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The hedgehog-patched signaling pathway and function in the mammalian ovary: a novel role for hedgehog proteins in stimulating proliferation and steroidogenesis of theca cells

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Abstract

The expression of hedgehog (Hh) genes, their receptor, and the co-receptor in mice, rat, and bovine ovaries were investigated. RT-PCR of ovarian transcripts in mice showed amplification of transcripts for Indian (*Ihh*) and desert (*Dhh*) Hh, patched 1 (*Ptch1*), and smoothened (*Smo*) genes. Semi-quantitative RT-PCR and northern blot analyses showed that whole ovarian *Ihh* and *Dhh* transcripts decreased 4–24 h after hCG versus 0–48 h after pregnant mares serum gonadotrophin treatment in mice, whereas mouse *Ptch1* and *Smo* transcripts were expressed throughout the gonadotropin treatments. Quantitative real-time RT-PCR (qRT-PCR) revealed that the expression of the Hh-patched signaling system with *Ihh* mRNA abundance in granulosa cells was greater, whereas *Smo* and *Ptch1* mRNA abundance was less in theca cells of small versus large follicles of cattle. In cultured rat and bovine theca-interstitial cells, qRT-PCR analyses revealed that the abundance of *Gli1* and *Ptch1* mRNAs were increased ($P<0.05$) with sonic hedgehog (SHH) treatment. Additional studies using cultured bovine theca cells indicated that SHH induces proliferation and androstenedione production. IGF1 decreased *Ihh* mRNA abundance in bovine granulosa cells. The expression and regulation of *Ihh* transcripts in granulosa cells and *Ptch1* mRNA in theca cells suggest a potential paracrine role of this system in bovine follicular development. This study illustrates for the first time Hh activation of Gli1 transcriptional factor in theca cells and its stimulation of theca cell proliferation and androgen biosynthesis.
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Introduction

The hedgehog (Hh) family of proteins was first cloned in *Drosophila* (Hammerschmidt *et al*. 1997), and its signaling pathway is highly conserved during evolution (Ingham & McMahon 2001, Lum & Beachy 2004, Wang *et al*. 2007). The Hh signaling pathway is triggered by the stoichiometric binding of Hh ligand to its receptor, patched 1 (PTCH1; Marigo *et al*. 1996, Stone *et al*. 1996, Fuse *et al*. 1999). In the absence of Hh protein, PTCH1 suppresses the constitutive activity of smoothened (SMO), the seven transmembrane G-protein-coupled co-receptor (Taipale *et al*. 2002). The secreted Hh protein inactivates the actions of PTCH1 expressed in adjacent cells (Ingham & McMahon 2001). Inactivation of PTCH1 following binding with the Hh protein removes the inhibition on the activity of SMO (Taipale *et al*. 2002, Gulino *et al*. 2007, di Marcotullio *et al*. 2007), and allows the activation of *Gli1*, a latent cytoplasmic transcription factor (GLI1, the mammalian homolog to *Drosophila* Ci protein), leading to the induction of target gene expression (Ingham & McMahon 2001, Lum & Beachy 2004). In mammals, three Hh proteins, Indian (IHH), desert (DHH), and sonic (SHH) hedgehog, are capable of binding to PTCH1 leading to signal transduction via derepression of the co-receptor, SMO (Ingham & McMahon 2001, Lum & Beachy 2004, Wang *et al*. 2007). All three Hh proteins bind to PTCH1 with equal affinity (Pathi *et al*. 2001) and have been used interchangeably to invoke biological responses (Vortkamp *et al*. 1996, Krishnan *et al*. 2001, Zhang *et al*. 2001, Deckelbaum *et al*. 2002). Hh proteins are expressed at epithelial–mesenchymal boundaries in several developing organs to activate PTCH1, essential for the growth, differentiation, and morphogenesis of the lung, gut, pancreas, hair follicle, and tooth (Ingham & McMahon 2001, Taipale *et al*. 2002, Lum & Beachy 2004).

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Although originally found to be important during embryonic development, recent studies have demonstrated the importance of the Hh signaling in many tissues during postnatal life. Extensive genetic and molecular evidence indicates that SHH controls the proliferation and differentiation of cells in the central and peripheral nervous systems, skin, limbs, and gut (Ingham & McMahon 2001, Lum & Beachy 2004, Wang *et al.* 2007). In addition, IHH has been implicated in the growth and differentiation of cartilage, yolk sac endoderm development, and hematopoiesis in mice (Belaoussoff *et al.* 1998, St-Jacques *et al.* 1999, Dyer *et al.* 2001). In reproductive processes in mice, DHH is required for the development of the testis (Pierucci-Alves *et al.* 2001, Yao *et al.* 2002), whereas IHH plays a crucial role in implantation during the early stages of pregnancy (Matsumoto *et al.* 2002, Takamoto *et al.* 2002, Lee *et al.* 2006). However, the role of Hh signaling in regulating reproduction of monotocous mammals such as cattle has not been studied.

Recently, the Hh system has been shown to be present in the mouse ovary (Wijgerde *et al.* 2005, Russell *et al.* 2007). Specifically, Hh target genes *Ptch1* (i.e. Hh receptor) and *Gli1* (i.e. an Hh activated transcription factor) are primarily expressed in theca cells, whereas *Ihh* and *Dhh* mRNAs are predominately located in granulosa cells (Wijgerde *et al.* 2005). Immunostaining of PTCH1 was found in both theca and granulosa cells of mice and SHH-stimulated mitosis of granulosa cells *in vitro* (Russell *et al.* 2007), but the potential role of Hh proteins in the ovary, and in particular theca cells, remains unclear. Moreover, the ovarian expression of genes of the Hh signaling pathway has not been evaluated in monotocous species. Therefore, we evaluated: 1) the expression and hormonal regulation of *Ihh* in granulosa cells and its receptor *Ptch1* in theca cells in both rat and bovine ovaries; 2) whether follicle size is associated with changes in *Ptch1* mRNA in theca and *Ihh* mRNA in theca and granulosa cells; 3) whether treatment with SHH stimulated the expression of the *Gli1* transcription factor in cultured theca cells in rats and cattle; and 4) whether treatment of cultured theca cells with SHH alters their proliferation and/or androgen biosynthesis.

