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SC1 sustains the self-renewal capacity and pluripotency of chicken blastodermal cells by inhibiting the phosphorylation of ERK1 and promoting the phosphorylation of Akt

Rongyang Li[1] | Xiaochuan Tang[2] | Shiyong Xu[3] | Qing Chen[3] | Baobao Chen[1] | Shuo Liu[1] | Bojiang Li[1] | Weijian Li[1] | Yilong Yao[1] | Wangjun Wu[1] | Honglin Liu[1]

[1]College of Animal Sciences and Technology, Nanjing Agricultural University, Nanjing, China

[2]College of Animal Sciences and Technology, Guang Xi University, Nanning, China

[3]College of Animal Sciences and Technology, Jingling Institute of Technology, Nanjing, China

Correspondence
Honglin Liu, College of Animal Sciences and Technology, Nanjing Agricultural University, Nanjing 210095, China.
Email: liuhonglin@njau.edu.cn

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Abstract

Small molecules discovered during the recent years can be used to regulate the growth of embryonic stem cells (ES cells). Chicken blastodermal cells (cBCs) play an important role in both basic and transgenic researches as an important ES cell. However, the regulatory mechanism of small molecules involved in the self-renewal and pluripotency of cBCs remains unknown. This study revealed that the small molecule, SC1, can maintain cBCs in an undifferentiated, pluripotent state in serum- and feeder-free E8 media without leukaemia inhibitory factor. Furthermore, SC1 inhibits downregulation of pluripotency-related genes caused by retinoic acid and promotes the proliferation of cBCs. Furthermore, the results of this study indicated that SC1 functions by inhibiting ERK1 phosphorylation and promoting Akt phosphorylation, thus promoting the expression of pluripotency-related genes and maintaining the pluripotency of cBCs. The results also demonstrated that SC1 sustains the self-renewal capacity and pluripotency of cBCs cells by inhibiting ERK1 phosphorylation and promoting Akt phosphorylation. This kind of regulatory mechanism might be conserved in avian ES cells. Other molecules, similar to SC1, might provide insights into the molecular mechanisms that control the fate of stem cells and ultimately help in-vivo stem cell biology and therapy.

KEYWORDS
chicken blastodermal cells, gelatin, SC1, ERK1, pluripotency

1 | INTRODUCTION

In scientific research, embryonic stem (ES) cells are employed to understand cell proliferation, differentiation and tissue and organ formation; they are the best model to study embryo development. In the field of medicine, ES cells are used to supply self-organizing organs. They are also used as an ideal substitute for necrosed tissue to treat diseases, including degenerative diseases (Mead et al., 2015). Transgenic chicken embryo is one of the most effective and exciting research tools in the field of agriculture and medicine (Cao et al., 2015; Li et al., 2014; Park et al., 2015; Zhu et al., 2005). Furthermore, chicken blastodermal cells (cBCs) can be utilized as a model to investigate self-renewal, pluripotency and epigenesis of ES cells (Busch, Krochmann, & Drews, 2013; Cloney & Franz-Odendaal, 2015; Jahanpanah et al., 2014; Kress, Montillet, Jean, Fuet, & Pain, 2016; Li et al., 2014). However, the regulatory mechanisms involved in the self-renewal and pluripotency of cBCs remain unclear.

Small molecules, such as IDE1, R-ABO and SB431542, discovered during the recent years can be used to regulate the growth of ES cells (Borowiak et al., 2009; Han et al., 2012; Kimura et al., 2015). During the growth of ES cells, small molecules can be used to not only maintain pluripotency, but also induce directional differentiation of ES cells. Studies have reported that SC1 inhibits the ERK1/2 signalling pathway and activity of RasGAP in mouse ES cells (mESCs). It not only inhibits the ERK1/2 pathway, and thus the differentiation of mESCs, but also promotes the phosphoinositide-3 kinase (PI3 K)/Akt signalling-mediated self-renewal of mESCs (Chen et al., 2006). However, it is unclear if similar mechanisms for maintaining self-renewal and pluripotency exist in cBCs.

This study (a) described the pluripotency of cBCs in E8 medium, (b) demonstrated that SC1 can promote the expression of pluripotency-related genes and (c) revealed that SC1 can activate the PI3 K/Akt signalling pathway and inhibit the ERK1/2 signalling pathway in cBCs. These pathways are necessary for maintaining the self-renewal capacity and pluripotency of stem cells.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experiments were in accordance with the guidelines of the regional Animal Ethics Committee, and the Institutional Animal Care and Use Committee of Nanjing Agricultural University approved all experiments.

