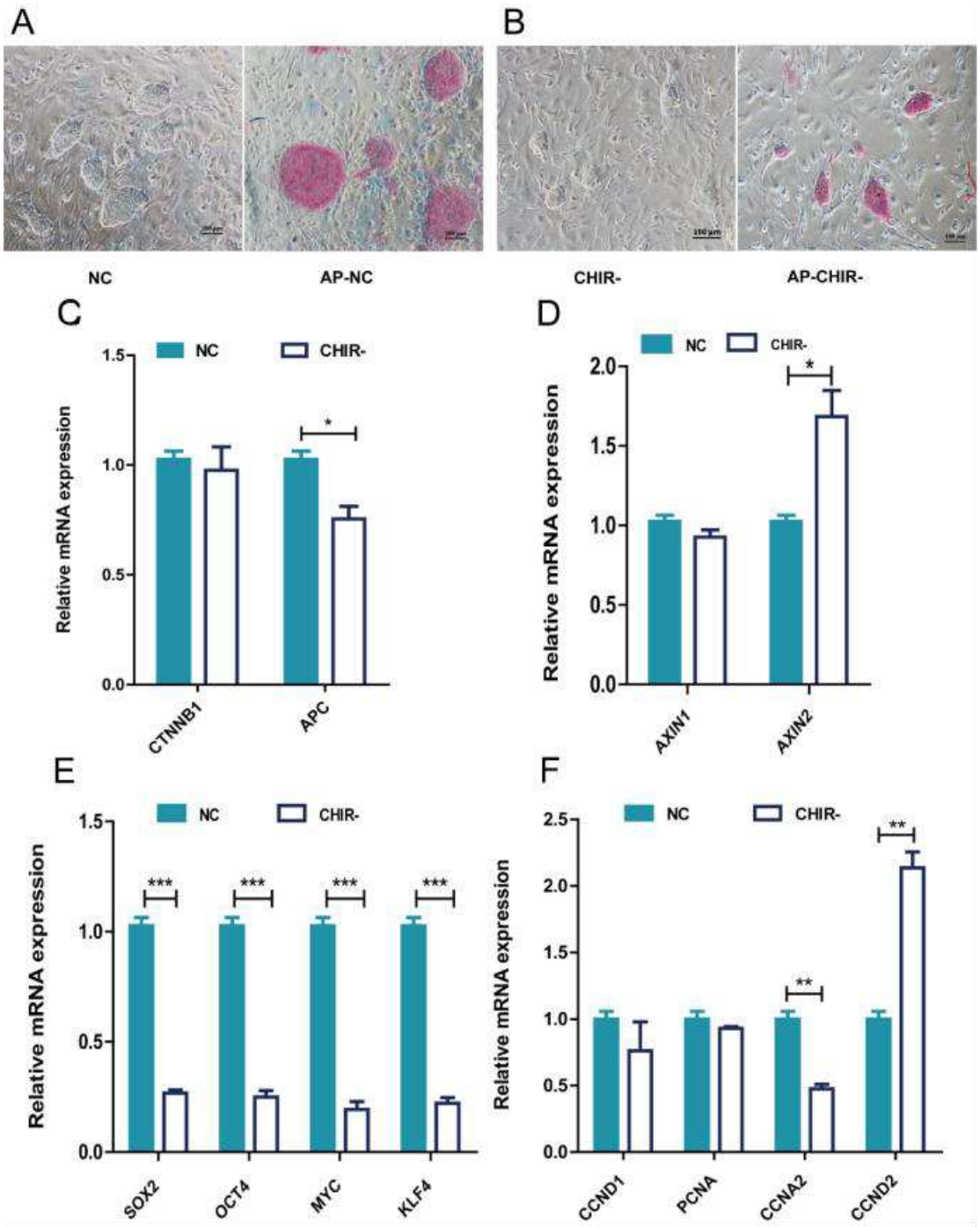
Author contribution

RZ and JH designed the research. RZ performed the main experiments. YS, QS, WZ, JZ, XW, ZZ, and XW helped with the experiments. RZ wrote the manuscript. All the authors have read and agreed to the published version of the manuscript.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Northwest A&F University.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CCND1
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CCND2

