Sonic hedgehog supplementation of oocyte and embryo culture media enhances development of IVF porcine embryos

Ngoc Tan Nguyen1,2, Neng-Wen Lo3, Sing-Ping Chuang1, Ya-Lan Jian1 and Jyh-Cherng Ju1

1Department of Animal Science, National Chung Hsing University, 250 Kuokuang Road, Taichung 402, Taiwan, ROC, 2Agricultural Biotechnology Center, Institute of Agricultural Sciences for Southern Vietnam, 121 Nguyen Binh Khiem Street, Ho Chi Minh City, Vietnam and 3Department of Animal Science and Biotechnology, Tunghai University, 181 Section 3, Taichung Harbor Road, Taichung 407, Taiwan, ROC

Correspondence should be addressed to J-C Ju; Email: jcju@dragon.nchu.edu.tw

Abstract

We investigated the expression of sonic hedgehog (SHH) receptor PTCH1 and its co-receptor smoothened (SMO) in fertilized porcine embryos. Effects of exogenous SHH on embryonic development and expressions of survival- and pluripotency-related genes were also determined. We found that PTCH1 and SMO are expressed from two-cell to blastocyst embryos. When oocytes or fertilized embryos were respectively cultured in the maturation or embryo culture medium supplemented with SHH (0.5 μg/ml), their blastocyst rates and total cell numbers increased (P<0.05) compared with the untreated control. When cultured simultaneously in the in vitro maturation (IVM) and in vitro culture (IVC) media supplemented with SHH, the oocytes gained increased blastocyst rates and total cell numbers in an additive manner, with reduced apoptotic indices (P<0.05). Interestingly, SHH treatment did not affect the expression of the BCL2L1 (BCL-XL) gene, yet reduced BAX expression. Blastocysts cultured with various SHH regimes had similar pluripotency-related gene (POU5F1 (OCT-4) and CDX2) expression levels, but blastocysts derived from SHH treatment during IVM had higher ZPF42 (REX01) expression (P<0.05). The highest ZPF42 expression was observed in the blastocysts derived from SHH-supplemented IVC and from dual IVM and IVC treatments. The levels of acetylated histone 3 (AcH3K9/K14) increased in the two-cell and the four-cell embryos when IVM and/or IVC media were supplemented with SHH (P<0.05). Our findings indicate that SHH conferred a beneficial effect on preimplantation development of porcine embryos, particularly when both IVM and IVC media were supplemented with SHH, and the effects may be further carried over from IVM to the subsequent embryonic development.

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Introduction

The porcine animal model has been broadly applied in many aspects of biochemical research (Prather et al. 2003), and porcine embryos hold great promise for embryonic stem cell technologies (Beebe et al. 2009). Despite efforts that have been made to optimize the in vitro production (IVP) system of embryos, the yield and the quality of IVP embryos are still low when compared with their in vivo-produced counterparts (Abeydeera et al. 1998, Kim et al. 2006, Ock et al. 2007). It is well known that oocyte and embryonic quality has been closely associated with the expression of a series of genes during early embryogenesis (Lonergan et al. 2006, Zheng & Dean 2007), exerting a significant influence on the pre- and the post-implantation embryonic development to term (Krisher 2004, Sirard et al. 2006, Mtango et al. 2008).

A great majority of improvements in oocyte exploitation rely heavily on the accumulated knowledge concerning the in vitro culture (IVC) system of the oocytes and embryos up to the blastocyst stage (Van Langendonckt et al. 1997, Lonergan et al. 2003, Rizos et al. 2003). By altering the culture conditions of oocyte maturation and embryo development, patterns of gene expression could be manipulated and reprogrammed to closely mimic in vivo conditions, and, as a result, enhance embryo quality. In spite of the common use of defined media for in vitro maturation (IVM) of oocytes and IVC of embryos in many species, porcine oocyte- and/or blastocyst-promoting agents to improve the yield and quality of IVP embryos are still under extensive investigation (Thompson 2000). However, little consensus has been reached regarding the mechanisms of action of growth factors, cytokines, vitamins, or amino acids added to culture media (Craig et al. 2004, Kim et al. 2006, Gupta et al. 2007, Uhm et al. 2007).

