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Sonic Hedgehog promotes *in vitro* oocyte maturation and term development of embryos in Taiwan native goats

De-Chi Wang^{a,b}, Jan-Chi Huang^b, Neng-Wen Lo^c, Lih-Ren Chen^{d,e}, Pascal Mermillod^f, Wen-Lung Ma^{g,h}, Hsin-I Chiang^a, and Jyh-Cherng Ju^{a,g,i,j,k}

^a Department of Animal Science, National Chung Hsing University, Taichung 402, Taiwan.

^b Hengchun Branch Institute, COA-LRI, Hengchun, Pingtung 946, Taiwan.

^c Department of Animal Science and Biotechnology, Tunghai University, Taichung 407, Taiwan.

^d Physiology Division, COA-LRI, Hsinhua, Tainan 712, Taiwan.

^e Biotechnology Institute, National Cheng Kung University, Tainan 701, Taiwan.

^f INRA, UMR7247, Physiologie de la Reproduction et des Comportements, INRA, CNRS, Université de Tours, Haras Nationaux, Nouzilly, France.

^g Graduate Institute of Biomedical Sciences, China Medical University, Taichung 404, Taiwan.

^h Sex Hormone Research Center, China Medical University Hospital, Taichung 404, Taiwan.

ⁱ Core Laboratory for Stem Cell Research, Medical Research Department, China

Medical University Hospital, Taichung 404, Taiwan.

^j Department of Bioinformatics and Medical Engineering, Asia University, Taichung,

Taiwan.

^k Corresponding author: Jyh-Cherng Ju. E-mail: jcju@dragon.nchu.edu.tw

Short title: Sonic Hedgehog promotes development of goat embryo

Abstract

The aim of this study was to investigate the effects of Shh (Sonic Hedgehog) protein on caprine oocyte maturation, early embryo development, and developmental competence after embryo transfer of vitrified-thawed *in vitro*-produced embryos. Cumulus-oocyte complexes (COCs) derived from abattoir were randomly allocated to the *in vitro* maturation (IVM) medium supplemented with 0 (Control), 0.125, 0.25, 0.5, or 1.0 $\mu\text{g}\cdot\text{mL}^{-1}$ recombinant mouse Shh protein. After IVM, COCs were fertilized with frozen-thawed semen and the presumptive zygotes were cultured on goat oviduct epithelial monolayers in M199 medium for 9 days. Our results showed that supplementation of Shh (0.25 or 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$) enhanced oocyte maturation as compared with the control group (92.4% and 95.0% vs. 86.2%, $P <$

0.05), yet the effect could be reversed by the simultaneous addition of cyclopamine (an inhibitor of Shh signaling by direct binding to the essential signal transducer Smo). Subsequently, an improved blastocyst rate (66.3 ± 10.9 , $P < 0.05$) was observed for the embryos derived from the oocytes matured in the presence of $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ Shh compared with the control group (41.4 ± 12.9). Expressions of *Shh*, *SMO* and *Gli1* were observed in the ovaries, granulosa cells, COCs, cumulus cells, oocytes and oviduct epithelia. Notably, *Ptch1* was expressed in nearly all of the aforementioned tissues and cells except cumulus cells. The embryos exhibited a higher survival rates in the Shh-supplemented group (37.5%) compared to those without Shh supplementation (14.8%; $P < 0.05$) after embryo transfer. This study demonstrated the beneficial effects of Shh supplementation on oocyte maturation and subsequent embryo development both *in vitro* and *in vivo*, suggesting a functional existence of Shh signaling during the final stage of folliculogenesis and early embryogenesis in caprine.

Keywords: Sonic Hedgehog, oocyte maturation, goat, embryo transfer, *in vitro* development

1. Introduction

Influenced by macrobiotics and traditional Chinese medicine, goat milk and meat consumption has been popular in Taiwan. Compared to the exotic breeds, Taiwan native goats have much better diseases- and coarse diets-resistant despite of their low productivity. In response to the ever-increasing demand, a large number of Nubian goats were imported to cross-breed with Taiwan native goats during the 1980s [1,2]. This is one of the main reasons for the decreased population of the pure Taiwan native goats, which may soon become endangered. Therefore, reproductive technologies could be an important tool for preserving genetic diversity and rescuing this caprine breeds in Taiwan.