Figure 1 CHIR99021 was crucial to maintain the pluripotency and proliferation of piPSCs

A, B, Representative image of colonies and AP staining after 5 days of clonal growth of piPSCs in the control (NC) and without CHIR99021(CHIR-); C, qPCR analysis of the expression levels of *CTNNB1* and *APC* in NC and CHIR- group; D, qPCR analysis of *AXIN1* and *AXIN2* in NC and CHIR- group; E, qPCR analysis of the expression levels of endogenous *OCT4*, *SOX2*, *KLF4*, *MYC* in NC and CHIR- group; F, qPCR analysis of the expression levels of *CCND1*, *CCND2*, *PCNA* and *CCNA2* in NC and CHIR- group; * represent $P<0.05$; ** represent $P<0.001$; *** represent $P<0.0001$.

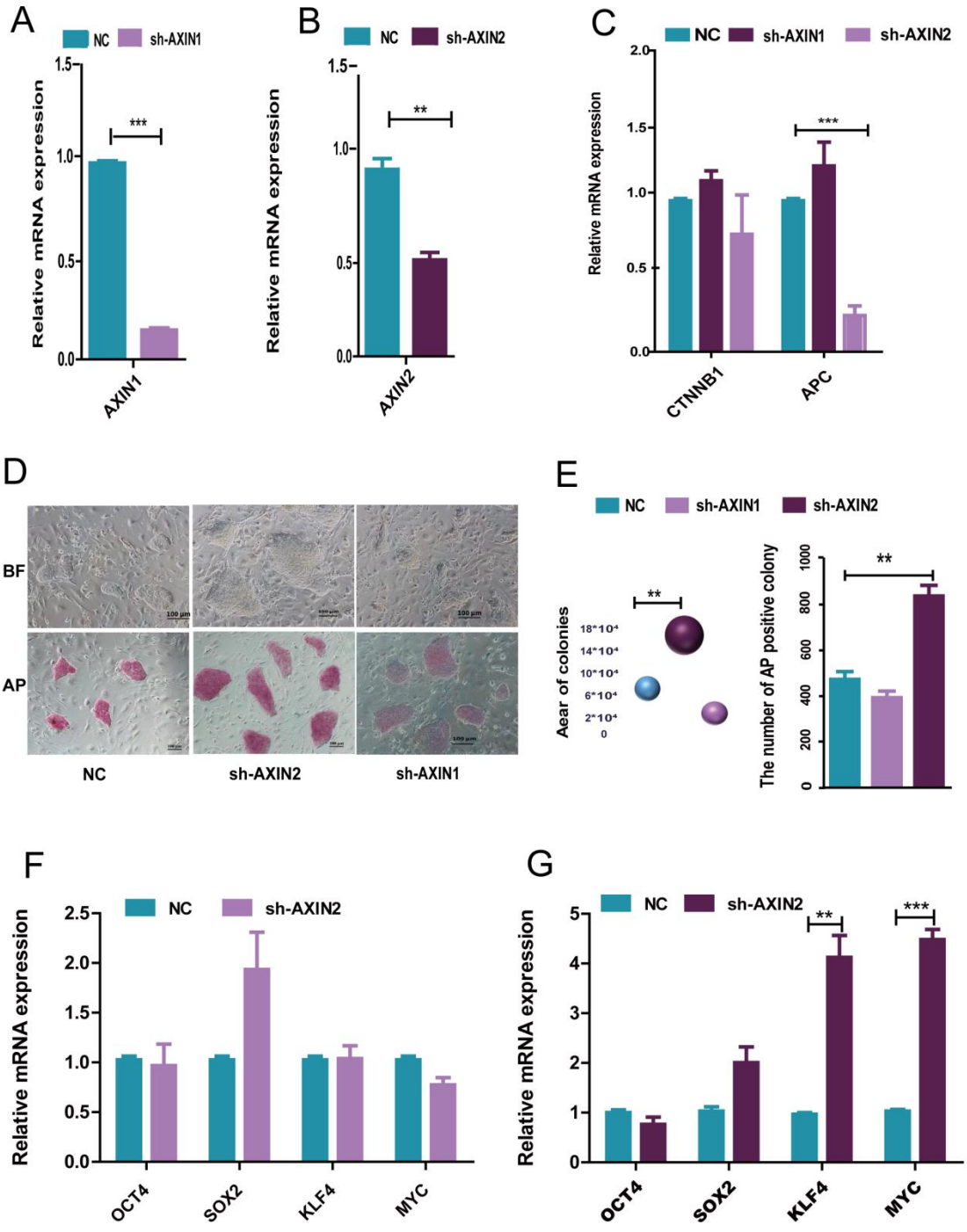
A
NC
sh-AXIN1
Relative mRNA expression
1.5
1.0
0.5
0.0
***
AXIN1
B
NC
sh-AXIN2
Relative mRNA expression
1.5
1.0
0.5
0.0
**
AXIN2
C
NC
sh-AXIN1
sh-AXIN2
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
***
CTNNB1
APC
D
BF
AP
NC
sh-AXIN2
sh-AXIN1
E
NC
sh-AXIN1
sh-AXIN2
Aear of colonies
18*10⁴
14*10⁴
10*10⁴
6*10⁴
2*10⁴
0
**
The number of AP positive colony
1000
800
600
400
200
0
**
F
NC
sh-AXIN2
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
OCT4
SOX2
KLF4
MYC
G
NC
sh-AXIN2
Relative mRNA expression
5
4
3
2
1
0
**
***
OCT4
SOX2
KLF4
MYC