Hedgehog (Hh) signaling molecules exist in the rodent (Wijgerde et al. 2005, Russell et al. 2007, Ren et al. 2009) and bovine (Spicer et al. 2009) reproductive
systems and are proposed to function as paracrine factors to promote cell proliferation and steroid hormone production. On the other hand, Hh signaling, particularly of Indian hedgehog (IHH), has been shown to promote the peri-implantation development of embryos in mice (Matsumoto et al. 2002, Takamato et al. 2002, Lee et al. 2006). Recent studies have also reported that ablation of IHH in murine uterus (Franco et al. 2010a) or consecutive activation of smoothened (SMO; Franco et al. 2010b) led to infertility in female mice. Our previous studies in porcine demonstrated that Hh signaling molecules, including patched1 (PTCH1), SMO, and GLI1, were present in the ovaries, oocytes, and parthenogenetic embryos at different stages. Inclusion of exogenous sonic hedgehog (SHH) in the IVM or IVC medium enhanced oocyte maturation and development of parthenogenetic embryos (Nguyen et al. 2009, 2010). Moreover, embryo culture medium containing SHH also promoted in vitro development of handmade cloned embryos (Nguyen et al. 2010). In this study, we investigated the combined effects of SHH treatment in both IVM and IVC media on the development of IVF porcine embryos.

Results

Experiment 1: expression of Hh signaling molecules at various developmental stages of porcine embryos

The expression of Hh receptor PTCH1 and co-receptor SMO at the protein level was detected by immunocytochemical staining. PTCH1 (left column) and SMO (middle column) were detected in the IVF-derived embryos from the two-cell stage to the blastocyst stage as shown in Fig. 1 (the positive staining is shown in green). The negative control was derived from embryos stained by secondary antibody, while the primary antibody was omitted. No detectable signal was observed for these embryos (right column).

Experiment 2: the collective effects of SHH on embryo development during IVM and IVC

We have previously reported that SHH enhances nuclear and cytoplasmic maturation of porcine oocytes. To investigate whether there is a combined or accumulative effect of SHH on oocyte maturation and embryo development, we tested the correlations of IVM and IVC media on the development of IVF porcine embryos.

![Figure 1 Detection of the Hh receptor in porcine embryos of different developmental stages by immunocytochemistry. After fixation in 4% paraformaldehyde, receptor PTCH1 and co-receptor SMO of individual embryos were detected using anti-PTCH1 (left column) and anti-SMO antibodies (middle column). Negative control embryos were probed directly with the secondary antibody instead of the primary antibody (right column). 2C, two-cell; 4C, four-cell; 8C, eight-cell; M, morula; BL, blastocyst. Green fluorescence indicates positive staining. Scale bar, 50 μm.](www.reproduction-online.org)
Table 1 Effects of sonic hedgehog supplementation in maturation and culture media on the development of porcine embryos in vitro.

<table>
<thead>
<tr>
<th>SHH (0.5 µg/ml)</th>
<th>IVM</th>
<th>IVC</th>
<th>Total (N)</th>
<th>Cleavage rate, % (n)</th>
<th>Blastocyst rate, % (n)</th>
<th>Cell number per blastocyst (%)</th>
<th>Apoptotic index, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>– –</td>
<td>226</td>
<td>79.3 ± 3.4a (179)</td>
<td>25.0 ± 1.4a (56)</td>
<td>46.4 ± 3.3a</td>
<td>4.5 ± 0.4a (19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ –</td>
<td>214</td>
<td>84.3 ± 3.1a (181)</td>
<td>37.3 ± 1.2b (79)</td>
<td>57.4 ± 3.7b</td>
<td>3.6 ± 0.3bc (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>– +</td>
<td>220</td>
<td>81.2 ± 2.1a (179)</td>
<td>33.8 ± 2.1b (73)</td>
<td>59.1 ± 2.2ab</td>
<td>3.2 ± 0.3ab (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>214</td>
<td>85.5 ± 1.8a (182)</td>
<td>42.8 ± 1.7a (90)</td>
<td>69.7 ± 3.2c</td>
<td>2.4 ± 0.2c (27)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the number of embryos in each category. Data are mean±S.E.M. from six replicates. aWithin a column, means without the same superscript letters differed (P<0.05). IVM, in vitro maturation; IVC, in vitro culture.

