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Inhibitor of Differentiation (Id) Genes Are Expressed in the Steroidogenic Cells of the Ovine Ovary and Are Differentially Regulated by Members of the Transforming Growth Factor-β Family

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Inhibitor of differentiation (Id) proteins act during embryogenesis and development to repress gene transcription required for lineage commitment, while promoting cell growth. Growth factors belonging to the TGFβ superfamily of signaling molecules, notably the bone morphogenetic proteins (BMPs) and activin, can regulate Id expression in these tissues. Id expression and function in adult physiology is less well determined, and we hypothesized a role for Id proteins in the adult mammalian ovary. Immunohistochemistry for Id1, Id2, Id3, and Id4 in the sheep ovary revealed consistent expression in granulosa and thecal cells of ovarian follicles throughout development. In atretic follicles, Id proteins were selectively down-regulated in thecal cells ($P < 0.0001$). Additionally, Id1 was universally up-regulated in the cumulus cells adjacent to the oocyte. Immunohistochemistry for phospho (p)-smad 1/5/8 signaling components (stimulated by BMPs) showed a punctate pattern of expression whereas p-smad 2/3 (stimulated by activin) was ubiquitously expressed in follicles. Neither pathway, however, displayed differential staining in line with Id1 cumulus-specific expression, suggesting a more complex relationship between Id1 expression and TGFβ signaling in these cells. Nevertheless, *in vitro*, stimulation of ovine granulosa cells with BMP6 or activin A led to a respective increase and decrease in *Id1* ($P < 0.0001$), *Id2* ($P < 0.0001$), *Id3* ($P < 0.0001$), and *Id4* ($P < 0.05$) transcripts, and *Id1* gene expression was further manipulated by the oocyte-secreted factors BMP15 and growth differentiation factor 9 ($P < 0.001$). These data confirm that TGFβ signaling can regulate *Id* gene expression in the sheep ovarian follicle and suggest a functional role for the Id family in the mammalian ovary. (*Endocrinology* 151: 1247–1256, 2010)

Τhe ovary, unlike other adult mammalian tissues, undergoes persistent cyclical remodeling with regulated proliferation, differentiation, and cell death. Inhibitors of differentiation (Id) proteins (also known as inhibitors of DNA binding) are a subfamily of regulatory dimeric basic helix-loop-helix transcription factors. These factors regulate many genes, including those required for growth and differentiation, through binding to E-box (CANNTG) sequences on the promoter region of target genes (1, 2). Id proteins lack a basic DNA-binding domain and can het-erodimerize with other basic helix-loop-helix proteins to block chromatin binding and thus subsequent transcriptional activity (3). Four known mammalian Id isoforms (Id1–4) have been identified and can regulate growth and differentiation across embryonic tissues (4–6), where both overlapping and nonredundant functions are reported (7, 8). Predictably, these properties have led to Id proteins being implicated in tumorigenesis in various adult tissues (9, 10).

Upstream mediators of Id expression include members of the TGFβ superfamily (11), which include the bone

Abbreviations: BMP, Bone morphogenetic protein; GDF, growth differentiation factor; Id, inhibitors of differentiation; p-, phospho-; qRT, quantitative real-time.

morphogenetic proteins (BMPs) and activin (12). Dimeric TGFβ ligands interact with a range of BMP type-1 and type-2 serine/threonine kinase receptor complexes to activate the smad signaling pathway (12). BMPs signal through the smad 1/5/8 pathway, whereas activin activates the smad 2/3 cascade (13), and both are antagonized by smad 6 and smad 7 (14). Signaling by TGFβ has been linked to *Id* gene regulation during development, controlling the timing of differentiation and lineage commitment (8, 15–18). This has particular significance for the adult ovary in which BMPs and activin are involved in many processes governing follicle development and oocyte maturation and competency (19–21). Furthermore, although not yet characterized in the adult mammalian ovary, *Id* mRNA is reported to be expressed in the hen (*Gallus domesticus*), and the Ids are speculated to be involved in the control and timing of follicle selection and granulosa cell differentiation in this avian species (22, 23).

Although the molecular regulation of proliferation and differentiation in the cycling ovary is not fully understood, members of the TGFβ family appear to have key mechanistic roles. We hypothesized that Id proteins have roles in the regulation of growth and differentiation of the steroidogenic cells of the adult ovary. We therefore studied the localization of Id protein expression (Id1, Id2, Id3, and Id4) in the pre- and postpubertal sheep ovary. In addition, we hypothesized that Id proteins are differentially regulated by the TGFβ family members through intracellular smad signaling pathways. We therefore localized the smad proteins in the ovine ovary *in vivo* and investigated the

effects of ovarian BMPs [BMP6, BMP15, and/or growth differentiation factor (GDF) 9] and activin A on the expression of *Id* genes in primary cultures of nonluteinized granulosa cells *in vitro*. Herein we report the consistent localization of the Id proteins in the ovine ovary *in vivo* and their differential gene regulation by BMPs and activin A *in vitro*.

Materials and Methods

Reagents

All reagents and chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK), unless otherwise stated.

Immunohistochemistry

Ovaries from Scottish Greyface lambs (n = 12), pregnant ewes (n = 15), and nonpregnant cycling ewes (n = 5), selected from the control cohort of additional studies, were obtained after local ethical committee and regulatory approval. Tissue was fixed in Bouin's solution for 24 h, transferred to 70% ethanol, and embedded in paraffin wax.