Results

Mouse and rat ovarian expression and gonadotropin regulation of Hh, Ptch1 *and* Smo *genes*

To elucidate the expression of Hh, *Ptch1* and *Smo* transcripts and to monitor their regulation by gonadotropins, semi-quantitative RT-PCR was performed using ovarian cDNAs from ovaries of mice treated with gonadotropins. As shown in Fig. 1, treatment with pregnant mares serum gonadotrophin (PMSG) caused

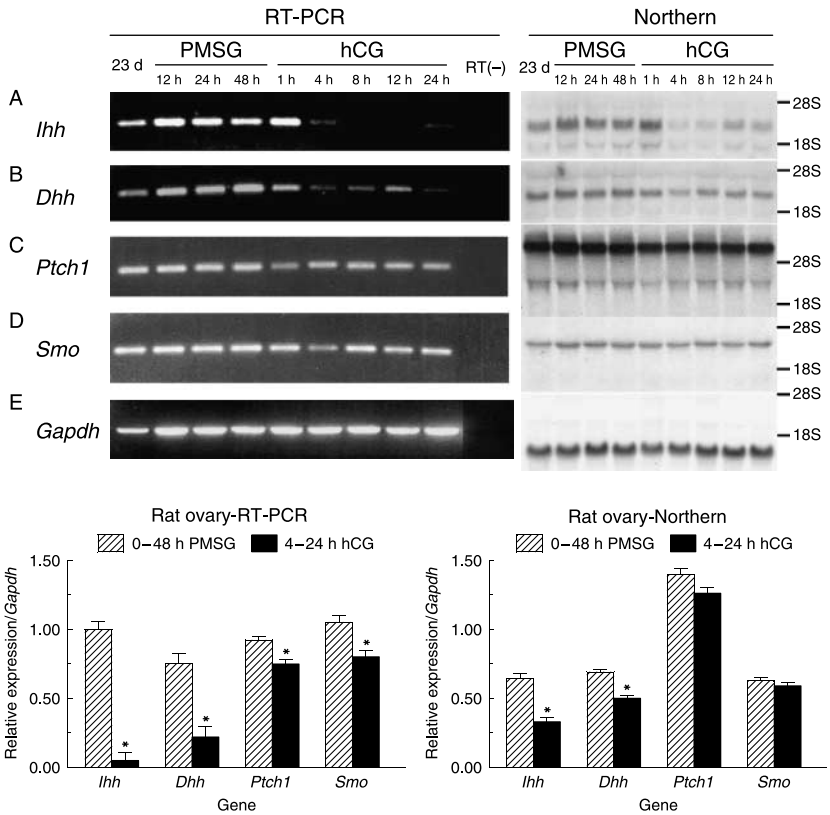

Figure 1 Expression of (A) *Ihh*, (B) *Dhh*, (C) *Ptch1*, (D) *Smo*, and (E) *Gapdh* in ovaries of gonadotropin-treated mice using RT-PCR and northern analyses. Ovarian RNA was extracted at different times after hormonal treatment before RT-PCR or northern blot analyses. Day 23 (23 d) is equivalent to 0 h prior to PMSG. *Gapdh* expression served as an internal control as described in Materials and Methods. The experiments for each gene were repeated thrice for *Ihh*, *Dhh*, and *Gapdh*, and twice for *Ptch1* and *Smo*. Representative results are shown from RNA pooled from two to three mice for RT-PCR and northern analysis. Bottom panels: band density was analyzed for 0–48 h post-PMSG and 4–24 h post-hCG treatment, and expressed abundance normalized to *Gapdh* mRNA band intensity; asterisk (*) indicates mean differs ($P<0.05$) from its respective 0–48 h post-PMSG value.

little change in *Ihh* mRNA abundance. Following hCG treatment, whole ovarian *Ihh* mRNA abundance appeared to decrease within 4-h post-hCG and was 95% lower ($P<0.05$) 4–24 h post-hCG versus 0–24 post-PMSG (Fig. 1). A similar trend (75% decrease) was found for whole ovarian *Dhh* mRNA abundance (Fig. 1). Regulation of *Ptch1* and *Smo* mRNA abundance by gonadotropins was less dramatic with *Ptch1* and *Smo* mRNA abundance 19 and 24% lower 4–24 h post-hCG than 0–48 h post-PMSG. These results suggest that the ligands of the Hh signaling system may be regulated by gonadotropins, but expression levels of receptor and co-receptor showed minimal changes. By contrast, other paralogs of Hh (*Shh*) and patched (*Ptch2*) could not be amplified under the same conditions (data not shown).

To extend the RT-PCR data, northern blot analyses were performed to further analyze the expression and regulation of mRNA levels for *Ihh*, *Dhh*, *Ptch1*, and *Smo* (Fig. 1). The sizes of major transcripts for *Ihh*, *Dhh*, *Ptch1*, and *Smo* in rat ovaries were 2.5, 2.5, 7.9, and 3.7 kb respectively, consistent with earlier findings in other tissues (Goodrich *et al.* 1997, Traiffort *et al.* 1998). Similar to RT-PCR results, semi-quantitative whole ovarian *Ihh* and *Dhh* mRNA abundance was 48 and 27% lower ($P<0.05$) respectively, 4–24 h post-hCG than 0–40 h post-PMSG treatment. By contrast, whole ovarian *Ptch1* and *Smo* mRNA abundance did not appear to change.

Bovine ovarian expression and developmental regulation of Hh, PTCH1 and SMO genes

To investigate the ovarian cell types expressing *IHH*, *PTCH1*, and *SMO* mRNA in small and large follicles, quantitative real-time RT-PCR (qRT-PCR) analyses were performed on granulosa and theca cells collected from two sizes of bovine follicles (Fig. 2). Theca cell *PTCH1* (Fig. 2A) and *SMO* (Fig. 2B) mRNA levels were greater ($P<0.05$) in large follicles than small follicles. Also, *PTCH1* and *SMO* mRNAs were detectable in granulosa cells but at a much lower abundance than in theca cells, and did not differ between small and large follicles. The ligand *IHH* mRNA abundance was the greatest ($P<0.05$) in small-follicle granulosa cells and similar in large-follicle theca and granulosa cells (Fig. 2C).

