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 androstenediene 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.

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 Marco tullo 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).
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...
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 Ptc1 and Smo mRNA abundance by gonadotropins was less dramatic with Ptc1 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 (Pch2) 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, Ptc1, and Smo (Fig. 1). The sizes of major transcripts for Ihh, Dhh, Ptc1, 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 Ptc1 and Smo mRNA abundance did not appear to change.

Bovine ovarian expression and developmental regulation of Hh, Ptc1 and Smo genes

To investigate the ovarian cell types expressing Ihh, Ptc1, 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 Ptc1 (Fig. 2A) and Smo (Fig. 2B) mRNA levels were greater (P<0.05) in large follicles than small follicles. Also, Ptc1 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 Ptc1 receptor in cultured rat theca-interstitial cells

To study the functional importance of Ptc1 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 Ptc1 with similar affinity (Zhang et al. 2001, Matsumoto et al. 2002). Treatment (24 h) with Shh stimulated the transcript level for Gli 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 Ptc1 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 Ptc1 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) Ptc1 and Gli1 mRNA abundance.
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 (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 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).

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...
(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, Pch1, 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 Pch1 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). Pch1 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...
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 monotoocous (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, and levels of PTCH1 and SMO mRNA existed in theca cells of large than small follicles (Aad et al. 2002). Our studies using sensitive qRT-PCR indicate that both granulosa and theca cells have detectable IHH 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 1

<table>
<thead>
<tr>
<th>Dose of IGF1 (ng/ml)</th>
<th>Dose of SHH (ng/ml)</th>
<th>Small follicle progesterone (ng/10⁴ cells/24 h)</th>
<th>Large follicle progesterone (ng/10⁵ cells/24 h)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>17.4±2.0</td>
<td>8.8±1.4</td>
</tr>
<tr>
<td>30</td>
<td>1000</td>
<td>38.9±2.5</td>
<td>17.5±3.5</td>
</tr>
<tr>
<td>0</td>
<td>1000</td>
<td>39.4±2.1</td>
<td>16.2±2.7</td>
</tr>
</tbody>
</table>

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

### Table 2

<table>
<thead>
<tr>
<th>Plating density (cells/well)×10⁵</th>
<th>Duration of treatment (h)</th>
<th>Dose of insulin (ng/ml)</th>
<th>Dose of LH (ng/ml)</th>
<th>Dose of IGF1 (ng/ml)</th>
<th>PtcH1 mRNA (relative abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>2.8±0.6</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>1.5±0.2</td>
</tr>
</tbody>
</table>

*No significant (P>0.10) treatment effects were observed (means ±S.E.M).
conditional deletion of the
ment or induce theca cell pathogenesis such as PCOS
in the Hh system could alter theca interna develop-
from large and small follicles, but whether alterations
proliferation and androstenedione production in cells
mRNA predominates, SHH stimulated theca cell
the proliferation of granulosa cells under certain
the ovarian Hh signaling system could be involved in
role of Hh signaling during follicle development.

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. Further-
more, 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 back-
grounds, Dhh-null mice also exhibited discrete defects
in testis organization, including abnormal develop-
ment 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 develop-
ment or induce theca cell pathogenesis such as PCOS
will require further study. Also, further studies using
conditional deletion of the Ihh or Ptc1 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
Diagnosics 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
E2 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 Transcribe (Qiagen) and oligo(dT)12-18 (Invitrogen Co). Transcripts of different genes were amplified using primers as follows; Ihh (209 bp): 5'-TATCAACCTCA- GACTGTGAC-3' and 5'-ACCGGTCTCTGACATGA-3', Dhh (209 bp): 5'-AGCCGATTGTTCGCTTAC-3' and 5'-GGTCGGAGAAGGACGACTG-3', Shh (209 bp): 5'-CCGAGTGTGACGAACTCAG-3' and 5'-CACGTACGGTAAAG-3', Ptch1 (210 bp): 5'-CCATACACCAGGAGACTTCTGG-3' and 5'-GGAGGCTGGATGAGTAAGT-3', Ptch2 (231 bp): 5'-CCAGGGGAGTATGATCAG-3' and 5'-GTCGGTGACCTGGCTTTACAG-3', Smo (210 bp): 5'-CTGACTGTCGGAAACTCCATG-3' and 5'-CATGGGCTGAGTCATTTG-3', Ihh (209 bp): 5'-GAAGCTGACGGACTACCTCATG-3' and 5'-CTGTGGCTTCCACAATCACTT-3', Gli1 (209 bp): 5'-AGTCATGACCTAGCAGCG-3' and 5'-GACGTGACTACAC-3', Gli2 (209 bp): 5'-AAGCCTGCTCCACAATCTCTC-3' and 5'-GACAGATTACGAGTACT-3', Gli3 (209 bp): 5'-AGTCATGACCTAGCAGCG-3' and 5'-GACGTGACTACAC-3', SMO (290 bp): 5'-CCGAGTGTGACGAACTCAG-3' and 5'-CACGTACGGTAAAG-3'.

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).

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), RNA preparations were derived from theca-interstitial cells. For qRT-PCR, rat Gli1, Gli2, Gli3, Ptch1, and β-actin cDNAs were amplified using the Quantitec 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 were designed as follows; Gli1: 5'-AGCTCCTGTTATGATCTCTCC-3' and 5'-CTGCTGTGACAACTTGGAGC-3', Gli2: 5'-AAGGCTCTGCACATACTTGT-3' and 5'-ACCTTCTCTTGACGACG-3', Gli3: 5'-GACAGCAGTGGAGGACG-3' and 5'-CCAGATTACGAGTACT-3', Ptch1: 5'-GACTCGCCAGTACGCTCGAC-3', and 5'-CTGTGGCTTCCACATACATG-3', and 5'-GAGTGGCTGTCAGCAGCTTCTGAC-3', and 5'-GAGTGGCTGTCAGCAGCTTCTGAC-3', and 5'-GAGTGGCTGTCAGCAGCTTCTGAC-3', and 5'-GAGTGGCTGTCAGCAGCTTCTGAC-3'.
same well 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 a ng or pg per 10⁶ 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 ³H-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 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 CGGCTTCGACTGG-TGTATTAC, AGGGAACGACCCACCTGCT, CAAGGCC-CACGTTGCAATTGCTCC respectively; and for Ptc1 (Accession XM_601000) were TGCCCCAGGCTACGAGGACTA, CCGGA-CACGTTGCAATTGCTCC respectively; and for Smo (Accession XM_869803) were TCCCCAGCTACGAGGACTA, CCGGA-CATATAAGGCCACATG, and TGACCAGGCGGTGTGTTGAG-GACC respectively; and for 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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