Generation of high quality goat embryos is an essential step towards further development and application of *in vitro*-produced embryos [3,4]. Previous studies have demonstrated that the growth of preimplantation embryos can be enhanced by supplementation of cytokines and/or growth factors in the culture medium [5]. Hedgehog (Hh) protein, which attracts considerable attention over the past few years, is a paracrine factor that enhances embryonic development [6]. In vertebrates, there are at least three Hh members, namely, Sonic Hh (Shh), Indian Hh (Ihh) and Desert Hh. The signaling of the Hh family peptides is mediated through a membrane

bound surface receptor Patched (Ptc) and a membrane associated signal transducer Smoothed (Smo). In the absence of Hh ligand, Ptc suppresses Smo so that no downstream signaling occurs [7,8]; whereas in the presence of Hh, the suppression of Smo is lifted, leading to the activation of intracellular transcription effectors Gli1, Gli2 and Gli3 in vertebrates [9].

In the mouse, *Ihh* and *Dhh* are expressed in the granulosa cells of preantral and antral follicles, with the receptor Ptc and the signal transducer Smo expressed in thecal cells. Previous studies also suggested that paracrine signaling of Hh exist between granulosa and theca cells [10,11]. Similarly, Russell *et al.* [9] reported that Hh ligands, including *Ihh*, *Dhh* and *Shh*, are expressed in both immature and adult mouse ovaries, where the expression of Ptc (*Ptc1*, *Ptc2*) and Smo are found in all ovarian tissues. Therefore, the Hh signaling pathway is most likely to be involved in granulosa cell proliferation and oocyte maturation. Spicer *et al.* [12] demonstrated that the mRNA expression of the Hh-patched signaling molecule *Ihh* in granulosa cells increased, but mRNA expressions of *Smo* and *Ptch1* levels decreased in theca cells of small follicles compared to large follicles in cattle. In cultured bovine theca-interstitial cells, qRT-PCR analyses revealed that the abundance of *Gli1* and *Ptch1* mRNAs increased with *Shh* treatment. Additional studies have shown that *Shh* induces proliferation and androstenedione production of cultured bovine theca

cells. Moreover, expression and regulation of *lhh* mRNA in granulosa cells and *Ptch1* mRNA in theca cells may also suggest a potential paracrine role during bovine folliculogenesis. Nguyen *et al.* [13,14,15] have also reported that the Shh signaling pathway is active or at least partially active in the porcine ovary, which is likely associated with cytoplasmic and nuclear maturation of oocytes as well as with subsequent embryonic development *in vitro*. Therefore, we propose that this paracrine factor also promotes oocyte maturation and early embryogenesis in goats. To test this hypothesis, the effects of Shh treatment during goat oocyte maturation on both *in vitro* and *in vivo* developmental competence, including oocyte maturation, embryonic development, pregnancy and kidding rates as well as the Shh-related gene expression in reproductive cells/tissues, were all determined.

2. Materials and Methods

2.1. Chemicals

All chemicals used in the present study were purchased from Sigma-Aldrich (St Louis, MO, USA), unless stated otherwise.

2.2. Oocyte recovery and *in vitro* maturation (IVM)

During the breeding season, ovaries of adult goats were collected from a local

abattoir and transported to the laboratory in saline at 38 °C within 2.5 h. Ovaries were further washed in warm saline, and oocytes were harvested by slicing all visible follicles (1 to 5 mm in diameter) with a blade and then flushed with TCM 199 (Gibco, 12340-030, Grand Island, USA) supplemented with 100 UI/mL heparin, 40 mg/mL gentamycin and 10 mM/mL HEPES. Only oocytes surrounded by multilayered, unexpanded cumulus cells and finely granulated ooplasm (Grades 1 and 2) were used for IVM [16]. The cumulus-oocyte complexes (COCs) were washed three times in the maturation medium (TCM 199 supplemented with 10% FCS, 10 μ g/mL FSH, 10 μ g/mL LH, 0.2 mM sodium pyruvate, 1 μ g/mL estradiol 17 β , 10 ng/mL EGF and 100 μ M cysteamine), and then cultured in 4-well dishes (Nunc, Roskilde, Denmark) containing 0.5 mL of maturation medium and 20 to 30 oocytes per well. The COCs were incubated for 24 h at 38.5 °C in a humidified atmosphere containing 5% CO₂ in air [17].

