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**—Original Article—**

# Liver receptor homolog 1 influences blastocyst hatching in pigs

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**Abstract.** Liver receptor homolog 1 (*Lrh1*, also known as *Nr5a2*) belongs to the orphan nuclear receptor superfamily and has diverse functions in development, metabolism, and cell differentiation and death. *Lrh1* regulates the expression of *Oct4*, which is a key factor of early embryonic differentiation. However, the role of *Lrh1* in early development of mammalian embryo is unknown. In the present study, the localization, *Lrh1* mRNA expression, and LRH1 protein levels in porcine early parthenotes were examined by immunofluorescence and real-time reverse-transcription polymerase chain reaction. To determine the role of *Lrh1* in porcine early embryo development, the parthenotes were treated with the specific LRH1 antagonist 505601. The immunofluorescence signal for LRH1 was only observed in the nucleus of blastocysts. The blastocyst developmental rate in the presence of 50 and 100 μM 505601 was significantly lower than that in the control group. The blastocyst hatching rate was also reduced in the presence of 50 and 100 μM 505601 than that under control conditions. The latter effect was possibly due to the decreased expression of hatching-related genes such as *Fn1*, *Itgα*5, and *Cox2* upon the inhibition of *Lrh1*. Incubation with the LRH1 antagonist also increased the number of apoptotic cells among the blastocysts. Moreover, LRH1 inhibition enhanced the expression of the pro-apoptotic genes *Bax* and *Casp3*, and reduced the expression of the anti-apoptotic gene *Bcl2*. *Lrh1* inhibition also led to significant decrease in the expression levels of *Oct4* mRNA and octamer-binding transcription factor 4 (OCT4) protein in the blastocysts. In conclusion, *Lrh1* affects blastocyst formation and hatching in porcine embryonic development through the regulation of OCT4 expression and cell apoptosis.

**Key words:** Apoptosis, Hatching, LRH1, Octamer-binding transcription factor 4 (OCT4), Pig

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Blastocyst hatching from the zona pellucida is a critical event during the attachment of the blastocyst to the uterine epithelium before implantation [1]. Any dysregulation of the hatching process causes implantation failure, which leads to infertility [2]. Blastocyst hatching is controlled by an extremely complicated interplay of various cellular factors and biomolecules, which are regulated in a defined spatio-temporal fashion [3].

Liver receptor homolog 1 (*Lrh1*, also known as *Nr5a2*) belongs to the orphan nuclear receptor superfamily and has diverse functions in the development, metabolism, and cell differentiation and death. *Lrh1* plays important roles in bile acid biosynthesis [4], cholesterol metabolism [5], and glucose sensing [6] in the liver. In the ovaries, *Lrh1* regulates steroid synthesis [7], pregnancy time course [8], maturation of ovarian follicles, and ovulation [9]. *Lrh1* is also a critical factor in embryonic development. Mice with a homozygous null mutation of the *Lrh1* gene die on around embryonic day 7.5 and exhibit pathological features typical of visceral endoderm dysfunction [10].

Several studies have demonstrated that *Lrh1* is highly expressed in embryonic stem cells (ESCs). In cooperation with its partner *Dax1*, *Lrh1* activates the transcription of *Oct4*, which encodes the key factor for maintaining the pluripotency of mouse ESCs [11-13]. *Lrh1* acts upstream of *Oct4* and regulates *Oct4* expression during the epiblast stage of mouse embryonic development [14]. In addition, *Lrh1* not only increases the reprogramming efficiency, but also substitutes for exogenous *Oct4* in reprogramming mouse embryonic fibroblasts to induced pluripotent stem cells (iPSCs), which further underscores the relationship between the two genes and highlights the important role of *Lrh1* in regulating *Oct4* and maintaining pluripotency [15]. On the other hand, *Oct4* is essential for porcine embryo development, as can be inferred from the effects of *Oct4* small interfering RNA treatment on porcine blastocyst formation [16]. Overexpression of *Oct4* enhances the proliferation of porcine embryos [17], however, the function of *Lrh1* in porcine blastocyst formation is still unknown. We hypothesized that *Lrh1* plays a critical role in porcine early embryonic development by regulating the expression of the octamer-binding transcription factor 4 (OCT4).