Figure 2 Knockdown of AXIN2 showed higher pluripotency in piPSCs.

A,, qPCR analysis of the expression levels of *AXIN1* in the control (NC) and knockdown AXIN1 (sh-AXIN1) group; B, qPCR analysis of the expression levels of *AXIN2* in the control (NC) and knockdown AXIN2 (sh-AXIN2) group; C, qPCR analysis of the expression levels of *CTNNB1* and *APC* in NC, sh-AXIN1 and sh-AXIN2 group; D, Representative image of colonies and AP staining after 5 days of clonal growth in the NC, sh-AXIN1 and sh-AXIN2 group; The scale bar represents 100 μm; E, The area of NC, sh-AXIN1 and shAXIN2 colonies; the quantifies of AP positive colonies in the NC, sh-AXIN1 and sh-AXIN2 group; F, qPCR analysis of endogenous *OCT4*, *SOX2*, K*LF4*, *MYC* in NC and sh-AXIN1; G, qPCR analysis of endogenous *OCT4*, *SOX2*, K*LF4*, *MYC* in NC and sh-AXIN2 group; * represent $P<0.05$; ** represent $P<0.001$; *** represent $P<0.0001$.

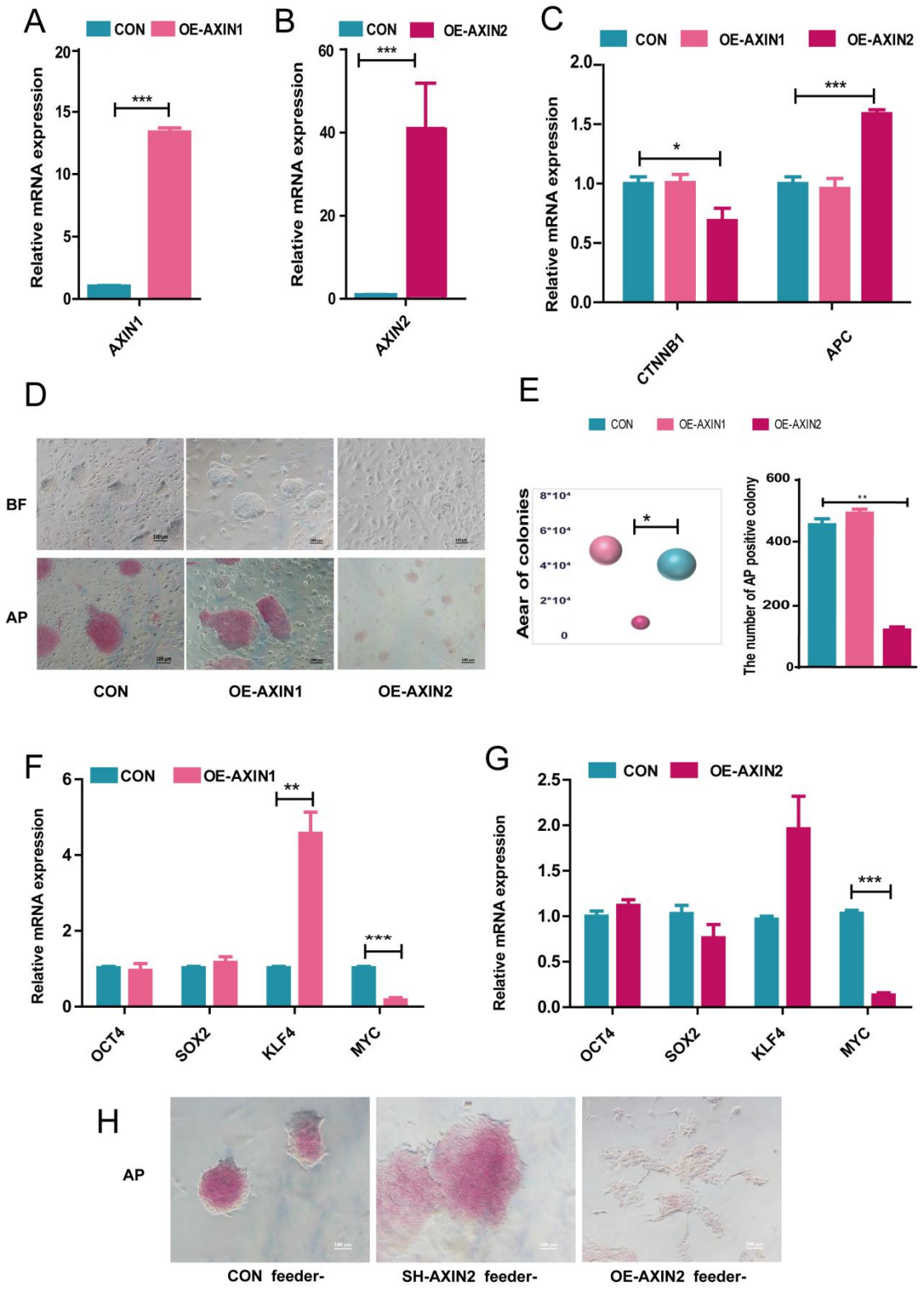
A
CON
OE-AXIN1
Relative mRNA expression
20
15
10
5
0
***
AXIN1
B
CON
OE-AXIN2
Relative mRNA expression
60
40
20
0
***
AXIN2
C
CON
OE-AXIN1
OE-AXIN2
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
*
***
CTNNB1
APC
D
BF
AP
CON
OE-AXIN1
OE-AXIN2
E
CON
OE-AXIN1
OE-AXIN2
Aear of colonies
8*10⁴
6*10⁴
4*10⁴
2*10⁴
0
*
