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 divergent 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 parthenogenotes were examined by immunofluorescence and real-time reverse-transcription polymerase chain reaction. To determine the role of Lrh1 in porcine early embryo development, the parthenogenotes 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, Itga5, 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 (J. Reprod. Dev. 62: 297–303, 2016)
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% CO2 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% CO2 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)12-18 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−ΔΔCT 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).

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
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 \( \text{Fn1}, \ \text{Itg}\alpha_5, \ \text{and Cox2} \) 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 (12.66 ± 3.13% and 20.10 ± 2.81%, respectively) from the hatching rate in the control (40.59 ± 0.59%).

**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 R:TCGGCCCTTACCGCTTCTT | 60 | 193 |
| Fn1  | F:AGGGCGATGAAACACAGT R:GCTCCAGCGAACGACAAT | 60 | 221 |
| Itg\(\alpha_5\) | F:TGTAGCACAGTTTGGGCTAC R:CAAAGTCTCTGGCTTCT | 60 | 100 |
| Cox2  | F:GGCTGGGGGAACAAATAAGA R:GCAAGCTCTGGGTCAAATTC | 55 | 183 |
| Bcl2  | F:GCCGAAATGTTTGTCTGAC R:GCCGATCTGGAAGAAAT | 60 | 154 |
| Bax   | F:GATCGGAGAGGCGAATTG R:GGGCCCTTGAGCAACGTTTA | 60 | 277 |
| Casp3 | F:GCCGCCGCTTGAGGAAGAAGA R:GCAGCTCTGGGTCAAATTC | 60 | 101 |
| Oct4  | F:CCCCGGGTTATGACTTC R:TAGAGCTTGGGCAAATTTGGTC | 60 | 269 |
| F-actin | F:AGTTCACCATCACACACCACCATCA | 60 | 146 |
| Gapdh | F:TTCCACGGCAGAAGCTGAAGA R:CTTGGCCGCTTTGGGTCATTC | 60 | 117 |

In order to understand the mechanism by which Lrh1 exerts this effect on apoptosis, the expression of three critical apoptotic genes,
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.
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.
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...
development, especially during blastocyst formation and hatching. The present study demonstrated that Lrhl 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 Lrhl 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.

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