Several studies have addressed the function of *Lrh1* in the regulation of ESC pluripotency and differentiation, however little information is available on the actions of *Lrh1* in porcine early embryo development. It has been demonstrated that porcine parthenogenetic diploids can develop to the blastocyst stage just as in fertilized porcine embryos [18]. Furthermore, researchers have shown the successful development of parthenogenetic porcine embryos to the post-implantation stage [19]. However, porcine embryos of homogeneous quality are difficult to obtain because of the relatively high incidence of polyspermy during *in vitro* fertilization (IVF) [20]. Therefore, parthenogenetic

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diploids could be used as model embryos for early development studies in pigs [21]. To investigate the developmental role of *Lrh1*, parthenotes were treated with a specific LRH1 antagonist and the blastocyst formation and OCT4 expression levels were analyzed. We demonstrated that *Lrh1* may play an important role in blastocyst formation and hatching through the regulation of OCT4 and apoptosis. Therefore, *Lrh1* is possibly a critical factor in early porcine embryo development.

## Materials and Methods

*Oocyte collection, in vitro maturation, and embryo culture*

Ovaries from prepubertal gilts were obtained from a local slaughterhouse, maintained in saline at 37°C, and transported to the laboratory. Cumulus oocyte complexes (COCs) were isolated from follicles and washed three times in Tyrode's lactate-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The COCs were cultured in tissue culture medium 199 (TCM 199) supplemented with 10% porcine follicular fluid, 0.1 g/l sodium pyruvate, 0.6 mM L-cysteine, 10 ng/ml epidermal growth factor, 10 IU/ml luteinizing hormone, and 10 IU/ml follicle stimulating hormone at 38.5°C for 44 h in a humidified atmosphere of 5% $CO_2$ and 95% air. After maturation, cumulus cells were removed by treatment with 0.1% hyaluronidase for 2–3 min and repeated pipetting. For parthenogenetic activation, oocytes with polar bodies were selected and activated by two direct current (DC) pulses of 1.1 kV/cm for 60 μsec and then incubated in the porcine zygote medium (PZM-5) containing 7.5 μg/ml of cytochalasin B for 3 h. Finally, the embryos were cultured in PZM-5 medium for 8 days at 38.5°C in a humidified atmosphere of 5% $CO_2$ and 95% air. On the fifth day, fetal bovine serum (FBS) was added to the medium to a total concentration of 4%. To observe the effect of LRH1 on porcine early embryo development, the LRH1 antagonist 505601 (Merck Millipore, Darmstadt, Germany) was added to the medium used for parthenogenic activation to obtain final concentrations of 50 or 100 μM.

*Real-time reverse-transcription polymerase chain reaction*

Twenty oocytes and 40 embryos were collected initially (at 0 h) and after culturing the oocytes for 48 h. The oocytes had been activated for 24, 30, 43, or 144 h corresponding to the GV, MII, one-cell, two-cell, four-cell, and blastocyst stages, respectively. mRNA was extracted from 10 oocytes per group with a Dynabeads mRNA Direct Kit (DynalAsa, Oslo, Norway) according to the manufacturer's instructions. cDNA was obtained by reverse transcription of the mRNA using the Oligo $(dT)_{12\text{-}18}$ primer and SuperScript TM III Reverse Transcriptase (Invitrogen, Grand Island, NY, USA). The amplification cycles used were as follows: 95°C for 3 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 20 sec, and a final extension at 72°C for 5 min. The relative quantification of gene expression was normalized to internal porcine *Gapdh* mRNA levels using the $2^{-\Delta\Delta CT}$ method. The primers used to amplify each gene are shown in Table 1.

*Immunofluorescence and confocal microscopy*

Oocytes and embryos were fixed in 3.7% paraformaldehyde for 20 min at room temperature, permeabilized with phosphate buffered saline/polyvinyl alcohol (PBS/PVA) containing 1.0% Triton X-100 at 37°C for 1 h, and then incubated in PVA-PBS containing 3.0% bovine serum albumin at 37°C for 1 h. Subsequently, the oocytes and embryos were incubated overnight at 4°C with an anti-LRH1 antibody (ab153944, 1:100; Abcam, Cambridge, UK) and an anti-OCT4 antibody (sc8628, 1:100; Santa Cruz Biotechnology, CA, USA). After washing three times in PBS/PVA, the oocytes and embryos were incubated at 37°C for 1 h with either goat anti-rabbit IgG or rabbit anti-goat IgG. The oocytes and embryos were then stained with Hoechst 33342 for 5 min, washed three times in PBS/PVA, mounted onto slides, and examined using a confocal microscope (Zeiss LSM 710 META, Jena, Germany). Images were processed using Zen software (version 8.0, Zeiss, Jena, Germany).