IVC media in the presence or absence of SHH with the end point development of porcine embryos in vitro. As shown in Table 1, consistent with what we had found previously, presence of SHH in IVM or IVC medium alone enhanced blastocyst formation (37.3 and 33.8% respectively) compared with the control group (25%, P<0.05). The improvement of blastocyst formation reached the highest level when SHH was included in both IVM and IVC media (IVM+/IVC+) compared with the control groups (25%, 58.2%). IVM alone enhanced blastocyst formation (37.3 and 33.8%) compared with the control group (25%, 58.2%). Similarly, a significant improvement of normal fertilization rate was also found when SHH was added in IVM medium alone (72.6%) or in both media (74.2%; control group, 58.2%). Among the SHH-treated groups, the AcH3K9/K14 levels in the dual SHH-treated embryos (IVM+/IVC+) were significantly higher (P<0.05) at the two-cell stage in the presence of SHH, either in IVM (130.5±3.7) or in IVC (134.6±3.2) medium alone, and in both media (161.1±4.7) compared with the control group (108.2±4.2). Among the SHH-treated groups, the AcH3K9/K14 levels in the dual SHH-treated embryos (IVM+/IVC+) were significantly higher than those in the embryos with single SHH treatment (IVM+/IVC− or IVM−/IVC+). At the four-cell stage, embryos derived from IVM−/IVC+ (214.1±5.8) and IVM+/IVC+ treatments (223.3±8.0) had a significantly higher level of acetylation pattern than those of the control (178.9±5.3) or from IVM+/IVC− treatment (182.9±5.0). However, embryos developing up to the blastocyst stage had only a similar level of acetylation between the control and the treatment groups.

Table 2 Effects of sonic hedgehog supplementation to in vitro maturation and culture media on IVF rates.

<table>
<thead>
<tr>
<th>SHH (0.5 µg/ml)</th>
<th>IVM</th>
<th>IVC</th>
<th>Number of oocytes examined</th>
<th>Penetration rate, % (n)</th>
<th>Monospermic rate, % (n)</th>
<th>Normal fertilization rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>– –</td>
<td>96</td>
<td>86.4 ± 0.5 (73)</td>
<td>67.4 ± 2.7a (57)</td>
<td>58.2 ± 2.5a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ –</td>
<td>92</td>
<td>85.5 ± 2.2 (80)</td>
<td>85.0 ± 2.1b (68)</td>
<td>72.6 ± 2.3b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>– +</td>
<td>88</td>
<td>87.5 ± 1.7 (77)</td>
<td>68.7 ± 5.5a (54)</td>
<td>60.0 ± 4.2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>95</td>
<td>86.5 ± 1.7 (83)</td>
<td>83.7 ± 1.4b (71)</td>
<td>74.2 ± 1.2b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the actual number of oocytes examined. Data are mean±S.E.M. from four replicates. a,bWithin a column, means without the same superscript letters differed (P<0.05). IVM, in vitro maturation; IVC, in vitro culture.

Monospermic rate (%): number of monospermic oocytes/total penetrated oocytes×100. bNormal fertilization rate (%): number of monospermic oocytes/total inseminated oocytes×100.
Experiment 4: effects of SHH supplementation during IVM and/or IVC on gene expressions of porcine embryos

Expressions of cell survival-related genes (BCL2L1 and BAX) and pluripotency-related genes (POU5F1, CDX2 and ZPF42 (REX01)) were analyzed using quantitative real-time PCR at the blastocyst stage on day 7. As shown in Fig. 5, no significant difference in BCL2L1 gene expression was found among treatment groups. However, BAX gene expression was significantly reduced in the blastocysts derived from the IVM_C/IVC_C (0.51-fold) or IVM_K/IVC_C (0.58-fold) group compared with those derived from the IVM_C/IVC_K (0.83-fold) or the control groups. There was also a detectable difference in the BAX expression between the IVM+/IVC− group and the control group. Taken together, differential BAX expression levels in the blastocyst embryos between the SHH-treated groups and the control group were obvious.

In the case of the pluripotency-related genes, the levels of CDX2 and POU5F1 expressions were similar (P>0.05) between the control and the treatment groups (Fig. 6A and B). However, the levels of ZPF42 expression were significantly higher in the IVM+/IVC− (1.13-fold), IVM−/IVC+ (1.61-fold), or IVM+/IVC+ (1.58-fold) treatment groups compared with the untreated control group (P<0.05). However, no difference was found between the SHH-treated embryos in the IVM−/IVC− group and the IVM+/IVC+ group (Fig. 6C, P>0.05).
Similarly, with the presence of SHH in both IVM and IVC media, expression levels of BAX and ZPF42 were significantly enhanced. In contrast, the beneficial effect of SHH on BAX and ZPF42 expression levels was neutralized by the presence of cyclopamine in the medium (Fig. 7).