2.3. *In vitro* fertilization (IVF) and embryo culture

Motile spermatozoa from frozen-thawed semen of Taiwan native goats were separated by washing and centrifugation (10 min at 900 $\times$ g) with washing medium (2 mM CaCl₂.2H₂O, 521 μ M MgCl₂.6H₂O, 112 mM NaCl, 4 mM KCl, 819 μ M NaH₂PO₄.H₂O, 3.69 mM NaHCO₃, 1.25 mM Sodium pyruvate, 13.9 mM D-glucose, 6 mg/mL Bovine serum albumin, 50 μ g/mL Gentamycin, and 20% Goat serum).

135 Viable spermatozoa were diluted with an appropriate volume of fertilization medium
136 (the washing medium supplemented with 20 µg/mL heparin and 0.8 µg/mL caffeine)
137 to a concentration of 1×10^7 spz/mL and then capacitated for 15 min at 38.5 °C in
138 an incubator containing 5% CO₂ in humidified air. Cumulus cells were removed by
139 gentle pipetting and oocytes were washed three times with fertilization medium.
140 Groups of 20 to 30 oocytes were transferred into a 4-well dish containing 450 µL of
141 fertilization medium covered with 350 µL of mineral oil. For fertilization, capacitated
142 sperm (50 µL) were added into the wells at the final concentration of 1×10^6 spz/mL
143 and then co-incubated for 18 h [17]. After IVF, fertilized embryos were co-cultured
144 with goat oviduct epithelial cell (GOEC) monolayer in a 4-well dish containing 500 µL
145 culture medium (TCM 199 plus 10% FCS) for 9 days and half of the culture medium
146 was renewed every 48 h. The GOEC monolayer was prepared based on a previous
147 study by Mermillod *et al.* with some modifications [18]. Briefly, the mucosa layer was
148 mechanically expelled by squeezing the oviduct, collected from the abattoir, with a
149 sterile microscope slide onto the bottom of a Petri dish. The epithelial fragments
150 were washed three times in TCM199 medium and placed in 4-well dishes containing
151 500 µL of TCM 199 supplemented with 10% (v/v) FCS and 80 µg/mL of gentamycin
152 and then cultured at 38.5 °C in a humidified atmosphere of 5% CO₂. The GOEC
153 monolayers were established 2 days prior to being used for embryo co-culture. A

half of the embryo culture medium was renewed every 48 h during *in vitro* development.

2.4. Recipients does

Crossbred does at ages of 3 to 4 years were used as recipients for embryos transfer. The does were housed indoors and fed with 0.3 kg/day of concentrates with free access to quality hays and water. Experiments were carried out during the breeding seasons (spring and autumn) of goats in Taiwan. Recipient animals were synchronized for estrus by inserting a vaginal releasing device containing 366 mg progesterone (Controlled Internal Drug Release, CIDR, EAZI-breed, Rydalmere, Australia) for 11 days. Two days before CIDR removal, the recipients received 500 IU eCG (Sera-Gona, China Chemicals, Taipei, Taiwan) and 125 mg cloprostenol (Estrumate, Schering-Plough, Baulkham, Australia). Behavioral estrus was observed 15 to 30 h following CIDR removal. Preoperative treatment, anesthesia, surgery for embryo transfer and post-operation care were performed as described previously according to the IACUC guidelines (approval number# LRIIACUC 101003) [19,20].

2.5. Experiment 1: RT-PCR analyses for the expression of *Shh* signaling molecules

RT-PCR assays were performed to determine whether ovarian tissue express

174 components of the Shh signaling molecules. Total RNA was isolated from ovaries,
 175 granulosa cells, COCs, cumulus cells, oocytes, and oviduct epithelia using an RNA
 176 extraction kit (Bio-Mi kit, Bio-Mi, Taiwan) according to the manufacturer's
 177 instructions. The extracted RNA was analyzed using a semi-quantitative one-step
 178 RT-PCR (Qiagen) procedure by Liu *et al.* [21] and manufacturer's instructions, with
 179 minor modifications. To detect the transcripts of Shh signaling molecules, i.e., *Ptc*,
 180 *Smo*, and *Gli1*, the total RNA was isolated from each sample and dissolved in 20 μ L
 181 of RNase-free water. For detection of gene expressions of Shh signaling molecules,
 182 5 μ L (100 ng) of total RNAs was used for each analysis. The thermo-profiles for
 183 amplification cycles were as follows, reverse transcription at 50 °C for 30 min, initial
 184 PCR activation at 95 °C for 15 min, denaturation at 94 °C for 1 min, annealing for 1
 185 min at 54 to 58 °C (depending on the primer pair used, see Table 1), and extension
 186 at 72 °C for 1 min, with a total of 40 cycles. The primer sequences for Shh-related
 187 transcripts (*Ptch 1*, *Smo*, *Gli1*), *GAPDH* are shown in Table 1.

