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Functional link between bone morphogenetic proteins and insulin-like peptide 3 signaling in modulating ovarian androgen production

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Bone morphogenetic proteins (BMPs) are firmly implicated as intraovarian regulators of follicle development and steroidogenesis. Here we report a microarray analysis showing that treatment of cultured bovine theca cells (TC) with BMP6 significantly (>twofold; $P < 0.01$) up- or down-regulated expression of 445 genes. Insulin-like peptide 3 (*INSL3*) was the most heavily down-regulated gene (−43-fold) with cytochrome P450, subfamily XVII (*CYP17A1*) and other key steroidogenic transcripts including *s*teroidogenic acute regulatory protein (*STAR*), cytochrome P450 family 11, subfamily A1 (*CYP11A1*) and 3 beta-hydroxysteroid dehydrogenase type 1 (*HSD3B1*) also down-regulated. BMP6 also reduced expression of nuclear receptor subfamily 5A1 (*NR5A1*) known to target the promoter regions of the aforementioned genes. Real-time PCR confirmed these findings and also revealed a marked reduction in expression of INSL3 receptor, relaxin/insulin-like family peptide receptor 2 (*RXFP2*). Secretion of INSL3 protein and androstenedione were also suppressed suggesting a functional link between BMP and INSL3 pathways in controlling androgen synthesis. RNAi-mediated knockdown of *INSL3* reduced *INSL3* mRNA (75%) and protein (94%) level and elicited a 77% reduction in *CYP17A1* mRNA and 83% reduction in androstenedione secretion. Knockdown of *RXFP2* also reduced CYP17A1 expression (81%) and androstenedione secretion (88%). Conversely, treatment with exogenous (human) INSL3 increased androstenedione secretion ∼twofold. The CYP17A1 inhibitor abiraterone abolished androgen secretion and reduced expression of both *INSL3* and *RXFP2*. Collectively, these findings indicate a positive autoregulatory role for INSL3 signaling in maintaining thecal androgen production, and *visa versa*. Moreover, BMP6-induced suppression of thecal androgen synthesis may be mediated, at least in part, by reduced INSL3-RXFP2 signaling.

growth factor | ovary | reproduction

Ovarian androgen synthesis is obligatory for normal ovarian follicle development, estrogen production, ovulation, and female fertility in animals and humans. However, ovarian hyperandrogenism, a feature of the widespread polycystic ovarian syndrome (PCOS) in women, is detrimental to fertility and has other adverse consequences for the affected individual (e.g., hirsutism, acne, and metabolic manifestations) (1, 2). Although pituitary luteinizing hormone (LH) has long been recognized as a primary driver of androgen synthesis by theca interna cells of the mammalian ovarian follicle (3, 4), evidence has emerged implicating a variety of intraovarian factors as having modulatory roles in this process, including inhibins, activins, and bone morphogenetic proteins (BMPs) (reviewed in refs 5–8). Additional proteins, including insulin-like growth factors (IGFs), EGF, TGF-α, and TNF-α, have also been shown to modulate thecal androgen production in different in vitro model systems (9–12).

Previous studies using a primary bovine ovarian theca interna cell culture model showed that different BMPs (BMP2, BMP4, BMP6, and BMP7) potently suppress basal and LH-induced androgen secretion (13) and that their action can be antagonized by inhibin (14). Because multiple BMPs, along with their signaling receptors, antagonists (e.g., inhibin), and extracellular binding proteins (e.g., gremlin, chordin, and noggin) are widely expressed in different intraovarian compartments, including theca (TCs), granulosa cells (GCs), and ovarian stroma (14–18), it is likely that BMPs exert autocrine/paracrine actions to regulate follicular steroidogenesis, as well as other aspects of ovarian function (5, 8). Our previous findings led us to hypothesize that a functional deficit in thecal BMP signaling could be a contributory factor in the etiology of hyperandrogenemic disorders such as PCOS (13). Conversely, a functional excess of thecal BMP signaling could contribute to androgen insufficiency. Both situations compromise normal follicle development, leading to subfertility or infertility in animals and humans, as well as other extraovarian consequences arising from the perturbation of sex hormone output.

To gain further insight into the mechanism through which BMP suppresses ovarian androgen production, we undertook a microarray analysis of global gene expression in bovine TCs exposed to BMP6 under both basal and LH-stimulated conditions. As documented here, this experiment led to the unexpected identification of insulin-like factor 3 (INSL3) as the most heavily down-regulated transcript in BMP-treated cells. INSL3 has a well-documented role in the development of the

Significance

Ovarian androgen synthesis is essential for normal ovarian follicle development and female fertility in animals and humans. However, ovarian androgen excess, a feature of the widespread polycystic ovarian syndrome in women, is detrimental to fertility and has other pathophysiological consequences. Our findings reveal the importance of the intraovarian growth factor insulin-like peptide 3 signaling for maintaining androgen production by ovarian theca cells and show that the suppressive action of bone morphogenetic proteins on androgen production is linked to their inhibitory effect on insulin-like peptide 3 signaling, likely mediated via down-regulation of the nuclear transcription factor steroidogenic factor-1.

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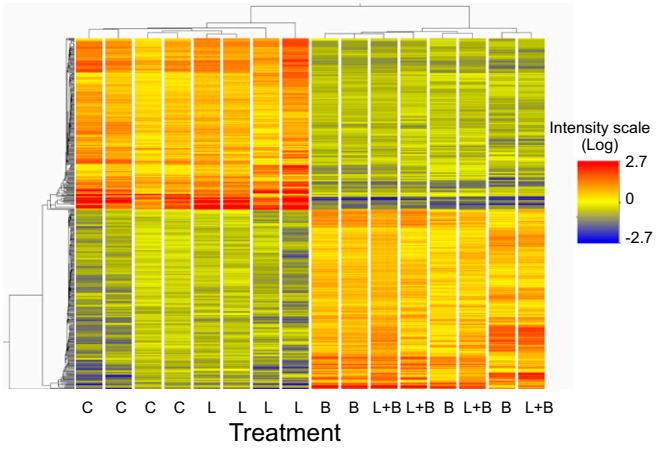

Fig. 1. Hierarchical cluster analysis and heatmap representing 498 differentially expressed genes (>twofold up- or down-regulation, $P < 0.01$ in at least one condition pair) among the four treatment groups (C, control; L, LH; B, BMP6; L+B, LH + BMP6; $n = 4$ arrays per treatment). Note the major effect of BMP treatment in comparison with the much more limited effect of LH treatment.

male reproductive system, being involved in the first (transabdominal) phase of testicular descent (19). Although it has been known for some time that INSL3 is also expressed in the adult ovary, primarily in TCs (20) and luteal tissue (21, 22), its potential physiological role in the regulation of ovarian function and steroidogenesis is poorly defined. On the basis of our microarray findings presented here, we therefore designed and executed a further series of experiments to (*i*) confirm that BMP treatment suppresses INSL3 secretion; (*ii*) establish the dose- and time-dependency of the response to BMP6; (*iii*) explore the possibility that INSL3 signaling is a requirement for thecal androgen production; (*iv*) investigate whether INSL3 signaling actually mediates the suppressive effect of BMP on thecal androgen production or, alternatively; (*v*) whether BMP comodulates expression of INSL3 along with one or more key components of the steroidogenic pathway.

Results

Microarray Analysis: Global Effect of BMP6 Treatment on TC Gene Expression. The 24,128 probesets on the array represented 19,023 unique transcripts, of which 15,464 remained on the list filtered to exclude those with low (unreliable) hybridization signal intensity. After filtering the data to enrich transcripts displaying a significant difference (corrected $P < 0.01$) in at least one of four condition pairs, 2,550 transcripts remained. After filtering this list to only retain transcripts satisfying a fold change cutoff ≥2 [corrected $P < 0.01$; ANOVA with post hoc Tukey's honestly significant difference (HSD) test] in at least one of the four condition pairs, there were 498 transcripts. Clustering analysis generated a hierarchical condition tree and heatmap representing this set of transcripts (Fig. 1). This shows a strong effect of BMP6 treatment on the gene expression profile, with a much weaker effect of LH. None of these differences in response to LH treatment was found to be statistically significant after applying Benjamini-Hochberg correction, despite the fact that, on a priori grounds, several of these genes, including steroidogenic acute regulatory protein (*STAR*) (2.8-fold up-regulation) and cytochrome P450, subfamily XVII (*CYP17A1*) (2.5-fold up-regulation) were expected to respond to LH treatment, being well-established targets of LH.