Tissue sections (5 μm) were dewaxed and rehydrated through an alcohol series before undergoing antigen retrieval by pressure cooking in 0.01 M sodium citrate buffer (pH 6.0) for 5 min. Sections were washed in PBS (twice for 5 min) and placed in 3% H_2O_2 diluted in distilled water for 10 min, followed by PBS washes (twice for 5 min). Tissue was then blocked with 20% normal goat serum and 5% BSA diluted in PBS for 1 h. Primary antibodies (Table 1) were diluted in blocking solution and incubated with tissue overnight at 4 C. Sections were washed with PBS plus 1% Tween 20 (twice for 5 min) before incubation with a biotinylated goat antirabbit IgG secondary antibody (Dako,

TABLE 1. List of primary, secondary, and tertiary antibodies used for immunohistochemistry (IH) and immunofluorescence (IF)

Antigen	Antibody clone/source	Dilution	Secondary antibody[a]	Tertiary antibody[b]
Cleaved caspase-3	Polyclonal rabbit (Asp175, 9661; Cell Signaling Technology Inc., Beverly, MA)	IH 1:100	1	
Id1	Polyclonal rabbit (C20; sc-488, BP sc-488 P) Santa Cruz Biotechnology Inc. (Santa Cruz, CA)	IH 1:1000; IF 1:500	1	A
Id2	Polyclonal rabbit (C20; sc489, BP sc-489 P; Santa Cruz	IH 1:500	1	
Id3	Polyclonal rabbit (C20; sc490, BP sc-490 P; Santa Cruz	IH 1:400	1	
Id4	Polyclonal rabbit (L20; sc491) BP (sc-491 P; Santa Cruz)	IH 1:200	1	
p-Smad 2/3	Polyclonal rabbit (Ser465/46; 3101; Cell Signaling)	IH 1:500	1	
p-Smad 1/5/8	Polyclonal rabbit (Ser463/465)/(Ser463/465)/ (Ser426/428; 9511; Cell Signaling)	IH 1:50; IF 1:300	2	B
Smad 6	Polyclonal rabbit (IMG-555; Imgenex Corp., Cambridge BioScience, Cambridge, UK)	IH 1:100; IF 1:600	2	B
Smad 7	Polyclonal rabbit (IMG-531; Imgenex)	IH 1:100	1	

[a] 1, Biotinylated goat antirabbit IgG (Dako, Glostrup, Denmark) 1:500; 2, peroxidase goat antirabbit (Dako) 1:200.
[b] A, Streptavidin Alexafluor 546 (s-11225; Invitrogen, Paisley, UK) 1:200; B, tyramide fluorescein (PerkinElmer) 1:50.

Glostrup, Denmark) at a 1:500 dilution. After additional washes in PBS (twice for 5 min), tissue was incubated with Vectastain ABC Elite tertiary complex (PK-1600 series; Vector Laboratories, Peterborough, Cambridgeshire, UK) for 1 h, and washed in PBS (twice for 5 min) before colorimetric visualization by incubation with 3,3′-diaminobenzidine (Dako) for 30 sec. Tissue was rinsed in distilled water, dehydrated in alcohol, counterstained with hematoxylin, and mounted.

Primary antibodies were incubated with blocking peptide before application, or in the absence of a specific blocking peptide (smad 6 and smad 7), negative controls consisted of incubation with nonspecific rabbit Igs and omission of the primary antibody. To ensure the binding specificity of smad 6 and smad 7, Western blotting was additionally carried out as previously described (24), using protein extracted from ovine granulosa cells. Specific bands were visualized for smad 6 and smad 7 at approximately 52 and 42 kDa, respectively, as anticipated (data not shown). Histological images were captured using an Olympus Provis BX2 microscope (Olympus America Inc., Center Valley, PA) equipped with a Canon E0S 30D Microcam camera (Canon Inc. Headquarters, Tokyo, Japan).

Immunofluorescence

Sheep ovary sections were treated in the same way as for immunohistochemistry but for the following alterations to the protocol. After incubation with the secondary antibody on d 2 and washing, the appropriate tertiary antibody was applied (Table 1) for 1 h if using streptavidin or 10 min if using tyramide detection systems. Sections were subsequently retrieved by microwaving for 2.5 min in 0.01 M sodium citrate buffer (pH 6.0) (tyramide detection system only) and then blocked in normal goat serum for 1 h before overnight incubation with the second primary antibody at 4 C. On d 3, the appropriate secondary and tertiary complexes were applied as described above, and the sections were counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen, Paisley, UK) diluted 1:1000 in PBS for 10 min before mounting with PermaFluor (Thermo Fisher Scientific, Cheshire, UK). Images were captured using an LSM 510 Meta confocal microscope and Zen 2008 software (Carl Zeiss Ltd., Welwyn Garden City, UK).

Quantification of thecal Id expression in healthy and atretic follicles

To obtain a quantitative measurement of thecal Id expression in follicles, staining intensity for Id1, Id2, Id3, and Id4 proteins were classified in three groups: absent, partial, or intense stain. Follicles were classified as atretic or nonatretic based on immunostaining for activated caspase-3 in a serial section. Forty nonatretic and 40 atretic follicles (50:50 preantral and antral) were examined across six to eight different ovaries, and a count was performed categorizing follicles into the appropriate staining class based on clear visual reference pictures agreed by three observers. Proportional data were presented as a percentage of the total number of follicles examined in each group and examined using a χ^2 test.