*Terminal deoxynucleotidyltransferase-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling (TUNEL) assay*

After the oocytes were treated with different concentrations of the LRH1 antagonist, the blastocysts were collected. The blastocysts were fixed in 3.7% paraformaldehyde for 30 min at room temperature and then permeabilized by incubation in 1% Triton X-100 for 1 h at 37°C. The embryos were incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyltransferase enzyme (*In Situ* Cell Death Detection Kit, Roche, Mannheim, Germany) for 1 h at 37°C and then washed three times in PBS/PVA. Embryos were treated with Hoechst 33342 for 5 min to stain the DNA, washed three times in PBS/PVA, and mounted onto glass slides. Images were captured using a confocal microscope.

*Statistical analysis*

All data were analyzed with a one-way analysis of variance and differences between the treatment groups were assessed by the least significant difference test using Statistical Package for the Social Sciences (SPSS) software. Each experiment was performed at least in triplicate and differences were considered to be significant if $P < 0.05$.

## Results

*Expression and localization of Lrh1 in porcine embryos*

Before investigating the function of *Lrh1* in early embryo development, the expression level and subcellular localization of this protein were initially examined. As shown in Fig. 1A, *Lrh1* mRNA was expressed throughout porcine oocyte maturation and the early embryo developmental stages, although, these expression levels were significantly higher at the blastocyst stage than during other embryonic stages. To investigate the subcellular localization of the LRH1 protein, porcine embryos at different stages were cultured and processed for immunofluorescent staining. An anti-LRH1 antibody was used to detect LRH1 localization (Fig. 1B). An LRH1 immunofluorescence signal was only observed in the nucleus of the blastocysts, but not during other stages. On the basis of these findings, we concluded that *Lrh1* may play a role during the pre-implantation stage.

*Effect of Lrh1 on the development of porcine parthenotes*

In order to investigate the functions of *Lrh1* in development, parthenotes were treated with different concentrations of the LRH1-specific

**Table 1.** List of primers used for real-time reverse-transcription polymerase chain reaction

| Gene | Primer Sequence (5′–3′) | Annealing temperature (°C) | Product size (bp) |
|---|---|---|---|
| *Lrh1* | F:GGTACCACTATGGGCTCCTCAC<br>R:TCGGCCCTTACCGCTTCT | 60 | 193 |
| *Fn1* | F:AGGGCGATGAACCACAGT<br>R:GCTCCAGCGAACGACAAT | 60 | 221 |
| *Itgα5* | F:TGATGACAGTTATTTGGGCTAC<br>R:CAAAGTCCTCGCTGCTCT | 60 | 100 |
| *Cox2* | F:GGCTGCGGGAACATAATAGA<br>R:GCAGCTCTGGGTCAAACTTC | 55 | 183 |
| *Bcl2* | F:GCCGAAATGTTTGCTGAC<br>R:GCCGATCTCGAAGGAAGT | 60 | 154 |
| *Bax* | F:GATCGAGCAGGGCGAATG<br>R:GGGCCTTGAGCACCAGTTTA | 60 | 277 |
| *Casp3* | F:GACGGACAGTGGGACTGAAGA<br>R:GCCAGGAATAGTAACCAGGTGC | 60 | 101 |
| *Oct4* | F: CCCCGCCCTATGACTTCT<br>R: TAGGAGCTTGGCAAATTGTTC | 60 | 269 |
| *F-actin* | F:AGTTCACCATCACACCACCTACA<br>R:CTTGGCAGTTTGGGCTTCATTC | 60 | 146 |
| *Gapdh* | F: TTCCACGGCACAGTCAAG<br>R: ATACTCAGCACCAGCATCG | 60 | 117 |

antagonist 505601. No significant differences in the developmental rates for the two-cell and four-cell stages were observed between the treatment and control groups. However, the blastocyst development rates in the presence of 50 and 100 μM of 505601 (37.30 ± 3.67% and 20.10 ± 2.81%, respectively) were significantly lower than that of the control group (53.43 ± 3.67%, Fig. 2A). The blastocyst hatching rates were also reduced ($P < 0.01$) by the treatment with 50 or 100 μM 505601 (12.66 ± 3.13% and 20.10 ± 2.81%, respectively) from the hatching rate in the control (40.59 ± 0.59%).

In order to detect the mechanism of *Lrh1*'s influence on blastocyst hatching, the expression of hatching-related genes was examined. As illustrated in Fig. 2C, the expression levels of the hatching-related genes *Fn1*, *Itgα5*, and *Cox2* were significantly lower in the treatment group than in the control group ($P < 0.01$).