Given these results and to simplify further analysis focusing on the effect of BMP6, a decision was reached to combine basal and LH treatment groups to give two groups ($n = 8$ each), comparing cells cultured in the presence or absence of BMP6. This generated a filtered list of 445 unique transcripts that were significantly down- or up-regulated (>twofold; corrected $P < 0.01$) in response to BMP6 treatment. Tables 1 and 2 show the top 20 transcripts from these lists that were down- or up-regulated. The full gene lists may be found in Tables S1 and S2. Notable at the top of the list of genes down-regulated by BMP6 was *INSL3* (43-fold), with inhibin α (*INHA*; 14-fold; third place), *CYP17A1* (11-fold; sixth place), *STAR* (5.5-fold; 17th place), and *CYP11A1* (5.3-fold; 19th place) also heavily suppressed. Genes prominently up-regulated by BMP6 included uroplakin 1B (*UPK1B*; 7.2-fold), sterile α motif

Table 1. Rank order list showing top 20 of 202 transcripts identified by microarray analysis as being significantly ($P < 0.01$) down-regulated >twofold in bovine TCs in response to BMP6 treatment

Order	Affymetrix_ID	Entrez ID	Gene symbol	Gene title	Fold change
1	Bt.12186.1.S1_at	281870	*INSL3*	Insulin-like 3 (Leydig cell)	−43.47
2	Bt.11438.1.S1_at	511094	*TNNI3*	Troponin I type 3 (cardiac)	−15.24
3	Bt.4897.1.S1_at	281254	*INHA*	Inhibin, alpha	−14.44
4	Bt.16678.1.S1_at	767968	*BEX2*	Brain expressed X-linked 2	−12.21
5	Bt.1922.1.S1_at	NA	NA	NA	−11.90
6	Bt.3985.1.S1_at	281739	*CYP17A1*	Cytochrome P450, family 17, subfamily A1	−11.07
7	Bt.10630.1.S1_at	506584	*NMB*	Neuromedin B	−10.72
8	Bt.643.1.S1_at	NA	NA	NA	−10.57
9	Bt.13796.1.S1_at	783399	*LOC783399*	Major allergen Equ c 1-like	−9.59
10	Bt.12916.1.S1_at	281210	*GPX3*	Glutathione peroxidase 3 (plasma)	−7.73
11	Bt.23393.1.S1_at	515161	*ATP1A2*	ATPase, Na^+/K^+ transporting, alpha 2 polypeptide	−7.37
12	Bt.10150.1.S1_at	NA	NA	NA	−7.24
13	Bt.25052.1.S1_at	539360	*CAPN6*	Calpain 6	−7.05
14	Bt.21896.1.S1_at	NA	NA	NA	−6.22
15	Bt.12405.1.S1_at	538276	*LOC538276*	Similar to Janus kinase 3	−6.11
16	Bt.4760.1.S1_at	281824	*HSD3B1*	Hydroxy-steroid dehydrogenase, type 3 beta	−5.77
17	Bt.4709.1.S1_at	281507	*STAR*	Steroidogenic acute regulatory protein	−5.54
18	Bt.2122.1.S1_at	282604	*FDXR*	Ferredoxin reductase	−5.34
19	Bt.7190.1.S1_at	338048	*CYP11A1*	Cytochrome P450, family 11, subfamily A1	−5.28
20	Bt.25738.1.A1_at	505855	*ERP27*	Endoplasmic reticulum protein 27	−5.23

NA, not applicable.

Table 2. Rank order list showing top 20 of 243 transcripts identified by microarray analysis as being significantly ($P < 0.01$) up-regulated >twofold in bovine TCs in response to BMP6 treatment

Order	Affymetrix ID	Entrez ID	Gene symbol	Gene title	Fold change
1	Bt.4089.1.S1_at	282113	*UPK1B*	Uroplakin 1B	7.23
2	Bt.19485.1.A1_at	100140500	*SAMD11*	Sterile α motif domain containing 11	6.63
3	Bt.1730.1.A1_at	497011	*ID1*	Inhibitor of DNA binding 1	6.51
4	Bt.29871.1.S1_at	282497	*BOLA-DYA*	MHC, class II, DY alpha	6.51
5	Bt.25769.1.A1_at	539199	*LOC539199*	Similar to MAM domain containing 2	6.22
6	Bt.22389.1.S1_at	617336	*SHISA2*	Shisa homolog 2 (*Xenopus laevis*)	5.58
7	Bt.19909.1.A1_at	NA		NA	5.38
8	Bt.24625.1.S1_at	618793	*FOXO4*	Forkhead box O4	5.34
9	Bt.5007.1.A1_at	508098	*LOC508098*	Hypothetical LOC508098	5.33
10	Bt.23126.1.S1_at	282876	*NOS2*	Nitric oxide synthase 2, inducible	5.25
11	Bt.20501.1.S1_at	NA		NA	4.77
12	Bt.18639.1.A1_at	513776	*FBXO32*	F-box protein 32	4.62
13	Bt.26774.1.S1_at	537487	*IGSF10*	Ig superfamily, member 10	4.60
14	Bt.24212.1.S1_at	534650	*RSPO3*	R-spondin 3 homolog (*Xenopus laevis*)	4.55
15	Bt.22746.1.S1_at	505366	*WFDC1*	WAP four-disulfide core domain 1	4.34
16	Bt.20148.1.S1_at	NA		NA	4.30
17	Bt.13363.1.A1_at	NA		NA	4.23
18	Bt.15758.1.S1_at	282023	*PTGS2*	Prostaglandin-endoperoxide synthase 2	4.22
19	Bt.262.1.S1_at	404105	*TKDP3*	Trophoblast Kunitz domain protein 3	4.20
20	Bt.3546.1.S1_at	785924	*ROR2*	Receptor tyrosine kinase-like orphan receptor 2	4.14

NA, not applicable.

domain containing 11 (*SAMD11*; 6.6-fold), and inhibitor of differentiation/DNA binding 1 (*ID1*; 6.5-fold), the latter being a well-established BMP-responsive target gene.

The list of 445 genes significantly ($P < 0.01$) down- or up-regulated >twofold in response to BMP6 treatment was subjected to Gene Ontology (GO) enrichment analysis. A total of 89 GO classification terms were significantly ($P < 0.05$) overrepresented among these 445 transcripts: 12 terms in the Cellular Component category, 54 terms in the Biological Process category, and 23 terms in the Molecular Function category. This list showing the percentage of the 445 transcripts significantly overrepresented under each GO term is presented in Table S3.

RT–Quantitative PCR Validation of Microarray Data. To verify the microarray findings we carried out RT–quantitative PCR (RT-qPCR) analysis of a selection of transcripts, including *INSL3*, *STAR*, *CYP11A1*, 3 beta-hydroxysteroid dehydrogenase type 1 (*HSD3B1*), *CYP17A1*, 17-beta-hydroxysteroid dehydrogenase type 1 (*HSD17B1*), luteinizing hormone/choriogonadotropin receptor (*LHCGR*), inhibin alpha (*INHA*), nuclear receptor subfamily 5A1 (*NR5A1*), and *ID1*. Genes found to be significantly up- or down-regulated by BMP6 by microarray were correspondingly shown to be up- or down-regulated by RT-qPCR (Fig. 2). In addition, the cognate receptor for INSL3, relaxin/insulin-like family peptide receptor 2 (*RXFP2*), which was not represented on the microarray, was found by RT-qPCR to be heavily down-regulated by BMP6 under both basal and LH-stimulated conditions.