2.2 | Isolation and culture of cBCs

The area pellucida from stage X (EG&K) embryos of Hyline chicken (*Gallus gallus*) were isolated as previously described (van de Lavoir & Mather-Love, 2006), and washed twice with Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco, USA) and then dispersing it gently into a single cell suspension using a 1,000-μl pipette. We centrifuged the cell suspension at 350 *g* for 5 min and removed the supernatant. We subsequently resuspended the pelleted cells in complete E8 medium (Gibco) containing 100 μg/ml penicillin and 100 μg/ml streptomycin (Gibco). We seeded the cells on 0.1% gelatin (Millipore, USA)-coated culture plates (Costar, Corning, NY). When the cells attained 70%–80% confluence, we performed passaging by washing and pipetting the cells with calcium/magnesium ion (Ca^{2+}/Mg^{2+})-free phosphate-buffered saline (PBS) (Gibco, USA).

2.3 | Immunofluorescence detection

We cultured chicken blastodermal cells for 48 hr until the cell density reached 70%–90%. We washed adherent cells with PBS, fixed in 4% paraformaldehyde at 25°C for 20 min and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 10 min. After incubating with blocking buffer containing 5% newborn bovine serum albumin (BSA) (Solarbio, Beijing, China) for 20 min, we stained the cells with anti-SSEA-1 antibodies (1:100) (Abcam, Cambridge, UK) and anti-Sox2 antibodies (1:100) (Abcam, Cambridge, UK), respectively, at 4°C overnight, and then rewarmed at 37°C for 45 min. Alexa Fluor 488 goat anti-mouse IgG (1:1,000) (Abcam) as anti-SSEA-1 antibody and Alexa Fluor 488 goat anti-Rabbit IgG (1:1,000) (Abcam) as anti-Sox2 antibody were added, respectively, to the samples and incubated at room temperature for 1 hr. We stained the nuclei with 10 μM 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 30 min. We obtained the fluorescence images using a fluorescence microscope (IX71; OLYMPUS, Tokyo, Japan).

2.4 | Reverse transcription and quantitative polymerase chain reaction analyses

We isolated total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the instructions of the manufacturer. PrimeScript RT Master Mix reverse transcription kit (TaKaRa, Japan) was used for complementary DNA (cDNA) synthesis. We amplified reverse transcription products using the SYBR Premix Ex Taq PCR kit (TaKaRa). We conducted the PCR amplification using an automated StepOne system (Applied Biosystems, Carlsbad, CA, USA). We analysed relative gene expression data by $2^{-\Delta\Delta Ct}$ method. We designed primers using Primer 6.0 software. Table 1 presents primer sequences and PCR product lengths.

TABLE 1 Primers for PCR analysis

Genes	Accession no.	Primer sequences (5′ to 3′)	Product length (bp)
PouV	NM_001110178	GCCAAGGACCTCAAGCACAA	511
		ATGTCACTGGGATGGGCAGA	
Nanog	NM_001146142.1	CAGCAGACCTCTCCTTGACC	586
		AAGCCCTCATCCTCCACAGC	
Sox2	NM_205188.2	AGGCACAGGCAACTCCAACTC	472
		GCCGAGCTGCTCTTGCATCAT	
β-actin	NM_205518	GAACCCCAAAGCCAACAGA	185
		GGAGGGCGTAGCCTTCATAGA	

2.5 | Treatment of cultured cells with the drugs

We treated the cells with 1 μM SC1 (Selleck, Houston, TX, USA) and with 3 and 10 μM LY294002 (Selleck, Houston, TX, USA). During cotreatment with SC1 and the inhibitor LY294002, we treated the cells with either SC1 or 3 μM retinoic acid (RA) (Sigma-Aldrich) for 6 hr or 12 hr. The chemicals were dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich) and diluted with E8 medium. The final concentration of DMSO in the medium was less than 0.1%.

2.6 | EdU staining

We measured cell proliferation using Cell-LightTM EdU Apollo567 In Vitro Kit (RiboBio, China) according to instructions of the manufacturer.