### *Effect of Lrh1 on the porcine blastocyst quality*

The quality of the blastocysts was further evaluated based on Gardner's criteria [22]. Blastocysts were graded on a scale of 1 to 6 and the grade depended on their degree of expansion and hatching status as follows: 1, an early blastocyst without a full blastocoel volume, which occupies less than a half of the embryo; 2, a blastocyst with a blastocoel volume, which occupies at least half (or more) of the embryo; 3, a full blastocyst with a blastocoel completely filling the embryo; 4, an expanded blastocyst with a blastocoel, which is larger than the volume of the early embryo, with a thinning zona pellucida; 5, a hatching blastocyst with the trophectoderm, which begins to hatch from the zona pellucida; and 6, a hatched blastocyst, which completely escaped from the zona pellucida. The distribution of the blastocyst quality scores is shown in Fig. 3A. In the present study, there was a significant decrease in the number of high quality blastocysts and an increase in the number of low quality blastocysts following the inhibition of LRH1 (Fig. 3B). A blastocyst score from 4 to 6 was a sign of a high quality blastocyst. Blastocysts of lower quality received scores from 1 to 3. The fraction of high quality blastocysts was significantly lower in the presence of 50 and 100 μM of 505601 (32.48 ± 0.81%; 34.85 ± 1.5%, respectively) than that fraction in the control group (56.41 ± 6.41%).

### *Effect of Lrh1 on apoptosis in porcine blastocysts*

Previous studies showed that blastocyst hatching was influenced by the blastocyst cell number [23], therefore the effect of *Lrh1* on this parameter was investigated (Fig. 4A). The total blastocyst cell number in the control group (40.67 ± 2.84) was significantly higher ($P < 0.05$) than that observed in the presence of 50 (28.25 ± 2.53) or 100 μM of 505601 (27.00 ± 4.95). Thus, the blastocyst hatching after treatment with an LRH1 antagonist may be associated with the total blastocyst cell number.

Since the inhibition of LRH1 by 505601 decreased the total blastocyst cell number, we next investigated if this pharmacological manipulation affected apoptosis. The number of apoptotic cells per blastocyst was checked on the eighth day after parthenogenetic activation in the treatment and control groups. The number of apoptotic cells was significantly lower ($P < 0.05$) in the control group (3.00 ± 0.82) than in the groups treated with 50 (6.00 ± 0.71) or 100 μM (6.00 ± 0.75) of 505601 (Fig. 4B). Therefore, we concluded that the inhibition of LRH1 increased the apoptotic rate of the blastocysts.

In order to understand the mechanism by which *Lrh1* exerts this effect on apoptosis, the expression of three critical apoptotic genes,