Effect of BMP6 on Secretion of INSL3 Protein. Fig. 3 provides confirmation that the inhibitory action of BMP6 on expression of INSL3 mRNA detected by microarray analysis and RT-qPCR is accompanied by a corresponding reduction in secretion of INSL3 protein by the cells.

Do Other BMPs Suppress Expression of INSL3 and RXFP2? Fig. 4 shows a comparison of the effects of BMP2, -4, -6, and -7 on androstenedione (A4) and INSL3 secretion and expression of mRNA for *INSL3* and its receptor *RXFP2*. At the dose level tested (10 ng/mL) all four BMPs had comparable inhibitory effects on each of the above endpoints.

Time Course of BMP6 Action on Expression of INSL3, RXFP2, and Other Transcripts. To examine the time course of the response to BMP6, cells were lysed at a fixed time on day 6 of culture, having been exposed to BMP6 (10 ng/mL) for the preceding 0-, 4-, 24-, 48-, or 96-h periods. As shown in Fig. 5*A* expression of *ID1*, a classic BMP-responsive gene, increased markedly in response to BMP6, with a significant increase (~fourfold; $P < 0.05$) detected as early as 4 h after starting treatment. At the same time point expression of *RXFP2* was significantly reduced (38%, $P < 0.05$). Expression of *INSL3*, *CYP17A1*, *STAR*, *CYP11A1*, *HSD3B1*, *LHCGR*, *NR5A1*, and nuclear receptor subfamily 0, group B, member 1 (*NROB1*) was down-regulated by BMP6, with each transcript following a broadly similar time course but with none showing a significant decline at 4 h after treatment. In each case the maximal decline was observed after the full 96-h period of BMP6 treatment. Fig. 5*B* shows the corresponding time course for A4 and INSL3 secretion.

Does Knockdown of Endogenous INSL3 or RXFP2 Affect CYP17A1 Expression and Androgen Secretion? As shown in Fig. 6*A*, transfection of TCs with an siRNA duplex targeted at *INSL3* reduced *INSL3* mRNA level by 75% ($P < 0.01$) and secreted INSL3 protein level by ~94% ($P < 0.01$) relative to values in cells exposed to nonsilencing control duplex. This was accompanied by a 77% reduction in *CYP17A1* mRNA level ($P < 0.01$) and 83% reduction in A4 secretion ($P < 0.05$). Fig. 6*B* shows that transfection with an siRNA duplex targeted at *RXFP2* reduced *RXFP2* mRNA level by 80% ($P < 0.01$) and that this also led to a marked reduction in *CYP17A1* mRNA level (81%; $P < 0.01$) and A4 secretion (88%; $P < 0.01$).

Does Synthetic Human INSL3 Modulate Androgen Secretion by Bovine TCs? As shown in Fig. 7 treatment of bovine TCs with LH elicited the anticipated biphasic response in terms of A4 secretion ($P < 0.001$), with a maximal response observed at 150 pg/mL Treatment with synthetic human INSL3 promoted a modest (~twofold) increase in A4 secretion under basal and LH-stimulated conditions ($P < 0.05$), although the effect was not dose dependent, being evident at the lowest dose level tested (0.5 ng/mL). There was no LH × INSL3 treatment interaction ($P = 0.9$, two-way ANOVA).

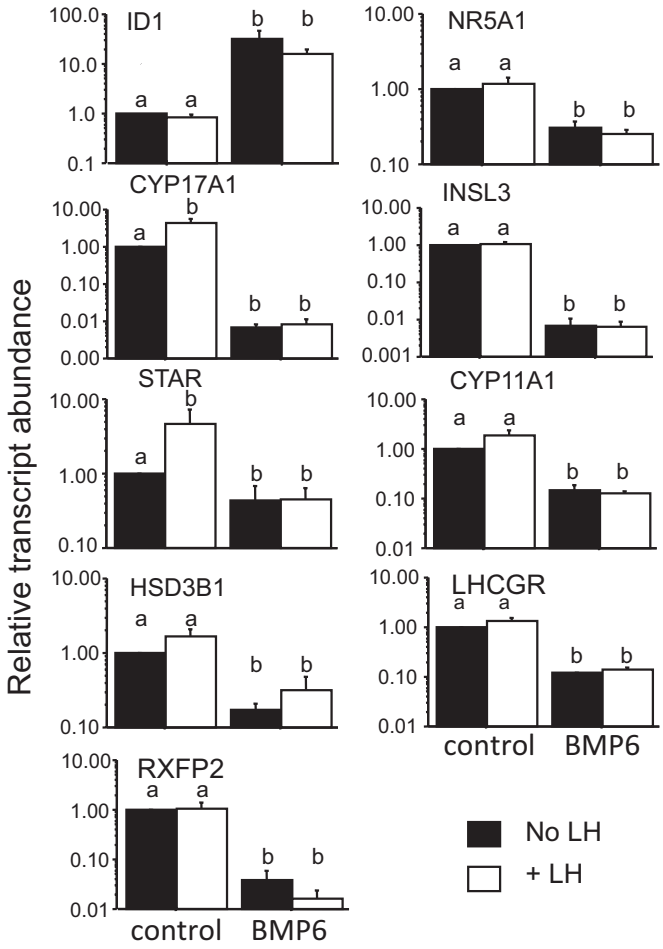

Fig. 2. RT-qPCR confirmation of microarray findings for selected transcripts shown to be affected by BMP6 treatment. Note that *RXFP2* was not represented on the microarray. Values are means, and vertical bars indicate SEM (n = 4 independent cultures). Means without a common letter are significantly different ($P < 0.05$).

Does Direct Inhibition of Androgen Synthesis Affect Expression of INSL3 or RXFP2? Exposure of TCs to abiraterone abolished A4 secretion ($P < 0.001$), confirming its action as a potent inhibitor of CYP17A1 (Fig. 8). Expression of mRNA for *INSL3* and its receptor *RXFP2* were both reduced by ~50% ($P < 0.05$) in abiraterone-treated cells. Mean INSL3 protein secretion level was also ~50% lower in abiraterone-treated cells, but the difference was not statistically significant.

What Other Intraovarian Factors Affect TC Expression of INSL3? As shown in Fig. 9 treatment of TCs with LH alone promoted a robust (eightfold; $P < 0.05$) increase in A4 secretion but did not affect *INSL3* mRNA level or INSL3 protein secretion. Treatment with EGF, TGF-α, or TNF-α promoted marked reductions ($P < 0.05$) in A4 secretion, *INSL3* mRNA level, and INSL3 protein secretion under both basal and LH-stimulated conditions. In addition, cotreatment of cells with inhibin-A reversed the suppressive effects of BMP4, -6, and -7 on *INSL3* mRNA level and INSL3 protein secretion (Fig. 10).

Ingenuity Pathway Analysis Suggesting Functional Associations Between TC Genes Up- or Down-Regulated by BMP6 Treatment. The list of 445 genes shown to be up-or down-regulated by BMP6 (>twofold; $P < 0.01$) in the microarray study was exported to Ingenuity Pathway Analysis (IPA) software for pathway analysis. This generated a network that linked 88 of the 445 genes on the basis of previously reported functional interactions (Fig. 11). Multiple nodes were evident in the gene network, but it is notable that NR5A1 (steroidogenic factor 1, SF-1) formed one prominent node radiating direct links to *INSL3*, *INHA*, *CYP17A1*, *CYP11A1*, *STAR*, *LHCGR*, scavenger receptor class B, member 1 (*SCARB1*), and *NR0B1*. Expression of each of these genes was significantly down-regulated by BMP6 treatment.