The number of AP positive colony
600
400
200
0
**
F
CON
OE-AXIN1
Relative mRNA expression
6
4
2
0
**
***
OCT4
SOX2
KLF4
MYC
G
CON
OE-AXIN2
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
***
OCT4
SOX2
KLF4
MYC
H
AP
CON feeder-
SH-AXIN2 feeder-
OE-AXIN2 feeder-

Figure 3 Overexpression of AXIN1, AXIN2 in piPSCs

A, qPCR analysis of the expression levels of *AXIN1* in the control (CON), overexpressing AXIN1 (OE-AXIN1) group; B, qPCR analysis of *AXIN2* in the control (CON) and overexpressing AXIN2 (OE-AXIN2) group; C, qPCR analysis of *CTNNB1* and *APC* in CON, OE-AXIN1 and OE-AXIN2 group; D, Representative image of bright and AP positive colonies after 5 days of clonal growth in the CON, OE-AXIN1 and OE-AXIN2 group; The scale bar represents 100 μm; E, The area of CON, OE-AXIN1and OE-AXIN2 colonies; the quantifies of AP positive colonies in the CON, OE-AXIN1 and OE-AXIN2 group; F, qPCR analysis of endogenous *OCT4*, *SOX2*, K*LF4*, *MYC* in NC and OE-AXIN1; G, qPCR analysis of endogenous *OCT4*, *SOX2*, K*LF4*, *MYC* in CON and OE-AXIN2 group; H, Representative image of AP staining after 5 days of clonal growth without feeder in the CON, sh-AXIN2 and OE-AXIN2 group; The scale bar represents 100 μm. * represent $P<0.05$; ** represent $P<0.001$; *** represent $P<0.0001$.