2.7 | Western blotting

We performed western blotting according to a previously described method (Yu, Guan, & Zhang, 2011). We lysed adherent cells using RIPA buffer (50 mM Tris, pH 8.0; 150 mM sodium chloride; 1% NP-40; 0.5% deoxycholic acid; and 0.1% sodium dodecyl sulphate) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA). We centrifuged the lysate and estimated the concentration of protein in the supernatant using a BCA protein assay kit (Beyotime Biotechnology, China) following the instructions of the manufacturer. After blocking with 5% BSA at room temperature for 2 hr, we incubated the membrane with primary antibodies overnight at 4°C and with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr the following day. After ECL application (Millipore, USA), we performed imaging using Lumimager LAS-3000 (FujiFilm, Japan). We used the following antibodies for western blotting: SOX2 (Abcam, USA), GAPDH (Abcam, USA), Phospho-ERK1/2 (Cell Signaling Technology), α-Tubulin (Cell Signaling Technology, USA) and Phospho-Akt (Ser473) (Cell Signaling Technology, USA). All antibodies were used at a dilution of 1:1,000.

2.8 | Statistical analysis

We used Student's *t* test in IBM SPSS 20.0 software (SPSS Inc., Chicago, IL) to analyse data sets. *p*-Values less than 0.05 were considered to be significantly different, while *p*-values less than 0.01 were considered to be extremely significantly different. All experimental data are shown as mean, and error bars represent the experimental standard error.

3 | RESULTS

3.1 | Identification of pluripotency of chicken blastodermal cells in E8 medium

To detect the pluripotency of cBCs in E8 medium, antibodies SSEA-1 and Sox2 were used to detect pluripotent makers within 24 hr after cBCs were seeded and attached firmly to culture plates. The expression of *SSEA-1* (Figure 1a) and *Sox2* (Figure 1b) in cBCs was confirmed by immunostaining. The results showed that the culturing system used can sustain the pluripotency of cBCs.

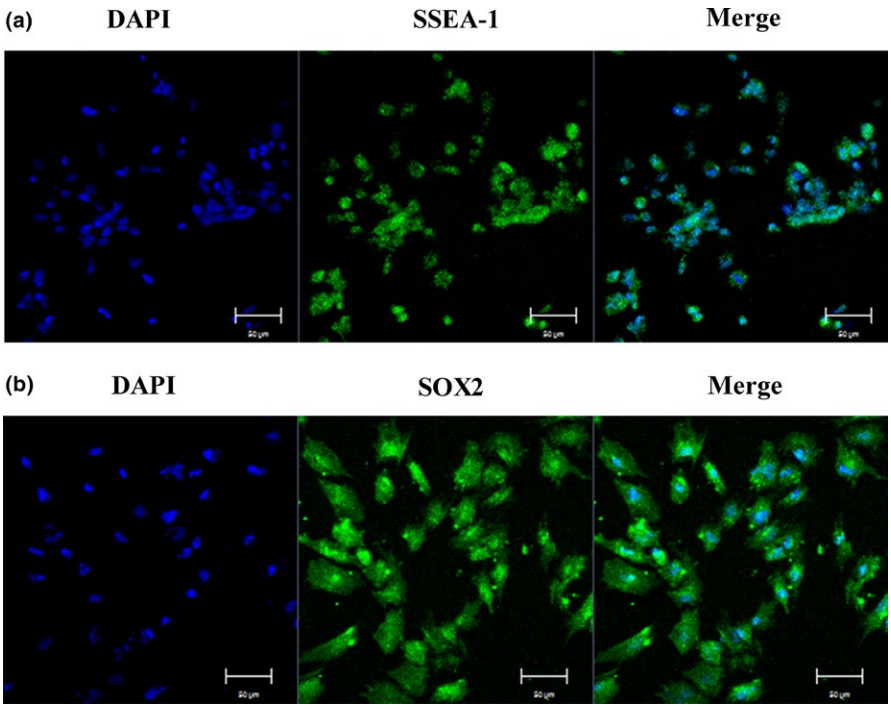

FIGURE 1 The pluripotent state of chicken blastodermal cells (cBCs) in E8 medium in the absence of leukaemia inhibitory factor (LIF). (a) Immunofluorescence staining of SSEA-1 (green), nuclei were counterstained with DAPI (blue), and cBCs were cultured for 48 hr. (b) Immunofluorescence staining of Sox2 (green), nuclei were counterstained with DAPI (blue), and cBCs were cultured for 48 hr (Scale bar, 20 μm)