**Discussion**

Several lines of evidence indicated the involvement of Hh signaling during oogenesis. It was reported that Hh signaling molecules were present in mouse (Wijgerde et al. 2005, Russell et al. 2007, Ren et al. 2009) and bovine ovaries (Spicer et al. 2009), particularly in the theca and granulosa cells. Ren et al. (2009) reported that a major activation of co-receptor SMO resulted in an alteration of theca cell proliferation and prevention of ovulation in mice, although follicles developed normally to preovulatory stage in response to LH stimulation. In addition, we previously reported that SHH and its related signaling molecules (PTCH1, SMO, and GLI1) were expressed in porcine ovaries, specifically in the cumulus–oocyte complexes (COCs) and oocytes (Nguyen et al. 2009). In contrast, Wijgerde et al. (2005) reported that no SMO was detected in mouse oocytes. Interestingly, we detected SMO expression in denuded porcine oocytes by western blot (Nguyen et al. 2009), suggesting that SMO expression could be species-specific. Furthermore, we have recently reported that Hh signaling receptor PTCH1 and SMO are also expressed in porcine parthenotes from the two-cell stage throughout the blastocyst stage at both mRNA and protein levels (Nguyen et al. 2010). In this study, we also found the expressions of PTCH1 and SMO from two-cell to blastocyst stages of fertilized porcine embryos detected by immunocytochemistry.

Since the embryonic genome activation (EGA) occurs at the four-cell stage of porcine embryos (Jarrell et al. 1991), the presence of PTCH1 and SMO at the two-cell stage is most likely a maternal legacy. Our previous study documented the existence of SHH protein in porcine follicular fluids from follicles of various sizes (Nguyen et al. 2009). Since Hh signaling was detected in the oocytes and embryos of different developmental stages, we hypothesize that Hh signaling is involved in folliculogenesis as well as in early embryogenesis. In fact, our previous study had demonstrated that addition of SHH to the IVM medium promoted nuclear and cytoplasmic maturation of porcine oocytes, and subsequently enhanced parthenogenetic embryo development (Nguyen et al. 2009). Consistently, we also found that SHH addition during IVM increased
the blastocyst rate and total cell number per blastocyst in this study.

The potential role of SHH during oogenesis is evident in several species including pigs. During in vivo maturation, oocytes are exposed to SHH in the follicular fluid, because SHH was present in porcine follicular fluid. This was supported by our previous studies with solely parthenogenetic embryos (Nguyen et al. 2009, 2010). Interestingly, the yield and quality of IVF-derived embryos were also further enhanced by the inclusion of SHH in the IVC medium in addition to the IVM medium, suggesting that an additive or accumulative effect has been exerted by SHH during these courses of development in porcine embryos.

In vitro-produced porcine embryos were particularly vulnerable to free radicals or subsequent oxidative stresses, due to their high lipid contents (McEvoy et al. 2000). This phenomenon may have led to poor embryo viability and, in turn, to progressive embryo losses during IVP, as evidenced by high incidence of apoptotic rates in response to suboptimal culture conditions (Nanassy et al. 2008). In this study, we demonstrated that the presence of SHH in culture medium effectively reduced apoptosis, although the underlying molecular mechanisms remained unclear. Presumably, SHH might act as a survival factor, perhaps by altering the expression of some cell survival-related proteins such as AKT (Nguyen et al. 2010). Other reports indicated that PTCH1, a negative regulator of SHH signaling, may trigger an apoptotic pathway in the absence of SHH (Thibert et al. 2003, Mille et al. 2009). Similar to the findings in parthenogenetic embryos, SHH also reduced apoptotic indices, improved blastocyst rates, and increased cell numbers of fertilized embryos in this study. The presence of SHH in IVM and/or IVC media has no influences on the expression of the anti-apoptotic gene BCL2L1, but the expression of pro-apoptotic gene BAX was reduced with the presence of SHH either in IVC medium alone or in both IVM and IVC media. Collectively, our data supported the notion that SHH modulated or prevented apoptosis of blastomeres, which, in turn, improved the blastocyst rate and total cell numbers of porcine embryos.