Cell culture and quantitative real-time (qRT)-PCR

Granulosa cells were collected and cultured as previously described (25). Briefly, follicles (<3.5 mm) were collected from ovaries of 10 Scottish Greyface ewes during the estrous cycle. Follicles were hemisected and the granulosa cells collected by flushing thecal shells using a 1-ml syringe. The supernatant containing granulosa cells was removed and cells collected by centrifugation, washed, and resuspended in culture medium containing McCoys 5a medium with sodium bicarbonate, supplemented with 0.1% BSA, 0.5 × penicillin/streptomycin, 3 mM L-glutamine, 5 μM transferrin, 0.3 mM testosterone, 4 nM selenium, 0.01 μM insulin, 0.01 μM ovine IGF-I LR3 (Novozymes Biopharma AU Ltd., Adelaide, Australia), and 1 nM FSH (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Three separate experiments consisted of pooling granulosa cells from three to four animals to obtain sufficient numbers. Around 75,000 granulosa cells per well were cultured in 200 μl medium with the addition of 100 ng/ml activin A and/or BMP6, BMP15, and/or GDF9 (R&D Systems, Abingdon, UK) in treatment wells, or carrier only in control wells at 37 C/5% CO$_2$ for 24 h. These concentrations were chosen based on the existing literature regarding BMP and activin effects on ovine steroidogenic cells (26, 27).

Cells were lysed, and RNA was extracted using the QIAGEN (West Sussex, UK) RNeasy Micro Kit. Lysed cells from two to three wells were pooled to obtain adequate RNA concentration and purity (A$_{260}$/A$_{280}$ ratio), which were measured using a

TABLE 2. List of primer sequences used for qRT-PCR and amplicon size

| Gene (accession)	Nucleotide sequence (5′–3′)	Product size (bp)
Id1 (NM_001097568)		
Forward	TCTGGGATCTGGAGTTGGAG	151
Reverse	ATACGATCGTCCGCTGGAA	
Id2 (NM_001034231)		
Forward	CATCTTGGACTTGCAGATCG	185
Reverse	AGAGAGCTTTGCTGTCATTTG	
Id3 (NM_001014950)		
Forward	ACTCAGCTTAGCCAGGTGGA	160
Reverse	TTTGGTCGTTGGAGATCACA	
Id4 (NM_001546)		
Forward	TCACTGCGCTCAACACCGACC	132
Reverse	TTCCCCCTCCCTCTCTAGTGCTCCTG	
GAPDH (NM_001034034)		
Forward	GGCGTGAACCACGAGAAGTATAA	229
Reverse	AAGCAGGGATGATGTTCTGG	
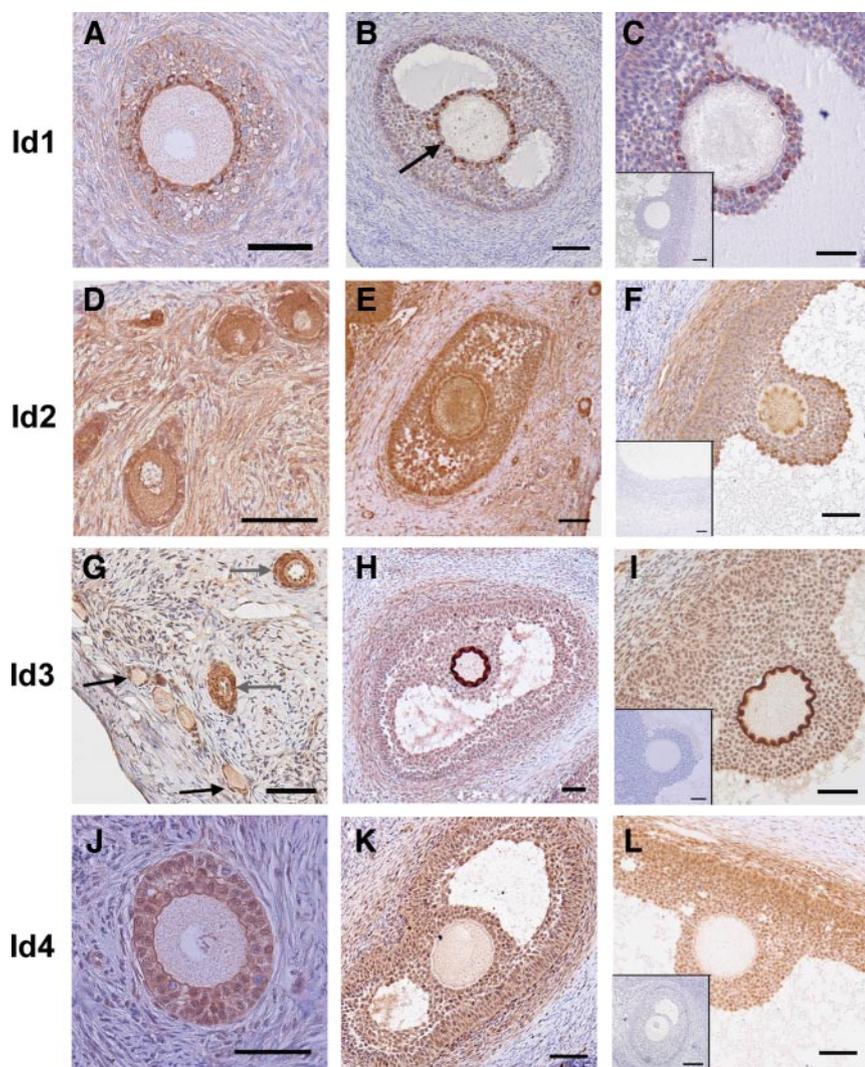
FIG. 1. Immunohistochemistry for Id1 (A–C), Id2 (D–F), Id3 (G–I), and Id4 (J–L) protein (*brown*) in the sheep ovary illustrates, from *left to right*, staining in primordial or primary, secondary or early antral, and tertiary or late antral follicles. *Arrow* in B indicates intense peri-oocytic Id1 expression. *Insets* in C, F, I, and L are negative controls using the respective Id antibody binding protein to confirm antigen-specific binding. *Scale bars*, 50 μm.

cDNA with nuclease-free water. The relative expression level of each target gene to *GAPDH* was quantified using the $\Delta\Delta Ct$ method, and the control data were standardized between runs. Data are presented as the mean ± SEM, and statistical analysis was performed using a one-way ANOVA with Bonferroni pair-wise comparison after logarithmic transformation. *P* values < 0.05 were regarded as significant.