Regulation of the expression of Gli1 transcriptional factor and Ptch1 receptor in cultured rat theca-interstitial cells

To study the functional importance of PTCH1 in theca interna of rats, we isolated theca-interstitial cells and treated them with a recombinant amino-terminal peptide of mouse SHH (Matsumoto *et al.* 2002). This mature region of SHH is 91% identical to the corresponding region of IHH and binds to PTCH1 with similar affinity (Zhang *et al.* 2001, Matsumoto

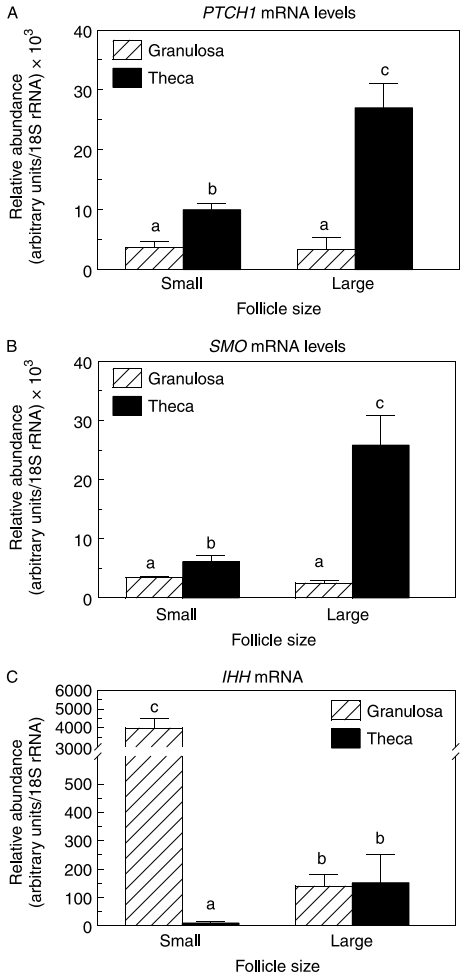

Figure 2 Effect of the size of follicle on the abundance of granulosa and theca cell *PTCH1* (hedgehog receptor; (A)), *SMO* (the hedgehog co-receptor; (B)) and *IHH* (C) mRNA in bovine follicles. Granulosa and theca cells from small (2–6 mm) and large (8–22 mm) follicles were collected, RNA isolated, and qRT-PCR used to quantify mRNA levels. Values are normalized to constitutively expressed 18S rRNA. ^{a,b,c}Means ($n=6$) without a common superscript differ ($P<0.05$).

et al. 2002). Treatment (24 h) with SHH stimulated the transcript level for *Gli1* in a dose-dependent manner with 1000 ng/ml leading to >37-fold increases (Fig. 3). Although requiring higher doses, treatment with 1000 ng/ml SHH also stimulated the expression of *Ptch1* mRNA leading to a 1.9-fold increase in its abundance (Fig. 3). By contrast, SHH treatment did not alter the transcript levels for *Gli2* and *Gli3* (Fig. 3).

Hh regulation of the expression of GLI1 transcriptional factor and PTCH1 receptor in cultured bovine theca cells

In bovine theca cells cultured in the presence of 10 ng/ml insulin-like growth factor-I (IGF1) and 10 ng/ml LH, treatment of 1000 ng/ml SHH for 5 h increased ($P<0.05$) *PTCH1* and *GLI1* mRNA

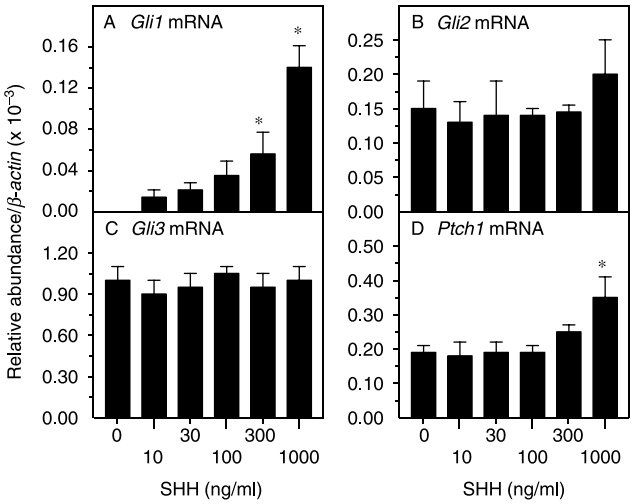

Figure 3 Regulation of *Gli* transcriptional factor and *Ptch1* expression by recombinant mouse SHH amino terminal peptide (SHH) in cultured rat theca-interstitial cells. Enriched theca-interstitial cells (1–2×105 cells/500 μl) were obtained from ovaries of at least ten rats following a Percoll discontinuous density centrifugation procedure and incubated in McCoy 5A medium supplemented with 10% FBS for 3 h. Media were then replaced by the serum-free McCoy 5A medium, and cells were incubated for 24 h with or without increasing doses of SHH. After washing with PBS twice, cells were harvested to extract RNA, before determination of transcript levels for different genes by using qRT-PCR. (A) *Gli1*, (B) *Gli2*, (C) *Gli3*, and (D) *Ptch1*. Mean±S.D. of triplicates from four experiments. *$P<0.05$ versus respective control (0 ng/ml).

abundance, but had no effect ($P>0.10$) on the abundance of *SMO* mRNA (Fig. 4A). Also, the levels of *LHCGR*, *CYP11A1*, and *CYP17A1* mRNA were not affected ($P>0.10$) by 1000 ng/ml SHH (Fig. 4B).

Hh regulation of proliferation and steroidogenesis of bovine theca cells

SHH increased ($P<0.05$) IGF1-induced numbers of theca cells from small (by 21%) and large (by 37%) follicles (Fig. 5). In the absence of IGF1, SHH also increased (by 21%; $P<0.05$) the numbers of theca cells from large follicles but had no effect ($P>0.10$) on the numbers of theca cells from small follicles (Fig. 5B). In addition, treatment of bovine theca cells from large follicles with 10 ng/ml IGF1 increased ($P<0.05$) ^{3}H-thymidine incorporation by twofold and 100 ng/ml SHH had no effect ($P>0.10$) on this response (Fig. 5C). However, 1000 ng/ml SHH further increased ($P<0.05$) IGF1-induced ^{3}H-thymidine incorporation by 39% (Fig. 5C).

In cultured bovine theca cells, IGF1-induced androstenedione production was increased 19 and 32% ($P<0.05$) by SHH (1000 ng/ml) treatment in cells from large (Fig. 6A) and small (Fig. 6B) follicles respectively. SHH also stimulated basal androstenedione production by twofold ($P<0.05$) in theca cells of small follicles (Fig. 6B), but had no effect ($P>0.10$) on basal androstenedione production by theca cells from large follicles (Fig. 6A). Progesterone production by small- and large-follicle theca cells was not affected ($P>0.10$) by 1000 ng/ml SHH (Table 1).