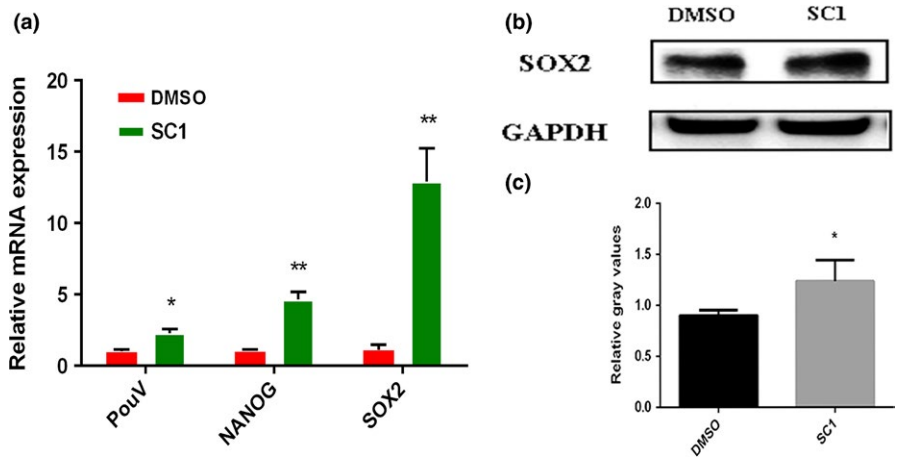

FIGURE 2 Effect of SC1 on the pluripotency of cBCs in E8 medium without leukaemia inhibitory factor (LIF). (a) The expression level of the pluripotency-related genes *PouV*, *Nanog* and *Sox2* was measured by qRT-PCR after treating the cells with 1 μM SC1 for 6 hr; DMSO was used as a control. Values are represented as mean ± *SEM* ($n = 3$). Data are representative of results of three independent experiments, and each condition was normalized to β-actin abundance. (*$p < 0.05$; **$p < 0.01$) (b) The expression level of *Sox2* was analysed by western blotting 6 hr after SC1 (1 μM) treatment in the E8 media. Chicken blastodermal cells cultured with DMSO were used as negative control. (c) Quantification of Sox2 to GAPDH conversion. Data are represented as mean ± *SEM*; $n = 3$ (*$p < 0.05$; **$p < 0.01$)

3.2 | SC1 contributes to the pluripotent character of cBCs

In chicken, *PouV*, *Sox2* and *Nanog* have been reported as the core transcriptional regulators of pluripotency (Jean et al., 2013; Lavial et al., 2007). To evaluate the role of SC1 in the pluripotency of cBCs, the expression of the genes *PouV*, *Sox2* and *Nanog* in cBCs treated with SC1 was determined. The results revealed that the mRNA expression of *PouV* and *Nanog* was increased by SC1 (Figure 2a). Furthermore, SC1-treated cBCs also expressed high levels of Sox2 as determined by western blotting (Figure 2b,c). These results suggest that SC1 can promote the expression of multifunctional factors to maintain the pluripotency of cBCs.

3.3 | SC1 inhibits downregulation of pluripotency-related genes caused by retinoic acid

Retinoic acid (RA), an active metabolite of vitamin A, alters the expression of target genes to regulate different growth and differentiation processes during embryogenesis and organogenesis (Zieger, Candiani, Garbarino, Croce, & Schubert, 2017). RA is used as an efficient inducer of neuron-like cell differentiation (Guleria, Pan, Dipette, & Singh, 2006; Jacobs et al., 2006). Therefore, SC1-treated cBCs were subjected to RA treatment to verify whether SC1 can inhibit the downregulation of pluripotency-related genes caused by RA. The results revealed that the expression of these genes was suppressed at the mRNA level. However, when compared with those of the groups treated with only RA, there was a significant increase ($p < 0.01$) in the expression of *Sox2*, *Nanog* and *PouV* in the groups treated with both RA and SC1 (Figure 3a,b). Furthermore, western blotting revealed that SC1 restrained the downregulation of *Sox2* caused by RA (Figure 3c). This suggests that SC1 might inhibit the differentiation of cBCs through RA-related signalling pathway.