Discussion

According to the "two cell-two gonadotropin" model of ovarian estrogen synthesis (23–25) pituitary LH acts on TCs to promote the synthesis of androgens that diffuse to neighboring GCs to act as substrate for cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1*) (CYP19A1), whose expression is induced by pituitary follicle stimulating hormone. CYP19A1 then converts androgens into estrone and estradiol-17β. Because GCs do not express *CYP17A1* they are unable to synthesize androgens de novo, and thus TC–GC cooperation is essential for the follicular unit to fulfill its all-important role of secreting estrogens, which are vital for multiple aspects of female reproduction and other physiological processes. Although this basic model remains valid, there are many aspects of the mutual interplay between GCs and TCs that have yet to be resolved, and it is increasingly evident that local autocrine/paracrine signaling must play a prominent role and that GCs, in turn, modulate TC function (25). Compelling evidence, largely from in vitro studies in rodents, indicates that locally produced growth factors, including various members of the TGF-β superfamily, are expressed by GCs, TCs, and oocytes in a developmental stage-related manner and contribute to the regulation of follicle development and steroidogenesis (reviews in refs. 5, 6, 8, and 26). Notable among these are BMPs, activins, and inhibins. As discussed here, another peptide unrelated to the TGF-β family, INSL3, is known to be expressed abundantly in ovarian TC and, to a lesser extent, corpus luteum (20, 22, 27, 28), but its functional role in the ovary has remained obscure.

The finding from our microarray study that treatment of bovine TCs with BMP6 promotes a profound reduction in expression of *INSL3* concomitantly with reduced androgen secretion and diminished expression of *CYP17A1* and other transcripts involved in steroidogenesis suggests a functional link between INSL3 signaling and thecal steroidogenesis and prompted further

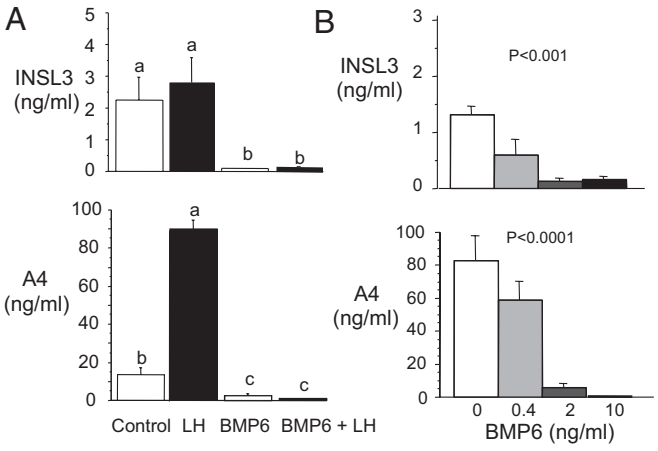

Fig. 3. (*A*) BMP6 under basal and LH-stimulated conditions reduces secretion of INSL3 and A4 by bovine TCs. Means without a common letter are significantly different ($P < 0.01$). (*B*) Dose-dependent suppressive effect of BMP6 on secretion of INSL3 and A4. Values are means ± SEM (n = 4 independent cultures).

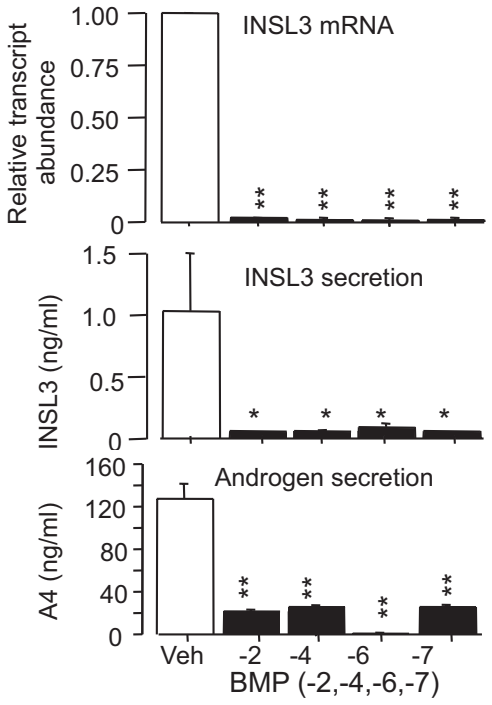

Fig. 4. BMP2, -4, -6, and -7 all suppress TC expression of *INSL3* mRNA and protein secretion. Values are means ± SEM (n = 4 independent cultures). *P < 0.05, **P < 0.001 vs. control.

investigation. Having confirmed that the BMP6-induced reduction in *INSL3* mRNA expression was accompanied by a corresponding inhibition of INSL3 protein secretion by the cells, we showed that three other BMPs (BMP2, BMP4, and BMP7) known to be expressed in bovine follicles (14, 17, 29) exerted a similar suppressive action on *INSL3* expression, at both the mRNA and protein level. Moreover, BMP treatment suppressed thecal expression of *RXFP2*, the cognate signaling receptor for INSL3 (30) that is coexpressed with INSL3 in follicular theca cells (28, 31). On the basis of these findings, we hypothesized that the BMP-induced suppression of thecal androgen production might actually be mediated via an inhibition of thecal INSL3-RXFP2 signaling acting in an autocrine/paracrine manner. To address this we first examined the time course of the response to BMP treatment with the prediction that a BMP-induced decline in *INSL3* and/or *RXFP2* expression would precede the corresponding fall in expression of *CYP17A1* and other steroidogenesis-related transcripts. If so, this would support our hypothesis. In the event, a significant BMP-induced down-regulation of *INSL3* mRNA was first detected after 24 h of treatment, coincident with a significant decline in *CYP17A1*, *CYP11A1*, *HSD3B1*, and *LHCGR* expression, as well as A4 secretion. Although the mean INSL3 protein secretion level was ∼40% lower at 24 h, this decline was not statistically significant. However, a significant fall in *RXFP2* expression was detected just 4 h after BMP treatment, coincident with a significant up-regulation of the classic BMP-responsive gene *ID1* (32, 33). As such, this observation provides partial support for the above hypothesis.

Next we used an RNA interference approach to investigate the dependency of TC androgen production on INSL3-RXFP2 signaling. Transfection of cells with *INSL3*-targetted siRNA successfully knocked down levels of *INSL3* mRNA by ∼75% and secreted protein by >90%. This was accompanied by a substantial decline in *CYP17A1* expression and A4 secretion, supporting an obligatory role for INSL3 in maintaining TC androgen output. Likewise, *RXFP2*-targeted siRNA reduced *RXFP2* mRNA level by 80%, and this was also accompanied by a marked fall in *CYP17A1* expression and A4 secretion, reinforcing this concept. Interestingly, female mice with null mutations in *insl3* reportedly show reduced fertility associated with increased follicle atresia, accelerated luteolysis, and extended estrous cycles (34, 35). However, whether ovarian androgen production is affected in *insl3* null mice has not been reported.

To complement the findings of our INSL3/RXFP2 knockdown experiments we treated TCs under basal and LH-stimulated conditions with various concentrations of exogenous INSL3 (synthetic human), the prediction being that this would boost androgen secretion. A small (<twofold) although statistically significant effect of INSL3 was seen, but there was no LH × INSL3 treatment interaction (P = 0.9), reinforcing the view that autocrine/paracrine INSL3-RXFP2 signaling has a positive influence on TC androgen production under both basal and LH-stimulated conditions. The relatively modest effect of exogenous