Hormonal regulation of PTCH1 mRNA in bovine theca cells and IHH mRNA levels in bovine granulosa cells

To determine whether *PTCH1* mRNA was regulated by hormones, the ability of insulin, IGF1, and LH to alter abundance of *PTCH1* mRNA was analyzed in cultured theca cells. Neither plating density nor insulin affected ($P>0.10$) the abundance of *PTCH1* mRNA in large-follicle theca cells (Table 2). Similarly, neither LH nor IGF1 affected ($P>0.10$) abundance of *PTCH1* mRNA in theca cells (Table 2). By contrast, insulin increased ($P<0.05$) *LHCGR* mRNA abundance by twofold (data not shown), and LH plus IGF1 increased ($P<0.05$) *CYP11A1* mRNA by 1.8-fold (data not shown).

To determine whether *IHH* mRNA was regulated by hormones, small-follicle granulosa cells were treated with IGF1 and/or FSH for 24 h (Fig. 7). FSH had no effect

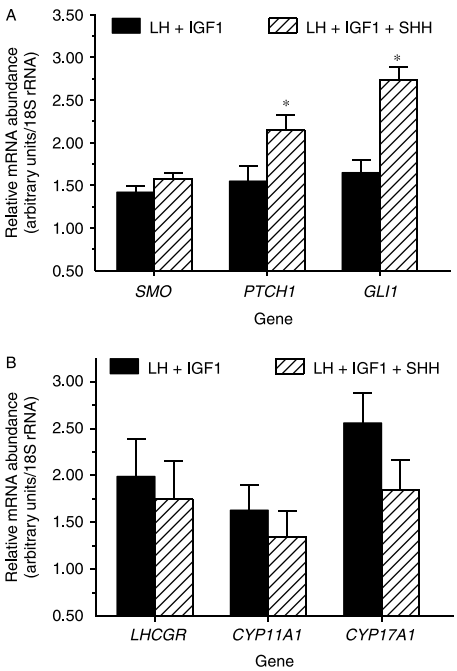

Figure 4 Effect of sonic hedgehog (SHH; 1000 ng/ml) on *SMO*, *PTCH1*, *GLI1*, *LHCGR*, *CYP11A1*, and *CYP17A1* mRNAs in bovine theca cells. Theca cells from large follicles were cultured for 2 days in the presence of 10% FCS, and then cells were washed and incubated in serum-free medium in the presence of 10 ng/ml IGF1 and LH for 24 h. Medium was changed and cells incubated in the absence or presence of 1000 ng/ml SHH with 10 ng/ml IGF1 and LH for an additional 5 h and cellular RNA collected. Real-time qRT-PCR was used to quantify mRNA levels. Values are means of three separate experiments and normalized to constitutively express 18S rRNA. *Within gene type, mean ($n=6$) differs ($P<0.05$) from control (LH+IGF1).

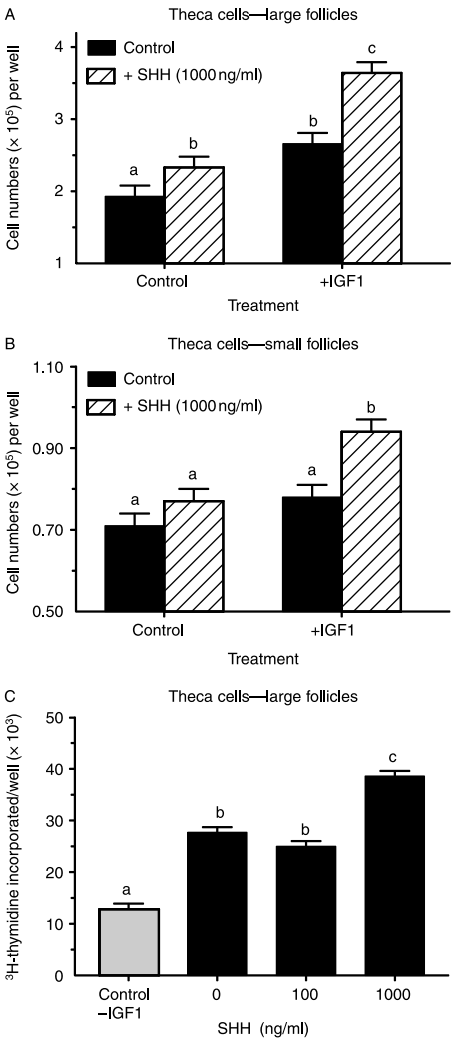

Figure 5 Effect of treatment of sonic hedgehog (SHH) and IGF1 on bovine theca cell proliferation. (A) and (B) Theca cells from large (8–22 mm; (A)) and small (2–6 mm; (B)) follicles were cultured for 2 days in the presence of 10% FCS, and then cells were washed and incubated in serum-free medium for an additional 48 h in the absence or presence of 1000 ng/ml SHH with 30 ng/ml IGF1 and 100 ng/ml LH. (C) Theca cells from large follicles were cultured for 48 h in 10% FCS, serum-starved for 24 h in serum-free medium, and then cultured in serum-free medium with no treatments (control) or with IGF1 (30 ng/ml, black bars) and SHH (0, 100, 1000 ng/ml) for 40 h in the presence of 1 μCi of ^{3}H-thymidine to measure DNA synthesis. Values are means±S.E.M. of three separate experiments (n=9). ^{a,b,c}Within a panel, means without a common superscript differ (P<0.05).

(P>0.10) on *IHH* mRNA in granulosa cells, whereas IGF1 decreased (P<0.05) the abundance of *IHH* mRNA in granulosa cells (Fig. 7).

Discussion

The present study documents novel functional Hh signaling in theca cells of rats and cattle, and further extends previous investigations demonstrating the cellular localization of Hh system in mouse ovaries (Wijgerde *et al.* 2005) and SHH induction of *Gli1* mRNA in mouse granulosa cells (Russell *et al.* 2007), and *GLI1* and *PTCH1* mRNA in other mammalian cells (Goodrich *et al.* 1996, Kenney & Rowitch 2000). Moreover, these studies demonstrated hormonal and developmental regulation of the transcripts for *Ihh*, *Ptch1*, and *Smo* in the rodent and bovine ovary, and discovered that SHH treatment stimulated proliferation of bovine theca cells and augmented androstenedione production.