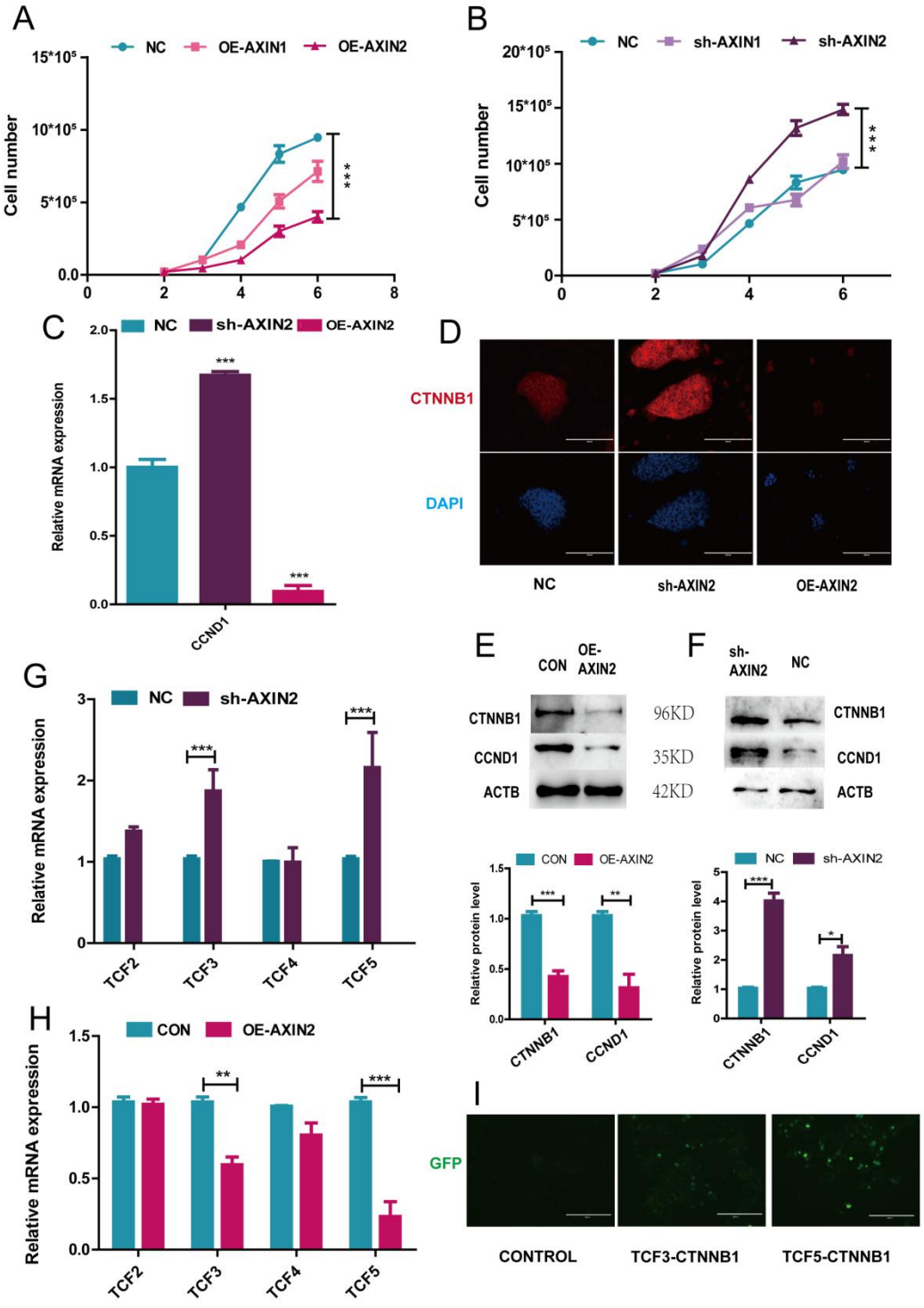

Figure 4 AXIN2 effects cell proliferation through CCND1

A, Cell growth curve of CON, OE-AXIN1, OE-AXIN2 group; *** represent *P*<0.0001; B, Cell growth curve of NC, sh-AXIN1, sh -AXIN2 ; *** represent *P*<0.0001; C, qPCR