It is still unknown whether a certain degree of polyspermy is physiologically permissive in porcine,

Figure 5 Expression of cell survival-related genes BCL2L1 and BAX in porcine blastocysts cultured in SHH-supplemented IVM and/or IVC media. a–cWithin category, bars with different letters differ (P<0.05). –/–, absence of SHH in IVM and IVC media (control); +/-, presence of 0.5 μg/ml SHH in IVM medium but absence of SHH in IVC medium; –/+ , absence of SHH in IVM medium, but presence of 0.5 μg/ml SHH in IVC medium; +/+ , presence of 0.5 μg/ml SHH in both IVM and IVC media.

Figure 6 Expression of pluripotency-related genes POU5F1 (A), CDX2 (B), and ZPF42 (C) in porcine blastocysts cultured under various regimes of SHH supplementation. a–cWithin each gene, bars with different letters differ (P<0.05). –/–, absence of SHH in IVM and IVC media (control); +/-, presence of 0.5 μg/ml SHH in IVM medium but absence of SHH in IVC medium; –/+ , absence of SHH in IVM medium, but presence of 0.5 μg/ml SHH in IVC medium; +/+ , presence of 0.5 μg/ml SHH in both IVM and IVC media.
and whether porcine oocytes have any capacity to remove the excessive sperm in the ooplasm (Xia et al. 2001). Regardless of the IVC conditions, we found that the presence of SHH during oocyte maturation enhanced the monospermic rate, i.e., normal fertilization rate. It is conceivable that the improved oocyte quality or ooplasmic maturation would potentially reduce polyspermy in the medium containing SHH. Our previous study revealed that oocytes matured in medium containing SHH had higher intracellular calcium release induced by IP_3 and, in particular, a prolonged duration of calcium transient (Nguyen et al. 2009). These physiological cues may be of importance for preventing the polyspermic fertilization in porcine oocytes.

Histone acetylation/deacetylation is a dynamic process during embryogenesis and differentiation. It is closely associated with the chromatin structures and, hence, with transcriptional regulation (Kim et al. 2003). We examined whether SHH altered the pattern of histone acetylation in various stages of embryos, particularly by the time of EGA of developing embryos. Intriguingly, the results revealed that the levels of acetylated H3K9/K14 were higher in SHH-treated embryos in IVM⁻/IVA⁻ and IVM⁺/IVA⁺ groups at two- and four-cell stages compared with the control group. The elevated levels of acetylation of H3k9/K14 in the SHH-treated embryos during IVM might carry an accumulative effect of SHH from IVM via an improved oocyte over to a development-enhanced embryo. It is also known that oocyte maturation helps to carry out a proper epigenetic remodeling of parental chromatin, as reflected by pronuclear methylation and acetylation (Gioia et al. 2005). Ma & Schultz (2008) also noted that oocyte maturation was accompanied by changes in histone modification, and the maturation-associated reduction in acetylation was due to the histone deacetylase activation and a lack of histone acetyltransferase activity in the oocytes (Kim et al. 2003). Cervera et al. (2009) reported that high levels of histone acetylation at the four-cell stage of porcine embryos helped to render sufficient chromatin accessibility for initiation of gene transcriptions during subsequent stages of development. In this study, it appeared that acetylation levels at the four-cell stage were correlated with the reduced expression of pro-apoptotic BAX gene and the enhanced expression of ZPF-42 in the blastocyst stage. In addition, we found that expression of POU5F1 and CDX2 transcripts was not changed at the blastocyst stage regardless of the presence of SHH. In contrast to POU5F1 and CDX2, ZPF-42, a developmentally regulated gene containing zinc finger motifs (Hosler et al. 1989), was expressed at a higher level in the SHH-treated embryos, although the exact function and mechanisms are not clear. This might have altered the ratio of inner cell mass to trophectoderm cells at the blastocyst stage, given that enhanced histone acetylation led to reallocation of the ICM and TE cells in bovine blastocysts (Cervera et al. 2009, Ikeda et al. 2009). It is likely that the pattern of histone acetylation at the four-cell stage is associated with those changes found in this study (Figs 3 and 4). This inference is further supported by Ikeda et al. (2009), whose report showed that epigenetic modifications to the genome during fertilization exerted a carryover effect on cell proliferation and differentiation of preimplantation embryos. Furthermore, the level of POU5F1 protein expression would be critical to ZPF-42 expression due to a POU/octamer site found in its promoter region (Cervera et al. 2009). However, it is unclear why the ZPF-42 level was increased without a change in the POU5F1 transcript level as observed in this study. More complicated mechanisms may be involved in modulating ZPF-42 expression of porcine embryos cultured in the medium supplemented with SHH. Previous studies have shown that SHH promotes the nuclear and cytoplasmic maturation and the embryonic development through the trio PTCH1–SMO–GLI1 action (Nguyen et al. 2009, 2010). Similarly, in this study, we found that the beneficial effects of SHH on BAX and ZPF-42 gene expression were prevented by cyclopamine inhibition. However, the correlations of the histone acetylation at the four-cell stage embryo with the expressions of the survival- and pluripotency-related genes (BAX and ZPF-42) have not been documented.