Results

Id proteins are expressed in ovine follicles

Id proteins were localized in sheep ovaries from different functional stages including prepubertal, pregnant, and nonpregnant states. Specific immunostaining was observed for Id1, Id2, Id3, and Id4 in the follicles of all ovaries examined (Fig. 1). These were consistently localized to granulosa cells in follicles throughout development from primary to preovulatory stages. In addition, consistently weaker staining was observed in the theca cells of these follicles (Fig. 1). Id2 immunostaining was also apparent in the oocytes at all follicular stages, and there was strong antibody binding observed across the ovarian stroma (Fig. 1, D–F). Weak Id3 expression was visible in the oocytes of primordial follicles (Fig. 1G; *black arrows*), whereas the zona pellucida exhibited a high level of Id3 expression across follicle development (Fig 1, H and I). Specific immunostaining was also observed in endothelial cells for each Id protein examined (Fig. 1), particularly Id3 where there was strong blood vessel localization of this protein (Fig. 1G; *gray arrows*).

Differential localization of Ids in granulosa cells

The Id1 antibody revealed intense staining of peri-oocytic cumulus cells (Fig. 1, A–C), a striking observation that was reproduced across follicular development and in all specimens, including prepubertal lamb and pregnant sheep ovaries as well as cycling ovaries. Although Id4 was more intensely immunostained than Id3, their localization was similar across the granulosa cells and was maintained throughout follicle development (Fig. 1, G–L). However, unlike Id1, Id3, and Id4, immunoreactivity for Id2 was consistently most intense in those mural granulosa imme-

NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA was stored at −80 C until cDNA was synthesized from 200 ng total RNA per reaction using the high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA) and thereafter stored at −20 C.

Primer sets were designed for amplification of partial regions of target genes by qRT-PCR (Table 2). Primers were prevalidated in the sheep using conventional PCR, and DNA sequencing was performed to confirm the authenticity of the product. In addition to this, primer efficiency was tested by generating standard curves (cDNA diluted 1:2, 1:4, 1:8, and 1:16) in qRT-PCR. A 10-μl final reaction volume was prepared using 2× PowerSYBR Green PCR Master Mix (5 μl; Applied Biosystems), 5 μM primer pairs (0.5 μl), cDNA (1 μl), and nuclease-free water. The qRT-PCR cycling program consisted of a denaturing step (95 C for 10 min), annealing and extension step (95 C for 15 sec, 60 C for 1 min) repeated 40 times, and a dissociation step (95 C, 60 C, and 95 C for 15 sec each). Each reaction was carried out in duplicate. Negative controls included a reaction using cDNA prepared with the omission of reverse transcriptase and a reaction substituting
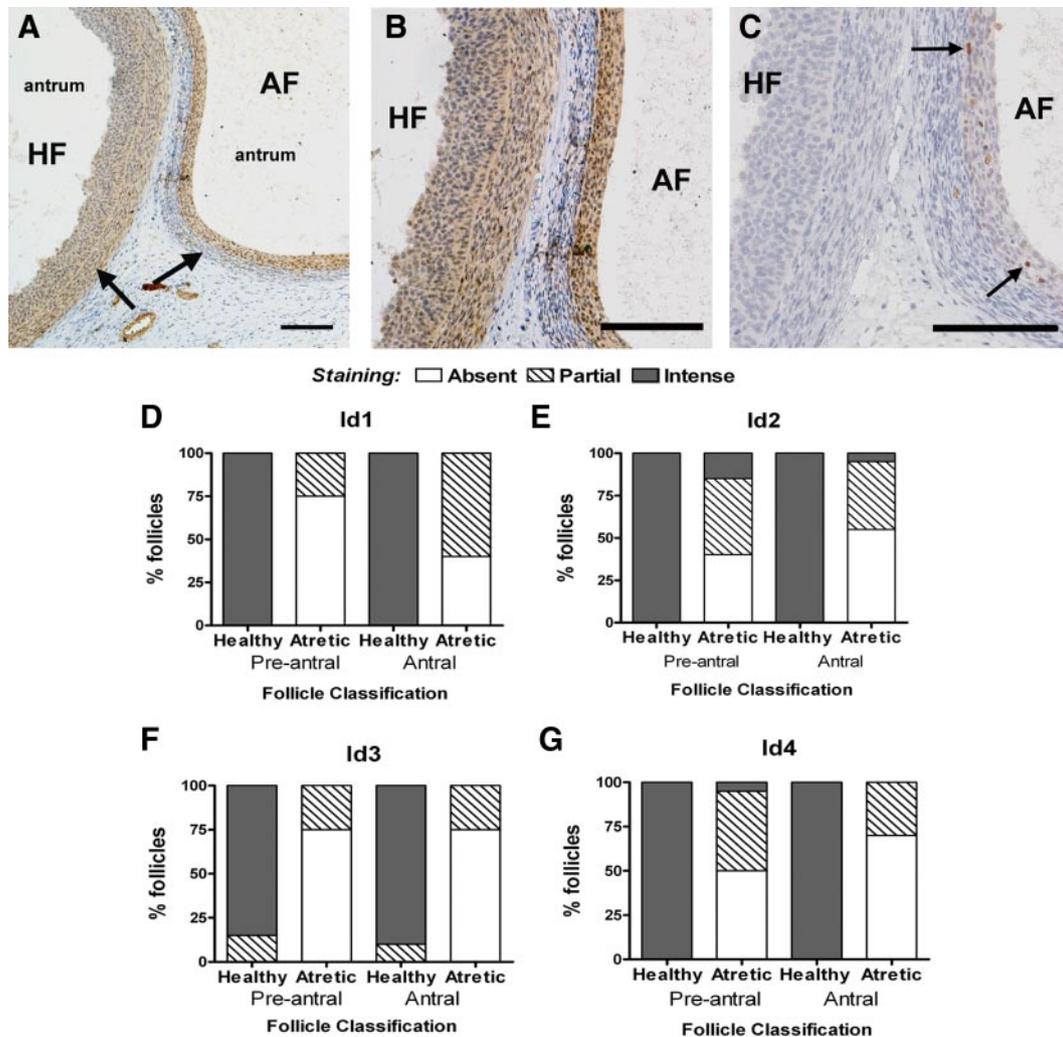
FIG. 2. Id2 expression in adjacent healthy and atretic follicles at low (A) and high (B) magnification and activated caspase-3 protein expression (C) in the corresponding ovine follicles. *Arrows* in A show thecal layer and in C depict positive caspase immunoreactivity indicating apoptosis in those cells. *Scale bars*, 50 μm. Absent, partial or intense Id1 (D), Id2 (E), Id3 (F), and Id4 (G) expression was classified in thecal cells of healthy and atretic, preantral, and antral follicles and is illustrated for each Id. The χ^2 statistical analysis showed a significant difference between the healthy and atretic categories for Id1 ($P < 0.0001$), Id2 ($P < 0.0001$), Id3 ($P < 0.0001$), and Id4 ($P < 0.0001$).