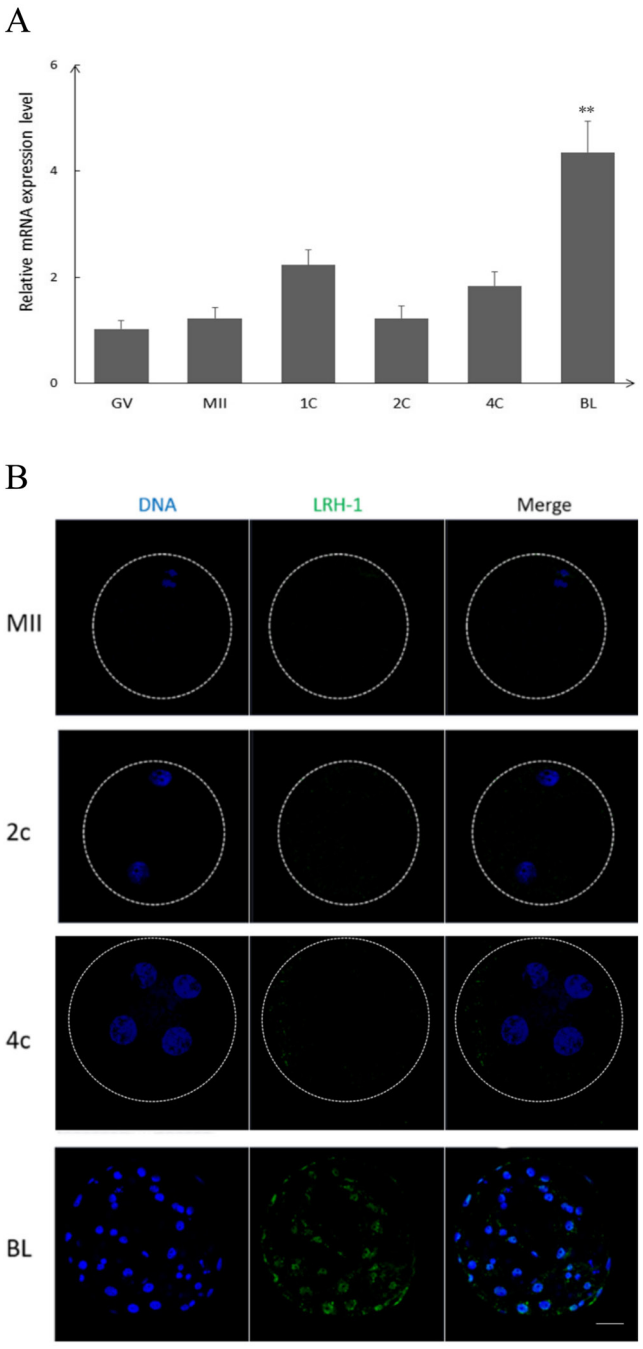



**Fig. 1.** *Lrh1* mRNA expression and localization in porcine oocytes and early embryos at various developmental stages. (A) mRNA was collected during different phases of porcine embryo development. (B) Laser scanning confocal microscopy images of liver receptor homolog 1 immunostaining during the MII, 2C, 4C, and blastocyst stages. GV, MII, 1C, 2C, 4C, and BL indicate germinal vesicle, meiosis II, one-cell stage, two-cell stage, four-cell stage, and the blastocyst, respectively. ** indicates statistically significant difference ($P < 0.01$) from GV. Data are presented as the mean (± standard deviation of the mean) of three independent experiments.

*Bax*, *Casp3*, and *Bcl2*, was analyzed. LRH1 inhibition enhanced the expression of the pro-apoptotic genes *Bax* and *Casp3* ($P < 0.05$) and reduced the expression of the anti-apoptotic gene *Bcl2* ($P < 0.01$). Thus, *Lrh1* could regulate apoptosis via modulation of the expression of the apoptosis-related genes *Bax*, *Casp3*, and *Bcl2*.

*Effect of Lrh1 on the expression of OCT4 in porcine blastocysts*

The expression levels of OCT4 mRNA and protein were analyzed following treatment with the LRH1 antagonist 505601. The *Oct4* mRNA expression was significantly lower in the LRH1 antagonist-treated embryos than in the control, untreated embryos ($P < 0.001$, Fig. 5A). Consistent with the data on mRNA expression, weaker expression of the OCT4 protein was observed at the blastocyst stage in the LRH1 antagonist-treated embryos than in the untreated embryos (Fig. 5B).

## Discussion

In mammals, blastocysts must hatch from the zona pellucida before implantation for further development. Blastocyst hatching is an essential event for the subsequent viability and development of the embryo [24]. Although it is vital to understand the mechanism of hatching in detail, in-depth studies of this critical process are still scarce. In the present study, we discovered that the activity of *Lrh1*, a novel transcription factor, affects blastocyst hatching. It has been reported previously that mice bearing a homozygous null mutation in the *Lrh1* gene die on embryonic days 6.5–7.5. This observation shows that *Lrh1* is likely to play an important role in embryo development [10]. The structure-based discovery of LRH1 antagonists has identified ligands that inhibit *Lrh1* transcriptional activity and diminish the expression of the receptor's target genes [25, 26]. Therefore, several studies have used LRH1 antagonists to investigate the receptor's biological function[25]. In the present study, the treatment of porcine parthenotes with an LRH1-specific antagonist had a negative effect on blastocyst formation and quality; therefore, our data support the notion that *Lrh1* affects the developmental capacity of the embryo. Furthermore, decrease in the hatching rate that we observed upon LRH1 inhibition can lead to implantation failure.

. In the present study, the mRNA expression levels of several hatching-related genes, i.e. *Fn1*, *Itgα5*, and *Cox2*, were significantly smaller in the experimental groups treated with an LRH1 antagonist than in the control, untreated groups. The FN1 protein is produced by the trophoblast cells of the blastocyst, and the interaction of this protein with integrins is critically important for the attachment of the embryo to the maternal endometrium during successful implantation [27]. FN1 is an important bridging ligand providing the Arg-Gly-Asp integrin recognition site for apically-expressed integrins in the endometrium and the embryo [28]. *Itgα5* is a major fibronectin receptor, and *Cox2* is another key factor for blastocyst hatching. Prostacyclin synthesized by *Cox2* plays an important role in embryo development during preimplantation and blastocyst hatching [29]. The selective pharmacological inhibition of *Cox2* completely blocks blastocyst hatching [30]. Hence, we postulate that *Lrh1* regulates blastocyst hatching through its effect on the expression of *Fn1*, *Itgα5*, and *Cox2*, which are followed by the attachment of the embryo to the uterine endometrium.