Taken together, these findings suggested that SHH played a positive role in the development of porcine pre-implantation embryos. Ooplasmic maturation and epigenetic modifications such as histone 3 acetylation

**Figure 7** Effects of SHH on the expression of BAX and ZPF-42 genes in porcine blastocysts in the presence or absence of the inhibitor cyclopamine. a, b: Within each gene, bars with different letters differ (P<0.05). –/–, absence of SHH in IVM and IVC media (control); +/+, presence of 0.5 µg/ml SHH in both IVM and IVC media; +/- plus cyclopamine, presence of 0.5 µg/ml SHH in both IVM and IVC media plus 1 µM/ml cyclopamine.
of the genome pre- and post-fertilization could be persistent and carried over to the subsequent embryonic development, in particular, when both IVM and IVC media were supplemented with SHH to direct cell survival, proliferation, and differentiation during the preimplantation development of porcine embryos.

Materials and Methods

Chemicals

All chemicals used in this study were purchased from Sigma Chemicals, unless otherwise specified.

Oocyte maturation and embryo culture

Procedures for collection of ovaries and oocytes were described previously (Nguyen et al. 2009, 2010). In brief, freshly harvested COCs were rinsed several times and cultured for 22 h in the NCSU-23 medium supplemented with 10% porcine follicular fluid, cysteine (0.1 mg/ml), eCG (10 IU/ml), and hCG (10 IU/ml). The COCs were further cultured for an additional 22 h in the same medium without hormones.

Sperm preparation, IVF, and embryo culture

Diluted semen from two boars of proven fertility was supplied by a porcine artificial insemination center (Taichung, Taiwan), stored at 15 °C, and used for 2 days. Motile sperm were obtained by centrifugation at 700 g with Percoll (P1644) discontinuous gradient (2 ml of 45% over 2 ml of 90%) for 15 min. Spermatozoa were collected from the bottom of the 90% fraction and washed in Dulbecco’s PBS (DPBS) supplemented with 10% fetal bovine serum, followed by centrifugation at 100 g for 5 min. After centrifugation, the sperm pellet was resuspended in modified tris-buffered medium (mTBM) containing 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2, 2H2O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, and 0.2% (w/v) BSA. After 44 h of maturation, oocytes were freed from cumulus cells, and matured oocytes with first polar body were washed three times in mTBM that had been pre-equilibrated for at least 12 h at 39 °C under 5% CO2. After washing, groups of 30 oocytes were randomly placed into 45 μl droplets of mTBM covered with pre-warmed mineral oil. After dilution (10×), 5 μl of sperm suspension were added to a 45 μl droplet of fertilization medium (mTBM) to give a final sperm concentration of 5×105 sperm/ml. The gametes were co-cultured for 6 h at 39 °C in an incubator containing 5% CO2 in humidified air. After 6 h, oocytes were removed from the fertilization medium and washed three times in porcine zygote medium-3 (PZM-3) to remove excessive sperm. The presumptive zygotes were then cultured in 100 μl droplets (20–30 oocytes per droplet) of PZM-3 covered with mineral oil for 7 days. Cleavage and blastocyst rates were evaluated under a stereomicroscope at days 2 and 7 after insemination (insemination day = day 1).

An additional experiment was designed to evaluate the fertilization rate. At 10 h post-insemination, some oocytes were selected and fixed in the fixative. The fixed oocytes were subjected to triple staining and observed under a fluorescence microscope for nuclear evaluation, and were classified into three categories, i.e. unfertilized oocytes, monospermic oocytes (two pronuclei, 2PN), and polyspermic oocytes (more than 2PN or 2PN plus swollen sperm heads). The representative images are presented in Fig. 2.