3.4 | SC1 maintains the pluripotency and promotes the proliferation of cBCs through PI3K/Akt signalling pathway

EdU staining revealed that the EdU-positive cells in the group treated with SC1 was higher than that in the control group treated with DMSO (Figure 4a). The results revealed that SC1 improves the proliferation of cBCs. In order to study the mechanism of SC1 involved in maintaining the pluripotency of cBCs, cBCs were treated with the PI3K phosphorylation inhibitor, LY294002—the first synthetic molecule known to inhibit PI3Kα/δ/β (Wang et al., 2016). Further, western blotting showed that the expression of *Sox2* increased after treatment with SC1, while treatment with LY294002 inhibited the expression of *Sox2* in a dose-dependent manner (Figure 4b). The phosphorylation of Akt did not significantly increase after treatment with 1 μM SC1. However, treatment with 3 μM SC1 decreased the phosphorylation of Akt (Figure 4c). These results indicate that SC1 maintained the pluripotency and promoted the proliferation of cBCs through PI3K/Akt signalling pathway.

3.5 | SC1 inhibits phosphorylation of ERK1 in cBCs

In mouse, prior gastrulation, the FGF-ERK1/2 signalling becomes highly active in extraembryonic ectoderm, thus promoting the growth of trophoblast stem cells in vitro (Corson, Yamanaka, Lai, & Rossant, 2003). Western blotting analysis showed that SC1 inhibited the phosphorylation of ERK1. On the contrary, leukaemia inhibitory factor (LIF) had no significant effect on the phosphorylation of ERK1/2 (Figure 5).