analysis of *CCND1* in the NC, sh-AXIN2 and OE-AXIN2 group; D, Representative image of CTNNB1 immunofluorescence in the NC, sh-AXIN1 and OE-AXIN2 group; The scale bar represents 200 μm; E, Western blot analysis of CTNNB1 and CCND1 in the CON and OE-AXIN2 group; F, Western blot analysis of CTNNB1 and CCDN1 in the NC and sh-AXIN2 group; G, qPCR analysis of *TCF2, TCF3, TCF4,TCF5* in the NC and sh-AXIN2; H, qPCR analysis of *TCF2, TCF3, TCF4,TCF5* in the CON and OE-AXIN2; I, Representative image of Split fluorescent protein in CONTROL, TCF3-CTNNB1 and TCF5-CTNNB1 293T cells; The scale bar represents 200 μm. * represent $P<0.05$; ** represent $P<0.001$; *** represent $P<0.0001$.

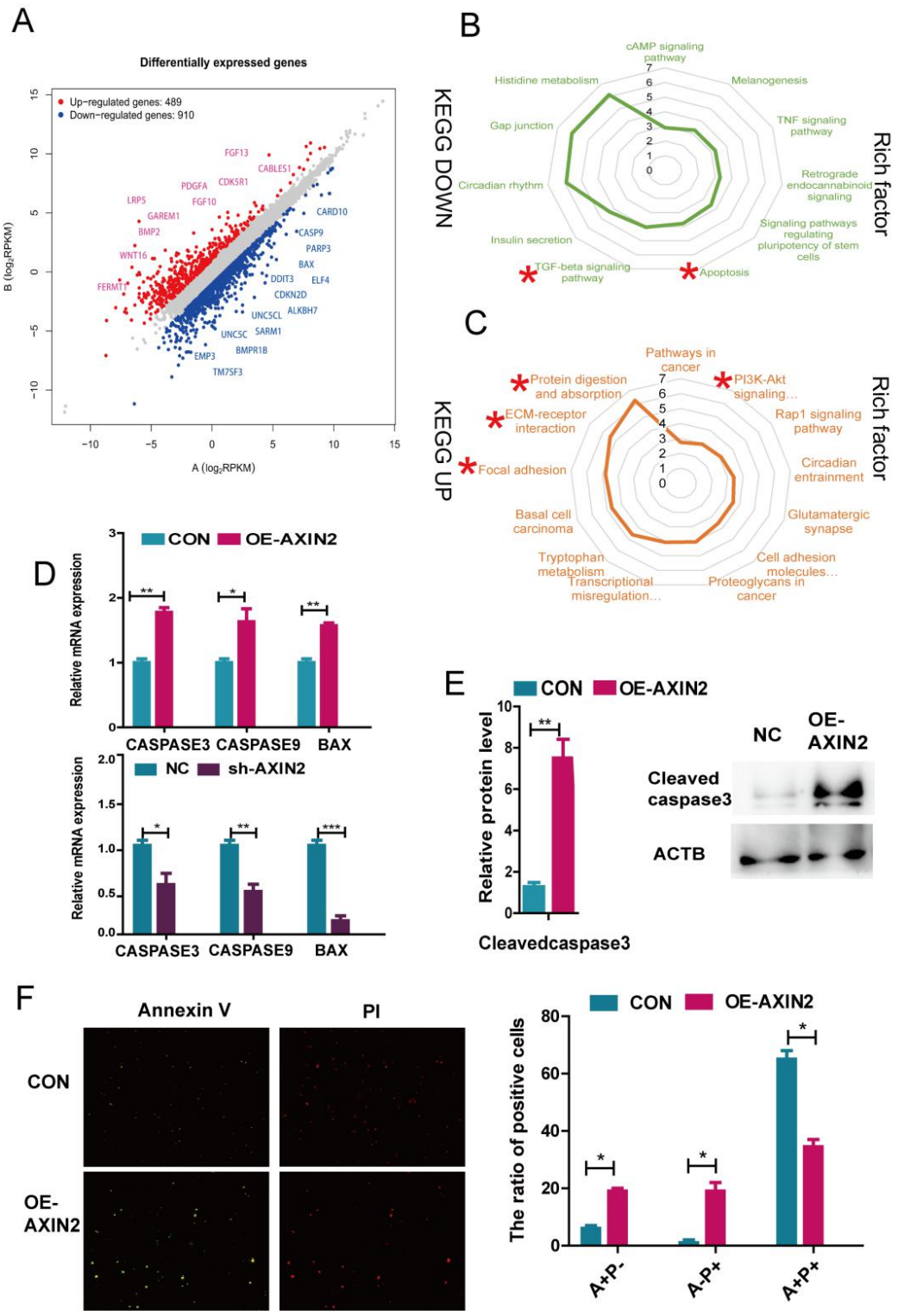
A
Differentially expressed genes
Up−regulated genes: 489
Down−regulated genes: 910
B (log2RPKM)
A (log2RPKM)
FGF13
CABLES1
PDGFA
CDK5R1
LRP5
FGF10
GAREM1
CARD10
BMP2
CASP9
WNT16
PARP3
BAX
FERMT1
DDIT3
ELF4
CDKN2D
UNC5CL