For the first time, we demonstrated that *Smo* mRNA is not altered with Hh stimulation, and that *Smo* mRNA abundance in theca cells is greater in large than small follicles. Consistent with the present study, Hh treatment increases the expression of *Ptch1* itself in cell types other than theca cells including mouse neuronal cells (Kenney & Rowitch 2000) and mouse medulloblastoma cells (Briggs *et al.* 2008). PTCH1 is a key component of the Hh signaling pathway, which controls cell fate determination during development (Hammerschmidt *et al.* 1997). *Ptch1* mutations cause derepression of target genes, cell fate changes, and excessive growth in some tissues (Ingham *et al.* 1991). Results of the present study identified for the first time a potential functional role of SHH in theca cell function (i.e. steroidogenesis and proliferation) of mammals. Effects of Hh proteins on steroidogenesis, although a novel finding for ovarian

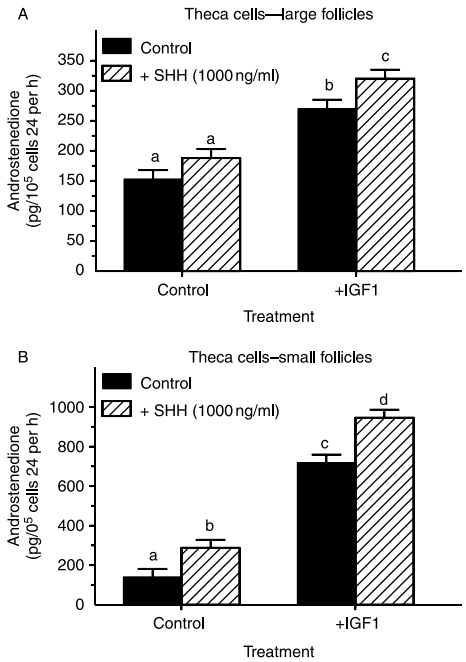

Figure 6 Effect of 2-day treatment of IGF1 and sonic hedgehog (SHH) on bovine theca cell androstenedione production. Theca cells from large (8–22 mm; (A)) and small (2–6 mm; (B)) follicles were cultured for 2 days in the presence of 10% FCS, and then cells were washed and incubated in serum-free medium for an additional 48 h in the absence or presence of 1000 ng/ml SHH with 30 ng/ml IGF1 and 100 ng/ml LH. Values are means±S.E.M. of three separate experiments (n=9). ^{a,b}Means without a common superscript differ (P<0.05).

Table 1 Effect of 2-day treatment of insulin-like growth factor-I (IGF1), sonic hedgehog (SHH), or both on progesterone production by theca cells from small (2–6 mm) and large (8–22 mm) bovine follicles.

Dose of IGF1 (ng/ml)	Dose of SHH (ng/ml)	Small follicle progesterone (ng/10^5 cells/24 h)	Large follicle progesterone (ng/10^5 cells/24 h)
0	0	$17.1^{a}\pm2.0$	$8.8^{a}\pm1.4$
30	0	$38.9^{b}\pm2.5$	$17.5^{b}\pm3.5$
0	1000	$17.4^{a}\pm0.7$	$8.9^{a}\pm0.9$
30	1000	$39.4^{b}\pm2.1$	$16.2^{b}\pm2.7$

[a,b]Within a column, means (±S.E.M.) without a common superscript differ ($P<0.05$).

cells, are not without precedence. In fetal Leydig cell precursors, PTCH1 signaling up-regulates P450 side-chain cleavage enzyme (*CYP11A1*) expression (Yao *et al.* 2002). Because the theca interna plays a key role in the pathology of polycystic ovarian disease, these results raise the possibility for examining a potential role of Hh signaling in the pathogenesis of PCOS.

The present study revealed that both rat theca-interstitial and bovine theca cells respond to Hh with increased *Gli1* mRNA, but further study will be required to more clearly define how Hh proteins may regulate ovarian follicular function particularly as it pertains to monotocous (e.g. cattle) versus polytocous (e.g. rats) species. Recently, we have reported that *PTCH1* mRNA in theca cells were lower in cattle selected for double versus single ovulations, suggesting that increased *PTCH1* expression may be involved with the development of multiple dominant follicles (Aad *et al.* 2008). Because systemic and follicular fluid IGF1 are greater in cattle with double ovulations (Echternkamp *et al.* 2004), perhaps IGF1 regulates theca *PTCH1* mRNA. However, *PTCH1* mRNA abundance was unaltered by IGF1, LH, or insulin in the present study. It is possible that IGF1 decreases theca *PTCH1* mRNA indirectly by reducing granulosa IHH production (see next section). In *Drosophila*, the role of Hh signaling in the adult ovary is well characterized and Hh was found to drive proliferation of somatic and germline stem cells (Forbes *et al.* 1996, Zhang & Kalderon 2000). By contrast, a role of Hh signaling in the vertebrate ovary has only recently been described using *in situ* hybridization and immunohistochemistry in mice (Wijgerde *et al.* 2005, Russell *et al.* 2007), and results of the present study support a mitogenic role for Hh in bovine theca cells. Because dramatically greater abundance in *SMO* and *PTCH1* mRNAs existed in theca cells of large than small follicles in cattle, and levels of *PTCH1* and *SMO* mRNA in bovine granulosa cells did not differ between small and large follicles, results of the present study indicate that the theca layer may be the primary site of the Hh response system (i.e. PTCH1 and SMO) in the bovine ovary.

Of interest, the quantitative differential expression of ligand and receptor of the Hh-PTCH1 signaling system in cell types of different embryonic origins as observed in the present and previous (Wijgerde *et al.* 2005) studies is similar to that found in the testis (Bitgood *et al.* 1996, Pierucci-Alves *et al.* 2001). Evidence indicates that Hh ligands secreted by epithelial cells (e.g. IHH in granulosa or DHH in Sertoli cells of the testis) interact with PTCH1 in mesenchymal cells (e.g. theca cells or Leydig cells of the testis) at their cellular boundaries in a paracrine context (Bitgood *et al.* 1996, Wijgerde *et al.* 2005). A recent study in mice has indicated that both Hh and its PTCH1 response system may also exist in an autocrine context within granulosa cells (Russell *et al.* 2007). Apparently, which ligand (i.e. IHH, DHH, SHH) is produced is not critical because all Hh proteins bind to PTCH1 with similar affinities (Pathi *et al.* 2001) and similar biological responses (Vortkamp *et al.* 1996, Krishnan *et al.* 2001, Zhang *et al.* 2001, Deckelbaum *et al.* 2002). Our studies using sensitive qRT-PCR indicate that both granulosa and theca cells have detectable *PTCH1* and *SMO* mRNA, but granulosa cells contain significantly less abundance than in theca cells of cattle. By contrast, granulosa and theca cells had detectable *Ihh* mRNA, but theca cells contained significantly less than granulosa cells of small follicles. Not previously reported for any cell type, we found that *IHH* mRNA abundance was suppressed by IGF1, linking the ovarian Hh system with the IGF1 system at least in cattle. As mentioned, cattle selected for double

Table 2 Lack of effect of insulin, LH, and insulin-like growth factor-I (IGF1) treatments on transcript levels for *Ptch1* mRNA in large-follicle (8–22 mm) theca cells*.