Detection of histone acetylation and quantification

The effect of SHH on the acetylation of histone 3 at lysine 9 and 14 (AcH3K9/K14) during pre-implantation development was studied. Embryos at different developmental stages were fixed with 4% paraformaldehyde (PFA; EMS, Hatfield, Pennsylvania) in DPBS containing 0.1% polyvinyl alcohol (PVA) for 1 h at RT. They were then washed in washing solution (DPBS containing 0.1% (w/v) PVA, 1% (w/v) BSA, and 0.1% (v/v) Tween-20) for 1 h, and were permeated with 0.5% (v/v) Triton X-100 in DPBS for 30 min at room temperature and blocked in working solution (DPBS-PVA supplemented with 2% BSA) at 4 °C overnight. The embryos were then incubated with anti-acetyl histone 3 (1:300, Cat #9677, Cell Signaling Technology, Inc., Beverly, MA, USA) for 1 h at 37 °C, washed in washing solution for 30 min, and then incubated with Alexa Flour 488-conjugated secondary antibody (1:200, Invitrogen) for 1 h at room temperature (darkness), followed by two washes in the washing solution. The samples were mounted on slides with mounting medium containing Hoechst. Immunofluorescent labeling of AcH3K9/K14 was evaluated under an epifluorescent microscope (Nikon, Tokyo, Japan), and micrograph images were captured. Fluorescence intensity was measured by analysing the micrographs with ImageJ Software (Image Processing and Analysis in Java, Version 1.42 for Windows, NIH, Bethesda, MD, USA) after background subtraction. Data are presented as a mean of relative fluorescence intensity (pixels).

Immunocytochemical staining

Patched (PTCH1) and SMO proteins

To detect PTCH1 and SMO, embryos at two-cell, four- to eight-cell, morula, and blastocyst stages were fixed in 4% (w/v) PFA in DPBS supplemented with 0.1% (w/v) PVA for 1 h at room temperature. After washing twice in washing solution, embryos were permeated with 0.5% (v/v) Triton X-100 in DPBS for 30 min. The embryos were then blocked in working solution supplemented with 5% (v/v) chicken serum at 4 °C overnight. After being washed twice in washing solution, the embryos were incubated with primary antibody for PTCH1 (1:130; sc-13943; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or SMO (1:330; sc-9016; Santa Cruz) at 4 °C overnight. They were then washed for 30 min and incubated with Alexa Fluor 488-conjugated secondary antibody (1:200, Invitrogen) for 1 h (darkness) and then washed twice with washing solution. Samples were mounted on slides with mounting medium containing Hoechst, and images were captured under an epifluorescence microscope (Nikon).

Nuclear and cytoskeleton: triple staining

Triple staining procedure was performed as described by Ju et al. (2003). In brief, microtubule stabilization buffer (MTS,
RNA purification, reverse transcription and quantitative real-time PCR

Total RNA was extracted by TRIzol Reagent (Invitrogen) and purified by RNeasy Mini Kit (Qiagen). Purified RNA was quantified using a ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA) and qualitatively analyzed by Bioanalyzer 2100 (Agilent Technology, USA). To prepare a cDNA pool from each RNA sample, total RNA (19 ng) was reverse transcribed using MMLV reverse transcriptase (Promega). The resulting samples were diluted 2 × 10^3 times with DNase-free water. Each cDNA pool was stored at −20°C until further real-time PCR analysis.

Real-time PCR were performed on the Roche LightCycler Instrument 1.5 using LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Cat. 03 515 885 001). Briefly, the 10 μL reaction mixture contained 0.5 M PIPES, 25 mM magnesium chloride, 125 mM EGTA, 0.01% (w/v) aprotinin, 1 mM dithiothreitol, 50% deuterium oxide, 1 mM paclitaxel, 0.1% (v/v) Triton X-100, and 2% (v/v) formaldehyde. Mounting medium contained 10 μg/ml Hoechst 33342, 50% glycerol, and 25 mg/ml sodium azide in DPBS. Oocytes were incubated in water bath (38°C) for 1 h, and stored at 4°C for at least 1 week before staining. For staining, the fixed oocytes were incubated with anti-α- and anti-β-tubulin primary antibodies at 4°C overnight. The oocytes were then washed three times with washing solution containing 2% (w/v) BSA, 2% goat serum, 0.2% (w/v) milk powder, 0.2% (w/v) sodium azide, and 0.1% (v/v) Triton X-100 in DPBS. The oocytes were subsequently incubated with the FITC-conjugated secondary antibody at 4°C overnight. After being washed three times, microfilaments of the oocytes were stained with rhodamine-phalloidin (R147; Molecular Probes, Invitrogen) for 1 h for labeling the filamentous actin and then washed three times again. Finally, the oocytes were mounted on the slide with mounting medium containing Hoechst 33342 and then sealed with fingerprint polish, and examined under an epifluorescence microscope (Nikon) with magnification at 40×.