diately adjacent to the antral cavity of tertiary follicles (Fig. 1F).

Id immunostaining is reduced in the theca cells of atretic follicles

There was differential staining of Id proteins in the theca cells of some follicles (Fig. 2A). Id1, Id2, Id3, and Id4 protein expression was reduced in the thecal layer of atretic follicles, by reference to activated caspase-3 immunostaining in serial sections (Fig. 2C), whereas granulosa cell expression was maintained (Fig. 2, A and B). This phenomenon was encountered consistently in atretic follicles and could be quantified so that intense antibody staining in the thecal layer of healthy follicles was lost, resulting in partial or absent expression of Id1, Id2, Id3, and Id4 proteins in follicles undergoing atresia (Fig. 2, D–G; $P < 0.0001$). No significant differences for Id pro-

tein expression or alterations in thecal cells in atresia were observed between preantral and antral follicles (Fig. 2).

Lack of specific peri-oocyte phospho-(p-)smad immunostaining in ovine follicles

The most striking feature of Id localization in the ovine ovary was the intense staining for Id1 in the cumulus granulosa cells closest to the oocyte. We therefore hypothesized that an oocyte-derived paracrine factor may regulate the expression of Ids in the neighboring cells. Because members of the TGFβ superfamily are excellent candidates for an oocyte-secreted factor, we investigated the localization of the main TGFβ signaling pathways in the follicles. The p-smad 1/5/8 proteins could be localized to the nucleus of both theca and granulosa cells (Fig. 3, A and B). Indeed, the punctate pattern of more intense staining (Fig. 3B) suggests a specific regulated role for this pathway
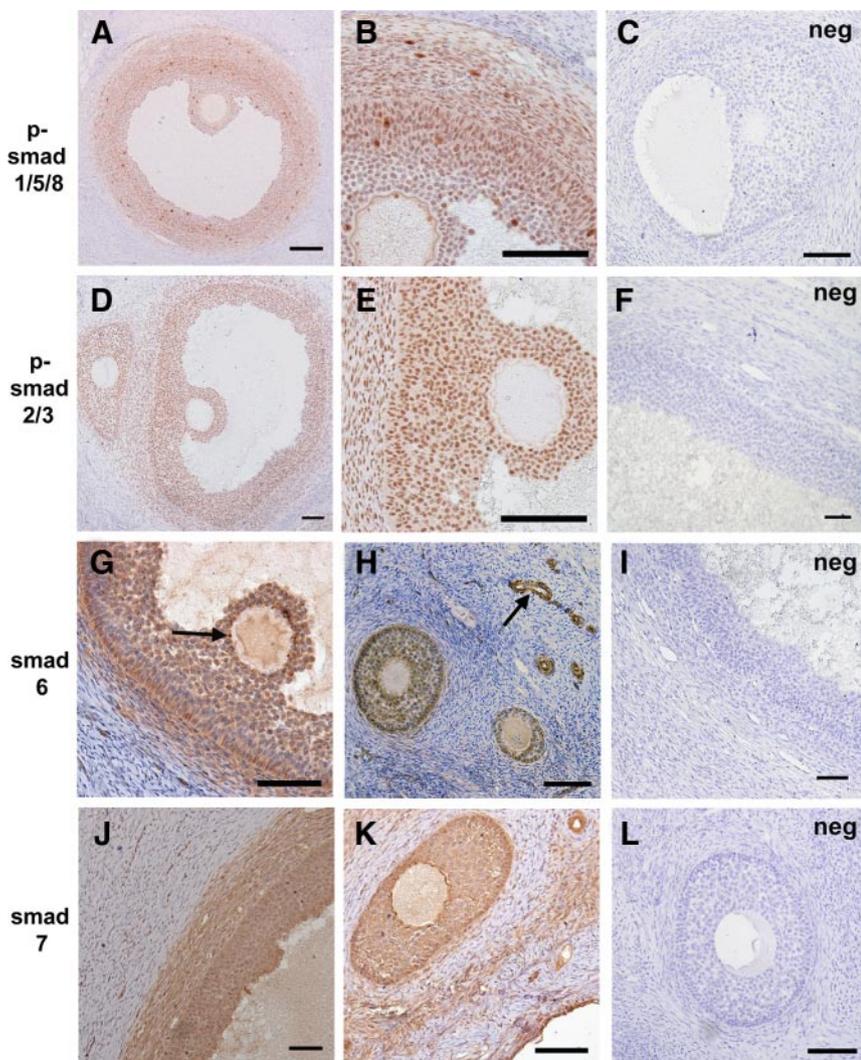
FIG. 3. Immunohistochemistry (*brown*) for p-smad 1/5/8 (A and B), p-smad 2/3 (D and E), smad 6 (G and H), and smad 7 (J and K) in the sheep ovary. *Arrow* in G identifies smad 6-positive immunostaining in granulosa cells and in H indicates staining in a blood vessel. Respective negative controls in C, F, I, and L represent omission of the primary antibody. *Scale bars*, 50 μm.