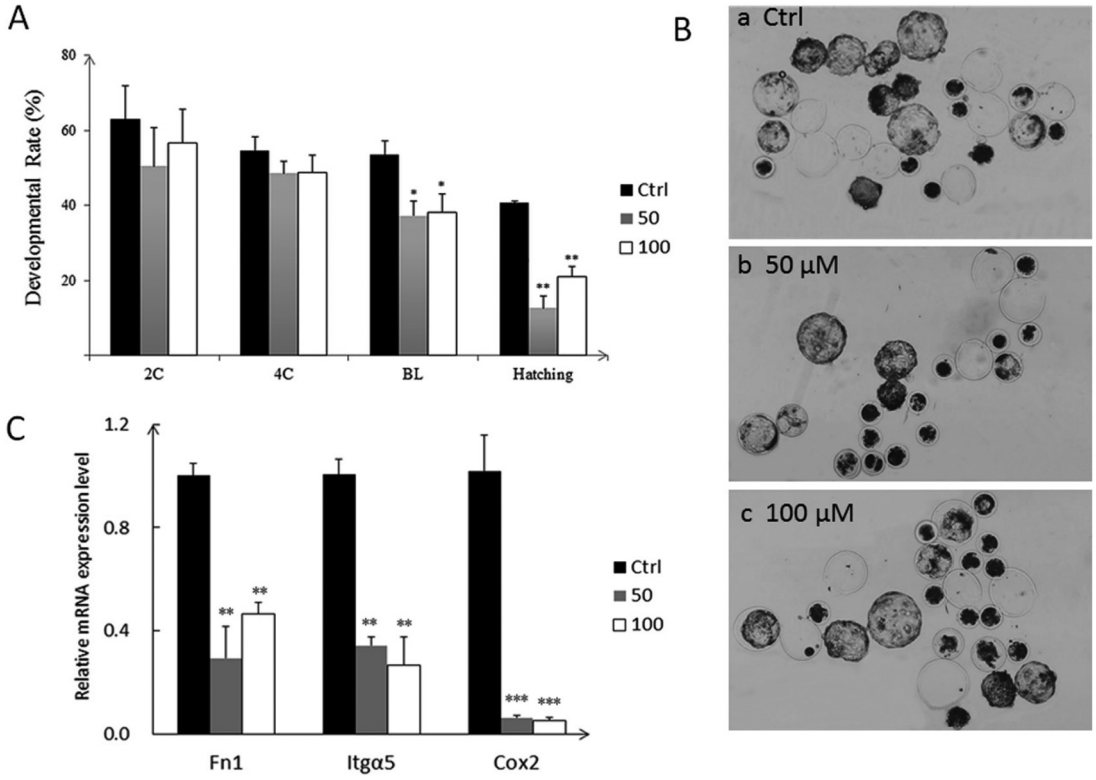
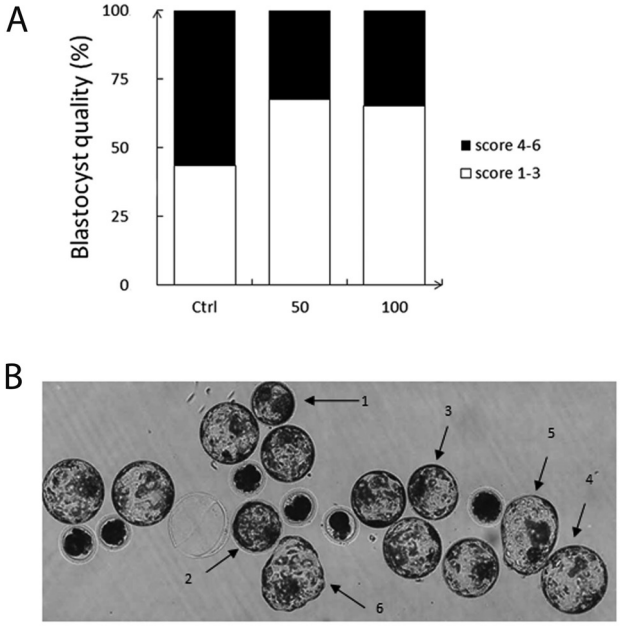