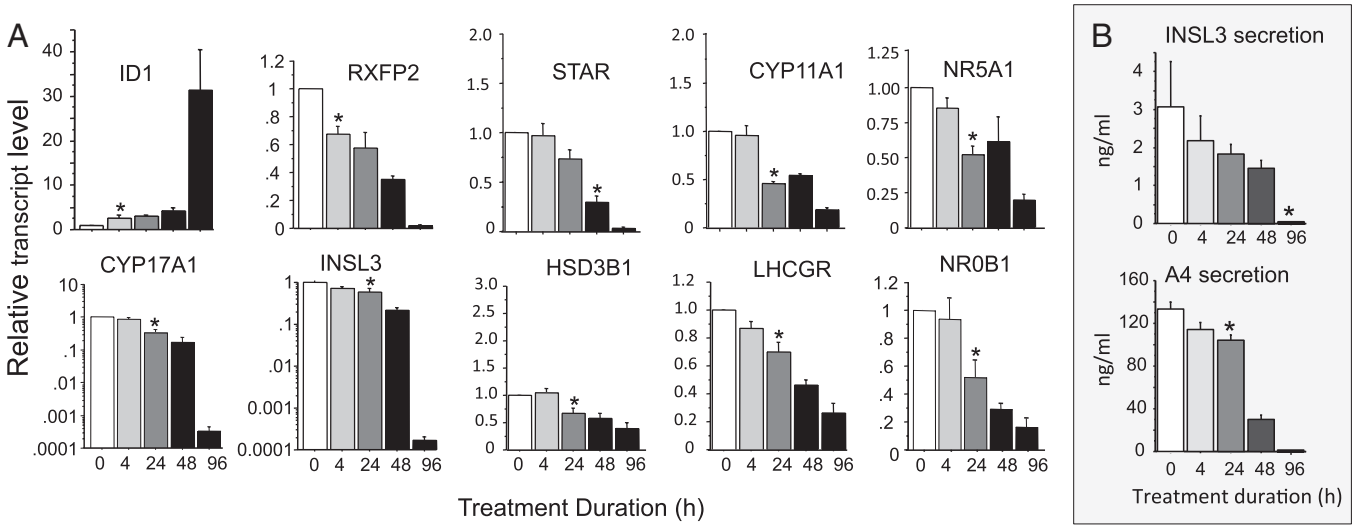

Fig. 5. Time course of BMP6 action on (*A*) relative abundance of 10 selected mRNA transcripts and (*B*) secretion of INSL3 and A4 by bovine TC. Note log scales for *CYP17A1* and *INSL3* expression. Values are means ± SEM (n = 4 independent cultures). *The first time point at which a significant change was detected (P < 0.05 vs. time 0).

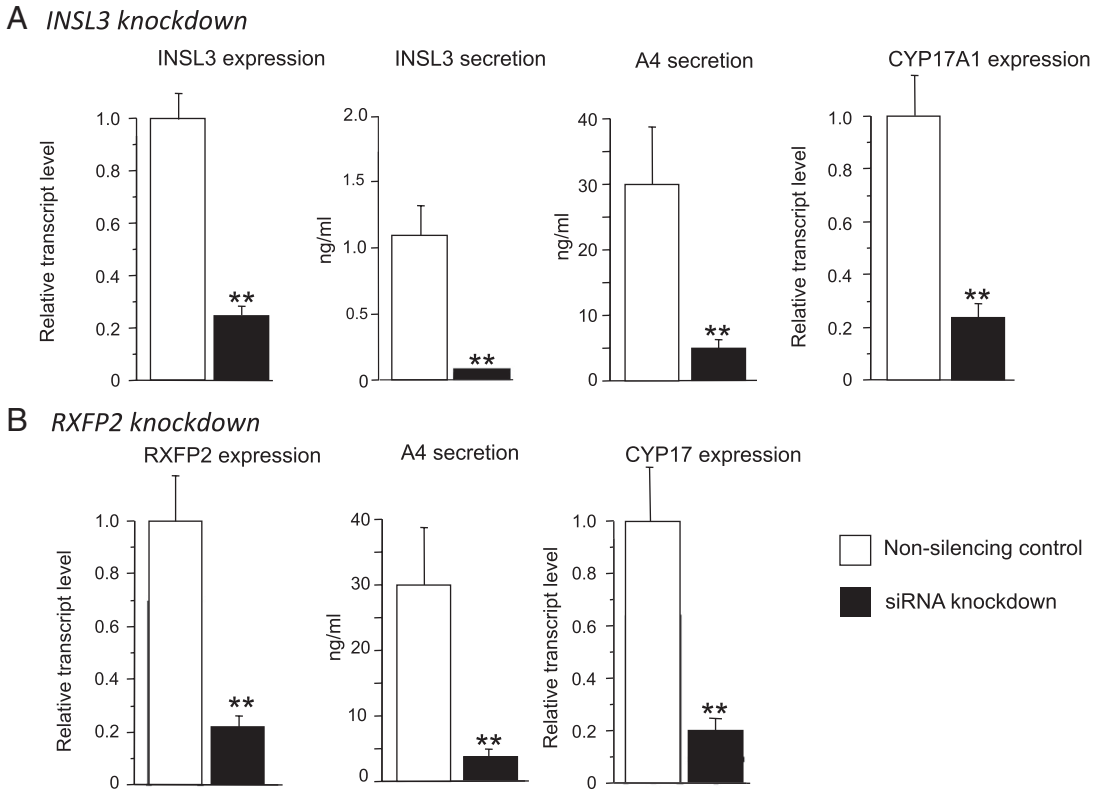

Fig. 6. siRNA-mediated knockdown of (*A*) INSL3 and (*B*) RXFP2 suppresses CYP17A1 expression and A4 secretion by bovine TCs in vitro. Relative transcript abundance is expressed as a proportion of the average value in control cells transfected with nonsilencing control siRNA. Values are means ± SEM ($n = 4$ independent cultures). $^{**}P < 0.01$ vs. control.

INSL3 on androgen secretion most likely reflects the high level of endogenous INSL3 generating concentrations, particularly near the cell membrane, sufficient to promote near-maximal INSL3 receptor (RXFP2) occupancy. In addition, because the amino acid sequences of bovine and human INSL3 are only about 75% homologous (22), human INSL3 may be a poor agonist at the bovine receptor.

Given the above evidence that autocrine/paracrine INSL3-RXFP2 signaling has a positive role in promoting TC androgen production, we considered the possibility that, in a reciprocal fashion, androgen might promote INSL3-RXFP2 signaling. Because high levels of endogenous androgens are already present in these cultures, rather than treating cells with exogenous androgen, we used a pharmacological approach to inhibit endogenous androgen production. Abiraterone, a potent inhibitor of CYP17A1 enzyme (36), abolished A4 secretion and reduced expression of *INSL3* and *RXFP2* mRNA by ∼50%, supporting the notion of a mutual, positive autoregulatory loop linking thecal INSL3-RXFP2 signaling and androgen synthesis. Our evidence that ovarian (thecal) expression of RXFP2 is positively regulated by androgens concurs with the recent observation in male mice that testosterone administration to androgen-deficient *LHCGR*-null mutants up-regulated RXFP2 expression in the gubernaculum (37).

TC expression of *INSL3* is acutely up-regulated by LH/CG treatment in rats (38), whereas longer-term treatment of bovine TCs with a high dose level of LH or with serum (to promote luteinization) down-regulates *INSL3* expression (28, 39, 40). In this study, involving nonluteinized TCs, no consistent stimulatory effect of LH on *INSL3* mRNA expression or secretion of INSL3 protein was seen, despite a clear-cut stimulation of A4 secretion. This concurs with a recent human study showing that a gonadotropin-releasing hormone agonist-induced LH rise promotes androgen secretion but does not raise circulating INSL levels in either control or PCOS subjects (41). Treatment of bovine TCs with EGF, TGF-α, and TNF-α, growth factors previously shown to suppress thecal androgen production (10, 42, 43), uniformly suppressed *INSL3* mRNA level and INSL3 protein secretion, reinforcing the positive association between androgen and INSL3 production by the cells. Moreover, inhibin-A cotreatment could reverse the BMP-induced inhibition of INSL3 expression, reinforcing previous evidence that granulosa-derived inhibin contributes to the physiological regulation of BMP signaling and, in turn, thecal androgen synthesis (14). Although not examined in the present study, it should also be mentioned that insulin and IGF1 have also been shown in several species to promote thecal androgen production in vitro (9, 12).