Smad 6 revealed a similar localization to smad 1/5/8 where staining was limited to some granulosa and thecal cells only (Fig. 3, G and H). However, more intense nuclear staining was observed in some cumulus cells, notably around the oocyte, and also in cortical blood vessels (*arrows* in Fig. 3, G and H, respectively). Smad 7 staining was more uniform in the granulosa and thecal cells of all of the follicles examined (Fig. 3, J and K). To ensure the specificity of smad 6 and 7 antibody binding in the sheep, Western blotting for these proteins was also performed in ovine granulosa cell protein extracts. Specific bands were visualized for both smad 6 and 7 at the expected molecular weight, confirming the expression of these proteins in this cell population and supporting the specificity of the immunohistochemistry.

The relationship of the smads to Id1 was subsequently investigated using co-immunofluorescence (Fig. 4). The p-smad 1/5/8 did not colocalize in the cumulus layer with Id1 (Fig. 4A). There was, however, some colocalization between smad 6 and Id1 staining in the peri-oocyte granulosa cells (Fig. 4B). This suggests that manipulation of the smad signaling system may yet have a role in the specific staining pattern of Id1 in the peri-oocyte granulosa cells.

in such cells. However, the staining was not increased or decreased in the granulosa cells next to the oocyte in any follicle examined. The alternative p-smad 2/3 pathway could also be detected in nuclei of both theca and granulosa cells of the follicle (Fig. 3, D and E). Although this staining was more uniform, it also did not demonstrate a differential staining pattern in the granulosa cells surrounding the oocyte. Although it is clear that the cells surrounding the oocyte operate both smad 1/5/8 and 2/3 pathways, the consistent immunostaining pattern did not reveal a direct relationship with the peri-oocyte Id1 expression in cumulus granulosa cells.

Peri-oocyte localization of smad 6 in granulosa cells

Both of the inhibitory smads, smad 6 and smad 7, were found to have widespread ovarian expression (Fig. 3).

Activin and BMP signaling regulates *Id* gene expression in ovine granulosa cells *in vitro*

To investigate the effect of stimulating the smad 2/3 and smad 1/5/8 pathways on *Id* expression, ovine granulosa cells were cultured with activin A and/or BMP6 and the levels of Id gene mRNA were analyzed by qRT-PCR. Overall, ANOVA revealed that *Id1* ($P < 0.0001$), *Id2* ($P < 0.0001$), *Id3* ($P < 0.0001$), and *Id4* ($P < 0.05$) were all changed by the treatments in the same pattern (Fig. 5). Stimulation with activin A led to the down-regulation of *Id1* and *Id3* mRNA expression, whereas conversely, BMP6 treatment up-regulated these genes (Fig. 5; Bonferroni pair-wise analysis $P < 0.001$). It was particularly notable for *Id1* expression where activin A and BMP6 treatment led to a 5-fold decrease and 7-fold increase in mRNA, respectively (Fig. 5A). Furthermore, treatment
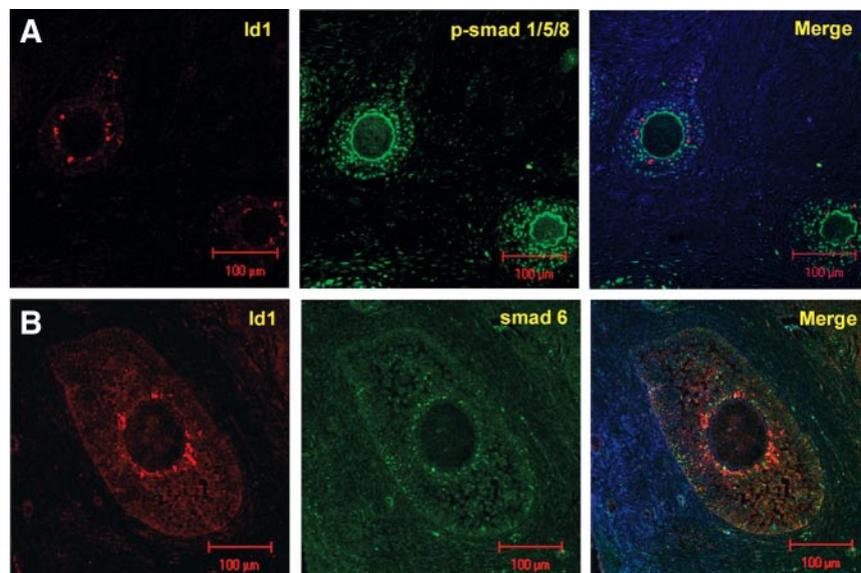
FIG. 4. Co-immunofluorescence of Id1 (*red*) with p-smad 1/5/8 (*green*) (A) and smad 6 (*green*) (B) in follicles of the sheep ovary. Merged pictures include 4′,6-diamidino-2-phenylindole nuclear counterstain (*blue*). *Scale bars*, 100 μm.

with activin A significantly negated the BMP6-induced up-regulation of the *Id*1 (Fig. 5A; Bonferroni pair-wise analysis $P < 0.05$).