ALKBH7
UNC5C
SARM1
BMPR1B
EMP3
TM7SF3
B
KEGG DOWN
Rich factor
cAMP signaling pathway
Melanogenesis
TNF signaling pathway
Retrograde endocannabinoid signaling
Signaling pathways regulating pluripotency of stem cells
Apoptosis
TGF-beta signaling pathway
Insulin secretion
Circadian rhythm
Gap junction
Histidine metabolism
C
KEGG UP
Rich factor
Pathways in cancer
PI3K-Akt signaling...
Rap1 signaling pathway
Circadian entrainment
Glutamatergic synapse
Cell adhesion molecules...
Proteoglycans in cancer
Transcriptional misregulation...
Tryptophan metabolism
Basal cell carcinoma
Focal adhesion
ECM-receptor interaction
Protein digestion and absorption
D
CON
OE-AXIN2
Relative mRNA expression
CASPASE3
CASPASE9
BAX
NC
sh-AXIN2
Relative mRNA expression
CASPASE3
CASPASE9
BAX
E
CON
OE-AXIN2
Relative protein level
Cleavedcaspase3
NC
OE-AXIN2
Cleaved caspase3
ACTB
F
Annexin V
PI
CON
OE-AXIN2
CON
OE-AXIN2
The ratio of positive cells
A+P-
A-P+
A+P+