Earlier immunolocalization studies of bovine follicles found that not all healthy follicles were immunopositive for INSL3 (20). Nearly half of the follicles over 10 mm in diameter lacked immunoreactvity. However, recent RT-qPCR data (28) indicate a progressive increase in thecal expression of mRNA for both *INSL3* and its receptor *RXFP2* with antral follicle growth from 1 to 2 mm to 9 to 10 mm in diameter. The more limited sensitivity of immunohistochemistry compared with RT-qPCR for detecting INSL3 expression is one possible explanation for this apparent discrepancy. Satchell et al. (28) also reported that plasma INSL3 levels peak during the preovulatory phase of the bovine estrous cycle and then fall concomitantly with estrogen after the LH surge as follicle luteinization occurs. This agrees with the finding of a striking inverse relationship between the presence of INSL3 in the theca and 3 beta-hydroxysteroid dehydrogenase type 1 protein in the membrana granulosa (20), because 3βHSD is up-regulated during follicle luteinization. In mouse ovary, *INSL3* expression is higher in the follicular phase than in the luteal phase, further suggesting a positive correlation of *INSL3* with follicular maturation (44). Regarding other possible intraovarian roles of *INSL3*,

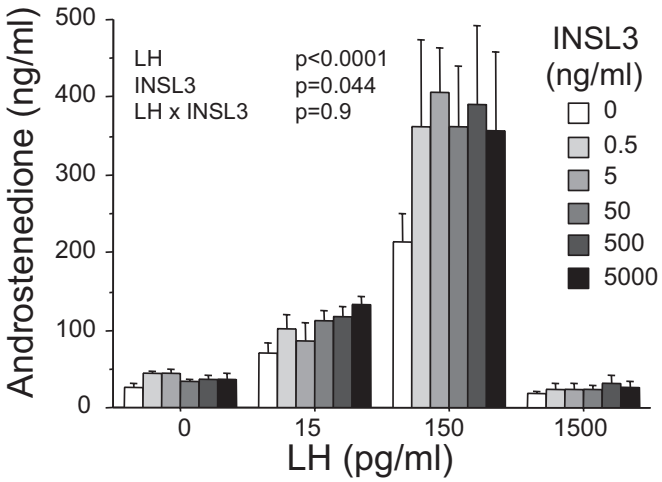

Fig. 7. Effect of synthetic human INSL3 on thecal A4 secretion under basal and LH-stimulated conditions. Values are means ± SEM (n = 4 independent cultures). Results of two-way ANOVA are indicated.

intrabursal injection of INSL3 promoted oocyte maturation in pregnant mare serum gonadotropin-primed rats but, unlike LH or chorionic gonadotropin, did not induce ovulation (38). In the bovine ovary, expression of *RXFP2* mRNA is most abundant in TCs, consistent with an autocrine/paracrine action of TC-derived INSL3 on the same cell type (28, 45). *RXFP2* is also expressed to a lesser degree in GC and corpus luteum tissue. Serum INSL3 levels are higher in women with PCOS compared with controls (41, 46), being positively correlated with testosterone and LH in both groups. Moreover, in the PCOS group, INSL3 levels were positively correlated with ovarian follicle number and "hyperandrogenemia index" (46).

Taken together with the bovine microarray finding reported here that BMP6 concomitantly reduces TC *INSL3* expression and androgen production, the above reports highlight the potential importance of INSL3 and BMPs in the etiology of LH-dependent ovarian hyperandrogenism and indicate the need for further research to elucidate the contribution of INSL3-RXFP2 signaling and BMP signaling to ovarian physiology, particularly in the context of PCOS. Despite extensive research over many decades the etiology of this widespread although complex and heterogeneous syndrome remains poorly understood (reviewed in refs. 1, 2, 47, and 48). Both genetic and environmental factors are implicated, and there is an association with insulin resistance and hyperinsulinemia, giving rise to the experimentally supported concept that heightened exposure of theca cells to insulin amplifies LH-dependent androgen production by theca cells (47, 48). It has also been proposed that excessive exposure to androgens in utero and/or adolescence could be a contributory factor in triggering insulin resistance in later life, or that androgen excess in utero exposure induces a persistent abnormality of follicle development (49). Such an alteration could involve, or lead to, perturbation of one or more intraovarian signaling systems known to positively (e.g., INSL3, inhibin, IGF?) or negatively (e.g., BMP, activin, TGF-α?) regulate androgen biosynthesis.

In conclusion, this study has established the importance of INSL3-RXFP2 signaling for maintaining androgen production by ovarian theca cells. The potent suppressive action of BMP on thecal androgen production seems to be linked to its suppressive action on INSL3-RXFP2 signaling and may be mediated via down-regulation of the nuclear transcription factor NR5A1 (SF-1), which is known to interact with the promoter/enhancer regions of the *INSL3* gene and multiple genes involved in the steroidogenic response, including *STAR*, *CYP11A1*, *CYP17A1*, and *LHCGR*. We suggest that a functional deficit in intraovarian BMP signaling could lead to ovarian androgen excess by promoting increased INSL3-RXFP2 signaling and up-regulation of the above steroidogenic genes. Further research is warranted to evaluate this possibility, particularly in the context of PCOS.

Materials and Methods

Bovine Ovaries and Primary Culture of TCs. Theca interna cells were isolated from adult bovine ovaries obtained from the slaughterhouse, as described previously (13, 17). TCs pooled from ~50 individual 4- to 6-mm follicles were plated out in either 96-well plates (7.5×10^4 viable cells per 0.2 mL per well) for evaluation of hormone secretion (immunoassay) and viable cell number (neutral red uptake assay), or in 24-well plates (0.5×10^6 viable cells per mL per well) for analysis of gene expression (RT-qPCR or microarray analysis). Cells were routinely cultured for 6 d under defined serum-free conditions, with treatments present on days 3–6 inclusive. For RNA interference experiments cells were cultured for 7 d in total. The culture medium used routinely was McCoy's 5A modified medium supplemented with 1% (vol/vol) antibiotic-antimycotic solution, 10 ng/mL bovine insulin, 2 mM L-glutamine, 10 mM Hepes, 5 μg/mL apotransferrin, 5 ng/mL sodium selenite, and 0.1% (wt/vol) BSA (all purchased from Sigma). Antibiotic-antimycotic was omitted from the culture medium used during transfection of cells with RNAi in accordance with the protocol provided by the supplier (Dharmacon). Media were replenished on days 3 and 5 with fresh media containing treatments as appropriate. Conditioned media were retained for hormone assay, and at the end of culture cell lysates were prepared using the lysis buffer component of the RNA isolation kit. Pooled lysates from replicate wells were stored at −80°C until total RNA isolation.

Treatments. Highly purified ovine LH (oLH-S-16) was obtained from the National Hormone and Pituitary Program. Recombinant human (rh) BMP2, BMP4, BMP6, and BMP7 were purchased from R&D Systems. Human INSL3 was prepared by optimized solid-phase synthesis of the separate A- and B-chains (26 and 31 residues, respectively), followed by sequential formation of each of the three disulfide bonds via regioselective chemical formation (50). The peptide was comprehensively chemically characterized by analytical RP-HPLC and MALDI-TOF MS to confirm its high purity. Treatments stock solutions were sterilized using 0.2-μm membrane filters before dilution in sterile culture medium. The CYP17A1 inhibitor abiraterone was purchased from Selleck Chemicals and stock solution (50 mM) prepared in DMSO. For experiments involving knockdown of endogenous *INSL3* and *RXFP2*, siRNAi duplexes against bovine *INSL3* (sense strand: GGCAAGACCUGCUGACCCUUU; antisense strand: AGGGUCAGCAGGUCUUGCCUU) and bovine *RXFP2* (sense strand: CCUGAAGUGUCUACAGAAAUU; antisense strand: UUUCUGUAGACACUUCAGGUU) were custom-designed and synthesized by Dharmacon Thermo Scientific. Controls included cells transfected with a nonsilencing control RNAi (NSC3; Dharmacon), as well as cells exposed to transfection