189 2.6. Experiment 2: detection of extracellular signal-regulated kinase 1/2 (ERK1/2)

190 After maturation, cumulus-free oocytes (50 oocytes per treatment) were
 191 collected into sample buffer (100 mM Tris-HCl (pH 6.8), 200 mM β -mercaptoethanol,
 192 0.4% sodium dodecyl sulfate (SDS), 0.002% bromophenol blue, 20% glycerol) and

193 then stored at -80°C until use. For analysis, samples were boiled for 5 min, cooled
 194 on ice and then loaded onto 10% SDS–polyacrylamide gels for electrophoresis.
 195 After electrophoresis, separated proteins were transferred from the gel to
 196 nitrocellulose membranes (Cat. no. HAHY0010; Millipore, Billerica, Ireland) that had
 197 been blocked for 1 h in TBS buffer (20 mm TRIS-HCl, 500 mm NaCl, 0.1% Tween
 198 20) containing 10% chicken serum. The membranes were then incubated with
 199 primary antibody against ERK1/2 phosphorylation (1 : 500; Cat. no. 9101; rabbit
 200 polyclonal phospho-p44/p42 mitogen-activated protein kinase (Thr202/Tyr204)
 201 antibody; Cell Signaling Technology) at 4°C for 6 h. After incubation, membranes
 202 were washed five times for 10 min each and then incubated with secondary antibody
 203 (1 : 1000; HRP-labelled anti-rabbit immunoglobulin; Cell Signaling Technology) for 1
 204 h at room temperature. After washing for 5 min, proteins were detected using
 205 Super® SignalWest Pico Chemiluminescent Subs kits (Pierce Biotechnology,
 206 Rockford, IL, USA) and visualized by X-ray film. The membrane was probed with
 207 polyclonal rabbit anti-ERK1/2 antibodies (1 : 100; Cell Signaling Technology),
 208 incubated with HRP-labelled anti-rabbit immunoglobulin (1 : 1000; Cell Signaling
 209 Technology), and then subjected to the procedures described above. β -actin was
 210 used as an internal control. Band intensities were measured with Scion Image
 211 software (ver. Beta 4.0.3; 1997–2005 Scion Corporation) for Windows and then

normalized against β -actin prior to statistical analysis.

2.7. Experiment 3: cyclopamine treatment to inhibit Shh signaling

To confirm whether nuclear maturation was specifically enhanced by Shh supplementation, COCs were cultured in IVM medium with or without recombinant mouse Shh protein (461-SH, R&D Systems, Inc., Minneapolis, MN, USA) with cyclopamine (GR-344, Biomol, Farmingdale, NY, USA). Therefore, COCs were randomly allocated to various treatment groups, i.e., Control (without Shh), 0.5 $\mu\text{g/mL}$ Shh, 0.5 $\mu\text{g/mL}$ Shh plus 0.5 μM cyclopamine, and 0.5 $\mu\text{g/mL}$ Shh plus 1 μM cyclopamine. After 24 h of maturation, the nuclear status of the oocytes was assessed.

2.8. Experiment 4: effect of Shh supplementation on the nuclear maturation of oocytes

COCs were randomly allocated to IVM medium supplemented with 0 (control), 0.125, 0.25, 0.5, or 1.0 $\mu\text{g/mL}^{-1}$ recombinant mouse Shh protein. After IVM, the surrounding cumulus cells were stripped off and oocytes were then fixed for immunocytochemical staining for the examination of their nuclear stages, i.e., germinal vesicle (GV), MI, or MII stages, under an epifluorescence microscope.

To determine whether the Shh protein has a direct effect on oocyte maturation or acting indirectly through cumulus cells, denuded oocytes were cultured in the medium without or with Shh ($0.5 \mu\text{g}\cdot\text{mL}^{-1}$). The nuclear stages of oocytes were assessed 24 h after IVM as described previously.

2.9. Experiment 5: in vitro developmental competence of embryos derived from oocytes matured in Shh-containing media

COCs were randomly allocated to the IVM medium supplemented with 0 (control), 0.125, 0.25, 0.5, or $1.0 \mu\text{g}\cdot\text{mL}^{-1}$ recombinant mouse Shh protein. After oocyte IVF, the effects of Shh supplementation in the maturation medium on subsequent preimplantational embryo development including the rates of cleavage (Day 2) and blastocyst formation (days 6 to 9), and the total cell number per blastocyst were determined. Blastocysts were mounted onto glass slides and stained with Hoechst 33342 for detection of the total cell counts.