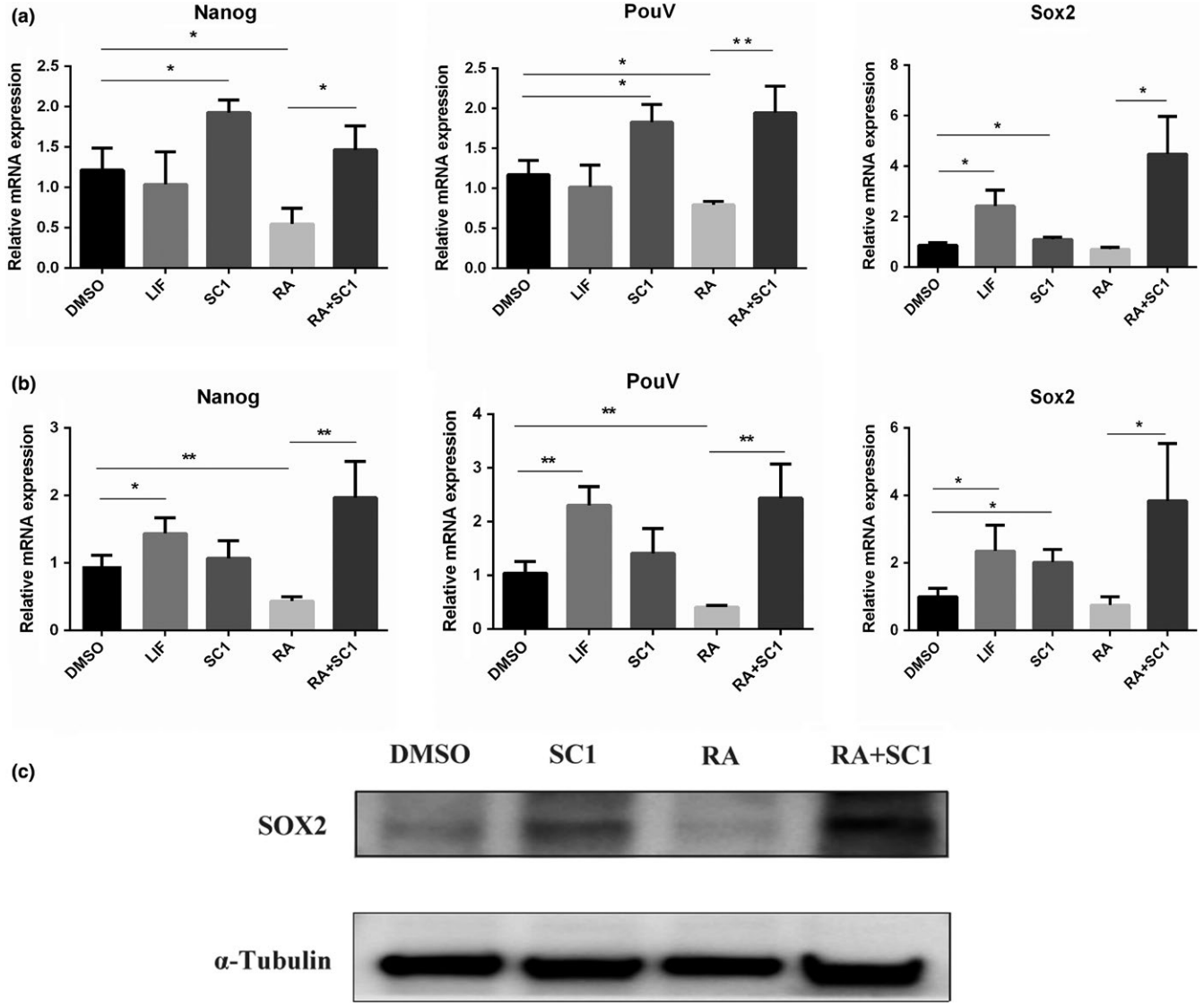

FIGURE 3 SC1 inhibits the differentiation of chicken blastodermal cells caused by retinoic acid (RA). Cells cultured in E8 medium with 3 μM RA and with or without SC1 for 6 hr (a) or 12 hr (b); cells cultured in E8 medium with leukaemia inhibitory factor (LIF) were used as positive control, and those cultures with DMSO were used as negative control. The expression level of *Sox2*, *Nanog* and *PouV* was examined by real-time PCR. β-Actin was used to normalize template levels. Data are presented as mean ± *SEM* of three independent experiments (**p* < 0.05; ***p* < 0.01). (c) The expression level of *Sox2* was analysed by western blotting 6 hr after treatment with DMSO, 1 μM SC1, 3 μM RA, 1 μM SC1 with 3 μM RA, respectively

4 | DISCUSSION

In chicken, cBCs are considered as pluripotent cells that can give rise to all somatic tissues and germline, when injected into the subgerminal cavity of stage X (EG&K) recipient embryos. In stem cells, the Ras protein is activated by the binding of guanosine triphosphate (GTP). Consequently, activating the enzyme PI3K may promote the self-renewal of cBCs. Furthermore, the activated Ras protein activates the enzymes ERK1 and ERK2, which promote cell differentiation. The Ras protein can be deactivated by the enzyme RasGAP, which converts GTP to guanosine diphosphate (GDP) (Letso & Stockwell, 2006). Interestingly, recent studies based on microarray analysis have revealed that chicken ES (cES) cells resemble mouse ES cells (mESCs) more closely than mouse epiblast SC at the transcriptome level, and cBCs show a highly similar profile of gene expression as cES cells (Jean et al., 2015). Further, SC1, as a dual inhibitor of ERK1 and RasGAP, can be utilized to maintain the self-renewal capacity of mESCs. The results of the present study were used to explore the mechanism of SC1 in maintaining the pluripotency of cBCs. The data indicated that SC1 promoted the phosphorylation of Akt by enhancing stem cell renewal via PI3K pathway. PI3K is a lipid kinase activated by these growth factors. PI3K produces phosphatidylinositol (3,4,5)triphosphate (PIP3) from phosphatidylinositol (4,5)bisphosphate (PIP2) and transmits signals via downstream effector proteins, such as serine/threonine kinase Akt, and small GTPases Rac1 and Cdc42. Furthermore, Akt promotes physiological and