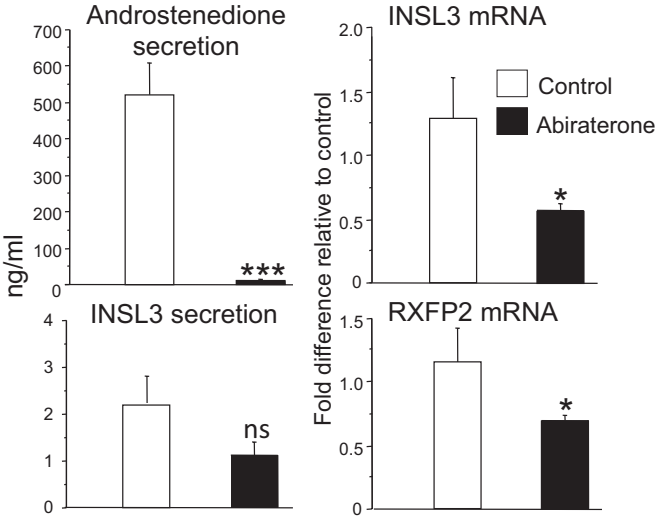

Fig. 8. CYP17A1 inhibitor abiraterone abolished A4 secretion ($P < 0.001$) and reduced expression of both *INSL3* and *RXFP2* mRNA. *$P < 0.05$, ***$P < 0.001$ vs. corresponding control. Values are means ± SEM (n = 3 independent cultures).

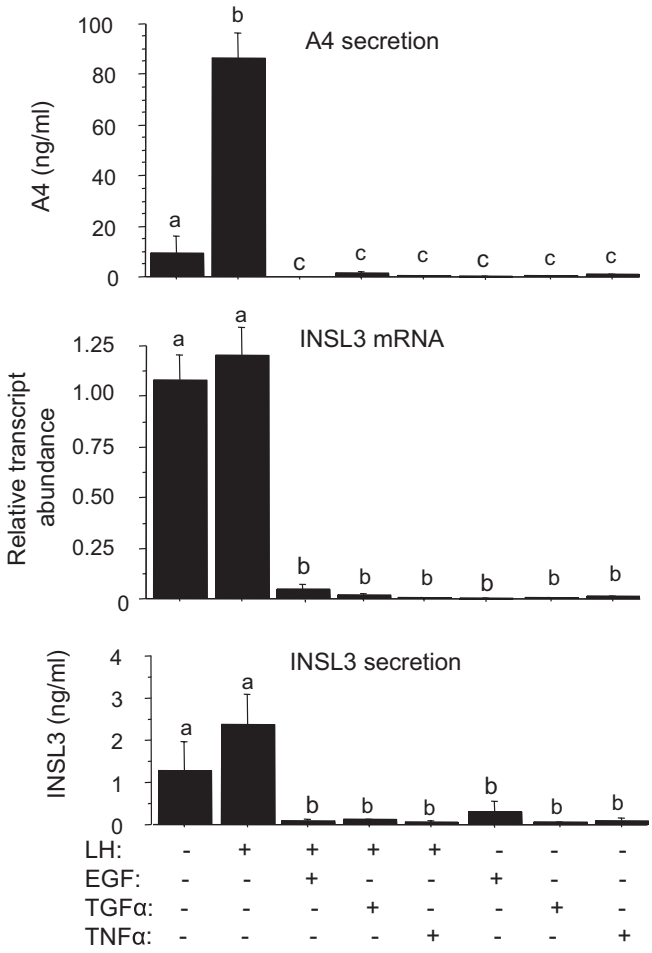

Fig. 9. Suppressive effect of EGF, TGF-α, and TNF-α, alone and in combination with LH, on secretion of A4 and INSL3 and on expression of *INSL3* mRNA by bovine TCs. Values are means ± SEM (n = 4 independent cultures). Means without a common letter are significantly different ($P < 0.05$).

reagent only (DharmaFECT 2; Dharmacon). All cell culture experiments were repeated three to four times using TC prepared from independent batches of follicles.

Microarray Analysis of Global Gene Expression in TCs Treated with BMP6. For the microarray study TCs were treated for 4 d with BMP6 (10 ng/mL) under both basal and LH-stimulated conditions (±160 pg LH/mL). These dose levels of BMP6 and LH were chosen because they elicited optimal responses in our previous studies (13, 14). The experiment was replicated four times with TCs from different batches of ovaries. Total RNA was isolated using the Ribopure RNA isolation kit (Ambion) according to the manufacturer's instructions. RNA yield and quality were evaluated by spectrophotometry at 260/280 nm and agarose gel electrophoresis before submitting 5 μg of each RNA sample (n = 16 constituting four biological replicates × four treatment conditions) to an accredited Affymetrix service provider (Almac Diagnostics Ltd) for microarray analysis using the bovine genome array GeneChip (Affymetrix). There are 24,128 probe sets on the array, representing more than 23,000 bovine transcripts and ∼19,000 UniGene clusters. Raw hybridization data were stored as .cel files and loaded into Genespring GX (v12.1; Agilent) for probe summarization (robust multiarray averaging algorithm), filtering, and statistical analysis using the GeneSpring "biological significance" workflow utility for a gene-level experiment. Listed entities passing the expression level filter (hybridization signal >20th percentile in at least one sample in one treatment group) were filtered on statistical significance (pairwise treatment comparisons reaching corrected $P < 0.01$ using the Benjamini-Hochberg false discovery rate correction for multiple comparisons) and then on fold difference (>twofold between treatments) to ensure robust identification of up- or down-regulated transcripts. The resultant list of >twofold up- or down-regulated genes (corrected $P < 0.01$; unpaired t test with Benjamini-Hochberg correction) was subjected to GO analysis and subsequently exported to the IPA program (Ingenuity) for gene network analysis. The raw microarray dataset from this experiment has been submitted to the Gene Expression Omnibus database (accession no. GSE44704).

RT-qPCR Validation of Microarray Findings. RT-qPCR was used to analyze the relative abundance of selected transcripts shown to be up- or down-regulated by BMP6 in the microarray analysis. cDNA samples were prepared from the same set of total RNA samples used for microarray analysis and used at a 1/50 dilution for qPCR amplification as described below.

Effect of BMP6 on Secretion of INSL3 Protein. To verify that the profound effect of BMP6 treatment on *INSL3* mRNA abundance was accompanied by a corresponding change in the secretion of INSL3 protein by the cells, we assayed media samples using an immunoassay for bovine INSL3 recently developed in one of our laboratories (27).

Do Other BMPs Suppress Expression of INSL3 and RXFP2? To ascertain whether BMPs other than BMP6 also affect TC expression of INSL3 and its receptor we directly compared the effects of BMP2, -4, -6, and -7 (each at 10 ng/mL, 4-d treatment) on the secretion of INSL3 protein and A4 by the cells and on the relative expression of a range of transcripts, including *INSL3*, *RXFP2*, and *CYP17A1*.

Time Course of BMP6 Action on Expression of INSL3, RXFP2, and Other Transcripts. Because a routine 4-d treatment protocol was used in the above experiments, it was of interest to establish the time course of the TC response to BMP6. To achieve this we designed a "reverse" time course experiment in which cultures were terminated and cells were lysed at a fixed time on day 6 of culture, having been exposed to BMP6 (10 ng/mL) for the preceding 4-, 24-, 48-, or 96-h period.