2.10. Experiment 6: embryo vitrification, thawing and transfer

Expanded and hatched blastocysts derived from the control and $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ Shh-supplemented group at Day 8 post-fertilization were vitrified as described by Huang *et al.* [19]. Briefly, the holding medium for vitrification was TCM199

250 supplemented with 20% FBS (v/v). Vitrification Solution 1 (VS1) contained 10% EG,
251 10% DMSO and 10% FBS in TCM-199, and Solution 2 (VS2) contained 16.5% EG
252 and 16.5% DMSO. Three blastocysts were initially placed in the holding medium for
253 5 min followed by VS1 for 45 s, VS2 (2 μ L) for 25 s, and then were aspirated into a
254 glass pipette. Immediately, a microdrop containing three embryos was generated at
255 the tip of the pipette and then directly dropped onto liquid nitrogen (LN₂) by gently
256 flipping. The vitrified droplets were collected into a cryovial and stored in LN₂. For
257 thawing embryos, microdrop were picked up and plunged into 38.5 °C TCM-199
258 containing 0.5 M sucrose and 20% FBS for 30 s, followed by serial dilution in 0.25 M
259 sucrose solution for 2 min and 0.15 M sucrose solution for 5 min to remove
260 cryoprotectants. Thereafter, embryos were transferred into TCM-199 containing 5%
261 FBS and were incubated at 38.5 °C in an incubator at 5% CO₂ with humidified
262 atmosphere for 1 h. All vitrified-thawed embryos were surgically transferred via a
263 midline incision at the abdomen of the recipient goat. Every 2-3 embryos were
264 deposited into the uterine horn ipsilateral to the corpus luteum of synchronized
265 recipients on day 8 after behavioral estrus. Pregnancy diagnosis was performed by
266 transrectal ultrasonography 60 days after embryo transfer. Numbers of transferred
267 embryos that survived to term were recorded.

268

2.11. Statistical analysis

All data, including western blots, maturation rates, cleavage rates and blastocyst rates derived from 4-6 replicates, were subjected to ANOVA, using the general linear model procedure in SAS (version 9), followed by the Tukey's test. Percentile data were arcsine-transformed before ANOVA and the probability at $P < 0.05$ was considered as significant. Pregnancy, parturition and embryo survival rates (number of kids born/number of transferred embryos) were compared by *Chi-square* test.

3. Results

The RT-PCR analyses showed expressions of *Shh*, *Smo*, *Ptc1*, and *Gli1* were detected in the whole ovaries, granulosa cells, COCs, cumulus cells, oocytes and oviductal epithelial cells; whereas no *Ptch1* expression in cumulus cells was observed (Fig. 1).

The effect of *Shh* on ooplasmic maturation showed that mRNA levels of phosphorylated ERK1/2 were increased 1.38-fold and 1.25-fold in oocytes matured in the presence of 0.5 $\mu\text{g/mL}$ and 1.0 $\mu\text{g/mL}$ *Shh*, respectively, compared to those of the control group ($P < 0.05$; Fig. 2).

To further confirm the effect of *Shh* on nuclear maturation, oocytes harvested

from the same batch of abattoir ovaries were randomly allocated to cyclopamine, an Shh inhibitor, and/or Shh treatments with four replicates. (Fig. 3). We found that oocytes (n=106) maturing in the presence of 0.5 $\mu\text{g/mL}$ Shh had a greater proportion to reach the MII stage than those in the control (n=103) group (93.5 ± 1.5 vs. 78.2 ± 4.5 , $P < 0.05$). However, oocytes that matured in the same medium with the presence of 0.5 (n=110) or 1.0 μM (n=131) cyclopamine had their nuclear maturation compromised compared to those with Shh only or without cyclopamine. (80.9 ± 1.1 , 69.6 ± 2.5 vs. 93.5 ± 1.5 , respectively, $P < 0.05$).