**Table 3** Primer sequences, GenBank accession numbers, and expected product sizes for the qRT-PCR analysis.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primers</th>
<th>Sequences (5' to 3')</th>
<th>GenBank accession number</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>BCL2L1</td>
<td>Forward</td>
<td>GCAGGTATTTGAACGAATCTTCGCC</td>
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<tr>
<td>BAX</td>
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<tr>
<td>CDX2</td>
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<td>CTCTCCACCAACATATCTAC</td>
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<tr>
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<tr>
<td>POU5F1</td>
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<tr>
<td>ACTB</td>
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<td>168</td>
</tr>
</tbody>
</table>

SMH enhances development of IVF porcine embryos

Experiment 1: expression of Hh signaling molecules at different developmental stages of IVF-derived porcine embryos

The expressions of PTCH1 and SMO in IVF-derived two-cell, four- to eight-cell embryos, morulae and blastocysts were investigated by immunocytochemical staining. At least thirty embryos in each stage were used for detection.

Experiment 2: the collective effects of SHH on embryo development accumulated during IVM and IVC

The optimized dose usage of SHH at 0.5 μg/ml was determined from our previous studies (Nguyen et al. 2009, 2010) and used throughout all treatments in this study. Matured oocytes derived from IVM with or without SHH treatment were subjected to IVF. After 6 h co-culture with sperm, the presumptive zygotes were cultured in PZM-3 and randomly allocated into the following culture groups: no SHH in both IVM and IVC, 0.5 μM SHH in IVM and 5 μM SHH in IVC, 5 μM SHH in both IVM and IVC, and 10 μM SHH in both IVM and IVC.
(control, IVM−/IVC−), IVM with SHH and IVC without SHH (IVM+/IVC−), IVM without SHH and IVC with SHH (IVM−/IVC+), and SHH in both IVM and IVC (IVM+/IVC+). The cleavage and blastocyst rates were recorded at days 2 and 7 respectively. The blastocysts were subjected to Hoechst staining and TUNEL labeling for the determination of the total cell number and apoptotic cells respectively (Nguyen et al. 2010). Apoptotic indices were calculated as follows:

Apoptotic index (%) = (number of positive nuclei/total cell number) × 100.

At 10 h post-fertilization, a proportion of presumptive zygotes from each treatment were randomly selected for evaluation of fertilization rate.

Experiment 3: effects of SHH treatment in IVM and IVC media on acetylation profile of IVF embryos

Embryos from each treatment as in Experiment 1 were collected at two-cell, four-cell, and blastocyst stages for histone acetylation assay. At least 25 embryos at each stage were used, and the representative numbers were given as shown in the Fig. 4.

Experiment 4: effects of SHH treatment in IVM and IVC on gene expressions of IVF embryos

Embryos collected and pooled at day 7 from each treatment (ten embryos per treatment) were incubated with cell lysis buffer at 42 °C for 1 h, and then stored at −80 °C. Cell survival-related genes (BCL2L1 and BAX), pluripotency marker gene POUSF1, trophoderm lineage marker CDX2, and inner cell mass lineage marker ZPF42 were analyzed by quantitative real-time PCR.

An additional experiment was designed to confirm the beneficial effect of SHH on BAX and ZPF42 expression. After insemination, matured oocytes under the presence of 0.5 µg/ml SHH were divided into two groups, in which one group was cultured in the IVC medium supplemented with 0.5 µg/ml SHH, and the other cultured in the medium with 0.5 µg/ml SHH plus 1 μM cyclopaamin, an SHH inhibitor. The matured oocytes cultured without SHH in both IVM and IVC media were the control group. The blastocysts harvested at day 7 as mentioned earlier were used to determine the expression levels of BAX and ZPF42 genes.

Statistical analysis

All data were subjected to ANOVA using the general linear model procedure in SAS version 9 (SAS Institute, Cary, NC, USA), followed by Tukey’s test. Percentile data were arcsine transformed before statistical analysis, and the P<0.05 was considered significantly different between treatment groups.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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