Does Knockdown of Endogenous INSL3 or RXFP2 Affect CYP17A1 Expression and Androgen Secretion? To investigate the effect of knockdown of endogenous INSL3 and RXFP2, TCs were exposed to siRNA duplexes (50 nM final concentration), nonsilencing control duplex (NSC3; Dharmacon; 50 nM), or transfection reagent only (DharmaFECT 2; 0.25 μL per well). siRNA duplexes were prepared for transfection as recommended in the supplier's protocol (Dharmacon) and added to cells, together with LH (150 pg/mL), after medium

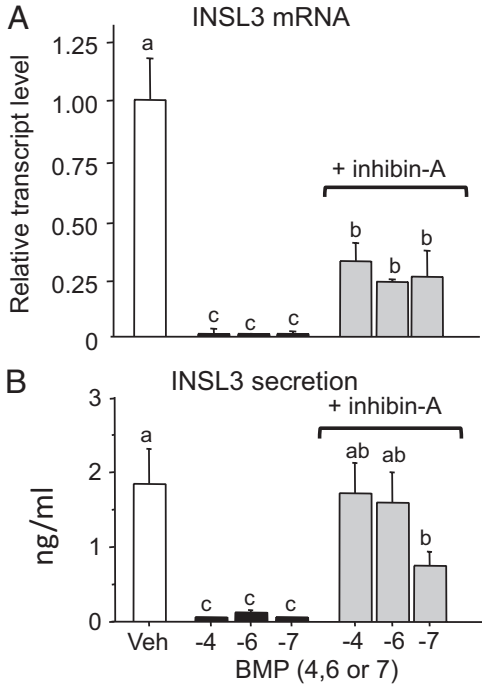

Fig. 10. Inhibin-A reverses the suppressive effect of BMP4, -6, and -7 on TC expression of (*A*) *INSL3* mRNA and (*B*) INSL3 protein secretion. Values are means ± SEM (n = 4 independent cultures). Means without a common letter are significantly different ($P < 0.05$).

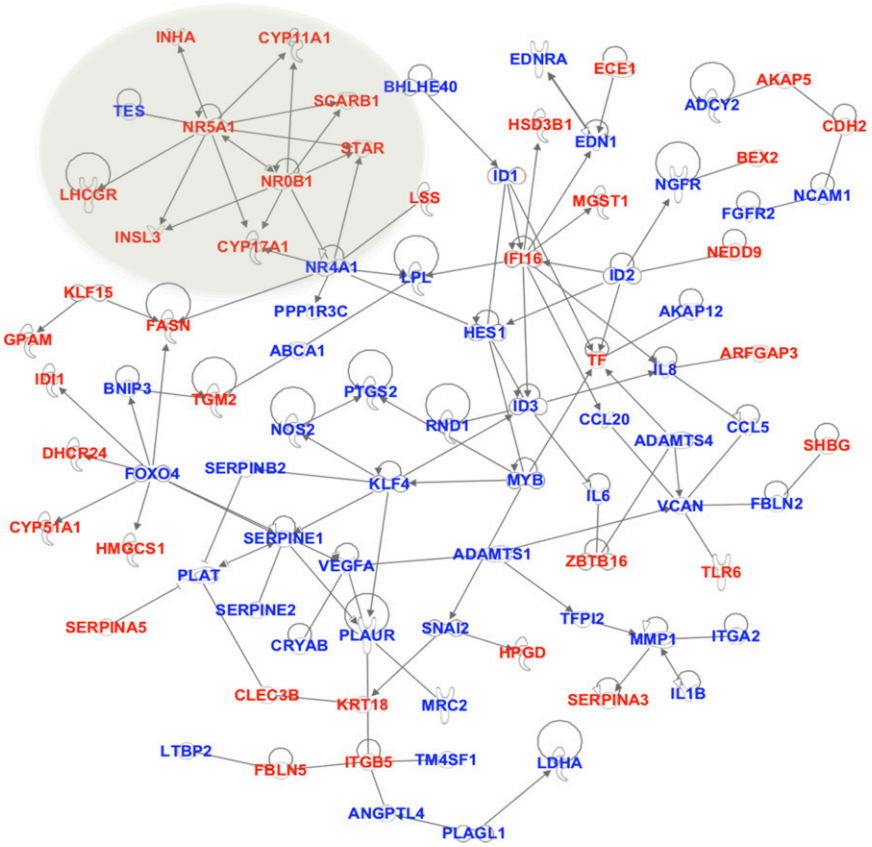

Fig. 11. IPA-generated gene network linking 88 of 445 genes shown by microarray analysis to be significantly ($P < 0.01$) up- (blue) or down- (red) regulated >twofold in bovine TCs in response to BMP6 treatment. Note the node centered on *NR5A1* (SF-1) linking *INSL3*, *INHA*, *CYP17A1*, and several other components of the steroidogenic pathway that were heavily down-regulated by BMP6 (green highlight). To aid clarity dashed lines indicating indirect interactions have been excluded.

changes on days 2 and 4 of culture. Cells were lysed 3 d later for RNA extraction and qPCR analysis; media were retained for steroid and INSL3 assay.

Does Synthetic INSL3 Modulate Androgen Secretion by Bovine TC? TC cultured in the presence and absence of LH (0, 15, 150, or 1,500 pg/mL) were treated for 4 d with synthetic human INSL3 at five dose levels, ranging from 0.5 to 5,000 ng/mL. Media were changed and treatments replenished after 2 d. Viable cell number at the end of culture and concentrations of A4 and progesterone in conditioned media were determined.

Does Pharmacological Inhibition of Androgen Synthesis Affect Expression of INSL3 or RXFP2? TC were cultured for 4 d under under LH-stimulated conditions in the presence and absence of abiraterone (100 nM), a potent CYP17A1 inhibitor. The effects on viable cell number and secretion of A4 and progesterone were determined.

What Other Intraovarian Factors Affect TC Expression of INSL3? TCs were cultured for 4 d under basal and LH-stimulated conditions in the presence and absence of EGF, TGF-α, and TNF-α each at 10 ng/mL We also examined whether cotreatment of cells with inhibin-A (50 ng/mL) could antagonize the suppressive effects of BMP4, -6, and -7 (10 ng/mL) on expression of *INSL3* mRNA and INSL3 protein secretion.

Real-Time PCR. cDNA was synthesized from 1 μg of total RNA using the Applied Biosystems high-capacity cDNA reverse transcription kit used according to the manufacturer's instructions (Applied Biosystems; Life Technologies). Primers (Table S4) were designed using Primer Express software (Applied Biosystems) or Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR assays were carried out in a volume of 14 μL, comprising 5 μL cDNA (1/50 dilution), 1 μL each forward and reverse primers (final concentration 360 nM), and 7 μL SYBR Green 2× Master Mix (QuantiTect; Qiagen). Samples were processed on an Applied Biosystems StepOne Plus real-time PCR instrument (Life Technologies) with the following thermal cycling conditions: 15 min at 95 °C (one cycle), followed by 15 s at 95 °C and 1 min at 60 °C (40 cycles). Melt curve analysis confirmed amplification of a single product of the predicted melting temperature and absence of nonspecific products.

The ΔΔCt method (51) was used for comparison of relative transcript abundance. Transcript Ct values in a given sample were first normalized to β-actin Ct value (which was uniform across all experimental groups: ANOVA $P > 0.1$). Resultant ΔCt values for individual replicates within each treatment group were then normalized to the mean ΔCt value of vehicle-treated controls. ΔΔCt values were finally converted to fold differences using this formula: fold difference = $2^{(-\Delta\Delta Ct)}$.

Hormone Assays. A4 was determined by an in-house ELISA as fully described in *SI Materials and Methods*. The assay detection limit was 30 pg/mL, and mean intra- and interassay coefficients of variation (CVs) were 7% and 10%, respectively. Progesterone was determined by ELISA (52), with a detection limit of 0.1 ng/mL and mean intra- and interassay CVs of 6% and 9%, respectively. Bovine INSL3 was measured using a recently established time-resolved fluorescent immunoassay (27). This homologous assay had a detection limit of 20 pg/mL and had mean intra- and inter-CVs of <2% and <10%, respectively.

Statistical Analysis of Immunoassay and RT-qPCR Data. Hormone secretion data were analyzed using one- or two-way ANOVA and are presented as means ± SEM on the basis of four independent culture experiments. Where indicated, Fischer's protected least significant difference (PLSD) test was used for post hoc pairwise comparisons. To reduce heterogeneity of variance, hormone data were log-transformed before statistical analysis. RT-qPCR data (from at least four independent TC batches) were analyzed (ANOVA and post hoc Fisher's PLSD test) as ΔCt values before conversion to fold difference values for graphical presentation.

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