The direct effect of Shh protein on nuclear maturation was evaluated by using denuded oocytes (Fig. 4), which were randomly allocated to all the treatment groups in the same batch with 4 replicates. With the presence of 0.5 $\mu\text{g/mL}$ Shh, 72.0% of denuded oocytes (n=144) progressed to the MII stage, which was significantly higher ($P < 0.05$) than those matured in the medium without Shh (60.5%, n=121). Therefore, supplementation of Shh (0.25 or 0.5 $\mu\text{g} \cdot \text{mL}^{-1}$) significantly enhanced oocyte maturation than that of the control group (92.4%, n = 67 and 95.0%, n = 62 vs. 86.2%, n = 64, respectively, $P < 0.05$; Table 2).

For subsequent embryo development, an improved blastocyst rate was obtained when embryos were derived from the oocytes matured in the presence of 0.5 $\mu\text{g/mL}$ Shh compared to the control group (66.3 ± 10.9 , n = 135 vs. 41.4 ± 12.9 ,

n = 137; Table 3).

After embryo transfer, although their pregnancy rates were similar, the parturition and embryo survival rates (number of kids born/number of transferred embryos) of vitrified embryos derived from the 0.5 µg/mL Shh supplemented group were 56.3 and 31.3%, respectively, higher ($P < 0.05$) than those without Shh supplementation (37.5 and 14.8%; Table 4).

4. Discussion

In mouse gonads, Hh signaling has been shown to play an active role in Sertoli cells and granulosa cells during steroidogenesis and oogenesis [10,22]. Nguyen *et al.* [13] have also reported that Hh signaling is active in oocytes, cumulus cells and granulosa cells during oogenesis in porcine species. To our best knowledge, there is no report on the role of Hh in caprine embryos, particularly for their *in vivo* development. In the present study, we clearly confirmed the gene expressions of Hh signaling molecules, *Ptc*, *Smo* and *Gli1*, in the ovarian, oviductal and oocyte-associated cells by RT-PCR. Of these molecules, the expression of *Gli1* was most evident, which presence is regarded as an indicator for the full activation of Hh signaling [23]. As in porcine species, *Gli1* expressed in caprine COCs, cumulus cells and granulosa cells indicated that Shh is likely involved in paracrine and/or

326 autocrine regulation during oocyte maturation in caprine.

327 It has been generally accepted that the quality of oocytes is one of the key
 328 determinants for the subsequent development of embryos [3] ; the blastocyst rate
 329 and the total cell number per blastocyst also very much depend on the quality of the
 330 developing embryos [24]. Clearly, *in vitro* culture systems could not fulfill all the
 331 required conditions to maximize embryonic development compared to that in the
 332 female reproductive tracts [25]. It is known that MAPKs are a family of
 333 serine/threonine protein kinases that are activated through
 334 phosphorylation-dependent events [26]. Two isoforms of the canonical MAPKs,
 335 namely ERK1 and ERK2, are expressed in mammalian oocytes [27]. Several
 336 studies have reported that ERK1/2 or their upstream MEK1/2 are associated with
 337 the function of meiotic spindles [28]. It has also been reported that Shh can activate
 338 ERKs in somatic cells [29] and that ERKs participate in regulation of cyclin B1
 339 expression [30]. Given the finding that Shh increases ERK1/2 phosphorylation
 340 levels in adult muscle cells [29], Shh also enhances the nuclear and cytoplasmic
 341 maturation of oocytes and thus promotes subsequent embryonic development.

342 Our results clearly showed that the maturation rate determined by polar body
 343 extrusion was enhanced in the Shh-supplemented medium. By contrast, in the
 344 presence of cyclopamine, effects of Shh on nuclear maturation were compromised

to the degree close to the control group, suggesting Shh signaling could be a superimposed factor to further enhance caprine oocyte maturation and subsequent embryo development. In the mouse, however, oocyte maturation was not affected by Shh or cyclopamine addition to the culture medium [22], different from the results in goats and pigs. In developing mouse ovaries, oocytes expressed Shh receptor, and *Ptch1* genes, but lacked signal transducer *Smo* [10]. These studies suggested that mouse oocytes at the diplotene stage of the meiotic prophase did not respond to granulosa-derived Ihh and Dhh. It is possible that at least another distinct pathway exists and involves oocyte maturation and MPF and MAPK signalings [29,31]. In contrast, our results indicated that several members of Hh signaling pathway were all expressed in oocytes and cumulus cells. This diverse phenomenon in response to Shh stimulation during oocyte maturation appears to be species-dependent.

To further clarify whether the beneficial effect of Shh protein on oocyte maturation is through a direct action on oocytes or indirectly through cumulus cells, cumulus-free oocytes were matured in the presence of Shh. We found that a relatively higher percentage of denuded oocytes reached the MII stage in the presence of Shh. These results suggested that *in vitro* maturation of a proportion of caprine oocytes could be enhanced directly by Shh treatment.