**Fig. 2.** The effect of liver receptor homolog 1 (LRH1) inhibition on the embryo development. The parthenotes were treated with the specific LRH1 antagonist 505601 in concentrations of 50 or 100 μM. (A) The percentages of those in the two-cell, four-cell, blastocyst, and hatching blastocyst stages are shown by bars. (B) The images of embryos treated with different concentrations of 505601 are shown. (C) Expression of *Fn1*, *Itgα5*, and *Cox2* mRNA in hatching blastocysts in the presence or absence of the LRH1 antagonist. Statistical significance of the differences from the control group is indicated as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Data are presented as the mean (± standard deviation of the mean) of three independent experiments.




There is a direct correlation between blastocyst quality and blastocyst hatching [22]. In the presence of an LRH1 antagonist in the culture medium, the fraction of good quality blastocysts was smaller than that in control conditions. Furthermore, blastocyst hatching is influenced by the blastocyst cell number [23], and apoptosis in the early embryo has an important impact on embryo development [31]. It is possible that LRH1 inhibition caused a failure of embryo competence because of the enhancement of the apoptotic rate. In support of this, LRH1 inhibition upregulated the expression of the proapoptotic genes *Bax* and *Casp3* and decreased the expression levels of *Bcl2*, an anti-apoptotic gene. These observations suggest that the natural activity of intact *Lrh1* is to enhance blastocyst vitality.

Blastocysts escape from the zona pellucida and form a compact inner cell mass (ICM) [32]. *Oct4* is a key factor in ICM formation.

**Fig. 3.** Effect of pharmacological inhibition of liver receptor homolog 1 (LRH1) on porcine blastocysts. (A) Blastocyst quality was examined in the presence or absence of the LRH1 antagonist. Black and white bars indicate high and low quality blastocysts, respectively ($P < 0.05$). (B) Blastocysts were graded on a scale from 1 to 6 as illustrated. Different scores were indicated by the respective numbers. Ctrl, control; 50, 50 μM; 100, 100 μM.

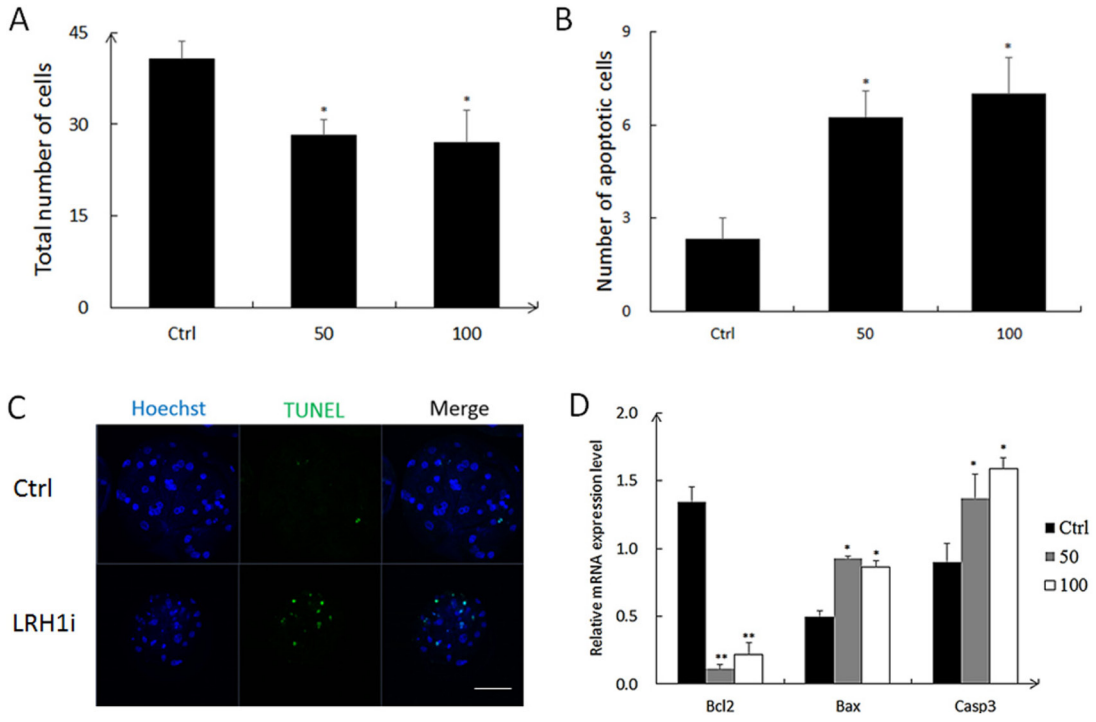
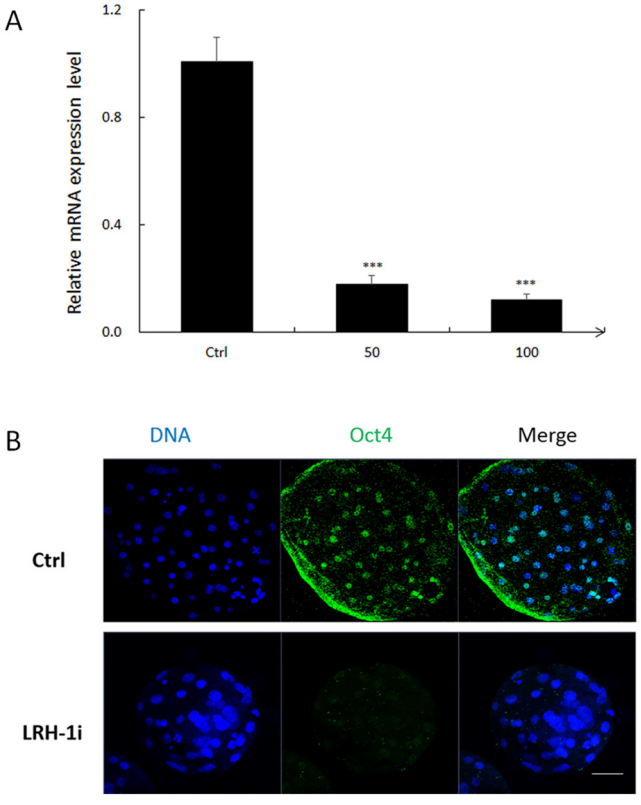