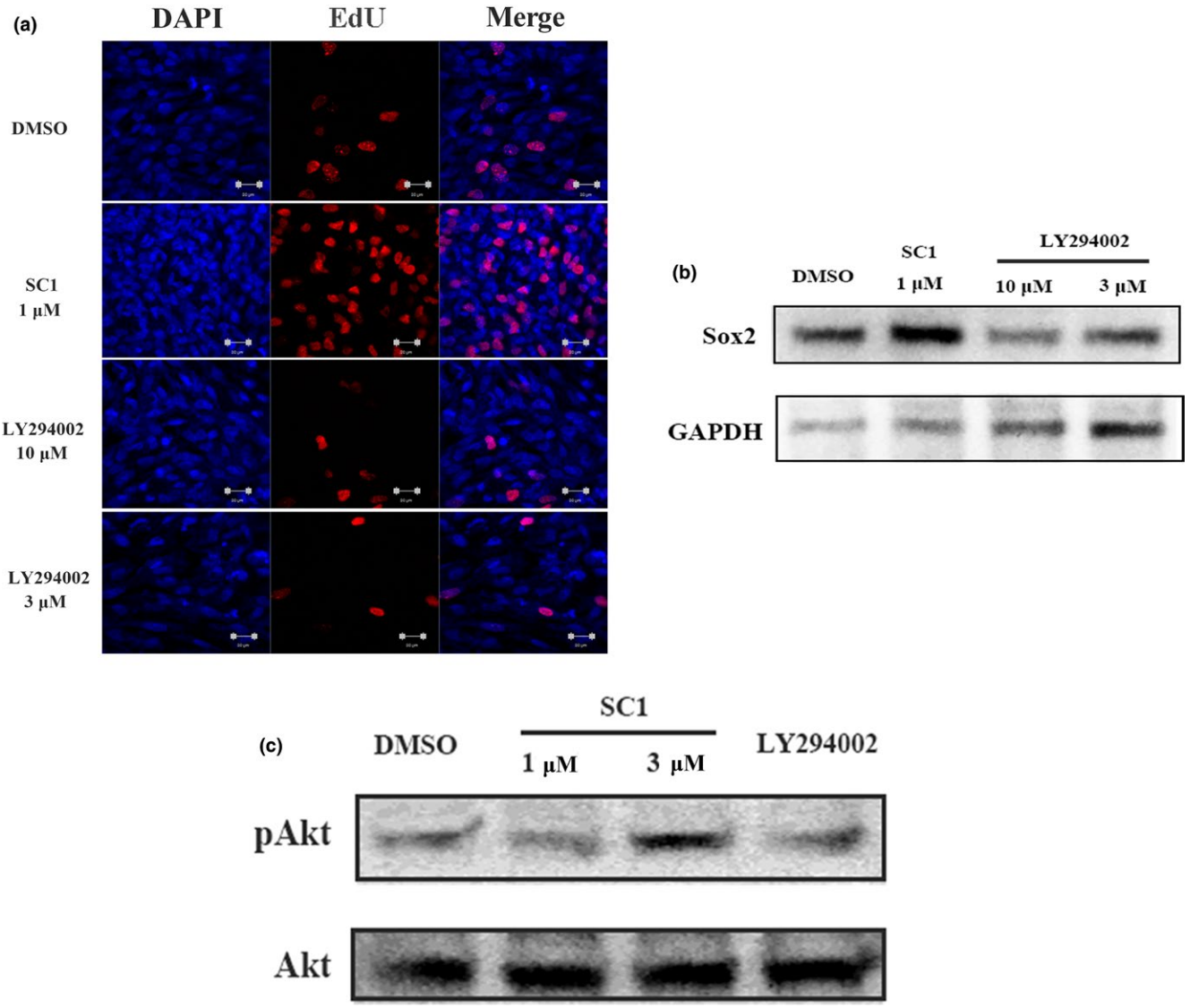

FIGURE 4 Effect of SC1 on pluripotency and proliferation of cBCs through PI3K/Akt signalling pathway. (a) EdU staining of cBCs cultured in E8 medium treated for 6 hr with DMSO, 10 ng/ml leukaemia inhibitory factor (LIF), 1 μM SC1, 10 μM LY294002 and 3 μM LY294002, respectively. (b) The expression level of *Sox2* was analysed by western blotting 6 hr after treatment with DMSO, 1 μM SC1, 10 μM LY294002 and 3 μM LY294002, respectively. (c) Western blot of the phosphorylation of Akt. cBCs were treated with 1 μM SC1, 10 μM and LY294002 for 20 min

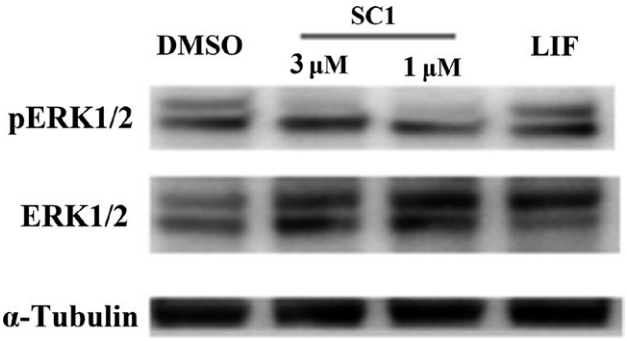

FIGURE 5 SC1 inhibits phosphorylation of ERK1/2. Western blot of the phosphorylation of ERK1/2. Chicken blastodermal cells cultured in E8 medium was treated with DMSO, 1 μM SC1, 3 μM LY294002 and 10 ng/ml LIF for 20 min, respectively