By using IVF embryos, we demonstrated that treating oocytes with 0.5 $\mu\text{g/mL}$ Shh increased blastocyst rates and total cell counts per blastocyst. Given the findings that Shh increases ERK1/2 phosphorylation, it may therefore enhance the cytoplasmic and nuclear maturation of oocytes which also improve embryo development. However, when the concentration of Shh was increased to 1.0 $\mu\text{g/mL}$, the blastocyst rate of embryos declined to a similar level as in the control group, suggesting a potentially de-sensitization effect may exist at higher doses of Shh.

In generation of IVP embryos, the culture system has been among those most critical factors to determine the quantity and quality of the resultant blastocyst, and, in turn, their ability to survive after cryopreservation and embryo transfer [32,33]. It has been reported that IVP embryos are more sensitive to freezing processes than *in vivo* embryos [18]. In the present study, we showed that IVF embryos derived from IVM oocytes treated with 0.5 $\mu\text{g/mL}$ Shh greatly increased their blastocyst rates and total cell counts per blastocyst (Table 3). The increase in the cryo-survival rates of more advanced embryonic stages are found to be correlated with a higher number and smaller cell size of blastomeres [34,35]. Therefore, the beneficial effect reflects upon the kidding rates from Shh-treated embryos seems to be via cell proliferation and cryo-tolerance of the blastocysts (Table 4).

As shown in Table 4, transfer of the vitrified IVP embryos derived from

Shh-treated oocytes resulted in the kidding rate and embryo survival rate (56.3% and 31.3%, respectively) similar to those in the previous studies [36,37] on direct transfer of *in vivo*- or *in vitro*-derived vitrified embryos (52% and 34%; 56% and 33%, respectively). Although the mechanisms behind are not completely understood, in this study, Shh was shown to have a beneficial effect on IVP embryo quality, and consequently improved their survival rates comparable to that of the vitrified IVP and *in vivo*-derived embryos in this study.

Taken together, our results clearly demonstrate that Shh protein promotes maturation of caprine oocytes as evidenced by cytoplasmic parameters, which ultimately contribute to the subsequent development of the *in vitro* fertilized oocytes. This study also evidences that the Hh signaling pathway remains active or can be reactivated in caprine oocytes which might offer another spare alternative to oocyte maturation, and in turn, to enhance the *in vivo* development of the frozen-thawed caprine embryos. Although the underlying mechanisms for Hh-induced oocyte maturation and subsequent embryo development require further elucidation, we report for the first time that Taiwan native goat semen was used to fertilize the Shh-enhanced caprine oocytes; through embryo transfer with such *in vitro* produced embryos, healthy kids were successfully born.

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- 515

516

517 **Table 1**

518 Primer sequences, annealing temperatures, and expected amplicon sizes for
 519 one-step RT-PCR analysis of Shh signaling molecules in caprine

Target gene	Primer	Sequences (5' to 3')	Gene bank accession no.	Annealing temperature (°C)	Product size (bp)
<i>SMO</i>	Forward	GCCACTTCTA	BC_009989	58	301
		TGACTCTCA			
	Reverse	ATCATCTGCT			
		CTTCTTGATC			
<i>Ptch 1</i>	Forward	CAGACCCCC	XM_599250	58	504
		AAGGAAGAA			
	Reverse	ACACCCACC			
		ATCAAAACAA			

<i>Gli 1</i>	Forward	CCAGAGTCC	BC_146090	58	104
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AGAGGTTCA

AG

Reverse	TGAGTAGAC
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AGAGGTTGG

GAG

<i>GAPDH</i>	Forward	AGGCCATCA	AF_017079	54	325
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CCATCTTCCA

G

Reverse	GGCGTTGGA
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CAGTGGTCA

TAA

520

521

Fig. 1. Gene expressions of the Shh signaling molecules in ovarian, oviductal and oocyte-associated cells. A representative ethidium bromide-stained gel of a RT-PCR assay is shown for the gene expression in various cell types. M: 100 bp ladder marker; 1: ovary; 2: ampulla; 3: ampullar-isthmic junction; 4: isthmus; 5: cumulus-oocyte complexes; 6: cumulus cells; 7: granulosa cells; 8: oocytes; 9: negative control.