To further explore the potential roles of TGFβ-mediated regulation of Id1 expression in the cumulus cells, an experiment was conducted where the granulosa cell population received doses of oocyte-secreted factors BMP15 and/or GDF9, and *Id1* gene expression was measured (Fig. 5B; ANOVA $P < 0.001$). Similarly to the effects produced with the BMP6 cultures, BMP15 treatment also led to an increase in *Id1* transcript (Fig. 5B; Bonferroni pair-wise analysis $P < 0.01$). GDF9 alone did not significantly alter *Id1* gene expression; however, in combination with BMP15, this increased 8-fold (Fig. 5B; Bonferroni pair-wise analysis $P < 0.001$).

Discussion

This study describes Id protein expression across the developing mammalian ovary and provides evidence for functional roles in normal adult tissue. We have shown for the first time that Id1, Id2, Id3, and Id4 are expressed in the granulosa and theca cells of healthy mammalian ovarian follicles across development. The Ids have two main functions: 1) to maintain proliferation and 2) to inhibit differentiation (4, 6). Such regulation during development ensures that the appropriate level of growth leading to patterning can occur before lineage commitment and the terminal differentiation of cells. The adult ovary similarly undergoes persistent tissue remodeling, resulting in the growth and differentiation of somatic follicular cells that

house the oocyte. Bidirectional paracrine communication between theca cells, granulosa cells, and the oocyte result in the accumulation of genetic and developmental competence for ovulation, fertilization, and subsequent embryogenesis. Id proteins are differentially expressed throughout development in many species including the mouse and *Xenopus laevis* and can have nonredundant actions as well as cell-specific roles (8, 28). We propose a role for the Id proteins in the sheep ovary that might involve transcriptional regulation critical for normal folliculogenesis that may be analogous to those observed during development.

Cumulus cell-specific Id1 expression as well as the somatic cell-wide Id protein expression was hypothesized to be regulated by activin and/or BMPs, present in the mammalian ovary, which are known to promote growth and differentiation in granulosa and thecal cells of various species (29–31). The steroidogenic cells of the adult ovary are fairly transient and possess similar characteristics to embryonic tissues or progenitor cells of which there are widespread reports of TGFβ-mediated Id mRNA and protein regulation (15, 16, 18). Here, we show that activin and BMPs can alter *Id* gene expression in ovine granulosa cells *in vitro*, and moreover, follicles have the cellular machinery for TGFβ signaling *in vivo*. This was demonstrated in ovine granulosa and thecal cells by the expression of p-smad 1/5/8 and p-smad 2/3 and the presence of smad 6 and smad 7 inhibitors indicating regulation of TGFβ signaling.

Id proteins may be regulated in ovine granulosa cells by various BMPs and activin that act in an opposing way to balance growth and differentiation appropriately. *Id1* and *Id3* expression was significantly increased after BMP6 stimulation of granulosa cells *in vitro*, whereas activin A down-regulated these genes. Therefore, Id protein regulation in the sheep ovary may be a target not only of BMPs but also of activin. The effect of activin on Id function is not well described; however, our findings are consistent with a previous study reporting a negative association with activin treatment and *Id* gene expression (17). Reports of the specific expression of BMPs in the ruminant follicle vary (30, 32, 33); however, it is probable that BMP4, -6, and -7 have actions in these cells and may all regulate Id expression, although this is yet to be established. We also found that treating cells with both activin A and BMP6 reduced the BMP-mediated up-regulation of
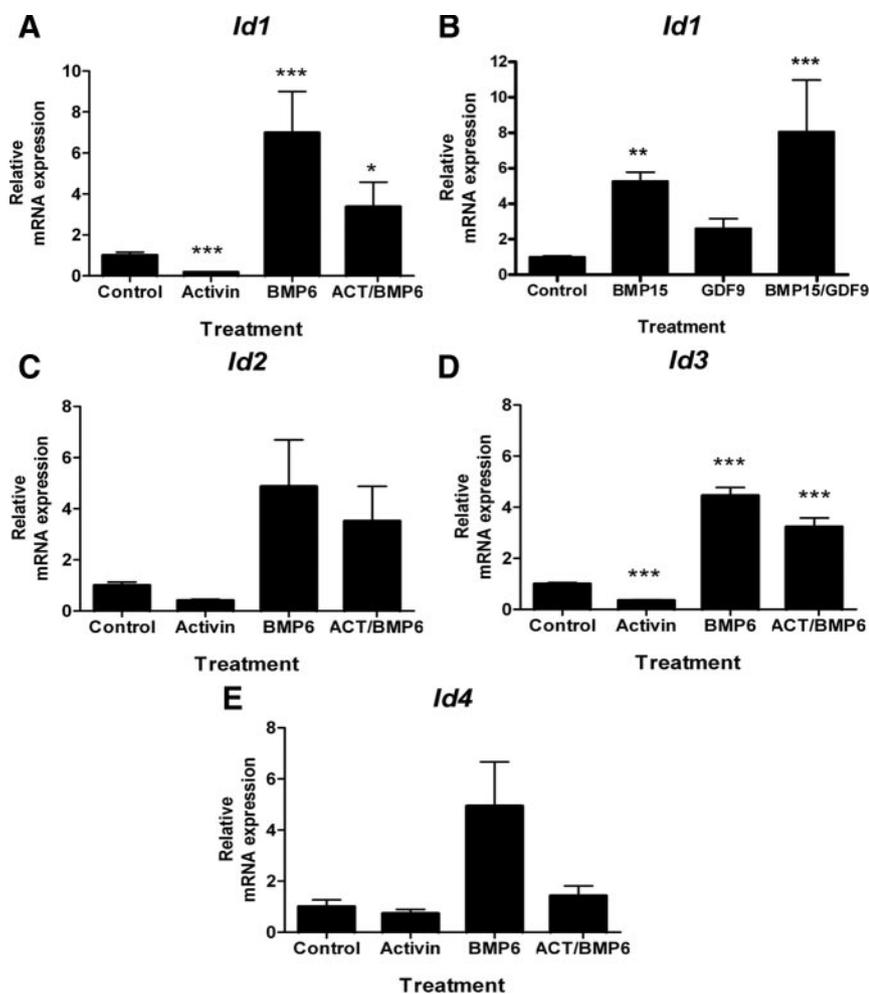
FIG. 5. Effect of activin A and/or BMP6 (A) and GDF9 and/or BMP15 (B) on *Id1* gene expression and activin A and/or BMP6 on *Id2* (C), *Id3* (D), and *Id4* (E) gene expression in ovine granulosa cells cultured for 24 h. Values are mean ± SEM of relative mRNA levels standardized to control values (n = 3; three experiments using cells from a total of 10 sheep). An ANOVA revealed overall significant differences for each gene: *Id1* (A, $P < 0.0001$, and B, $P < 0.001$), *Id2* ($P < 0.0001$), *Id3* ($P < 0.0001$), and *Id4* ($P < 0.05$) and a pair-wise comparison with the control column using the Bonferroni post test are illustrated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Id genes, suggesting a competing regulatory role for these paracrine molecules.