Plating density (cells/well)×10^5	Duration of treatment (h)	Dose of insulin (ng/ml)	Dose of LH (ng/ml)	Dose of IGF1 (ng/ml)	***Ptch1* mRNA** (relative abundance)
1	24	0	0	0	1.5±0.2
1	24	100	0	0	2.2±0.5
3	24	0	0	0	1.5±0.1
3	24	100	0	0	1.7±0.1
2	24	0	0	0	2.8±0.6
2	24	0	0	30	2.2±0.5
2	24	0	30	0	2.4±0.3
2	24	0	30	30	2.2±0.3
1	48	0	30	0	1.5±0.2
1	48	0	30	30	1.5±0.2

*No significant ($P>0.10$) treatment effects were observed (means±S.E.M).

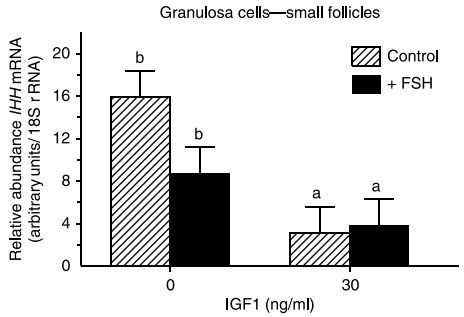

Figure 7 Effect of FSH and IGF1 treatments on transcript levels for *IHH* mRNA in small-follicle granulosa cells of cattle. Small-follicle (2–6 mm) granulosa cells of cattle were treated for 24 h with the indicated treatments in serum-free medium and the various mRNAs were quantified by qRT-PCR as described in Materials and Methods. [a,b]Means (±S.E.M.) without a common letter differ ($P<0.05$). Relative mRNA abundance was normalized to constitutively expressed 18S rRNA and expressed in arbitrary units from three replicate experiments ($n=6$).

ovulations versus single ovulations have greater IGF1 levels in blood and follicular fluid and have recently been reported to contain lower amounts of *PTCH1* mRNA in theca cells (Aad *et al*. 2008). Thus, IGF1 may indirectly reduce theca PTCH1 by reducing granulosa IHH production. In mice, qualitative RT-PCR and northern analyses revealed that ovarian *Ihh* and *Dhh* mRNA abundance was down-regulated by hCG. Furthermore, research will be required to resolve the paracrine versus autocrine context by which the Hh system operates within the ovarian follicle of various mammals as well as clarify the species differences that may exist in terms of hormonal regulation of the Hh system.

It is known that the *Dhh*-null male mice lack mature sperm (Bitgood *et al*. 1996). On select hybrid backgrounds, *Dhh*-null mice also exhibited discrete defects in testis organization, including abnormal development of peritubular myoid cells, apolar Sertoli cells, absence of basal lamina, and anastomotic testis cords (Pierucci-Alves *et al*. 2001, Park *et al*. 2007). Defects in adult Leydig cell differentiation were also reported (Clark *et al*. 2000). Studies have indicated that high levels of *Ihh* expression in granulosa cell tumors of mice overexpress a long acting gonadotropin (Owens *et al*. 2002), and that SHH increases mouse granulosa cell proliferation *in vitro* (Russell *et al*. 2007). Thus, the ovarian Hh signaling system could be involved in the proliferation of granulosa cells under certain conditions. In bovine theca cells where *PTCH1* mRNA predominates, SHH stimulated theca cell proliferation and androstenedione production in cells from large and small follicles, but whether alterations in the Hh system could alter theca interna development or induce theca cell pathogenesis such as PCOS will require further study. Also, further studies using conditional deletion of the *Ihh* or *Ptch1* gene in the ovary could reveal the exact paracrine or autocrine role of Hh signaling during follicle development.

In conclusion, the expression and regulation of *IHH* transcripts in granulosa cells and *PTCH1* mRNA in theca cells suggest a potential paracrine role of this system in bovine follicular development. These studies illustrate, for the first time, Hh stimulation of theca cell proliferation and androgen biosynthesis.

Materials and Methods

Biological materials and cell culture

Mouse and rat tissues

To investigate the expression and regulation of ligands and receptors of the Hh system in the ovary, 23-day-old female Swiss-Webster mice were obtained from Charles River Breeding Laboratories (Wilmington, MA, USA), and injected with 4 IU of PMSG (Calbiochem, San Diego, CA, USA) s.c., followed by 10 IU of hCG (Sigma Chemical Co.) i.p. 48 h later. Animals were housed in accordance with institutional and NIH guidelines for the care and use of experimental animals.

Theca-interstitial cells were prepared from 28- to 29-day-old female Sprague–Dawley rats as previously described (Ohnishi *et al*. 2001). Individual ovaries were cut into four to six pieces, and many granulosa cells and oocytes were removed following needle puncture in L-15 Leibovitz medium (Life Technologies Inc.) to enrich theca-interstitial cells. Ovaries were incubated for 60 min at 37 °C (0.25 ml/ovary) in 2.5 mg/ml collagenase (type I; Sigma Chemical Co.) and 100 μg/ml DNase I (Roche Diagnostics Corp). The incubated ovaries were pipetted every 30 min and dispersed cells were washed thrice with L-15 Leibovitz medium (Life Technologies Inc.) and passed through cell strainers of 40 μm pore size (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Theca-interstitial cells were then purified by a modified discontinuous density centrifugation procedure with 42 and 56% Percoll (Ohnishi *et al*. 2001) in 17×100 mm polystyrene Falcon tubes. Dispersed cells were layered on top of the Percoll and centrifuged at 400 ***g*** for 30 min at 4 °C. After centrifugation, the theca-interstitial cells were collected from the interface between 42 and 56% Percoll layers.