Fig. 2. Phosphorylation of mitogen-activated protein kinases (MAPKs), namely extracellular signal-regulated kinases (ERK) 1/2, of caprine oocytes matured for 24 h in the medium containing various concentrations of Sonic Hedgehog (Shh) protein (1, without Shh; 2, 0.125 μ g/mL Shh; 3, 0.25 μ g/mL Shh; 4, 0.5 μ g/mL Shh; 5, 1.0 μ g/mL Shh). The immunoblot analysis was performed with anti-phosphorylated MAPK and total MAPK antibodies; β -actin (45 kDa) was served as the internal control. Densitometry analysis of phosphorylated MAPK was normalized and performed using the Scion Image Analysis System. Data are given as fold of control and show the Mean $\pm$ s.e.m. from four replicates. Within categories, values with different superscript letters differ ($P < 0.05$).

Fig. 3. Effect of Sonic Hedgehog (Shh) on the nuclear maturation of caprine oocytes.

Cumulus–oocyte complexes (COCs) were collected from ovarian follicles and then randomly allocated to various treatment groups for IVM (24 h). 1, without Shh; 2, 0.5 µg/mL Shh; 3, 0.5 µg/mL Shh+0.5 µM cyclopamine; 4, 0.5 µg/mL Shh+1 µM cyclopamine. Data are the Mean±s.e.m. of four replicates. Values with different superscript letters differ ($P < 0.05$).

Fig. 4. Direct effect of Sonic Hedgehog protein (Shh) on the IVM of denuded caprine oocytes. Oocytes were collected from the follicles and denuded of cumulus cells before being cultured for 24 h in medium without (control; n=121) or with 0.5 $\mu\text{g/mL}^{-1}$ Shh (n=144). Data are the Mean $\pm$ s.e.m. of four replicates. Values with different superscript letters differ ($P < 0.05$).

Table 2

Nuclear stages of goat oocytes matured in the medium supplemented with various concentrations of Sonic Hedgehog (Shh)

Shh, $\mu\text{g/mL}$	No. of oocytes	MII stage oocytes, %
0	64	86.2 $\pm$ 2.9 ^b
0.125	71	89.9 $\pm$ 4.7 ^{ab}
0.25	67	92.4 $\pm$ 2.5 ^a
0.5	62	95.0 $\pm$ 1.2 ^a
1.0	64	88.3 $\pm$ 0.9 ^b

^{a, b} Values in the same column without the same alphabetic letters differ ($P < 0.05$).

Data are Mean $\pm$ s.e.m. of four replicates.

Table 3

Development of IVF goat embryos derived from oocytes matured in the medium supplemented with various concentrations of Sonic Hedgehog (Shh)

Shh, $\mu\text{g/mL}$	No. of oocytes fertilized	Cleavage rate, %	Blastocyst rate, %	Cell count per blastocyst
0	137	63.4 $\pm$ 11.7	41.4 $\pm$ 12.9 ^b	128.8 $\pm$ 27.5
0.125	145	67.5 $\pm$ 10.2	56.1 $\pm$ 8.5 ^{ab}	140.1 $\pm$ 44.4
0.25	135	68.5 $\pm$ 19.8	56.6 $\pm$ 6.4 ^{ab}	136.6 $\pm$ 47.9
0.5	135	72.5 $\pm$ 11.9	66.3 $\pm$ 10.9 ^a	172.1 $\pm$ 58.5
1.0	135	68.1 $\pm$ 18.9	51.4 $\pm$ 11.4 ^{ab}	157.7 $\pm$ 53.3

^{a, b}Values in the same column without the same alphabetic letters differ ($P < 0.05$).

Data are Mean $\pm$ s.e.m. of six replicates.

594 **Table 4**595 *In vivo* development of vitrified-thawed goat embryos derived from the oocytes

596 matured in the medium supplemented with Sonic Hedgehog (Shh)

Parameters	Control	Shh*
No. of recipients (embryos)	16 (54)	16 (48)
No. of pregnancy (%)	10 (62.5)	13 (81.3)
No. of parturition (%)	6 (37.5)	9 (56.3)
No. of kids born (%)	8 (14.8)	15 (31.3)
No. of embryos losses (%)	46 (85.2)	33 (68.7)

597 *Shh supplementation: 0.5 µg/mL.

Highlights

- Effects of Shh on oocyte maturation and embryo development of Taiwan Native Goats were examined.
- Both in vitro and in vivo development are improved by an initial addition of Shh in maturation medium.
- Functional Shh signaling during oocyte maturation and early embryogenesis exists in caprine.