**Fig. 4.** Effect of liver receptor homolog 1 (LRH1) on apoptosis. (A) The total number of blastocyst cells and the fraction of apoptotic cells were examined. (B) Laser scanning confocal microscopy images of nuclei and fragmented DNA in samples from the control group and the LRH1-antagonist-treated group. (C) Expression of *Bax*, *Bcl2*, and *Casp3* mRNA in porcine blastocysts during *in vitro* culturing with or without the LRH1 antagonist. Statistical significance of differences from the control group is indicated as follows: * $P < 0.05$, ** $P < 0.01$. Data are presented as the mean (± standard deviation of the mean) of three independent experiments. Ctrl, control; 50, 50 μM; 100, 100 μM.




It should be noted that mouse embryos with a homozygous null mutation in the *Oct4* gene die around the time of the implantation stage [33]. Previous studies have demonstrated that the expression of the *Oct4* gene was regulated by *Lrh1*, as knockdown of the *Lrh1* gene decreased *Oct4* expression [15]. In the present study, the pharmacological inhibition of LRH1 during early porcine embryonic development resulted in decreased expression of OCT4 protein and mRNA. Regulation of the *Oct4* expression level by *Lrh1* may be mediated by a canonical Wnt/β-catenin-dependent pathway, which has been shown to be involved in the hatching of pig blastocysts [34, 35]. Therefore, we hypothesized that *Lrh1* affects blastocyst hatching through the regulation of *Oct4* expression via the Wnt/β-catenin signaling pathway.

In summary, we revealed an important role of *Lrh1*, an orphan nuclear receptor superfamily member, in early porcine embryo

**Fig. 5.** The mRNA and protein expression levels of octamer-binding transcription factor 4 (OCT4). Relative *Oct4* mRNA expression level and laser scanning confocal microscopy images of immunostaining for the OCT4 protein in porcine blastocysts during treatments with different concentrations of the liver receptor homolog 1 (LRH1) antagonist. *** indicates statistically significant difference ($P < 0.001$) from the control group. Data are presented as the mean (± standard deviation of the mean) of three independent experiments. Ctrl, control; LRH1i, inhibition of LRH1; 50, 50 μM; 100, 100 μM.

development, especially during blastocyst formation and hatching. The present study demonstrated that *Lrh1* influenced early embryo development by regulating apoptosis and OCT4 expression. In the future, further experiments will be necessary to understand the pathways involved in the *Lrh1* regulation of blastocyst hatching; this will be particularly necessary to validate the application of LRH1 antagonists to improve porcine IVF cycles suffering from hatching problems.

## Acknowledgments

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