Bovine tissues

Ovaries of cattle obtained at slaughter from a nearby abattoir were brought to the laboratory on ice and processed as previously described for obtaining theca and granulosa cells from small (2–6 mm) and large (8–22 mm) follicles (Spicer & Chamberlain 1998, Spicer *et al*. 2008). Purity of these bovine theca cell preparations is ≥95% (Spicer *et al*. 2008). These follicle size categories were selected because: 1) previous studies indicate that granulosa cells from small follicles are less responsive to FSH and IGF1 than are cells from large follicles (Spicer & Chamberlain 1998, Spicer *et al*. 2002), 2) the observations that follicles larger than 8 mm have much greater E_2 concentrations than small follicles (Spicer *et al*. 1986, 2001, Stewart *et al*. 1996), 3) follicles that are destined to ovulate average 10±2 mm surface diameter (Marion *et al*. 1968), and 4) selection of the dominant follicle occurs at about 8 mm in diameter (Ginther *et al*. 2000).

RT-PCR and northern blotting of mouse and rat tissue RNA

Total RNA from mouse ovaries was isolated using the RNeasy Mini kit (Qiagen). Samples were transcribed into cDNAs using Omniscript Reverse Transcriptase (Qiagen) and oligo(dT)$_{12–18}$ (Invitrogen Co). Transcripts of different genes were amplified using primers as follows; *Ihh* (209 bp): 5′-TATCACCACCTCA-GACCGTGAC-3′ and 5′-ACCCGGTCTCCTGGCTTTACAG-3′, *Dhh* (209 bp): 5′-AGCCGGATTCGACTGGGTCTAC-3′ and 5′-GGTCCAGGAAGAGCAGCACTG-3′, *Shh* (290 bp): 5′-CCACTGTTCTGTGAAAGCAGAG-3′ and 5′-CAGCGTCTC-GATCACGTAGAAG-3′, *Ptch1* (210 bp): 5′-CCATACACCAGC-CACAGCTTCG-3′ and 5′-GGAGGCTGGAGTCTGAGAACTG-3′, *Ptch2* (231 bp): 5′-CCAGCAGCCAGCATGTAGTCAC-3′ and 5′-CTCGTGTCTGGAGCAGTAAAGG-3′, *Smo* (210 bp): 5′-CTGACTGGCGGAACTCCAATCG-3′ and 5′-CAGAC-TACTCCAGCCATCAAGG-3′, glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*; 983 bp): 5′-TGAAGGTCGGTGT-GAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTC-CACCAC-3′ as an internal control. The only primers that spanned exon–exon junctions were the *Shh* primers. PCRs were performed for 25–35 cycles at 94 °C, 30 s for denaturation; 62 °C, 30 s for annealing, and 72 °C, 45 s for elongation. The PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide. Amplicons were sub-cloned into pGEM-T easy vector (Promega Corp.), and used as probes for northern blot analyses. Northern blotting was performed as described previously (Chun *et al*. 2001) using ^{32}P-labeled probes for mouse *Ihh*, *Dhh*, *Ptch1*, and *Smo* gene fragments derived from RT-PCR and verified based on DNA sequencing. For data normalization, blots were stripped by boiling in 0.1×SSC and 0.5% SDS for 30 min before reprobing with a cDNA probe for mouse *Gapdh*.

Rat theca-interstitial cell culture and qRT-PCR for Gli transcription factors and Ptch1

Theca-interstitial cells were washed thrice with McCoy's 5A medium (Life Technologies Inc.) and cell viability (∼90%) determined using trypan blue exclusion. After culturing under different conditions with or without recombinant mouse SHH amino-terminal peptide (SHH; R&D Systems, Inc., Minneapolis, MN, USA), cDNA preparations were derived from theca-interstitial cells. For qRT-PCR, rat *Gli1*, *Gli2*, *Gli3*, *Ptch1*, and *β-actin* cDNAs were amplified using the QuantiTect Probe PCR Kit (Qiagen), and analyzed using the Smart Cycler II System (Cepheid, Sunnyvale, CA, USA). Webtool Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi) was used for designing PCR primers and probes. Primers and Taqman probes are as follows: *Gli1*: 5′-AGCTCCTGTGTAAT-TACGTTCAGTC-3′, 5′-GGCTCTGACTAACTTGAGAACCTC-3′, and 5′-6-FAM-CAACCAGGAACTTCCATATCAGAGCC-TAMRA-3′; *Gli2*: 5′-AAGCCTGCTCCACAATCTCTC-3′, 5′-AACTTGTTCTCTTCAGCCAAGC-3′, and 5′-6-FAM-AGAA-TTCCTCACGCCTCACCACAC-TAMRA-3′; *Gli3*: 5′-GACCAG CACAGTTGACAGCTT-3′, 5′-CCAGATTAGGCTGGTATGGTC-3′, and 5′-6-FAM-AGTCATGACCTAGAAGGCGTGCAGA-TAMRA-3′; *Ptch1*: 5′-GACTCCGAGTACAGCTCTCAGAC-3′, 5′-CTGTGGCTTCCACAATCACTT-3′, and 5′-6-FAM-CAG-TGAGGAGCTCAGGCACTATGAA-TAMRA-3′; and *β-actin*: 5′-GGACCTGACGGACTACCTCATG-3′; 5′-TCTTTGATGTC-ACGCACGATTT-3′, and 5′-FAM-CCTGACCGAGCGTGGC-TACAGCTTC-TAMRA-3′ as an internal control. None of the rat primers spanned exon–exon junctions. Thermal cycling condition was 95 °C for 15 min followed by 40 cycles at 94 °C for 15 s and 60 °C for 60 s.

Bovine theca cell culture and qRT-PCR for GLI1, PTCH1, and SMO

To determine the developmental changes in the Hh signaling system, cells were collected as described earlier except that cells were not cultured and immediately after isolation, cells were lysed with TRIzol and frozen for later extraction of RNA (see below).

To determine the effect of SHH on the abundance of *GLI1*, *PTCH1*, and *SMO* mRNAs, theca cells were isolated from large follicles as previously described (Stewart *et al*. 1995, Spicer & Francisco 1997, Spicer & Chamberlain 1998, Spicer *et al*. 2008). Briefly, medium was a 1:1 mixture of DMEM and Ham F12 containing gentamicin, glutamine, and sodium bicarbonate (Sigma Chemical Co.) and 2×10^5 viable cells were seeded in plastic 24-well plates containing 1 ml of 10% FCS medium. Prior to plating, cells were resuspended in medium containing 1.25 mg/ml collagenase and 0.5 mg/ml DNase. Cultures were kept at 38.5 °C in a 95% air–5% CO_2 atmosphere, and for all experiments medium was changed every 24 h. After the 2-day plating period, this culture system utilizes serum-free medium so that specific effects of growth factors can be ascertained; LH has little or no effect alone but when concomitantly treated with insulin or IGF1, LH consistently stimulates steroidogenesis (Stewart *et al*. 1995, Spicer & Francisco 1997, Spicer & Chamberlain 1998, Spicer *et al*. 2008).