Figure 5 AXIN2 affected the survival of piPSCs though Caspase family

A, Transcriptional changes of NC and sh-AXIN2 piPSC group; B, KEGG enrichment of down-differentially expressed (DE) genes in sh-AXIN2 cell lines; C, KEGG enrichment of UP- DE genes in sh-AXIN2 cell lines; D, qPCR analysis of *CASPASE3, CASPASE 9* and *BAX* in NC, sh-AXIN2 and CON, OE-AXIN2 group; * represent $P<0.05$, ** represent $P<0.001$, *** represent $P<0.0001$; E, Western blot analysis of Cleaved caspase 3 in the NC and OE-AXIN2 group; F, Representative images of piPSCs stained with PI (P) and Annexin V (A) after 5 d of culture in the NC and OE-AXIN2 group. A+/P+: non-viable apoptotic cell or necrotic cells; A−/P+: mechanic injury; A+/P−: viable apoptotic cell. The quantitative analysis is shown on the right. n = 3 independent experiments. The scale bar represents 400 μm; H, Ratio of positive cell of PI (P) and Annexin V (A) staining; * represent $P<0.05$.

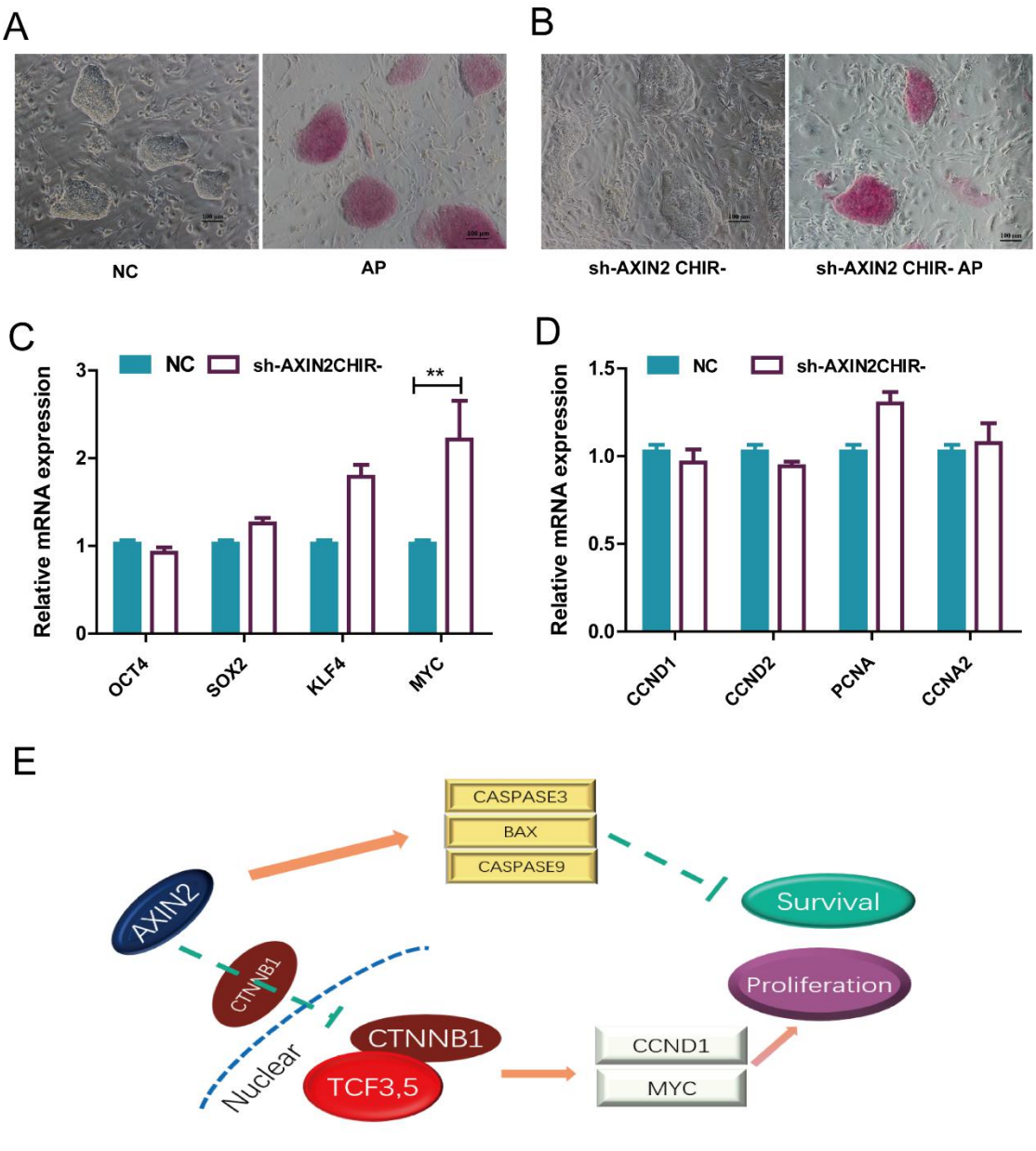

Figure 6 sh-AXIN2 could maintain the proliferation and pluripotency of piPSCs without CHIR99021

A, Representative image of bright and AP staining in the NC group withdrawing CHIR99021; The scale bar represents 100 μm; B, Representative image of bright and AP staining in sh-AXIN2 group withdrawing CHIR99021; The scale bar represents 100 μm; C, qPCR analysis of endogenous *OCT4*, *SOX2*, *KLF4*, *MYC* in NC,

sh-AXIN2CHIR- group; ** represent *P*<0.001; D, qPCR analysis *CCND1, CCND2, PCNA* and *CCNA2* in NC, sh-AXIN2CHIR- group; E, The diagram of the function of AXIN2 in piPSCs.

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