pathological processes, such as proliferation, survival, metabolism and tumorigenesis, through the phosphorylation of various target proteins. It has been reported that SC1 promotes phosphorylation of Akt of mESCs via PI3K/Akt signalling pathway (Chen et al., 2006). The phosphorylation of ERK1/2 was inhibited by SC1. Consequently, the differentiation of cBCs was inhibited, which is similar to the results for mESCs. It is noteworthy that RA is used to not only induce the differentiation of germ cell from cBCs through BMP-Smad1/5 signalling pathway (Tang et al., 2017), but also induce neural phenotypes in various stem cells in vitro. However, to understand whether BMP-Smad1/5 and other signalling pathways are affected in cBCs by SC1, further studies are necessary.

Embryonic stem cells have attractive prospects in the biomedical field. However, the instability of in-vitro growth and natural

differentiation limits the utilization of stem cells in certain cells, tissues and organs. For the clinical application of ES cells, the self-renewal and differentiation of ES cells must be controlled. Small molecules discovered during the recent years can be used for regulating the growth of ES cells. During the growth of ES cells, the small molecules can be used to not only maintain the pluripotency of ES cells, but also induce directional differentiation of ES cells. It has been found that the small molecules maintain the pluripotency of ES cells. Further, BIO is an inhibitor of the Gsk3-beta pathway in ES cells. The inhibition of Gsk3-beta activity can not only reduce the expression of differentiation-related genes, but also increase the expression of pluripotency-related genes (Sato, Meijer, Skaltsounis, Greengard, & Brivanlou, 2004). Both PD0325901 and CHIR99021 are inhibitors of MEK and Gsk3-beta, respectively. During the cultivation of the ES cells, PD0325901 and CHIR99021 not only inhibit the function of MEK-ERK1/2 pathway, and thus the differentiation of ES cells, but also promote PI3K signalling-mediated self-renewal of ES cells. Consequently, ES cells maintain their undifferentiated state in the serum- and LIF-free media (Buehr et al., 2008). 5-Aminoimidazole-4-carboxy-amide-1-beta-D-ribofuranoside (AICAR) is an activator of MAPK pathway; AICAR can not only upregulate the expression of pluripotent-related genes, but also restrain the differentiation of ES cells induced by RA (Shi et al., 2013). During differentiation, small molecules can also induce directional differentiation of ES cells into pancreatic, motor neuron, insulin-producing, retinal, myocardial and other types of cells (Chen et al., 2009; Graichen et al., 2008; Kunisada, Tsubooka-Yamazoe, Shoji, & Hosoya, 2012; Osakada et al., 2009). Furthermore, during embryonic development, the distinct intracellular signalling pathways that influence the orderly directional growth of ES cells are activated in response to the surrounding environment factors. A few studies have shown that these inhibitors can also be used in nonmammals. Studies on cBCs reveal that SC1 can maintain the pluripotency of cBCs via the PI3K/Akt and ERK1/2 signalling pathways, which suggests that this kind of regulation mechanism is relatively conserved in avian ES cells. However, it is not clear if this regulatory mechanism is also conserved in other nonmammal ES cells, which necessitates further studies. Due to the limitations in experimental condition, the activity of Ras was not determined in the present study. It has been reported that cellular α-ketoglutarate/succinate ratio contributes to the ability of ES cells to suppress differentiation (Carey, Finley, Cross, Allis, & Thompson, 2015). Therefore, we are exploring the change of α-ketoglutarate/succinate ratio in E8 medium, and its role in the transcriptional and epigenetic state in cBCs.

5 | CONCLUSION

In summary, the present study revealed that the small molecule, SC1, can sustain the self-renewal capacity of cBCs in serum- and feeder-free culturing systems. This molecule appears to work by inhibiting the phosphorylation of ERK1 and promoting the phosphorylation of Akt, thus maintaining the pluripotency of cBCs. Other molecules, similar to SC1, might provide insights into the molecular mechanisms that control the fate of stem cells and ultimately help in-vivo stem cell biology and therapy.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Honglin Liu and Xiaochuan Tang conceived and designed the experiments; Rongyang Li performed the experiments and analysed the data; Shiyong Xu, Qing Chen, Baobao Chen, Bojiang Li, Weijian Li, Yilong Yao and Shuo Liu contributed to reagents and materials; and Wangjun Wu revised the manuscript.

ORCID

Rongyang Li http://orcid.org/0000-0003-4107-8237

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