After the 48-h plating period, cells were washed twice with 0.5 ml serum-free medium, and treated for an additional 24 h with 10 ng/ml IGF1 (recombinant human IGF1; R&D Systems) and ovine LH (NIDDK-oLH-26; activity: 1.0×NIH-LH-S1 U/mg; National Hormone & Pituitary Program, Torrance, CA, USA) in serum-free medium to maintain theca cell phenotype. Medium was then changed and cells treated for an additional 5 h with 0 or 1000 ng/ml recombinant human SHH amino terminal peptide (R&D Systems) in the presence of ovine LH (10 ng/ml) and recombinant human IGF1 (10 ng/ml) in serum-free medium. Cells were then lysed with TRIzol and frozen for later extraction of RNA (see below).

To determine the effect of SHH on theca cell proliferation and steroidogenesis, theca cells from small and large follicles were isolated and cultured as described earlier, and after the 48-h plating period, cells were treated for an additional 48 h in serum-free medium with either 0 or 1000 ng/ml SHH in the presence of 0 or 30 ng/ml IGF1 and 100 ng/ml LH. To maximize steroid production, IGF1 and LH were included in the culture medium. Medium was collected and stored at −20 °C until RIAs previously validated in our laboratory (Stewart *et al*. 1995, Spicer & Francisco 1997, Spicer & Chamberlain 1998) were conducted to quantify progesterone and androstenedione concentrations. Numbers of cells (in the

same wells that medium was collected) were determined using a Coulter counter as previously described (Stewart *et al*. 1995, Spicer & Chamberlain 1998, Spicer *et al*. 2008), and used to calculate steroid production on an ng or pg per 10^5 cell basis.

To further verify that the effect of SHH on cell numbers was due to cell proliferation, theca cells from large follicles were cultured for 48 h in 10% FCS, serum-starved for 24 h by culturing in serum-free medium, medium changed, and then cells cultured for an additional 40 h in serum-free medium with either no treatment, 10 ng/ml IGF1, 10 ng/ml IGF1 plus 100 ng/ml SHH, or 10 ng/ml IGF1 plus 1000 ng/ml SHH in the presence of 1 μCi of ^{3}H-thymidine to assess DNA synthesis as previously described (Spicer *et al*. 2008).

To determine whether *PTCH1* mRNA was regulated by hormones, theca cells were obtained from large bovine follicles and cultured for 48 h in 10% FCS, followed by treatments arranged in three experiments. The first experiment evaluated the effect of 24-h treatment of insulin (0 or 100 ng/ml) and plating density (1 or 3×10^5 cells/well) on *PTCH1* mRNA. The second and third experiments evaluated the effect of 24-h and 48-h treatment respectively, of IGF1 (0 or 30 ng/ml) and/or LH (0 or 30 ng/ml) on *PTCH1* mRNA. After the first 48 h, cells were washed twice with 0.5 ml serum-free medium, and treated for an additional 24 or 48 h in serum-free medium with the indicated treatments. Cells were then lysed with TRIzol and frozen for later extraction of RNA (see below).

To determine whether *IHH* mRNA was regulated by hormones, granulosa cells were obtained from small bovine follicles and cultured for 48 h in 10% FCS. After the first 48 h, cells were washed twice with 0.5 ml serum-free medium, and treated for an additional 24 h in serum-free medium with IGF1 (0 or 30 ng/ml) and/or FSH (0 or 30 ng/ml). Cells were then lysed with TRIzol and frozen for later extraction of RNA (see below).

Bovine theca and granulosa cells were lysed in 0.5 ml TRIzol Reagent (Life Technologies Inc.), RNA extracted, and RNA quantity determined spectrophotometrically at 260 nm as previously described (Voge *et al*. 2004, Spicer *et al*. 2008). The target gene primers (forward, reverse) and probe sequences for *Ihh* (Accession XM_601000) were CGGCTTCGACTGGGTGTATTAC, AGGGAAGCAGCCACCTGTCT, CAAGGCCCACGTGCATTGCTCC respectively; for *Ptch1* (Accession XM_869803) were TGCCCAGGCTACGAGGACTA, CCGGACATTAAAAGGCACATG, and TGACCACGGCCTGTTTGAGGACC respectively; and for *Smo* (conserved regions of Accession SM_876452 and XM_586374) were CACCTGCTCACGTGGTCACT, CAAAACAGATGCCGCTCACA, and ACTGTGGCAATCCTCGCCGTGG respectively. The primers that spanned exon–exon junctions were the *Ihh* and *Smo* primers. Sequences for the *LHCGR*, *CYP11A1*, and *CYP17A1* primers and probes have been reported (Spicer *et al*. 2008). A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) was also conducted to insure the specificity of the designed primers and probe and to assure that they were not designed from any homologous regions coding for other genes.

The differential expression of target gene mRNA in theca and granulosa cells along with 18S rRNA (for normalization of target gene expression) was quantified using the one-step multiplex qRT-PCR for Taqman Gold RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) as previously described (Voge *et al*. 2004, Spicer *et al*. 2008). All samples were run in duplicate. Relative quantification of target gene mRNAs was expressed using the comparative threshold cycle method as previously described (Voge *et al*. 2004, Aad *et al*. 2006, Spicer *et al*. 2008).

Statistical analyses

Experimental data are presented as least-squares means ± S.E.M. of measurement from replicated experiments. For rat studies, means were analyzed by one-way ANOVA and *t*-tests conducted to compare means. For bovine studies, each experiment was replicated three or more times, and within each experiment, treatments were applied in triplicate culture wells. Each experiment was conducted on a separate pool of theca or granulosa cells obtained from five to eight cows or heifers. The main effects and their interactions on the variables measured were assessed by general linear models procedure of SAS (SAS Institute Inc., Cary, NC, USA). To correct for heterogeneity of variance, androstenedione production, *Ptch1* mRNA, *Ihh* mRNA, *Smo* mRNA, and *Gli* mRNA were analyzed after transformation natural log $(x+1)$. Specific differences among treatments were tested using Fisher's protected least-significant difference procedure (Ott 1977). Significance was declared at $(P<0.05)$ unless noted otherwise.

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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