The universally increased Id1 protein expression observed in peri-oocytic cumulus cells in follicles throughout development suggests a specific regulatory mechanism exerted by Id1 that is likely mediated by paracrine signaling from the oocyte. Although this study revealed TGFβ-mediated signaling via smad 1/5/8 and/or smad 2/3 across the whole follicle, neither pathway could be directly linked to the cumulus-specific Id1 up-regulation. The p-smad 2/3 was strongly but not differentially localized in granulosa cells, and although p-smad 1/5/8 expression was more limited to certain cells, this was not specific to the cumulus layer. In fact, co-immunofluorescence established that in the cumulus cells, Id1 and p-smad 1/5/8 were not present in the same cell and thus led to a hypothesis that smad 1/5/8 and/or smad 2/3 may indirectly regulate Id1 in these

cells. Intermediary factors might include the inhibitory smads, and it was observed that smad 6 displayed a more differential expression pattern with some up-regulation in the cumulus cells as well as some colocalization with the Id1 protein. Smad 6 may repress smad signaling in some of these cells, although the factors regulating such an interaction are unknown, and further work is required to investigate this. Specific oocyte-secreted factors in the sheep include TGFβ signaling molecules such as BMP6 (30) and BMP15 (GDF9b) (34) that activate smad 1/5/8 signaling in granulosa cells (35). BMP15 stimulates granulosa cell proliferation while inhibiting differentiation (36, 37) and is essential for folliculogenesis in the sheep (34).

Alternatively, the lack of colocalization observed between Id1 and p-smad 1/5/8 raises the possibility that the smad 2/3 pathway could in fact be responsible for the up-regulation of Id1 in perioocytic cumulus cells. Activin, an activator of smad 2/3, led to a decrease in *Id1* message in granulosa cell culture experiments. However, this effect could be cell specific because the majority of granulosa cells in these cultures are mural, and the response may well be different in cumulus granulosa cells modulated by factors secreted by the oocyte. GDF9, an oocyte-secreted TGFβ member closely related to BMP15 (38), is an excellent candidate for triggering such a response. Its signaling pathway is less clear because it can influence both smad 1/5/8 and smad 2/3 responses (39). GDF9 signaling has been shown to be essential for cumulus cell function where activation of smad 2/3 brings about the up-regulation of cumulus-specific genes (40). This paracrine signaling through smad 2/3 is crucial for normal cumulus cell expansion, resulting in a local milieu enabling the acquisition of oocyte developmental competence (41–43). We showed *in vitro* that GDF9 (not significant) and BMP15 can in fact increase *Id1* gene expression in cultured ovine granulosa cells, and both ligands together enhance this response further, although we reiterate that these findings are limited to a largely mural granulosa cell population that may differ from cumulus cells. In context, this latter finding is interesting because the synergy between these

growth factors is known to be important for granulosa cell proliferation in the sheep (44). We hypothesize that although the surrounding granulosa cells produce activin that might negate Id1 expression via smad 2/3, TGFβ signal from the oocyte such as BMP6, BMP15, and/or GDF9 may lead to Id1 up-regulation in these cumulus cells.

We also demonstrate the down-regulation of Id proteins in the thecal cells of atretic ovine follicles, which are not altered in the granulosa cells. Follicular atresia occurs when FSH levels decrease during the follicular phase of the estrous cycle, leading to apoptosis in subordinate follicles (45). The present study identifies a differential change in Id protein distribution in these follicles that may be triggered by autocrine or intracrine thecal signaling, which is potentially inhibitory and does not effect granulosa cell expression. Because most changes associated with atresia are detected in the granulosa cell population, these theca-specific changes are of interest. However theca-specific Id protein down-regulation in atretic follicles is merely associative with no evidence at this stage of a causal relationship with further work required to clarify the significance of such findings.

This study is the first to our knowledge to carry out a comprehensive immunohistochemical examination of the Id proteins in the adult mammalian ovary. Id1, Id2, Id3, and Id4 are present in the ovine ovarian follicle and may be regulated by BMPs and/or activin via smad signaling. Further studies are required to establish how important the Ids are for the regulation of proliferation and differentiation of steroidogenic cells occurring during folliculogenesis and the physiological significance for fertility and reproductive diseases.

Acknowledgments

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